Regulatory role of ambient pH in the expression of pathogenicity determinant gene products of *Beauveria bassiana* and *Metarhizium anisopliae*

A Thesis
Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Applied Microbiology and Food Science
University of Saskatchewan
Saskatoon
By

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GENERAL ABSTRACT

Entomopathogenic fungi (EPF) are the one of the potential cause of the morbidity and mortality of insects. In agro-forestry uses, they are applied mainly in the form of conidial preparations in dry, aqueous or oil formulations. This approach, while practical, works in a hit and miss fashion leading to a frustrating dilemma of why successes and failure perpetuate. The fundamental solution is to bridge gaps in our knowledge about conidia of EPF in varied environments where they confront a diversity of insect hosts to start their pathogenesis.

This thesis was undertaken to examine the effects of hydration and the regulatory role of ambient pH on proteases which are the primary pathogenicity determinants in *Beauveria bassiana* and *Metarhizium anisopliae*. The approaches used were those of biochemical, proteomics and functional proteomics.

Novel aspects of pH regulation/homeostasis during the soaking of conidia in water, (type II water, which had a maximum electrical conductivity of *1μS/ cm* at 298K/ 25° C) were identified. Hydrated conidia showed swelling in type II water as assessed by (Multisizer III^{TM} (Coulter Counter^{TM}). Release of proteases, metabolic activity through liberation of ammonia and citrate and synthesis of protein, RNA and DNA was established. It was deduced that conidial enzymes are either attached by loose hydrogen bonding or were associated to the spore membranes. Water soaked or hydrated conidia can secrete citrate and ammonia to modify the ambient pH and maximize the activity of secreted proteases.

Pr1- and- Pr2-like proteases were liberated by washing conidia in tween (Tw), water (Ww) and buffer. The washing of conidia in buffers (pH 4-10) affected the release/activity of Pr1 and

Pr2. The thesis shows a newly designed native IPG strip zymography to identify the release of 4 and 8 isoforms of proteases, respectively from conidia. The 2-DE zymography (copolymerized gelatin) of protease from Tw of *B. bassiana* and *M. anisopliae* indicated one band (Mr 70 kDa; pI 6.3) and six isozymes (Mr 115-129 kDa; pI 3.7-9.0), respectively, which were identified using mass spectrometry (MALDI-TOF) as a serine-like protease.

Six metalloprotease isozymes from *M. anisopliae* but only one from *B. bassiana* was documented by 1-DE native zymography combined with 2-D spot densitometry scans. Cationic PAGE native zymography separated two basic protease isozymes from Tw extract of *M. anisopliae* depending upon the pH of the incubation buffer. However, one activity band was identified from *B. bassiana*. Furthermore, only one activity band was apparent during 1st and 2nd Ww up to day 2 for both EPF. SDS PAGE (non-dissociating) zymogram of secreted protease isozymes from Tw of *B. bassiana* revealed three bands of Mr100, 60, and 36.3 kDa. The isozymes observed at day 2 and 3 had a Mrs of 35.4 and 25 kDa, and 24.7 and 20.3 kDa at day 4. The SDS PAGE zymograms for *M. anisopliae* indicated two isozymes of Mr 103 and 12 kDa, respectively. During the 1st Ww and incubation of spores at day 2 and 3, a 12 kDa band was observed. These results confirm the presence of diversity of proteases and their isozymes with unique molecular sizes.

This thesis research discovered and characterized a diversity of proteins/enzymes not previously reported from any other fungi. A newly designed enzyme overlay membrane (EOM) technique revealed three isoforms of Pr1-like subtilisin from Tw of *M. anisopliae* (pI 8.1-9.7) and *B. bassiana* (pI 8.4-9.7). Conversely, only one isoform of Pr2-like trypsin was identified from *M. anisopliae* and no Pr2-like activity was observed from *B. bassiana*. Use of metalloprotease (MEP) inhibitors in conjunction with EOM analysis revealed their release during

treatment in Tw. In *M. anisopliae* four activities (pI 4.4-7.5) of thermolysin-like MEP were observed. However, Tw of *B. bassiana* showed one activity band (pI 5.5). In addition, an isozyme of neutral MEP containing Zinc from *M. anisopliae* (pI 6.1) and one from *B. bassiana* (pI 6.5-7.6), respectively, was identified. MALDI-TOF and Q-TOF analysis revealed the presence of proteins similar to ROD 1, ά- and β-glucanases, elastase, lipase 5 and galectin 7, which are important during the initial phase of germination and pathogenesis.

In addition subtilisin (Pr1-like), trypsin (Pr2-like) and NAGase synthesis from the germinating conidia and mycelia under the supply of different carbon and nitrogen (C/ N) sources was studied. The regulation of the synthesis of cuticle-degrading enzymes (CDE) from germinating conidia and mycelia was hypothesized to be controlled through regulatory derepression and nutritional starvation. Pr1 and Pr2 are regulated in a different manner in conidia and mycelia. Both enzymes are regulated through a multiple control mode. It was concluded that C/ N repression occurs only when it is necessary for infective structures to establish a nutritional relationship with the host cuticular structures. In addition, C/ N sources have a significant effect upon pH modulation, ammonia production and protease secretion. Furthermore, the synthesis of Pr1 and Pr2 from germinating conidia was affected by the (inducer pH) pH*i* of the growth media. Growing mycelia of *B. bassiana* under acidic (4.0), neutral (7.0) and basic (11.0) pH conditions produce ammonia which modifies the pH thereby creating environments suitable for protease. Growth, morphology, radial extension rate and conidiation at different pH*i* revealed that both EPF modify the pH of growth medium effectively as opposed to the saprophytic fungus, *Aspergillus nidulans*.

The presence of MEPs and Pr2-like trypsin suggests that these enzymes can act as a back up system for Pr1 to breach the cuticle and facilitate penetration before appressoria formation.

The diversity of isozymes released from conidia suggests that the EPF are pre-adapted to pathogenic mode of life style, further contributing complexity to their interaction with host insects. Such isozymes can circumvent protease inhibitors present in the insect cuticle and the hemolymph. In addition, these isozymes may offer selective advantages in exploring new habitats (substrates) either as pathogen or saprophyte.

ACKNOWLEDGEMENTS

I am indebted to the following individuals and organizations for their aid or understanding during the course of my degree.

A most special note of appreciation is for Dr. George G. Khachatourians, who gave me an opportunity of training in his lab. I am grateful to him for his friendship and guidance over the years. I am very much impressed by his seemingly endless scientific knowledge, imaginations and philosophical thoughts behind science, which I am sure, is an important and special aspect of GGK-lab training. It was his wisdom and mentorship that has led him to advise and observe me at appropriate times. I am sure that this training will help me as a scientist and in due course as a philosopher. Let neither of us bound by those who attempt to trivialize either our writings or our ideas.

I wish to acknowledge the members of my advisory committee for their time, efforts, patience, and positive criticism. I would like to formally thank Drs. Darren R. Korber, Susan M. Boyetchko and Sabine Banniza for their suggestions and comments during the preparation of this thesis. I am also grateful to Dr. Mike Ingledew and Dr. Vikram Misra for being on my advisory committee and their periodic inputs. I am thankful to Dr. Robert T. Tyler for serving as a Graduate Chair. His organizational talents have certainly helped to expedite the completion of my degree. I am also grateful to Dr. Michael J. Bidochka, Biology Department, Brock University, St. Catharine, Ontario for being an external examiner, his positive criticism and advice.

I am also grateful to the generous financial support that I received during my studies, including Graduate Scholarship from the University of Saskatchewan and Agriculture

Biotechnology Scholarship. I also acknowledge the generous funding provided to me through the NSERC grant of Dr. Khachatourians. In addition, I offer my thanks to College of Agriculture and Bioresources for Educational Enhancement Fund (EEF), College of Graduate Studies and Research for Alumni Travel Award(s) and NSERC travel grants of GGK, which provided me an excellent opportunities to communicate the findings of my thesis to scientific community.

I am also thankful to the current and past members of GGK labs: Daniel Uribe, Linda Chelico, Sandra Espitia and Adrienne Woytowich, Darcy Pawlik and Kelly Aasan for their friendship and help during my studies. I would like to extend my special thanks to Dinka Besic for her cheerful company, help and friendship over the years. I would remember conversations each day we had during lunch and tea, which has allowed us to share our projects and problems. Thanks are also due for her and Dubravko Besic for the technical help in the preparation of the Fig. 9.1-9.5. I am thankful to Proteomics Facility, National Research Council of Canada-Plant Biotechnology Institute (Saskatoon), for providing Mass spectrometry services.

I am thankful to Dr. Dwayne D. Hegedus and Dr. Martin Erlandson for their comments and suggestions on chapter no. 4 of the thesis. I am also indebted to Lorraine McGrath for critical comments on chapter no. 10 of the thesis. I am grateful to Dr. Larry Gusta and Dr. Fiona C. Buchanan for lending me the Computerized Power Pack and Bio Rad Mini Protean-II apparatus, respectively. Also special thanks to Lorna Mead and Dr. Adrienne Woytowich for their disposition to share materials and equipments.

I am indebted to Dr. Herbert N. Arst, Imperial College, London, UK and Dr. Christian P. Kubicek, Technische Universitat Wien, Austria for sharing their personal experiences and results during the course of my project. I also, am grateful to all the authors, which are cited in the thesis and for others, whose work was not fit into the thesis context, but was valuable in shaping the

project. I am thankful to Dr. Naheed Fatima, Quaid-e-Azam University, Islamabad, Pakistan for her encouragement and moral support during my Ph.D. studentship.

I acknowledge my family, especially my parents. I can only say that I have made you proud. I also, am thankful to the financial support that I receive from my parents time to time. Special thanks for my best friend and wife Dr. Ayesha Sohail, who has done an incredible job of raising Hamna, supporting me personally and morally. I only hope that I can make it up at some day for you. Love and thanks are due for Hamna Qazi, whom I owe much time and attention, which I was not able to provide because of my research.

Finally, I thank the faculty, staff and fellow students. I am indebted to Cathy, Patty and Sharon for their caring attitude and help during the course of my study.

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ABBREVIATIONS

µmhos/ cm	micromhos per centimeter
***µS*/ cm**	microsiemens per centimeter
1-DE nzPAGE	One-dimensional native zymography
1-DE nzSDS PAGE	One-dimensional SDS non-dissociating zymography
1-DE	One-dimensional electrophoresis
2-DE nrPAGE	Non-reducing 2-DE zymography
2-DE	Two-dimensional electrophoresis
ADP	Adenosine-di-phosphate
APHA	American public health association
APS	Ammonium persulfate
ATP	Adenosine-tri-phosphate
BOD	Biochemical oxygen demand
bp	Base pairs
CCR	Carbon catabolite repression
CDE	Cuticle-degrading enzymes
CHAPS	3-[(3- cholamidopropyl) dimethylammonio]-l-propanesulphonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dTTP	Deoxythymidime-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EP	Extracellular protease

EPF	Entomopathogenic fungi
EST	Expressed Sequence Tag
ExM	Exuviae medium
FAN	Free amino nitrogen
g	Gravitational force
h	Hour/ hours
HPC	Heterotrophic plate count
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IPM	Insect pest management
KDa	kiloDalton(s)
LC-MS/ MS	Liquid chromatography-mass spectrometry
mA	Milliampere
MALDI/ TOF	Matrix-assisted laser desorption or ionization/Time of flight
MEP	Metalloprotease
min	Minute/ minutes
MΩ.cm	Mega ohm/ cm
NAD	Nicotine adenine di-nucleotide
NADH	Nicotine adenine di-nucleotide, reduced
nIPG	Native IPG strip zymography
NMR	Nitrogen metabolite repression
no.	Number
NTU	Nephelometric turbidity units

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH*f*	Final pH of the spent supernatant
pH*i*	Inducer pH
PMSF	Phenylmethylsulfonyl fluoride
PC	Protease activity against casein as a substrate
PG	Protease activity against gelatin as a substrate
Q-TOF	Quadrupole time-of-flight mass spectrometry
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphosim
RNA	Ribonucleic acid
rpm	Revolution per minute
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetra methylethylenediamine
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
YPG	Yeast extract peptone glucose
YPGA	Yeast extract peptone glucose agar

1.0 GENERAL INTRODUCTION

The current insect pest management programs heavily rely on the use of synthetic chemical based insecticides or pesticides and herbicides, which is a multibillion dollar industry. The main driving force behind chemical insecticides is the fast speed of kill, high efficacy and political influence of the companies involved in this business. On the other hand, biological control is the use of biological control agents (BCA: fungi, bacteria, viruses, protozoa) to control the insects, weeds and fungal pathogens, which are safe and environmentally friendly. Among BCA, entomopathogenic fungi (EPF) have an important place because of their percutaneous mode of entry into the host and multiple modes of actions that decrease the chance of development and selection of resistant insect populations (Khachatourians, 1996). Approximately 700 species of EPF can infect insects thereby overcoming the cuticular barrier. Fungi are particularly important for controlling sap sucking insects (e.g., white flies) for which there is no alternative available in the present BCA.

Developments in biotechnology have generated interest and means necessary for displacement of synthetic chemical insecticides which currently dominate the market place. BCA currently occupy a very small niche in the insecticide industry, most of which is based on *Bacillus thuringiensis*-derived insecticides, including transgenic crops. However, various estimates indicates that BCA, which currently represent $260 million (based on 2005 sales) of the $28 billion global market for pesticides will increase to grow $350-$400M by the year 2015 (Warrior, 2006). The potential for wide spread use of BCA is timely because of their relative host specificity, ecologically non-disruptive nature and legislative aspects.

In a true sense, biological control is not a new concept. *B. bassiana* has been known as a silk worm pathogen since 1835. Furthermore, Elie Metchnikoff in 1879 demonstrated the use of *Metarhizium anisopliae* (*Entomophthora anisopliae*) against the wheat grain beetle, *Anisoplia austriaca*. Both EPF are members of the Ascomycotina (Hypocreales) (Seifert and Gams, 2001), which produce conidia as an infectious propagule. Upon availability of suitable physical and biological conditions, conidial germination may takes place. Therefore, conidia act as a vehicle of transmitting infection between saprophytic and pathogenic states.

For successful fungal infection, the first few critical steps in fungal spore germination and penetration of insect cuticle have been described as: (i) attachment, (ii) hydration, (iii) germ tube formation, (iv) secretion of mucilage substances, (v) formation of appressoria, and (vi) subsequent production of hydrolytic enzymes (mainly proteases and chitinases).

The synthesis of extracellular enzymes is crucial for the infection process of EPF. Successful infection primarily relies on the synthesis of molecular scissors such as extracellular proteases, chitinases and esterases (Clarkson and Charnley, 1996). The insect infection relies on protease(s) action because 75% of the cuticle is made up of proteins. Furthermore, chitinase(s) help degradation of *N*-acetyl-D-glucosamine moieties present in the cuticle (Khachatourians, 1996; Charnley, 1997; Khachatourians et al., 2007).

Due to the significance of proteases in breaching the insect cuticle, they have received more attention from researcher's world wide. During more than three decades of research on EPF, several investigators have established that two proteases, namely subtilisins and trypsins, as important virulence factors (St. Leger et al., 1986a; Bidochka et al., 1987; St. Leger et al., 1988). *M. anisopliae* is one of the best studied mycopathogens with regard to its proteases. It produces at least two well-characterized subtilisins (Pr1a and Pr1b) (St. Leger et al., 1994). Both Pr1a and

Pr1b show similar physical and biochemical properties. Pr1 has a great affinity for hydrophobic amino acids of the cuticle and is regarded as a key enzyme for cuticle penetration (St. Leger, 1995). Two isoforms of Pr2 are known from *M. anisopliae* (pI 4.2 and pI 4.9), which have high activity against solubilized cuticular proteins. In contrast to Pr1, Pr2 has little or no activity against covalently bound cuticular protein due to its poor adsorption (Bidochka and Khachatourians, 1993).

Fungi are considered as champions among scavengers because of their ability to acquire nutrients from the environment by using extracelluar enzymes. Bailey and Arst (1975), and Marzluf (1977) have shown that the carbon (C) and nitrogen (N) sources regulate the synthesis of extracellular enzymes in the filamentous fungi, *Neurosporra crassa* and *Aspergillus nidulans,* respectively. Similarly, proteases and chitinases from the growing mycelia of *B. bassiana* and *M. anisopliae* were shown to be regulated by food cues (C/ N ratios) (St. Leger et al., 1988; Bidochka and Khachatourians, 1988a; St. Leger, 1995). In addition, there is strong evidence of pH regulation in filamentous fungi, especially in *A. nidulans*. It has been found that *pacC* is responsible for pH-mediated gene expression. PacC is a transcription factor which is processed under alkaline and acidic conditions in a fashion that can regulate the delicate balance of pH and ensures that the metabolites, permeases and enzymes, are secreted in the habitat where they can perform optimal function. A *pacC* homolog has been identified in filamentous fungi and several phytopathogenic fungi (Penalva and Arst, 2004). Amongst EPF, St. Leger et al. (1998) showed that the ambient pH in *M. anisopliae* can regulate expression of pathogenicity determinant genes. In subsequent study they reported that *M. anisopliae* can regulate the pH and protease activity by secreting ammonia (St. Leger et al., 1999). In a similar context, Qazi and Khachatourians (2007) and Khachatourians et al. (2007) have provided the first report on the release of metabolites

(ammonia and citrate) and multiple proteases (Pr1, Pr2 and MPr) from the conidia of EPF during the swelling phase in type II water, which is the first stage of germination.

In bacterial and other opportunistic human pathogens such as *Candida albicans,* pH-mediated gene expression has been shown to be required for virulence and survival of pathogens in host cells (Mekalanos, 1992; De Bernardis et al., 1998). These results strongly suggest pH has a key role in homeostasis, secretion of metabolites and enzymes in fungi and higher eukaryotes.

Due to the importance of pH in the expression of pathogenicity genes/proteins this thesis project was initiated with the intent to delineate its role in growth and development of *B. bassiana* and *M. anisopliae.* Molecular biological and biochemical methods were used to provide insight into the pathogenesis for *B. bassiana* and *M. anisopliae*. Since, the dissolution of the cuticle occurs in the vicinity of conidia, very few investigators have attempted to study conidial enzymes. Hence, a holistic approach was adopted to study the enzymes and their regulation.

Regulation of the extracellular enzymes from germinating conidia and mycelia was studied under starvation, supply of C/ N sources and ambient pH. A novel observation regarding conidial pH adaptation was made, which suggests that the conidia are pre-adapted to the pathogenic mode and do not require any additional C/ N source to release those proteases in the surrounding environment at the initiation of the infection process. Evidence is provided that Pr1 alone, while being crucial, is not the most critical pathogenicity factor for the infection process of EPF. Indeed, results show that the multiple protease isozymes released by hydrated conidia of EPF are evolved to maximize the potential of cuticle degradation before host defenses become active and to avoid protease inhibitors present in the insect cuticle (Yoshida et al., 1990) and haemolymph (Tong and Kanost, 2005).

The results of this thesis provide a new insight into the biochemical aspects of fungal pathogenesis/disease development. It is hoped that this information will help improve the design and use of mycoinsecticides.

The thesis research was based on the following hypothesis and objectives:

1.1 Hypothesis

Ambient pH is a signal for the expression of proteins necessary for disease process and the developmental cycles of *B. bassiana* and *M. anisopliae.*

1.2 Aims and Objectives

The present thesis focused on the following objectives:

a) To determine if ambient pH is a major regulator of metabolite synthesis [organic acid(s) and/ or ammonia].

b) To determine if EPF mycelia can modify the pH of their surrounding environment by secreting ammonia and regulate the release and activity of protease.

c) To identify ambient pH-responsive expression for extracellular enzymes.

d) To determine the relative importance of C, N and/ or pH regulation for expression of extracellular enzymes.

e) To understand the role of pH in the growth and development cycle of *B. bassiana* and *M. anisopliae.*

f) In general, to understand mechanism(s) of biocontrol at the biochemical and molecular level with emphasis on pH and use this information to predict the biocontrol capabilities of this broad range mycoinsecticide.

1.3 Organization of thesis

The organization of this thesis was according to "chapterized or manuscript-style" option of the College of Graduate Studies and Research (CGSR).

1.4 Convention on nomenclature of proteases and chitinase

In this thesis the nomenclature used is consistent with the accepted rules of official Enzyme Convention, including use of trivial names and the published literature.

14.1 Proteases

The terms describing protease(s) (Pr), such as, subtilisin (Pr1-like) or trypsin (Pr2-like) are used for identity of proteases. In the theses abbreviations "Pr1" and "Pr2" were used in context of Pr1-like subtilisin and Pr2-like trypsin. Similarly, **m**etallo**pr**oteses abbreviated, as "MPr" is consistent with published literature (St. Leger, 1995). On two occasions in the thesis the terms used are names based on substrates, gelatin and casein (PG and PC) to represent "gelatinase and caseinase". While these terminologies are contrary to Enzyme Commission's recommendation, it is justifiable because literary continuity makes it necessary. These terms do not imply anything about the catalytic mechanism, but the substrates of these two proteases. In addition, several investigators (Kocholaty and Weil, 1938; North, 1982; Evans and Ridell, 1985; Hastings and Ellis, 1985; Titball and Munn, 1985; Shams et al., 1994; St. Leger et al., 1994; Jobin and Grenier, 2003) have used both terms to describe these two proteases on the basis that they degrade casein and gelatin as substrates. There is still the precedent of North (1982) who has described the classification of proteases on the basis of substrate specificity.

14.2 Chitinase

The terms chitinase or *N*-acetyl-D-glucosaminidase (NAGase) were used interchangeably in the thesis.

2.0 GENERAL LITERATURE REVIEW

2.1 Biochemical aspects of disease development

The EPF confront their hosts to create a unique niche both to support their growth and disease development. EPF confrontation starts with the attachment and germination of the conidia that subsequently facilitate their entry into the haemocoel after breaching the insect integument. Once inside the host, EPF may encounter and often evade host defenses that results in its multiplication and finally exit from the host. Apart from pathogenic life style EPF live as saprophytes or endophytes (Bing and Lewis, 1991), which may create another option for their survival in another unique niche without a host. The severity of invasion and degrees of disease development depend on the particular biochemical reactions. For successful EPF infection, several critical steps are required: (i) attachment, (ii) invasion, (iii) production of cuticle-degrading enzymes, (iv) *in vivo* growth, (v) utilization of host nutrients and establishment within the host, and (vi) production of toxins and virulence factors. The successful EPF infection is the cumulative effects of several biochemical reactions that may alter host food consumption, growth and behavioral responses (Fargues et al., 1994).

2.2 Physico-chemical aspects of disease development

The physico-chemical aspects of the disease development require favorable interaction of the pathogen with the host outer tissues. Thereafter, pathogenesis involves; 1) growth of the germ tube on the cuticle with the concomitant production of extracellular hydrolytic enzymes, 2) production of adhesive mucilagenous substance(s), 3) formation of appressoria on the cuticle surface, and 4) penetration of infectious peg into the epicuticle layer, then the procuticle and

finally the hemocoel and hemolymph. Inside the hemoceoel and hemolymph fungal growth and multiplication leads to the production of hyphal bodies. These bodies distribute themselves throughout the hemocoel, produce toxic metabolites and interact with and/ or evade insect defense mechanisms**.**

2.2.1 Spore adhesion/conidial attachment

EPF produce several distinct types of spores depending upon growth conditions of the fungi. In liquid media, *B. bassiana* produces thin-walled, single-celled hyphal bodies or blastospores. On solid media it produces conidia (aerial conidia), whereas under certain special nutritional conditions, submerged conidia are produced (Thomas et al., 1987; Hegedus et al., 1990a). All spores vary in morphology and biochemical properties that may reflect their specific role in fungal growth and development.

Conidia are resilient, environmentally stable and UV tolerant, which makes them propagule of choice for the mycoinsecticide manufacturing industry. In general, conidia are formulated in water or invert emulsions before being sprayed. After spraying they must adhere to, germinate and breach, the cuticle. The cuticle forms the primary defense barrier against the invading fungus. The insect integument is comprised of three layers, namely, the epicuticle, the procuticle and the epidermis. As a prerequisite to the infection process, EPF conidia (spores) should adhere to the cuticle.

2.2.2 Non-specific interactions

Several mechanisms could be ascribed to non- specific spore-cuticle interaction. James and O'Shea (1994) reported that the electrostatic and electrodynamic forces determine the non-

specific adhesion of microbial cells to surfaces. As general rule, electrostatic forces result from the simple ion-ion interaction of the charged group present on the spore and epicuticle. On the other hand, electrodynamic forces result from a combination of distance, such as van der Waals interactions, London dispersion forces and the hydrophobic effect.

The nature of cell surface charges and their role in nonspecific microbial interactions has been reviewed (James, 1991). Most microbial cell surfaces possess a negative electric charge (Jones and O'Shea, 1994; Jones et al., 1995) which is found at the cell liquid/solid interface. This electrostatic charge is due to ionization of cell wall components as well as a dispersal process that preferentially attracts negative ions to any particle in solution (Muller et al., 1986; James, 1991).

The biological surfaces have a net negative charge. The extent and level of this charge determine that how it would attract and help proliferation, survival and adhesion of microbes (Klotz et al., 1985). In addition Neihof and Nichols (1973), and James (1991) have documented that pH can influence microbial surface charge, which can affect adhesion to the substrate.

2.2.3 Specific interactions

Specific interactions are considered irreversible and occur through concerted action of physicochemical forces and chemical binding of lectins. Lectins or adhesion moieties are present within the microbial cell wall and bind to carbohydrate receptors in host cell walls. These processes have been well characterized for mycoparasites and other insect and human fungal pathogens (Cole and Hoch, 1991; Khachatourians, 1996; Manocha and Sahai, 1993; Inbar and Chet, 1994).

The most important mechanism is that of the hydrophobic interactions (Boucias et al., 1988). Hydrophobicity of EPF spores is, in part, responsible for their attachment to the host cuticle. As well, the outer hydrophobic layer is known to protect the spore from dehydration in the environment (Boucias et al., 1988). Conidia of *B. bassiana,* whether produced under aerial or submerged conditions, showed similar hydrophobic characteristics but blastospores were less hydrophobic suggesting that conidia can bind to the cuticle much more efficiently (Hegedus et al., 1992).

Once spores are hydrophobically attracted, other specific recognition systems, such as lectins, may strengthen the attachment by mucilagenous substances (Lecuona et al., 1991) and facilitate subsequent germination (Latge et al., 1988). The EPF surface includes carbohydrates and glycoproteins implicated in a number of roles, such as, chemotaxis, adhesion and infection (Pendland and Boucias 1986; Pendland et al., 1987). Lectins labelled with fluorescein isothiocyanate (FITC) show that carbohydrates present on the surface of spores vary (Pendland and Boucias 1986; Hegedus et al., 1992). Boucias and Pendland (1993) showed variability in the ability of galactose and mannose specific lectins to bind to cell wall surfaces during growth of *Paecilomyces farinosus*. Hegedus et al. (1992) indicated that the D-mannose/ D-glucose specific lectin, concanavalin A (Con A), and the ß-galactosamine specific lectin, *Ricinus communis* agglutinin (RCA), showed somewhat stronger reactions with aerial and submerged conidia. They further showed that blastospores produced in the absence of exogenous carbohydrate or in the presence of mannitol, maltose, sorbitol or glycerol, showed weak levels of fluorescence with the wheat germ agglutinin (WGA) FITC-conjugate. However, if glucose, fructose, *N*-acetyl-D-glucosamine or trehalose were added to the medium, the resultant blastospores did not fluoresce when treated with WGA. Regardless of the carbohydrate source in the growth medium,

blastospores treated with Con A always fluoresced. The exogenous carbohydrates in general do not influence the types of glycoprotein production on the cell surface (Hegedus et al., 1990b; 1992).

Jansson (1993) showed that the conidia of the endoparasitic nematophagous fungus *Drechmeria coniospora*, adhered to the sensory organs of many nematode species and in certain instances, the adhesion phase was followed by penetration through the nematode cuticle. The ability of the spores to make adhesive knobs in this fungus depended on fungal physiology and population density, but was independent of the host nematode. However, maturation of the adhesive knob formation was an autonomous process (van den Boogert et al., 1992). In contrast to the studies of Boucias et al. (1988) with *B. bassiana* spores, those of Jansson (1993) suggested that hydrophobicity and electrical charges were not involved in conidial adhesion, but specific lectins acted as adhesins to specific proteins excreted from the sensory organs of the nematodes.

2.3 Fungal spore swelling or pre-germination phase

The first step in the fungal spore germination and penetration must be spore hydration, activation and production of hydrolytic enzymes. Ironically, conidial hydration has not been thoroughly studied in fungi, especially EPF, which is a crucial phase of germination.

The swelling phase of conidia has been known since long time, but has not been taken seriously by fungal physiologists and applied mycologists beyond a so called “physical phenomenon”. Brodie and Neufeld (1942) and Cochrane (1958) defined isotropic growth or spore swelling as a phase of the germination process. Yarwood (1936, 1950) described swelling process of fungal spore as an inanimate uptake of water. Tomkins (1932) characterized swelling as a latent period of germination, the time interval before germ tube formation or emergence.

Hawker (1955) stated that if the other conditions are favorable, a ripe fungal spore may swell up before germination upon contact with moist substrate and water.

Similar observations were stated by Van Ettan (1983) and Schmit and Brody (1976) in their classical reviews. The investigators stated that conidia do not form germ tube in de-ionized water, but a pre-incubation period does reduce or affect the rate of germination in the glucose minimal medium. On the other hand, Griffin (1994) reported that during the soaking period there is also a growth of the fungal wall. Sussman and Halvorson (1966) have stated that swelling of fungal spores is sometimes, but not always, a characteristic of the germination process. They further reported that the spores of fungi imperfecti and ascospores of several genera do not swell perceptibly.

Metabolic activation of conidia soaked in de-ionized water (pre-germination phase) for *M. anisopliae* was described by Dillon and Charnley (1985, 1990). They reported that unless provided with exogenous nutrients, the conidia of EPF did not swell in distilled water. Swelling due to hydration is the first sign of initiation of germination of conidia of *B. bassiana* and *M. anisopliae* (Bidochka et al., 1987; Uribe, 2004). In contrast to EPF, isotropic growth or a swelling phase has been well documented in *A. nidulans* and *Neurospora crassa*. d' Enfert (1997) has reported the activation of several genes involved in ribosome assembly and amino acid biosynthesis during isotropic growth of *N. crassa*.

2.4 Fungal spore germination-growth

The ability of spores to germinate requires the presence of carbon as an energy source, attachment to a substratum or cuticle, and the production and utilization of precursors of cellular growth and macromolecular synthesis derived from the insect host. Spores of *B. bassiana*

following attachment to cuticle surfaces undergo hydration, germination and growth. For these processes, the production of extracellular cuticular degradative enzymes is a prerequiste. Many EPF grow directly *in vitro* on the cuticle with or without augmentation of some kind of growth medium (Hassan et al., 1989; Bidochka and Khachatourians 1992; El-Sayed et al., 1992, 1993a, 1993b). Cuticular hydrocarbons of *Ostrinia nubilalis, Melolontha melolontha* and *Pyrusta nubilals* were degraded by *B. bassiana* within a matter of days or hours respectively (Lecuona et al., 1991; Fargues et al., 1994). Degradation of monomethyl alkanes by *B. bassiana* germinating spores was a prerequisite to germ tube penetration in the host (Lecuona et al., 1991), whereas in the non-host insects, hyphal growth over the cuticle was observed. Finally, there is a relationship between the nitrogen source and lipid/carbohydrate reserves of *B. bassiana* and spore adhesion, germination and virulence (Lane et al., 1991a; 1991b).

2.5 Fungal extracellular enzymes

Extracellular enzymes are the hallmark of the infection process of EPF. They are responsible for the catabolism of complex substrates required for growth *in vitro* and *in vivo* (Khachatourians, 1991; Charnley and St. Leger, 1991). *In vivo,* the enzymes degrade and penetrate the cuticle as the primary host barrier and facilitate fungal penetration. It is generally accepted that insect-EPF interactions are mediated by both mechanical force and enzymatic processes and perhaps certain metabolic acids (Bidochka and Khachatourians 1991). Cuticular penetration by the germ tubes of EPF (Bidochka and Khachatourians 1991; 1992) showed a zone of clearing surrounding the penetration peg before invasion by hyphae (Khachatourians, 1991; Charnley and St. Leger, 1991). In EPF, extracellular proteases are produced in the vicinity of the degradation zone by the conidia (St. Leger et al., 1987), germ tube (Pendland and Boucias, 1992), appressoria

and growing mycelia (Khachatourians, 1991; St. Leger, 1995). Cuticle-degrading enzymes released by the infective structures are absorbed to target tissues and possessively degrade these substrates into utilizable monomeric units or precursors. In addition, extracellular proteases (Pr1 and Pr2), chitinases, lipases, DNAses and other cellulolytic enzymes, have important roles in the haemocoel, tissue degeneration and the immune system of insects.

2.5.1 Proteases

Insect cuticle is primarily composed of protein and chitin. Neville (1975) reported that the 75% of the insect cuticle contain protein-embedded chitin layers. Due this fact, proteases of EPF have received considerable attention from insect pathologists, geneticists, microbiologists and molecular biologists alike. The role of protease(s) from EPF has been well-documented (Khachatourians, 1991; St. Leger, 1995, Khachatourians, 1996, Charnley, 1997). The protein-degrading enzymes include hydrolytic enzymes that have catalytic features similar or identical to collagenases, chymoleastases, metalloenzymes chymotrypsin and trypsin-like enzymes. Most of these have been identified and characterized from a variety of entomopathogenic fungi, including *Aphelinus aelyrodis, B. bassiana, Entomophthora coronata, Erynia spp., Lagenidium giganteum, M. anisopliae, Nomureyi rileyi* and *Verticillium lecanii* (Khachatourians, 1991, 1996). In addition, proteases and peptidases are also required for the saprophytic growth of EPF.

2.5.2 Biochemical and molecular analysis of proteases

In the past two decades several investigators (St. Leger, 1995; Clarkson and Charnley, 1996; Khachatourians, 1996) have contributed enormously to the field of EPF biochemistry and molecular biology playing a vital role in the understanding of EPF pathogenesis and their

evolutionary significance. This information has also improved our understanding about the isozymes, which may have helped in the design of recombinant mycoinsecticides. In the subsequent section an in-depth review of proteases is provided.

The subtilisin/chymoelastase (Pr1) plays a crucial documented role in fungal pathogenesis. *M. anisopliae* produces at least two well-characterized subtilisins, namely Pr1a and Pr1b (St. Leger et al., 1994). Both Pr1a and Pr1b show similar physical and biochemical properties. In addition, they share 53% amino acid sequence homology. Characterization of a cDNA clone revealed that Pr1a is synthesized as a large precursor (43.3kDa), which contains an 18 amino acid signal peptide, an 18 amino acid pro-peptide and a mature protein (28.6 kDa) containing 281 amino acids. The sequence shows considerable similarity with other serine endoproteases, all having the serine, histidine and aspartate residues at the active site of the enzyme (St. Leger et al., 1992).

On the other hand, Pr2 occurs as two isoforms (pI 4.2, Mr 30 kDa and pI 4.9, Mr 27 kDa) with high activity against solubilized cuticular proteins. In comparison to Pr1, Pr2 has little or no activity against covalently bound cuticular protein due to poor adsorption properties (Bidochka and Khachatourians, 1993). The N-terminal sequences of the two isoforms were 56% homologous revealing that both were related, but the product of different genes. Analysis of cDNA clones has revealed that Pr2 isozymes are more homologous to *Streptomyces* (bacterial) trypsins and mammalian proteases than to other fungal enzymes (St. Leger, 1995).

Apart from *M. anisopliae,* Pr1 has been documented from *B. bassiana.* In this context, Joshi et al. (1995) have reported the cloning of an extracellular subtilisin-like serine endoprotease from *B. bassiana.* A cDNA clone of the protease was isolated from a mycelial culture of *B. bassiana* grown on cuticle/chitin cultures. The deduced amino acid sequence homology with *M. anisopliae* (Pr1) *and Tritiruchium album* (proteinase K) was 53.6% and 59.1%, respectively. The

data revealed that the *B. bassiana* Prl is synthesized as a large precursor (Mr 37.5 kDa) containing a signal peptide for translocation, a propeptide and the mature protein predicted to have a Mr, of 27 kDa. In a subsequent study Joshi et al. (1997) have identified an unsuspected differentially expressed subtilisin-like protease (Pr1B) encoding gene by using reverse transcription differential display PCR (RT-DD-PCR). The deduced amino acid sequence had 54% homology to the previously identified key virulence determinant (Pr1A). Electrophoretic karyotypic analysis revealed that *Pr1A* and *Pr1B* were located on different chromosomes. They found Pr1B being synthesized as a large precursor of 1158 nucleotides with a deduced molecular mass of 40 kDa. It contained a signal peptide, a propeptide and the mature protease (283 amino acids with a deduced molecular mass of 28.7 kDa). In contrast to Pr1 and other subtilitin-like proteases from *A. fumigatus* and mammalian insulinoma PC2 protease, Pr1B possesses substitution of Thr ^{220} by serine as well as Asn^{155} by glycine. Both of these amino acid substitutions have not been reported in any other known subtilisins. Screen and St. Leger (2000) have recently reported the existence of chymotrypsins (CHY1) from *M. anisopliae* by using expressed sequence tag (EST) analysis. They identified two trypsins of S1 family. CHY1 resembles actinomycete (bacterial) chymotrypsins of S2 family rather than other eukaryotic enzymes, which belong to family S1. The enzyme was synthesized as a precursor species (374 amino acids, pI/ MW: 5.07/ 38.2 kDa) containing a large N-terminal fragment (186 amino acids).

Gene structure and expression of a novel *B. bassiana* protease (Bassianin I) was described (Kim et al., 1999). The bassianin I gene was 1137 bp (379 amino acids) long and had 3 introns which are 69, 62 and 68 bp long. They also found a high homology between the deduced amino acid sequences of bassianin I, *M. anisopliae* (Pr1) and proteinase K. Finally, upon expression of the bassianin I in *Escherichia coli*, a clear proteolytic degraded zone on LB-skim milk was

reported, representing the activity expression of the cloned gene. The pI of the purified enzyme was 9.5 where as the optimum pH for activity was reported as 10.5.

Comparative analysis between *B. bassiana* Pr1 and bassianin I gene indicated 82.1 % homology, whereas protein identity (bassianin I) was 78.2% with the Pr1 of *B. bassiana*. An extracellular *B. bassiana* protease, designated BBP, has also been purified and characterized (Urtz and Rice, 2000). BBP was produced at late stages of growth from gelatin containing media. Inhibition by PMSF and chymostatin indicated that it is serine protease with chymotrypsin and elastase-like activity. Urtz and Rice (2000) identified another protease from the same strain, and identified it as Pr1 on the basis of pI, amino acid sequence identity and substrate specificity. In comparison to Pr1, BBP had a lower isoelectric point (pI 7.5) than Pr1 and was 0.5 kDa smaller. Both proteases had different substrate specificity Pr1 was more active against Suc-Ala-Ala-Pro-Phe-pNA, whereas BBP had high activity against MeOSuc-Ala-Ala-Pro-Met-pNA. However, both proteases had equal activity against cuticle, although the time of expression was different.

Fang et al. (2002) reported the cloning and characterization of cuticle-degrading protease (CDEP-1) from *B. bassiana.* The protease gene (CDEP-1) was isolated from a cDNA library. Further analysis indicated that it contained 1134 bp ORF, predicting a protein of 377 amino acids (Mr, 38.6 and pI, 8.3). Its amino acid sequence showed 57.9%, 83.3% and 54.7% identity to *M. anisopliae* Pr1, *B. bassiana* Pr1 and proteinase K, respectively. Southern analysis indicated that CDEP-1 was present as single copy in *B. bassiana.*

2.6 Molecular probing into the disease process

Due to advances in molecular biology in the last decades it is now possible to dissect the genetics of the EPF infection process. Several laboratories have contributed in this field, the result

of which shows that the insect-fungus interaction is more complicated than ever believed. The diversity of proteases and the complex response of fungus after interacting with insect cuticle partially answered why there is more failure than success? On the other hand, such information would help to solve the critical issues regarding successful design of mycoinsecticides.

Wang et al. (2002) isolated and characterized three spontaneous single spore mutants of *M. anisopliae* (V275). They identified three genetically-identical spontaneous *Pr1A* and *Pr1B* mutant strains using the nested PCR approach. Sequencing comparison of the 28S rDNA (domain 9-11) confirmed that the isolates were *M. anisopliae* yet each isolate had its own morphological and genetic differences (from the parent strain). Random amplified polymorphic DNA (RAPD) data revealed that the overall similarity between wild type and mutant stains was less than 70%. Enzyme assays proved that the mutants had low levels of Pr1A and elastase activity. They reported the reduced lethal activity of the mutants (20%) on *Tenebrio molitor*. Conversely, there was no difference between the lethal activities between the wild-type parent and mutants on the larvae of *Galleria mellonella.*

Freimoser et al. (2003) studied the insect pathogen, *Conidiobolus coronatus* (Zygomycota) during the growth on insect cuticle. Expressed sequence tags (ESTs) cDNA clones were sequenced to analyze gene expression. They found approximately 60% of the genes that encoded chitinases and multiple subtilisins, trypsin, metalloprotease and aspartyl protease activities with the potential to degrade host tissues and disable anti-microbial peptides. However, Freimoser and coworkers found fewer (in comparison with *M. anisopliae*) hydrolases, antimicrobial agents and secondary metabolites. Conversely, *C. coronatus* showed much higher proportion of ESTs encoding ribosomal proteins whereas they could not find ESTs with any putative role in antibiotic synthesis. These results are consistent with *C. coronatus* having adapted a modification of the saprophytic

ruderal-selected strategy using rapid growth to overwhelm the host and exploit the cadaver before competitors overrun it.

Bagga et al. (2004) classified subtilisins by using ESTs. They reported 11 subtilisins during growth on insect cuticle from *M. anisopliae sf. anisopliae* (strain 2575), a broad host range pathogen. Polymerase chain reaction amplified 10 orthologs from *M. anisopliae sf. anisopliae* (strain 820) and 7 from the locust specialist *M. anisopliae sf. acridum* (strain 324). Bagga and coworkers grouped *M. anisopliae* subtilisins into four major groups based on sequence similarity and exon-intron structure. Class I (bacterial) subtilisin (Pr1C), and three clusters of proteinase K-like class II subtilisins: extracellular subfamily 1 (Pr1A, Pr1B, Pr1G, Pr1I and Pr1K), extracellular subfamily 2 (Pr1D, Pr1E, Pr1F and Pr1J) and an endocellular subtilisin (Pr1H). The roles of these subtilisins have yet to be elucidated in the infection process of the EPF.

Freimoser et al. (2005) have elucidated the molecular basis of the infection process of *M. anisopliae* infecting cuticles from different insect hosts. The gene expression responses to diverse insect cuticles were measured by using cDNA microarrays constructed from an ESTs clone collection of 837 genes. They found 273 genes upregulated during growth on *Manduca sexta* cuticle, some of which had unknown functions. However, the upregulated genes with identified functions included cuticle-degradation (e.g., proteases), amino acid/peptide transport and transcription regulation. Conversely, there were 287 down-regulated genes, including a large set of ribosomal proteins. Freimoser and coworkers also identified some overlapping gene responses from *Manduca* cuticle with other studied cuticles. Interestingly, they reported a unique expression pattern in response to cuticles from *Lymantria dispar, Blaberus giganteus* and *Popilla japonica*. This feature of the pathogen demarcates that there is a precise and specialized way that *M. anisopliae* can interact and adapt to differing environmental conditions. Sequence comparison data

from *Trichoderma reesei* and *Saccharomyces cerevisiae* also highlighted the differences in the regulation of glycolysis-related genes and citric acid cycle/oxidative phosphorylation functions.

Subtilisin-like (Pr1) protease is considered to be a key virulence determinant of EPF, once believed to be upregulated during appressorium formation (St. Leger, 1995). Small and Bidochka (2005) reported the upregulation of the Pr1 gene during conidiogenesis. They sequence identified seven conidiation associated genes (*cag*) in *M. anisopliae* by using subtractive hybridization. Out of seven identified genes they found *cag*7 vital for cuticle evasion that encodes an extracellular subtilisin-like proteinase (Pr1). Reverse-transcription polymerase chain reaction (RT-PCR) analysis confirmed that *cag* cDNA are expressed during conidiation under nutrient starved conditions. RT-PCR analysis was also performed for Pr1 during infection of *G. mellonella*. Data revealed the up-regulation of subtilisin like-Pr1 upon infection of insect when the mycelia appear on the surface of cuticle and produce conidia on the surface of the cadaver.

2.7 Evolutionary significance of proteases in EPF pathogenesis

The evolutionary genetics of EPF and phytopathogens are intriguing. As proteases are best studied, several investigators have attempted to elucidate their evolutionary role. However, their involvement during *in vivo* infection is far from clear. All is known about them is a preference of certain amino acids at, or in, the proximity of catalytic site. Nonetheless, there are efforts from many investigators which have improved our understanding of EPF evolution either as a pathogen or saprophyte.

St. Leger et al. (1997) showed adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and EPF to the requirements of their ecological niches. They studied fungal isolates which included saprophytic, opportunistic human pathogen, an opportunistic insect

pathogen, plant pathogens, a mushroom pathogen, and entomopathogens. The pathogens showed enzymatic adaptation to the polymers present in the integument of their particular hosts. Thus, the plant pathogens produced high levels of enzymes capable of degrading pectic polysaccharides, cellulose and xylan, as well as cutinase substrate, but secreted little or no chitinase and showed no proteolytic activity against elastin and mucin. The entomopathogens and *V. fungicola* degraded a broad spectrum of proteins (including elastin and mucin). In contrast, cellulose, cutinase and chitinase were produced by *V. lecanii* and *V. fungicola*. In addition, low levels of polysaccharidases were produced by *B. bassiana*. These results indicate adaptation of fungi to the requirements of their ecological niches.

Bidochka et al. (1999) found that members of the genus *Verticillium*, which are pathogenic towards insects and plants, have some commonality in extracellular proteases and carbohydrases. They examined phylogenetic relationships of 18 isolates in the genus *Verticillium*. These isolates represented 13 species of diverse eco-nutritional groups; pathogens of insects, plants, mushrooms, nematodes and spiders, and saprobes. The phylogenetic analyses were both based on DNA sequences and abilities to infect larvae of *G. mellonella* and to cause necrosis in alfalfa and for their proteolytic, chitinolytic and pectinolytic activities. They suggested that the ability to infect insects might have evolved independently many times. The insect and mushroom pathogens, and several nematode pathogens were distinguishable from the plant pathogens in their ability to produce chitinases.

Segers et al. (1999) described the distribution and variation between the subtilisins of entomogenous and phytopathogenic fungi. They reported multiple isoforms with unique N-terminal sequences in single strains that might represent the presence of subtilisin gene families. Restriction fragment length polymorphisms (RFLPs) (*Pr1* probe from *M. anisopliae*) confirmed

qualitative differences between subtilisin-like genes within and between species. Neither subtilisin nor homologous genes from plant pathogenic *Verticillium* spp. were obtained. Conversely, Pr1 like enzymes and the corresponding genes from weakly plant pathogenic species or saprotrophs were observed in this study.

Bidochka et al. (1999) have evaluated the insect and plant pathogenic species of the genus *Verticillium* for the production and regulation of hydrolytic enzymes. Bidochka and coworkers have used zymopheretograms and API-ZYM strip analysis during this evaluation. Phytopathogenic and entomopathogenic fungi species of *Verticillium* were found well adapted to the integuments of their respective host. The facultative plant pathogens including *V. albo-atrum* and *V. dahliae* showed greater production of cellulase and xylanase than the facultative insect pathogen, *V. lecanii.* Conversely, *V. lecanii* produced extracellular subtilisins (Pr1) upon induction in insect cuticle supplemented medium. No subtilisins were detected in the plant cell wall supplemented medium while growth of *V. lecanii.* The opportunistic plants pathogens such as *V. fungicola* and *V. coccosporum* and saprophytic species like *V. rexianum* were less specific in production and regulation of the proteases, cellulases and xylanases.

Bidochka and Melzer (2000) reported on genetic polymorphisms in three subtilisin-like protease isoforms (Pr1A, Pr1B, and Pr1C) from *Metarhizium* strains by restriction fragment length polymorphisms (RFLP) studies in several isolates of *M. anisopliae*. RFLP variation was not observed in any of the Pr1 genes from isolates within the same genetically related group. Between genetically related groups and between isolates from disparate geographical areas, the greatest variation in RFLP patterns was observed for Pr1A. When variation does occur at Pr1B and Pr1C, it was generally observed at an EcoRI site. On the other hand, *M. anisopliae* var. *majus* strain 473 and a *M. flavoviride* isolate were most dissimilar in RFLP patterns at all Pr1 genes when compared

to the *M. anisopliae* strains. Bidochka and Melzer (2000) suggest that Pr1 genes represent a gene family of subtilisin-like proteases and that the Pr1A gene encodes for the ancestral subtilisin-like protease which has subsequently duplicated and rearranged within the genome.

2.8 Chitinases

Chitinases perform critical functions both in EPF and their host insects by their involvement in the growth and degradation of the fungal cell wall and insect cuticle. This is because chitin is a major component of both. Khachatourians (1991, 1996) reviewed the literature on chitinases, showing the diversity of chitinolytic activity found in many EPF. The classification of chitinases indicates the presence of both endo-and exo-chitinases. These refer to cleavage of chitin polymer (*N*-acetyl-D-glucosamine polymer, $NAGA_n$) into smaller units or degradation and release of monomers of NAGA, in a processive manner. Chitinases can be interacellular or extracellular, which are released into cellular environments for catabolic purposes.

Extracellular chitinases have been suggested to be virulence factors in fungal entomopathogenicity (Khachatourians, 1991, 1996; Charnley, 1997). A great deal more remains to be learned about the complexity of involvement between chitinases and their host substrates.

2.8.1 Biochemical and molecular analysis of major chitinases

The role of chitinases in cuticle degradation is secondary. Therefore, they are not well pursued as their counterpart, namely proteases. St. Leger (1995) reported the presence of *N*-acetyl-D-glucosaminidases and endochitinases (chitinolytic enzymes) from the *M. anisopliae*, *M. flavoviride*, and *B. bassiana* during growth in media supplemented with insect cuticle. However, *M. flavoviride* also secreted the 1,4-β-chitobiosidases into the cuticle media. They further reported

that the chitinase from *M. anisopliae* was acidic (pI 4.8). It resolved into two major bands (43.5 and 45 kDa) on SDS PAGE. The identified N-terminal sequences of both bands were similar to an endochitinase from the mycoparasite *Trichoderma harzianum*.

Valadares-Inglis and Peberdy (1997) have studied the location of chitinolytic enzymes in enzymatically produced protoplasts and whole cells (mycelia) of *M. anisopliae***.** No significant induction of chitinolytic enzymes was observed by the soluble carboxymethylated (alkaline) chitin from mycelia. However, protoplasts induced these enzymes significantly. At day 1, protoplasts secreted 76% of *N*-acetyl-D-glucosaminidase, whereas less than 10% of chitinase was secreted into the medium. At day 2, *N*-acetyl-D-glucosaminidase secretion dropped to 34% of total activity, while the proportion of chitinase activity secreted by protoplasts continued to rise. At day 2, the majority of chitinolytic enzyme activity had become cell-bound in both protoplast preparations and whole cells. In protoplasts, this activity was mainly located in the membrane developing wall fraction.

An endochitinase (Bbchit1) was recently purified to homogeneity from colloidal chitin containing liquid cultures of *B. bassiana* (Fang et al., 2005). Bbchit1 had a molecular mass of about 33 kDa (pI, 5.4) as determined by SDS-PAGE. The chitinase gene, *Bbchit*1, and its upstream regulatory sequence were cloned based on N-terminal amino acid sequence. *Bbchit1* contained no introns and is present as a single copy in *B. bassiana* genome. The regulatory sequence of *Bbchit1* contains putative CreA/ Crel (analogous to *creA* of *Aspergillus*) binding elements, which regulate carbon metabolism in fungal kingdom (carbon catabolite repression or glucose repression). The amino acid sequence of Bbchit1 is significantly similar to endochitinase of *Streptomyces avermitilis, S. coelicolor* and *T. harzianum* (Chit36Y). However, it revealed very low homology to other characterized chitinase genes isolated todate from other EPF. This feature reflects that

Bbchit1 is a novel chitinase. Fang and co-worker constructed a *B. bassiana* transformant (*gpd-Bbchit1),* which can overproduce *Bbchit1*. Insect bioassays with transformed strain revealed enhanced virulence for aphids as indicated by 50% LC_{50} and 50% LT_{50} of the transformants compared to the values for the wild-type strain.

Nahar et al. (2004) found that the extracellular constitutive chitin deacetylase produced by *M. anisopliae* converted chitin, a β-1,4-linked *N*-acetyl-D-glucosamine polymer, into its deacetylated form chitosan, a glucosamine polymer. When grown in a yeast extract-peptone medium two chitin-metabolizing enzymes, viz. chitin deacetylase (CDA) and chitosanase were produced. Chitinase activity was induced in chitin-containing medium. Staining of 7.5% native polyacrylamide gels at pH 8.9 revealed CDA activity in three bands. SDS-PAGE showed that the apparent molecular masses of the three isoforms were 70, 37, and 26 kDa, respectively. Solubilized melanin (10 µg/ ml) inhibited chitinase activity, whereas CDA was unaffected. Following germination of *M. anisopliae* conidia on isolated *Helicoverpa armigera* cuticle revealed the presence of chitosan by staining with 3-methyl-2-benzothiazoline hydrazone. Hydrolysis of chitin with constitutively produced enzymes of *M. anisopliae* suggested that CDA along with chitosanase contributed significantly to chitin hydrolysis.

Kang et al. (1998, 1999) reported the isolation and characterization of a chitinase cDNA from *M. anisopliae* grown in a medium containing chitin as the sole carbon source. This chitinase had a 70 kDa molecular mass. However, the deduced amino acid sequence indicated a 58 kDa protein. These molecular masses were different from values of 33 kDa for an endochitinase and 110 kDa for an exochitinase (*N*-acetyl-D-glucosaminidase) from *M. anisopliae* published previously. The chitinase was purified by DEAE-Sephacel, CM-Sepharose CL-6B ion-exchange chromatography, and gel filtration with Superose 12HR. The molecular mass of the enzyme

determined by SDS-polyacrylamide gel electrophoresis was approximately 60 kDa and the optimum pH of the enzyme was 5.0.

Screen et al. (2001) reported the cloning of chitinase gene (*Chit*1) from the *M. anisopliae* sf. *acridum* ARSEF strain 324 in *M. anisopliae* sf. *anisopliae* ARSEF strain 2575 *(Chit*1). They used the promoter of *Aspergillus* (*gpd)* for constitutive expression. In non-transformed strains, both EPF secrete 44 kDa basic and acidic isoforms of endochitinase upon induction. However, in transgenic strain 2575-Chit the expression of CHIT1 was in a non-inducing medium. Screen and coworkers were unable to detect the native, acidic form of chitinase on zymogram gels (isoelectric focusing) from either 2575 or 2575-Chit strain in non-inducing medium. Conversely, the 2575-Chit strain produced chitinase earlier than the wild type strain in chitin-containing medium. Screen and coworkers proposed that the formation of soluble chitin inducer after hydrolysis of chitin by CHIT1 was necessary for its production. During bioassays, the chitinase overproducers did not revealed any altered virulence to *M. sext*a in comparison to the wild-type fungus, suggesting that wild-type levels of chitinase are not limiting for cuticle penetration.

Baratto et al. (2003) reported the expression and characterization of the 42 kDa chitinase of the biocontrol fungus *M. anisopliae* in *E. coli.* They characterized *Chit1* gene, thereby expressing the active form of the protein (CHIT 42) in *E. coli* using a T-7- based promoter expression vector. The recombinant protein was reported to be active against glycol chitin and synthetic *N*-acetyl-D-glucosamine (GlcNAc) dimer and tetramer substrates.

2.9 Regulation of protease and chitinase production

Fungi in general have a unique ability to identify and utilize the nutrients on the basis of their nutritional components and ease of synthesis (Arst, 1995; Marzluf, 2001). Three genes, *areA*,

creA, and *pacC* encode transcriptional factors that in fungi regulate carbon and nitrogen metabolism. The signal transduction pathway helps identify for the fungus as to what is present as a food source. The global acting elements provide signal(s) to pathway specific elements to permit the biosynthesis from complex metabolic sources. Bidochka and Khachatourians (1988a) presented the first comprehensive study of the *in vitro* regulation of extracellular protease synthesis in *B. bassiana.* The presence and types of extracellular carbon-and nitrogen-containing substrates play roles in cellular metabolism. The repression of extracellular enzyme synthesis was due to the growth substrates (or product such as GlcNAc) and mycelial protein molar ratios (Khachatourians, 1996).

In stark contrast, the production of proteases is regulated under *creA* and *areA* genes, which regulate carbon catabolite repression and nitrogen metabolite repression in fungi. Homologs of both genes that encode zinc finger transcription factors have been identified in *M. anisopliae* (Screen et al., 1997, 1998). There is convincing evidence of pH regulation by the *pacC* (encoding the PacC zinc finger transcription factor) and its involvement in regulating such enzymes from other fungi including *A. nidulans, A. niger, A. fumigatus Penicillium* spp. and *Fusarium* spp.

Furthermore, evidence of the involvement of PacC in the regulation of virulence in *A. nidulans* has been demonstrated recently (Bignell et al., 2005). Using mutational analysis it was shown that blocking of the pH signaling pathway or altered PacC proteolytic processing would lead to attenuated virulence. It also resulted in limited growth of the fungi and reduction of inflammatory cell infiltration. Ironically, the role of *pacC* has not been fully pursued in any of the EPF studied to date. St. Leger et al. (1998) have demonstrated for the first time that the ambient pH is a major determinant in the expression of the cuticle-degrading enzymes and hydrophobin by the EPF fungus, *M. anisopliae*. Northern hybridization analysis and/or enzyme assays of Pr1, Pr2,

metalloprotease, aspartyl protease, aminopeptidase and chitinase revealed different expression pattern due to ambient pH. Moreover, they found hydrophobins were equally expressed at pH 5 and 8 but not at pH 3. In conclusion, they stated the alkalinity of the infected cuticle represented a physiological signal that triggers the production of virulence factors. In a subsequent study evidence of pH regulation in the gene expression of *M. anisopliae* was further explained (St. Leger et al., 1999). They reported that the *M. anisopliae, N. crassa* and *A. fumigatus* regulated the ambient pH thereby secreting ammonia and then released subtilisin-like proteases, whose activities were observed at basic pH only. They also reported the secretion of TCA cycle intermediates such as citric, oxalic and succinic acid for the modulation of ambient pH.

Many EPF secrete subtilisin proteases to acquire nutrients and breach host barriers. The regulation of protease synthesis (Pr1-like) was first reported by Bidochka and Khachatourians (1988a) and regulatory genetic details were reported in a study by Screen et al. (1997), indicating that the synthesis of the Pr1 gene product of *M. anisopliae* is subject to both carbon and nitrogen repression. The Pr1 promoter region was sequenced revealing the presence of putative CREA- and AREA-binding sites. Using a PCR-based strategy, the *M. anisopliae crr*1 gene was identified; it encodes a putative C2H2-type DNA-binding protein with significant sequence similarity to *A. nidulans* CREA. Complementation experiments with an *A. nidulans* strain carrying creA204 mutation demonstrated that CRR1 can partially substitute for CREA function. In addition, Screen et al. (1998) identified an *M. anisopliae nrr*1 (nitrogen response regulator 1) gene using a PCR-based strategy. This regulatory element encoded a putative DNA-binding protein with a single zinc finger motif defined by the C-X2-C-X17-C-X2-C sequence with a significant sequence similarity to *N. crassa* NIT2.

2.10 Lipases and Lipoxygenases

The epicuticle of the insect integument contains lipoproteins, fats and waxy layers. Without action of lipases and lipoxygenases, some of these materials would be barriers to EPF for entry as some have anti-fungal activity and would be of no use as substrates to EPF (Bidochka and Khachatourians, 1992). The growth of some EPF can be inhibited with various short chain saturated fatty acids (Khachatourians, 1991). Lipoxygenases and various cyclooxygenases of EPF have roles in spore germination, early aspects of infection, induction and maturation of the sexual stage (Kerwin and Washino, 1986). Lord et al. (2002) showed a role for the lipoxygenase pathway through eicosanoid-mediated cellular immune response to *B. bassiana*. Indeed, Kerwin (1984) showed that the regulation of *Erynia variabilis* conidia on adult and puparia of the lesser house fly *Fannia canicularis* was dependent on the presence of sufficient amounts of oleic acid to induce germination. In spite of the above, the detailed biochemical role for the stimulatory or inhibitory aspects of lipids and respective metabolic enzymes, whether *in vitro* or *in vivo,* on the host remains to be established.

Napolitano and Juarez (1997) reported the degradation of epicuticular hydrocarbons of *Triatoma infestans* by EPF. James et al. (2003) also showed that cuticular lipids and silverleaf whitefly (*B. argentifolii*) affected conidial germination of *B. bassiana* and *P. fumosoroseus*. They found differential susceptibility of different instars of whiteflies to both fungi. Cuticular lipids were found to have toxic or inhibitory effects *in vitro* on conidia of *B. bassiana* and *P. fumosoroseus* when the spores were germinated on nutrient agar in the presence of the lipids. On the other hand, in the absence of added nutrients, *P. fumosoroseus* conidial germination increased in the presence of the lipids. To test if the inhibitory effects of the lipids were due solely to hydrophobicity of conidia Napolitano and coworkers tested the effects of synthetic long-chain wax

esters. The synthetic wax esters inhibited germination of *P. fumosoroseus* to a degree that was similar to the effect of the cuticular lipid extracts, but the synthetic lipids did not have a significant effect on *B. bassiana*. Thus, the thick coating of long-chain wax esters produced by whitefly nymphs affected spore germination, but whether they play a significant role in defense against disease remains to be established. Lord and Howard (2004) proposed the cuticular fatty amides of *Liposcelis bostrychophila* to have a role in preventing adhesion of dry-conidial preparations of *B. bassiana, P. fumosoroseus, A. parasiticus* or *M. anisopliae,* and hence producing low (16%) mortality. Of the cuticular lipids of this insect, saturated C: 14 and C: 16 fatty acids did not reduce the germination rates *of B. bassiana* or *M. anisopliae* conidia, and therefore did not contribute to tolerance. Saturated C: 6 to C: 12 fatty acids that have not been identified in *L. bostrychophila* cuticular extracts significantly reduced germination, but the reduction was mitigated by the presence of stearamide. Cis-6-hexadecenal did not affect germination rates. Mycelial growth of either fungal species did not occur in the presence of caprylic acid, was reduced by the presence of lauric acid, or was not significantly affected by palmitic acid. *Liposcelis bostrychophila* is the only insect for which fatty acid amides have been identified as cuticular components. Stearamide, its major fatty amide, did not reduce germination of *B. bassiana* or *M. anisopliae* conidia or growth of their mycelia. Adhesion of conidia to stearamide preparations did not differ significantly from adhesion to the cuticle of *L. bostrychophila.* Pretreatment of a beetle, *Oryzaephilus surinamensis* known to be fungus-susceptible with stearamide significantly decreased the adhesion of *B. bassiana or M. anisopliae* conidia to their cuticles. This evidence indicates that cuticular fatty amides may contribute to insect tolerance for entomopathogenic fungi by decreasing hydrophobicity and static charge, thereby reducing conidial adhesion.

2.11 Non-peptide Toxins and Pigments

Oxalic acid is an important virulence factor since EPF penetrating through an external barrier are assisted by it (Bidochka and Khachatourians, 1991). The EPF, *B. brongniartii* produces crystals of ammonyl oxalate in liquid cultures. The oxalic acid secreted by *B. bassiana* during hyphal penetration is thought to aid the solubilization of cuticular protein (Bidochka and Khachatourians, 1991). However, hyperproducing isogenic mutants isolated to date do not have higher virulence (Bidochka and Khachatourians, 1991). The organic acids kojic and oxalic acid have been shown to reduce over all biological fitness when fed at 1500 ppm to the *Latrodectus hesperus* (Alverson, 2003). Oxalic acid, when applied directly to the lone star tick *Amblyomma americanum* at 50 mM, induces > 60% mortality after two weeks (Kirkland et al., 2005). Kirkland et al. (2005) confirmed the role of oxalic acid in insect pathogenesis. They found cell-free culture supernatants of *B. bassiana* induced mortality in several tick species, including adult *A. americanum* (L.), *A. maculatum* Koch, and *Ixodes scapularis* Say.

Asaff et al. (2005) isolated dipicolinic acid from *P. fumosoroseus* and it showed to have insecticidal toxicity to whiteflies *B. tabaci* and *B. argentifolii* nymphs by topical applications. The most abundant metabolite was purified by HPLC and identified by mass spectrometry and NMR as dipicolinic acid. Dipicolinic acid was detected after 24 h when the fungus started growing; and production was directly correlated with fungal growth. In general, these nonpeptide toxins are produced in rich medium *in vitro*, and to a lesser extent *in vivo* (Strasser et al., 2000a, b).

2.12 Proteomics

The term “proteome” was first coined in 1994 by Australian post doctoral fellow, Marc Wilkins. Proteome refers to the total set of proteins expressed in a given cell at a given time, the

study of which is referred as “Proteomics”. This emerging field promises to bridge the gap between genome sequence and cell behavior. In reality, it aims to study the dynamic protein products of the genome, interactions and functions rather then focusing on static DNA blueprint of a cell.

The rapid resurgence of proteomics in the modern biotechnology era is largely being driven by the development, integration and automation of large-scale analytical tools, such as 2-DE and mass spectrometry. The field is equally powered by the bioinformatics tools that have improved the characterization and reliability of tandem mass spectrometry.

Proteins are diverse molecules that can be studied in various different contexts such as: (i) sequence, (ii) structure, (iii) interactions, (iv) expression, (v) localization and (vi) modification. Hence, proteomics can be divided into different major sub-disciplines, which help to understand the role of proteins in the biological system.

Structural proteomics is often termed as “structural genomics”. Sequence and structural proteomics is concerned with high-throughput determination of protein structures. The field relies on the wealth of genomic and protein sequences present in the sequence databases like GenBank, EMBL (The European Molecular Biology Laboratory Nucleotide Sequence Database, DDBJ (DNA Data Base of Japan) and Swiss-Prot (Swiss Institute of Bioinformatics) and TrEMBL (Translated European Molecular Biology Laboratory Nucleotide Sequence Database). The study of three-dimensional protein structure is underpinned by technologies such as X-ray crystallography and nuclear magnetic resonance (NMR) and bioinformatics softwares.

Expression proteomics is the analysis of protein abundance and involves the separation of complex mixtures of proteins by 2-DE combined with tandem mass spectrometry. On the other hand, interaction proteomics considers genetic and physical interactions among proteins as well as interactions between proteins and nucleic acids or small molecules. In functional proteomics protein

functions are directly tested, but on a large scale. It is a relatively new development in the field of proteomics.

2.13 Definition of Isozyme

Before discussing the various separation technologies, it is worth mentioning what is mean by the term “isozymes”. In 1959, the term “isozymes” or “isoenzymes” was first introduced by Markert and Moller to designate different molecular forms of the same enzyme occurring with in a single individual or in different numbers of species (Markert and Moller, 1959). Several years of work in the laboratories of scientists around the world have revealed that there are three main causes of the multiple molecular forms of enzymes:

(a) The presence of the one gene locus coding for an enzyme.

(b) The presence of more then one allele at a single gene locus coding for the enzymes.

(c) Post-translational modifications of the formed enzymatic polypeptides resulting in the formation of non-genetic or so called secondary, isozymes (Kenney, 1974).

According to the recommendations of the Commission on the Biological Nomenclature of IUPAC-IUB, isozymes are defined as genetically-determined multiple molecular forms of an enzyme (Anonymous, 1977). Hence, the term isozyme, or isoform, is used to represent multiple molecular forms derived from different genetic loci. In contrast, the term “allozymes” is used to designate multiple molecular forms deriving from different alleles of the same genetic locus. Some isozymologists have used the term “allelic isozymes” for the later case. In the subsequent section, some of the key functional proteomic methods will be presented that can separate isozymes.

2.14 Two-dimensional electrophoresis (2-DE): An historical perspective

The key technology that is considered as the back bone of proteomics is 2-DE, and was pioneered almost three decades ago by O'Farrell (1975) and Klose (1975). It separates proteins on the basis of pI and molecular weight in two discrete steps, usually referred to as dimensions:

(a) During the first step, isoelectric focusing (IEF) is performed under high voltage, separating proteins according to their isoelectric point (pI).

(b) In the second step, standard sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weights.

In the original technique, the first-dimension separation was performed by casting polyacrylamide gels along with carrier ampholytes in narrow tubes. During the pre-run, carrier ampholytes would generate a pH gradient under the influence of an electric current. Although 2-DE is the most effective means of resolving complex protein mixtures, it was not widely used for many years after it was first introduced in the early 1970s. This was due to three main reasons:

(a) The relative technical difficulty of performing the IEF step.

(b) Transferring the focused proteins into the SDS-PAGE gel.

(c) The inability to characterize minute quantity of proteins.

In tube gels, the major problem was to obtain reproducibility during the pH gradients formation while using IEF. Thus, 2-DE was technically challenging to perform and even more difficult to perform reproducibly. However, after the advent of immobilized pH gradients,

reproducibility between different labs became possible. Essentially, an IPG strip is a combined mix of polyacrylamide gel on a plastic support with ampholytes (polycarboxylic acid) immobilized on supports to reproducibly create stable pH gradients. Due to this invention it is now possible to have stable pH gradients from 3-11 (Gorg et al., 1988).

Indeed, 2-DE is the only available high throughput screening tool in proteomics to differentially study protein maps. The 2-DE are combined with mass spectrometry to study the function of the protein, which is based on the sequence comparison of the peptide fragments deposited in NCBI, Swiss-Prot etc. (Gorg et al., 1988).

2.15 A bottle neck for 2-DE: Sample preparation

Over the past decade, there have been several improvements in the design and automation of 2-DE instrumentation and software. The technique is still demanding technically and requires careful sample preparation and is dependant on basic protein chemistry/biochemistry principles known over a half century or more.

A critical problem often encountered while performing 2-DE is sample preparation. Solubilization of proteins from the biological sample is crucial in order to obtain a complete picture of the proteome or secretome. Immobilized pH gradients (IPGs) are based on the principle that the pH gradient is generated by a limited number (6-8) of well defined chemicals (Immobilines) which are copolymerized with the acrylamide matrix. This would help eliminate cathodic drift, enhance reproducibility, pattern matching and allow laboratory comparison with ease (Bjellqvist et al., 1993). In addition, it also helps protein solubilization, scavenge cyanate ions and precipitate nucleic acid during centrifugation (Righetti, 1990). They are also useful in inhibiting interactions between sample proteins and immobilines of the IPG strips (Gorg and Weiss, 2000).

The most frequent bottle neck for the efficient 2-DE does not lie in the initial protein solubilization, but in maintaining the solubility during the IEF step. There is no universal protocol for complete solubilization. However, the key is to remove any interfering compounds that may be present in the sample. Rabilloud et al. (2007) pointed out that blocking of protease action and disruption of in-frequent interactions (severe ionic bonds) is of significance. Breaking of such interactions would depend on the type of sample used, the protein of interest and amount to be separated. Hence, the optimal solubilization protocol can vary greatly from one sample to another (Rabilloud et al., 1997). The solubilization of protein also depends on breaking of various complex interaction/forces that exist between the protein moieties and other molecules (lipids, polysaccharides and nucleic acids) present in the sample.

Rabilloud and Chevallet (2000) reported the constraint for a good solubilization medium for 2-DE as breaking of: (i) hydrogen bonds, (ii) ionic bonds, (iii) hydrophobic interactions and (iv) disulphide bridges under conditions compatible with IEF. Hence, the use of the correct detergent and chaotropes is of vital significance; the role of detergent in IEF is to coat the proteins and make them more hydrophilic, thereby preventing isoelectric precipitation. Improvement in the analysis of hydrophobic proteins by zwitterionic detergents (4% CHAPS and A2% SB-14) in combination with chaotropes and reducing agents has been documented (Chevallet et al., 1998). However, these urea-reducing-detergent mixtures are not effective for more hydrophilic proteins, which are lost during IEF because of poor binding.

In contrast, there are certain situations (i.e., functional proteomics) during which breaking of all the interactions of protein molecule would render protein non-functional. Hence, the functional state of the protein cannot be analyzed by using protocols for the complete disruption of protein molecule by using chaotrope-detergent-reducing agent mixtures. Such cases require

careful selection of one or more of the above to analyze the functional form/state of protein, which again vary from sample to sample. The discussion of such cases is provided in below.

2.16 Zymography

Detection of enzymes on electrophoretic gels means visualization of gel areas occupied by specific enzyme molecule after electrophoretic separation and subsequent digestion of the substrate. Hence, zymography is a very powerful biochemical tool to identify enzymes after 1-DE, IFE or after 2-DE.

Due to the broad implications of proteases in prokaryotic and eukaryotic biological processes (cancer, metastasis, development, pathogenesis, signal transduction and growth etc), zymography of proteases have received considerable attention. Zymograms can detect isoforms of enzymes after electrophoretic separation in one-dimensional (1-DE) gels (Kleiner and Stetler-Stevenson, 1994; St. Leger et al., 1996a). Furthermore, zymography after two-dimensional electrophoresis (2-DE) can resovle complex biological samples. It theoretically separates over 10000 proteins in comparison to HPLC that can separate 100 proteins (Klose, 1999).

Despite the high throughput screening ability of 2-DE, few investigators have attempted to separate functional proteins (i.e., in their active form) from biological samples. Sanderink et al. (1986) demonstrated 2-DE zymography of human sera dissolved under non-denaturing conditions in triton X-100, nonidet P-40 and carrier ampholytes. Ong and Chang (1997) used cell extract dissolved in urea, nonidet P-40 and 2% carrier ampholytes to resolve proteins from *Photorhabdus luminescens* by using 2-DE zymography. In contrast, Zhao and Russel (2003) used purified bovine trypsin dissolved in multiple surfactant solution (urea, thiourea, CHAPS, SB- 3-10, carrier ampholytes) to identify proteases on 1-DE and 2-DE zymography. In EPF, St. Leger et al. (1994) used IPG strip

zymography to reveal isofoms of Pr1, Pr2 and metalloprotease from *M. anisopliae*. They used purified enzyme preparation mixed with ampholytes to resolve proteases. In a subsequent study, St. Leger et al. (1996a) have used detergent, ampholytes and urea to solubilize purified enzyme sample. St. Leger and co-workers also have resolved trypsin isozymes by using 2-DE zymography. Choi et al. (2004) have used urea and ampholytes during 2-DE zymography of proteases from crude extract of *Bacillus* sp. An interesting nature of sample behavior was reported by Park et al. (2002) in their classic paper on 2-DE zymography. Park and co-workers were unable to identify clear protease band under non-denaturing conditions. However, after addition of urea and DTT to the crude sample, similar to native IEF separation, clear patterns of zymography was observed. However, after omitting DTT all the zymography bands were lost except one. Indeed, the results from different labs clearly show that every sample has its own intricacies, especially when they are crude samples. It is worth mentioning here that the ultimate goal of any proteomic technology is to analyze most, if not all, proteins present in the samples. Therefore, use of purified enzymes or commercially-available enzymes is not representative of the complex biological samples that form the starting point of any proteomics investigation.

2.17 Enzyme overlay membranes (EOM)

EOM help determination of enzyme activities (isozymes) without the need of purification of the samples. Essentially, crude samples from tissue homogenates, body fluids or microorganisms can be successfully analyzed by this method. In addition, in combination with enzyme inhibitors, the use of EOM would be able to identify the specific class of enzyme in question. Primarily, the samples to be analyzed should be submitted to electrophoresis (native PAGE or IEF) and then sandwiched to

the EOM. EOM can be incubated in buffer containing substrate. However, this procedure would render left over substrate useless and add cost to the assay.

Several investigators (Haverback et al., 1960; Bundy, 1962; Bieth et al., 1974; Nakajima et al., 1979; Del Mar et al., 1979; Del Mar et al., 1980; Ohlsson et al., 1986) have reported that the commercially-available substrates, such as amino acids and peptide substrates containing the terminal group β-naphthyl amide or β-naphthyl ester, are not as specific or sensitive as substrates containing the *p*-nitroanilide group. The *p*-nitroanilide substrates can be cleaved by the proteases and the yellow product, *p*-nitroanaline, can be measured spectrometrically. However, *p*-nitroanaline is too faint to be directly visualized on the gel, but it can be diazotized with naphthylethylenediamine and then seen as red colour (Ohlsson et al., 1986). The major problem with this method is that the dye does not precipitate within the gel and hence the zymogram becomes useless. This problem for 1-DE gels have been solved by Ohlsson et al. (1986). The investigators used enzymoblotting to visualize active proteases on nitrocellulose membrane after diazotization. Unfortunately, this method has not been demonstrated after isoelectric focusing, which is the key method to identify the isozymes. In contrast, Smith (1984) has used fluorogenic substrates (7-amino-4-trifluoromethylcoumarin; AFC) impregnated into cellular membranes for the identification of protease isozymes after analytical isoelectric focusing. However, AFC substrates are expensive and EOM after development require specialized equipment for image capturing.

2.18 Mass spectrometry for identification and characterization of proteins

Mass spectrometry (MS) is now an indispensable tool for the rapid protein and peptide structure analysis. In addition, widespread use of MS is a reflection of its ability to solve structural problems not previously resolved by other conventional tools. Current mass

spectrometry instrumentations are highly sensitive and robust that can reliably analyze biomolecules, especially proteins/peptides (Twyman, 2004).

Mass spectrometers have three principal components: a source of ions, a mass analyzer and an ion detector. The ionization source converts the analyte into a gas phase ions under vacuum. The ions are then accelerated in an electric field towards the analyzer that separates them according to their m/z ratios on their way to detector. The function of the detector is to record the impact of individual ions. Such detectors are equipped with modern computers and bioinformatics software's that allow identification of peptides/proteins with unraveled matching accuracy. In general, MS instrumentation provides two different types of analysis, which are vital in field of proteomics

a) The analysis of intact peptide ions

It can help calculate the masses of intact peptides, which can be used to identify the proteins in a sample by searching correlative databases.

(b) The analysis of fragmented ions

This allows the masses of peptide fragments to be determined that can be used to derive de novo sequences or to search against correlative data bases.

Indeed, MS sequence data provide the most powerful and unambiguous approach to protein and peptide identification. Applications of proteomics technologies are impressive, but are expensive, labor intensive and require trained personals. Proteomics has four principal applications as described below:

2.18.1 Mining proteomes

Proteome mining entails the identification of all or most of the peptides/proteins present in a sample. The point of mining is to catalog the proteome directly, rather than to rely on the static blueprint, DNA, by using cDNA microarrays and RT-PCR etc., (Twyman, 2004).

2.18.2 Protein-expression profiling

Protein-expression profiling helps identify peptides/proteins in a given sample as a function of a particular state of the organism or cell e.g., analysis of the cell during various stages of development or effect of a certain drug on proteins synthesized by the cell (Twyman, 2004).

2.18.3 Protein-network mapping

Many, if not all, proteins function in close association with other proteins. Protein-network mapping mainly deals with the identification of proteins and their interacting counterpart protein molecules in living cells. Many proteins in a signal-transduction cascade and complex biosynthetic pathways function in close association with other proteins. The current state of knowledge about protein-protein interactions mainly came from *in vitro* studies with either purified proteins or by using state of the art technology such as the yeast two-hybrid system (Twyman, 2004). In contrast, proteomic approaches offer the advantage to identify and characterize more complex networks of proteins, which are vital for cellular functions.

2.18.4 Mapping of protein modifications

It is well known that during protein synthesis one of the crucial components is to add modified residues to protein moieties (post-translational modifications) that are vital for the functionality of the proteins. One such modification is phosphorylation, which governs protein targeting, structure, function, and turnover. Such modification of proteins can be accurately identified by using proteomic approaches. Hence, mapping of protein modification in reality is the task of identifying how, and where, such modification on the proteins exists (Twyman, 2004).

The introduction of MS methods to analyze peptides now offers the best means to characterize protein modifications. However, in order to identify protein modification successfully, the MS spectrum should be very good quality with very high sequence coverage. Many new instruments such as Q-TOF can provide quite reliable results in this context (Twyman, 2004).

2.19 Conclusion

In this section, I attempted to provide an in-depth review of the extracellular enzymes, their regulation and potential role in the pathogenicity of EPF. In addition, a brief review of the separation technologies was provided, which are important to resolve protease isozymes or proteins.

There is a wealth of literature on the biochemical/molecular and applied research in the field of bicontrol agents. However, in the author's opinion, there is a lack of interaction between the leading contemporary scientists involved in basic and applied research. Therefore, one neglected aspect, which is studied during this thesis, is the conidial enzymes. This series of

investigation show that there was in-adequate knowledge regarding germinating-conidia and their fate in causing fungal pathogenesis.

The author feels that there is a strong need to establish research projects that has mutual interests for academia and industry. In other words, how the bench top research can be linked to the needs of industry or end user. In this context, a fundamental change could be started by designing curriculum based on basic and applied aspects for the students. More importantly, at a professional level this task can be achieved through the help of scientific societies.

3.0 Choice of water for the hydration of conidia of *Beauveria bassiana*

3.1 Abstract

Entomopathogenic fungi (EPF) are an important insect pest management tool. Formulation of EPF spores requires suspension in water, oil or invert emulsions of oil and water. However, there is inadequate knowledge about the spore swelling phase or initiation of germination. Prior to germination, EPF spores absorb water and swell through an active process whereby activation of certain metabolic processes must occur. However, it is unknown whether soaking of spores in water can initiate the swelling process. An investigation was conducted on the conidia of *B. bassiana* to show that they can undergo a swelling phase in water alone. The results confirmed the conidial swelling in type II water. In addition, rate of swelling of conidia was different in type I and tap water. These results suggested that water not only becomes a carrier necessary for swelling, but also its source and ionic content and contact time affects spore swelling/initiation of germination. During spore hydration, change of pH was observed ensuing from metabolic activities of spores, which was affected by the type of water used. Moreover, radioisotope uptake analysis revealed that freshly hydrated (0.25 h) conidia have different metabolic rates than germinating and 24 h nutritionally-starved conidia. To date, this is the first report demonstrating that the choice of water sources can impact spore swelling or the initiation of germination.

3.2 Introduction

Entomopathogenic fungi are emerging as an important pest management tool in modern agriculture. The multiple mode of action of EPF along with percutaneous entry into the host has

made them superior candidates over other biological control agents. The EPF belong to Ascomycotina (Hypocreales) (Seifert and Gams, 2001), which produce infective propagules called conidia. As a prerequisite to infection by EPF, conidia must adhere to, and germinate before breaching the cuticle. However, before germination, a very crucial phase is hydration of the fungal spore which activates several metabolic processes with in conidia (Schmit and Brody, 1976). Fungal spore germination is well documented, whereas spore swelling or isotropic growth is poorly described in EPF.

Historically, Tomkins (1932) has characterized swelling as the latent period of germination, defined as time interval before germ tube formation or emergence. Brodie and Neufeld (1942), and Cochrane (1958) have defined isotropic growth or spore swelling as a phase during the germination process. In contrast, Yarwood (1936, 1950) described the swelling process of fungal spores as inanimate uptake of water. Hawker (1955) reported that a mature fungal spore swells before germination upon contact with moist substrate and water. Van Ettan et al. (1983) and Schmit and Brody (1976) reported that conidia do not form germ tubes in de-ionized water, but pre-incubation period does reduce or affect the rate of germination in a glucose minimal medium. Griffin (1994) has showed that during the swelling phase there is also growth of fungal cell wall.

Various views exist regarding the swelling phase or isotropic growth with regard to biological activity in the fungi. Yangaita (1957) distinguished endogenous swelling referring to an initial increase in volume which occurs in de-ionized water alone. Yangaita termed this phase as non metabolic swelling. The investigator reported that the metabolic swelling depends upon the nutrients and hence the term “exogenous swelling” was used. However, Sussman and Halvorson (1966) have stated that swelling of fungal spore is sometimes, but not always, a

characteristic of the germination process. They further reported that the spores of fungi imperfecti and ascospores of several genera do not swell perceptibly. Barnes and Parker (1966, 1967) described swelling as a two phase process i.e., metabolic and non-metabolic swelling. They further characterized non-metabolic swelling as a hydration state or osmotic phenomenon.

A very fundamental and novel observation regarding spore swelling and change in pH of the incubation supernatant is reported in this chapter. The soaking of spores in water simulated the nutritionally starved condition. Such conditions may occur upon aqueous formulations of the biopesticides. The main objective of this study was to show that conidia of *B. bassiana* upon soaking in water undergo swelling or pre-germination phase. It has been shown that water soaked conidia were metabolically active as determined by increase in volume and change in pH of the water supernatant. Interestingly, a potential difference in swelling rate and pH was observed when different types of water (type I, type II and tap) were used.

3.3 Materials and methods

3.3.1 Fungal isolates and culture conditions

Beauveria bassiana was obtained from the BioInsecticide Research Laboratory, Department of Applied Microbiology and Food Science, College of Agriculture and Bioresources, University of Saskatchewan, Canada. The strain was maintained on YPG agar at 27° C.

Two weeks old cultures were pooled with 0.03% (v/v) tween to collect the conidia. A 5 ml of the tween suspension was dispensed into Petri plate and conidia were disrupted gently by using a bent glass rod. The conidia were filtered twice through glass wool. Next, spores were washed with the type II water (maximum electrical conductivity of 1$\mu S/$ cm at 25° C) and then

centrifuged at 8500g (Sorvall RC, 5C Plus; Rotor no. SS-34; Global Medical Instrumentation, Inc., Minnesota, USA) to collect the pellet. Three successive washes with type II water were employed to get rid of any traces of growth medium. The final concentration of spores was adjusted to 10^6/ ml and incubated at 27° C in a water bath rotatory shaker incubator at 150 rpm (New Brunswick Scientific Co., New Jersey, USA). A comparative analysis of swelling rate of conidia was undertaken during this study in different types of water. Therefore, conidia were incubated in type II, type I (resistivity of 18.2 MΩ·cm at 25 °C) and tap water (conductivity 344 µmhos/ cm at 25°C). The rationale was to observe, if the difference in the ionic content of the water could influence the swelling rate of conidia. Samples were pooled from 0.25 h to 40 h and subsequently analyzed for pH and volume analysis.

3.3.2 pH analysis

pH measurements were performed on Accumnet, AR 50 digital research pH meter (Fisher Scientific Co., Pittsburgh, USA). A 5 ml sample was obtained and pH was measured by using Lazar research microelectrode (Fisher Scientific Co., Pittsburgh, USA).

3.3.3 Volume analysis

Volume analysis was performed by using a Multisizer III™ (Coulter Counter™; Beckman Coulter, Inc., California, USA). Multisizer III™ (Coulter Counter™) was calibrated with standard latex bead (5, 10 and 20 µm; Beckman Coulter, Inc., California, USA) as indicated by the manufacturer instructions. Background noise was analyzed each time before performing samples measurement. A 500 µl sample of the spore suspension was dispensed in 19.5 ml of Isotone-II (Beckman Coulter, Inc., California, USA) in accuvates. Subsequently, few drops of

the dispersant II C™ (Beckman Coulter, Inc., California, USA) were added to facilitate the dispersion of conidia in the Isotone-II (Beckman Coulter, Inc., California, USA). A 50 µm aperture tube was used to determine the volume of spores of *B. bassiana*. Conidia at concentration of 2-3 X 10^4/ ml were used for Multisizer III (Coulter Counter™ ; Beckman Coulter, Inc., California, USA) analysis. Median volume attained by conidia during soaking period was determined from 0.25 h to 40 h. Each point was based on the median volume of four different runs (quadruplicate). The experiment was repeated twice.

3.3.4 Radioisotope precursor uptake

Conidia were collected with 0.03% tween and were washed thrice in type II water. Next, conidia were re-suspended in the type II water at 10^7/ ml concentration. The radioisotopes were provided at various set time intervals to hydrated conidia (HC; exposed to water for approximately 15 min), nutritionally starved conidia (NSC; pre-soaked in type II water/starved for 24 h) and germinating conidia (GC; conidia pre-soaked in 0.1% glucose and 0.05% peptone for 24 h), respectively. All radioisotopes were purchased from Amersham Biosciences, (Buckinghamshire, United Kingdom). Radioisotopes fed to all three types of propagules include: ^{14}C-uracil (sp. act. 2 GBq/mmol), ^{14}C-cholesterol (sp. act. 1.85MBq/mmol), ^{3}H-deoxythymidine triphosphate ammonium salt (^{3}H-dTTP; sp. act. 185 GBq/mmol) and ^{3}H-leucine (sp. act. 6.59 TBq/mmol). However, ^{3}H-maltose (sp. act. 1.85 MBq/mmol) and ^{14}C -methyl thiogalactopyranoside (^{14}C-TMG; sp. act. 1.85 GBq)) were only provided to HC. A 25 µl of sample of each radioisotope was pooled and vacuum filtered on the filter paper (Millipore MF™ Membrane filter; 0.45 µm pore size HA type, Millipore, Billerica, USA) after which time a wash with cold type II water (4 ml) was employed. Filter papers were dried for one hour at 60° C and

placed in a scintillation vial. After addition of 5 ml of scintillation fluid (Beckman Ready-Solv scintillation liquid; Beckman Coulter, Inc., California, USA), scintillation spectrometry (Beckman LS1801 scintillation counter; Beckman Coulter, Inc., California, USA) was performed for total counts per minute (cpm) analysis. As a control empty scintillation vials and fluid filled vials were run to detect the background noise level. In all experiments the background noise was negligible with both conditions.

3.3.5 Statistical analysis

Analysis of variance was performed by using one way completely randomized ANOVA. The CoStat version 6.204 (CoHort Software, Monterey, CA, USA) was used. In case of conidial swelling, ANOVA was applied on median values (volume) attained during soaking period. The level of significance was determined by using LSD methods of mean separation where $p < 0.05$ and $n = 4$. All experiments were repeated twice.

3.4 Results

3.4.1 Comparison between type I, type II and tap water

Interestingly, swelling of conidia was observed in type II water alone under nutritionally starved conditions. Conidia undergo swelling phase in all the three water types. In addition, shrinkage of the conidial volume was also observed during incubation period. Results reveal that the rates of swelling of conidia in type I, type II and tap water, respectively (Fig. 3.1 and table 3.1) was different ($p < 0.05$) during incubation period from 0.25 to 40 h. Variability in spore swelling rate in different waters could be due to different buffering actions or their ionic

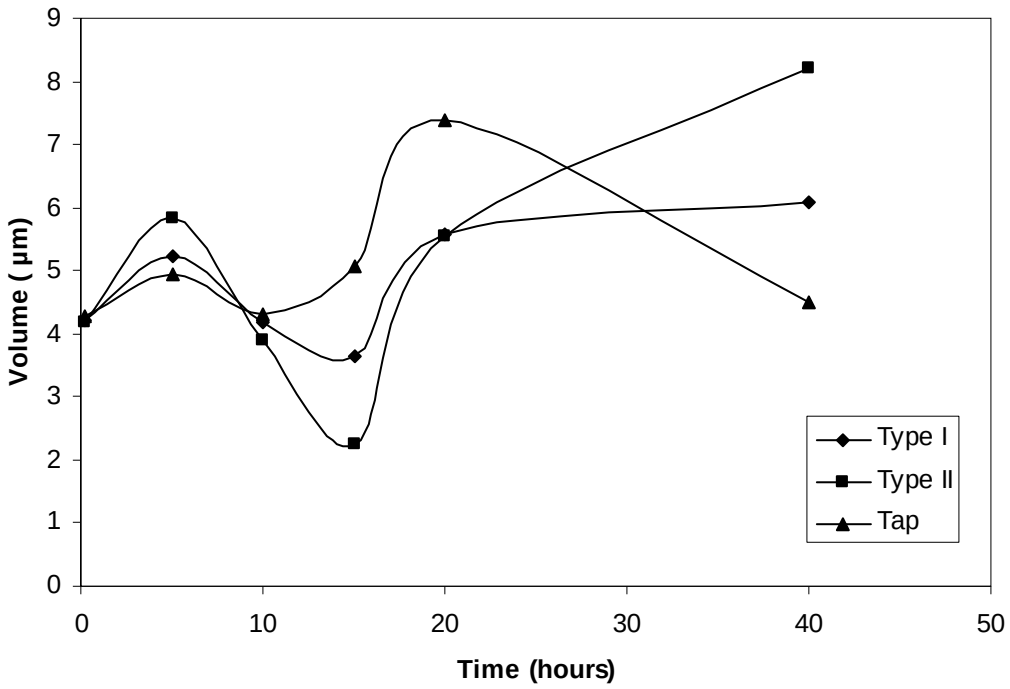


Figure 3.1: Rate of swelling of conidia of *B. bassiana* in type I, type II and tap water. Each point represents mean of four runs, where median conidial volume increase was measured by using Multisizer-II^{TM} (Coulter Counter^{TM}). During each run 2-3 X 10^4 conidia were counted for determining the percentage volume at given time intervals. Level of significance is indicated in Table 3.1.

Table 3.1 Level of significance during swelling of conidia of *B. bassiana* in type I, type II and tap water.

	Time (hours)					
Treatment	**0.25**	**5**	**10**	**15**	**20**	**40**
Type I	hij	def	hij	j	cde	c
Type II	hij	cd	ij	k	cde	a
Type II	efgh	efgh	ghij	defg	b	fghi

Legend:

Analysis of variance was performed by using one way completely randomized ANOVA. Level of significance was determined by using LSD method of mean separation where $p < 0.05$ and $n = 4$. The mean values are those depicting the Fig. 3.1. Same letters listed in columns and rows for each treatment are not significantly different.

contents. In addition, results revealed significant difference ($p < 0.05$) in the pH of the three categories of spent water washes (Fig. 3.2) over the incubation period. The difference in pH, along with swelling rate of conidia in various types of water implies difference in metabolic rate. The difference in pH was due to release of metabolites in the wash supernatant as previously showed by Khachatourians et al. (2007).

3.4.2 Comparison of radioisotope uptake

To elucidate that hydrated conidia were metabolically active during soaking period in type II water, radioisotopes uptake was conducted. Uptake of lactose non-hydrolysable (non-metabolizable) analog ^{14}C-methyl thiogalactopyranoside (^{14}C-TMG) linearly increased for 60 min of the soaking period of HC (Fig. 3.3a). In contrast, high uptake of ^{3}H-maltose (Fig. 3.3b) for HC was evidenced at 15 min, which showed significant decline after 30 min of incubation period. Results showed significantly high uptake of ^{3}H-leucine immediately after soaking of HC in type II water (Fig. 3.4). The uptake declined significantly at 30 min after which time the uptake of ^{3}H-leucine significantly increased again (Fig. 3.4a). In NSC uptake of ^{3}H-leucine was significantly lower in comparison to HC. At 30 min a slight increase in the uptake was observed. In GC and NSC significantly low uptake of ^{3}H-leucine was observed in contrast to HC. The uptake of ^{3}H-leucine by GC continued till 60 min with no significant change in the uptake of ^{3}H-leucine.

A rapid uptake of ^{14}C-uracil (Fig. 3.4b) was observed in HC at 15 min after which time a significant decline was observed. In contrast a very low uptake was evident for NSC and GC, respectively at 30 min. The uptake of ^{14}C-uracil showed significant increase in GC after 30 min that rose up to 60 min. In contrast, uptake of ^{14}C-uracil was low in NSC.

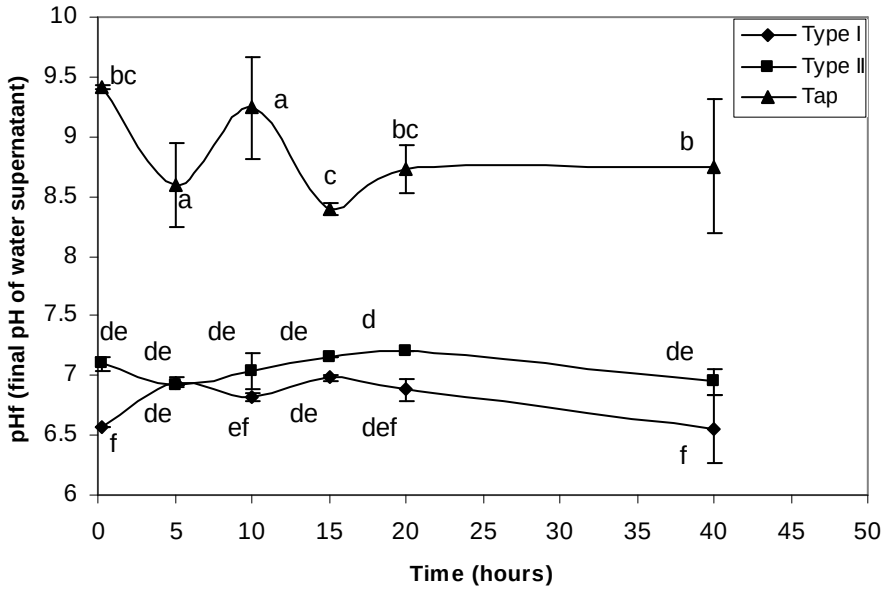
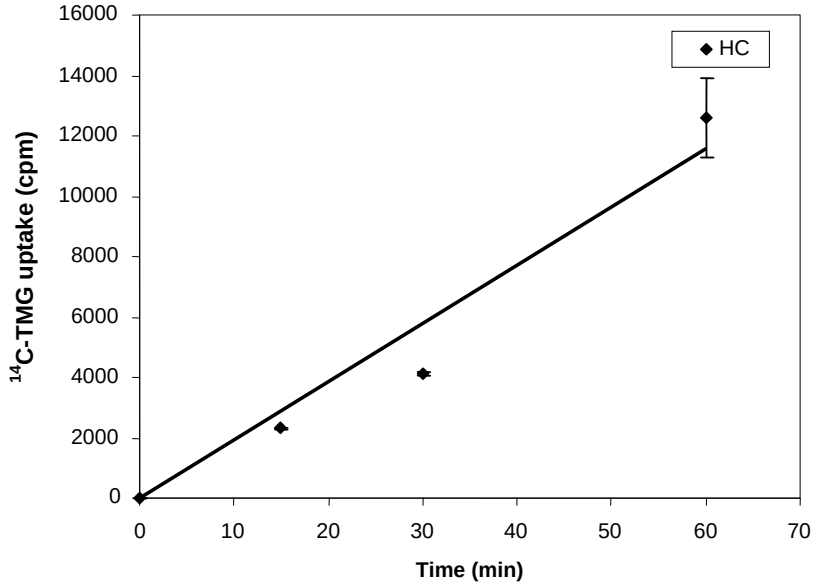
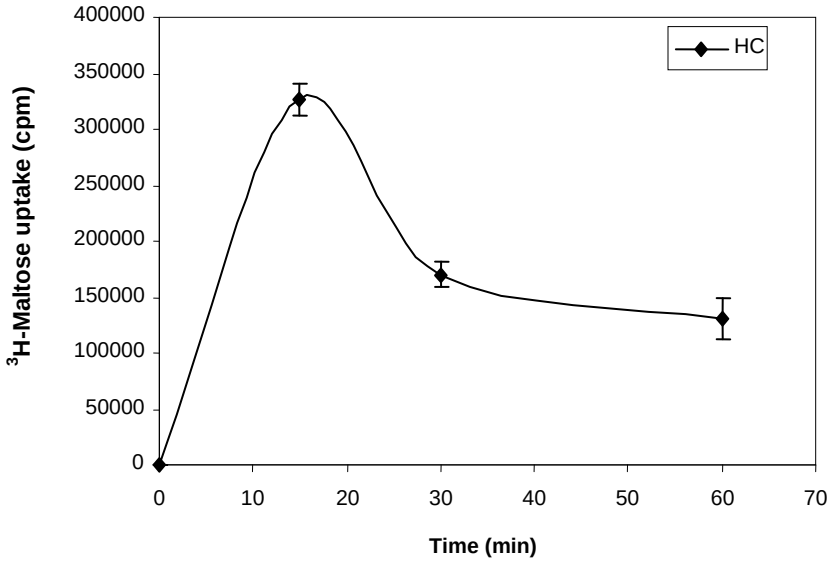


Figure 3.2: Change of pH during swelling phase of conidia of *B. bassiana* in type I, type II and tap water. Each point represents mean (N = 4), where error bars represents S.D. Analysis of variance was performed by using one way completely randomized ANOVA. Level of significance was determined by using LSD method of mean separation where $p < 0.05$ and $n = 4$. Mean points followed by same letters are not significantly different.

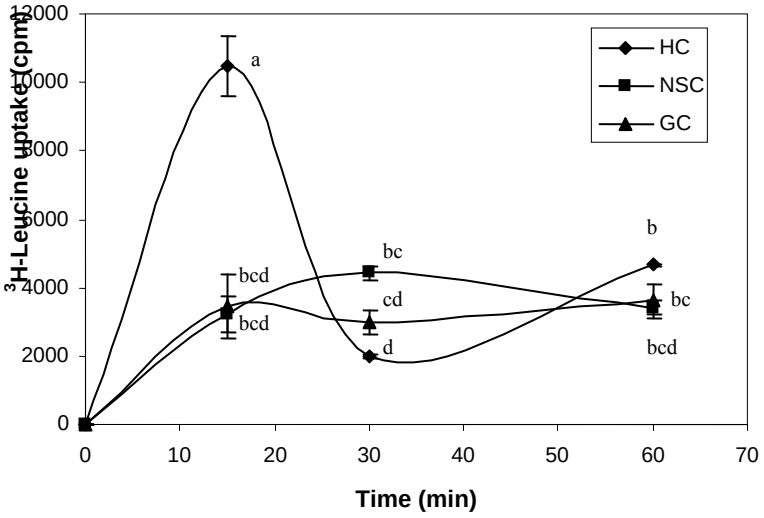


(a)

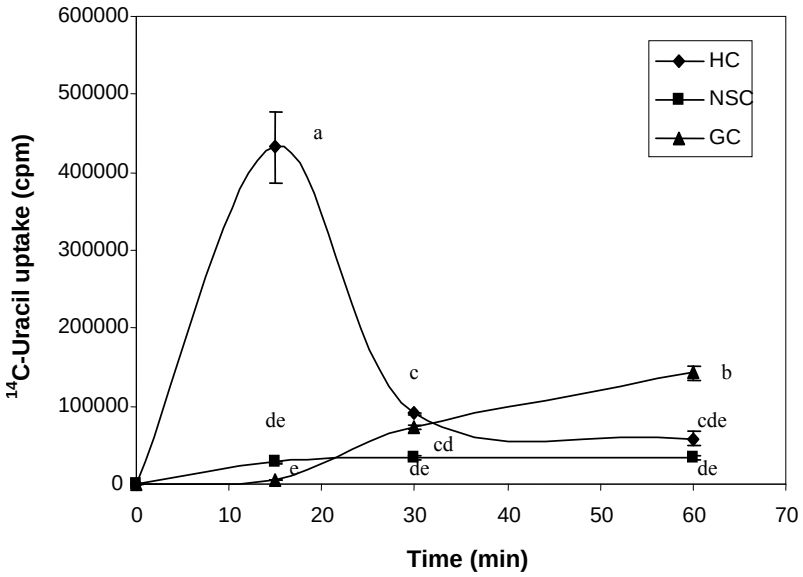


(b)

Figure 3.3: Rate of ^{14}C-TMG (a) and ^{3}H-maltose (b) uptake by the conidia of *B. bassiana* during soaking period in type II water. Each point represent the mean, where n = 3 and error bars represents S.D.



(a)



(b)

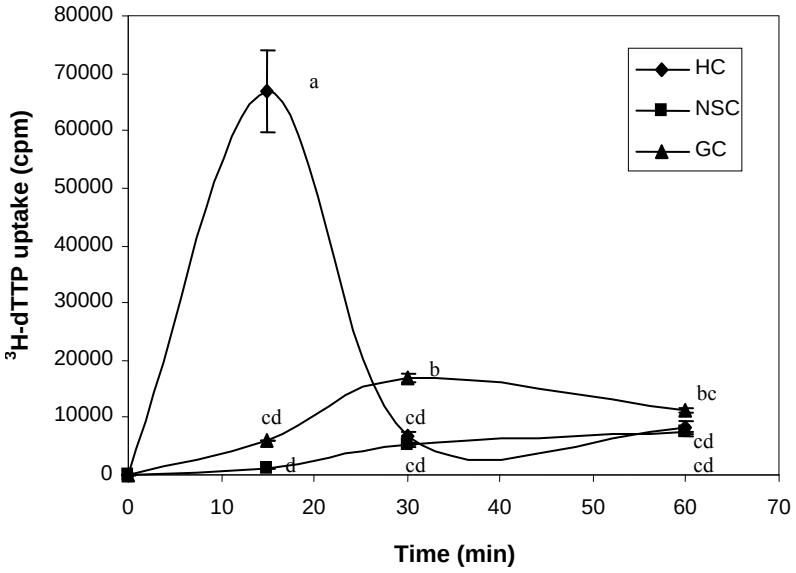
Figure 3.4: Rate of ^{3}H-leucine (a) and ^{14}C-uracil (b) uptake by the conidia of *B. bassiana* during soaking period in type II water, where HC: hydrated conidia, NSC: nutritionally starved conidia and GC: germinating conidia. Each point represent the mean, where n = 3 and error bars represents S.D. Analysis of variance was performed by using one way completely randomized ANOVA. Level of significance ($p < 0.05$) was determined by using LSD method of mean separation. Means followed by same letters are not significantly different.

Results also revealed a rapid uptake of ^{3}H-dTTP (Fig. 3.5a) by HC at 15 min of incubation in type II water, followed a significant drop in uptake by 30 min. Conversely, a significant low uptake of ^{3}H-dTTP was observed at 15 min in NSC and GC, respectively. The uptake of ^{3}H-dTTP showed a significant increase at 15 min after which time a significant drop in uptake was observed. In addition, an important observation was made regarding ^{14}C-cholesterol uptake (Fig. 3.5b). It appeared that HC and NSC conidia have ^{14}C-cholesterol uptake in contrast to germinating conidia. This could be related to the hydrophobicity of the conidial wall. In contrast, a very low uptake of ^{14}C-cholesterol was also observed for GC.

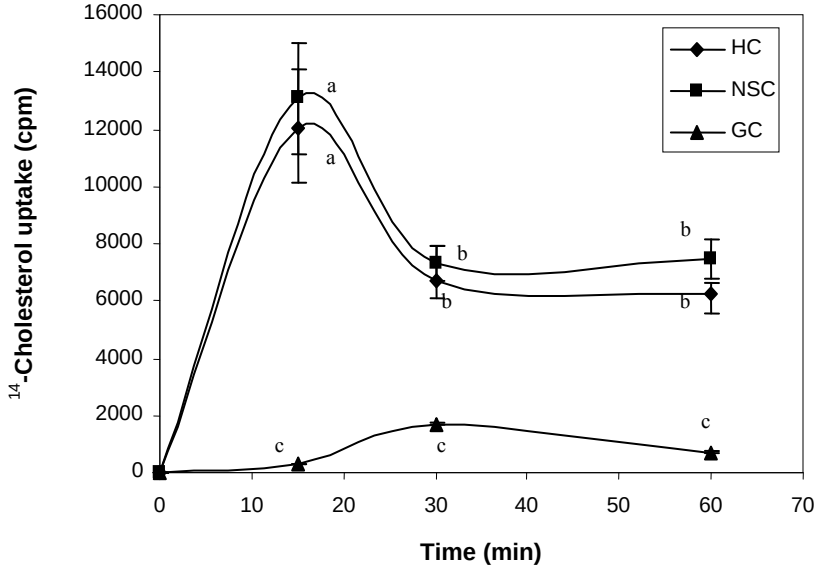
3.5 Discussion

Soaking of conidia of *B. bassiana* in water resulted in the swelling and metabolic activation. Swelling rate of conidia was measured by using Multisizer- III™ (Coulter Counter™). The method involves the sensing of electric pulse or determination of electronic sizing of spores. The method is extremely sensitive to measure the swelling of spores long before germ tube emergence or significant changes in respiration were observed.

Measurement of volume is considered to be the most significant approach to assess the biological cells and follow their growth (Barnes and Parker, 1968). Coulter Counter™ has been used in counting and cell determination of blood cells (Mattern et al., 1957; Wisecup and Crouch, 1963), bacterial growth (Lark and Lark, 1960), yeast (Zellner et al., 1963) and cells in tissue culture (Cooper and Jordon, 1963). Barnes and Parker, (1966, 1967) have used Coulter Counter™ to measure spore size of a mold during germination and the effects of antiseptics. Similarly, Fargues (2001) have measured the spore size during germination of *M. anisopliae.* The technique is unrivaled in its efficacy and statistical accuracy for measuring biological cells.



(a)



(b)

Figure 3.5: Rate of ^{3}H-dTTP (a) and ^{14}C-cholesterol (b) uptake by the conidia of *B. bassiana* during soaking period in type II water, where HC: hydrated conidia; NSC: nutritionally starved conidia and GC: germinating conidia. Each point represent the mean, where n = 3 and error bars represents S.D. Analysis of variance was performed by using one way completely randomized ANOVA. Level of significance ($p < 0.05$) was determined by using LSD method of mean separation. Means followed by same letters are not significantly different.

Change of pH of different types of water clearly demonstrates that the *B. bassiana* have the ability to alter their microhabitat during spore the swelling phase. *B. bassiana* significantly ($p < 0.05$) altered the pH of the water supernatants of all three types of water. The level of significance becomes more apparent when the buffering capacity of these water types is considered (see materials and methods). The change in pH may play an important role during isotropic growth because the transport of amino acids and ions, both cations and anions depends upon pH of the medium.

In a similar context, Schmit and Brody (1976) reported that the conidia contain storage compounds such as glutamic acid, trehalose and perhaps phospholipids. They stated that conidia contain readily-available energy sources that can be mobilized as soon as the conidia become hydrated. The biochemical events that do not require exogenous carbon source include: i) formation of γ- aminobutyric acid, ii) simultaneous degradation of glutamate and formation of aspartate, iii) the assembly of polysomes into ribosomes, and iv) limited amount of protein and RNA synthesis. In similar vein, extrusion of ions, amino acid, metabolic acids, coenzymes (notably NADPH) and extracellular enzymes were reported from hydrated conidia of *N. crassa* during incubation in de-ionized water (Schmit and Brody, 1976).

It is well known that distilled water can contain organic and inorganic contaminants in addition to dissolved ionized gases (e.g., chlorine, ammonia and carbon dioxide). It also contains dissolved organics which do not volatilize at low temperature. In contrast, de-ionized water contains dissolved organics, particulates, bacteria and pyrogens (Barnstead/Thermolyne Co., Barnstead International, Iowa USA). The importance of ions (Ca^{++}, K^{+}, Na^{+}, Mg^{++}) in enhancing germination has been documented for *Blastocladiella emersonii* zoospore (Soil and Sonneborn,

1972; Harold and Harold, 1980; Van-Brunt and Harold, 1980), alkalophilic *Bacillus* spp. (Kudoi and Horikoshii, 1983) and *M. anisopliae* (St. Leger et al., 1990).

Several enzymes that have an important role in fungal growth and metabolism are affected by millimolar and micromolar quantities of ionic species as previously reported by Griffin (1994). The intent here is not to assign a specific role to any of the essential trace elements. However, tap water quality analysis (Appendix 3: Water quality details, City of Saskatoon) suggest that H^+, Ca^{++}, Mg^{++}, Fe, and K^+ and other nutrients are present in sufficient quantities that could affect the swelling process. Moreover, in tap water the amount of bicarbonate and carbonate is also very important in considering its impact on pH. The pH change during isotropic growth could be attributed to the CO_2 being produced as a by-product of metabolism in addition to CO_2 in the atmosphere. At a pH between 5 and 9 it dissociates into carbonic acid which forms carbonate and bicarbonate ions affecting the pH of a habitat. In addition, tap water contains ammonia and citrate, which can affect the pH of the water and conidial metabolism.

pH affects a number of metabolic processes, for instance, metallic ions at certain pH ranges become insoluble. Magnesium and phosphate coexist in their free form at low pH but at higher pH forms insoluble complexes, thereby reducing the ability to the growing fungus. Besides iron, calcium, copper and zinc deficiency may occur at alkaline pH. All these ions affect different metabolic enzymes and germination (Griffin, 1994). Furthermore, pH also affects the cell permeability and this effect is particularly important for compounds that ionize.

The uptake of radioisotopes revealed that the HC were ready for the uptake of ^3H maltose immediately after the soaking period (15 min) which stopped at 30 min. This was not evident with ^{14}TMG. The uptake of ^{14}TMG was linear in comparison to maltose. The uptake of ^{14}C-

cholesterol indicated that HC conidia have high uptake of ^{14}C-cholesterol. These results put *B. bassiana* into fungal group, which can utilize sterol derivatives for growth. Similarly, some genera of Phytophathora and Pythium have the ability to use sterols for growth purpose (Griffin, 1994). In addition, Khachatourians (1996) reported that EPF have the ability to utilize sterol derivatives.

Results showed that *de novo* RNA synthesis was required immediately upon hydration. This also suggested that the conidia could initiate protein synthesis during hydration without exogenous supply of C/ N that could be due to the polyribosomes present within conidia. Similarly, Hitchcock (1967) has demonstrated the incorporation of ^{14}C-uracil into RNA during an incubation period in de-ionized water. Schmit and Brody (1976) have described that RNA synthesis probably begins with in 5 min after the initiation of conidial germination. Bhagwat and Mahadevan (1970) demonstrated that the conidia incorporated a significant amount of radioactive ^{3}H-leucine into protein within the first 5 min. In another investigation, mitochondrial as well as cytoplasmic protein synthesis was observed within first 15 min after the initiation of germination (Greenawalt et al., 1972).

It is evident that the uptake of ^{14}C-uracil was significantly higher than ^{3}H-leucine at 15 min of hydration. These results put conidia of *B. bassiana* into the 'third category' of spores as described by Lovett (1968). Fungi in this category initiate RNA synthesis prior to protein synthesis during the germination process. Other notable examples of fungi in this category includes, *A. niger* (Yangaita, 1957), *Rhizophlyctis rosea* (Le John and Lovett, 1966), *B. emersoni* (Lovett, 1968) and *A. nidulans* (Bainbridge, 1971). The uptake of ^{3}H-leucine may imply that conidia contain fully active ribosomes to initiate protein synthesis during soaking period of conidia in water (Bonnen and Brambl, 1983; Brambl et al., 1987).

d' Enfert (1997) reported that the activation of several genes involved in ribosome assembly and in amino acid biosynthesis occur very rapidly at the onset of isotropic growth. In a subsequent study, Binisti and d' Enfert (d' Enfert, 1997, cited as manuscript in preparation) reported the cloning of genes of *A. nidulans* that were expressed at a significantly higher level in conidia than in mycelia. They found 10 cDNAs encoding ribosomal proteins that were rapidly activated upon activation of spores for germination in comparison to genes involved in polarized growth or chromatin structure. d' Enfert stated that the activation of ribosomal genes during conidial germination is likely because *de novo* synthesis of ribosomes is pre-requisite to achieve a growth rate appropriate for the germ tube emergence that could not be obtained by the sole use of the translation machinery stored in conidia.

Different views exits about the swelling phase of spores. Yangaita (1957) reported that *A. niger* spores did not swell in de-ionized water after 9 h of incubation. Chung and Wilcoxson (1969), and Macko (1981) have reported that soaking removed auto-germination inhibitor. French and Gallimore (1972) on the other hand described the release of germination initiator as germination promoting factor. Dillon and Charnley (1985, 1991) reported that *M. anisopliae, B. bassiana, V. lecani* and *N. rileyi* do not swell altogether in distilled water. Very recently, Qazi and Khachatourians (2007) and Khachatourians et al. (2007) showed that the hydrated conidia of *B. bassiana* and *M. anisopliae* secreted ammonia and citrate along with release of multiple proteases, which have the ability to degrade cuticle prior to the formation of germ tube or appressoria.

Indeed, these results show that the water soaked conidia of *B. bassiana* were swollen and metabolically active as changes in pH of the incubation supernatant was observed. In addition, the rate of swelling of conidia and change in pH of various types of water were different. These

results imply that the ionic composition of the waters have an influence on the swelling or the pre-germination phase. Results also revealed that HC has a different metabolic rate than NSC and GC. HC were metabolically active i.e., protein, DNA, RNA synthesis and nutrient uptake.

4.0 Hydrated conidia of *Beauveria bassiana* and *Metarhizium anisopliae* release a family of metalloproteases

4.1 Abstract

Beauveria bassiana and *Metarhizium anisopliae* spores released a family of metalloproteases during hydration over a four day incubation period. The proteases were identified and characterized by using one-dimensional native PAGE (1-DE nzPAGE) and one-dimensional SDS non-dissociating (1-DE nzSDS PAGE) zymography. The ability of these isozymes to degrade gelatin varied as revealed by 2-D spot densitometry. In *B. bassiana,* 1-DE nzPAGE zymography showed the presence of one activity band up to day 4. In contrast, 1-DE nzPAGE zymopheretograms revealed five isoforms of gelatinase from tween wash of conidia. One to three activities with different intensities appeared on gel from wash (tween and water) and incubation supernatants up to day 4. The relative migrations of these activities on 1-DE nzPAGE zymograms appeared as fast, medium and slow on the gel. The 2-D spot densitometry of zymograms indicated isoforms have different proteolytic activities as quantified by pixel intensities. In *B. bassiana,* SDS-PAGE zymography revealed that the tween fraction had three bands of 100, 60, and 36.3 kDa, respectively. The molecular weight for the protease observed at day 2 and 3 was 35.4 and 25 kDa. However, the two bands of 24.7 and 20.3 kDa were observed at day 4. In *M. anisopliae,* SDS PAGE zymography indicated the release of two isozymes of Mr 103 and 12 kDa during tween treatment of conidia. However, during the first washing step with water and incubation of spores at day 2 and 3, respectively only12 kDa protein was evident. In *B. bassiana,* the major protease activity in the band was inhibited by EDTA, $CaCl_2$ and $MgCl_2$. In contrast, the majority of the proteases from *M. anisopliae* were inhibited by EDTA, but

stimulated by $CaCl_2$, and $MgCl_2$. The presence of isozymes in conidia and their release during hydration must have functional significance for fungi, and should provide advantages to the EPF in its saprobic or pathogenic modalities. This is the first report describing the release of metalloprotease isozymes from conidia.

4.2 Introduction

Entomopathogenic fungi (EPF) infect insects primarily by breaking through the cuticular barrier. The insect cuticle is comprised of protein and chitin and there is strong agreement that chitin is embedded in the protein fibrils forming the protective barrier. Moreover, cuticle contains more protein than the chitin (75 to 80%). Extracellular enzymes, whether of mycelial or spore origin, are the hallmark of the fungal infectious process. The successful infection relies upon the efficient release of exoenzymes, which have the potential to degrade insect cuticle (Khachatourians, 1996). Among these, proteases are important as they are among the first to appear during the infection process (St. Leger et al., 1986a; St. Leger, 1995). The role of proteases in the fungal infection process has been the subject of many reviews (Khachatourians, 1991, 1996; Clarkson and Charnley, 1996), and many investigators have studied the properties of fungal proteases (Bidochka and Khachatourians, 1987, 1988a; St. Leger et al., 1994; Urtz and Rice 2000; Khachatourians et al., 2007).

St. Leger et al. (1991) provided evidence that conidia are pre-adapted to the pathogenic mode of life. They found Pr1, chitinase and esterase release from conidia after treatments with buffer, detergent and reducing agents which suggested that the enzymes membrane bound. Boucias and Pendland (1991) screened spores of *Beauveria bassiana, Metarhizium anisopliae* and *N. rileyi* for the presence of alkaline phosphatase, esterase (C8), lipase (C14), leucine

aminopeptidase, acid phosphatase, phosphohydrolase, ß-glucosidase, *N*-acetyl-D-glucosaminidase and α-manosidase upon direct application of 10^6 spores to APIZYM strip. However, to date there are no published reports dealing with characterization of purified enzyme(s) obtained from conidia under prolonged starvation conditions.

In vitro, water soaked spores simulate the extreme starvation of conidia, which may occur upon spraying of bioinsecticide. In most field applications of EPF, conidia are sprayed in oil, water or as invert emulsion in oil. Despite their importance, the knowledge of spore-associated cuticle-degrading enzymes is incomplete. The objective of this study was to identify and characterize protease(s) associated with conidia and released during hydration or soaking period in water. It has been shown that (i) gelatinase-like multiple proteases are released by the hydrated spores of *B. bassiana* and *M. anisopliae* immediately upon hydration, and (ii) their activity is present up to four days in aqueous supernatants. This is the first report of the characterization of metalloproteases released from conidia upon hydration.

4.3 Materials and methods

4.3.1 Spore preparation

Beauveria bassiana (GK 2016) was obtained from the BioInsecticide Research Laboratory, Department of Applied Microbiology and Food Science, College of Agriculture and Bioresources, University of Saskatchewan, Canada. *Metarhizium anisopliae* (MA 2038) was generously provided by the USDA, Entomopathogenic Fungus Collection, Ithaca, NY, USA. Fungal strains were maintained on YPGA for 12 days at 27° C. Conidia were pooled with 0.03% tween 80 (v/v) in water which had a maximum electrical conductivity of *1µS/ cm* at 298K (25°C). This water will hereafter be referred to as type II water. To prepare spores, 5 ml of the

tween suspension was dispensed into Petri plate and conidia were disrupted gently by using a bent glass rod. Conidia were filtered twice through glass wool column. This was the first tween fraction. Furthermore, two washes of 15 ml each were employed with type II water, and fractions saved as type II water 1st and 2nd wash, respectively. Spores were counted by using a haemocytometer, and the final concentration of spores was adjusted to 10^8/ ml after inoculation into sterilized type II water placed in an Erlenmeyer flask. Spores were incubated at 27° C at 150 rpm in rotary shaker incubator (New Brunswick Scientific Co., New Jersey, USA). After set time intervals, 4 ml of the sample was taken from the Erlenmeyer flask and processed as described earlier.

4.3.2 Enzyme preparation from spores

Conidia were spun down at 8500g (Sorvall RC, 5C Plus; Rotor no. SS-34; Global Medical Instrumentation, Inc., Minnesota, USA) for 10 min at 4° C. Supernatant was passed through 0.22 µm pore size filter. This filtrate was collected in a sterile 15 ml Falcon tube and was referred as the enzyme filtrate/preparation. The spore enzyme filtrate was stored at –30° C subject to further analysis.

4.3.3 Initial screening of effective inhibitor

Enzyme filtrate (Tween wash) was initially tested against protease class specific inhibitors as indicated in the Fig. 4.1 for *B. bassiana* and *M. anisopliae*, respectively to obtain the maximum inhibition. Protease inhibitors were purchased from Sigma (St. Louis, USA) and were used at concentrations as follows: 10 mM EDTA, (iii) 100 µM iodoacetamide, (iv) 10 mM PMSF, (v) TPCK 10 mM, and (vi) STI (soya bean trypsin inhibitor) µg/ ml.

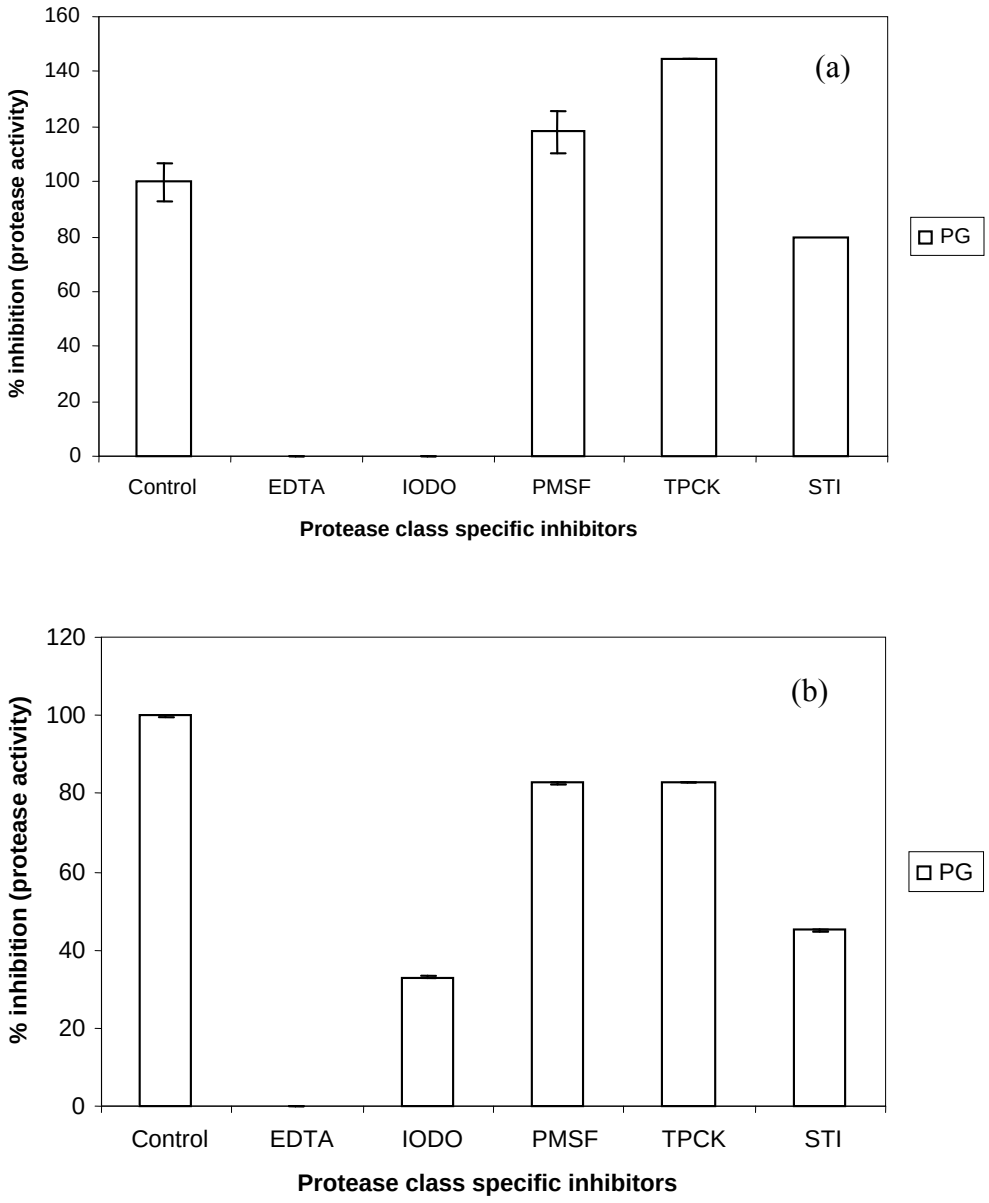


Figure 4.1: Proteolytic activity released by the conidia of *B. bassiana* (a) and *M. anisopliae* (b), respectively. Enzyme filtrate was tested against the different class specific protease inhibitors after Beynon and Salvesen (2001). (i) Control. The inhibitors used are as follows: (ii) 10 mM EDTA (iii) 100 µM iodoacetamide (iv) 10 mM PMSF (v) TPCK 10 mM and (vi) STI (soya bean trypsin inhibitor) µg/ ml. Error bars represents S.D., where n = 4.

The most effective inhibitor was chosen for further electrophoretic characterization. Briefly, protease screening medium comprised of 1% gelatin in 1.5% agar plates. Essentially, each plate contained 18 ml of the screening medium. Effective inhibitor concentration (50 µl) was dispensed into 0.5 mm well, which was constructed by using sterile Pasteur pipette. After 30 min of incubation at room temperature to allow diffusion, enzyme filtrate (50 µl) was loaded in each well. Finally, plates were sealed and incubated at 27° C for 24 h. The zone of clearing was measured and percentage maximum inhibition was obtained against control.

4.3.4 SDS non-reducing PAGE zymography

To estimate the relative molecular weight of the protease, non-reducing SDS PAGE (1DE-nzSDS PAGE) zymography was performed with co-polymerized gelatin (1 mg/ ml). Sample buffer was made after Lantz and Ciborowski (1994). Briefly, 10 mg bromophenol blue was added to 4 ml of 2 M Tris/ HCl, pH 8.8. Then, 25 ml of 60% (w/v) sucrose and 17.5 ml of 20% SDS was added with gentle stirring. Finally, the volume of the solution was brought up to 50 ml with type II water. Seventeen microliters of enzyme filtrate/preparation was loaded in each well. Electrophoresis was conducted at a constant voltage (150 V), at 4° C unless otherwise noted. Electrophoresis was performed for approximately 55 min or until the tracking dye was within the 2 mm of the bottom of the gel.

4.3.5 Native zymograms

Native zymography (1-DE nzPAGE) was performed with co-polymerized gelatin (1 mg/ ml) as a substrate (Difco carbohydrate free gelatin; BD Diagnostics, Sparks, USA). Sample buffer of the native gel was similar to SDS sample buffer, except that it contained 5 ml of 10%

triton X 100 instead of SDS. The high pH discontinuous buffer system of Reisfeld et al. (1962) was adapted and modified for copolymerization of gelatin as a substrate. Gelatin at a concentration of 1 mg/ ml was used as a substrate. The gel recipe composition was adapted from Hames (1997) except that TEMED was used a catalyst instead of riboflavin**.** Pre-electrophoresis was performed for 20 min without sample in order to avoid the deleterious effects of APS and TEMED on enzyme activity. Next, samples of enzymes were applied to SDS-containing gels for as described earlier. Electrophoresis was performed at 4° C at a constant current of 15 mA for approximately 55 min, or until the tracking dye was within the 2 mm of the bottom of the gel.

4.3.6 Enzyme activity in native gels

Enzyme activity was detected by placing the gel in 0.05 M TRIS/ HCl buffer, pH 7.4 at 37° C for 2.5 h. To study the effects of inhibitors and activators on protease, gels were incubated in buffer supplemented with the desired amount of protease inhibitors and activators. The effective concentrations of the inhibitors were selected/adapted from (Beynon and Salvesen, 2001). After incubation period the gels were fixed for 10 min in fixative and subsequently stained for 10 min with Commassie brilliant blue R-250 (0.1% dissolved in 10% acetic acid and methanol, respectively). To reveal the zones of clearing gels were placed in a de-staining solution (10% acetic acid and methanol, respectively in type II water) and incubated at 22-23° C on rotary shaker (5 rpm). Gels were photographed by using Kodak digital camera (DC 290 zoom) under fluorescent bright light. Images were converted to grey scale and adjusted by using Adobe Photoshop Professional Software (San Jose, CA, USA). 2-D spot densitometry was performed using Alpha Imager Software 5.5 to configure a quantitative relationship between clearing regions/area on the gels.

4.3.7 Development of SDS containing gels

SDS containing gels were washed for 10 min with 2.5% triton X 100 in water with two changes (50 ml portion of each). The gels were then subsequently washed with triton X 100 in TRIS/ HCl buffer, pH 7.4 with two changes (50 ml protein each). Finally, two washes (50 ml per gel) were performed with TRIS/ HCl buffer, pH 7.4 and gels were incubated in fresh TRIS/ HCl buffer, pH 7.4 for 1.5 h at 37° C. After incubation period, gels were fixed for 10 min in fixative and stained with Commassie brilliant blue R-250 for 10 min as described in section 4.3.6. Gels were de-stained to reveal the zones of clearing of proteolytic degradation.

4.3.8 Proteolytic activity in gels

A proteolytic unit is defined as the amount of the enzyme that can produce a net (background free) clearing zone (degradation of gelatin) of one pixel. Mathematically* the relationship can be summarized as follows:

AVG = IDV/ AREA (*Anonymous, Alpha Imager^{TM} user manual; Genetic Technologies, Inc., Miami, USA)

Where:

- AVG: Average value of the pixels (enclosed) after background subtraction.
- IDV: Sum of all the pixel values after background correction.
- IDV= $\sum$ (each pixel value – background or black).
- AREA: Size (in pixels) of the region enclosed by box, ellipse or free hand drawing.

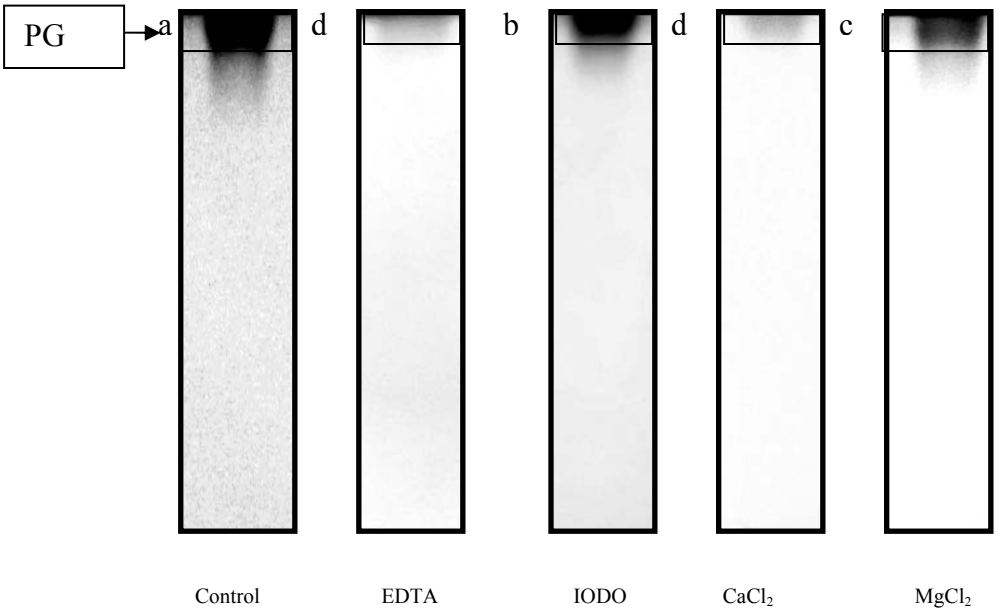
4.3.9 Statistical analysis

Gelatinase-like protease activities from three separate gels were obtained by using 2-D spot densitometry (as explained above) and were represented in pixels values to indicate the activity on control and treated bands. The results obtained were examined using one-way completely randomized analysis of variance (ANOVA) to determine which activities/bands were not significantly different. The statistical program CoStat, Version 6.204 (CoHort Software, Monterey, CA, USA) was used.

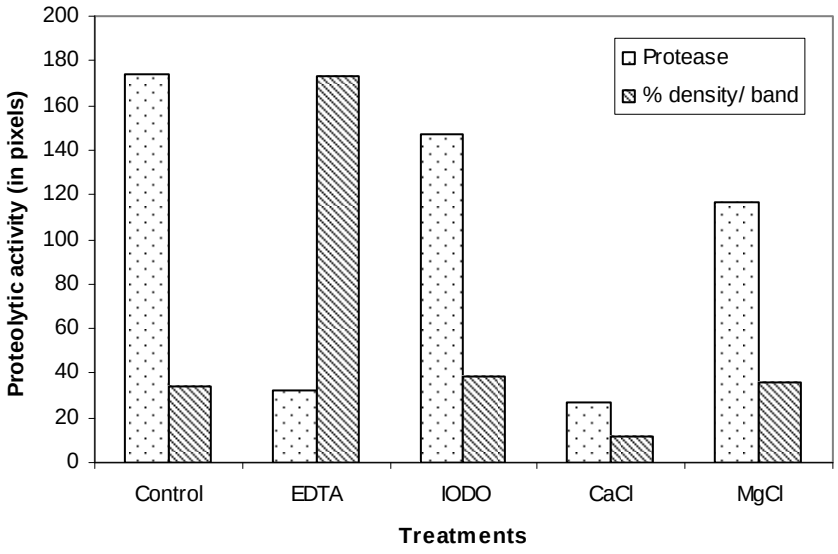
4.4 Results

4.4.1 1-DE zymography of *B. bassiana*

Conidia of *B. bassiana* showed swelling during incubation in type II water. More than 3 fold increase in conidial volume was observed after 24 h (Chapter 4). One activity band was identified by using 1-DE gels however, use of inhibitors and activators help identified activities resembling metalloproteases and cysteine proteases. In addition, spot 2-D densitometry was used to show the difference in the protease activity during treatment of the gel strips. It can be appreciated from Figure 4.2 that washing of conidia in 0.03% tween resulted in the appearance of one band of metalloprotease, which was inhibited by EDTA. Treatment of gel strips with $CaCl_2$ and $MgCl_2$ showed strong and slight inhibition, respectively. Washing of conidia in water 1^{st} (Fig. 4.3) and 2^{nd} wash (Fig. 4.4), respectively showed similar results. However, treatment of gel strip with $CaCl_2$ and $MgCl_2$ in the two water washes showed strong inhibition. At day 1 (Fig. 4.5) an activity band observed was a metalloprotease at which time treatment of gel with $CaCl_2$ and $MgCl_2$ resulted in approximately 50% inhibition of protease activity. At day 2 (Fig. 4.6) strong inhibition was observed with EDTA, whereas slight inhibition

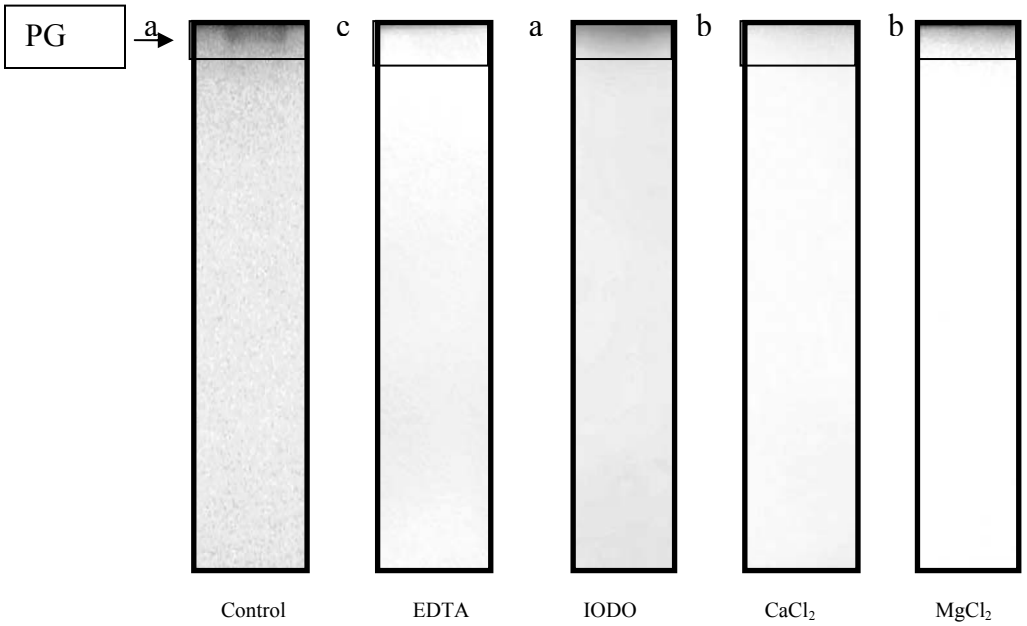


(a)

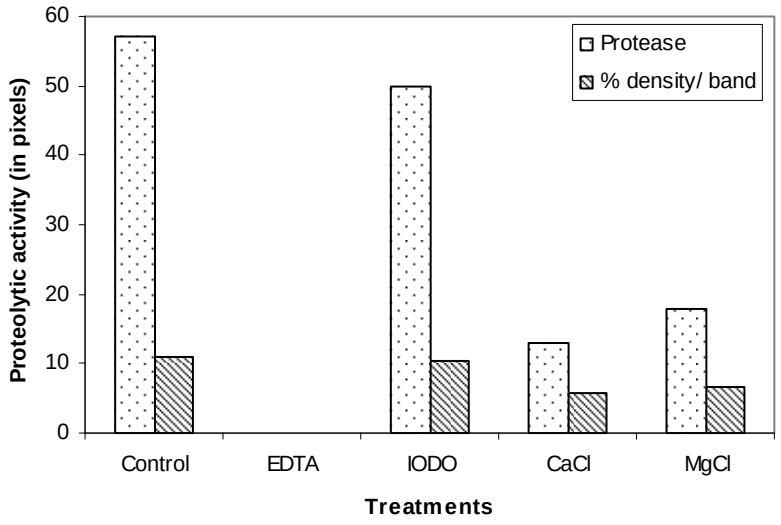


(b)

Figure 4.2 a, b: 1-DE native zymogram of proteases of *B. bassiana* after washing of conidia in 0.03% tween. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM iodoacetamide (IODO) (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).

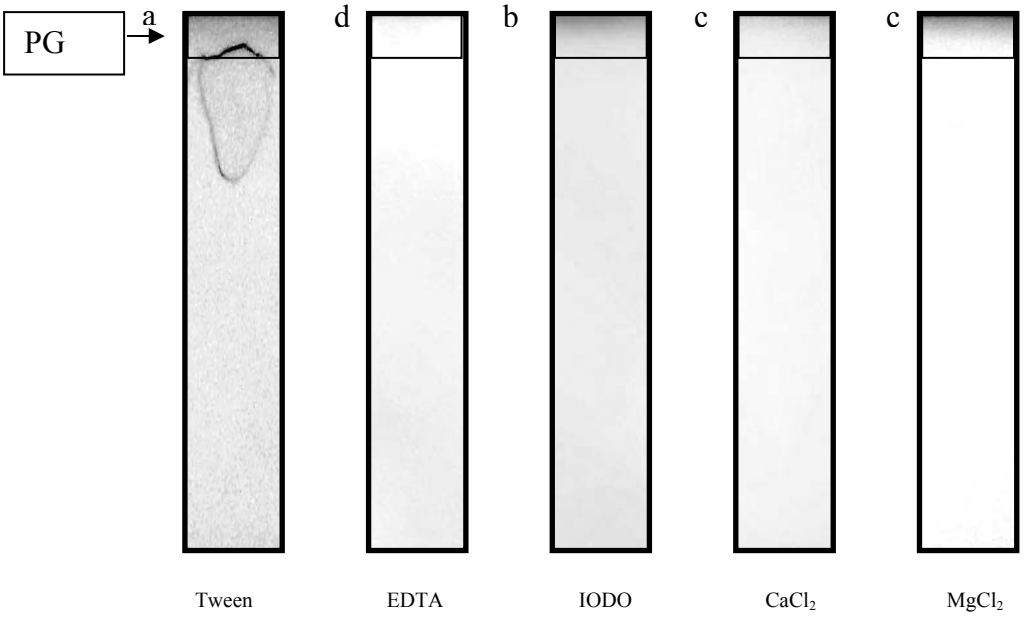


(a)

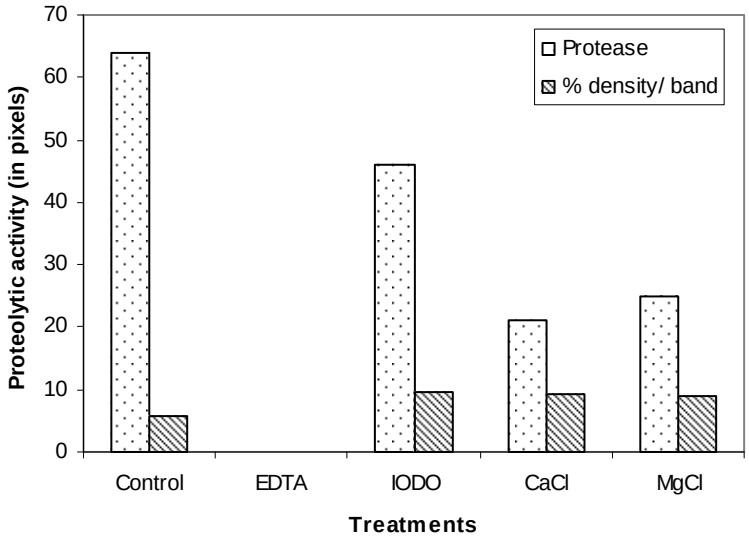


(b)

Figure 4.3 a, b: 1-DE native zymogram of proteases of *B. bassiana* after washing of conidia in (first wash) type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).

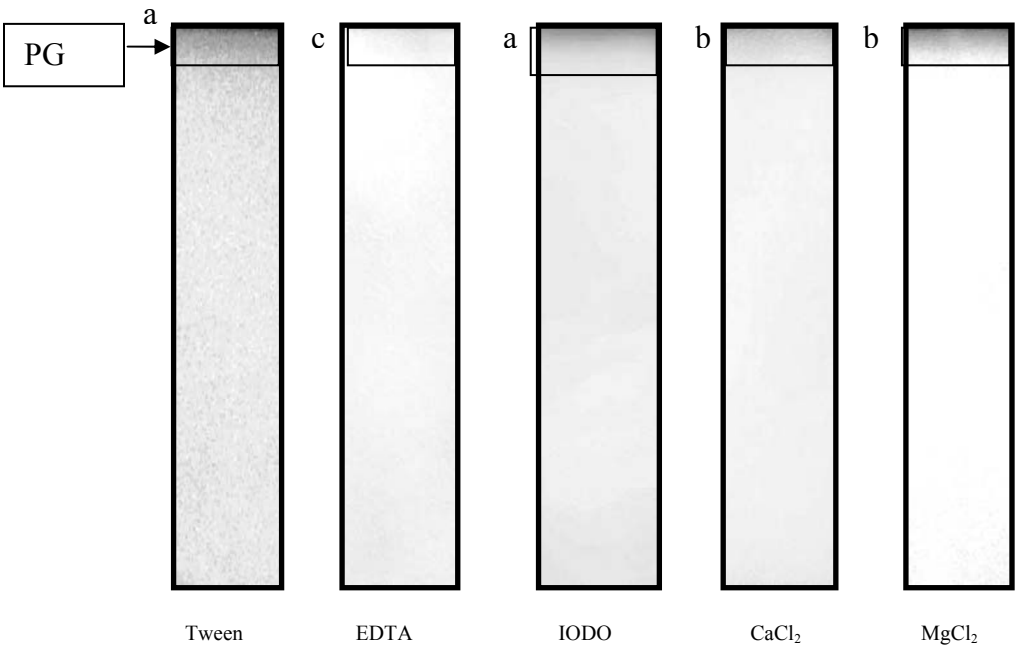


(a)

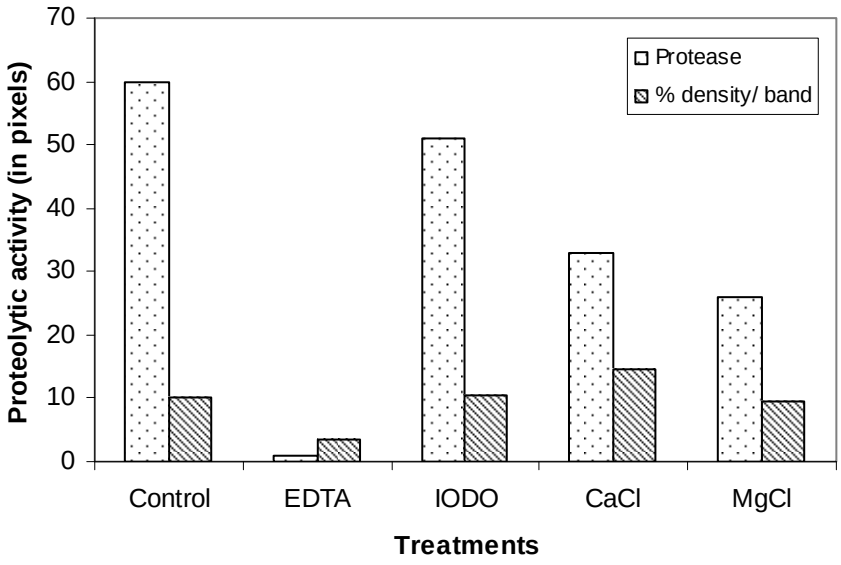


(b)

Figure 4.4 a, b: 1-DE native zymogram of proteases of *B. bassiana* after washing of conidia in (2^{nd} wash) type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).

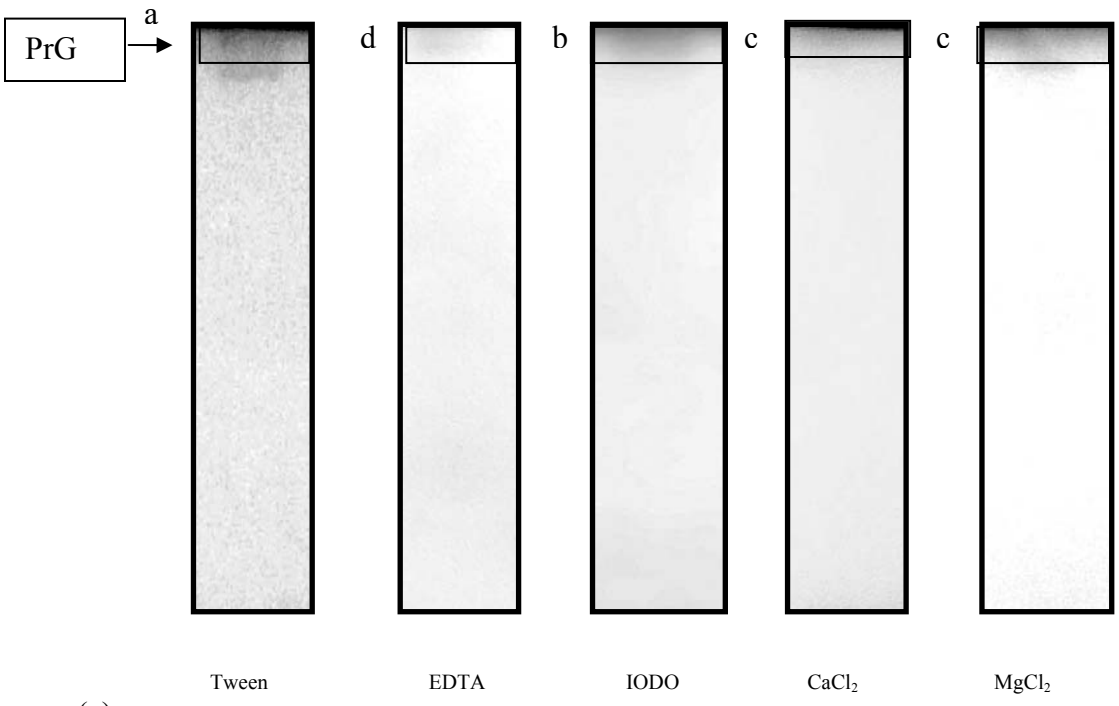


(a)

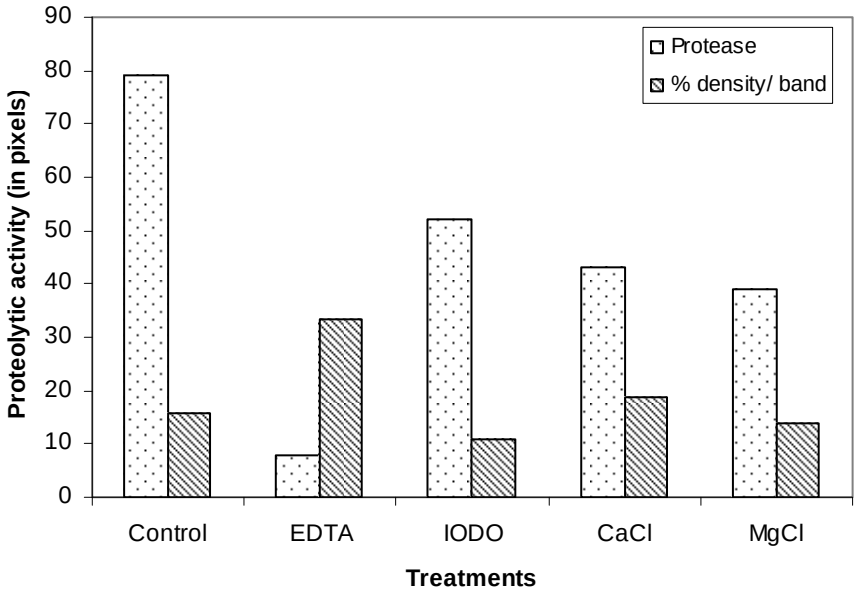


(b)

Figure 4.5 a, b: 1-DE native zymogram of proteases of *B. bassiana* after incubation of conidia at day 1 in type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).



(a)

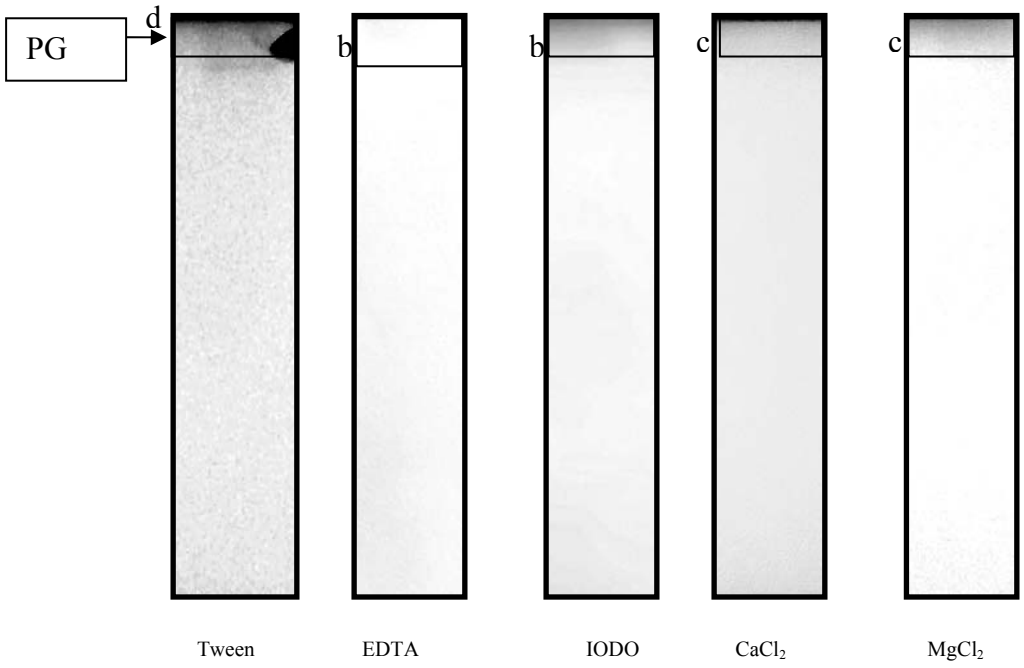


(b)

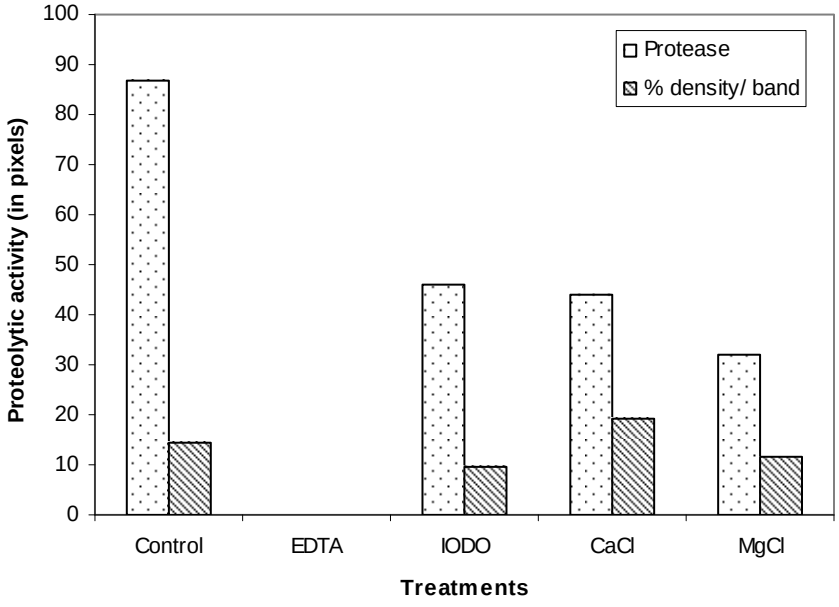
Figure 4.6 a, b: 1-DE native zymogram of proteases of *B. bassiana* after incubation of conidia at day 2 in type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).

of the band was evident with iodoacetaminde. Further treatment of the gel with $CaCl_2$ and $MgCl_2$ caused inhibition of the activity band. At day 3 (Fig. 4.7) inhibition of the activity band was evident with treatment of the gel strip with EDTA. Incubation of gel strip with iodoacetamide also caused approximately 50% inhibition of the band. Similarly, treatment of the gel with $CaCl_2$ and $MgCl_2$ showed 50% loss of the protease activity. At day 4 (Fig. 4.8) major protease activities in the band were of metalloproteases. Treatment of the gel strips with iodoacetamide, $CaCl_2$ and $MgCl_2$ were ineffective in causing inhibition or activation of the band.

The SDS PAGE zymography for *B. bassiana* was used to determine the molecular weights of protease secreted at various time intervals. The tween fraction (Fig. 4.9) showed three bands of Mr 100, 60 and 36.3 kDa, respectively. No band was observed during the washing steps of conidia with water and at day 1. It indicates that the SDS has irreversible effect during these steps. On the other hand after day 2 and 3, 25.4 - 25-kDa proteases were observed on the gels. However, after day 4 two bands detected on SDS PAGE zymography were 24.7 kDa and 20.3 kDa. The upper high molecular weight region had clearing region that was not identifiable as a band. 1-DE nzPAGE gel for first fraction with tween resulted in one band. After subjecting the same sample to SDS-PAGE zymogram it showed 3 bands. However, the subunits retained the activity in buffer supplemented with triton X during incubation period. The high molecular weight band appeared on the gel during washing treatments and incubation time. However, the low molecular weight band appeared only from day 2 and 3. At day 4 two bands of low molecular weights were observed on SDS PAGE gels.

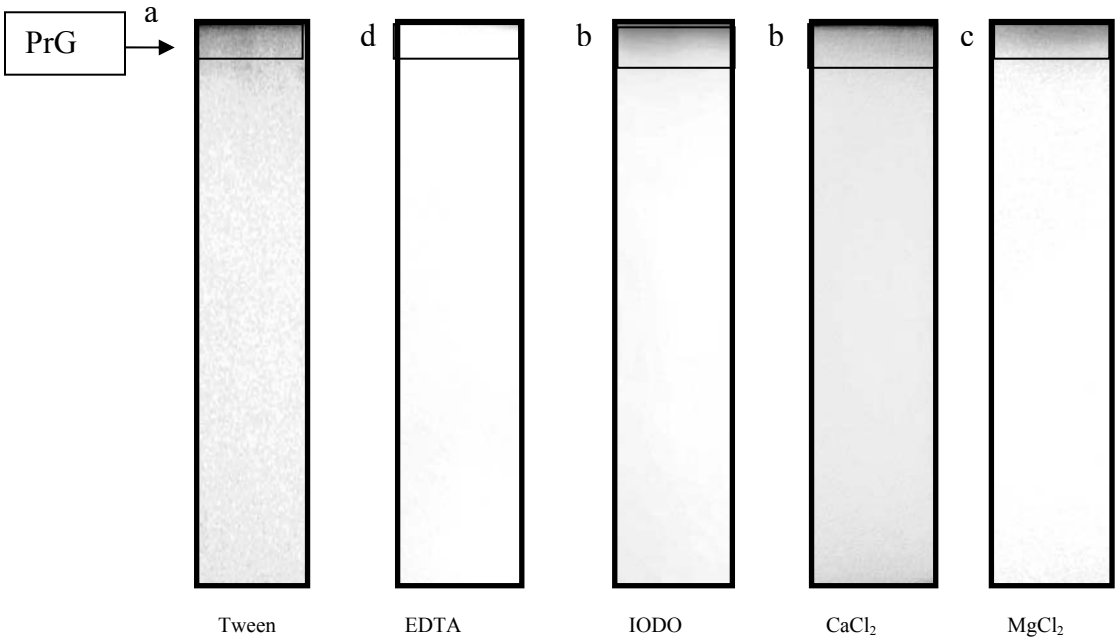


(a)

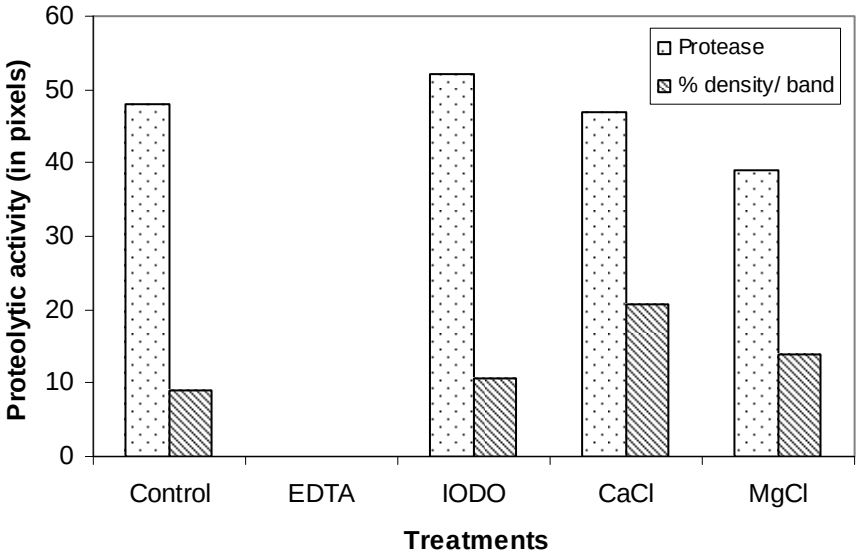


(b)

Figure 4.7 a, b: 1-DE native zymogram of proteases of *B. bassiana* after incubation of conidia at day 3 in type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).



(a)



(b)

Figure 4.8 a, b: 1-DE native zymogram of proteases of *B. bassiana* after incubation of conidia at day 4 in type II water. The gel strips showed the activities of proteases after incubation as follows: (i) control gel (ii) 10 mM EDTA (iii) 10 mM IODO (iv) 10 mM $CaCl_2$ and (v) 10 mM $MgCl_2$. b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to protease bands between different treatments are not significantly different ($p < 0.05$).

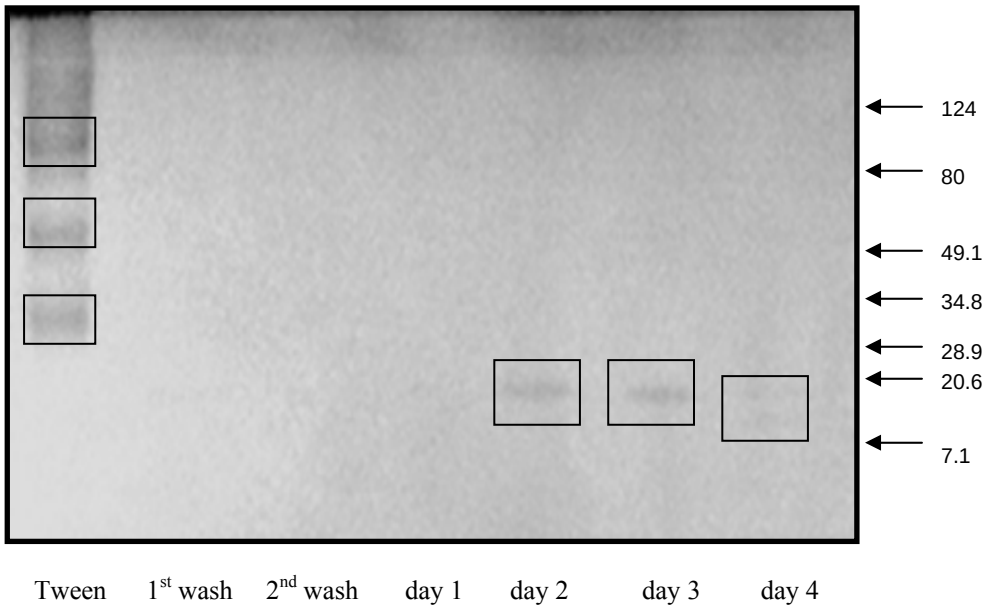


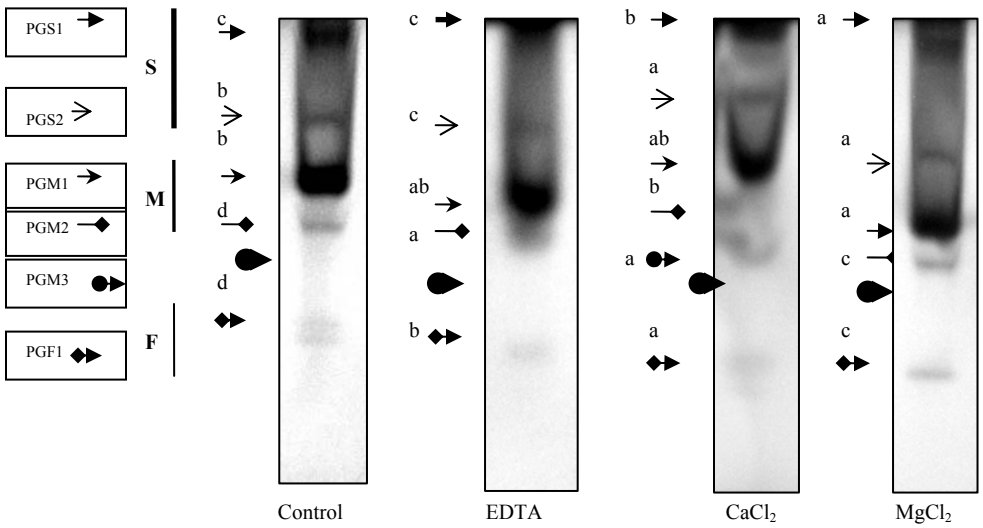
Figure 4.9: SDS PAGE (7.5%) zymography of *B. bassiana* proteases. Treatment of conidia with tween revealed three bands of Mr 100, 60 and 36.3 kDa. Incubation of supernatant at day 2 and 3 revealed two bands of 25.4 and 25 kDa, respectively. However, at day 4, two bands appeared on gel of Mr 24.7 and 20.3 kDa. Bio-Rad broad range standards (kDa) are indicated on right.

4.4.2 1-DE zymography of *M. anisopliae*

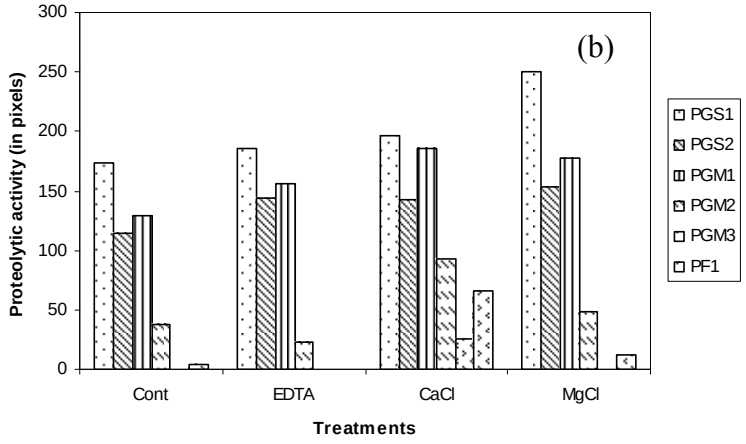
Conidia of *M. anisopliae* (MA 2038) under go through swelling phase before germ tube formation in YPG liquid medium (Uribe, 2004). During the present investigation conidia of MA 2038 showed swelling in type II water as measured by Multisizer- III^{TM} (Coulter Counter^{TM}) (Chapter 7; section 7.4.1). Hydration of the spores did not induce germ tube formation due to the absence of nutrients. Therefore, the incubation of spores in water represented a nutritional starvation allowing us to examine the release of exoenzymes under aforementioned conditions. A new gel based technology was adopted to study the release of isozymes because of its sensitivity and high throughput screening ability in comparison to conventional purification or chromatographic separations. The isozymes were compared among different treatments on the basis of relative mobility on 1-DE gels after Weiland (2004). In this case, three broad categories of isozymes were scored semiquantatively as slow (PGSn), medium (PGMn) and fast (PGFn) (where n denotes the band position on the gel with reference to its mobility) due to their mobility on gels. The gels were cut into individual strips and aligned with the control strip for the ease of comparison and clarity of presentation.

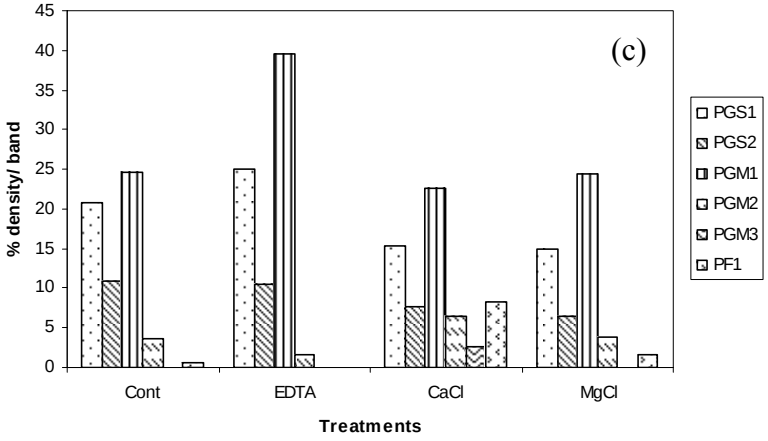
Following washing of conidia in 0.03% tween, five isozymes of gelatinase each with different proteolytic activities appeared on the control (untreated) gel strip (Fig. 4.10a-c). This demonstrated that each isozyme had different gelatin degrading ability and could be identified on native gel because of its relative motility due to difference in charge-to-mass ratio. The same sample strips were incubated with metalloprotease inhibitor EDTA and activators like $CaCl_2$ and $MgCl_2$. 2-D spot densitometry revealed that the protease activity was significantly altered by EDTA, $CaCl_2$ and $MgCl_2$ for slow (PGS1, PGS2) and medium migrating isozymes (PGM1,

Figure 4.10a-c: 1-DE native zymogram of proteases of *M. anisopliae* after washing of conidia in 0.03% tween. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGM3) on gel strip is indicated as (◀). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
PGS2
PGM1
PGM2
PGM3
PGF1
S
M
F
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
300
250
200
150
100
50
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGM1
PGM2
PGM3
PF1

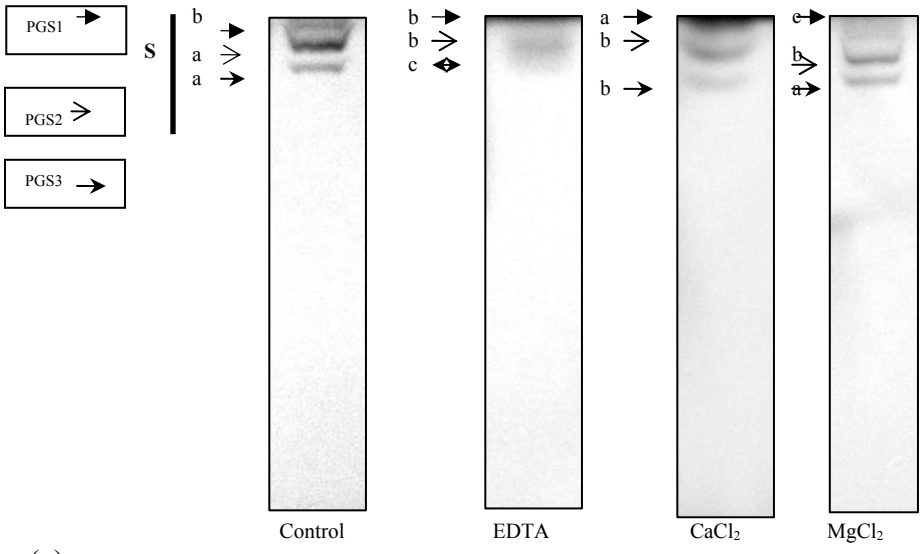

(c)
% density/ band
45
40
35
30
25
20
15
10
5
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGM1
PGM2
PGM3
PF1

PGM2). Furthermore, addition of $CaCl_2$ at 10 mM activated a third isozyme (PGM3) not observed in control or treated strips.

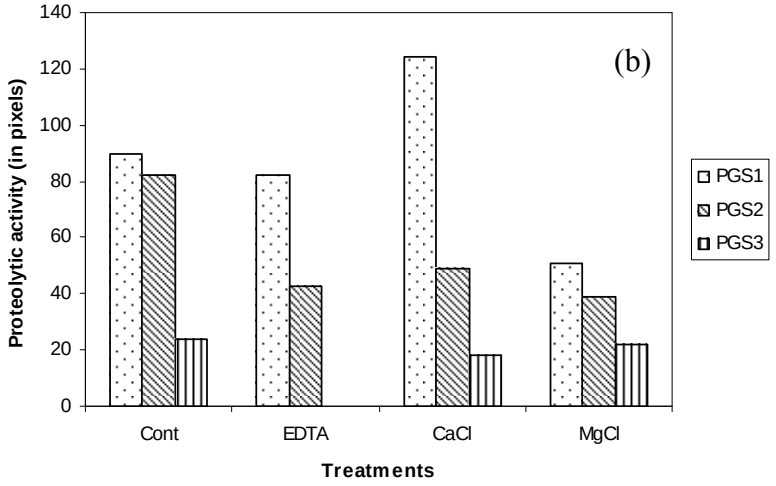
During the first water wash of conidia three isozymes were identified on control strips with different proteolytic units (PUs) as determined by 2-D spot densitometry (Fig. 4.11a-c). Similar strip upon incubation with inhibitors and activators yielded different relative intensities/proteolytic units. Incubation of the gel strip with EDTA inhibited one of the isozyme (PGS3) altogether indicating its identity as metalloprotease. Second washing step of conidia showed only one isozyme (PGS1) which was activated by the $CaCl_2$ in the buffer during incubation. Second washing of conidia in water revealed only one PGS1, which was activated by $CaCl_2$ as can be appreciated from Fig. 4.12a-c.

After the second wash conidia were incubated in type II water at 27° C on shaker bath at 150 rpm. At day 1 three isoforms (PGS1-PGS3) were identified on control strips (Fig. 4.13a-c). Incubation of the similar sample with EDTA caused inhibition for PGS1 and PGS2. Conversely, $CaCl_2$ acted as a strong stimulator for both these isozymes. Furthermore, treatment with $CaCl_2$ induced or activated the PGS3, not stimulated by $MgCl_2$. At day 2 three slow migrating isozymes (PGS1-PGS3) of gelatinase appeared on gel strips (Fig 4.14a-c). However, the proteolytic activity of those isoforms differed as indicated by spot densitometry results. All three activities were inhibited by the EDTA, whereas $CaCl_2$ showed stimulatory effects on PGS1 and PGS2. Isozyme, PGS3 was not observed when gel was treated with EDTA. Interestingly, a slight activation for this isoform was observed by the addition of $CaCl_2$. Incubation of conidia at day 3 presented an unusual pattern of inhibition by EDTA for PGS1 and PGS2, respectively as can be appreciated from Fig 4.15a-c. However, metal ions were unable to create any stimulatory effect on those isozymes.

Figure 4.11a-c: 1-DE native zymogram of proteases of *M. anisopliae* after washing of conidia (first wash) in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGS3) on gel strip is indicated as (◆). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments


PGS1
PGS2
PGS3
S
b
a
a
b
b
c
a
b
b
c
b
a
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
140
120
100
80
60
40
20
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

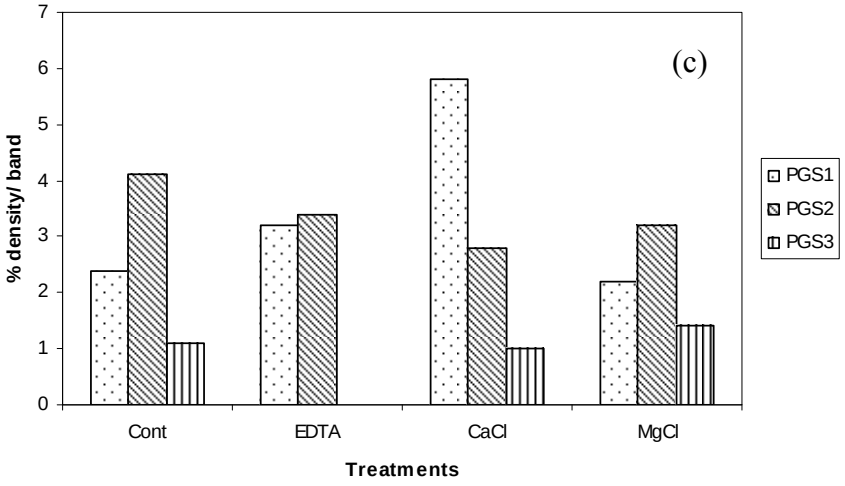
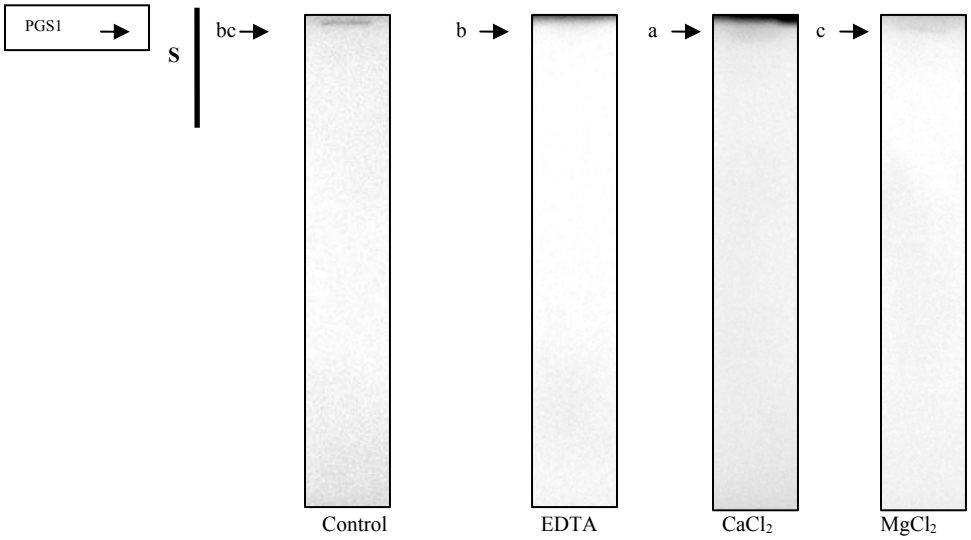
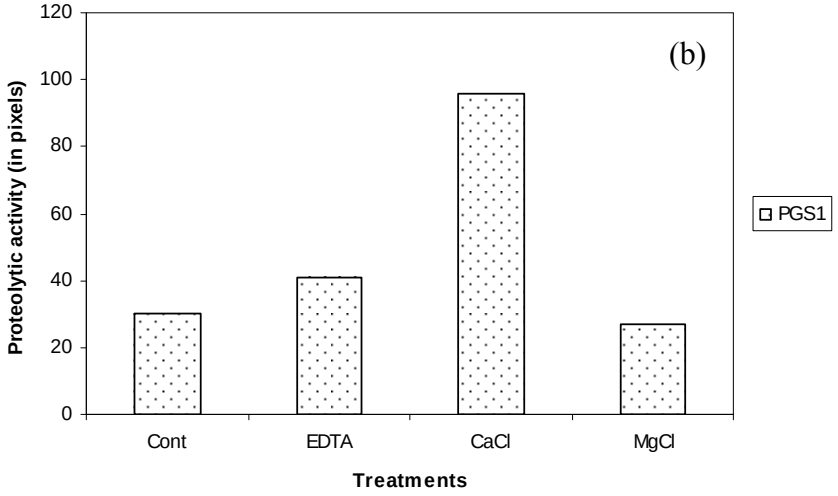

(c)
% density/ band
7
6
5
4
3
2
1
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

Figure 4.12a-c: 1-DE native zymogram of proteases of *M. anisopliae* after washing of conidia (second wash) in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
S
bc
b
a
c
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
120
100
80
60
40
20
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1

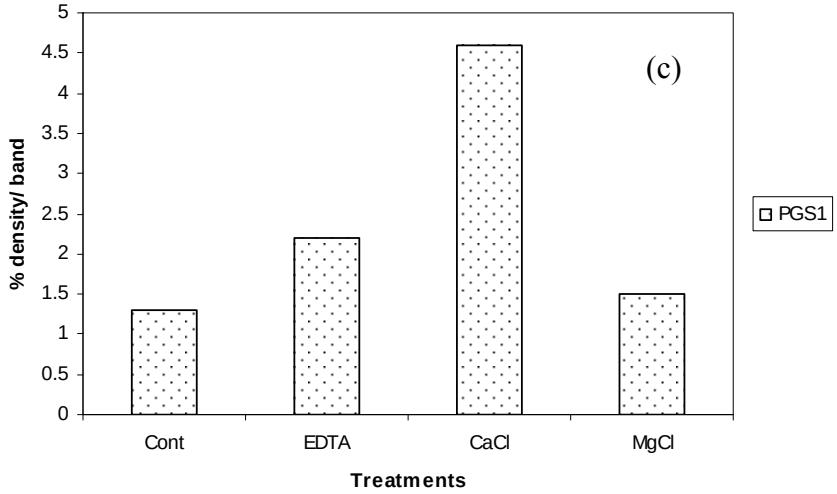
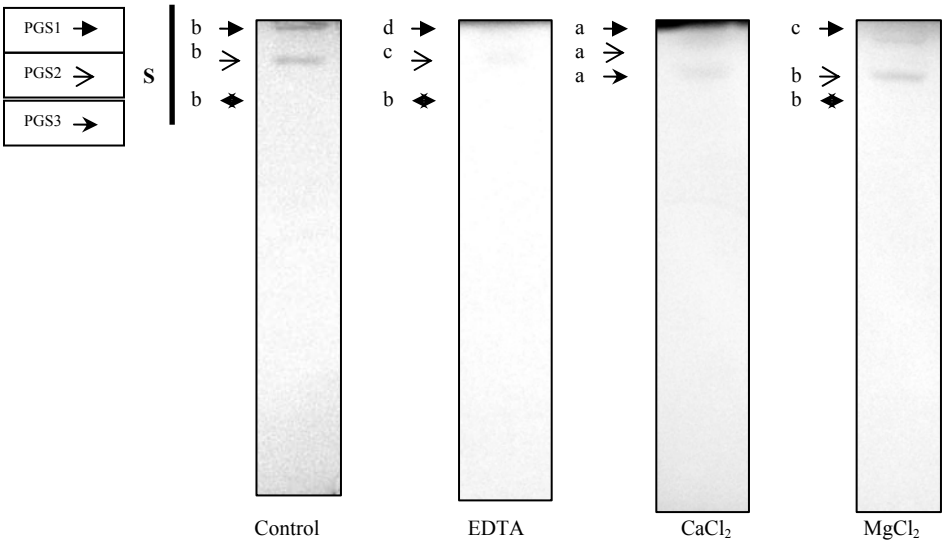
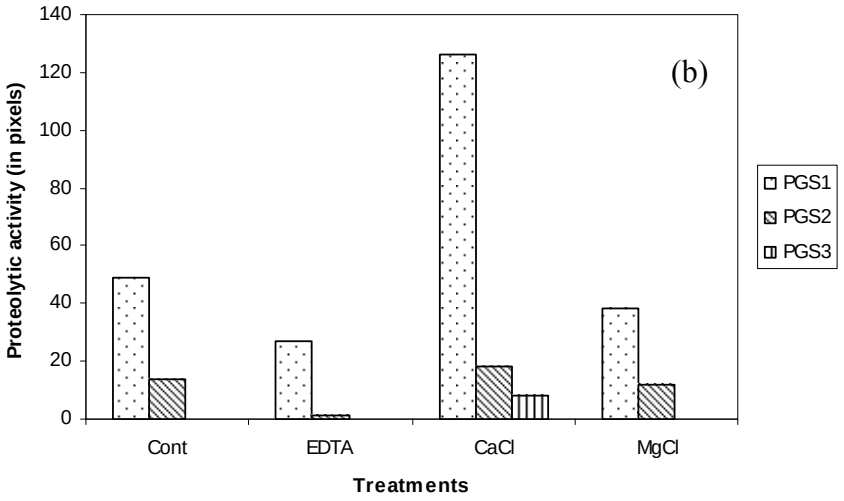

(c)
% density/ band
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1

Figure 4.13a-c: 1-DE native zymogram of proteases of *M. anisopliae* after incubation of conidia at day 1 in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGS3) on gel strip is indicated as (◂▸). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
PGS2
PGS3
S
b
b
b
d
c
b
a
a
a
c
b
b
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
140
120
100
80
60
40
20
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

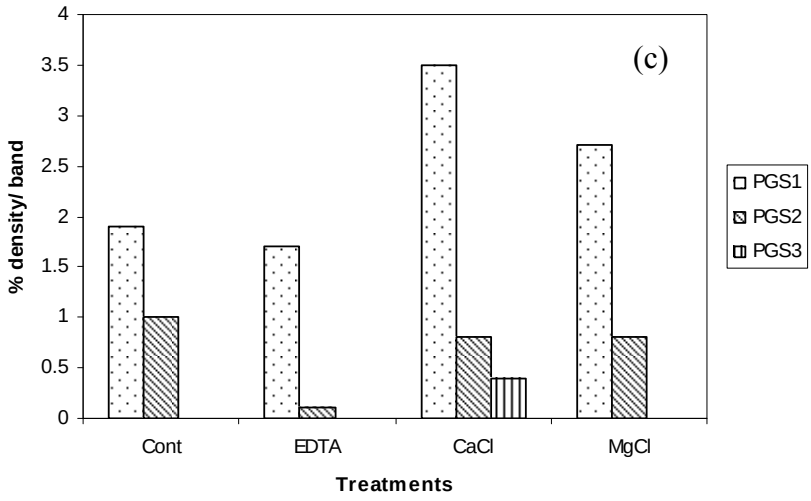
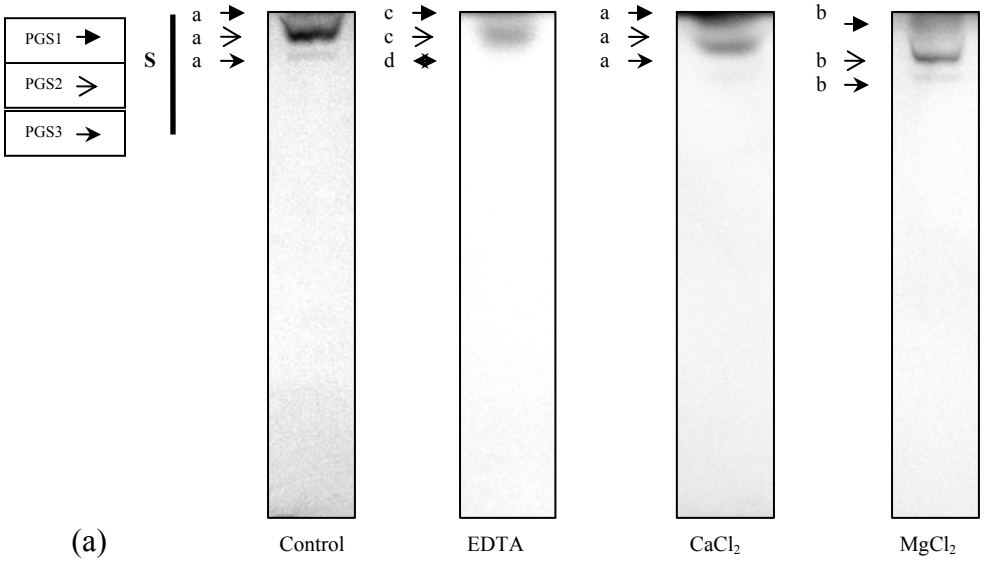
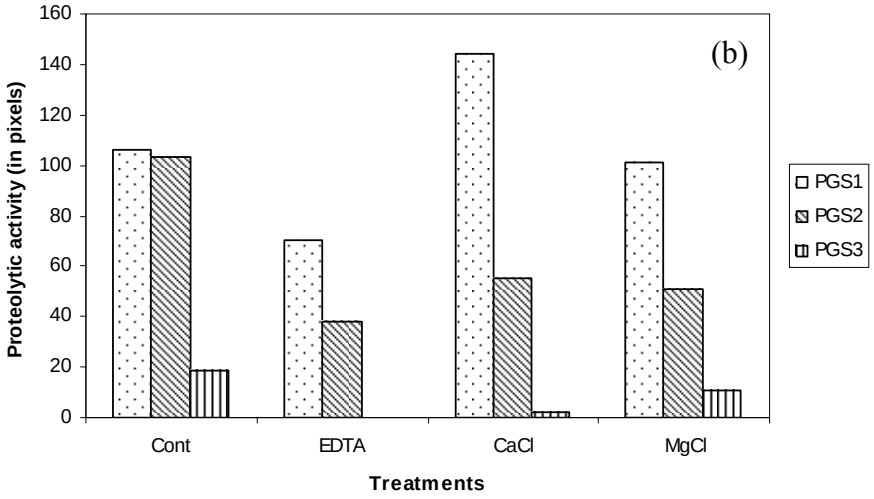

(c)
% density/ band
4
3.5
3
2.5
2
1.5
1
0.5
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

Figure 4.14 a-c: 1-DE native zymogram of proteases of *M. anisopliae* after incubation of conidia at day 2 in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGS3) on gel strip is indicated as (◂▸). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
PGS2
PGS3
S
a
a
a
c
c
d
a
a
a
b
b
b
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
160
140
120
100
80
60
40
20
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

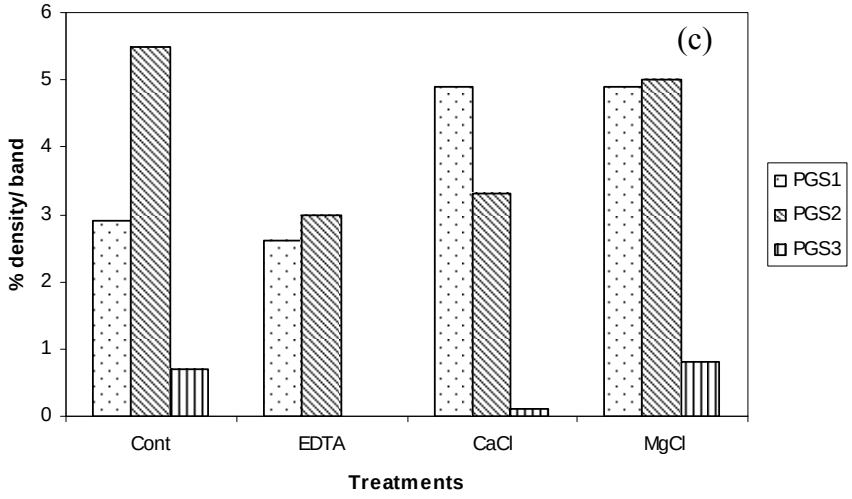
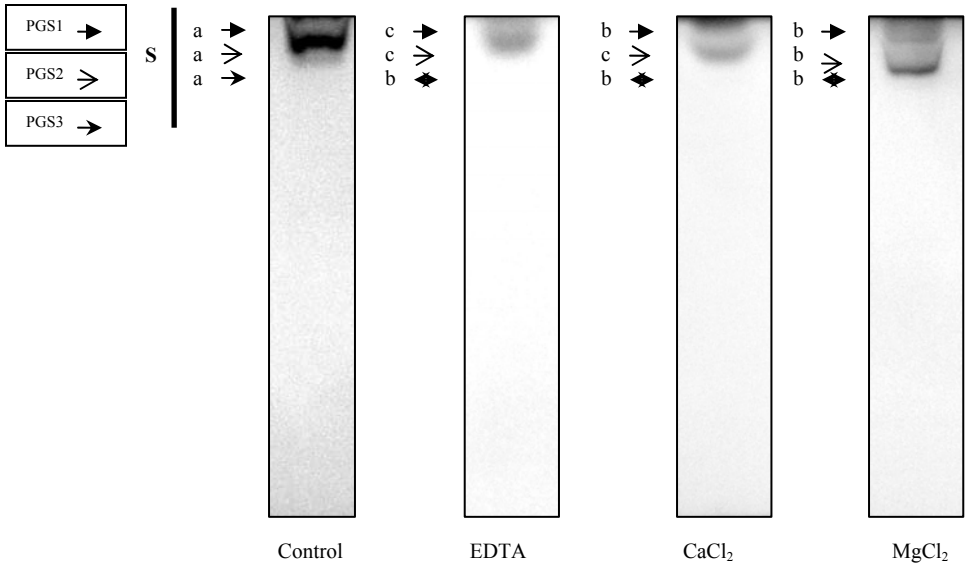
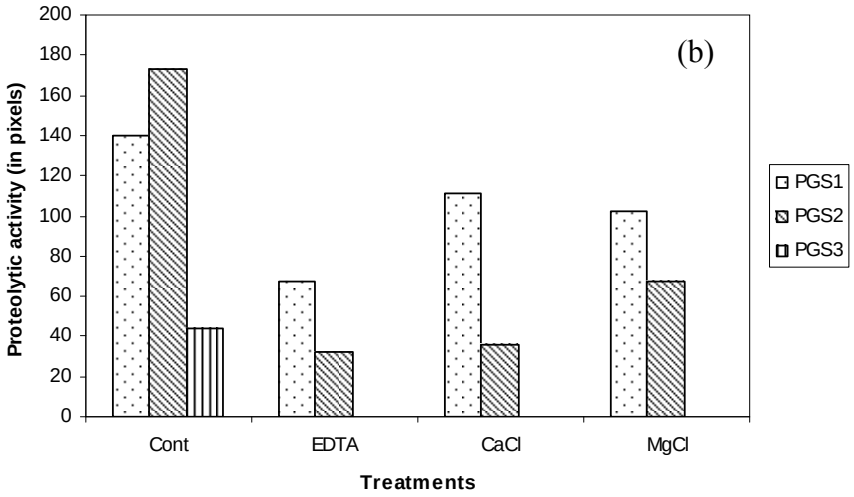
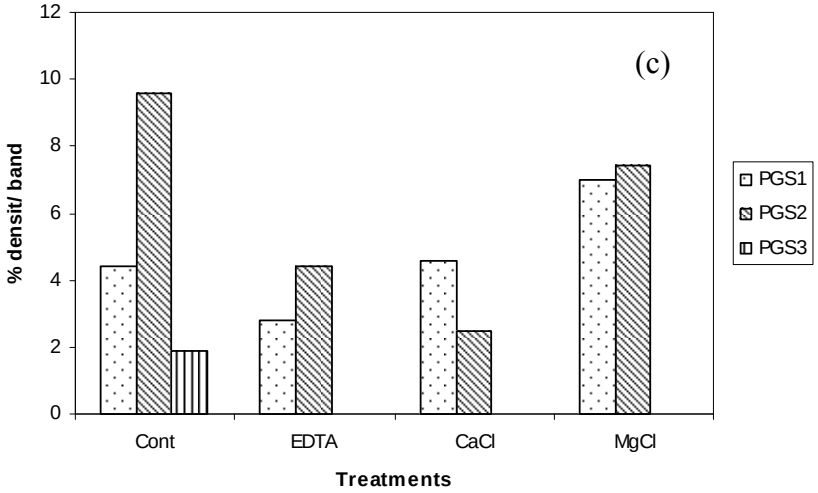

(c)
% density/ band
6
5
4
3
2
1
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

Figure 4.15a-c: 1-DE native zymogram of proteases of *M. anisopliae* after incubation of conidia at day 3 in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGS3) on gel strip is indicated as (◆). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
PGS2
PGS3
S
a
a
a
c
c
b
b
c
b
b
b
b
Control
EDTA
$CaCl_2$
$MgCl_2$

(a)


(b)
Proteolytic activity (in pixels)
200
180
160
140
120
100
80
60
40
20
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

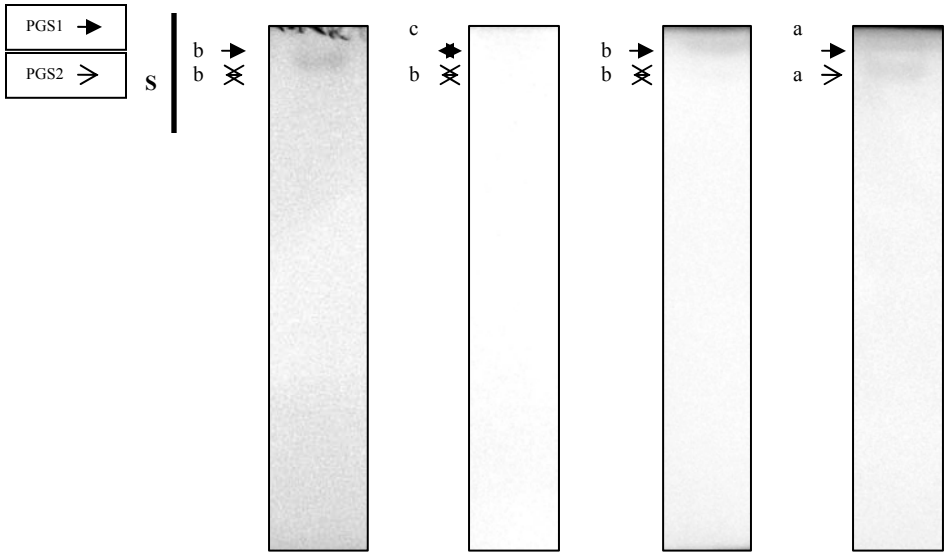

(c)
% densit/ band
12
10
8
6
4
2
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2
PGS3

Similarly, slow migrating isozyme PGS3 was not revealed on gel by the addition of EDTA, $CaCl_2$ and $MgCl_2$ to incubation buffer. This kind of inhibition pattern may indicate the presence of other released proteases (serine/trypsin/cysteine), which could not be separated by 1-DE zymography as a separate band. Incubation of conidia at day 4 resulted in the appearance of two slow migration isozymes, PG1 and PGS2 (Fig. 4.16a-c). Moreover, PGS1 was absent when treated with EDTA. Conversely, $MgCl_2$ activated another isoform of slow migrating isozyme (PGS2).

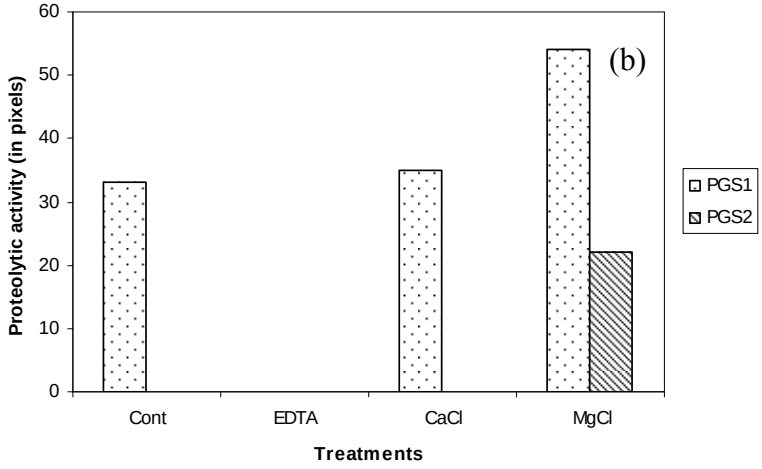
Use of SDS PAGE with copolymerized gelatin helped determination of the relative molecular weights of the enzymes released. As seen in Fig 4.17 that the treatment of conidia with tween released two protein bands of Mr 103 and 12 kDa. During first washing step the relative molecular size of the protein was identified at 12.1 kDa. No bands were observed after second washing step and after day 1, indicating the irreversible effects of SDS. However, the protease identified after day 2 and day 3 were of Mr12.2 and 12.4 kDa, respectively.

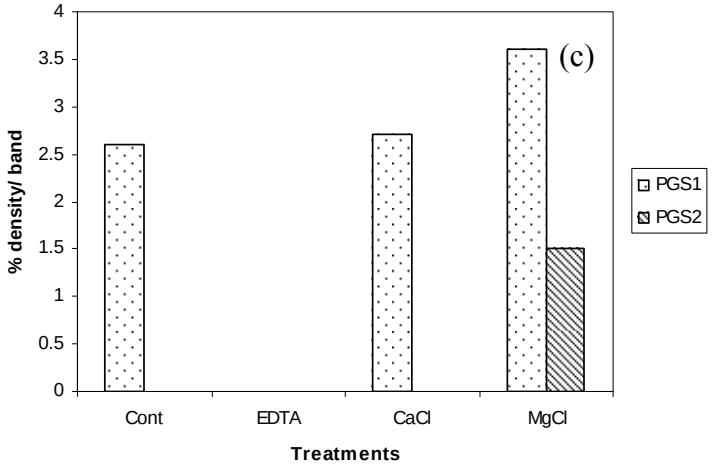
The pattern of the secretion/release of protease isoforms differed between control and the gels treated with the protease inhibitor EDTA and metal ions. There was a difference in the appearance of protein band patterns (presence/absence of band) as well as levels of activity (Percentage proteolytic activity/band measured in pixels by spot densitometry). The number and migration of bands during washes (tween and water) and incubation period (days 1- 4) was different, which may indicate that the isozymes have different size and charge to mass ratios that appeared on gel with different relative mobility/migration, presumably due to post translational modifications. Furthermore, these protease activities were detected without concentrating the enzyme filtrate, indicating that the isozymes were abundantly released by conidia with washing effects of tween, water and upon incubation till day 4.

Figure 4.16a-c: 1-DE native zymogram of proteases of *M. anisopliae* after incubation of conidia at day 4 in type II water. The gel strips showed the activities of protease isozymes (indicated by arrows of different shapes) after incubation as follows: (i) control gel, (ii) 10 mM EDTA, (iii) 10 mM $CaCl_2$, and (iv) 10 mM $MgCl_2$. Each arrow shape represents a specific protease activity on each strip with reference to its migration on gel strip. Same alphabets appearing adjacent to arrows (specific shape) between different treatments are not significantly different. The level of significance was determined by one way completely randomized ANOVA design. Means were separated by using Duncan's methods where $p < 0.05$. Absence of band (PGS1 and 2) on gel strip is indicated as (✦, ⋇). (b) Proteolytic units as determined by 2-D spot densitometry scans represented in pixels after incubation of gel strip with inhibitors and activators as indicated. (c) Percentage density of each band enclosed in a box, eclipse or free hand drawing after back ground subtraction. Results are the representative of three different experiments.


PGS1
PGS2
S
b
b
c
b
b
b
a
a
Control
EDTA
CaCl2
MgCl2

(a)


(b)
Proteolytic activity (in pixels)
60
50
40
30
20
10
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2


(c)
% density/ band
4
3.5
3
2.5
2
1.5
1
0.5
0
Cont
EDTA
CaCl
MgCl
Treatments
PGS1
PGS2

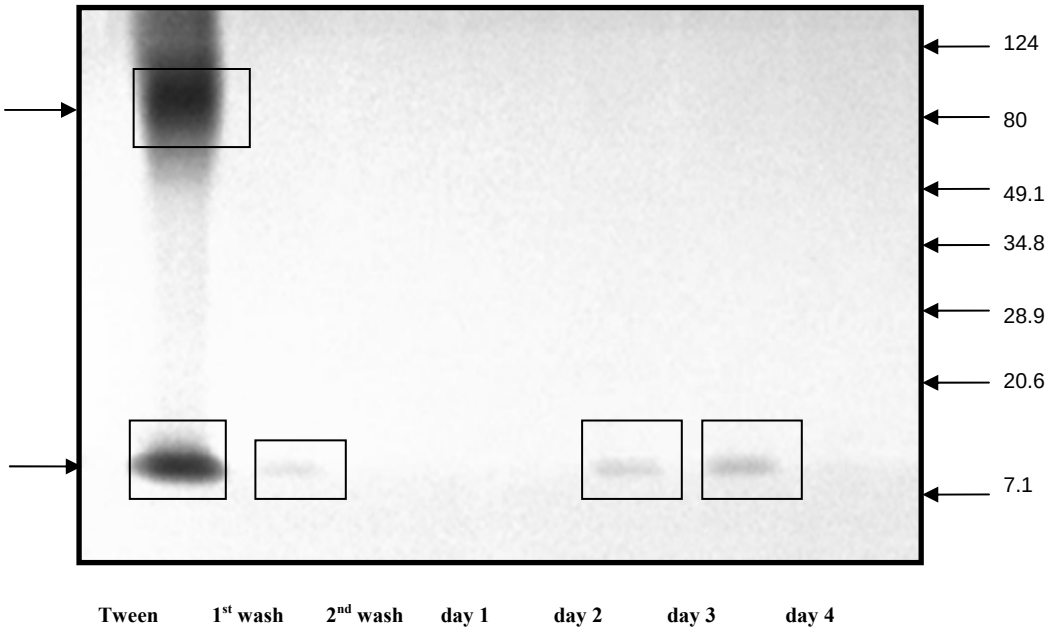


Figure 4.17: SDS PAGE (7.5%) zymography of *M. anisopliae* proteases. Bio-Rad broad range standards (kDa) are indicated on right. Upper and lower arrows indicate 103 and 12 kDa bands, respectively.

4.5 Discussion

Proteases are key virulence determinants for the infection process of entomopathogenic fungi (Clarkson and Charnley, 1996; Khachatourians et al., 2007). Amongst EPF, *M. anisopliae* produce multiple protease isozymes during the host-pathogen interaction (St. Leger, 1995). However, there is ultrastructural evidence that the degradative effects of the pathogen enzymes are limited to the vicinity of conidia (Zacharuk, 1970), mucus (Brey et al., 1986) and germ tubes (Persson et al., 1984). Ironically, fungal spore associated enzymes have received little attention despite their vital role. David (1967) provided histochemical evidence that the modification of the epicuticle occurred before the germ tube emergence. St. Leger (1987) reported aminopeptidase being secreted was localized and concentrated in the vicinity of *M. anisopliae* infection propagules. Similarly, Goettel et al. (1989) showed that *M. anisopliae* chymoelastase (Pr1) binds directly to the cell wall. However, much of these works has been with the appressorial or mycelial stages and therefore do not account for pre-germination events, that are, from spores to pre-appressorial stage (St. Leger et al., 1986a; Bidochka and Khachatourians, 1987).

The present study revealed the presence of multiple metalloproteases associated with the spores of the two EPF. Conidial proteases were released during the pre-germination phase or swelling phase. Metabolic activation in de-ionized water soaked conidia (pre-germination phase) for *N. crassa* and *M. anisopliae* was described respectively by Schmit and Brody (1976) and Dillon and Charnley (1985, 1990). Swelling due to hydration is the first sign of initiation of germination of conidia of *B. bassiana* and *M. anisopliae* (Bidochka et al., 1987; Uribe, 2004).

Soaking of conidia in water resulting in conidial swelling must simulate the pre-germination phase to become metabolically active (Chapter 6 and 7). Conversely, Dillon and

Charnley (1985, 1990) reported that unless provided exogenous nutrients the conidia of EPF do not swell in de-ionized water. Results presented here demonstrate the complexity of events associated with swelling phase, which may have implications for the initiation of infection process. Hydrated conidia, by releasing metalloprotease isozymes in addition to Pr1, provide a back-up system to enhance the potential for degrading the epicuticle/cuticle. This system would maximize the potential substrate for conidial contact, adherence and breaching.

The results revealed the variability in protease secretion from the spores of two EPF. In *B. bassiana,* only one band was evident on the 1-DE native gel. Use of the inhibitor/activator in conjuction with zymography revealed that the major protease released by *B. bassiana* was metalloprotease. The inhibition with EDTA, $CaCl_2$ and $MgCl_2$ is unusual. However, it is possible that the secreted protease belong to Zn-containing metalloprotease, which require $ZnCl_2$ as an activator. Inhibition with various metal ions of elastinolytic protease from *A. flavus* protease has been reported (Rhodes et al., 1990). A number of bacterial metalloproteases, e.g., elastase of *Vibrio vulnificus* and the neutral proteases of *Legionella pneumophilia* and *Serratia marcescens,* showed similar results with metal ions (Dreyfus and Iglewski, 1986; Kothary and Kreger, 1985; Lyerly and Kreger, 1979). In *M. anisopliae,* the protease isozymes identified by native zymography in the water supernatants were stable up to day 4. This represents an important functional feature of the *M. anisopliae* secreted proteins, which were stable and may have important role in the infection process. In *B. bassiana*, SDS zymography indicated that the initial secreted proteases had a molecular mass of greater than 36.3 kDa. Whereas, the protease present during the washing steps with water and incubation period was of approximately 20 to 25 kDa. This may represent autolytic cleavage of the parent molecule. Similarly, Mellon and Cotty (1996) have found a 23-kDa elastinolytic protease from *A. flavus* after degradation of 35-kDa

parent protein molecule. The 23-kDa protease was thermostable at 70° C (in comparison to the parent molecule) and had retained its activity in concentrated form upon storage at 4 °C for six months. Kobayashi et al. (1996) also reported that the degraded products of the alkaline protease from *Bacillus* strain were highly stable upon storage. Contrary to this, SDS PAGE zymography of *M. anisopliae* samples revealed two isozymes of Mr 103 and 12 kDa after treatment of spores with tween. However, during the first washing step with water and incubation of spores (at 48 and 72 h) only a 12 kDa protein retained its activity implying that it was stable in its activity upon incubation up to day 3.

Using a functional proteomic approach, release of four and eight isozymes of gelatinase from the conidia of *B. bassiana* and *M. anisopliae,* respectively, during hydration (0.03% tween wash) were identified (Chapter 5). The reported pI values of gelatinase isozymes in the aforementioned investigation represented activities resembling Pr1 and Pr2 on the basis of similarity in substrate specificity and pI to isozymes from mycelia of *M. anisopliae* (St. Leger et al., 1994). In a similar study, they reported one metalloprotease of pI 7.3 from *M. anisopliae* mycelial inocula during growth on chitin-based liquid medium. Indeed, 5-6 metalloprotease isozymes were identified from the mycelial inocula of *M. anisopliae* induced at pH 6 upon growth on cockroach cuticle (St. Leger et al., 1998). The complexity of isozymes observed here (in type II water) reflects the ability of *M. anisopliae* to respond to the environmental conditions of the host without the involvement of growth substrate. More importantly, metalloproteases could act a back-up system to complement the Pr1, which is considered as key virulence determinant. As stated by Powers and Harper (1986) metalloproteases have unique specificities, which allow them to catalyze certain peptide bond cleavages more efficiently than any other types of proteolytic enzymes suggest that metalloproteases can help evasion of protein moieties

in the cuticle/epicuticle. Further multiple proteases of different types could complement each other and hence digest most protein moieties present in the cuticle.

Results also indicated that hydrated conidia of EPF have the potential to modify the epicuticle by metalloproteases, and not by single protease serine as believed. The ease with which the enzymes associated with the hydrated conidia were released by incubation in water suggests that enzymes are located at the surface layers of the spores and probably mimic the release of enzymes under natural conditions. In this context, St. Leger et al. (1991) were the first to show time that the higher level cell bound enzymes (Pr1, NAGase and esterases) were released from the conidia of infected *Manduca sexta* cuticle in comparison to those harvested from sabouraud dextrose agar (SDA) upon washing in buffer, detergents and other chemicals. The role of exoenzymes during germination from the conidia of *N. crassa* has been reviewed (Schmit and Brody, 1976). Small and Bidochka (2005) have reported the up-regulation of *Pr1* (*cag* genes) in *M. anisopliae* at appressoria formation and upon conidiation. It is tempting to speculate that the diversity of proteases observed in this study may also be similar to other un-identified up-regulated protease genes during conidiation. As there is no precedent to this study therefore a definitive study leading to the characterization of other upregulated genes and proteins during conidiation is still an open and important question.

The diversity of isozymes found during hydration indicated that *M. anisopliae* is pre-adapted to its multiple hosts and environments, further contributing to the complexity of the host pathogen interaction. Endowed with multiple isozymes, the fungus can circumvent protease inhibitors present in the insect cuticle (Yoshida et al., 1990) and hemolymph (Frobius et al., 2000; Tong and Kanost, 2005). Moreover, the diversity of isozymes may offer a selective advantage in exploring new habitats (substrates) either as pathogen or saprophyte. For example,

features that determine specificity towards different insect taxa would co-relate to different chemical composition of the insect cuticle. Results showed that conidial bound multiple isozymes can upon hydration have the potential to act as a back-up system for Pr1 and Pr2. They can degrade cuticle by synergistic action or have an advantage of attacking different substrates because of their different binding ability. Indeed, this is the first report clarifying that conidia are endowed with a battery of proteases to start the infection process under extreme starvation conditions/swelling phase. Hence, conidial proteases can degrade insect integument prior to the formation of germ tube or appressoria.

5.0 Functional proteomic analysis of multiple protease isozymes from the genera, *Beauveria* and *Metarhizium* by using combination of IPG strip and two-dimensional zymography

5.1 Abstract

A new method for resolving protease isozymes of two entomopathogenic fungal species is described. This method uses of two-dimensional (2-DE) zymography with copolymerized gelatin and a functional proteomic approach for the detection of protease isozymes. Using a combination of immobilized pH gradient (IPG) strip and non-reducing 2-DE zymography protease isozymes (Gelatinase) were collected from the conidia of *Beauveria bassiana* (GK 2016) and *Metarhizium anisopliae* (MA 2038) after washing with 0.03% tween (w/v). Protein samples prepared in 2% carrier ampholyte were compared with 2% CHAPS in combination with 2% carrier ampholyte. Preparation using carrier ampholyte showed one and three isozymes with samples of isolates GK 2016 and MA 2038, respectively, whereas four and eight isozymes appeared on gel when carrier ampholyte in combination with CHAPS was used. Use of 2% CHAPS in combination with 2% carrier ampholyte was found to be crucial to resolve protein during IPG strip and 2-DE zymography. Copolymerized gelatin zymograms were more sensitive than overlay indicator gels. The combined use of IPG strip and 2-DE zymography should be a versatile and powerful tool to detect proteases. This is the first report of protease isozymes from hydrated conidia resembling acidic, Pr1, Pr2 and MPr activities on the basis of pI and substrate specificity. The role of the protease isozymes in relation to fungal pathogenicity is discussed.

5.2 Introduction

Proteases and their isozymes are key virulence determinants of fungal infection process of entomopathogenic fungi (EPF), *B. bassiana* and *M. anisopliae*. They help to degrade insect cuticle, facilitate penetration and promulgate secondary infection (Khachatourians, 1991; Charnley and St. Leger, 1991; Clarkson and Charnley, 1996; Butt et al., 1998; Khachatourians, 1996). Proteases have been isolated from fungi and higher eukaryotes that differ in their pI, thermostability, pH optima, pH stability and physico-chemical properties (North, 1982; Rao et al., 1998). Ironically, spore associated enzymes have received little attention whereas most of the proteases isolated and characterized to-date are from mycelia. Among the well-characterized proteases from EPF, chymoelastase (Pr1) or subtilisin-like protease, act as a first line molecular arsenal against cuticular proteins and help evade the chitin monomers (St. Leger, 1995) because of their basic pI and adsorption properties (Bidochka and Khachatourians, 1994). Metalloprotease (pI 7.3) is considered to be a back-up enzyme for cuticle evasion (St. Leger, 1994; St. Leger et al., 1998). However, the role of trypsins (Pr2) is poorly understood during fungal infection processes and it is regarded as a back up system for Pr1 or in the processing of Pr1 (St. Leger et al., 1996a). On the other hand, there is a large accumulation of data from ESTs and microarray approaches suggesting that there may be 11 proteases (subtilisins) or more excreted by *M. anisopliae* (Bagga et al., 2004). Such EST-based data could not delineate the functional role and the diversity of proteases as protein is a dynamic entity. The majority of such enzymes were isolated from mycelial inocula, which has little relevance to the initiation of the infection process. However, we have recently found the release of metalloprotease isozymes (MPr) from conidia of *M. anisopliae* during hydration (Qazi and Khachatourians, 2007), which suggests that the conidia are pre-adapted to the pathogenic mode of life. Hence, they do not

require C/ N source to release the proteases which are embedded in the spore wall (St. Leger et al., 1991; Khachatourians et al., 2007). Furthermore, hydrated conidia have the ability to modify the epicuticle/cuticle of the insect integument by the releasing multiple proteases (MPr, Pr1, Pr2) (Qazi and Khachatourians, 2007; Khachatourians et al., 2007).

Zymographic techniques are unique tools that can detect proteolytic enzymes (and other enzymes) following electrophoretic separation in one-dimensional (1-DE) gels (Kleiner and Stetler-Stevenson, 1994; St. Leger et al., 1996a; Kim et al., 1998, Choi and Kim, 1999; Qazi and Khachatourians, 2007). However, 1-DE gels have limitations in resolving complex mixture of proteins; moreover, it cannot be used to determine the pI of the proteins. This limitation has been overcome by the two-dimensional electrophoresis (2-DE), which is a powerful array technology in the field of proteomics (Wilkins et al., 1996). It also holds much promise in the growing field of functional proteomics either when combined with zymography or with mass spectrometry. Essentially, the method combines the use of isoelectric focusing during the first step and finally proteins are separated by conventional SDS-PAGE in the second step. The technique is versatile and can theoretically separate over 10000 proteins in comparison to other separation technologies including HPLC which can separate 100 proteins at maximum (Klose, 1999). One major draw back of 2-DE as a separation technology has the difficulty in obtaining reproducibility with carrier ampholytes (Hames, 1997). However, this limitation has been addressed by the development of immobilized pH gradients (IPG) whereby reproducibility between different laboratories is possible (Gorg et al., 1988). By combining 2-DE with zymography, which is one of the most important tools for protein identification at nanograms quantities (Sanderink et al., 1986; Park et al., 2002; Zhao and Russel, 2003; Choi et al., 2004), proteases or other enzymes in complex biological fluids can be identified/analyzed with high

throughput screening ability in less time with limited amount of sample(s). It is also a useful tool to compare multiple proteases in complex biological fluids in a purified form. Despite the high throughput screening ability of 2-DE, few investigators have attempted to separate functional proteins i.e., in their active form, in biological samples.

During the studies on the diversity of protease associated with conidia of EPF by functional proteomic gel based technology, a number of approaches were compared. Results indicated the need for the development of a new approach. Herein a new method is presented, which combines isoelectric focusing (IEF) in the first dimension and preparation of zymograms (copolymerized substrate and overlay indicator gel) in the 2nd dimension. It was noted that sample preparation steps are critical in protein resolution during analytical IEF. This methodology has high throughput screening ability, substrate specificity and higher sensitivity than conventional chromatographic separation technologies in place. This technique can be successfully adopted for the simultaneous determination of protease catalytic specificity (or other enzyme), molecular weight and isoelectric point.

5.3 Materials and methods

5.3.1 Reagents

Carbohydrate-free gelatin was purchased from Difco (BD Diagnostics, Sparks, USA). Acrylamide, bisacrylamide, carrier ampholyte (Biolyte; 3/10), ammonium persulfate (APS), glycine and TEMED were purchased from Bio-Rad (Hercules, USA). IPG strips [7 cm; 3/10] and immobline dry strip cover fluid was from Amersham Bioscience (Buckinghamshire, United Kingdom), triton X 100, bromophenol blue, SDS and 3-[(3- cholamidopropyl) dimethylammonio]-l-propanesulphonate] (CHAPS) were from, BDH (Poole, England), Sigma

(St. Louis, USA), VWR International (West Chester, USA) and ICN Biomedical (Aurora, USA), respectively.

5.3.2 Fungal isolates and culture conditions

Beauveria bassiana (GK 2016) our laboratory isolate and *Metarhizium anisopliae* (MA 2038) generously provided by USDA, USA were maintained on YPGA for 12 days at 27° C. Conidia were pooled with 0.03% tween 80 (v/v) in type II water. Type II water used in this study has maximum electrical conductivity of *1μS/ cm* at 298K (25°C). Briefly, 5 ml of the tween suspension was dispensed into petri plate and conidia were disrupted gently by using a bent glass rod. Conidia were filtered twice through glass wool column. This was the first tween fraction of conidia. Finally, the spores were counted by using a haemocytometer and the final concentration of spores was adjusted to 10^8/ ml by adding 0.03% tween to the fraction of conidia as indicated above. Spores were incubated at room temperature (20-22° C) for about 20 min. Finally, sample was taken from the test tube and processed as described in the later section.

5.3.3 Preparation of conidial protease

Conidia were spun at 8500g (Sorvall RC, 5C Plus; Rotor no. SS-34; Global Medical Instrumentation, Inc., Minnesota, USA) for 10 min at 4° C. Supernatant was passed through a pore size of 0.22 μm (Millipore™ Billerica, USA) nitrocellulose membrane. This filtrate was collected in a sterile 15 ml Falcon tube and was referred as the spore enzyme filtrate/preparation. The spore enzyme filtrate/preparation was dialyzed for 24 h with two changes of Milli-Q water. The dialysis (Spectra-pore, Spectrum Laboratories Inc., USA; molecular weight cut off 12000) tubing was prepared as described by the Spectrum Laboratories. Enzyme filtrate/preparation was

concentrated against polyethylene glycol (MW 20000) and the protein quantity was determined by using Bradford (1976) method before applying to IPG strips (7 cm; 3/10). The concentrated enzyme preparation was stored at –30° C.

5.3.4 Native IPG strip zymography

Native IPG (nIPG) zymography was performed in order to separate the proteases on the basis of their isoelectric points. Two different approaches were adopted for preparing samples. In first category, 2% carrier ampholyte and 2% CHAPS were used to prepare the sample. In the second category, samples were mixed with 2% carrier ampholytes. The frozen (-30° C) IPG strips were thawed at room temperature (20-22° C) for 10-15 min and 125µl (3X) of the sample was applied. The strips were incubated at room temperature for 12-14 h. Focusing was carried out in stepped fashion (100V for 15 min, 200V for 15 min, 450V for 1 h) as previously described (St. Leger et al., 1994) by using a Multiphor-II^{TM} apparatus (Amersham Biosciences, Buckinghamshire, United Kingdom). Following IEF the strip was washed two times with 20 ml of 0.05 M TRIS/ HCl buffer, pH 7.5 (supplemented with 10 mM $CaCl_2$) for 20 min each. The IPG strip zymography was performed by sandwiching the IPG strip with 7.5% polyacrylamide gel co-polymerized with 1 mg/ ml of gelatin (pre-incubated with two changes of the 50 ml buffer for 30 min). The IPG strip was overlaid on the gel, which was placed on a clean glass plate already washed with same buffer composition as described earlier. The IPG strip overlay zymogram was placed in a plastic box (Ziploc^{TM}; SC Johnson, Brantford, Canada), used as a humid chamber, for 30 min. IPG strip was removed and the zymogram of the overlay gel was performed as described for co-polymerized zymography.

5.3.5 Non-reducing 2-DE zymography

Non-reducing 2-DE zymography (2-DE nrPAGE) was performed for the estimation of the molecular weight of the isozymes. Briefly, IPG strips (7 cm; 3/10) were equilibrated for 30 min in rehydration buffer [2% SDS, 50 mM TRIS/ HCl (pH 8.8), 30% glycerol and 0.02% bromophenol blue]. The IPG strip was overlaid on the 7.5% polyacrylamide gel co-polymerized with or with out gelatin (1 mg/ ml) as indicated below. Electrophoresis was performed at 4° C with the constant voltage in stepped fashion on a Mini protein-II apparatus (Bio-Rad, Hercules, USA; 50V for 15min, 100V for 20 min, 150V for 40 min or until the tracking dye is within the 2 mm of the bottom of the gel). For gels without co-polymerized gelatin the electrophoresis was performed at room temperature (20-22° C) in the same running conditions as described earlier. Zymography after second dimension was performed as described in section 5.3.6.

5.3.6 Development of zymograms

Overlay indicator gels containing gelatin were washed twice with TRIS/ HCl buffer, pH 7.4 (10 min each with 50 ml per gel). However, 2-DE gels copolymerized with gelatin and second dimension gel (to be sandwiched with overlay indicator gel) were washed for 10 min with 2.5% triton X 100 in water (50 ml per gel). The gels were then subsequently washed with triton X 100 in TRIS/ HCl buffer, pH 7.4 (50 ml per gel). Final two washes (50 ml per gel) were performed for both types of zymograms with TRIS/ HCl buffer, pH 7.4. The overlay indictor gel was sandwiched with the second dimension gel. Finally, gels (copolymerized and overlay indicator) were incubated in fresh TRIS/ HCl buffer, pH 7.4 for 1.5 h at 37° C. After the incubation period, gels were fixed for 10 min in a fixative and stained with Commassie brilliant blue R 250 for 10 min to reveal the zones of clearing gels were de-stained on rotary shaker (5

rpm) in de-staining solution. Gels were scanned on flat bed scanner (HP 2200; Hewlett-Packard Company, Palo Alto, USA) at 600 dpi and files were saved in TIFF format for image analysis. Finally, the image was inverted and adjusted by using Adobe Photoshop version 6 (San Jose, CA, USA).

5.3.7 2-D spot densitometry and proteolytic activity in gels

2-D spot densitometry and proteolytic activity in gels were determined after Qazi and Khachatourians (2007). Briefly, 2-D spot densitometry was performed using Alpha Imager Software 5.5 (Genetic Technologies, Inc., Miami, USA) to configure a quantitative relationship between clearing regions/area on the gels. A proteolytic unit (PU) is defined as the amount of the enzyme that can produce a net (background free) clearing zone (degradation of gelatin) of one pixel.

5.3.8 Peptide sequencing

5.3.8.1 MALDI-TOF

Mass spectrometry (MALDI-TOF/ LC-MS/ MS) was performed by the National Research Council of Canada-Plant Biotechnology Institute, Saskatoon. Selected spots were excised from the 2-DE gels manually and placed in a 96 well microtiter plate. The protein spots were ingel digested using a MassPREP station robotic protein handling system Waters/Micromass (Waters, Milford, USA), following the standard digestion protocol of destaining, reduction of cysteines with dithiothreitol, alkylation with iodoactemide, digestion with TPCK-treated trypsin, and three extractions of the resulting peptides into a 96 well PCR plate.

Peptide Mass Fingerprinting (PMF) analysis of the digested peptide samples was carried out on an Applied Biosystems Voyager DE-STR (Foster City, USA) equipped with a nitrogen laser operated at 337 nm, 3 ns pulse. The instrument was operated in positive ion reflectron mode. The samples were analyzed as follows.

To the MALDI plate 0.75 µl of CHCA matrix (5mg/ ml in 75% acetonitrile/ 0.1% TFA) and 0.75 µl of protein digest was added, mixed on the plate and air dried under a gentle stream of warm air. The MALDI plates had previously been mass accuracy optimized with the OptiPlate software in the Voyager 5.1 software (Applied Biosystems, Foster City, USA). PMF analysis of the digested proteins was done in the Automatic Control Mode with recalibrations every 4 samples using a mixture of Angiotensin 1 (M+H 1296.6853), ACTH 1-17 (M+H 2093.0867), and ACTH 18-39 (M+H 2465.1989), and when present the spectra were internally calibrated with the autolytic fragment from trypsin (MH^+ 842.5100 m/z). Two hundred laser shots were averaged, and then smoothed, background corrected and converted to the monoisotopic values with Data Explorer (Applied Biosystems, Foster City, USA). The resulting peak lists were submitted to MASCOT for database searching against NCBI data bases.

5.4 Results

5.4.1 2-DE analysis of proteins

Gelatin zymography demonstrated that washing of spores with 0.03% tween releases protease isozymes (Gelatinase-like) from the conidia of EPF. Tween treatment, because of its membrane disrupting characteristics, appears to disassociate the enzymes which are attached to spore wall-membranes by loose hydrogen bonding or hydrophobic interactions (St. Leger et al, 1991).

5.4.2 IPG strip zymography

Two types of zymography were used in order to resolve the proteases released after the treatment of conidia with tween on nIPG strip zymography. In the first case 2% carrier ampholyte was used to prepare the sample for analytical IEF after which time it was sandwiched with indicator gel containing gelatin (1 mg/ ml). This strategy was referred as IPG strip zymography. The enzyme filtrate/preparation from isolate GK 2016 mixed in 2% carrier ampholyte produced only one protease band of pI 8.3 (Fig. 5.1a) of 4 PU as determined by 2-D spot densitometry. However, sample prepared with 2% carrier ampholyte and 2% CHAPS revealed four protease isozymes (Fig. 5.1b) of PU as shown in Fig. 5.2.

In the case of isolate MA 2038, sample prepared with 2% carrier ampholyte yielded three isozymes of protease (Fig. 5.1c) of PU as indicated (Fig 5.4a) and preparation with 2% carrier ampholyte and 2% CHAPS indicated the presence of eight isozymes (Fig. 5.1d) of PU as shown in Fig. 5.4b. Isozymes are more clearly separated when carrier ampholyte was used in combination with detergent. These results demonstrated that carrier ampholytes (biolyte) and CHAPS best resolve the membrane associated or complex mixture of proteins.

5.4.3 2-DE zymography

To determine the molecular weights of the protease isozymes, a duplicate strip was run on the second dimension gel of both EPF in two ways. Firstly, IEF strip (sample prepared in carrier ampholyte and CHAPS) was run on the gels copolymerized with gelatin (1 mg/ ml). Secondly, IEF strip (sample prepared in carrier ampholyte) was run on gel without substrate in the second dimension, and then subsequently sandwiched with other gel containing gelatin

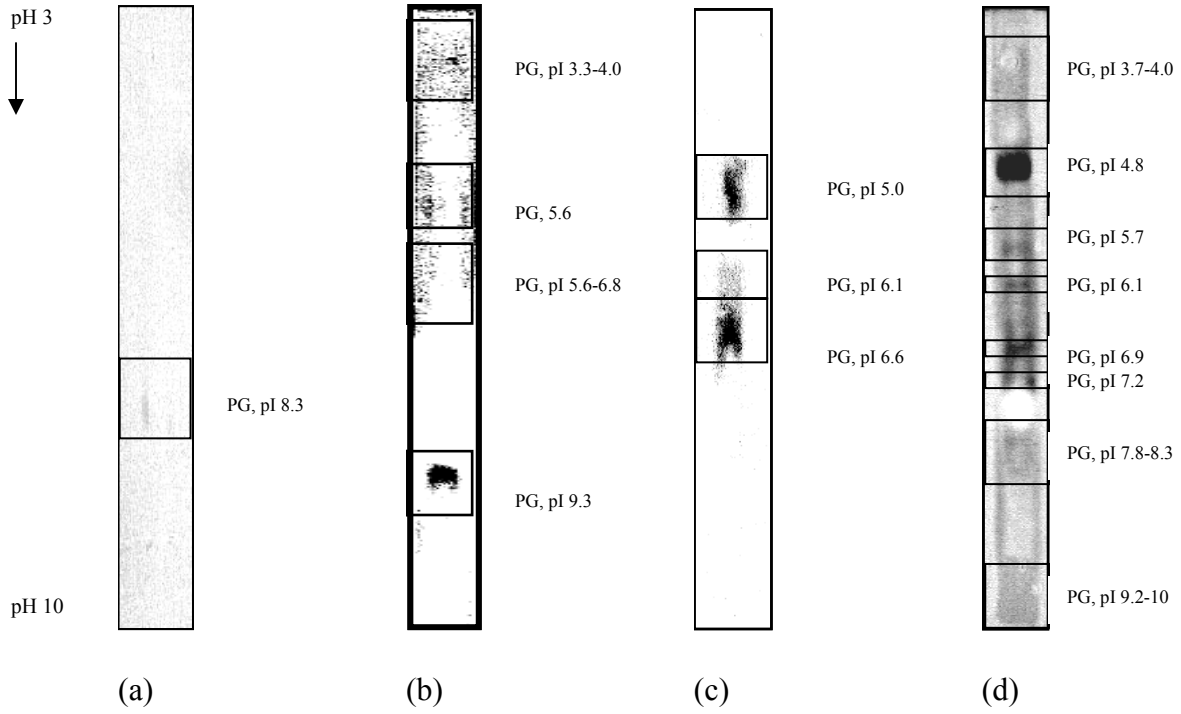


Figure 5.1a-d: Native IEF zymograms of protease isozymes of *B. bassiana* (a, b) and *M. anisopliae* (c, d). Only one isoform of pI 8.3 was separated when carrier ampholyte was used to prepare sample (a) where as four isoforms (PG, pI 3.3-4.0; PG, pI 5.2; PG, pI 5.6-6.8; PG, pI 9.3) of protease appeared when carrier ampholyte and CHAPS were used to make sample (b). Isolate MA2038 showed three isoforms of protease (PG, pI 5.0; PG, pI 6.1; PG, pI 6.6 with carrier ampholyte (c) and eight isoforms of protease (PG, pI 3.7-4.0; PG, pI 4.8; PG, pI 5.7; PG, pI 6.1; PG, pI 6.9; PG, pI 7.2, PG, pI 7.8-8.3 and PG, pI 9.2-10) appeared on gel with carrier ampholyte and CHAPS (d).

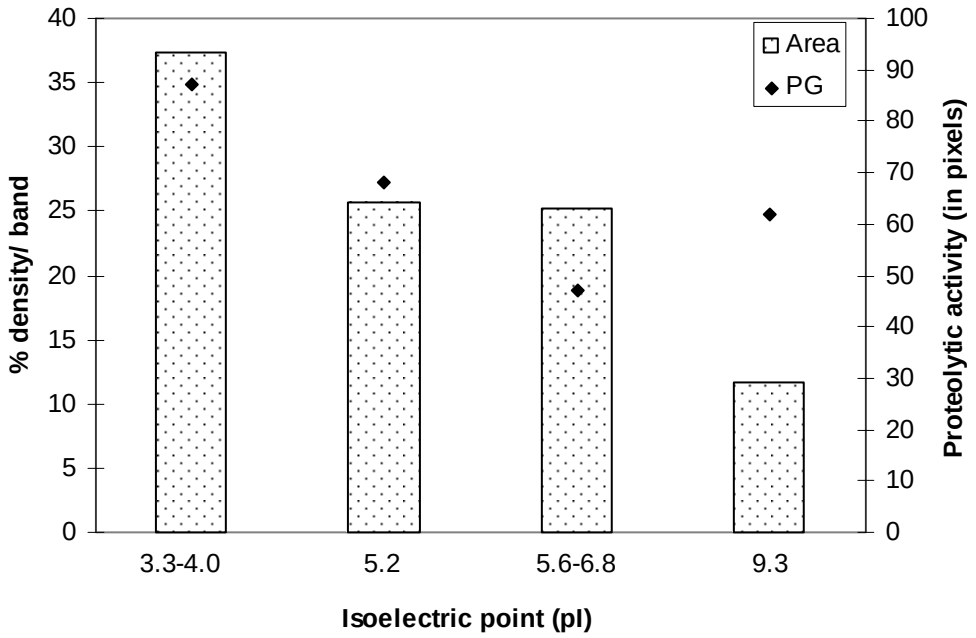


Figure 5.2: 2-D spot densitometry of the native IEF zymogram of *B. bassiana*. Samples were prepared in carrier ampholyte.

(1 mg/ ml) (also referred as overlay indicator gel). No clearance of protease activity appeared on 2-DE zymogram with overlay indicator gels for GK 2016 (Fig. 5.3a) and MA 2038 (Fig 5.3c).

On the other hand, 2-DE zymogram of protease released from isolate GK 2016 with copolymerized gelatin indicated one isozyme of Mr 70 kDa and pI 6.3 (Fig. 5.3b) of 163 PU. Conversely, 2-DE zymogram with copolymerized gelatin for isolate MA2038 showed six protease activities of pI 3.7-9.0 and Mr, as indicated in Fig 5.3d with PU as stated in Fig 5.4c. It can be appreciated from the activity spots shown in Fig. 5.3 that most are apparent in the gel (2-mm). Furthermore, enlarged view of the upper portion of gel for isolate MA 2038 is representing the six activity spots of protease in Fig. 5.5.

Due to some loss of protein recovery which occurred during transfer of proteins from IPG strip to the second dimension gel, not all proteases were resolved on second dimension gel even with copolymerized gelatin. Furthermore, urea was avoided in rehydration buffer in order to (i) retain maximum biologically active form of protease isozymes, and (ii) compensate for the loss of successful transfer of all the proteins from the IPG strip onto the second dimension gel. However, it was noted that protein spots with basic pI values were absent from 2-DE zymograms, indicating the near impossibility to resolve the proteins in the absence of denaturing agents and chaotropes. Yet, the 2-DE zymography with copolymerized gelatin is advantageous and more sensitive than the use of overlay indicator gels. The latter detection method suffers the disadvantage that in most cases the protease activity is weak and the manifest reaction is difficult to be co-localized with the activity on overlay indicator gels. Furthermore, during non-reducing 2-DE zymography, the extraction of corresponding material (spots) from parallel gel (lacking substrate) was performed after Park et al. (2002).

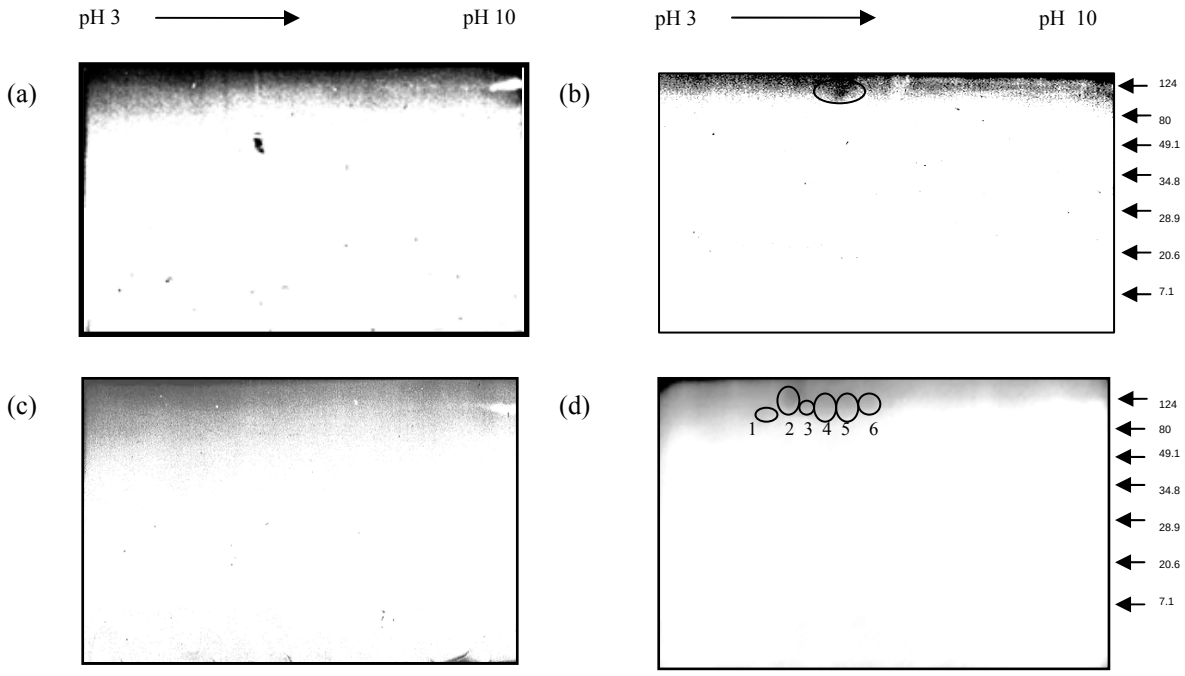
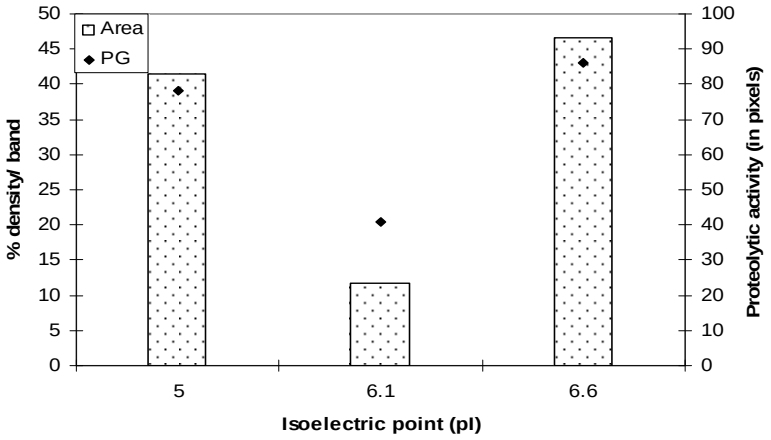
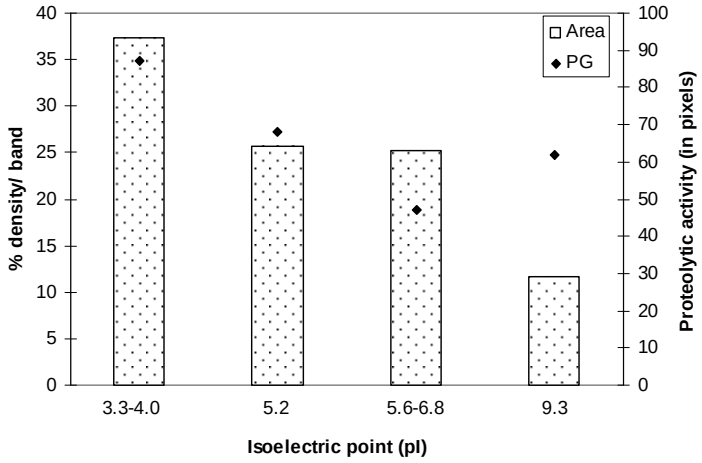


Figure 5.3a-d: Non-reducing 2-DE zymography of the *B. bassiana* (a, b) and *M. anisopliae* (c, d) isozymes (Gelatinase-like). Isolate GK 2016 with carrier ampholyte indicated (overlay indicator gel zymogram) no distinct spot (a). Conversely, sample prepared with carrier ampholyte and CHAPS (copolymerized zymogram) showed protease of 119 kDa, pI 6.2 (b). Non-reducing 2-DE zymogram of the *M. anisopliae* isozymes with overlay indicator gel had some degradation of gelatin by proteases, but no distinct spot (c). However, with combination of carrier ampholyte and CHAPS (copolymerized zymogram) six distinct proteases (spot 1: Mr 115 kDa, pI 4.9; spot 2: Mr 129 kDa, pI 5.4; spot 3: Mr 123 kDa, pI 5.9; spot 4: Mr 122 kDa, pI 6.2; spot 5: Mr 123 kDa, pI 6.5 and spot 6: Mr 124 kDa, pI 7.0) of gelatinase-like activity were identified. Bio-Rad broad range standards (kDa) are indicated on right (d).

(a)



(b)



(c)

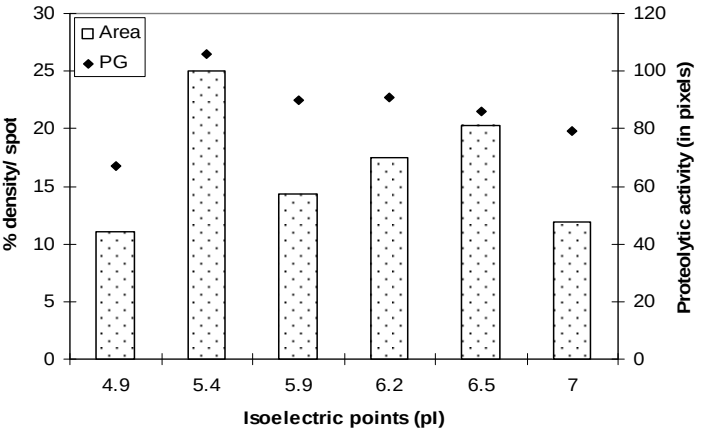


Figure 5.4a-c: 2-D spot densitometry of the native IEF zymogram of *M. anisopliae* (a), sample was prepared in carrier ampholyte. Conversely, densitometry of IEF zymogram of *M. anisopliae* prepared in carrier ampholyte and CHAPS (b). 2-D spot densitometric scan of non-reducing 2-DE zymography of the isolate *M. anisopliae* (c).

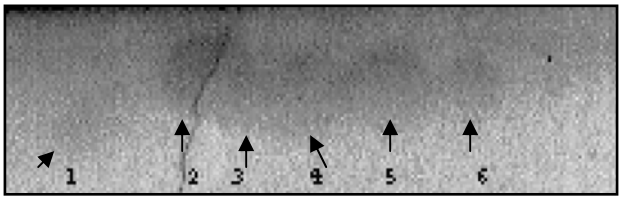


Figure 5.5: Enlarged view of the upper portion (Fig. 2d) of the resolving gel after 2-DE zymography showing spots of protease isozymes (Gelatinase) released by conidia of isolate MA 2038. The spot # 2 is 0.2 cm with in the resolving gel. Gel portion was sliced and adjusted by using Adobe Photoshop version, 6 to reveal the spots to proteases.

The mass spectrometry (MALDI-TOF) analysis showed that the protease activity spots from isolates GK 2016 and MA 2038 (Fig. 5.2) had elastase or serine protease-like activities previously reported from *M. anisopliae*. The technique described could be used to identify other enzymes by using different substrates, by combining with immunoblotting or for peptide sequence analysis by mass spectrometry.

5.5 Discussion

Analysis of functional proteins is a challenging task in proteomics. While denaturing 2-DE is a powerful tool for separating proteins, the use of chaotropes, detergents and reducing agents negates the determination of associated activities. The use of 2-DE zymography with copolymerized gelatin without denaturing agents (urea) has not been previously reported.

A functional proteomic analysis of the fibrinolytic enzymes from *Bacillus subtilis* 168 was described by using denaturing 2-DE zymography (Park et al., 2002). Similarly, the analysis of proteases from the different phase variants of *Photorhabdus luminescens* was reported by Ong and Chang (1997). In addition, the multiple forms of serum aminopeptidase under non denaturing conditions by using micro 2- DE was employed for their functional analysis (Sanderink et al., 1986). Contextual to EPF, St. Leger et al. (1996) reported use of urea during 2-DE zymography and characterization of four (two major and two minor) trypsin isozymes from *M. anisopliae* mycelia. When urea was used in our sample preparation it caused the loss of protease activity, indicating the sensitive nature of these proteases to urea. The reason for the inability of enzyme preparation to withstand the urea treatment and show its activity in the zymograms is not known. The discrepancy of results between the two reports therefore is a conundrum.

The solubilization of proteins in the sample buffer is critical for obtaining a true picture of the proteins present in the complex sample during IEF. The addition of carrier ampholytes or ampholytes to the sample buffer in this context is necessary, as it helps protein solubilization, scavenges cyanate ions and precipitation of nucleic acids during centrifugation (Righetti, 1990). Moreover, ampholytes are also useful in inhibiting interactions between sample proteins and the immobilines of the IPG strips (Gorg and Weiss, 2000). Use of cholic acid 3[(3-cholamidopropyl) dimethylammonio]-l-propanesulphonate (CHAPS) as a detergent of choice in addition to the carrier ampholyte helps to resolve complex proteins associated with membranes by producing clearly separated bands. Detergents are ideal for breaking hydrophobic interactions, which can play an important role in the structural integrity of proteins and protein/protein interactions, and which are very much essential for the analytical isoelectric focusing (Rabilloud et al., 1997). Such detergents help to disrupt membranes, solubilize lipids and delipidate proteins bound to vesicles or membranes (Rabilloud et al., 2007). However, the sulphobetaine derivative of CHAPS combines the advantageous characteristics of bile salts such as non-denaturing and dis-aggregating. In addition, CHAPS is neutral and efficient at disaggregating proteins like N-alkyl sulphobetaines (Hjelmeland, 1980).

The use of IPG strip, and 2-DE zymography aided in the identification of acidic, chymoelastase/subtilisins (Pr1), trypsin (Pr2) and MPr proteases from hydrated conidia. Furthermore, the results of mass spectrometry showed that the spots have elastase (Serine protease)-like activities previously reported from *M. anisopliae* (Shimizu and Yazawa, 2001; unpublished sequence, accession numbers, AB073327.1). Similarly, Qazi and Khachatourians (2007) have reported the release of 5-6 metalloprotease isozymes from the hydrated conidia of *M. anisopliae* by using 1-DE native and 1-DE SDS non-reducing zymography, respectively.

Collectively, these results indicated that conidia are pre-adapted to the pathogenic mode of life, further indicating the complex nature of the host-pathogenic-interaction. In this context, St. Leger et al (1991) provided the first evidence that conidia are endowed with the Pr1, NAGase and esterase-like enzymes. Such enzymes were released upon treatment with detergents, buffers and chemical agents. They further reported that the enzyme levels were significantly-higher when *M. anisopliae* was grown on insect cuticle, in contrast to SDA. Similarly, protease isozymes (acidic, Pr1, Pr2 and MPr) have been previously well documented from the appressoria and mycelial inocula of *M. anisopliae* during growth of insect cuticle in variety of conditions (St. Leger et al., 1994; 1996a; 1998). St. Leger et al. (1989) indicated that Pr1 is the major protein synthesized by appressorial cells vital for fungal penetration. However, results presented here show that prior to emergence of germ tube/appressoria, hydrated conidia themselves can secrete multiple proteases implying their direct role much earlier. Interestingly, during hydration phase, conidia of *B. bassiana* and *M. anisopliae* can secrete ammonia and citrate to modify the surrounding pH for the optimal function of the secreted proteases (Khachatourians et al. 2007). Similarly, St. Leger et al. (1998) found the increase in pH of the infected cuticle after 60 h post-infection by *M. anisopliae*.

It is not shown how these proteases are embedded in conidia. However, the ease with which the enzymes are removed by washing action of mild detergent and buffer is strongly in support of such an association (Khachatourians et al., 2007). The role of extracellular enzymes during hydration of conidia of *N. crassa* has been reviewed (Schmidt and Brody, 1976). Small and Bidochka (2005) identified the up-regulation of Pr1 (*cag*) genes during appressoria formation and conidiogenesis. Similarly, Shah et al. (2005) have reported the up-regulation of conidial Pr1 during growth of *M. anisopliae* in chemically defined media. Cho et al. (2006a)

have provided the evidence of stage specific gene expression from conidia, blastospore and aerial conidia of *B. bassiana.* Their EST analysis of cDNA libraries from aerial conidia showed transcripts of protein depolymerizing enzymes such as subtilisin protease, tripeptidyl-peptidase and thermophlic serine protease which is similar in sequence to that of *Bacills* Ak.1. It is speculated that there might be a similar up-regulation of other protease genes at conidiation to maximize the ability of conidia to degrade proteins under limited nutrient availability times.

Indeed, data presented here reveals that (1) breaking of the protein-protein and hydrophobic interactions by the combined use of carrier ampholyte and CHAPS is crucial to obtain clearer picture of the released isozymes during IEF and (2) the conidia of the both EPF are endowed with proteases similar to Pr1, Pr2 and MPr (on the basis of similarity in pI and substrate specificity) as previously described by St. Leger et al. (1994).

5.6 Conclusion

The method described should be useful in analyzing and functionally identifying the pI and molecular weight of proteases. Moreover, it could identify the substrate specificity of the proteases by using other protease substrates or to identify the inhibitors and activator. The method has the sensitivity to detect ng to pg amounts of proteins. Hence, the nIPG strip zymography and non-reducing 2-DE zymography (copolymerized gelatin) provide a unique tool for identifying and characterizing the novel proteases.

Conidia are endowed with the battery of multiple proteases implying that they may complement each other in devouring the cuticle, and not by a single protease (Pr1) as believed. The composition of insect cuticle is comprised up of complex proteins (Neville, 1975). As a more effective strategy, EPF must have evolved multiple genes to produce proteases that can

function under extreme starvation conditions. In such an event, the release of proteases from conidia has a synergistic role for acting on insect cuticles and environmental conditions for ultimate degradation. It would be expected that most of the hydrophilic (Arg-Y or Lys-Y) residues are attacked by isozymes of Pr2 whereas hydrophobic residues (Phe-Y or Leu-Y) are degraded by Pr1. Conversely, due to their unique specificities to catalyze certain peptide bonds, MPr can help evasion of protein moieties in the cuticle/epicuticle, while avoiding any one of the protease inhibitors present in the host cuticle/hemolymph (Tong and Kanost, 2005).

6.0 Biochemical analysis of proteases from the conidia of *Beauveria bassiana*

6.1 Abstract

A novel aspect of a change in pH upon hydration of the conidia of entomopathogen, *Beauveria bassiana* is reported. Conidia released ammonia and citrate during washes with either of tween (Tw) or water (Ww) and upon incubation in water for up to 2 days. Chymoelastase- (Pr1) and trypsin (Pr2)-like activities were detected in Tw, Ww and upon incubation till day 2. Cationic PAGE zymography demonstrated that Tw released one protease (Gelatinase-like; PGb) when treated with buffer of pH 7 or 7.4. One PGb was present during the washing steps and incubation period till day 2, where spot densitometry showed differences in these activities. A newly devised enzyme overlay membrane (EOM) technique helped identify three isoforms of Pr1 (pI 8.4-9.7) from Tw of conidia, whereas no Pr2 was identified. Use of metalloprotease (MPr) inhibitor in conjunction with EOM analysis revealed one activity band of pI of 5.5 that was inhibited by phosphoramidon implying similarity to thermolysin-like MPr. Further, one isozyme (pI 6.5-7.6) was inhibited by 1-10 phenanthroline indicating this as zinc-containing MPr. The washing of conidia in tween, buffer and water indicated that theses enzymes are attached to the spore wall or membrane, which in part could explain their localized action during initiation of infection. These results clearly indicate that the conidia are endowed with isozymes of Pr1, Pr2 and metalloproteases that can complement each other in cuticle degradation and may have potential to degrade the cuticle prior to the formation of germ tube/appressorium.

6.2 Introduction

Fungi pathogenic to humans, plants, nematodes and insects produce a wide array of extracellular proteases (EP) that can degrade the primary protective barrier of their host (Bowen, et al., 1998; Khachatourians, 1991; St. Leger, 1995). In comparison to other enzymes, EP forms an important group because of their functional/structural diversity and role in fungal pathogenesis (North, 1982; St. Leger, 1995). Elucidating the mechanism of the regulation of these hydrolytic enzymes is critical to understanding of the developmental biology and pathogenicity of these fungi. There is also the correlation that can be generated with other microorganisms; especially protozoa and bacteria that facilitate their infectivity because of the presence of proteases (North, 1982).

Protease production seems to be one of the first biochemical responses of *B. bassiana* and *M. anisopliae,* which are two commercially important entomopathogenic fungi (EPF) effective against a broad range of insects including sap sucking insects (Feng et al., 1994). The successful infection of EPF primarily relies upon the release of isoforms of the chymoelastase (Pr1) and trypsin (Pr2) during appressoria and mycelia formation (St. Leger, 1995). Pr1 and Pr2 in *B. bassiana* and *M. anisopliae* are regulated under the influence of food cues such as carbon and nitrogen (C/ N) sources and ambient pH (Bidochka and Khachatourians, 1987, Khachatourians, 1991; St. Leger et al., 1994; St. Leger, 1995). During the past two decades the conidial enzymes have received less consideration from microbiologists and applied mycologists on the account that mycelia have more relevance to the infection process of EPF. However, a question is raised whether mycelia have commercial relevance to the initiation of infection. Primarily mycelia are rarely formulated into the material that is sprayed as a mycoinsecticide. Secondly, C/ N catabolite repression becomes relevant once the conidia germinate and develop into mycelia,

which start growing in hemocoel or other internal tissues of insects. Although there is a dearth of knowledge about enzymes released or secreted by conidia, St. Leger et al. (1991) were first to show that the conidia, upon washing in buffers, detergents and chemical agents, release Pr1, chitinases (NAGase) and esterases. However, there are no reports on the isozymes associated with conidia. The recent investigations on conidia provided first evidence by using biochemical and functional proteomic analysis that they are endowed with the battery of metalloproteases, chymoelastase-and trypsin-like enzymes (Qazi and Khachatourians, 2007; Khachatourians et al., 2007).

The main objective of this study was to demonstrate and characterize the diversity of protease isozymes from conidia of *B. bassiana.* Further evidence is provided that conidia can modulate pH of the microenvironment in the nutrient starved conditions by secreting ammonia and citrate that can help to regulate the release and activity of EP. These results suggest that conidia associated protease isozymes were evolved to maximize their potential to establish successful infection.

6.3 Materials and methods

6.3.1 Fungal isolates and culture conditions

Beauveria bassiana (GK2016) was obtained from BioInsecticide Research Laboratory, Department of Applied Microbiology and Food Science. Fungal culture was maintained on YPG agar at 27° C. Spores were prepared from two weeks old cultures as described in Qazi and Khachatourians (2007).

6.3.2 Enzyme preparation from spores

Enzyme filtrate/preparation was made as previously described in Chapter 5 (section 5.3.3). The spore enzyme filtrate/preparation was concentrated (X 3) by freeze-drying. Concentrated samples were stored at –30° C till further use. Furthermore, Pr1, Pr2 and NAGase activities were assayed by using concentrated enzyme filtrate/preparation.

6.3.3 Conidial volume measurements

Swelling of the conidia of *B. bassiana* in type II water was determined by using Multisizer III (Coulter Counter^{TM} ; Beckman Coulter, Inc., California, USA). The system standardization was performed as per manufacturer instructions by using latex beads of 2, 5 and 10 µm. Briefly, 20 µl of the spore suspension was dispensed up to 20 ml of Isotone^{TM} (Beckman Coulter, Inc., California, USA). Further 2 -3 X 10^4 conidia were counted to determine the percentage volume of the conidia. Median volume attained by conidia during soaking period was determined from 0.25 h to 40 h (as shown in results). Each point was based on the percentage median volume of four different runs (quadruplicate).

6.3.4 Extraction of enzymes from conidia

Conidial protease (Gelatinase-like) was extracted by three washing procedures (WP) here after referred as WP-1, WP-2 and WP-3, respectively. In WP-1 conidia were washed three times, once in 0.03% tween and twice in water and after each wash conidia were collected by centrifugation. All supernatant fractions were assayed for protease activity. The conidia were incubated in water (10^8/ ml) in Erlenmeyer flask for one hour and supernatant assayed for protease activity. Conidia were spun down at 8500g (Sorvall RC, 5C Plus; Rotor no. SS-34;

Global Medical Instrumentation, Inc., Minnesota, USA) and incubated in water for another 24 h. Finally, water supernatant was assayed for protease and metabolites.

In WP-2 treatment conidia were washed by three successive centrifugation and supernatant fractions assayed for protease activity as described in WP-1. Next, conidia were incubated in 0.03 M TRIS/ HCl, pH 7 and 0.3 M TRIS/ HCl, pH 7, respectively for one hour in Erlenmeyer flasks and supernatant assayed for protease activity. Conidia were spun down at 8500g and incubated in fresh 0.03 M TRIS/ HCl and 0.3 M TRIS/ HCl, respectively for another 24 h. Finally, buffer supernatants were assayed for protease.

In WP-3 treatment conidia were washed by three successive centrifugations and supernatant fractions assayed for protease activity as described in WP-1. Conidia were incubated in 0.03% tween and 0.3% tween, respectively for one hour in Erlenmeyer flask and supernatant assayed for protease activity. Conidia were spun down at 8500g and incubated in new 0.03% tween and 0.3% tween for another 24 h after which time tween supernatants were assayed for protease and metabolites. The experiment was performed in triplicate.

6.3.5 Protease plate assay medium

Protease (Gelatinase-like) activity was determined by measuring the zone of clearing in 1.5 % (w/v) agar containing 1 % gelatin as a substrate. Essentially, each plate contained 18 ml of the medium as indicated earlier supplemented with 0.005% of phenol red. After cutting agar plugs by using 0.5 mm pipette, 50 µl of enzyme filtrate/preparation was dispensed in each well. After which time plates were incubated for 24 h to allow the development of a clearing zone. Three replicates were performed in each case.

6.3.6 Evaluation of ammonical by-products and organic acids

Agar plates showed colour change because of the presence of pH indicator (0.005% phenol red). Plates were monitored visually for color change (pH < 6 yellow color and pH > 8 red color). Yellow color indicated the release of organic acid secretion and pH < 6. Presence or absence of yellow color was observed and recorded with in < 60 min of dispensing enzyme filtrate. Yellow color was not observed after > 60 min. In contrast, ammonia release on plate was detected after 24 h due to the formation of red color (pH > 8). The intensity was assigned a score of red color as 1 (low), 2 (medium) and 3 (high) to assess the relative amounts of ammonia released.

6.3.7 Effect of buffers on conidial enzyme release/activity

The spores were inoculated into sterilized (120° C, 15 min) buffers at pH 4, 6, 8 and 10. The buffers employed in this study were 0.1 M citrate/ phosphate (pH 4 and 6), 0.1 M TRIS/ HCl, pH 8.0 and 0.1 M carbonate/ bicarbonate, pH 10. Buffered spore suspensions were incubated at 27° C at 150 rpm in rotary shaker incubator (New Brunswick Scientific Co., New Jersey, USA) for 6 h. After set time intervals 5 ml of the sample was taken from the Erlenmeyer flask and passed through Millipore^{TM} (Millipore, Billerica, USA; 0.45 µm pore size) filters. Culture filtrate was assayed of Pr1 and Pr2 as described in section 6.3.8.

6.3.8 Enzyme assays

Trypsin (Pr2)-like activity was measured with the chromogenic substrate N-ά-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; at 21.75 mg) that was dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) and made to a final volume of 50 ml with 0.05 M glycine-NaOH buffer (pH

8.5). Subtilisin or chymoelastase (Pr1)-like activity was measured against Suc-Ala-Ala-Pro-Phe-4-nitroanilide (15 mg), which was dissolved in 0.5 ml of DMSO and made to a final volume of 25 ml with 0.05 M TRIS/ HCl buffer (pH 8.0). A scale-down method was used for both Pr1 and Pr2 assays. The buffered substrate in each case (80 µl) was added to a 96-well microtiter plate with 20 µl of enzyme preparation. For control 20 µl of type II water was used instead of enzyme. The microtiter plate was incubated in incubator (Fisher Isotemp^{TM}, Standard Lab Incubator, Fisher Scientific Co., Pittsburgh, USA.) at 37° C for 10 min, and the absorbency was read at 411 nm on a Titertek Multiscan^{TM} (Titertek, Huntsville, USA) microtiter plate reader. Enzyme (Pr1, Pr2) activity is expressed as nmols NA (nitroanilide) released ml^{-1} min^{-1}.

Chitinase (NAGase) was assayed by measuring chromogenic substrate *p*-Nitrophenyl-*N*-acetyl-D-glucosaminide (2 mM) that was dissolved in 0.05 M citrate/ phosphate buffer, pH 4.8. The enzyme assay was performed with some modifications after Bowers et al. (1980) and Borooah et al. (1961). Briefly, 30 µl of both citrate phosphate buffer and substrate solutions were dispensed into 96 well microtiter plates. After gentle mixing, 20 µl of enzyme solution was added. The mixture was mixed by gentle shaking, and incubated for 10 min at 37° C. Reaction was stopped by adding 20 µl of 0.4 M sodium carbonate solution. In case of control, 20 µl of type II water was added instead of enzyme preparation. Finally, absorbency was read at 411 nm on a Titertek Multiskan™ microtiter plate reader (Titertek, Huntsville, USA). NAGase activity is expressed as nmols nitrophenol (NP) released ml^{-1} min^{-1}.

6.3.9 Analytical procedures

Ammonia (NH_3 and NH_4^+) production was estimated by the method of Chaney and Marbach, 1962). Essentially, to 1 ml of the sample 1 ml of the solution A (0.5 and 0.001M/ L of

phenol and sodium nitroprusside, respectively) and B (0.625 and 0.03M/ L sodium hydroxide and sodium hypochlorite, respectively) were added at 20-22º C. The color was developed at 60 °C for 3-5 min and spectrophotometric determinations were carried at 625 nm in suitable dilutions. Standard curve was prepared by using dilutions (10^{-7} – 10^{-1}) from 0.1 M ammonium chloride (Fisher Scientific Co., Pittsburgh, USA) in order to calculate the amount of ammonia in samples.

Citrate analysis was performed by the method of Marrier and Boulet (1958). A 250 µl of sample was added into the test tube. Next, 325 µl of pyridine was added to the tubes after which time the contents were mixed by vigorous shaking. Finally, 1.425 ml of the acetic anhydride was added and tubes were swirled. After wards tubes were immediately placed in the constant-temperature bath for 30 min for colour development. The spectrophotometric determinations were carried at 420 nm in suitable dilutions. Standard curve was prepared by using citric acid BDH (Poole, England) in order to calculate the amount of citrate in samples.

6.3.10 Cationic PAGE native zymography

Cationic PAGE (15%) native zymography was performed with co-polymerized gelatin (1 mg/ ml) as a substrate. Sample buffer was made after Lantz and Ciborowski (1994). Briefly, 6 ml of 1 N KOH and 360 µl of glacial acetic acid were added to 20 ml of glycerol. Next, 5 ml of 10% triton X-100 and 1% digitonin suspension were added with gentle stirring. Finally, 2 ml of 0.2% methyl green was added. Gelatin at a concentration of 1 mg/ ml was used as a substrate. The gel recipe composition was adapted from Lantz and Ciborowski (1994). Pre-electrophoresis was performed for 20 min without sample in order to avoid the deleterious effects of ammonium persulfate (APS) and riboflavin on enzyme activity. Electrophoresis was performed with reverse

polarity at 4° C at constant current 10 mA for approximately 55 min or until the tracking dye was within 2 mm of the bottom of the gel.

6.3.11 Enzyme activity in native gels

Enzyme activity was detected by incubating the gels in two different buffers. In first case protease activity was determined by placing the gel in 0.1 M phosphate buffer (PB) at pH 7.0. In the second case the gel was incubated in 0.05 M TRIS/ HCl buffer at pH 7.4. Gels were incubated at 37° C for 2.5 h. After incubation period, the gels were fixed for 10 min in fixative and subsequently stained for 10 min with Commassie brilliant blue R-250. To reveal the zones of clearing, gels were placed in a de-staining solution and incubated at 22-23 °C on rotary shaker (5 rpm). Gels were photographed by using Kodak digital camera (DC 290 zoom) under bright fluorescent light. For the ease of analyses, images were converted to gray scale and adjusted by using Adobe PhotoShop Professional Software. 2-D spot densitometric quantification was performed using Alpha Imager Software 5.5 (Genetic Technologies, Inc., Miami, USA) to configure a quantitative relationship between clearing regions/area on the gels.

6.3.12 Proteolytic activity in gels

A proteolytic unit is defined as the amount of the enzyme that can produce a background free (net) degradation of gelatin (clearing zone) of one pixel. Proteolytic activity after 2-D spot densitometry was determined as previously described (Qazi and Khachatourians, 2007).

6.3.13 Analytical isoelectric focusing

Isoelectric focusing (IEF) was performed in order to separate the proteases on the basis of their isoelectric points. The spore enzyme filtrate/preparation was prepared as previously described in chapter 5 (section 5.3.3). Concentrated samples (X 3) were dissolved in 2% biolyte and 2% CHAPS. The IPG strips (7cm; 3/10) were thawed at room temperature for 10-15 min and 125µl of the concentrated sample was applied. The strips were incubated at room temperature for over night. Focusing was carried out in stepped fashion (100V for 15 min, 200V for 30 min and 3000V for 1 h). IPG strips were stored at –30° C till further use.

6.3.14 Enzyme overlay membrane (EOM)

6.3.14.1 Preparation of nitrocellulose membranes (NM)

This method was specifically developed to detect proteases in complex biological samples. Instead of 7-amino-4-trifluoromethyl coumarin (AFC), the usual substrate, 4-Nitroanilide (NA) derived substrates were used which are sensitive, stable and less expensive. EOMs with NA can be stored at – 20°C for 6 month without significant loss in sensitivity. Additionally, best results for EOMs could be obtained by overlaying the membrane previously impregnated with the specific substrate (0.25 mg/ mL). Western blotting (capillary or electroblotting) with nitrocellulose membranes (with or with out substrates) were unsuccessful for separation of Pr1 and Pr2, possibly due to loss of activity during transfer from gel to membrane or due to incomplete transfer of proteins.

Nitrocellulose membranes (0.45 µm pore size, Scheider and Schuell Co., GmbH, Dassel, Germany) were cut to the size of 7.3 cm x 10.2 cm and were washed thrice in type II water i.e., after an interval of 60 min each. Washed NMs were inspected for imperfections (such as stain,

yellow tinge) and discarded if found defective. NMs were washed twice in buffer (Pr1: 0.05 M TRIS/ HCl, pH 8.0 and Pr2: 0.05 M Glycine/ NaOH, pH 8.5) for 30 min each after which time they were hung on plastic clamps to allow for air-drying. Specific substrates for chymoelastase (Pr1) was Suc-Ala-Ala-Pro-Phe-pNA and for trypsin (Pr2) was Bnz-Arg-pNA, which were prepared at a concentration of 0.25 mg/ ml in 0.05 M TRIS/ HCl (pH 8.0) and 0.05 M Glycine/ NaOH (pH 8.5) buffers, respectively. Air-dried NM was dipped (5 sec) in Pr1and Pr2 substrates. Next, NMs were air dried and stored in plastic Ziploc™ (SC Johnson, Brantford, Canada) bags till further use. The NM impregnated with substrates can be stored in a dissector chamber for 6 months safely.

6.3.14.2 Procedure for EOM

Following IEF, the IPG strip underwent (two changes of 20 ml for 20 min) in 0.05 M TRIS/ HCl buffer, pH 8.0 for Pr1 EOM. However, for Pr2 EOM, the same wash procedure was employed with 0.05 M glycine/ NaOH, pH 8.5. The EOM was performed by placing the buffer-soaked filter strips of Whatman no. 2 (previously washed in either Pr1, or Pr2 buffers as described above; strip dimension 0.5 cm X 8.0 cm) on buffer washed glass plate (7.3 cm x 10.2 cm). Substrate impregnated NM was soaked in buffer with or without added inhibitors (phosphoramidon and 1-10 phenanthroline) for the identification of metalloproteases. After IEF, the IPG strip was sandwiched with substrate-impregnated nitrocellulose membranes. The EOM was placed in a humid chamber (Ziploc™ box, SC Johnson, Brantford, Canada) for 30 min at 37° C. Subsequently, the IPG strip was removed and the EOM was diazotized after Ohlsson et al. (1986) by sequential incubations of 5 min each in 0.1 % sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl and 0.05% N- (1-naphthyl) ethylenediamine in 47.5% ethanol.

Immediately after the formation of pink bands of proteolytic activity was visible, membranes were placed in Ziploc™ (SC Johnson, Brantford, Canada) bags, and scanned on flat bed scanner (HP 2200) at 600dpi. Processed EOMs were stored at –20 °C.

6.3.15 Statistical analysis

For the statistical analyses, CoStat Version 6.204 program (CoHort Software, Monterey, CA, USA) was used. The level of significance was determined by using one way completely randomized design ANOVA. The whole study was repeated twice.

6.4 Results

6.4.1 Release of ammonia and citrate

Hydrated conidia during soaking period in type II water showed changes in mean cell volume as determined by Multisizer III (Coulter Counter^{TM}; Beckman Coulter, Inc., California, USA) analysis, to indicate swelling. It can be appreciated from Fig. 6.1 that conidia during soaking period in type II water showed a three-fold increase in median conidial volume (%) with in 5 h of incubation. After which time there was shrinkage of conidia at 60 h of incubation. In contrast, Dillon and Charnley (1985, 1990) by using Electrozone/Celloscope particle counter (Model II LTS, Particle Data Inc., Illinois, USA) reported that unless provided by exogenous nutrients, conidia of EPF do not swell in de-ionized water.

Soaking of conidia in water must stimulate the pre-germination phase to become metabolically active. Therefore, conidia during Tw or Ww washes, and upon incubation in water till day 2, indicated the release of ammonia or citric acid during incubation of conidia till day 2

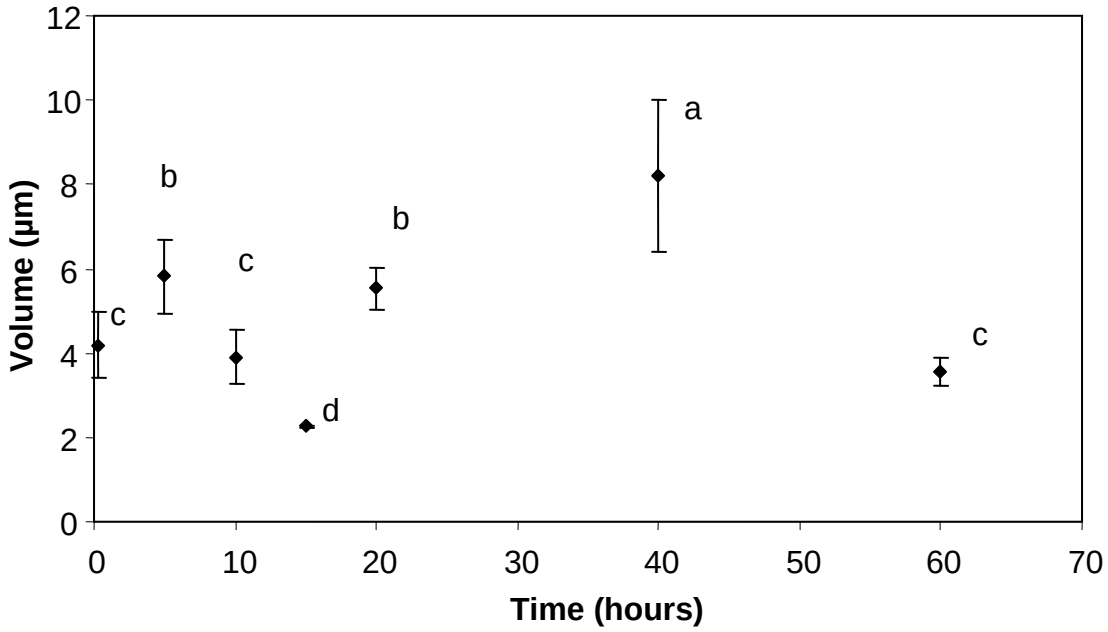


Figure 6.1: The rate of swelling of conidia of *B. bassiana* in type II water. Each point is based on the percentage median volume of 2-3 $X10^4$ conidia. Conidial volume was measured by using Multisizer III (Coulter Counter^{TM}). Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 4) were separated by using Duncan's method ($p < 0.05$). Values following same letters are not significantly different. The error bars represents S.D.

(Table 6.1). Citrate was in apparent in Ww fractions. The presence of these metabolites in the spent supernatant (washes and incubation) was taken to imply that hydration alone was sufficient to start metabolic activity within the conidia. Several metabolic reactions can lead to the formation of ammonia as end product (Stryer, 1995). Release of citrate in the spent wash and incubation supernatant from conidia also implies synthesis from the endogenous pool of amino acids or excretion of citrate from spore reserves. In this context, C.P. Kubiceck (personal communication) has considered spore swelling to be associated with the liberation of some organic- or amino- acids (e.g., glutamate) both present in the spores. The release of citric acid is not surprising because of its role in the initiation of infection cycle it could act as a C-source for the growing conidia or the germ tube emergence.

6.4.2 Release of Pr1 and Pr2 from conidia of *B. bassiana*

Results showed that the *B. bassiana* have released protein during washing steps and incubation period. Similarly, several years ago, Tom Alderson (Herbert Arst, personal communication) showed that conidia of *A. nidulans* release a germination inhibitory protein when incubated in distilled water. Upon further investigation, it was found that water washed supernatant contained activities resembling to chymoelastase and trypsin. It is apparent that Tw released the maximum amount of Pr1 and Pr2 like activities (Fig. 6.2). Subsequently, Pr1 and Pr2 activities were observed during the 1st wash and upon incubation till day 2. Pr1 activity was higher when Tw was used as washing agent for spores. After which time, more Pr2 activity was found during 1st and 2nd aqueous wash. Basal level of Pr1 and Pr2 were observed at day 1 and 2. The change in activity (Pr1and Pr2) may be explained as a result of change in the

Table 6.1 Estimation of ammonia, citrate, protein and pH in the wash and incubation supernatant of *B. bassiana* during isotropic growth/swelling phase.

Condition	Treatment	pH*	Ammonia (µM)	Citrate (µg)	Protein (µg)
Wash	Tween wash	7.98^{a}	150 (0.2)	27.5 (2.8)	2.0 (0.06)
	1^{st} dH_2O	7.71^{c}	75 (0.08)	0	3.0 (0.02)
	2^{nd} dH_2O	7.48^{d}	102 (0.20)	0	0.7 (0.09)
Incubation	24 h	7.74^{bc}	7.4 (0.1)	6.25 (0.1)	0
	48 h	7.76^{b}	102 (0.4)	1.25 (0.01)	1.0 (0.02)

Legend:

* Level of significance was determined by using one way completely randomized design ANOVA, where means were separated by using Duncan's method ($p < 0.05$). Values following same letters in each column are not significantly different. Values in brackets show S.D., where $n = 3$.

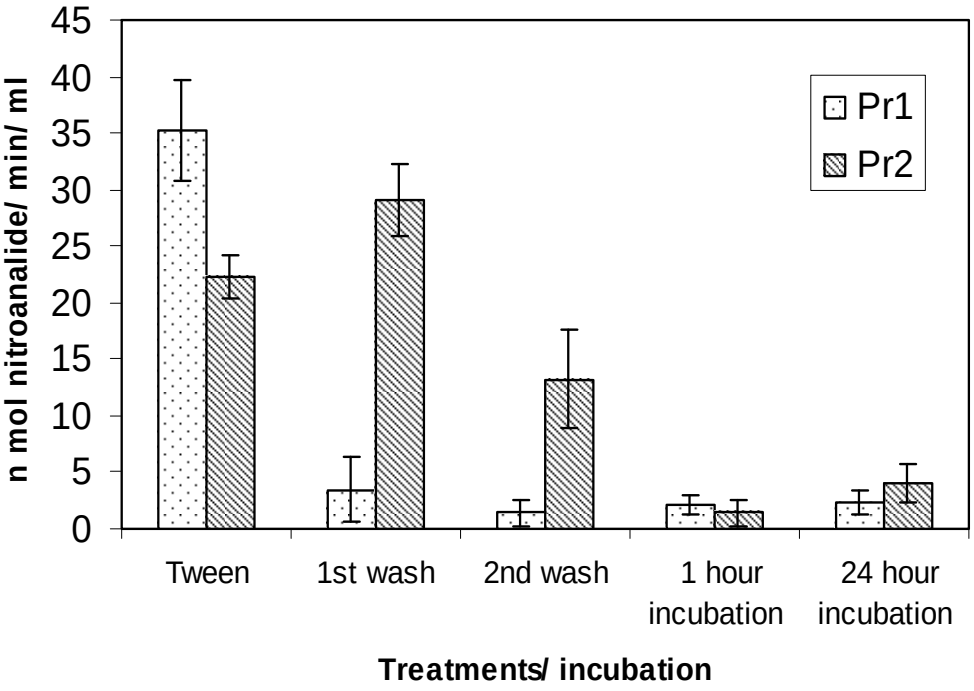


Figure 6.2: Subtilisin-like (Pr1) and trypsin-like (Pr2) enzyme activities from the conidia of *B. bassiana* during aqueous washes (Tw and Ww), and upon incubation in water up to day 2. Error bars represents SE., where n = 4.

pH of the wash and incubation supernatant. Significant reduction in the pH of Tw wash was evident in comparison to incubation period (day 2).

The Pr1 and Pr2 proteases were associated with conidial wall- membrane, as they could be released by the action of mild detergents (Fig. 6.2). The buffering action generated by ammonia and citrate must have loosened hydrogen bonding between Pr1 and Pr2 and spore wall to show they were stable and active till day 2. The conidial germination tested on YPGA plates showed that > 80% of the conidia were viable after day 2.

6.4.3 Effect of pH on conidial enzyme release/activity

The effect of pH on the conidial Pr1 and Pr2 release/activity was tested. Conidia were incubated in buffers of pH 4, 6, 8 and 10 (Fig. 6.3). A minimal change (0.3 units) in pH of the spent citrate/ phosphate buffer, pH 4 and 6 was observed. In contrast no change in pH of the TRIS/ HCl and carbonate/ bicarbonate buffers, pH 8 and 10, respectively was evident. Results showed the optimum activity for Pr1 at pH 6 and 10. In contrast, maximum Pr2 activity was observed at pH 10. Low activity expression of Pr1 and Pr2 at pH 4 was due to suboptimal pH for this enzyme. Interestingly, low Pr1 and Pr2 at pH 8 may imply the interfering effects of TRIS/ HCl buffer on the spore membrane and or release of proteases.

6.4.4 Release of proteases from conidia

Washing procedures (abbreviated as WP-1, WP-2 and WP-3, for details see section 6.3.4) were employed to dislodge conidial protease. The data presented in Fig. 6.4 showed the Tw released maximum amount of protease from conidia. The release of protease in Ww implies that some enzymes were spore-linked via electrostatic forces. Washing of conidia

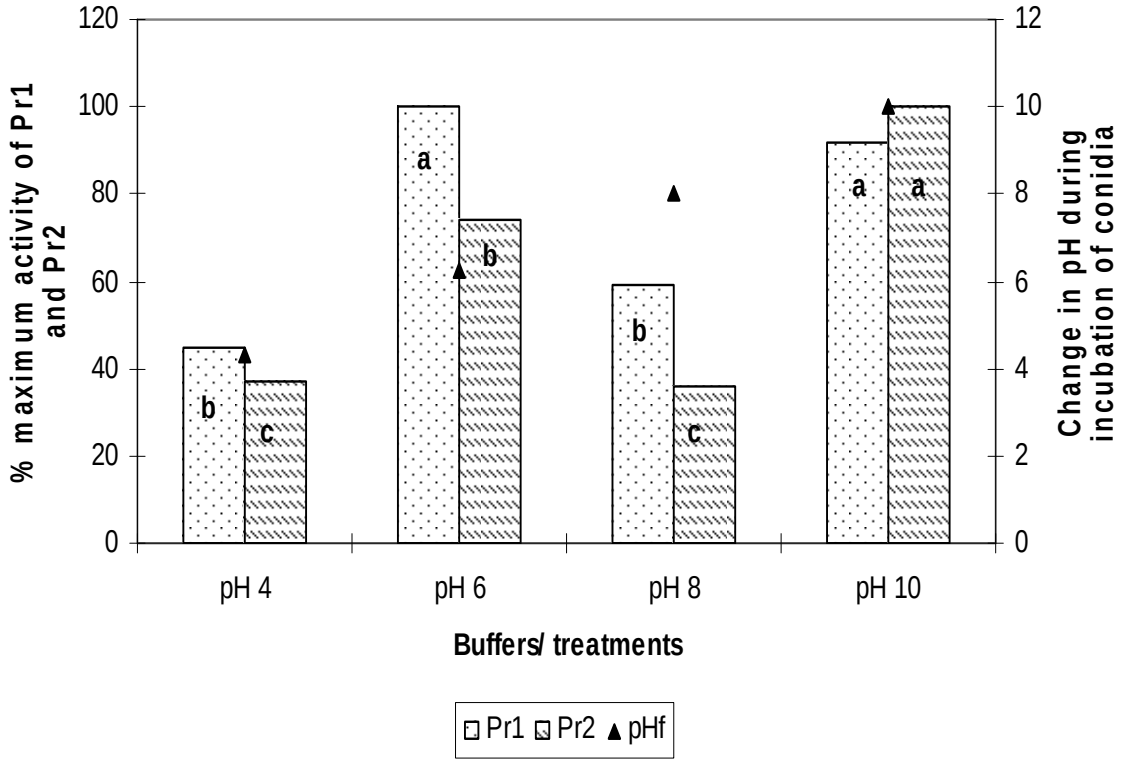
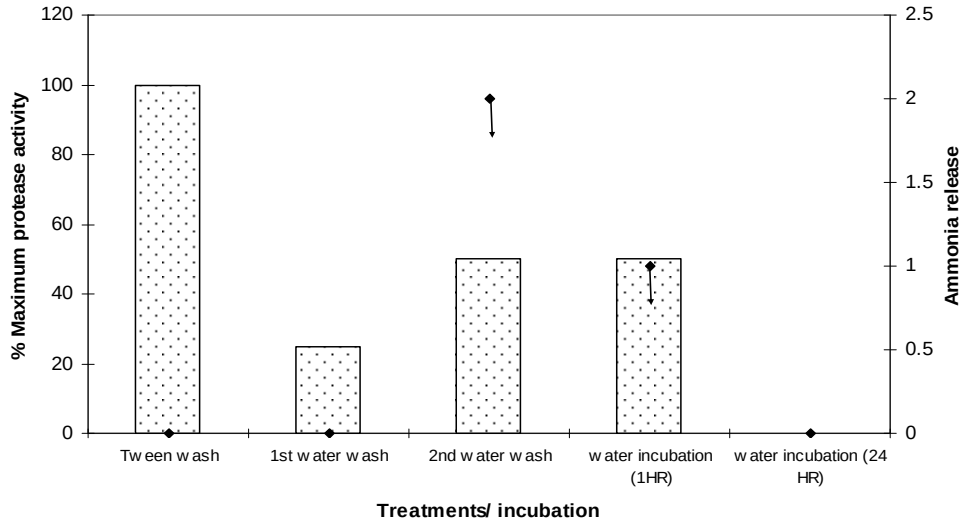
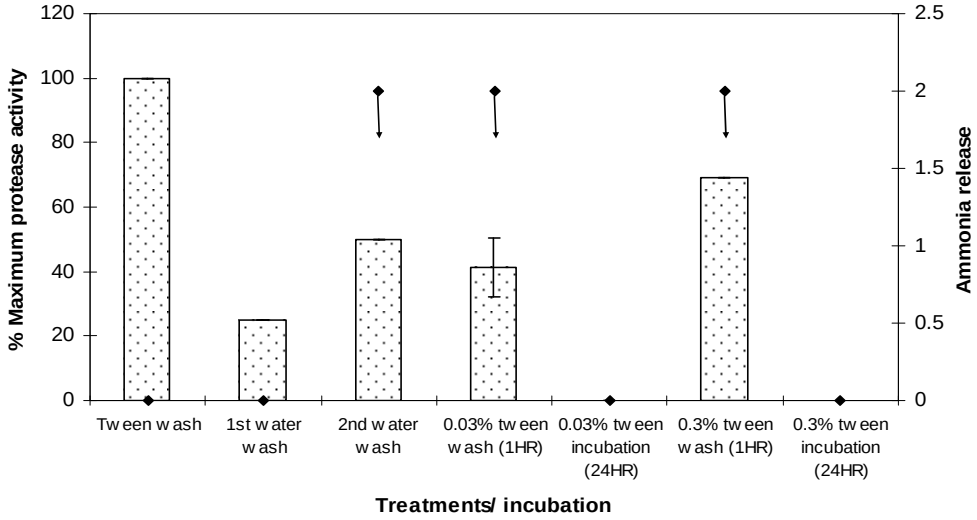
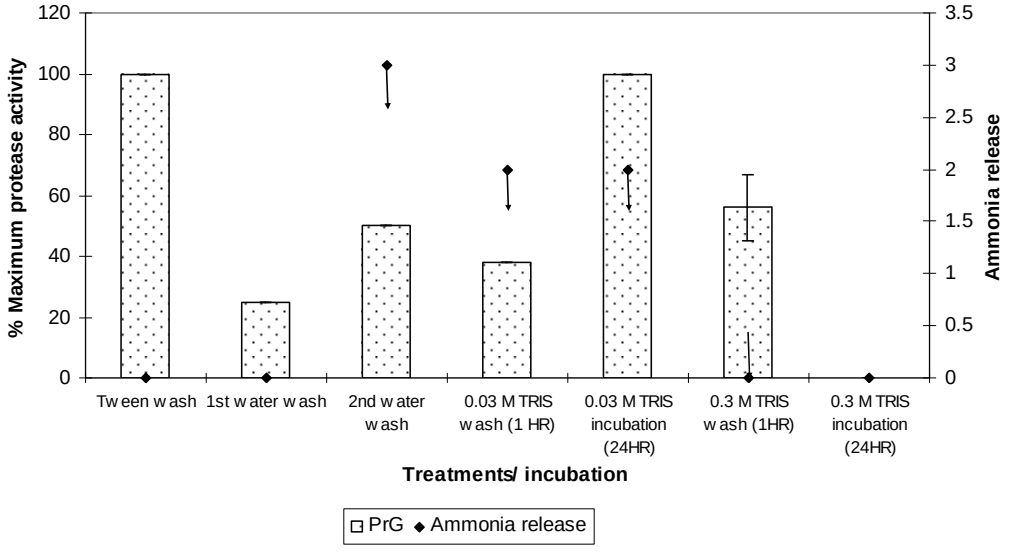


Figure 6.3: Release/activity of Pr1, Pr2 and change in pH of the buffer supernatants after 6 h of incubation of conidia of *B. bassiana.* The buffers employed in this study were: 0.1 M citrate/ phosphate (pH 4 and 6), 0.1 M TRIS/ HCl, pH 8.0 and 0.1 M carbonate/ bicarbonate, pH 10. Buffered spore suspensions were incubated at 27° C before being assayed for protease activities. Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 4) were separated by using Duncan's method ($p < 0.05$). Values following same letters are not significantly different.

Figure 6.4: Release/activity of protease, ammonia and citrate from the conidia of *B. bassiana* during different washing procedures designated as: WP-1, WP-2 and WP-3. In WP-1, WP-2 and WP-3 the total activity of the first tween wash of the conidia was set as 100% relative activity. The activities of the pooled water washes are represented as a proportion to this reference. Additionally, the sequential differential extractability with water [1 h and 24 h incubation supernatants, respectively] (a) 0.03% and 0.3% tween [1 h and 24 h incubation supernatants, respectively] (b) and 0.03 M and 0.3 M buffer [1 h and 24 h incubation supernatants, respectively] (c). Ammonia was scored visually as 1 (low), 2 (medium) and 3 (high), respectively after 24 h of incubation. The arrows represent release of citrate that was observed in < 60 min of dispensing enzyme filtrate on plate assay medium as described in material and methods section.


% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
water incubation (1HR)
water incubation (24 HR)
Treatments/ incubation


% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
0.03% tween wash (1HR)
0.03% tween incubation (24HR)
0.3% tween wash (1HR)
0.3% tween incubation (24HR)
Treatments/ incubation


% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
0.03 M TRIS wash (1 HR)
0.03 M TRIS incubation (24HR)
0.3 M TRIS wash (1HR)
0.3 M TRIS incubation (24HR)
Treatments/ incubation
PrG
Ammonia release

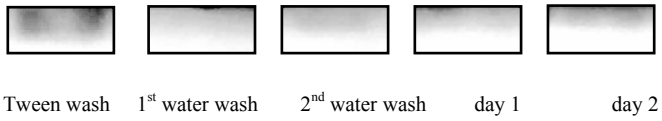
for one hour in 0.03% and 0.3% tween showed comparable results, indicating enzyme release. No activity was observed in 24 h tween (0.03% and 0.3%) incubation fractions. In addition, protease release/activity was found in TRIS/ HCl buffer (0.03 M and 0.3 M) washes after 1 h, which implies that some enzymes might be linked by weak hydrogen bonding. Interestingly, very high protease activity was evident in 0.03 M buffer wash after 24 h of incubation. In addition, plate assay showed colour change due to secreted metabolites (organic acid and ammonical by-products) by the water soaked conidia.

6.4.5 Cationic PAGE zymography

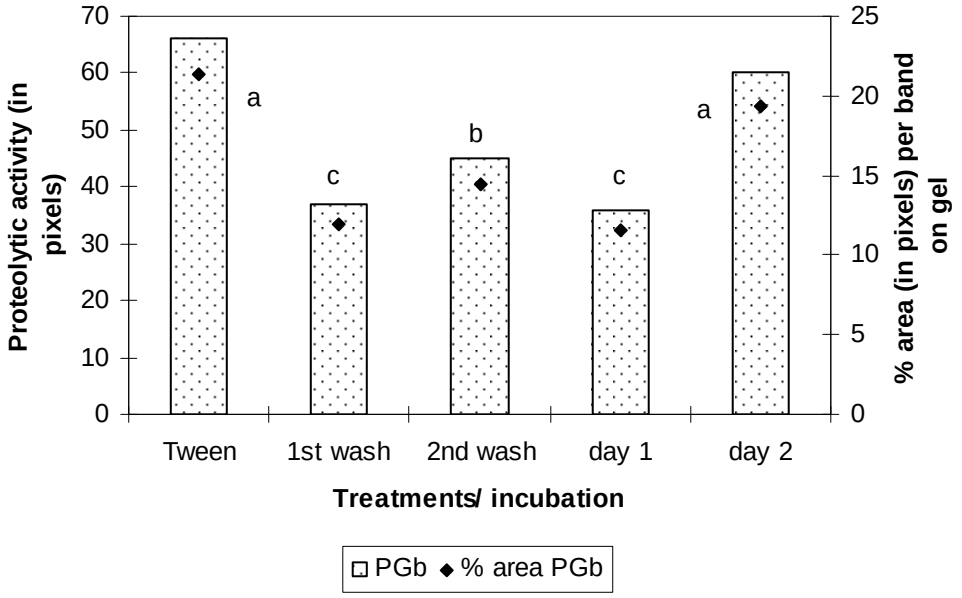
To identify the gelatinase-like isozymes, a cationic PAGE zymography was adopted. The basic proteases secreted by *B. bassiana* indicated the presence of only one band from Tw, Ww and from up to day 2 of 0.05 M TRIS/ HCl, pH 7.4 treatment (Fig. 6.5) and 0.1 M phosphate buffer, pH 7.0 (Fig. 6.6). However, 2-D spot densitometry revealed quantitatively that the proteolytic activity (represented in pixels values) of bands was different.

6.4.6 Enzyme overlay membrane (EOM) analysis

The Tw of conidia showed the presence of maximum proteolytic activity requiring further EOM analysis for isozymes of Pr1 and Pr2. IPG strips after isoelectric focusing were sandwiched with the Suc-Ala-Ala-Pro-Phe-pNA and Bnz-Arg-pNA substrates, which are specific for Pr1 and Pr2, respectively (St. Leger et al., 1994; St. Leger, 1995). Three isoforms of Pr1 (pI 8.4-9.7) were identified from *B. bassiana* (Fig. 6.7). Yet none was observed for Pr2 on EOM from tween wash. Use of MPr inhibitor phosphoramidon and 1-10 phenanthroline in conjunction with EOM analysis revealed MPr released during tween treatment. One activity

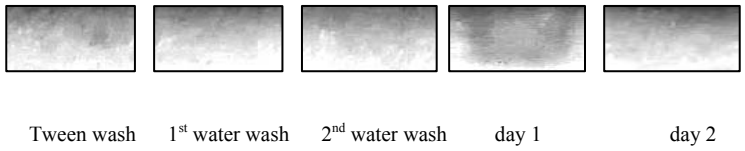


(a)

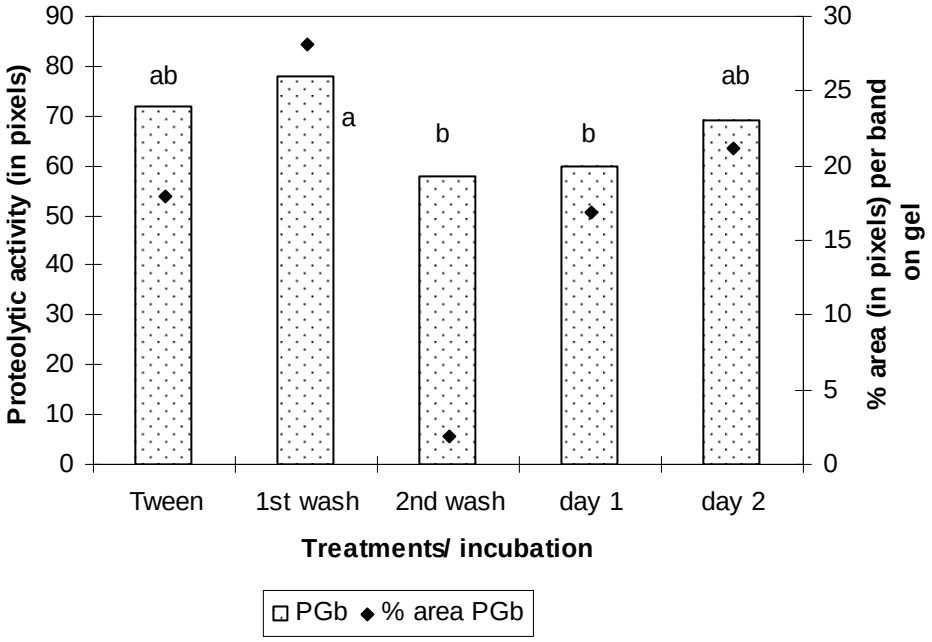


(b)

Figure 6.5: Cationic PAGE zymography of the basic proteases (PGb, gelatinase-like) released by conidia of *B. bassiana* (a). The gel was incubated in 0.05 M TRIS/ HCl buffer, pH 7.4. 2-D spot densitometry was used to determine the proteolytic activity, which are presented in pixels values (b). The percentage area of given zymography pattern per band on gel represents total density that each band has after background subtraction. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to hatched bars between different treatments are not significantly different ($p < 0.05$).



(a)



(b)

Figure 6.6: Cationic PAGE zymography of the basic proteases (PGb, gelatinase-like) released by conidia of *B. bassiana* (a) with copolymerized gelatin (1 mg/ ml) as substrate. The gel was incubated in 0.1 M phosphate buffer, pH 7. 2-D spot densitometry was used to determine the proteolytic activity, which are presented in pixels values (b). The percentage area of given zymography pattern per band on gel represents total density that each band has after background subtraction. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to hatched bars between different treatments are not significantly different ($p < 0.05$).

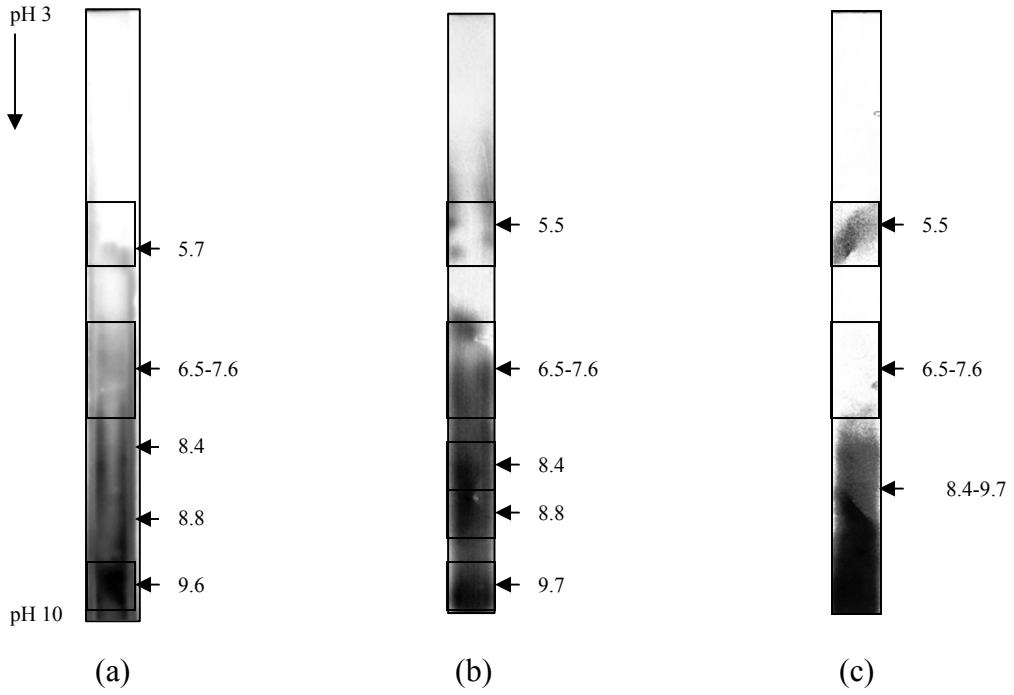


Figure 6.7: Enzyme overlay membrane sandwiched with IPG strips after isoelectric focusing of tween wash of conidia of *B. bassiana.* Chymoelastase specific substrate was used to detect the Pr1 isoforms (a). Same strip when incubated with phosphoramidon (1mM) (b) and 1-10 phenanthroline (5 mM) (c) revealed the absence of metalloprotease activities as indicated. Isoelectric points (pI) in each case are indicated along the strips.

Band with pI of 5.5 was inhibited by phosphoramidon implying its functional similarity to thermolysin like MPr. In addition, an activity band at pI 6.5-7.6 was inhibited by 1-10 phenanthroline indicating a Zn-containing neutral MPr.

6.5 Discussion

This chapter investigated the release of proteases from conidia of *B. bassiana* during hydration. Water-soaked conidia can modify the pH to create conditions for the optimal enzyme function. Hydrated conidia are endowed with the proteases (Pr1, Pr2 and MPr), which can be released into the micro-environment including that of insect cuticle-degrading activities for the onset of pathogenesis. From this perspective our findings have significance to entomopathogenic fungi. Table 6.1 showed secretion of ammonia and citrate by conidia. Similarly, evidence of ammonical by-products and organic acids on plate assay medium imply the significant pH changes for protease activity. In addition, washing of conidia in different buffers (pH 4-10) affected the activity/release of Pr1 and Pr2. There are several possibilities to explain the effects of pH on the enzyme release/activity such as change in stability, level of activities, pH inhibition and the interfering effects of buffer ions in addition to interference with the cell permeability.

The release of ammonia and citrate are unique in that they can destroy underlying tissue structure and facilitate fungal penetration. While there are several reactions, which could lead to the formation of ammonia as an end product (Stryer, 1995), and this has partially been determined in mycelia, it has not been studied in fungal conidia. Amongst EPF, St. Leger et al. (1999) reported increase in pH of the infected cuticle from 6 to 7.3 due to secretion of ammonia by *M. anisopliae* mycelia after 60 h of growth.

The more important question, however, is how does change in the pH aid conidia of EPF to initiate cuticular degradation in the first instance? In chapter 11 it has been shown that *B. bassiana* mycelia can modulate ambient pH in the presence or absence of C/ N sources thereby secreting ammonia. Among phytopathogens, ammonia is considered to be a major virulence factor for causing tissue destruction and disease development (Prusky and Yakoby, 2003). With respect to citrate, the precedent setting work of Bidochka and Khachatourians (1991) in EPF is contextual. They reported that the organic acids, especially citric and oxalic acids could aid in solubilization of the cuticular proteins when compared with inorganic acids. The role of citrate is well explained in the pathogenicity of phytopathogenic fungi (PF) and includes effects on gene expression (Prusky and Yakoby, 2003) and direct impact on host tissues. This data suggests that citric acid in addition to controlling the pH, could aid membrane destabilization helpful for enzyme release by conidia.

The identification of the isoforms of Pr1, Pr2 and MPr from tween wash of conidia by using EOM helps determine substrate preference of the individual proteases without purified enzyme preparations. EOM analysis help identify three isoforms of Pr1 (pI 8.4-9.7) from *B. bassiana*. Likewise, St. Leger et al. (1994) reported four isoforms of Pr1 (pI range 9.3-10.2) from growing mycelia of *M. anisopliae.* In another study, St. Leger et al. (1997) reported seven bands (pI range 6.8-9.6) of Pr1 from *Verticillium lecanii* (strain 1335) mycelial inocula. In a similar study on *Verticillium albo-atrum* and *Verticillium dahliae* low activity levels of neutral (pI 7) and basic (pI 9.5) subtilisin-like proteases were found.

EOM analysis revealed no Pr2 activity from *B. bassiana*; however, one band of Pr2 was identified from Tw of conidia of *M. anisopliae* (Chapter 7). In contrast, St. Leger et al. (1994) found two major isoforms with pI 4.4, 4.9 and two minor isoforms with a pI 5.2 of Pr2 from

growing mycelia of *M. anisopliae*, which might reflect that the synthesis of isoforms was due to the presence of inducer(s) derived from the cuticle. St. Leger et al. (1996a) further provided ultrastructural evidence that two isoforms (pI 4.4, 4.9) were secreted by appressoria and on the penetrating hyphae when grown on cockroach cuticle. St. Leger et al. (1997) also found high levels of Pr2 like activities (pI 4.5 and > 10) among PF, *V. albo-atrum* and *V. dahliae,* with plant cell walls as their inducers. MPr are active against a wide range of proteins (including elastin, gelatin and insect cuticle). North (1982) reported metalloproteases from *Aspergillus* spp. and *Penicillium* spp. Many MPr have rather unique specificities, which allow them to catalyze certain peptide bond cleavages more efficiently (Powers and Harper, 1986). EOM analysis revealed one activity band of thermolysin-like MPr (pI of 5.5). These results are similar to *Penicillium* spp. identified proteases (Gripon et al., 1980). In addition, one isozyme of a zinc-containing neutral MPr (pI 6.5-7.6) was identified. The neutral MPr I and II of *A. sojae* have pI of 4.7 and 4.2, respectively (North, 1982). However, only one isoform (pI 7.3) of thermolysin-like MPr was reported from *M. anisopliae* growing mycelia (St. Leger et al., 1994). Furthermore, analysis by IEF has revealed the presence of up to three isozymes of MPr in some strains of *M. anisopliae* (St. Leger et al., 1998) and some that are produced by *B. bassiana* (cited as unpublished data in St. Leger, 1995).

Insect cuticle has many proteins and other structural hydrophobic and hydrophilic residues useful for the provision of carbon and nitrogenous precursors for fungal growth. Thus, upon landing, conidia of EPF need to establish prerequisite conditions for functionality of proteases. To this end, citrate and ammonia indigenous to the conidia can become extracellular, due to the hydration and swelling. As a more effective strategy, EPF must have evolved multiple genes to produce isofunctional proteases. In such an event, release of isozymes of Pr1, Pr2 and

MPr from conidia have a synergistic role for acting on a diversity of cuticles and environmental conditions for ultimate degradation. It would be expected that most of the hydrophilic (Arg-Y or Lys-Y) residues are attacked by isozymes of Pr2, whereas hydrophobic residues (Phe-Y or Leu-Y) are degraded by Pr1. Conversely, due to their unique specificities to catalyze certain peptide bonds, MPr can help evasion of protein moieties in the cuticle/epicuticle, while avoiding any one of the protease inhibitors present in the host cuticle.

This chapter presented evidence on how conidia of EPF respond to, and modify the microenvironments in order to optimize the pH for the secreted proteases. pH-mediated gene expression is vital for pathogenicity of *A. fumigatus* (Penalva and Arst, 2004). Similarly, ambient pH regulates the expression of extracellular enzymes and hydrophobins from growing mycelia of *M. anisopliae* (St. Leger et al., 1998). In the past, investigators studying the role of pH have used mycelia. However, Herbert Arst (personal communication) found conidial pH adaptations to some extent by using a plate assay. Hence, current results address a larger question as to how these events are regulated and coordinated at the earliest phase of fungal life cycle. However, the important question is how these enzymes became associated with conidia. Small and Bidochka (2005) have reported the up-regulation of *Pr1* in *M. anisopliae* during appressoria formation and upon conidiation. Similarly, Shah et al. (2005) reported the upregulation of conidial Pr1 during growth of *M. anisopliae* in chemically defined media. Cho et al. (2006a) provided the evidence of stage specific gene expression from conidia, blastospore and aerial conidia of *B. bassiana.* Their EST analysis of cDNA libraries from aerial conidia showed the presence of subtilisin protease, tripeptidyl-peptidase and thermophlic serine protease. The diversity and functionality of conidial proteases at the initiation of the infection process may be similar to other unidentified up-regulated protease genes during conidiation.

7.0 Biochemical analysis of proteases from the conidia of *Metarhizium anisopliae*

7.1 Abstract

Proteolytic enzymes in conidia of entomopathogenic fungi (EPF) are potentially important as virulence factors since they degrade insect cuticle prior to the formation of germ tube/appressorium. When treated with either tween (Tw) or water (Ww), and upon incubation in water for up to 2 days, conidia release ammonia and citrate, resulting in a change in pH. Cationic PAGE zymography revealed two protease isozymes (Gelatinase-like), when gel was treated with phosphate buffer, pH 7. However, one activity band appeared on gel upon treatment with TRIS buffer, pH 7.4 from Tw. In addition, only one activity band appeared on the gel during the washing steps and incubation period till day 2, where spot densitometry showed differences in these activities. Further enzyme overlay membrane (EOM) analysis revealed three isoforms of (subtilisins) Pr1 (pI 8.1-9.7), one band of (trypsin) Pr2 (pI 5.2), four thermolysin-like activities (pI 4.4, 5.5, 6.5-7.0 and 7.5) and one band (pI 6.1) of zinc-containing MPr from Tw. It is speculated that the diversity of protease isozymes has evolved to maximize their potential for successful virulence in the insect hosts.

7.2 Introduction

Biological control agents (BCA) and particularly the entomopathogenic fungi (EPF) occupy a special niche in mycoinsecticide market because of their pathogenicity through contact mode and a broad host range (Khachatourians, 1991, Charnley and St. Leger, 1991; Butt, 2002). In bioinsecticide market, EPF are the only BCA available primarily being used to control the

plant injurious insects (especially sap sucking insects) associated with agro-ecosystem and forestry worldwide (Ferron, 1985; Feng et al., 1994; Valencia and Khachatourians, 1998; Khachatourians and Valencia, 1999; Qazi and Khachatourians, 2005). The mode of entry of most EPF is through direct penetration of the host cuticle. Ultrastructural and histochemical evidence has indicated that this occurs via a combination of enzymatic degradation and mechanical pressure (St. Leger, 1993; Clarkson and Charnley, 1996). *Beauveria bassiana* and *Metarhizium anisopliae* secrete a variety of hydrolytic enzymes with activity against insect cuticular components i.e., protein, chitin and lipids (St. Leger, 1993; Bidochka and Khachatourians, 1987). Several investigators have studied the properties of fungal proteases, especially Pr1 and Pr2 (St. Leger et al., 1986a; Bidochka and Khachatourians, 1987; Urtz and Rice, 2000), which are considered as key virulence determinants. Furthermore, the role of Pr1 and Pr2 in the fungal infection process has been the subject of many reviews (Khachatourians, 1991; St. Leger, 1995; Clarkson and Charley, 1996; Khachatourians, 1996).

Cuticle-degrading enzymes to date have been isolated from growing mycelia. However, in most of EPF field applications, conidia are sprayed in water or as an invert emulsion in oil. Ironically, the enzymes associated with spores have received little attention despite of their commercial significance. In precedent-setting work, St. Leger et al. (1991) reported the release of Pr1, chitinase and esterase from conidial wash after treatment with buffer, detergents and chemical agents, allowing their placement as membrane bound enzymes. Similarly, Boucias and Pendland (1991) screened spores of *B. bassiana, M. anisopliae* and *Nomurea rileyi* for the presence of alkaline phosphatase, esterase (C8), lipase (C14), leucine aminopeptidase, acid phosphatase, phosphohydrolase, ß-glucosidase, *N*-acetyl-D-glucosaminidase and α-manosidase upon direct application of spores (10^6) to APIZYM strip. Recent investigations on conidia has

provided first evidence by using biochemical and functional proteomic analysis that they are endowed with a battery of metalloproteases, chymoelastase and trypsin-like enzymes (Qazi and Khachatourians, 2007; Khachatourians et al., 2007).

The main objective of the present work is to demonstrate and characterize the diversity of protease isozymes from conidia of *M. anisopliae.* Further evidence has shown that conidia can modulate the pH of the microenvironment in the nutrient-starved conditions by secreting ammonia and citrate that can help to regulate the release/activity of extracellular proteases. These results suggest that conidia are adapted to the pathogenic mode of life style.

7.3 Materials and methods

7.3.1 Fungal isolates and culture conditions

Metarhizium anisopliae (MA 2038) was generously provided by USDA, Entomopathogenic Fungus Collection, Ithaca, NY, USA. The strain was maintained on YPG agar at 27° C. Spores were prepared from two weeks old cultures as previously described (Qazi and Khachatourians, 2007).

7.3.2 Enzyme preparation from spores

Enzyme filtrate/preparation was made as previously described in Chapter 5 (Section 5.3.3). Furthermore, Pr1, Pr2 and NAGase activities were assayed by using concentrated enzyme filtrate/preparation as described in chapter 6 (Section 6.3.8).

7.3.3 Sizing of conidia by using Multisizer III (Coulter Counter™)

Swelling of the conidia of *M. anisopliae* in type II water was determined by using Multisizer III (Coulter Counter™ ; Beckman Coulter, Inc., California, USA after system standardization. Percentage median volume of three runs was measured as previously described in chapter 6 (Section 6.3.3).

7.3.4 Extraction of enzymes from conidia

Conidial protease (Gelatinase-like) was extracted by three washing procedures (WP) as previously explained (Chapter 6, section 6.3.4). The experiment was performed in triplicate.

7.3.5 Protease plate assay medium

Protease (Gelatinase-like) activity was determined as described in chapter 6 (Section 6.3.5).

7.3.6 Evaluation of ammonical by-products and organic acids

Release of ammonical by-products and citrate was determined as previously described in chapter 6 (Section 6.3.6).

7.3.7 Effect of buffers on conidial enzyme release/activity

The effect of different pH on the release and activity of Pr1 and Pr2 was determined as previously described in chapter 6 (Section 6.3.7).

7.3.8 Enzyme assays

Trypsin (Pr2)-like activity was measured with the chromogenic substrate N-ά-benzoyl-DL-arginine-*p*-nitroanilide in 0.05 M glycine-NaOH buffer, pH 8.5. Subtilisin or chymoelastase (Pr1)-like activity was measures against Suc-Ala-Ala-Pro-Phe-4-nitroanilide in 0.05 M TRIS/ HCl buffer, pH 8.0. Trypsin and subtilisins activities were assayed as previously described (Chapter 6, section 6.3.8). Enzyme (Pr1, Pr2) activity is expressed as nmols nitroanilide (NA) released ml^{-1} min^{-1}.

Chitinase (NAGase) was assayed by was measuring chromogenic substrate *p*-Nitrophenyl-*N*-acetyl-D-glucosaminide as previously stated (Chapter 6, section, 6.3.8). NAGase activity is expressed as nmols nitrophenol (NP) released ml^{-1} min^{-1}.

7.3.9 Analytical procedures

Ammonia (NH_3 and NH_4^+) production was estimated by the method of Chaney and Marbach (1962) as described in chapter 6 (Section 6.3.9). Citrate analysis in the culture supernatant was performed by the method of Marrier and Boulet (1958) as stated in chapter 6 (Section 6.3.9).

7.3.10 Cationic PAGE native zymography

Cationic PAGE (15%) native zymography was performed with co-polymerized gelatin (1 mg/ ml) as a substrate. Sample buffer was made after Lantz and Ciborowski (1994) as previously described (Chapter 6, Section 6.3.10).

7.3.11 Enzyme activity in native gels

Enzyme activity was detected by incubating the gels in two different buffers. In first the instance, protease activity was determined by placing the gel in 0.1 M phosphate buffer (PB), pH 7.0. In second case, the gel was incubated in 0.05 M TRIS/ HCl buffer, pH 7.4. Gels were incubated at 37° C for 2.5 h. After incubation, the gels were fixed and processed as described in chapter 6 (Section 6.3.11).

7.3.12 Proteolytic activity in gels

A proteolytic unit is defined as the amount of the enzyme that can produce a background free (net) degradation of gelatin (clearing zone) of one pixel. Proteolytic activity after 2-D spot densitometry was determined as previously described (Qazi and Khachatourians, 2007)

7.3.13 Analytical isoelectric focusing

Isoelectric focusing (IEF) was performed as previously described in chapter 6 (Section 6.3.13).

7.3.14 Enzyme overlay membrane (EOM)

This method was specifically developed to detect proteases in complex biological samples. Instead of 7-amino-4-trifluoromethyl coumarin (AFC), 4-Nitroanilide (NA) derived substrates were used for EOM as previously described in chapter 6 (Section 6.6.14).

7.3.15 Statistical analysis

CoStat Version 6.204 program (CoHort Software, Monterey, CA, USA) was used for the statistical analyses. Levels of significance was determined by using one way completely

randomized design ANOVA, where means were separated by using Duncan's method ($p < 0.05$). The whole study was repeated two times.

7.4 Results

7.4.1 Release of ammonia and citrate

Hydrated conidia during soaking period in type II water have showed swelling as determined by Multisizer III (Coulter Counter™ ; Beckman Coulter, Inc., California, USA) analysis. An approximately 25% increase in the volume of conidia was observed after 48 h of incubation, after which time spore shrinkage was observed at 72 h (Fig. 7.1). As the determination of volume by Multisizer III (Coulter Counter™ ; Beckman Coulter, Inc., California, USA) is derived from changes in the conductivity, increase or decrease in volume could also reflect not just volume change but changes in particle zeta potential. Besides swelling, conidia during Tw, Ww washes and upon incubation in water till day 2 showed metabolic activity. Ammonia was detected during washing steps and incubation period up to day 1 (Table 7.1). Simultaneously, citric acid was also released by conidia in Tw and upon incubation till day 2. Citrate was not observed in Ww fractions. These results imply that the hydration alone is sufficient to start metabolic activity within the conidia. Several metabolic reactions can lead to the formation of ammonia as an end-product (Stryer, 1995), which may have toxic effects on cell (Doyle and Butler, 1990; Guerrini, 1997). The release of citric acid is not surprising as it may have multiple roles in the initiation of infection cycle; it could act as a source of nutrient to the growing conidia for the germ tube emergence by degradation of the cuticle to provide precursors necessary for growth. The conidial germination tested on YPGA plates showed that 70% of the conidia were viable after day 2.

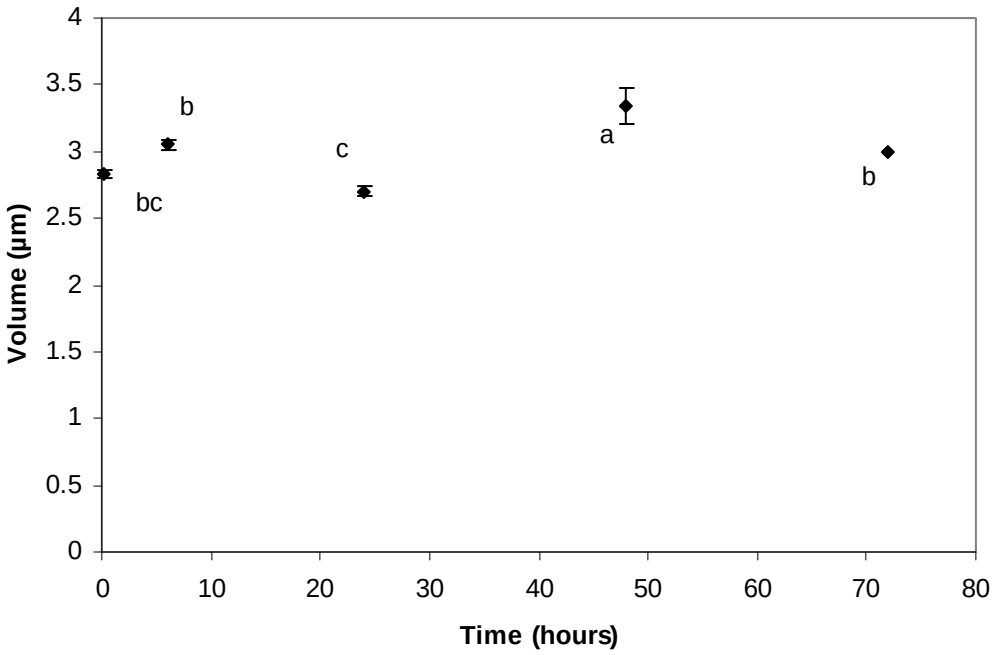


Figure 7.1: The rate of swelling of conidia of *M. anisopliae* in type II water. Each point is based on the percentage median volume of 2-3 $X10^4$ conidia. Conidial volume was measured by using Multisizer III (Coulter Counter^{TM}). Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 3) were separated by using Duncan's method ($p < 0.05$). Values following same letters are not significantly different. The error bars represents S.D.

Table 7.1 Estimation of ammonia, citrate, protein and pH in the wash and incubation supernatant of *M. anisopliae* during isotropic growth/swelling phase.

Condition	Treatment	pH*	Ammonia (µM)	Citrate (µg)	Protein (µg)
Wash	Tween	7.93^{a}	120 (3.0)	6.25 (1.85)	1.7 (0.08)
	1^{st} dH_2O	7.67^{b}	36 (0.9)	0	4.7 (0.08)
	2^{nd} dH_2O	7.69^{b}	12 (0.20)	0	0
Incubation	24 h	7.12^{c}	70 (1.0)	3.75 (0.1)	0
	48 h	7.05^{d}	0	6.5 (1.08)	0.75 (0.1)

Legend:

*Level of significance ($p < 0.05$) was determined by using one way completely randomized design ANOVA, where means were separated by using Duncan's method. Values following same letters in each column are not significantly different. Values in brackets show S.D., where n = 3.

7.4.2 Release of Pr1 and Pr2 from conidia of *M. anisopliae*

Results showed the presence of varying levels of protein during washing steps and incubation period (Table 7.1). In addition, Tw, Ww and incubation supernatant contained activities resembling to chymoelastase and trypsin. It was apparent that Tw released the maximum amount of Pr1-and Pr2-like activities (Fig. 7.2). Interestingly, more Pr2 activity was observed than Pr1 during both aqueous washes (Tw and Ww) and incubation period. Subsequently, Pr1 and Pr2 activities were observed during the 1st wash and upon incubation till day 2. Basal level of Pr1 and Pr2 were observed at day 1 and 2. The change in Pr1 and Pr2 activity may be explained as a result of change in the pH of the wash and incubation supernatant. Significant reduction in the pH of Tw wash was evident in comparison to incubation period (day 2).

The data also suggests that both proteases were associated with conidial wall-membrane, which could be disrupted by the action of mild detergents. Conversely, presence of both activities in water supernatant implies that the buffering action (due to released ammonia and citrate) could break hydrogen bonding between enzymes and spore wall. Interestingly, enzymes were active till day 2, which indicate that they were stable upon incubation, and have potential substrate degrading ability in the absence of freely available C/ N substrate.

7.4.3 Effect of pH on conidial enzyme release/activity

Results show a change in pH of the spent citrate/ phosphate buffer, pH 4 and 6 (Fig. 7.3). No change in pH of the TRIS/ HCl and carbonate/ bicarbonate buffers, pH 8 and 10, respectively was observed. Maximum activity for Pr1 was observed at pH*i* 10, whereas optimum activity of Pr2 was observed at pH 4 and 6.

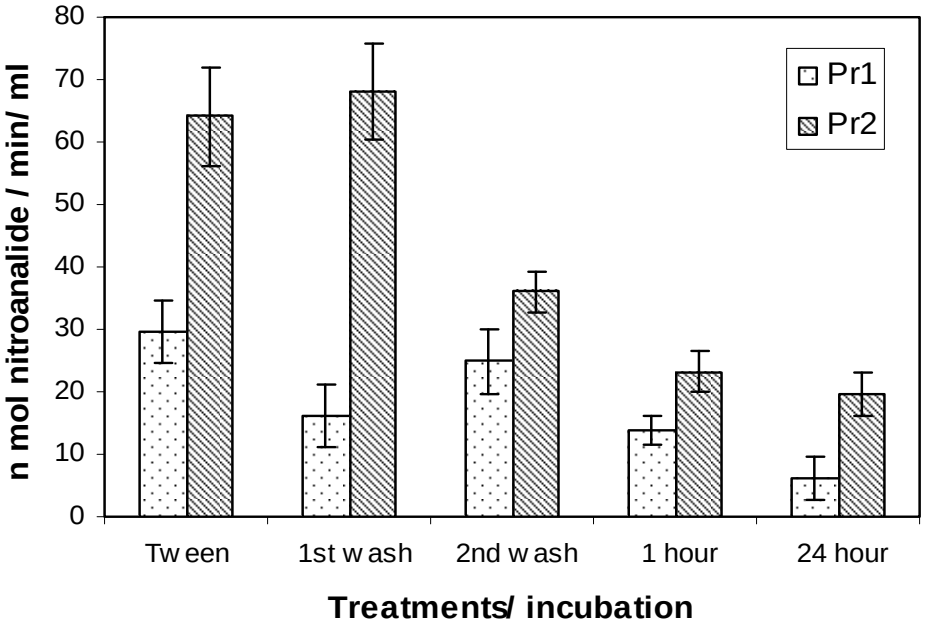


Figure 7.2: Subtilisin (Pr1) and trypsin (Pr2) activities from the conidia of *M. anisopliae* Tw and Ww, and upon incubation in water until day 2. Error bars represents S.E., where n = 4.

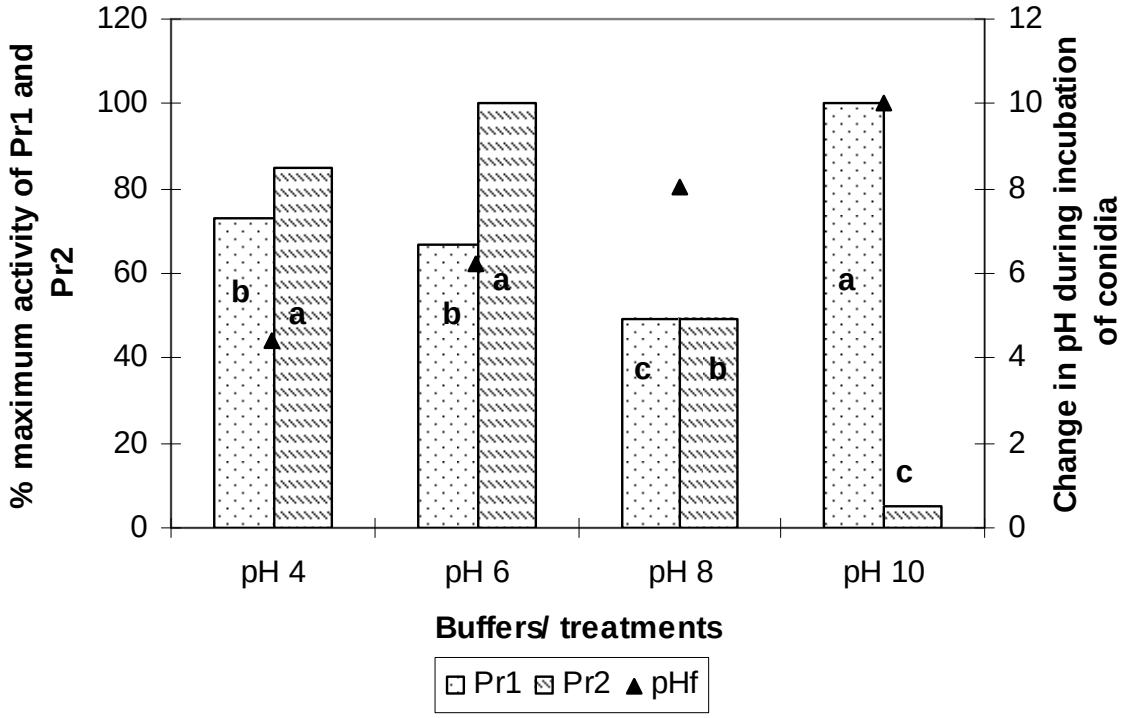


Figure 7.3: Release/activity of Pr1, Pr2 and change in pH of the buffer supernatants after 6 h of incubation of conidia of *M. anisopliae.* The buffers employed in this study were: 0.1 M citrate/ phosphate (pH 4 and 6), 0.1 M TRIS/ HCl, pH 8.0 and 0.1 M carbonate/ bicarbonate, pH 10. Buffered spore suspensions were incubated at 27° C before being assayed for protease activities. Level of significance ($p < 0.05$) are indicated showing values with same letters are not significantly different.

7.4.4 Release of proteases from conidia

Results presented in Figure 7.4 show that the washing of conidia in Tween released the maximum amount of protease. Similarly, the release of protease in Ww implies that the some activity was loosely bound to spore via electrostatic forces. Protease release/activity was also found in 0.03 M TRIS/ HCl buffer washes after 1 h of incubation. No protease activity present in TRIS/ HCl (0.03M and 0.3 M) spent buffer wash after 24 h. Washing of conidia in 0.03% and 0.3% tween revealed enzyme release after 1 h of incubation. No activity was observed in 24 h tween (0.03% and 0.3%) incubation fractions. Plate assay medium for the visual detection of metabolites and protease activity showed the conidial pH adaptations and protease activity.

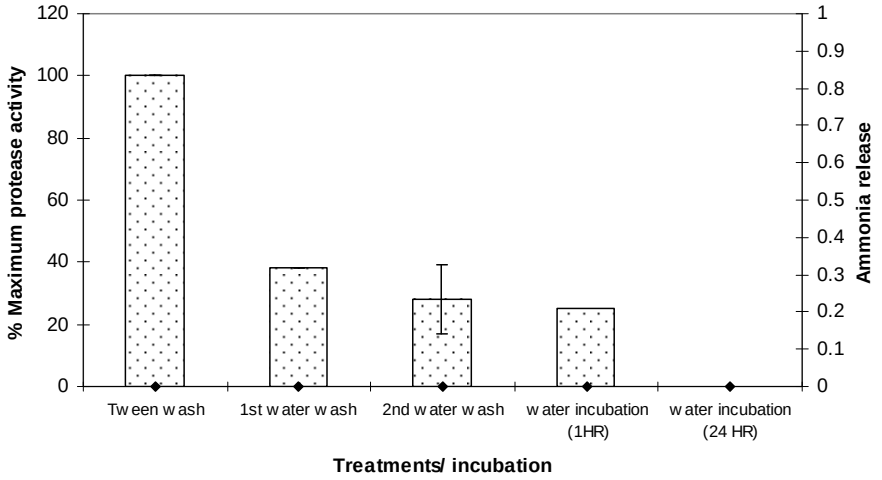
7.4.5 Cationic PAGE zymography

Cationic PAGE zymography was used to reveal the basic protease release during Tw, Ww and incubation period. Results indicated the presence of only one (Fig. 7.5) protease from Tw, Ww and incubation supernatant when gel was treated with 0.05 M TRIS/ HCl, pH 7.4. In contrast, two isozymes (Fig. 7.6) were observed with Tw and one activity band was evident from Ww and incubation supernatant up to day 2 when gel was treated with 0.1 M phosphate buffer, pH 7.0. Further 2-D spot densitometry revealed quantitatively that the proteolytic activity (represented in pixels values) released during different treatments and incubation periods was different.

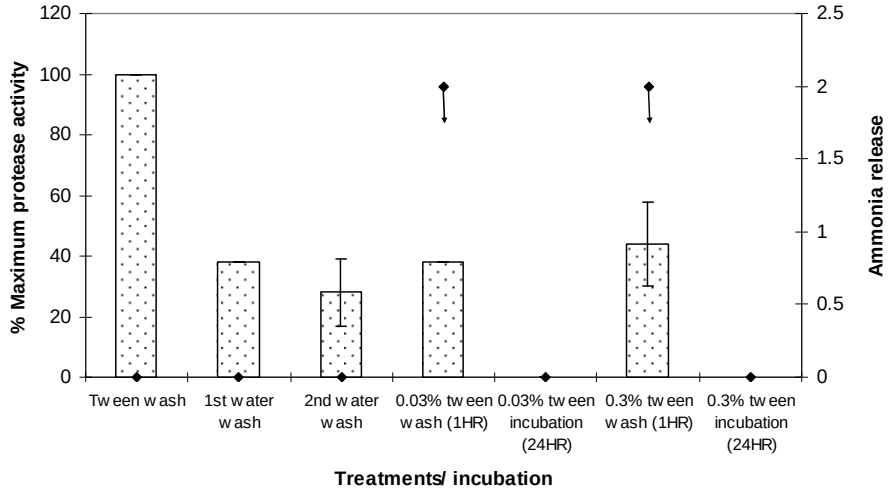
7.4.6 Enzyme overlay membrane (EOM) analysis

Tw of conidia was further subjected to EOM analysis for isozymes of Pr1 and Pr2. IPG strips, after isoelectric focusing, were sandwiched with the Pr1 (Suc-Ala-Ala-Pro-Phe-pNA)-and

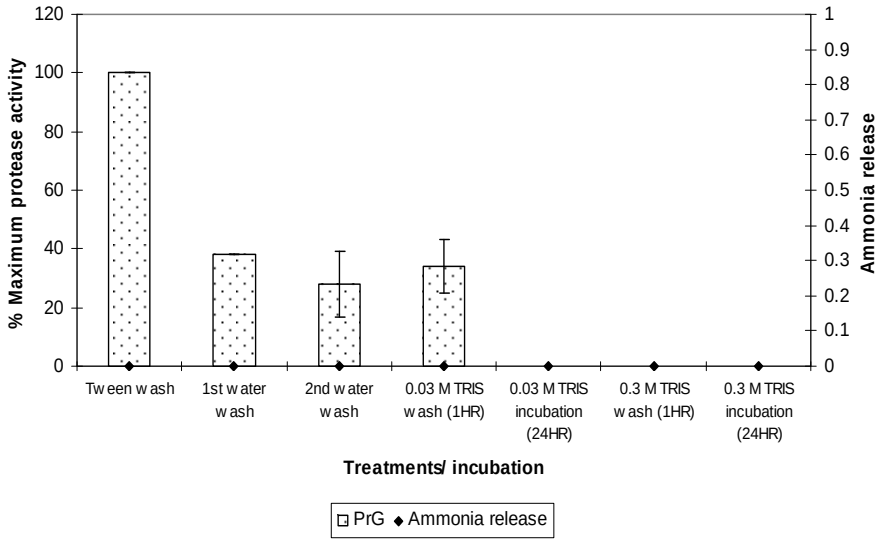
Figure 7.4: Release/activity of protease from the conidia of *M. anisopliae* during different washing procedures designated as: WP-1, WP-2 and WP-3. In WP1, WP-2 and WP-3 the total activity of the first tween wash of the conidia was set as 100% relative activity. The activities of the pooled water washes are represented as a proportion to this reference. Additionally, the sequential differential extractability with water [1 h and 24 h incubation supernatants] (a) 0.03% and 0.3% tween [1 h and 24 h incubation supernatants] (b) and 0.03 M and 0.3 M buffer [1 h and 24 h incubation supernatants] I. Ammonia was scored visually as 1 (low), 2 (medium) and 3 (high), respectively after 24 h of incubation. The arrows represent release of citrate that was observed in < 60 min of dispensing enzyme filtrate on plate assay medium.

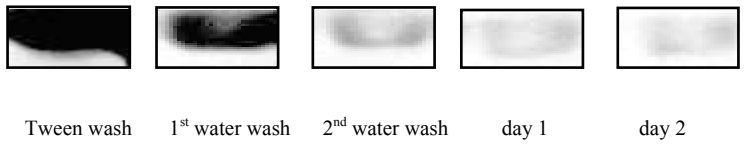

% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
water incubation (1HR)
water incubation (24 HR)
Treatments/ incubation

(a)

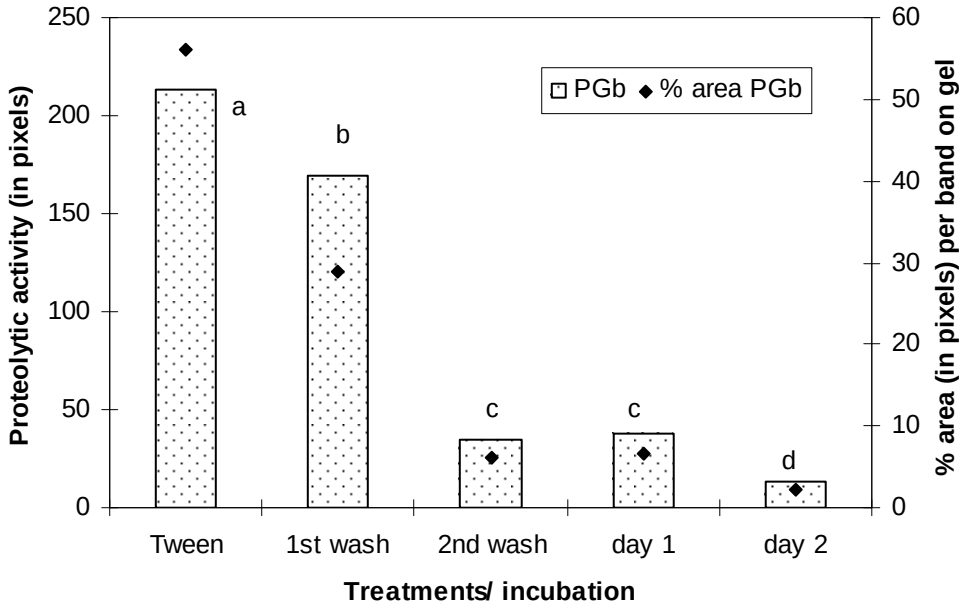

% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
0.03% tween wash (1HR)
0.03% tween incubation (24HR)
0.3% tween wash (1HR)
0.3% tween incubation (24HR)
Treatments/ incubation

(b)


% Maximum protease activity
Ammonia release
Tween wash
1st water wash
2nd water wash
0.03 M TRIS wash (1HR)
0.03 M TRIS incubation (24HR)
0.3 M TRIS wash (1HR)
0.3 M TRIS incubation (24HR)
Treatments/ incubation
PrG
Ammonia release

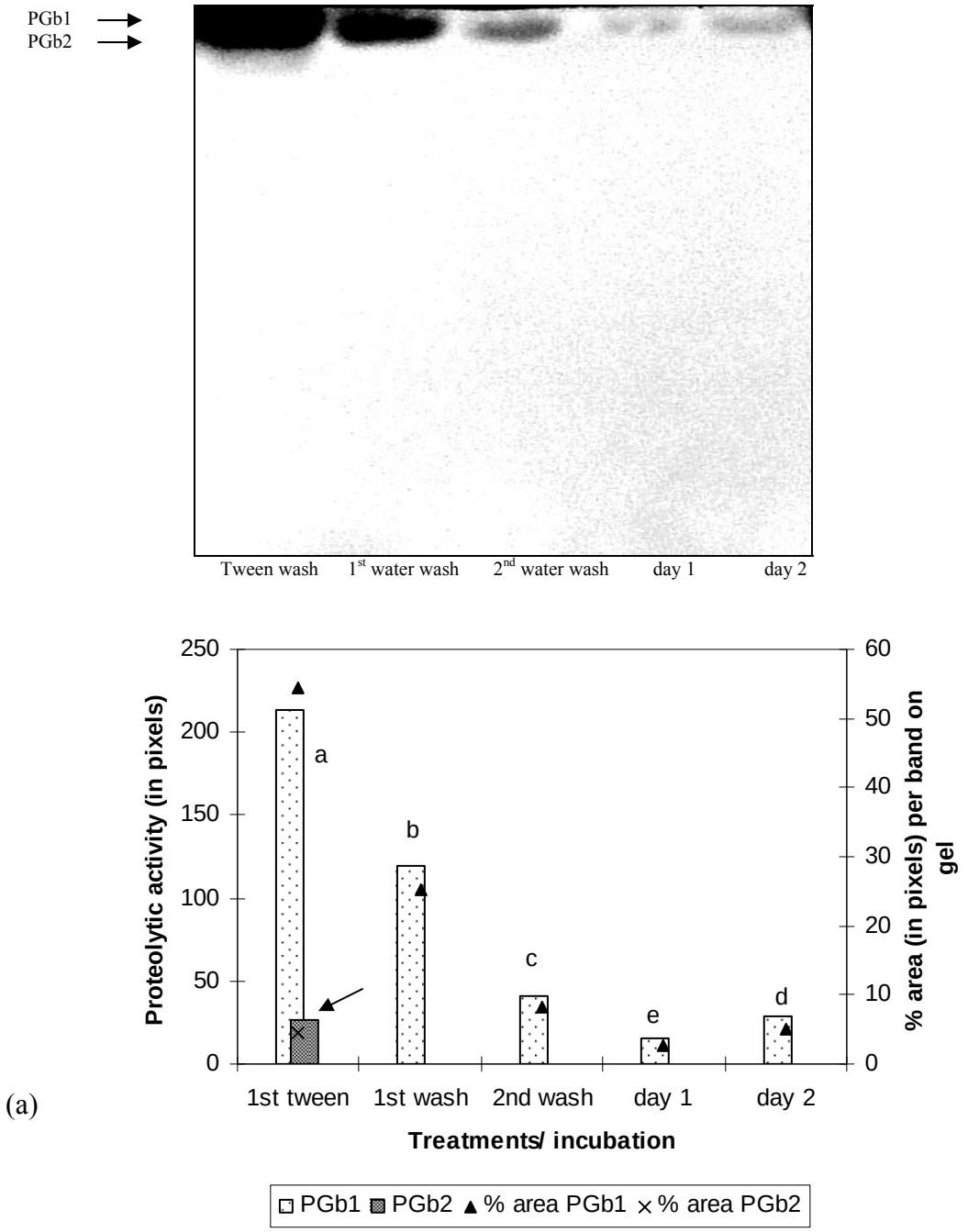


(a) Tween wash 1st water wash 2nd water wash day 1 day 2



(b)

Figure 7.5: Cationic PAGE zymography of the basic proteases (PGb, gelatinase-like) released by the conidia of *M. anisopliae* (a). The gel was incubated in 0.05 M TRIS/ HCl buffer, pH 7.4. 2-D spot densitometry was used to determine the proteolytic activity, which are presented in pixels values (b). The percentage area of given zymography pattern represents total density that each band has after background subtraction. Results are the representative of three different experiments. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to hatched bars between different treatments are not significantly different ($p < 0.05$).



(a)

Figure 7.6: Cationic PAGE zymography of the basic proteases (PGb, gelatinase-like) released by conidia of *M. anisopliae* (a). The gel was incubated in 0.1 M phosphate buffer, pH 7.0. The 2-D spot densitometry was used to determine the proteolytic activity, which are presented in pixels values (b). Arrow indicates a new activity band. The percentage area of given zymography pattern represents total density that each band has after background subtraction. The level of significance was determined by one way completely randomized ANOVA. Same letters appearing adjacent to hatched bars between different treatments are not significantly different ($p < 0.05$).

Pr2 (Bnz-Arg-pNA)-specific substrates (St. Leger et al., 1994). Three isoforms of Pr1 were identified from *M. anisopliae* (pI 8.1-9.7) (Fig. 7.7). Conversely, only one band of Pr2 (pI 5.0) was observed for *M. anisopliae* (Fig. 7.8). Use of the MPr inhibitor phosphoramidon and 1-10 phenanthroline in conjunction with EOM analysis revealed the release of MPr during Tw. Results showed four activities (pI 4.4, 5.5, 6.5-7.0, 7.5) inhibited by phosphoramidon implying their functional similarity to thermolysin-like MPr. In addition, an activity band of pI 6.1 was inhibited by 1-10 phenanthroline indicating its similarity to Zn-containing neutral MPr.

7.5 Discussion

In the preceding chapter, release of citrate, ammonia and proteases from conidia of *B. bassiana* was described. Herein similar work is extended with another broad range insect pathogen, *M. anisopliae* that can produce 11 subtilisins (Bagga et al., 2004). Hydrated conidia can modify the pH of the microhabitat and create conditions for the optimal enzyme function. Water-soaked conidia are endowed with the isoforms of Pr1, Pr2 and MPr, which can be released immediately into the micro-environment before the host defenses become active. This adaptation may enable entomopathogens to initiate degradation of the cuticular components prior to the formation of a germ tube/appressoria. There are several lines of evidence that pH changes affect the release/activity of conidial proteases. Firstly, ammonia and citrate release along with pH change is evident (Table 7.1). Secondly, Tw, Ww and incubation supernatant has varying level of Pr1 and Pr2 release/activity. Thirdly, washing of conidia in various buffers have affected the Pr1 and Pr2 activity/release. Finally, conidial pH adaptation along with protease release/ activity is evident from plate assay medium. Taken together, the results suggest that environmental pH modifications occur at the pre-germination phase that is important for

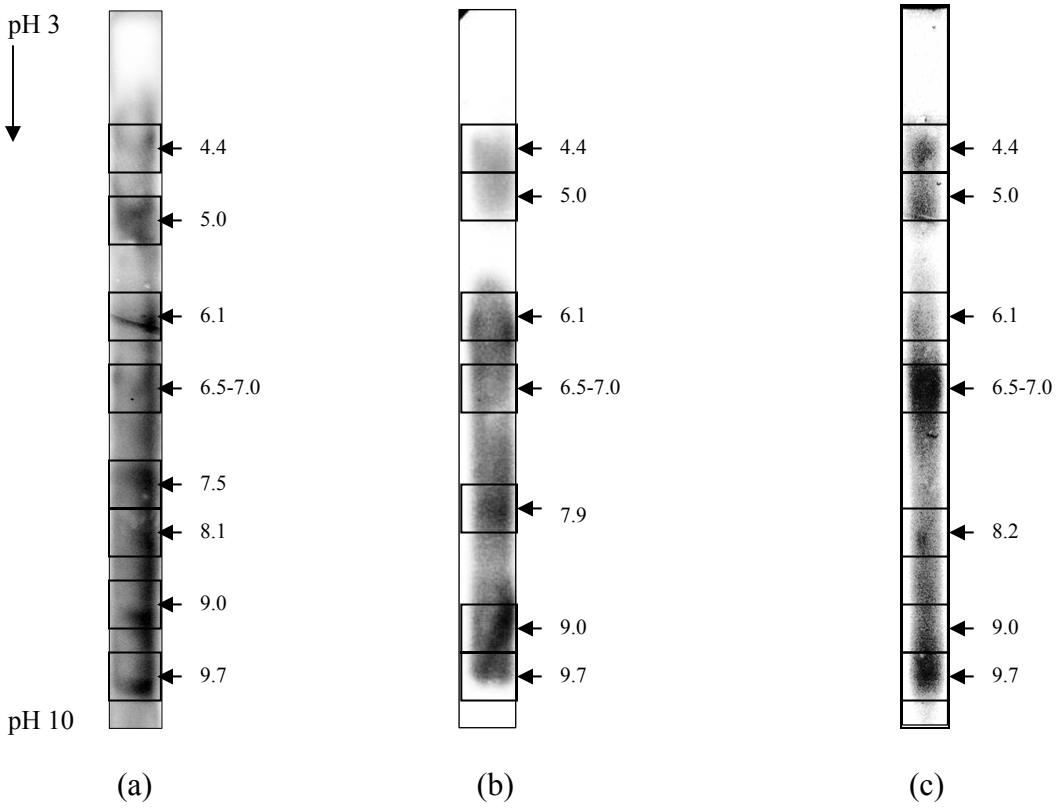


Figure 7.7: Enzyme overlay membrane sandwiched with IPG strips after isoelectric focusing of tween wash of conidia of *M. anisopliae*. Chymoelastase-specific substrate was used to detect the Pr1 isoforms (a). Same strip when incubated with phosphoramidon (1 mM) (b) and 1-10 phenanthroline (5 mM) (c) revealed the absence of metalloprotease activities as indicated. Isoelectric points (pI) in each case are indicated along the strips.

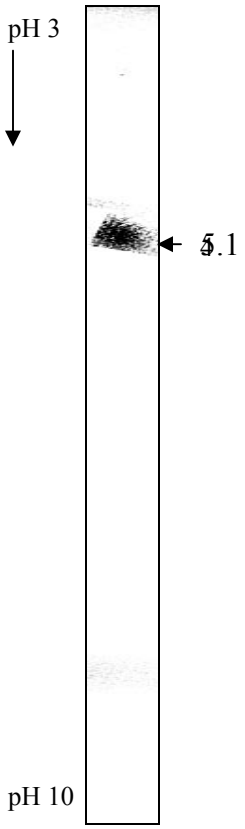


Figure 7.8: Enzyme overlay membrane sandwiched with IPG strips after isoelectric focusing of tween wash of conidia of *M. anisopliae*. Trypsin-specific substrate was used to detect the Pr2 isoform. Isoelectric point (pI) is indicated along the strip.

proteases(s) activity/release. Several hypotheses could explain the effect of pH on enzyme release/activity such as change in stability, level of activities, pH inhibition and the interfering effects of buffer ions in addition to interference with the cell permeability.

In addition to pH changes, ammonia and citrate release from phytopathogenic fungi could destroy host tissues and facilitate their penetration along with their effect on gene expression (Prusky and Yakoby, 2003). Amongst EPF, Bidochka and Khachatourians (1991) showed that the organic acids, especially citric and oxalic acids could aid in solubilization of the cuticular proteins in contrast to inorganic acids. Both metabolites, in addition to controlling the pH, may aid membrane destabilization helpful to enzyme release by conidia.

The release of the isoforms of chymoelastase (Pr1), trypsin (Pr2) and MPr from Tw of conidia revealed that they are pre-adapted for pathogenic life style. Three isoforms of Pr1 were identified from *M. anisopliae* (pI 7.5-9.7). Similarly, in chapter 6 three isoforms of Pr1 from conidia of *B. bassiana* was reported. In contrast, St. Leger et al. (1994) reported four isoforms of Pr1 (pI range 9.3-10.2) from mycelial inocula of *M. anisopliae.* In a subsequent study, St. Leger et al. (1997) reported seven isoforms (pI range 6.8-9.6) of Pr1 from *Verticillium lecanii* strain 1335. In a similar study on *Verticillium albo-atrum* and *Verticillium dahliae* low activity levels of neutral (pI 7) and basic (pI 9.5) subtilisin-like proteases were found. In nematophagous fungus, *Verticillium chlamydosporium* Segers et al. (1994) have purified a subtilisins protease VCP1 (pI 10.2) during submerged culture on soya peptone. Similarly, Segers et al. (1999) reported the pI of Pr1 like enzymes as 10, 10.4, 10.2 and 9.2 from *Verticillium chlamydosporium* strain no 10, 8, 65 and 11, respectively. In similar study they found at least four Pr1 isoforms from strain 26, ranging from very alkaline (pI 10.5, 10.2 and 9.1) to almost neutral (pI 7.8 to 7.4). EOM analysis revealed only one activity band of Pr2 from conidial Tw wash. Conversely,

Pr2 was not identified from Tw of conidia of *B. bassiana* (Chapter 6). St. Leger et al. (1994) found two major isoforms (pI 4.4, 4.9) and two minor isoforms (pI 5.2) of Pr2 from growing mycelia of *M. anisopliae.* St. Leger and co-workers further provided ultrastructural evidence that two isoforms (pI 4.4, 4.9) were secreted by appressoria on the penetrating hyphae when grown on the cockroach cuticle. In contrast to EPF, St. Leger et al. (1997) also found high levels of Pr2 like activities (pI 4.5 and > 10) among PF, *V. albo-atrum* and *Verticillium dahliae,* with plant walls as their inducers.

Metalloproteases are active against a wide range of proteins and have unique specificities allowing them to catalyze certain peptide bond cleavages more efficiently than other proteases (Powers and Harper, 1986). Four isoforms (pI 4.4, 5.5, 6.5-7.0, 7.5) of thermolysin-like MPr were released during Tw of conidia, which were insensitive to 1-10 phenanthroline (except pI 7.5). In this regard, North (1982) reported metalloproteases from *Aspergillus* spp. and *Penicillium* spp. Gripon et al. (1980) reported similar proteases from *Penicillium* spp. In addition, one isozyme of zinc-containing neutral MPr from *M. anisopliae* (pI 6.1) was identified. Similarly, North (1982) reported the neutral MPr I and II of *A. sojae* that have pI of 4.7 and 4.2, respectively. Amongst EPF, only one isoform (pI 7.3) of thermolysin-like MPr was reported from *M. anisopliae* mycelia grown in cockroach cuticle basal medium (St. Leger et al., 1994). In a subsequent study, St. Leger et al. (1998) reported the presence of up to three isozymes of MPr in some strains of *M. anisopliae.* Furthermore, analysis by IEF has revealed the presence of up to three isozymes of MPr in some strains of *M. anisopliae* (St. Leger et al., 1998) and some that are produced by *B. bassiana* (cited as unpublished data in St. Leger 1995).

Collectively, these results indicate that hydrated conidia of *M. anisopliae* can release Pr1, Pr2 and MPr. Their release upon washing in water, buffers and detergents imply that are located

at the surface layers of conidia. Similar, results were observed for *M. anisopliae* conidial Pr1, NAGase and esterase by St. Leger et al. (1991). It is probable that the same occurs under natural humid conditions. In addition, results show that citrate and ammonia release from hydrated conidia can provide buffering conditions for such proteases. In such an event, immediate release of conidial isozymes (Pr1, Pr2 and MPr) in the vicinity of insect integument provides an additive effect for the removal of cuticle monomers.

The role of extracellular enzymes from germinating conidia of *N. crassa* has been documented (Schmit and Brody, 1976). Penalva and Arst (2004) have shown that pH mediated gene expression in fungi is operated via *pacC*. Similarly, St. Leger et al. (1998) showed that the ambient pH regulate the expression of proteases, chitinase and hydrophobins in *M. anisopliae.* Contextual to the initiation of EPF infection process it has been shown (i) Pr1, Pr2 and NAGase synthesis during conidial growth of *B. bassiana* on aphid exuviae is different from growing mycelia (Qazi and Khachatourians, 2008), and (ii) ambient pH regulate the synthesis of Pr, Pr2 and NAGase from germinating conidia of *B. bassiana* and *M. anisopliae* (Chapter 10).

Results show that the synthesis of Pr1, Pr2 and MPr from germinating conidia is different from growing mycelia. Small and Bidochka (2005) have reported the up-regulation of *Pr1* (cag genes) in *M. anisopliae* at appressoria formation and upon conidiation. Similarly, Shah et al. (2005) have reported the upregulation of conidial Pr1 during growth of *M. anisopliae* in chemically defined media. More important work in the above context is of Cho et al. (2006a) who provided the first evidence of stage specific gene expression in *B. bassiana.* Their EST analysis of cDNA libraries from aerial conidia, blastospores and submerged conidia has 20-30% unique sequences to each library. They found unique EST sequences to conidia as subtilisin-like protease, tripeptidyl-peptidase and a thermophlic serine protease. Furthermore, Cho et al.

(2006b) described a comparative analysis of EST from aerial conidia with growing mycelia on chitin and oosporein producing media. They found eight subtilisins from *B. bassiana* with varying level of similarity to *M. anisopliae* enzymes (PR1A, PR1B, PR1C, PR1D, PR1G, PR1H, PR1J, PR1K), four of which (conidia_11-D01.e, conidia_22-C04.e, conidia_08-G10.e and conidia_12-H11.e) were unique to conidia. Taken together, these results raise a crucial question which is to further investigations on the genes and proteins up-regulated during the development cycle of these EPF. The diversity of protease isozymes reported in this study may also be similar to other unidentified up-regulated protease genes during conidiation.

8.0 Proteomic study of the hydrated conidia of *Beauveria bassiana* and *Metarhizium anisopliae*

8.1 Abstract

Conidia as infective propagules of *Beauveria bassiana* and *Metarhizium anisopliae* must adhere to substrate and germinate. However, information on proteins/enzymes that may take part at the initiation of germination/swelling phase is lacking. Tween wash of conidia of *B. bassiana* and *M. anisopliae* was analyzed by using high throughput 2-DE combined with mass spectrometry (MALDI-TOF/ LC-LC/ MS). Several proteins/enzymes were identified that may have an important role at the initiation of germination/swelling phase. Proteins similar to ROD 1, ά- and β-glucanases, elastase, lipase 5 and galectin 7, were identified. Results suggest that these conidia are pre-adapted to the pathogenic mode of life style. This is the first evidence of the proteome of the hydrated conidia of fungi, which suggests that these proteins have diverse functions during fungal metabolism/pathogenesis.

8.2 Introduction

Conidia, which are often used as infective propagules of EPF, are dormant, resilient and of commercial importance. In general, conidia are formulated in water or invert emulsion in oil before being sprayed. As a prerequisite to successful infection by conidia they must adhere to the cuticle and germinate (Boucias and Pendland, 1991; Jeffs et al., 1999). However, to the breach cuticle EPF produce key enzymes (i.e., proteases, chitinases and esterases) that serve as virulence factors (Charnley, 1997; Khachatourians, 1991). The role of proteases in this regard is vital because cuticle is composed up of 75% proteins (Neville, 1975). In *M. anisopliae,*

extracellular enzymes such as subtilisins (Pr1), trypsin (Pr2) were produced at the initial and late phases of growth by appressoria and growing mycelia (St. Leger, 1995). However, appressoria formation occurs after 12 h (or long) of growth of EPF if grown on nutrient-poor media. This raises a critical question as to how conidia tolerate starvation for approximately 12 h prior to germ tube/appressoria formation. Although there is inadequate knowledge of the conidial metabolism of EPF during swelling phase yet the biochemical and molecular event associated with water soaked conidia of filamentous fungi are well documented (de-Enfert, 1997).

Swelling of conidia of *Neurospora crassa* is the first stage of germination that was observed in de-ionized water (Schmit and Brody, 1976). In addition, soaking of conidia in de-ionized water synchronize germination. Several biochemical events associated with conidia activation have been documented (Schmit and Brody, 1976). Similarly in EPF, Dillon and Charnley (1991) have showed that soaking of conidia in distilled water synchronize germination, but they do not swell in water unless provided exogenous nutrients. D' Enfert (1997) reported that the activation of several genes involved in ribosome assembly and in amino acid biosynthesis occur very rapidly at the onset of isotropic growth or swelling phase of water soaked conidia of *Aspergillus nidulans* and *N. crassa*. Ironically, there is a dearth of knowledge of conidial swelling of EPF. Recently Qazi and Khachatourians (2007) and Khachatourians et al. (2007) provided the first evidence that the conidia of EPF show swelling in water, releasing multiple proteases and primary metabolites (citrate/ammonia). Indeed, after adherence and before germination of conidia there is a crucial missing link/step of swelling that occurs in the presence of water or may happen in nature due to water.

Hydration can switch on metabolic processes within conidia that may have vital role at the onset of germination/fungal pathogenesis. Therefore, hydrated conidia that are metabolically

active trigger conidial metabolism and release of proteases to start cuticle degradation prior to the emergence of germ tube/appressoria formation. High throughput 2-DE combined with mass spectrometry (MALDI-TOF or LC/LC MS) was used for the identification of the proteomic map of the hydrated conidia.

8.3 Materials and methods

8.3.1 Fungal isolates and culture conditions

Beauveria bassiana (GK2016) was obtained from BioInsecticide Research Laboratory, Department of Applied Microbiology and Food Science. *Metarhizium anisopliae* (MA 2038) was generously provided by USDA, USA. Fungal cultures were maintained on YPG agar at 27° C. Spores were prepared from two weeks old cultures as described in Qazi and Khachatourians (2007).

8.3.2 Enzyme preparation from spores

Spore-enzyme filtrate/preparation was made as previously described in chapter 5 (Section 5.3.3).

8.3.3 Analytical isoelectric focusing

Isoelectric focusing (IEF) was performed in order to separate the proteins on the basis of their isoelectric points as previously mentioned in chapter 6 (Section 6.3.13). Samples were made in two different buffers. Concentrated samples (X 3) were dissolved in 2% biolyte and 2% CHAPS for *M. anisopliae*. However, sample from *B. bassiana* was dissolved in 5 M urea, 2% biolyte and 2% CHAPS.

8.3.4 Two-dimensional Electrophoresis (2-DE)

Second dimension was performed for the separation of proteins on the basis of molecular weights. Sample preparation was performed in the following two ways. (i) For MA 2038 protein analysis, IPG strip (7 cm; 3/10) was equilibrated for 30 min in rehydration buffer [2% SDS, 50 mM TRIS/ HCl (pH 8.8), 30% glycerol and 0.02% bromophenol blue]. (ii) For GK 2016 protein profiles, IPG strip was equilibrated for 30 min in rehydraton buffer as described above except that it contained 5 M urea. IPG strip was overlaid on the 12% polyacrylamide. Electrophoresis was performed at room temperature (20-22° C) with the constant voltage in stepped fashion on Mini protein-II (Bio-Rad, Hercules, USA; 50V for 15min, 100V for 20 min, 150V for 40 min or until the tracking dye was within the 2 mm of the bottom of the gel. Next, gels were fixed for 30 min in a fixative and stained with blue silver for 3-5 h (Candiano et al., 2004). Gels were de-stained on rotary shaker (5 rpm) in de-staining solution. Gels were scanned on flat bed scanner (HP 2200; Hewlett-Packard Company, Palo Alto, USA) at 600 dpi and files were saved in TIFF format for image analysis. Finally, the image was inverted and adjusted by using Adobe Photoshop version 6 (San Jose, CA, USA).

8.3.5 Peptide sequencing

Mass spectrometry (MALDI-TOF/ LC-MS/ MS) service was performed by the National Research Council of Canada-Plant Biotechnology Institute (Saskatoon), which is described below.

8.3.5.1 MALDI-TOF

MALDI-TOF was performed as previously described in chapter 5 (Section 5.3.8.1).

8.3.5.2 Protein identification by LC-MS/ MS

Protein spots were excised manually from blue silver (Candiano et al., 2004) stained gels using a scalpel in the Molecular Microbiology laboratory, University of Saskatchewan and placed in a 96-well microtitre plate (Sigma, Milwaukee, USA). The proteins were then automatically de-stained, reduced with DTT, alkylated with iodoacetamide, and digested with porcine trypsin (Sequencing grade, Promega, Madison, USA) using a MassPREP protein digest station (Micromass, Manchester, UK). The digest was evaporated to dryness, then dissolved in 12 µl of 1% aqueous TFA, of which 6 µL were analyzed by LC-MS/ MS using a capLC ternary HPLC system (Waters, Milford, USA) interfaced to a Q-TOF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Micromass, Manchester, UK). Solvents A and C comprised 0.2% formic acid in water, while solvent B consisted of 0.2% formic acid in acetonitrile. The peptide digest sample was loaded onto a C18 trapping column (Symmetry 300, 0.35 x 5 mm Opti-pak; Waters, Milford, USA) and washed for 3 min using solvent C at a flow rate of 30 µL/ min. The flow path was then switched using a 10-port rotary valve, and the sample eluted onto a C18 analytical column (PepMap, 75 µm x 15 cm, 3-µm particle size; LC Packings). Separations were performed using a linear gradient of 5:95% to 60:40 % B:A over 43 min. The composition was then changed to 80:20 % B:A and held for 10 min to flush the column before re-equilibrating for 7 min at 0:100 % A:B. Mass calibration of the Q-TOF instrument was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the *m/z* range 50 to 1900. LC-MS/ MS analysis was carried out using data dependent acquisition, during which peptide precursor ions were detected by scanning from *m/z* 400 to 1900 in TOF MS mode. Multiply charged (2+, 3+, or 4+) ions rising above predetermined threshold intensity were automatically selected for TOF MS/ MS analysis, by directing these ions

into the collision cell where they fragment using low energy CID by collisions with argon and varying the collision energy by charge state recognition, product ion spectra were acquired over the *m/z* range 50 to 900. LC-MS/ MS data were processed using Protein Lynx v 2.15 software (Micromass, Manchester, UK) and searched against the NCBInr or Swissprot database using MASCOT (Matrix Science Inc., Boston, MA). Searches were performed using carbamidomethylation of cysteine as the fixed modification and oxidation of methionine as the variable modification, allowing for one missed cleavage during trypsin digestion.

8.4 Results and discussion

Hydrated conidia are active in metabolism and swell upon contact with water. Interestingly, hydrated conidia released proteases (acidic, Pr1, Pr2 and MPr) and metabolites (citrate/ammonia) during soaking period/under extremely starved conditions (Qazi and Khachatourians, 2007; Khachatourians et al., 2007). Proteomic analysis of the spent supernatant (0.03% tween) of the conidial wash was analyzed by using mass spectrometry (MALDI/ LC-MS/ MS).

This chapter provides the first evidence of the secreted protein profiles by the hydrated conidia of two EPF, *B. bassiana* and *M. anisopliae* that may have important role, in the initiation of infection process or during fungal growth. Selected spots from 2-DE gels were subjected to mass spectrometric analysis, as described in materials and methods section. Results showed that the conidia of *B. bassiana* (Fig. 8.1 and Table 8.1) and *M. anisopliae* (Fig. 8.2 and Table 8.2) have secreted different proteins as discussed below.

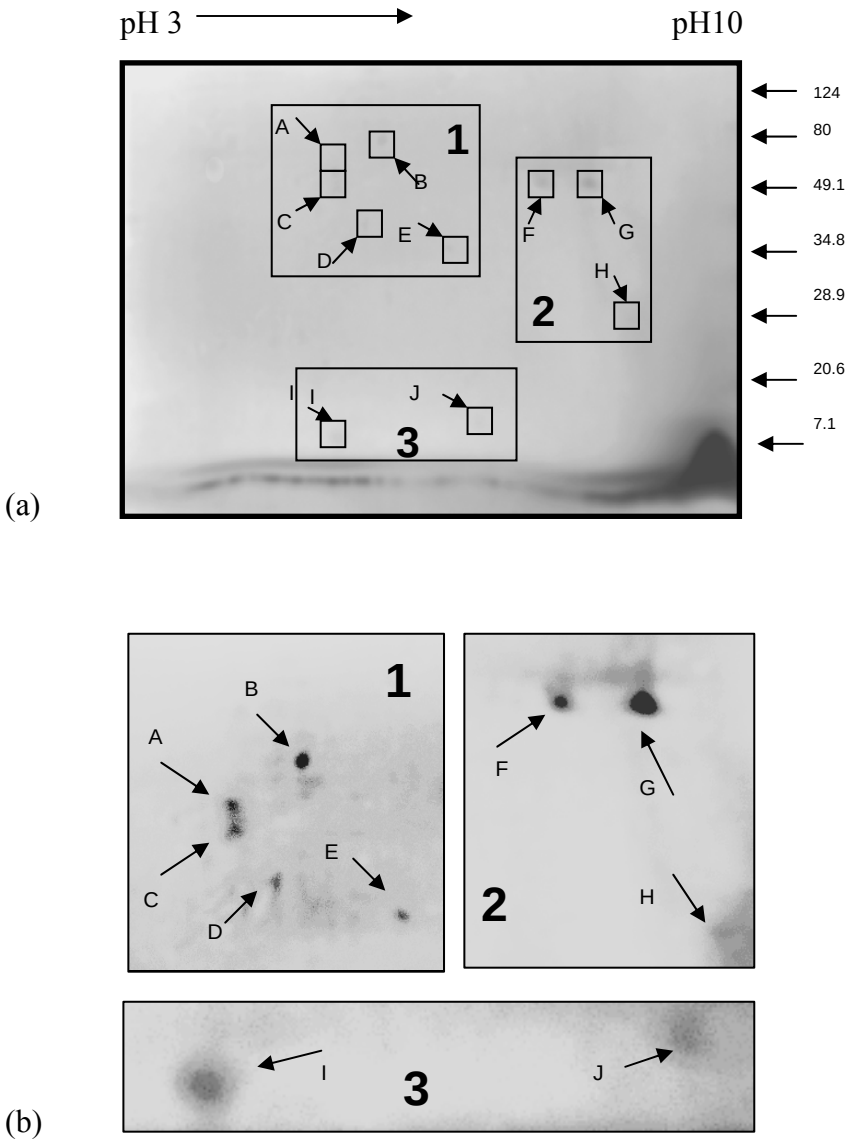


Figure 8.1: Protein profile of the tween wash of conidia of *B. bassiana* as determined using 2-DE (a). The protein spots enclosed in rectangular boxes (1, 2 and 3) are presented as enlarged view (b). The selected protein spots (A-E) were analyzed by mass spectrometry. Bio-Rad broad range standards (kDa) are indicated on right.

Table 8.1 Identification of selected proteins that were released during the tween wash of conidia of *B. bassiana*.

Protein identification (Spot number)	**Protein**	**Function/family**	**Molecular weight (kDa)**	**Isoelectric point (pI)**
A	Protein ROD 1 (yeast)	Membrane; Peripheral membrane protein, Mediates resistance to o-dinitrobenzene, calcium and zinc.	51.3	7.9
B	Elastase	Elastase, like trypsin and chymotrypsin, is a serine protease that can hydrolyze a variety of proteins especially elastin.	52.3	7.3
C	Uncharacterized membrane protein YBR235W	Membrane; Multi-pass membrane protein.	22.6	8.4
D	alpha-1,3-glucanase [*Aspergillus fumigatus* Af293]	ά-1,3-Glucanases (EC 3.2.1.59), also named mutanases, are extracellular enzymes able to degrade polymers of glucose bound by a-1,3-glycosidic links.	11.8	6.6
E	Protein ECM30 (Extracellular matrix protein 30)	Seems to be involved in cell wall organization and biogenesis.	10.8	5.2

Legend:
The protein spots were identified by MS analysis. Function and family analyses were based on search results from the National Center for Biotechnology Information database and Swiss Prot.

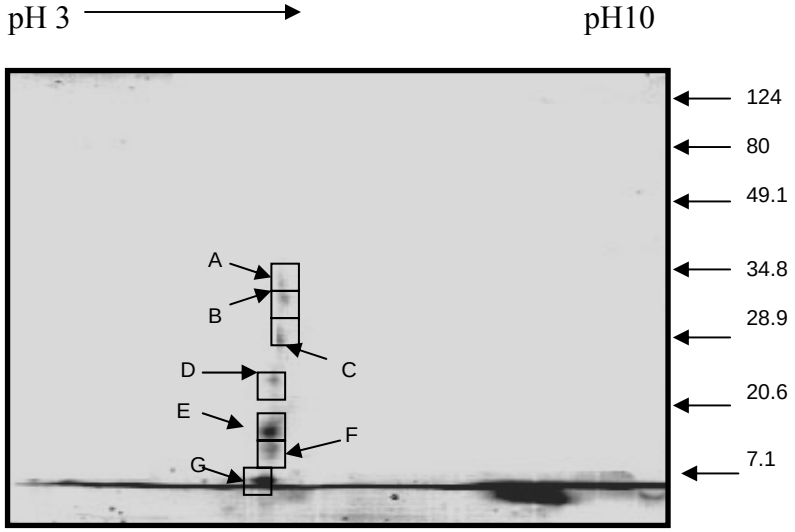


Figure 8.2: Protein profile of the tween wash of conidia of *M. anisopliae* as determined using 2-DE. The selected protein spots (A-F) were analyzed by mass spectrometry. Bio-Rad broad range standards (kDa) are indicated on right.

Table 8.2 Identification of selected proteins that were released during the tween wash of conidia of *M. anisopliae*.

Protein identification (Spot number)	**Protein**	**Function/family**	**Molecular weight (kDa)**	**Isoelectric point (pI)**
A	Un- named protein	Un-known	36	5.5
B	Elastase	Elastase, like trypsin and chymotrypsin, is a serine protease that can hydrolyze a variety of proteins especially elastin.	32.7	5.6
C	Lipase 5 (Triacylglycerol lipase)	Membrane; Single-pass membrane protein. Releases specific fatty acids from neutral lipid triacylglycerols (TAG) thereby supplying fatty acids to a general acylation process. May have a specific role in sporulation.	25.6	5.4
D	Glucan 1,3-beta-glucosidase precursor (Exo-1,3-beta-glucanase)	β-glucanases participate in the metabolism of beta-glucan, the main structural component of the cell wall. It could also function biosynthetically as a transglycosylase (By similarity).	20	5.4
E	Galectin-7	They are animal lectins, however fungal galectins are also known. Real physiological function not yet established. May be involved in cell adhesion, proliferation, apoptosis, metastasis, and immune function.	14.5	5.4
F	Un- named protein product (Homo sapiens)	Un known	13	5.3

Legend:
The protein spots were identified by MS analysis. Function and family analyses were based on search results from the National Center for Biotechnology Information database and Swiss Prot.

8.4.1 ROD 1

The Rod1 is a unique class protein and shares no significant homology with proteins of known function. Ai-Ling et al. (1996) have showed that the *ROD1* locus confer resistance to *o*-DNB, calcium, and zinc upon transformation of *Saccharomyces cerevisiae* with *I. orientalis* glutathione S-transferase (GST). In recombinant *S. cerevisiae,* elevated levels of GST were observed upon supply of *o*-DNB. GSTs have been known from plants (Ohtake et al., 1990; Rothstein, 1991) and insects (Towbin et al., 1979) where these enzymes are shown to be related to resistance to herbicides and pesticides, respectively. Ai-Ling and co-workers further showed that *ROD1* is not essential to the cell, but a ROD1 mutant strain possesses a hypersensitive phenotype to *o*-DNB, zinc, calcium, and diamide.

8.4.2 Elastase

Elastase is a serine protease which has specific affinity to degrade tissues rich in elastin. Amongst EPF, serine proteases have been shown to be involved in the cuticle degradation (Bidochka and Khachatourians, 1987; St. Leger et al., 1988). Elastinolytic serine protease from *A. fumigatus* has been shown to be involved in causing invasive aspergillosis in immunocompromised patient (Rhodes et al., 1990). The identification of elastase implies that the conidia have the ability to degrade elastin and other proteins present in the insect integument prior to the formation of appressoria.

8.4.3 ά-1,3-Glucanases

ά-1,3-Glucanases or mutanases are extracellular enzymes, which can degrade polymers of glucose bonded by ά-1,3-glycosidic links. Fuglsang et al. (2000) have grouped ά-1,3-

glucanases on the basis of their amino acid composition in family 71 of the glycosyl hydrolases. They have been isolated from bacteria (Takehara et al., 1981; Matsuda et al., 1998; Chung, 1998) and filamentous fungi, such as *A. nidulans* (Wei et al., 2001), *Penicillium purpurogenum* and *Trichoderma harzianum* (Fuglsang et al., 2000). The function performed by these enzymes in the fungal metabolism is not yet clear. However, evidence indicated that they are involved in the morphogenesis of the cell wall (Mellado et al., 2003), mobilization of ά-1,3-glucan from the cell wall in energy starvation conditions (Zonneveld, 1972; Wei et al., 2003), or the degradation of ά-1,3-glucan from other fungi during mycoparasitic interactions (Ait-Lahsen et al., 2001).

8.4.4 β-1,3-Glucanases

β-1,3-Glucanases are widely distributed among bacteria, fungi, and higher plants (Reese and Mandels, 1959). They are classified as exo-β-1,3-glucanases and endo-β-1,3-glucanases. The physiological functions of these enzymes depend on their source/origin. In fungi, β-1,3-glucanases seem to have different functions. Firstly, a physiological role in morphogenetic-morpholytic processes during fungal development and differentiation has been indicated (Peberdy, 1990). Secondly, β-1,3-glucanases have been related to the mobilization of β-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes (Rapp, 1992; Stahmann et al., 1992). Thirdly, β-1,3-glucanases are also involved in fungal pathogen-plant interactions, degrading callose (β-D-1,3-glucan) in the host's vascular tissues during pathogen attack (Schaeffer et al., 1994). Finally, a nutritional role in saprophytes and mycoparasites has been suggested (Chet, 1987; Sivan and Chet, 1989; Lorito, 1994). Contextual to EPF, recently, Choi et al. (2006a) have reported two ESTs from *B. bassiana* growing on chitin media similar to 1,3-β-glucanases that may entail its utility in cell wall remodeling. In a

subsequent study, Choi et al. (2006b) have reported the EST shared by conidia, blastospores and submerged conidia similar to a *S. cerevisiae* cell wall glycoprotein that displays lectin-like binding to 1,3-β-glucan and chitin, and possesses 1,3-β exoglucanase activity with laminarin as substrate (Woolford et al., 1986; Klebl and Tanner, 1989; Teter et al., 2001).

8.4.5 Lipase 5

Lipases are widely distributed among animals, plants and microorganisms (Borgstrom and Brockman, 1984; Jaeger et al., 1994). Lipase is produced by several EPF at the late stage of growth, yet convincing evidence is lacking for their involvement in insect mycosis. *B. bassiana* produces an extracellular lipase upon growth on a yeast extract peptone dextrose broth (Hegedus and Khachatourians, 1988). Lipase production was induced by olive oil and was inhibited by the addition of fatty acids, such as, myristic, palmitic, stearic, oleic, linoleic and arachidic acids. In addition, Iron and copper were inhibitory to lipase activity where as $MgCl_2$ slightly stimulate the activity of lipase. Lin and Liu (2004) have screened selected strains of *M. anisopliae, B. bassiana; Verticillium lecanii* and *Aspergillus* spp. All isolates produced lipase (triacylglycerol lipase) as analyzed by solid differential media. Similarly, during this study lipase activity against tween 20 was identified from tween wash of conidia of *M. anisopliae* as $CaCl_2$ precipitated zone on plate assay medium.

8.4.6 Galectin-7

Galectins are animal lectins that are related in amino acid sequence and belong to family of β-galactoside-binding lectins. They have affinity to specifically bind to β-galactoside carbohydrates such as lactose (Barondes et al., 1994). The galectin gene family has a conserved

carbohydrate-binding domain but have variable other domains. In addition, they have tissue-specific expression patterns. More distantly related, but conserving critical amino acid residues involved in carbohydrate-binding, are galectins in chicken, eel, frog, nematode, and sponge (Hirabayashi and Kasai, 1993). Galectins are known since last 20 years; however, their physiological functions have not yet been clearly established. They have affinity for oligosaccharides found on glycoconjugates on cell surfaces or in extracellular matrix, which suggests that galectins function extracellularly by binding to such ligands. Barondes et al. (1994) and Hughes (1994) have showed that certain galectins have particular affinity for specific glycoprotein ligands, such as polylactosamine chains on laminin. When added to cells or overexpressed after transfection, galectins can have major effects on cell adhesion, proliferation, apoptosis, metastasis, and immune function (Barondes et al., 1994; Hirabayashi and Kasai, 1993; Huges, 1994; Lotan et al., 1994; Perillo et al., 1995; Yang et al., 1996). Cooper et al. (1997) have reported lectins from mushroom, *Coprinus cinereus,* which expresses two lectins related in sequence and carbohydrate-binding specificity to other galectins. In EPF, lectins are known for their significance in spore attachment to the host insect, however, to date this is first proteomic evidence that show that the EPF lectins are related to galectin 7 family.

8.5 Conclusions

Results revealed the evidence of the multiple proteases and metabolites from the hydrated conidia of *B. bassiana* and *M. anisopliae* (Qazi and Khachatourians, 2007; Khachatourians et al., 2007). These results imply that hydrated conidia under extreme starved condition could initiate the degradation of insect cuticle/proteins. In order to get a complete picture, a proteomic analysis of hydrated conidia was undertaken. Several proteins were identified from hydrated conidia.

Results show that hydration alone is responsible for the activation of conidia that may have important role in fungal metabolism/pathogenesis. The proteome of tween wash indicated *ROD1* that was involved in the detoxification of xenobiotics in *S. cerevisiae*. In addition, extracellular enzymes like glucanses, elastase (serine protease) and lipase were identified. These enzymes were involved in several vital biological processes from growth, to cell wall remodeling and degradation of insect cuticle. Interestingly, sequences identified included galectin 7, which may have role in the attachment of conidia to substrate/insect cuticle. Indeed, conidia are endowed with the variety of proteins that have diverse functions during fungal metabolism/pathogenesis. This first proteomic evidence of the range of proteins may have important role in fungal metabolism and growth that reveals the significance of conidia in fungal infection and warrant further studies on the genes and proteins upregulated during conidiation.

9.0 Addition of exogenous carbon and nitrogen sources to aphid exuviae modulates synthesis of proteases and chitinase by germinating conidia of *Beauveria bassiana*

9.1 Abstract

Secretion of cuticle-degrading enzymes (CDE) is the hallmark of the infection of insects through the cuticle by entomopathogenic fungi (EPF). Results show that the germinating conidia of *Beauveria bassiana* regulate the synthesis of CDE through a multiple control mode during the initial stages of germination. Conidial growth of *B. bassiana* was tested on aphid exuviae with or without supplementation of additional carbon/ nitrogen (C/ N) sources. To understand the interrelation between conidial germination during growth the synthesis of CDE activity, free amino nitrogen (FAN), glucose and fungal dry weight biomass were measured. Immediately (0.25 h) upon incubation of conidia, activity expression of subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes and chitinase (NAGase) was observed in the culture filtrates. At 0.25 h, addition of exogenous C-source resulted in higher activities of Pr1 and Pr2. Conversely, at 0.25 h addition of N-source repressed the synthesis of Pr2, but Pr1. C/ N repression was observed only for exponentially-growing mycelia. NAGase activity remained at basal level, and unaffected by added C/ N. Results revealed that C/ N repression occurs only when it is necessary for the *B bassiana* infective structures to establish a nutritional relationship with the host structures.

9.2 Introduction

Biological control agents (BCA) have been, and in post-Kyoto environmental era will be, increasingly important tools in pest management in agricultural and forestry practices. Among BCA, the entomopathogenic fungi (EPF) impart important place because of their pathogenicity through ingestion and contact mode and a broad host range (Khachatourians, 1991, 1996; Valencia and Khachatourians, 1998; Khachatourians and Valencia, 1999; Butt, 2002). Furthermore, EPF are the only BCA available primarily being used to control the plant injurious insects (including sap sucking insects) associated with agro-ecosystem and forestry worldwide (Feng et al., 1994; Charnley, 1997; Valencia and Khachatourians, 1998; Khachatourians and Valencia, 1999; Qazi and Khachatourians, 2005). The mode of entry of most EPF is direct penetration of host cuticle. Ultrastructural and histochemical evidence indicated that this occur via combination of enzymatic degradation and mechanical pressure (St. Leger, 1993; Clarkson and Charnley, 1996). The entomopathogenic fungus *B. bassiana* secretes a variety of hydrolytic enzymes with activity against insect cuticular components i.e., protein, chitin and lipids (Smith et al., 1981; St. Leger et al., 1986a; Bidochka and Khachatourians, 1987; St. Leger, 1993).

Hydrolytic role of proteases is of paramount value to the degradation of the cuticle. Protein is the major cuticular constituents and comprises 55-80% of the insect cuticle (Neville, 1975) and some cuticular proteins are bound to the chitin fibrils. In this context, two proteases namely chymoelastase (Pr1) and trypsin (Pr2) are the key virulence determinants produced by *M. anisopliae* and *B. bassiana* (St. Leger et al., 1998). Biochemical and molecular properties of both Pr1 and Pr2 indicate that they are regulated by carbon and nitrogen derepression in mycelia of *M. anisopliae* (St. Leger et al., 1988; St. Leger, 1995).

The homologues of *creA* and *areA* genes that regulate carbon and nitrogen utilization in *Aspergillus nidulans* have been identified in *M. anisopliae* (Marzluf, 2001; Screen et al., 1997, 1998).

During the past three decades, most of the work has been done on the enzymes secreted by growing mycelia of EPF. In this context, the types of primary organic or inorganic nutrients, which contain elemental carbon and nitrogen, are crucial to the biosynthesis of macromolecules and metabolic regulation of growth. It is well established that as a carbon source, fungi prefer glucose over, or aldehyde or acetamide etc (Marzluf, 2001). Several investigators have highlighted the significance of catabolite repression (CR) during the fungal pathogenesis. Catabolite repression becomes more relevant if fungal mycelia are to be formed in a particular environment such as saprophytic versus external or internal to insect tissues. Here the sources of primary carbon I and nitrogenous (N) nutrients could be simple molecules or complex macromolecules (e.g., amino acids, carbohydrates, polyols, proteins, purines, ammonia) all of which can vary in their abundance. What follows next is the mechanisms responsible for processing, degrading and uptake for biosynthesis.

Very recently it has been shown that hydrated conidia of *B. bassiana* and *M. anisopliae* can release acidic proteases Pr1, Pr2 and metalloproteases under extreme starvation conditions (Qazi and Khachatourians, 2007; Khachatourians et al., 2008). Data suggests that conidia are endowed with a battery of proteases that can start cuticle degradation prior to the formation of appressoria/germ tube. Although conidial enzymes received very little attention from applied mycologists and microbiologists, St. Leger et al. (1991) were the first to show that the conidia of *M. anisopliae* are pre-adapted to the pathogenic mode of life style. They also reported that the conidia that grew on insect cuticle

have higher level of Pr1, chitinase or NAGase and esterase in comparison to Sabouraud dextrose agar. Furthermore, washing of conidia with tween and chemical agents have allowed their placement in the category of membrane bound enzymes.

Owing to the importance of the conidia during the initiation of germination on insect and in consideration of formulation for field applications, a study was initiated to delineate the role of CDE in conidia during fungal growth. The main objective of the present work is to study the regulation of synthesis of subtilisin, trypsin and NAGase on the aphid exuviae supplemented with or without carbon and nitrogen sources by *B. bassiana* at the initial and late phase of germination. In addition, comparison of the synthesis of CDE from growing conidia and mycelia was studied to understand the physiology of fungal growth during enzyme biosynthesis.

9.3 Materials and methods

9.3.1 Fungal isolates and culture conditions

Beauveria bassiana (GK 2016) was obtained from Department of Applied Microbiology, College of Agriculture and Bioresources, University of Saskatchewan. *B. bassiana* was maintained on Yeast Peptone Glucose Agar (YPGA) for 12 days at 27° C. Conidia were pooled with 0.03% tween 80 as previously described (Qazi and Khachatourians, 2007). The spores were counted by using haemocytometer and final concentration of spores was adjusted to 10^8/ ml before inoculation into sterilized (120° C, 15 min) 1% (w/v) aphid exuviae medium (ExM). The aphid exuviae consist entirely of exocuticle, which was collected from cages of fledged aphid (*Sitobion avenae* Fab). Before use, exuviae were grounded in mortar and pestle and powdered material was used in the preparation of ExM. The ExM was supplemented with or without

carbon I and nitrogen (N) sources as indicated: (i) addition of C and N (+CN), (ii) addition of C, but lacking N (+C-N), (iii) addition of N, but lacking C (-C+N), (iv) addition of *N*-acetyl-D-glucosamine, but lacking C and N (-CN+NAG), and (v) exuviae alone (Exo). In which case, C and N sources used were 1% sucrose and 0.2% NH_4Cl, respectively. In addition, 1% filter sterilized (Millipore^{TM}, 0.45 µm pore size; Millipore, Billerica, USA) *N*-acetyl-D-glucosamine (NAG) was added to the previously sterilized ExM. Cultures were incubated at 27° C at 150 rpm in rotary shaker incubator (New Brunswick Scientific Co., New Jersey, USA). After set time intervals 5 ml of the sample was taken from the Erlenmeyer flask and passed through Whatman no. 1 and Millipore^{TM} filter (0.45 µm pore size; Millipore, Billerica, USA). Culture filtrate was stored at -30° C subject to the further analysis as described in next section.

Fungal dry weight biomass was measured by their drying on Millipore^{TM} (Millipore, Billerica, USA) filters at 90° C for 4 h and cooled in a desicator for 4 h prior to weighing.

9.3.2 Enzyme assays

Trypsin (Pr2)-like activity was measured with the N-ά-benzoyl-DL-arginine-*p*-nitroanilide in 0.05 M glycine-NaOH buffer, pH 8.5. Subtilisin (Pr1)-like activity was measured against Suc-Ala-Ala-Pro-Phe-4-nitroanilide in 0.05 M TRIS/ HCl buffer, pH 8.0. Trypsin and subtilisins activities were determined as previously described (Chapter 6, section 6.3.8). Enzyme (Pr1, Pr2) activity is expressed as nmols nitroanilide (NA) released ml^{-1} min^{-1}.

Chitinase (NAGase) was assayed by was measuring chromogenic substrate *p*-Nitrophenyl-*N*-acetyl-D-glucosaminide dissolved in 0.05 M citrate/ phosphate buffer, pH 4.8. Enzyme assay was performed as previously described (Chapter 6, section 6.3.8). NAGase activity is expressed as nmols nitrophenol (NP) released ml^{-1} min^{-1}.

9.3.3 Metabolite assays

9.3.3.1 Glucose assay

Glucose was assayed by the enzymatic calorimetric procedure (Sigma Kit N. 510).

9.3.3.2 Free amino nitrogen (FAN)

Free amino nitrogen (FAN) was assayed by trinitrobenzene sulfonic acid method modified by Samples et al. (1984) using glycine as a standard. This procedure produces a trinitrophenylated amino-sulfite complex, which absorbs light at 411 nm.

Essentially, the original method was modified to micro scale for ease and cost saving. To 25 µl of cell-free culture supernatant in 1.5 ml microfuge tube (VWR International West Chester, USA), 225 µl-de-ionized water was added. To this 250 µl of the 24% TCA was added and the mixture was allowed to stand for 10 min. The samples were centrifuged for 10 min at 10000 rpm at 20-22° C (IEC-Micromax; Rotor no. 851; Thermo Fisher Scientific, Inc., Waltham, USA). After wards, 50 µl of each supernatant was transferred to another 0.5 ml microfuge tube and 50 µl of 5% sodium citrate was added. Next, 125 µl of 1.2 N sodium borate in 1.2 N sodium hydroxide solution was added. Tubes contents were mixed using a vortex for 2-3 sec. To this mixture, 250 µl of 0.2% trinitrobenzene sulfonic acid was added. The tubes were stoppered and incubated in the dark at 40° C for 40 min. The reaction was terminated by the addition of 0.5 µl of 2 N HCl followed by the addition of 0.5 µl of 0.001 N sodium sulfite in 0.1 N sodium phosphate. Finally, the absorbance was measured at 411 nm with a microtiter plate reader (Titertek Multiscan^{TM}; Titertek, Huntsville, USA). The results were expressed as mM glycine equivalents ml^{-1}. All assays were done in triplicate.

9.3.4 Statistical analysis

Analysis of variance (ANOVA) was performed by using one way completely randomized ANOVA design using CoStat version 6.204 (CoHort Software, Monterey, CA, USA). Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$. The whole study was repeated two times.

The regression analysis (Curve fitting) was performed by using Data Fit version 8.4 (Oakdale Engineering, Oakdale, USA). The graphs was plotted by using AutoCAD^{R} 2008, (Autodesk, Inc., San Rafael, USA)

9.4 Results

9.4.1 Growth of *B. bassiana*

Culture grown in the ExM supplemented with carbon and nitrogen (+CN) and carbon without nitrogen (+C-N) (Table 9.1) attained a higher dry weight per 5 ml culture in contrast to medium supplemented with nitrogen (-C+N), *N*-acetyl-D-glucosamine [NAG] (-CN+NAG) and exuviae alone. This clearly indicates that the presence of carbon source (1% sucrose) aids optimal fungal growth.

In the case of ExM supplemented with carbon (+C-N), high expression activity of Pr1 and Pr2 were observed. This suggests that the C/ N source was provided by the digestive action proteases to afford faster growth rate. In contrast, medium supplemented with nitrogen (–C+N) and NAG (–CN+NAG) attained similar fungal biomass, but had different FAN levels. Exuviae alone provided the lowest biomass and highest FAN levels, which is due high activity expression of Pr1 and lysis of mycelia because of starved conditions.

Table 9.1 Relationship between growth medium, secreted metabolites and extracellular enzymes activity in *B. bassiana* after 24 h of incubation in ExM.

Supplements	Subtilisin † (Pr1)	Trypsin † (Pr2)	NAGase †	Glucose *	FAN *§ (mM ml^{-1})	Biomass *¶ (mg 5 ml^{-1})
+CN	2.82^{d}	13.12^{b}	2.49^{b}	3.0^{b}	5.96^{b}	0.26^{a}
+C-N	10.22^{b}	18.4^{a}	3.12^{a}	3.96^{a}	2.95^{c}	0.25^{a}
-C+N	5.10^{c}	6.75^{c}	1.88^{bc}	0.62^{d}	5.96^{b}	0.21^{b}
-CN+NAG	9.24^{b}	0e	1.76^{c}	0.74^{c}	2.35^{d}	0.21^{b}
Exuviae	12.24^{a}	4.69^{d}	2.39^{b}	0.16^{e}	7.70^{a}	0.17^{c}

Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$. Means listed in columns followed by same alphabets are not significantly different. Results are representative of two separate experiments with similar results.

§ Free amino nitrogen.

¶ Fungal dry weight biomass.

*Each result is the mean of triplicate.

† Each result is the mean of quadruplicate.

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9.4.2 Effect of carbon and nitrogen sources on the synthesis of Pr1 and Pr2

B. bassiana (GK 2016) produces one extracellular serine protease in the gelatin containing liquid medium (Bidochka and Khachatourians 1987, 1988a). When freshly grown and harvested conidia were inoculated in 1% aphid exuviae supplemented with or without exogenous C/ N sources, both Pr1 (Fig. 9.1) and Pr2 (Fig. 9.2) enzyme activities appeared in the culture supernatant in less than 0.25 h. This result is indicating a very rapid synthesis under the influence of ExM and supplemented carbon and nitrogen (C/ N) sources.

The activity of Pr1, as shown in Fig. 9.1, was significantly high when the exuviae was supplemented with the C/ N sources in comparison to exuviae alone. After which time the expression Pr1 activity continued at varying level unaffected by any C/ N source except at 2 h. In contrast, at 24 h mycelia or exponentially grown cells showed different regulatory mechanisms for the synthesis of Pr1 and Pr2, respectively. Addition of carbon and nitrogen (+CN) to ExM resulted in the repression of Pr1. This repression could be attributed to the glucose levels in the medium. When exponentially growing mycelia were supplied with carbon source but deprived of nitrogen (+C-N), Pr1 activity expression was observed. However, upon the reversal of this regime, (-C+N), the expression of this activity was repressed. High levels of Pr1 activity was observed in medium supplemented with NAG (–CN+NAG) and in exuviae alone, most likely due to nutrition starved conditions. Similarly, Pr2 activity expression (Fig. 9.2) appeared in the culture supernatant in less than 0.25 h. There was a significant effect of the added C/ N sources for Pr2 activity expression. The results also revealed that Pr2 is regulated in a different way in response to the added nutrients. The Pr2 activity expression was significantly higher with all the C and N source till 2 h in comparison to exuviae alone, after which time ExM supplemented with carbon and

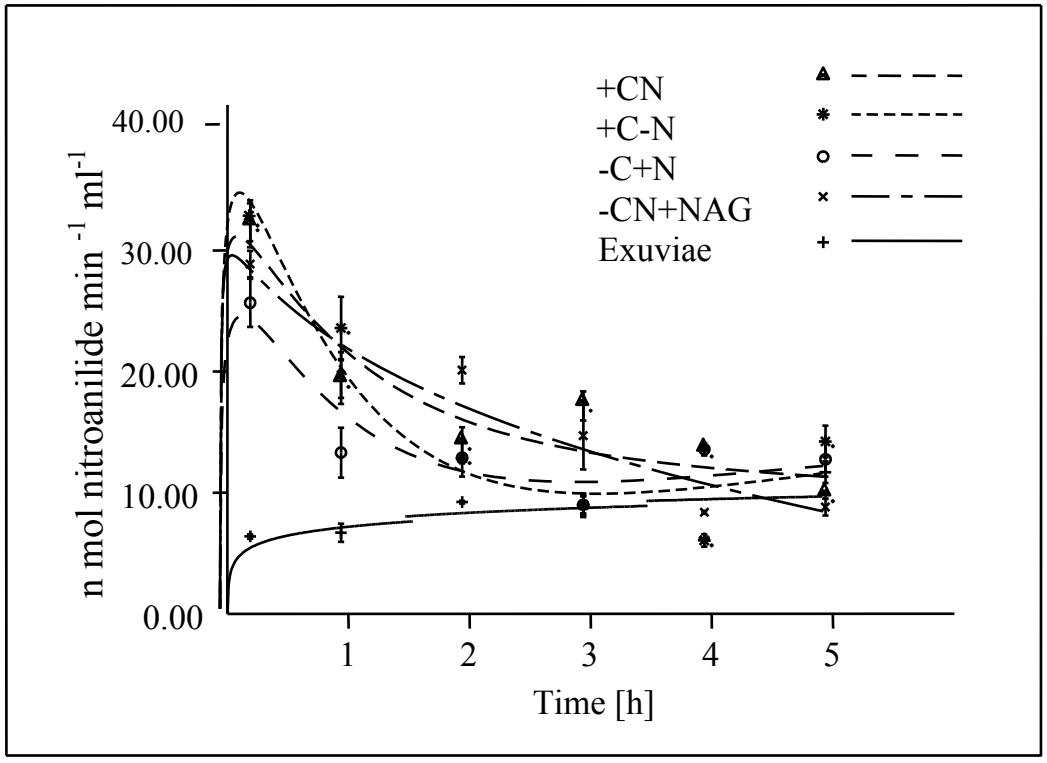


Figure 9.1: Time course of Pr1 release/synthesis during growth of *B. bassiana* in 1% aphid exuviae supplemented with or without C/ N sources, as indicated. Error bars indicate S.D., where n = 4. The regression equations (where y = Pr1 and x = incubation time) are: +CN: $y = 12.741-0.771*\log (x)^2+24.159*e^{(-x)}$ [$r^2 = 0.94$]; +C-N: $y = -2.987+8.574*\log(x)+62.28*e^{(-x)}$ [$r^2 = 0.94$]; -C+N $y = 2.097+ 5.893*\log(x)+38.53*e^{(-x)}$ [$r^2 = 0.93$]; -CN+NAG $y = 21.847-1.38*\log(x)^2-6.37*\log(x)$ [$r^2 = 0.96$]; Exo $y = 7191+1.048*\log(x)$ [$r^2 = 0.79$].

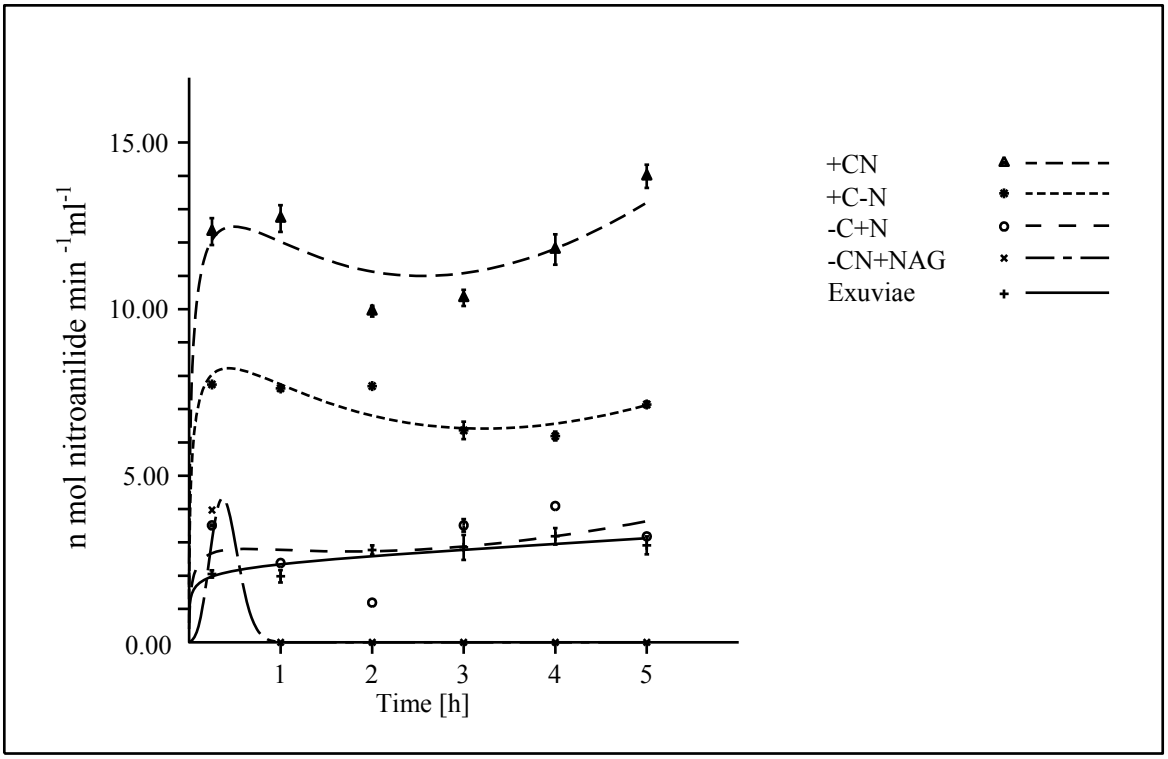


Figure 9.2: Time course of Pr2 release/synthesis during growth of *B. bassiana* in 1% aphid exuviae supplemented with or without C/ N sources, as indicated. Error bars indicate S.D., where n = 4. The regression equations (where y = Pr2 and x = incubation time) are: +CN y = -1.587+ 13.613*x-14.805*√x*log(x) [r^2= 0.98]; +C-N y = -0.842+8.584*x-9.713*√x *log(x) [r^2= 0.98]; -C+N y = -0.302+3.076*x-3.181*√x *log(x) [r^2 = 0.64]; -CN+NAG y = $0.0097*x^{(-17.358*x)}$ [r^2 = 0.90]; Exo y = 2.341+0.0467*x*log(x)+0.255*log(x) [r^2 = 0.96].

nitrogen only (+C-N) showed high Pr2 activity in comparison to the other treatments. Interestingly, addition NAG (-CN+NAG) to ExM inhibited the Pr2 activity and complete loss of enzyme activity was evident at 1 h. Similarly, basal level of activity expression of Pr2 was observed from 0-5 h for ExM supplemented with NAG (–CN+NAG) and nitrogen only (–C+N). When exponentially growing mycelia were supplied with carbon and nitrogen (+CN) sources Pr2 was unaffected (Table 9.1). This implies a different regulation of Pr2 in comparison to Pr1. Under nitrogen deprivation and carbon supply (+C-N) and carbon deprivation and nitrogen supplementation (-C+N), Pr2 activity expression was high and low, respectively. Conversely, ExM supplemented with NAG (-CN+NAG) alone resulted in the loss of the expression activity of Pr2.

9.4.3 **Effect of carbon and nitrogen sources on NAGase synthesis**

The expression of NAGase activity, which remained at a fixed basal level was not affected by any C/ N source added (Fig. 9.3). The NAGase activity appeared in the culture supernatant within 0.25 h, implying its presence on external spore layers that could be liberated by the washing. Similarly, St. Leger et al. (1991) have reported the NAGase being released from the spore of *M. anisopliae* upon washing in tween and triton X. In contrast, at 24 h the NAGase activity expression (Table 9.1) was at basal level except when supplemented with carbon only (+C-N). The utilization of amino sugar as C and N source to permit the faster growth rate is evident from high biomass and depleted FAN levels. Indeed, these data highlight the different modes for regulation of CDE synthesis for conidial and mycelial growth, which would have two separate roles in environments where EPF grow.

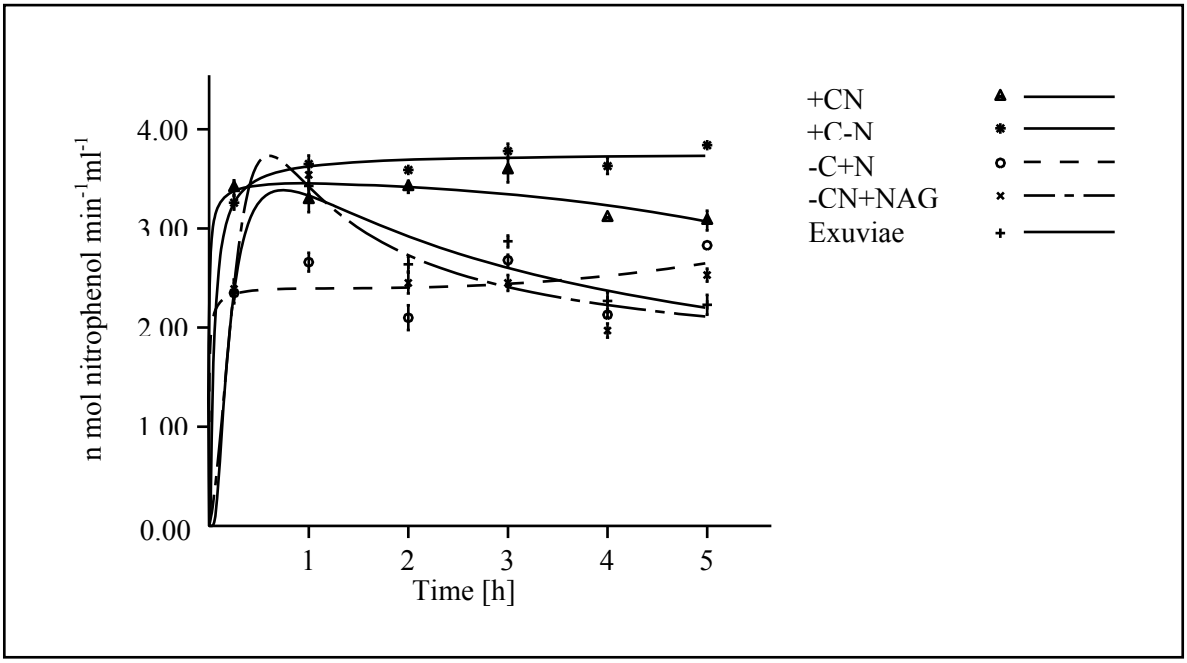


Figure 9.3: Time course of NAGase synthesis during growth of *B. bassiana* in 1% aphid exuviae supplemented with or without C/ N sources, as indicated. Error bars indicate S.D., where n = 4 for NAGase. The regression equations (where y = NAGase and x = incubation time) are: +CN y = $3.456-0.002*x^3-0.041*\log(x)^2$ [$r^2 = 0.99$]; +C-N y = $3.759*e^{(-0.036/x)}$ [$r^2 = 0.99$]; -C+N y = $2.394+0.008*x^2*\log(x)-0.028(x)^2$ [$r^2 = 0.99$]; -CN + NAG y = $x/(0.291+0.751*x-0.75*\sqrt{x}$ [$r^2 = 0.95$]; Exo y = $e^{(1.507-0.304/x-0.409*\log(x))}$ [$r^2 = 0.97$].

9.4.4 Effect of carbon and nitrogen on FAN and CDE synthesis

The glucose levels in the culture supernatant were measured by an enzymatic method. There was no catabolite/glucose repression effect at the initial phase of germination (Fig. 9.4). Hence, the Pr1 and Pr2 activity expression appeared rapidly in the supernatant even in the presence of added carbon source. This observation can be corroborated with the FAN levels of the medium (Fig. 9.5). It is also evident from the results that there is glucose (1-1.5 mg/ ml) in culture media, even in the absence of supplemented exogenous glucose. This suggests the degradation of exuviae by chitobiases and endochitinases, respectively. Reducing groups were also observed in *M. anisopliae* culture supernatant of chitin basal media (St. Leger et al. 1986b). The expression of Pr1 and Pr2 activity which began within 0.25 to 5 h at differing levels lead to degradation of cuticular proteins is evident from FAN analysis. In the case of 24 h exponentially growing mycelia, the addition of carbon and nitrogen (+CN) and carbon only (+C-N) to ExM resulted in high FAN levels (Table 9.1). In the first case it could be due to high Pr1 activity/fungal biomass due to rapid FAN uptake. However, in later case it can be corroborated with low Pr1 and Pr2 activity that yielded low fungal growth and high FAN levels in the culture. In the case of supplemented nitrogen source alone (–C+N) synthesis of both Pr1 and Pr2 was repressed. Low levels of glucose and FAN, which were observed, must be responsible for low fungal biomass in contrast to supplemented carbon (1% sucrose) sources. Similarly, exuviae alone and addition of NAG (–CN+NAG) to ExM alone showed low levels of FAN and biomass, respectively. Furthermore, differences in the FAN levels between different cultures was due to variety of reason such as nitrogen content of media or rapid uptake of FAN due to more mycelial growth.

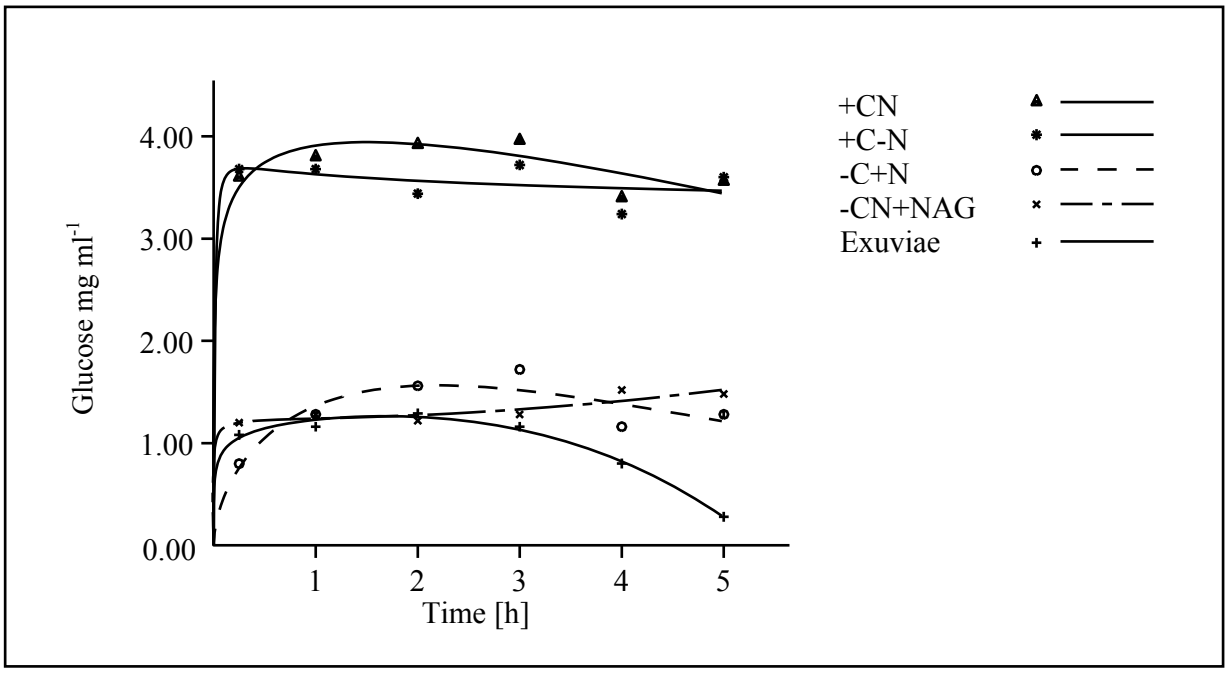


Figure 9.4: Time course of glucose levels during growth of *B. bassiana* in 1% aphid exuviae supplemented with nutrients, as indicated. Error bars indicate S.D., where n = 3. The regression equations (where y = glucose and x = incubation time) are: +CN $y = 4.212-0.301*x+0.456*\log(x)$ [$r^2 = 0.99$]; +C-N $y = e(1.299-0.01/x-0.033*\log(x))$ [$r^2 = 0.98$]; -C+N $y = 1.764*0.774^x * x^{0.564}$[$r^2 = 0.95$]; -CN+NAG $y = 1.227+0.013*x^2- 0.014*\log(x)^2$ [$r^2 = 0.98$]; Exo $y = 1.239-0.009*x^3+0.134*\log(x)$ [$r^2 = 0.96$].

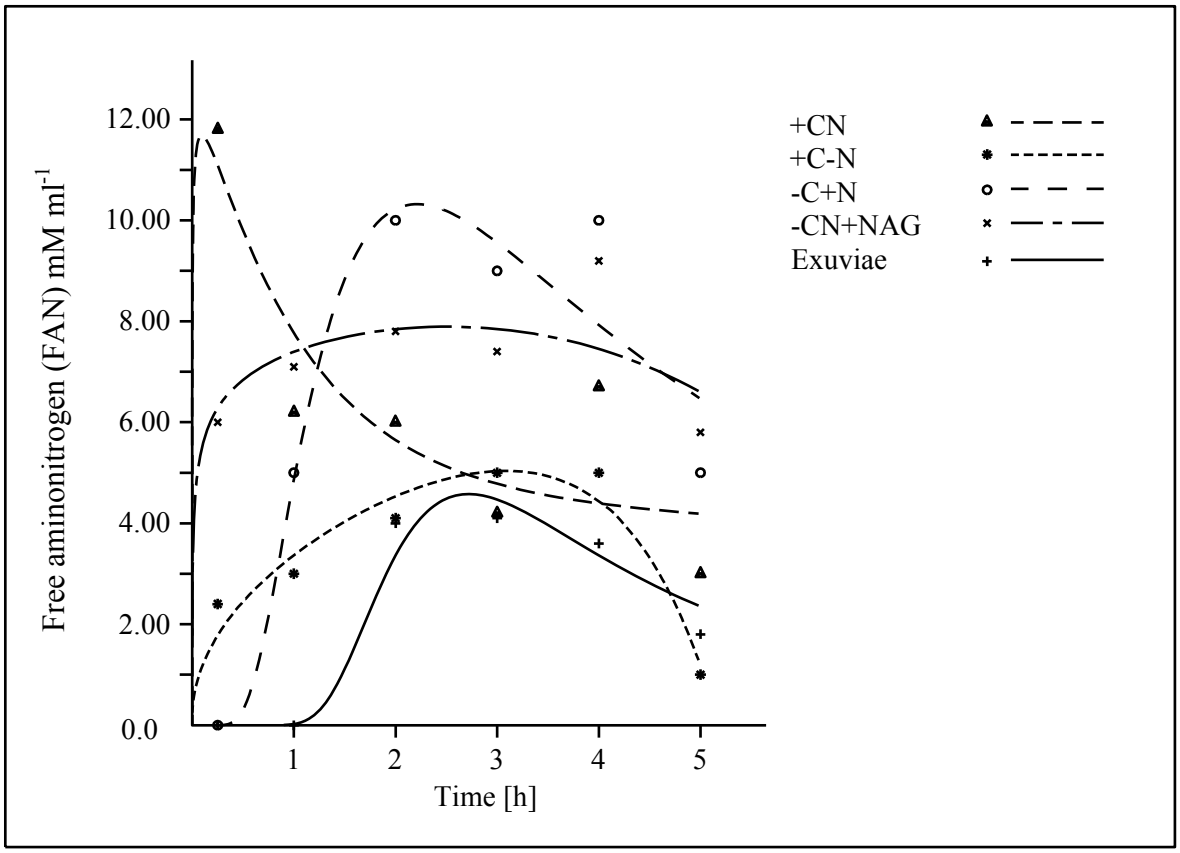


Figure 9.5: Time course of free amino-nitrogen (FAN) during growth of *B. bassiana* in 1% aphid exuviae supplemented with nutrients, as indicated. Error bars indicate S.D., where n = 3. The regression equations (where y = free amino nitrogen (FAN) and x = incubation time) are: +CN $y = 4.532-0.157*\log(x)^2+8.809*e^{(-x)}$ [$r^2 = 0.87$]; +C-N $y = 0.184-0.043*e^x+3.3*\sqrt{x}$ [$r^2 = 0.95$]; -C+N $y = 264.137*0.018^{(1/x)}*x^{-1.808}$ [$r^2 = 0.94$]; -CN+NAG $y = 7.412-0.017*x^3+0.817*\log(x)$ [$r^2 = 0.92$]; Exo $y = 0.022*x^{(14.475/x)}$ [$r^2 = 0.96$].

Contrary to the results reported by Bidochka and Khachatourians (1988a) there was no effect by the addition of glucose on FAN levels.

9.5 Discussion

St. Leger et al. (1986a) reported the synthesis of endoprotease, aminopeptidase and carboxypeptidase A in less then 24 h from EPF growing on insect cuticle. Subsequently, St. Leger et al. (1988) indicated that the synthesis of Pr1 and Pr2 occur in less than 2 h during C/ N derepression from 72 h mycelial inocula of *M. anisopliae* grown in minimal media. In the case of *B. bassiana* serine protease has been shown to be regulated under carbon and nitrogen limitation (Bidochka and Khachatourians, 1988a) and *N*-acetyl-D-amino sugar level in media (Bidochka and Khachatourians, 1988b). Contextual to the initiation of infection on host cuticle, St. Leger et al. (1989) provided first the evidence that Pr1 is the major protein synthesized from the appressoria of *M. anisopliae*. Upon supply of exogenous glucose to appressoria they found the C/ N derepression for Pr1. However, studies of CDE from mycelia cannot be extrapolated to the conidia and initial phase of host pathogen interaction especially when cuticle is not as nutrient rich to regulate the initial phase of entomopathogenic fungal growth.

The release of multiple proteases from the hydrated or swelling phase of conidia from *B. bassiana* and *M. anisopliae,* raise an important question as to that what are the syntheses levels of CDE during the initiation of germination/pre-germination phase (Qazi and Khachatourians, 2007; Khachatourians et al., 2007). The early events of entomopathogenic fungal interaction with host cuticle makes this question especially relevant in terms of CDE during the initiation of germination, that is, the short phase of growth (i.e., during swelling

phase of conidia or before the germ tube). For comparison, the CDE synthesis/release by exponentially growing mycelia was also studied.

These results clearly suggest that both Pr1 and Pr2 are regulated but in a different fashion. Secondly, both proteases were present in the initial phase of germination (i.e., from hydration to the germination tube formation) implying that the CDE associated with conidia have the ability to modify the epicuticle/cuticle for the germ tube formation prior to the appressoria/mycelia formation. As such, these results bridge the gap between initiation and promulgation of entomopathogenic fungal infection so far as the release/synthesis of the Pr1, Pr2 and NAGase is concerned. The appearance of these enzymes within 0.25 h indicates that the *B. bassiana* conidia are endowed with the multiple proteases. The release or synthesis levels were unaffected by the addition of C/ N sources, implies different regulatory mechanisms operative in conidia and mycelia.

Regulation of extracellular protease production in *A. nidulans* and *N. crassa* has been extensively studied (Arst, 1995; Marzluf, 2001). Synthesis of *A. nidulans* and *N. crassa* protease is under the control of multiple regulatory circuits (Cohen, 1973; Hanson and Marzluf, 1977; Marzluf, 2001). The protease in *N. crassa* has been identified as a glycoprotein (Abbot and Marzluf, 1984), where cysteine (Marzluf and Metzenberg, 1968), glutamine (Grove and Marzluf, 1981) and some product of glucose (Hanson and Marzluf, 1975) are repressed by three independent regulatory gene products. Hence, if preferred sources of nitrogen, carbon and sulfur are present, synthesis of all three regulatory products is repressed. Bidochka and Khachatourians (1988a) observed similar results from the 48 h growing mycelia of *B. bassiana*. They reported that the presence of glucose, glycerol,

trehalose, or mannitol, and ammonium together in a medium-containing gelatin repressed serine protease (Pr1 like) production.

Data presented in Figures 9.1 and 9.2 indicates that activity of both Pr1 and Pr2 was high when supplemented with the C/ N sources. The synthesis of Pr1 and Pr2 requires exogenous carbon and nitrogen sources essential for post-swelling stages of growth. In spite of supplementation with glucose, carbon catabolite repression (CCR) was not observed for both Pr1 and Pr2 at 0.25 h. On the other hand, nitrogen metabolite repression (NMR) was observed for Pr2 and not for Pr1. Hence, the C/ N produced regulation of Pr1 and Pr2 differ. These results are consistent with those reported by St. Leger et al. (1988) for exponentially growing mycelia of *M. anisopliae* in minimal media after 72 h of growth. Additionally, the results of measurements of Pr2 synthesis in ExM with nitrogen (-C+N) and NAG (-CN+NAG) supplementation indicate that the repression occurred in spite of the presence of cuticular proteins or the enhancing effects, which were expected. Similarly, St. Leger et al. (1988) found that synthesis of Pr1 and Pr2 in exponentially-growing mycelia of *M. anisopliae* was repressed from the 72 h upon supplementation with sucrose (1%), alanine (1%), *N*-acetyl-D-glucosamine (1%) or NH_4Cl (0.2%) in minimal media containing cuticle or cellulose.

During mycelial growth, CCR was observed at 24 h for Pr1 in ExM supplemented with both carbon and nitrogen sources (+CN), but not for Pr2. CCR was not observed for Pr1 and Pr2 when preferred nitrogen source was absent from (+C-N) ExM medium. Addition of preferred nitrogen source to the medium in the absence of added carbon (-C+N) repressed the synthesis of Pr1 and Pr2, indicating NMR to be operative. In addition, Pr2 synthesis was inhibited by the addition of NAG within one hour. In similar vein, Bidochka and

Khachatourians (1988b) have provided evidence that synthesis of serine protease (Pr1-like) in *B. bassiana* was repressed in gelatin medium containing GlcNAc at levels of > 1.07 μmol^{-1} mg of fungal dry $weight^{-1}$.

The role of sulfur starvation on *B. bassiana* protease production was not examined because sulfur-containing amino acids were not present in the insect cuticle (St. Leger et al., 1986a). The results show that the synthesis of Pr1 and Pr2 was under multiple model control circuit regarding carbon and nitrogen source. The *creC* and a*reA* control carbon and nitrogen regulation in *A. nidulans*, the homologue of both these genes (*crr1* and *nrr1*, respectively) has been identified in *M. anisopliae* that have domain upstream of *Pr1a* sequence (Screen et al., 1997, 1998). NAGase synthesis was at the basal level suggesting that their synthesis occur late during growth of EPF as previously described (St. Leger et al., 1986b; Bidochka and Khachatourians, 1993)

These results showed that the phenomenon of C/ N repression is not relevant during the initial interaction of conidia with exuviae or during pre-germination phase. Therefore, these results make a strong connection for the regulation of CDE as an ecologically adaptive mechanism for *B. bassiana* depending on whether the fungus proliferation occurs on insect cuticle or in hemolymph. The availability of nutrients containing preferred C/ N relative to proteins determines the amount and type of protease produced. Furthermore, it depends upon the morphogenetic phase of the fungus (conidia, appressoria and mycelia), and only when it is necessary to establish a nutritional relationship with the host tissue. Naturally, conidia have the potential for a robust mode of life. Upon attachment to the cuticle they require nutrients (C/ N) and suitable physical conditions to germinate. C/ N repression would be expected to operate during pathogenesis if the release of degradation products from the cuticle exceeds

the nutritional needs for fungal growth. This would occur only in nutrient rich hemolymph or other tissues of insect, but not during the pre-penetration phase/initiation of germination. Indeed, conidia being endowed with Pr1 and Pr2 and their isozymes illustrate the obvious advantage to the pathogen in acquiring nutrients to enable the onset of cuticle degradation and fungal penetration before host defenses become effective.

10.0 The synthesis of extracellular proteases and chitinase in *Beauveria bassiana* and *Metarhizium anisopliae* during growth on insect cuticle is regulated by initial ambient pH

10.1 Abstract

The environmental pH modulation for the regulation of cuticle-degrading enzymes (CDE) during the growth and development of entomopathogenic fungi (EPF) are crucial in initial and post-secondary infection. There is no information on how *Beauveria bassiana* and *Metarhizium anisopliae* regulate the synthesis of CDE during the conidial growth at different inducer pH (pH*i*) levels. Expression of subtilisin (Pr1), trypsin (Pr2) and chitinase (NAGase) activity was measured on cuticle under pH*i* 4-10 from 6 h of germination up to 24 h. Results show that the CDE activities were affected by the pH*i* and were also controlled through regulatory de-repression and nutritional starvation. In *B. bassiana* high Pr1 and Pr2 expression per mg dry weight was revealed with high pH*i* levels. Similarly, the same was observed for *M. anisopliae* at pH*i* 6 and 10. In both EPF, NAGase production at pH*i* 4 and 6 were optimal. Results imply that Pr1 and Pr2 action at pH*i* 10 cause the degradation of cuticular proteins and exposure of chitin layers enabling high levels of NAGase activity per mg dry weight. Ambient pH during conidial growth is a crucial element of coordination of CDE synthesis/activity for successful breaching of insect cuticle.

10.2 Introduction

It is a well known fact that proteolytic enzymes are of crucial importance in the metabolism and development fungi and higher eukaryotes (North, 1982; Rao et al., 1998). Amongst proteases, those which are extracellular form an important group of proteins because of

their functional and structural diversity (North, 1982; Rao et al., 1998; St. Leger, 1995). There is also accumulating evidence that the fungal pathogens of plants, humans, nematodes and insects produce proteases that can degrade the primary protective barrier of their host upon contact (Khachatourians, 1991; St. Leger, 1995; Bowen, 1998). As a result, elucidating the mechanism of the regulation of this group of enzymes is crucial to the understanding of the developmental cycle and pathogenicity of these fungi. Similarly, several laboratories have shown that protozoa and bacteria facilitate the infection of host because of the production of proteases (North, 1982; McKerrow et al., 1993; Gougen et al., 1995).

Protease production is one of the primary biochemical responses by many parasitic fungi, including *B. bassiana* and *M. anisopliae,* which are two important EPF (Feng et al., 1994; Butt, 2002; Qazi and Khachatourians, 2005). EPF are ubiquitous and can help maintain the balance of insect pest fauna in agriculture and forestry. EPF affect insects by multiple mode of action, which helps to avoid creation of insect resistance (Khachatourians, 1986). EPF cause infections primarily through the secretion of cuticle-degrading enzymes (CDE), proteases (chymoelastase and trypsin), chitinases (NAGase) (Khachatourians, 1991; St. Leger, 1995; Khachatourians, 1996) and esterases (Clarkson and Charnley, 1996). The insect cuticle is made up of 70-80 % of proteins (Neville, 1975), therefore the role of subtilisin (Pr1) and trypsin (Pr2) is considered as key factor in cuticle degradation (Charnley and St. Leger, 1991; St. Leger, 1995). In growing mycelia, both these enzymes are regulated by the carbon I and nitrogen (N) ratios (St. Leger et al., 1986a; Bidochka and Khachatourians, 1988a; St. Leger et al., 1988; Bidochka and Khachatourians, 1993). For the past two decades the regulation of the CDE synthesized by growing mycelia under the influence of C/ N cues is becoming established (St. Leger, 1995; Khachatourians, 1996; Khachatourians et al., 2007).

Research on the role of ambient pH on the regulation of CDE by EPF conidia, the primary interactive vehicle in infection, remains outstanding. In natural environment conidia will not only face different C/ N substrates, but also they will encounter various pH conditions. These changes demand efficient homeostatic mechanism in order to maintain the intracellular pH for cellular functions. One modality for pH control is through synthesis of metabolic acids and bases or pH stress proteins to accommodate fungal physiology, growth and differentiation. Additionally, synthesis and secretion of exoenzymes, permeases and exported metabolites will depend on pH values, which favour their optimal activities (Penalva and Arst, 2004). It is not surprising that in the human pathogen, *Candida albicans,* and phytopathogenic fungi, *Sclerotinia sclerotiorum, Colletotricum spp. and Alternaria spp.* pH regulation is associated with fungal virulence and pathogenicity (Arst and Penalva, 2003; Prusky and Yokoby, 2003). The prevalence of pH regulation in fungi indicates its importance as an ecological determinant for their distribution within the biological world.

Mycelia from EPF are seldom used for insect pest control and consequently do not have the same value as conidia for the initiation of infection or for commercialization for field application. However, St. Leger et al. (1998, 1999) used growing mycelia of *M. anisopliae* to show that three CDE, Pr1, Pr2 and NAGase, were regulated by the ambient pH on a medium containing cockroach cuticle. They reported that the pH of the infected cuticle was increased from 6 to 7.3 due to secretion of ammonia. It has been shown that the soaking of conidia of *M. anisopliae*, e.g., in hydrated formulations, prior to the formation of appressoria/germ tube could release a family of metalloproteases for cuticle degradation (Qazi and Khachatourians, 2007). It has been shown that *B. bassiana* can secrete ammonia to regulate the activity of gelatinase-like proteases under the influence of different C/ N sources Chapter 11). Finally, it was demonstrated

that the CDE levels can change during conidial growth, since their synthesis differs in growing conidia and mycelia of *B. bassiana* under the supply of C/ N sources (Qazi and Khachatourians, 2008).

Despite of the significance of pH regulation in fungi, its role during conidial growth of EPF is not documented. For example, what are metabolic activities that result in pH change for conidia and how is that regulated? It would be natural for the degradation of cuticular components by invading EPF to result in the release of ammonical by-products and organic acids, which in turn would change pH of the surrounding infected tissues. In such a scenario, it would be necessary for the growing fungus to coordinately regulate and modify ambient pH along with growth and development. The main objective of this study is to show that (i) *B. bassiana* and *M. anisopliae* can regulate the synthesis of CDE during growth on insect cuticle at four pH*i*, and (ii) that the synthesis of CDE from growing conidia and mycelia is under the concerted influence of cuticle and pH of the growth media.

10.3 Materials and methods

10.3.1 Fungal isolates and culture conditions

Beauveria bassiana (GK 2016) was obtained from Department of Applied Microbiology and Food Science and *Metarhizium anisopliae* (MA 2038) was generously provided by USDA, Entomopathogenic Fungus Collection, Ithaca, NY, USA. Fungal strains were maintained on yeast peptone glucose agar (YPGA) for 12 days at 27° C. Conidia were pooled with 0.03% tween 80 as previously described (Qazi and Khachatourians, 2007). The spores were counted by using haemocytometer and final concentration of spores was adjusted to 5 X 10^6/ ml before inoculation into sterilized (120° C, 15 min) 0.2 % (w/v) *Galleria*

mellonella cuticle medium (GcM). The cuticle was prepared after Anderson (1980). The pH of GcM was adjusted at four different inducer pH (pH*i*) values; 4, 6, 8 and 10. The buffers employed in this study were: 0.1 M citrate phosphate (pH 4 and 6), 0.1 M TRIS/ HCl, pH 8.0 and 0.1 M carbonate bicarbonate buffer, pH 10. Cultures were incubated at 27° C at 150 rpm in rotary shaker incubator (New Brunswick Scientific Co., New Jersey, USA). After set time intervals 5 ml of the sample was taken from the Erlenmeyer flask and passed through Whatman no. 1 and Millipore™ (0.45 μm pore size; Millipore, Billerica, USA) filters to obtain culture biomass or fluid. Culture filtrate was stored at -30° C subject to the further analysis as described in next section. Fungal dry weight biomass was measured by their drying on Millipore™ filters (Millipore, Billerica, USA) at 90° C for 4 h and cooled in a desicator for 4 h prior to weighing.

10.3.2 Enzyme assays

Subtilisin or chymoelastase (Pr1)-like activity was measured using Suc-Ala-Ala-Pro-Phe-4-nitroanilide. Trypsin (Pr2)-like activity was measured with the chromogenic substrate N-benzoyl-DL-arginine-*p*-nitroanilide. A scale-down method was used for both Pr1 and Pr2 assays, as previously described (Chapter 6, section 6.3.8). Enzyme (Pr1, Pr2) activity is expressed as nmols nitroanilide (NA) released ml^{-1} min^{-1}. Chitinase (NAGase) was assayed by measuring chromogenic substrate *p*-Nitrophenyl-*N*-acetyl-D-glucosaminide (2 mM) that was dissolved in 0.05 M citrate/ phosphate buffer, pH 4.8. Enzyme assay was performed as described in chapter 6 (Section 6.3.8). Finally, absorbance was read at 411 nm on a Titertek Multiscan™ (Titertek, Huntsville, USA). NAGase activity is expressed as nmols nitrophenol (NP) released ml^{-1} min^{-1}.

10.3.3 Glucose assay

Glucose was assayed by the enzymatic calorimetric procedure (Sigma Kit No. 510).

10.3.4 Free amino nitrogen (FAN)

Free amino nitrogen (FAN) was assayed by trinitrobenzene sulfonic acid method modified by Samples et al. (1984) using glycine as a standard. Essentially, the original method was modified to small scale down for ease and cost saving as previously described (Qazi and Khachatourians, 2008). The absorbance was measured at 411 nm with a microtiter plate reader (Titertek Multiscan^{TM} ; Titertek, Huntsville, USA). The results were expressed as mM glycine equivalents ml^{-1}. All assays were done in triplicate.

10.3.5 Statistical analysis

Analysis of variance (ANOVA) was performed by using one way completely randomized ANOVA design using CoStat version 6.204 (CoHort Software, Monterey, CA, USA). The level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$ unless stated otherwise. The whole study was repeated twice.

10.4 Results

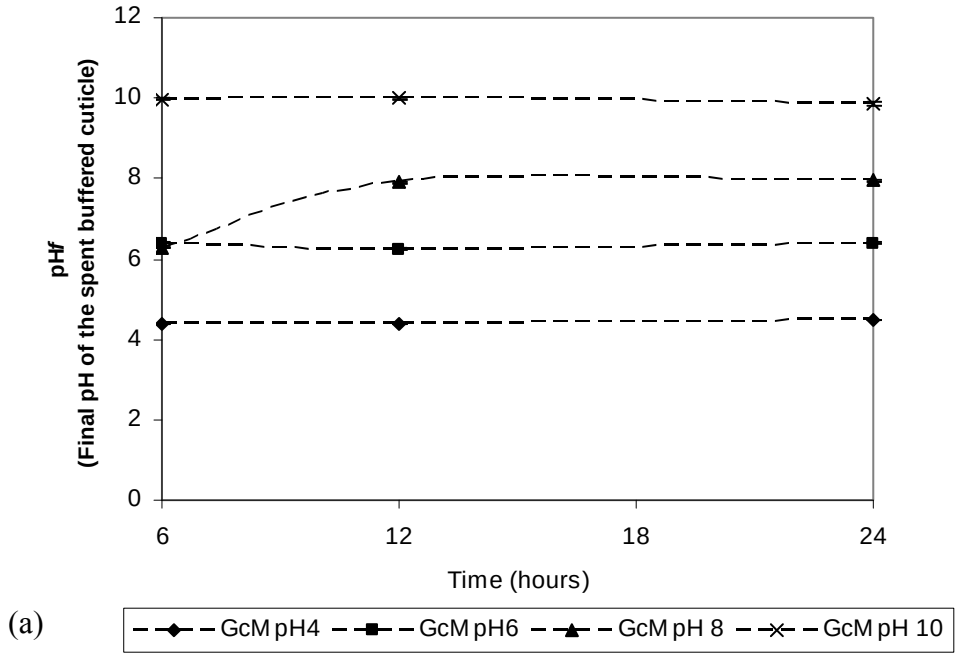
10.4.1 Cuticle-degrading enzymes: Synthesis on cuticle at varying pH*i*'s

During the growth of *B. bassiana* and *M. anisopliae* the synthesis of the proteases (Pr1 and Pr2) and chitinases (NAGase) in a 0.2% buffered suspension of GcM at four pH*i* set at 4, 6, 8, and 10 was measured. After 6 h *B. bassiana* altered the pH of the GcM adjusted at pH*i* 4-8,

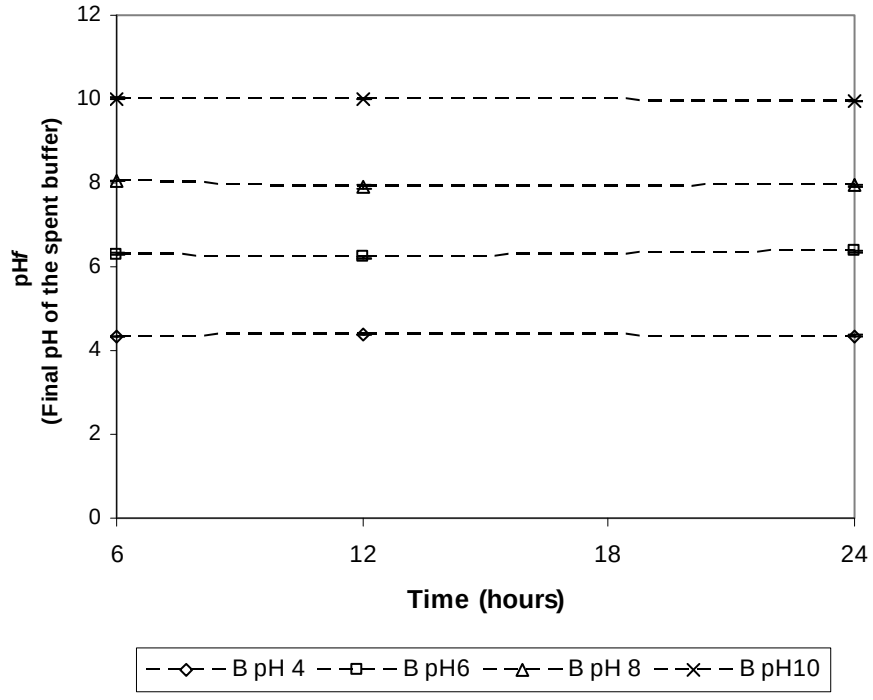
and buffers of pH*i* 4 and 6 (Fig. 10.1). In contrast *M. anisopliae* changed the pH of cuticle medium and un-supplemented buffer of pH*i* 4 and 6, respectively (Fig. 10.2) due to the release of ammonical by-products and organic acids.

B. bassiana showed varying levels of Pr1 activity in culture supernatant at the four pH*i* tested till 24 h. However, maximum activity was observed at pH*i* 10. Activity of Pr1 was observed after 6 h of incubation of conidia in GcM buffered at all pH*i* tested (Table 10.1) at which time higher levels of Pr1 activity was observed at pH*i* 4 and 6. This result indicates that conidia used citrate as a nutrient source. Pr2 synthesis was also detected after 6 h from the germinating conidia at all pH*i* tested (Table 10.2), continuing at varying levels up to 24 h. High levels of activity was observed at initial phase of germination, except at pH*i* 8, possibly due to a significant change in pH of the medium. NAGase activity in germinating conidia after 6 h was also under the influence of pH*i* (Table 10.3) till 12 h after which there was a decline.

In addition, incubation of conidia in buffers alone representing derepression conditions revealed the activities of Pr1 (Table 10.1), Pr2 (Table 10.2) and NAGase (Table 10.3). These results reaffirm our recent findings that even without nutritional cues, conidia can release multiple proteases during hydration that can modify the cuticle (Qazi and Khachatourians, 2007; Khachatourians et al., 2007). Activity of Pr1 was observed after 6 h of incubation of germinating conidia of *M. anisopliae* in all the pH*i* in the cuticle media (Table 10.4). High activity of Pr1 was observed at pH*i* 4-8 in contrast to that of pH*i* 10. At 12 h, high Pr1 activity was observed at pH*i* 8 and 10. At 24 h, the highest Pr1 activity was observed but only at pH*i* 10 in contrast to the other pH*i* tested. Expression of Pr2 activity was completely inhibited at pH*i* 4 (Table 10.5), while high activity was observed at pH*i* 6 and 8. At 12 h, Pr2 activity was not evident at any pH*i*

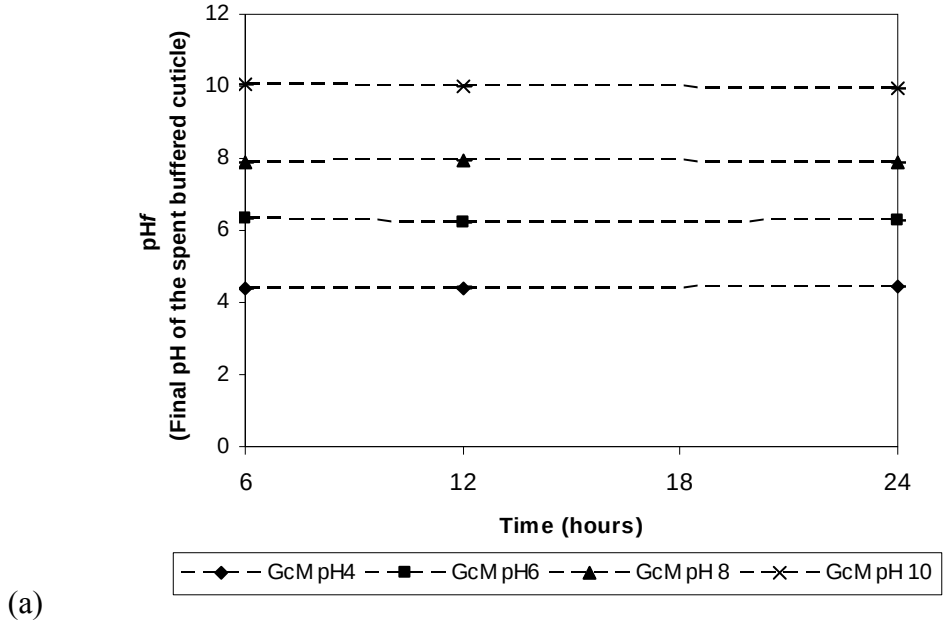


(a)

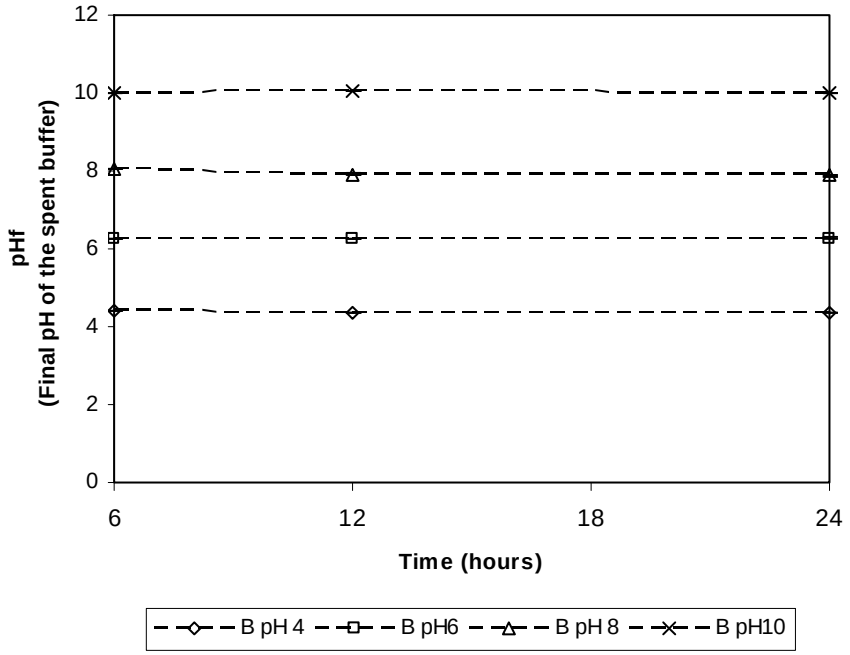


(b)

Figure 10.1: pH changes in the buffered cuticle media (a) and buffer (b) during growth of *B. bassiana* (GcM: Cuticle medium; B: buffer). Error bars represents S.D., where n = 3.



(a)



(b)

Figure 10.2: pH changes in the buffered cuticle media (a) and buffer (b) during growth of *M. anisopliae* (GcM: Cuticle medium; B: buffer). Error bars represents S.D., where n = 3.

Table 10.1 Expression of subtilisins/chymoelastase (Pr1) activity during the growth of *B. bassiana.*

	Pr1 activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	23.30[a]	16.75 [a]	19.02[b]	5.43[b]	2.44[c]	6.0[b]
6	22.22[a]	12.63[b]	16.10[c]	12.05[a]	5.83[b]	4.3[b]
8	18.88[b]	18.88[a]	15.83[c]	7.16[b]	11.58[a]	8.88[b]
10	12.85[c]	19.17[a]	26.80[a]	11.11[a]	10.94[a]	11.11[a]

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.2 Expression of trypsin (Pr2) activity during the growth of *B. bassiana.*

	Pr2 activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	29.57[a]	21.55[b]	23.07[b]	9.83[c]	3.82[c]	6.55[c]
6	30.50[a]	25.34[a]	27.85[a]	19.40[b]	13.48[b]	13.52[b]
8	19.75[b]	26.83[a]	22.86[b]	9.57[c]	12.98[b]	3.44[d]
10	31.35[a]	27.89[a]	22.53[b]	26.37[a]	16.82[a]	14.92[a]

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.3 Expression of NAGase activity during the growth of *B. bassiana*

	NAGase activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	4.91^{a}	3.06^{b}	2.54^{a}	1.50^{c}	0.27^{c}	0.39^{c}
6	4.36^{ab}	3.80^{ab}	2.68^{a}	3.06^{a}	0.83^{bc}	2.1^{b}
8	3.29^{c}	3.69^{ab}	2.01^{b}	1.84^{bc}	1.67^{a}	2.20^{ab}
10	3.64^{bc}	4.13^{a}	2.75^{a}	$2.60d^{b}$	1.41^{ab}	0.31^{c}

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.4 Expression of subtilisins/chymoelastase (Pr1) activity during the growth of *M. anisopliae.*

	Pr1 activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	30.69^{a}	24.58^{b}	34.10^{b}	11.27^{b}	13.63^{a}	3.52^{b}
6	26.52^{b}	23.55^{b}	33.67^{b}	10.27^{b}	5.5^{c}	4.97^{a}
8	28.47^{ab}	29.93^{a}	33.07^{b}	7.52^{c}	9.02^{b}	3.69^{b}
10	21.71^{c}	29.44^{a}	48.74^{a}	15.41^{a}	16.38^{a}	2.5^{b}

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.5 Expression of trypsin (Pr2) activity during the growth of *M. anisopliae.*

	Pr2 activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	0.0^{c}	0^{a}	31.54^{a}	5.25^{a}	0.40^{d}	9.96^{c}
6	8.07^{a}	0.40^{a}	29.81^{a}	6.17^{a}	1.85^{c}	14.73^{b}
8	7.82^{a}	0^{a}	23.46^{b}	3.05^{b}	6.11^{a}	2.12^{d}
10	3.85^{b}	0^{a}	10.07^{c}	0.32^{c}	3.01^{b}	25.49^{a}

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

tested. In contrast, at 24 h exponentially-growing mycelia, higher Pr2 activity expression was evident at all pH*i*. NAGase activity (Table 10.6) was observed in the culture supernatant after 6 h from the germinating conidia at all the pH*i*'s that continued till 24 h with higher activity observed at pH*i* 4 and 6.

In the absence of cuticle, *M. anisopliae* released significant activities of Pr1 (Table 10.4), Pr2 (Table 10.5) and NAGase (Table 10.6) in buffer supernatant after 6-24 h of incubation. These results showed that the CDE enzymes can be synthesized by conidia under nutritionally starved conditions. The activities of enzymes observed at various time intervals were different, implying the role of pH on stability and activity of enzymes along with their release from the starved conidia.

10.4.2 Fungal growth and CDE production

Growth of *B. bassiana* and *M. anisopliae* was tested at all pH*i* in order to elucidate that the low level of CDE synthesis/release was not merely due to non-permissive growth conditions for any isolate. Microscopic examinations at 6 h revealed that approximately 80 and 75%, respectively of *B. bassiana* and *M. anisopliae* conidia were swollen. At 12 h, young mycelia were produced by *B. bassiana* and *M. anisopliae* of approximately 10-15µm in length. Measurement and analysis of fungal dry weight showed that selected pH*i* supported the growth of *B. bassiana* (Table 10.7) and *M. anisopliae* (Table 10.8) to varying level, when compared with the dry weight attained in buffer alone. The relationship between the dry weight and CDE activities were also investigated to determine the increase in CDE levels at different pH*i* due to induction or increased fungal dry weight biomass. Fungal dry weight and enzyme activities were

Table 10.6 Expression of NAGase activity during the growth of *M. anisopliae.*

	NAGase activity/ time					
	Buffered 0.2% cuticle			**Buffer alone**		
pH*i*	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
4	4.11^{a}	3.94^{a}	3.61^{a}	2.47^{a}	0.67^{b}	1.32^{cd}
6	3.27^{b}	3.90^{a}	3.56^{a}	1.80^{a}	0.38^{b}	0.84^{d}
8	3.8^{ab}	3.94^{a}	2.31^{b}	1.82^{a}	1.60^{a}	1.55^{bc}
10	3.77^{ab}	3.52^{a}	2.47^{ab}	2.05^{a}	1.98^{a}	1.84^{ab}

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.7. Relationship between the pH*i* of the growth medium and growth, secreted metabolites, glucose and extracellular enzymes activity in *B. bassiana* after 24 h of incubation in GcM.

(pH*i*)	**DWBM*§ (mg^{-1})** +Cut/-Cut	**FAN†§ ($mM\ ml^{-1}$)** +Cut/-Cut	**Glucose § (mg^{-1})** +Cut/-Cut	**Pr1 A/DW $^{-1}$‡¶** +Cut	**Pr2 A/DW $^{-1}$‡¶** +Cut	**NAGase A/DW $^{-1}$‡¶** +Cut
4	0.0227a 0.0209c	2.4 7.2	0.04 0.012	838b	1016c	112b
6	0.0227a 0.0204c	3.8 6.0	0.02 0	709c	1226b	118b
8	0.0216b 0.0177d	2.0 4.2	0.02 0	733c	1058c	93c
10	0.0167e 0.0153f	4.8 7.5	0.08 0.08	1604a	1349a	165a

*DWBM dry weight biomass.
† Free amino nitrogen glycine equivalents.
‡ A/DW activity per unit dry weight
§ Each result is the mean of triplicate.
¶ Each result is the mean of quadruplicate.

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

Table 10.8 Relationship between the pH*i* of the growth medium and growth, secreted metabolites, glucose and extracellular enzymes activity in *M. anisopliae* after 24 h of incubation in GcM.

(pHi)	**DWBM*§ (mg^{-1})** +Cut/-Cut	**FAN†§ ($mM\ ml^{-1}$)** +Cut/-Cut	**Glucose§ (mg^{-1})** +Cut/-Cut	**Pr1 A/DW $^{-1}$ ‡¶** +Cut	**Pr2 A/DW $^{-1}$ ‡ ¶** +Cut	**NAGase A/DW $^{-1}$ ‡¶** +Cut
4	0.0222b 0.0164d	0 3.0	0.12 0	1536c	1421b	162b
6	0.0182cd 0.0158d	4.2 8.6	0.028 0.18	1850b	1638a	196a
8	0.0265a 0.0182cd	0 2.0	0.24 0.02	1247d	885c	87c
10	0.0190c 0.0162d	4.8 12	0 0.16	2565a	530d	130b

*DWBM dry weight biomass.
† Free amino nitrogen glycine equivalents.
‡ A/DW activity per unit dry weight
§ Each result is the mean of triplicate.
¶ Each result is the mean of quadruplicate.

Legend:
Analysis of variance was performed by using one way completely randomized ANOVA design. Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$ and $n = 4$. Means listed in columns for each enzyme followed by same letters are not significantly different.

measured at 24 h for buffered cuticle media. However, due to lysis of mycelia in buffer, enzyme activities per mg dry weight were not determined for buffer alone.

Results showed that Pr1, Pr2 and NAGase were regulated in a different manner in both *B. bassiana* and *M. anisopliae*. In *B. bassiana*, enzyme activities per unit of dry weight revealed that pH*i* 10 resulted in the highest activity for Pr1, Pr2 and NAGase (Table 10.7). Higher Pr1 and Pr2 activities were observed at pH*i* 10, reflecting the role of alkaline pH as inducer. In contrast, high NAGase activity per mg of dry weight at pH*i* 10 was due to the removal of protein layer embedding the chitin of insect cuticle because of high Pr1 and Pr2 action. Interestingly, higher levels of Pr1 and Pr2 were observed at pH*i* 4 and 6 in contrast to pH*i* 8. Higher NAGase activity per unit of dry weight at pH*i* 4 and 6 indicted the optimum pH range of two NAGases previously reported by Bidochka et al. (1993) in *B. bassiana.*

In *M. anisopliae,* significantly higher Pr1 (Table 10.8) activity per unit of dry weight was observed at pH*i* 10 in contrast to others, implying a role for alkaline pH in the synthesis of Pr1. In contrast, Pr2 activity per unit of dry weight was high at pH*i* 6 implicating the acidic pH as an inducer for this enzyme. Interestingly, incubation at pH*i* 8 produces in lower expression of Pr1 and Pr2 activity per mg of dry weight. High NAGase activity per unit of dry weight was observed at pH*i* 4 and 6, respectively implying optimal pH range for NAGase secreted by *M. anisopliae* (St. Leger et al., 1986b). NAGase activity per unit of dry weight at pH*i* 10 was a consequence of removed protein layer embedding chitin of insect cuticle due to higher protease (Pr1, Pr2) action. Both isolates showed low enzyme activities at pH*i* 8, which may entail the interfering role of TRIS buffer as it is a membrane permeating agents (Good et al. 1966).

10.4.3 Free amino nitrogen (FAN) and glucose analysis

To understand the particular metabolism of amino nitrogen during fungal growth chemical analysis of the culture supernatants were performed. After 24 h of growth, results for *B. bassiana* mycelia indicated that the different pH produced varying FAN levels, which imply the different activities of Pr1 and Pr2, respectively. Higher FAN levels were observed for the cultures grown at pH*i* 8 and 10, due to lysis of mycelia that can be corroborated with reduced fungal dry weight attained by EPF at these pH*i*'s. Microscopic examinations also corroborated these findings (data not presented). Similarly, after 24 h, growing mycelia of *M. anisopliae* had higher FAN levels for the culture buffered at pH*i* 6 and 10, indicating lysis of mycelia. After 24 h no FAN was observed for GcM at pH*i* 4 and 8, suggesting its rapid uptake corroborated with higher dry weight biomass. Glucose was also observed at 24 h in culture supernatant of both EPF. Reducing groups were also observed in *M. anisopliae* culture supernatant of chitin-basel media (St. Leger et al., 1986b).

10.5 Discussion

In fungi pH affects growth, development, secretion of extracellular enzymes and metabolites (Arst and Penalva, 2003). The pH response in relation to conidial growth and enzyme synthesis amongst EPF has not been documented. During this study synthesis of Pr1, Pr2 and NAGase was investigated during conidial growth in 0.2% buffered cuticle at four pH*i* (4, 6, 8, 10).

Results demonstrated that the buffered cuticle at selected pH*i* provided conditions for significantly ($p < 0.05$) high expression of the CDE. In addition, expression of the CDE was evident in buffers without cuticle implied the synthesis/release of the enzymes under

derepression conditions. High CDE activities in the buffered cuticle containing media corroborated previous findings that the synthesis by mycelia of EPF was induced by the degraded products of cuticle (St. Leger, 1995; St. Leger et al., 1998). Paterson et al. (1994a) showed that locust cuticle enhanced Pr1 production by *M. anisopliae* to a level approximately 10-fold that of the derepressed mycelium. In a subsequent study, Paterson et al. (1994b) reported partial characterization of the cuticular chemicals which are inducers (monomer Ala and Gly) and responsible for 2-2.7-fold high Pr1 induction from *M. anisopliae* inocula. Interestingly, they suggested that the inducer for Pr1 is the degraded product of the cuticle, as none of these inducers gave comparable levels of activities as seen with whole cuticle or enzymatically released peptides from locust cuticle.

Results presented here revealed a very complex picture for the regulation of Pr1 and Pr2, and one which is influenced mainly by two factors: (i) cuticle, and (ii) pH of the media. In *B. bassiana*, high levels of Pr1 and Pr2 were detected after 6 h of germinating conidia up to 24 h. In contrast for *M. anisopliae* high levels of Pr1 and Pr2 activity were observed after 6 h, after which time Pr1 activity continued up to 24 h, whereas complete loss of activity for Pr2 after 12 h. Insect cuticle can also influence the regulation of Pr1 and Pr2 in a unique way, presumably due to different inducers. In addition, Pr1 and Pr2 synthesis from germinating conidia of *M. anisopliae* on 1% aphid exuviae is regulated differently. Pr2 activity was observed immediately after 0.25 h, whereas Pr1 was not detected until 5 h of germination, after which time both activities were observed at varying levels in culture supernatant till 24 h (data not presented).

The relationship between enzyme activities and mycelial dry weight helps elucidate the levels of Pr1, Pr2 and NAGase synthesis whether it is due to induction or increase in fungal dry weight. The significant increase in the Pr1 and Pr2 activities (in comparison to other pH*i*

supplemented with cuticle) per mg dry weight at pH*i* 10 showed the role of alkaline pH in the induction of both enzymes for *B. bassiana.* Similarly, the significant increase in the Pr1 and Pr2 activities for *M. anisopliae* at pH*i* 10 and 6, respectively, indicated the role of induction due to pH rather than increase in fungal biomass. St. Leger et al. (1998) have reported the pH optima of *M. anisopliae* Pr1 and Pr2 as 8 and 6-8, respectively. Similarly, St. Leger et al. (1986c) also reported the optimum pH for Pr1 activity on locust cuticle to be 9. In similar vein, a subtilisin-type serine protease (*pepC*) produced by *Aspergillus niger* is expressed at equally high levels at pH 3 and 8, while the expression of aspartyl protease genes (*pepA* and *pepB*) is completely turned off under alkaline conditions (Jarai and Buxton, 1993). Production of NAGase at alkaline pH*i* could be relevant in terms of coordinated Pr1 and Pr2 release under alkaline conditions at infection sites. High productions of Pr1 and Pr2 at pH*i* 10 cause the degradation of proteinaceous components of cuticle that may have induced high NAGase activity. Both set of results are in agreement with those obtained for *M. anisopliae* (St. Leger, 1993). There is a significant role by cuticular proteins in determining the pH optima for Pr1 and Pr2, as during infection of the cuticle as they raise the pH (St. Leger et al., 1986c; St. Leger et al., 1998). pH shift, could also affect the hydration of the cuticle and thus interaction with protease (Reynolds, 1975).

Results also indicated that both EPF changed the pH of the buffered cuticle significantly from the initial set point of the buffer. Griffin (1994) reported that a difference of 0.3 units and 1.0 pH unit means two-fold and 10-fold alteration in the hydrogen ion concentration of the liquid medium for fungal growth. It is well documented that ambient pH is detrimental in the acquisition of nutrients by fungal hyphae (Jennings, 1995; Olsson, 2000; Griffin, 1994). pH also affects cell permeability, an effect that is particularly important for compounds that ionize. At low pH, the cell membrane becomes saturated with hydrogen ions limiting passage of essential

cations. On the other hand, at higher pH it become saturated with hydroxyl ions and limits the passage of essential anions (Moore-Landecker, 1996). As a result, the differences observed between the pH*i* during growth of EPF suggest a difference in metabolic activity. Ironically, selection of buffers and their buffering capacity has not been regarded as an important factor in previous investigations with EPF despite their proven significance with the phytopathogen, *Colletotricum* spp. (Prusky et al., 2001). The selection of TRIS as a buffer while predicated upon investigation with conidial Pr1 yielded surprisingly lower activity for enzymes, at pH*i* 8, for either EPF (St. Leger et al., 1991; Shah et al., 2005). It is also possible that reduced activity of Pr1 and Pr2 was a result of the interfering effects of TRIS (Good et al., 1966; Irvin et al., 1981a, Irvin et al., 1981b).

Results showed that the varying enzyme activities at different time intervals were due to the different levels of enzymes produced by the conidia and mycelia. In a similar context, White and Agabian (1995) have reported the synthesis of aspartyl protease isozymes from the human pathogen, *Candida albicans*. They showed that the pattern of isozyme(s) secretion was determined by the cell type (W and O cells) of strain WO-1, whereas the levels of isozymes released were determined by pH of the inducing medium. Contextual to EPF, very recently, Cho et al. (2006a) provided the first evidence of stage-specific gene expression in *B. bassiana.* Their EST analysis of cDNA libraries from aerial conidia, blastospores and submerged conidia showed 20-30% unique sequences to each library. They also reported unique EST sequences to conidia as a subtilisin-like protease, tripeptidyl-peptidase and a thermophlic serine protease (from *Bacillus* Ak 1). In the case of blastospores, unique EST included peptidases, two different amino-peptidases, an acid protease similar to that from yeast *Saccharamycopsis fibuligera* and an avian aminopeptidase H/ bleomycin hydrolase. No ESTs for CDE were observed from

submerged conidia. In this connection, it has been shown that conidial and mycelial Pr1 and Pr2 produced by *B. bassiana* on 1% aphid exuviae (supplemented with or without C/ N sources) are regulated differently (Qazi and Khachatourians, 2008). Collectively, these results raise a crucial question and warrant further investigations on the genes and proteins up-regulated during the development cycle of these EPF.

In conclusion, these results suggest that EPF have the ability to produce CDE under the influence of pH and media. The buffered cuticle along with induction pH has a significant effect on the synthesis and release of CDE. The release of enzyme activities in buffer without cuticle showed the synthesis of CDE due to derepression condition or nutritionally staved conditions. EPF have multiple proteases (Qazi and Khachatourians, 2007; Khachatourians et al., 2007), and have the ability to modify cuticle before germ tube emergence. These results suggest that the synthesis of CDE by EPF is an ecological adaptation. There is also evidence of the concerted action of pH and induction by cuticle on levels of enzyme production. At low pH the inductive effects of the cuticle (C/ N ratios) has overridden the pH mediated gene expression and hence Pr1 and Pr2 activities were observed for both EPF. In contrast high pH acted as an inducer of Pr1. This reflects a specializing adaptation by the pathogen during the course of evolution to invade host tissues in the presence of multiple environmental signals (pH and food cues). The concerted action of the host cuticle along with ambient pH may provide a dual control mechanism for the timed released of the CDE for the modification of host cuticle.

11.0 Regulation of the ambient pH by *Beauveria bassiana* determines the activity of extracellular proteases

11.1 Abstract

The purpose of this study was to determine how ambient pH affects optimal expression of mycelial extracellular protease (EP) activity in *Beauveria bassiana*. EP activity under acidic (4.0), neutral (7.0) and basic (11.0) pH conditions occurred when mycelia produced ammonia and modified the pH to optimize secretion and activity of EP. Fungal metabolism can serve to balance ambient pH, as the type of carbon and nitrogen sources used significantly affected pH modulation, ammonia production, and EP secretion. The significance of these findings extends into the role of EP in virulence determination for insect-hosts and pest management.

11.2 Introduction

Fungi are champions among scavengers and can use wide variety of compounds as carbon, nitrogen and sulfur sources. They and are capable of expressing, upon demand, the catabolic enzymes of many different pathways which are under the control of pathway specific and global gene regulatory networks (Marzluf, 2001). However, in certain circumstances pH can act as an ambient signal for the synthesis and release of catabolic enzymes (Penalva and Arst, 2004). As a consequence these fungi secrete either acid(s) or base(s) to modify pH of the medium to enable at secretion permissive level (Penalva and Arst, 2002).

The manner in which entomopathogenic fungi (EPF) respond to their microenvironments and modify for maximizing their establishment remains to be elucidated. Consider the catabolism of proteins by germinating spores in the cuticular environment of an insect host. The

compositions of insect cuticle or internal structures (Haemocoel) have many of the substrates useful for the providing carbon and nitrogenous precursors for growth. However, they may not have the optimum of physical conditions for onset or progression of infection. Thus, upon landing, spores of EPF need to establish prerequisite conditions for functionality of enzymes such as proteases on cuticle and the same within the body proper. The most obvious strategy from the fungal perspective would be to release metabolites indigenous to the spore during hydration or swelling phase of spore for the secretion permissive level of conidial enzymes. In such an event conidial enzymes can degrade cuticle to obtain nutrients for germination events. With mycelia the situation could manifest through leakage of ions, diffusion and release of metabolites (organic acids and ammonia) by exocytosis. Indeed, if such a scheme would make eminent evolutionary sense as timely action of proteases would instigate the breaching of the external layers of the host and first steps of pathogenesis.

How do mycelia during their development create the condition for optimal activity of proteases? What perceived value could we envisage in the protease interaction with substratum and polymers for survival and success as a pathogen for a specific host niche? In order for these questions to be answered, we need information on the effect of mycelial endogenous pH/ammonia content and their release on the pathogen while under nutritional deprivation. The main objective of this study was that *B. bassiana* mycelia can secrete ammonia to modify the pH of the surrounding environment to regulate the release/activity of gelatinase-like protease.

11.3 Materials and methods

11.3.1 Fungal isolates and culture conditions

Beauveria bassiana GK2016 was obtained from BioInsecticide Research Laboratory, Department of Applied Microbiology and Food Science, College of Agriculture and Bioresources, University of Saskatchewan, Canada. Strain was maintained on YPG agar at 27° C. Spores were prepared from two weeks old cultures as described in (Qazi and Khachatourians, 2007).

Standardized mycelial inocula from 48 h of growth in yeast extract peptone medium was transferred (in final volume of 2 % wet weight) to 2% crude crab chitin buffered medium or buffer alone (0.05 M citrate/ phosphate, pH 4.0; 0.05 M Na_2HPO_4/ NaH_2PO_4, pH 7.0; 0.05 M Phosphate/ Na OH, pH 11.0). Conversely, mycelial inocula were added to 1% crude crab chitin buffered medium (0.1 M citrate/ phosphate, pH 4.0; 0.1 M Na_2HPO_4/ NaH_2PO_4, pH 7.0; Phosphate/ Na OH, pH 11.0) supplemented with 0.5% glucose, glutamate, methionine, and $NaNO_3$ respectively. The mycelial inocula was incubated with shaking (New Brunswick rotatory shaker, 150 rpm: New Brunswick Scientific Co., New Jersey, USA) at 27º C. After set times, 5 ml of the sample was aseptically obtained and subsequently filtered through 0.22 µm pore size Millipore™ filters (Millipore, Billerica, USA). Protease and ammonia production and pH were measured as described in latter sections. Background ammonia release was tested in un-inoculated culture media, incubated with each assay. In all the cases three replicates were performed for statistical accuracy.

11.3.2 Protease activity

Protease activity (Gelatinase and caseinase) was determined by measuring the zone of clearing in 1.5% (w/v) agar mediums containing either 1% gelatin or 1% casein as substrates. Essentially, each plate contained 18 ml of the medium with the appropriate substrate supplemented with 0.005% of phenol red. After cutting agar plugs by using 0.5 mm pipette, 50 µl of enzyme filtrate was dispensed in each well. After which time plates were incubated for 24 h to reveal the clearing zone. Three replicates were performed in each case.

11.3.3 Ammonia analysis

Ammonia (NH_3 and NH_4^+) production was estimated by the method of Chaney and Marbach, 1962) as previously described (Chapter, 6, section, 6.3.9)

11.3.4 Intracellular pH determinations

Intracellular pH determinations were carried out by the method of Caddick et al (1986) after some modifications. Intracellular pH determination was carried out by obtaining the fungal mycelial inocula from 48 h YPG broth. The mycelia were washed (2.5gm) with 100 ml of type II water on Whatman no. 3 filter paper and subsequently transferred to the respective buffer (0.1 M citrate/ phosphate, pH 4.0; 0.1 M Na_2HPO_4/ NaH_2PO_4, pH 7.0; Phosphate/ NaOH, pH 11.0) of 50 ml portions each in triplicate. The mycelia were incubated for 2 and 24 h, respectively at 27° C, 150 rpm. After which time mycelia were filtered once again in same way as described earlier. The washed mycelia were transferred to 0.1% (vol/vol) triton X-100 in 20 ml portion per treatment. The mycelia in triton X-100 were placed at – 70° C for 48 h and disrupted by rapid thawing to room temperature. The pH of the mycelia (or any other sample in this experimental

series) was determined with glass electrode by using Accument Scientific AR50 (Fischer Scientific Co., Pittsburgh, USA) pH meter. The pH meter was standardized according to manufacturer instructions with three standard buffers before taking the experimental measurements.

11.3.5 Statistical analysis

Analysis of variance (ANOVA) was performed by using one way completely randomized ANOVA design using CoStat version 6.204 (CoHort Software, Monterey, CA, USA). Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$. The whole study was repeated two times.

11.4 Results

pH regulatory mechanisms ensure that the ambient pH is tailored to the requirement of the fungi for the synthesis and release of metabolites, permeases, and exoenzymes. Results revealed that *B. bassiana* regulates its ambient pH by secreting ammonia. The pH*i* or the initial set point pH of buffer changed (Table 11.1), indicating that ammonia production responded to carbon and nitrogen derepression at higher pH*i* (7 and 11). However, ammonia production on 2% buffered chitin-based medium was higher than buffer alone, which implies that the proteases are degrading the protein moieties present in the cuticle. Ammonia formation during incubation period was significantly higher at pH*i* 7 than any other treatment in chitin-based medium. Conversely, the production of ammonia was lowest at pH*i* 4, indicating acidic pH is less favorable for ammonia production. A protease assay showed that *B. bassiana* can produce gelatinase (PG) and caseinase (PC) activities under the chosen conditions. A slight

Table 11.1 Quantification of pH, ammonia and protease activity after 12 h incubation of mycelial inocula of *B. bassiana*.

Inducer pH (pH*i*)	**Final pH (pH*f*)**		**Ammonia (mM)**		**Gelatinase[a]**		**Caseinase[b]**	
	+	-	+	-	+	-	+	-
4.0	6.61[c]	6.47[c]	0.0073[c]	0[c]	7.3[c]	0[b]	0.55[a]	0
7.0	7.73[b]	7.02[b]	3.4[a]	0.007[b]	0.4[b]	0.4[c]	0	0
11.0	8.35[a]	7.89[a]	0.038[b]	0.06[a]	0.5[a]	0.4[a]	0	0

Legend:

Two day old mycelia from YPG were incubated in buffer or 2% crude crab chitin buffered medium at potential inducer pH*i* 4.0, 7.0 and 11. Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 3) were separated by using Duncan's method ($p < 0.05$). Values following same letters in each column are not significantly different.

[a] and **[b]**: protease activity measured against 1% gelatin and casein as substrate, respectively. Where (+) represents supplemented with cuticle and **(-)** indicates only buffer.

amount of PC activity was only evident at pH*i* 4 when cuticle was present as an inducer. Conversely, more gelatinolytic activity was observed at all pH*i* (4, 7 and 11) indicating that gelatinase-like enzymes have higher pH requirements (above 6.6). Furthermore, PG activity was not affected by the addition of chitin at pH*i* 7. Conversely, significant reduction in the activity of PG was apparent at pH*i* 4 and 11 when chitin was omitted from the medium. Therefore, pH has an impact on the synthesis or release of proteases (PG and PC).

The effect of different nutrients on the pH modulation, ammonia production and protease release/activity was tested. The addition of various nutrients has altered the pH of the buffered basal media (Table 11.2). Addition of glucose and methionine repressed the production of ammonia at all three pH*i*'s indicating that C/ N and S regulation has more importance than ambient pH regulation. Furthermore, methionine can be utilized as a preferred sulfur source in fungi that is under the control of sulfur regulatory gene network (Marzluf, 2001). Conversely, significant higher level of ammonia was detected by supplementing chitin-based medium with glutamate and sodium nitrate except at pH*i* 4 (sodium nitrate). Furthermore, the ammonia formation at these pH*i*'s was also significantly ($p < 0.05$) different.

Protease activity (Fig. 11.1) was significantly ($p < 0.05$) higher at pH*i* 4 when supplemented with glucose and glutamate and could change significantly following the addition of certain other nutrients. Interestingly, PC activity was completely inhibited at pH*i* 4 when supplemented with $NaNO_3$ and methionine, respectively. This may imply that these two preferred nitrogenous sources are readily available to the fungi making protease secretion was unnecessary. Conversely, at pH*i* 7 and 11 (with added nutrients), respectively, 4 to 2 fold reduction in the activity of PC was observed in comparison to maximum protease activity at pH*i* 4 in the presence of glucose.

Table 11.2 pH and ammonia measurements after 24 h of incubation of mycelial inocula of *B. bassiana*.

	Supplement/ pH*f*-NH_3 measurements							
Inducer pH	**Glucose**		**Glutamate**		**Methionine**		**Sodium Nitrate**	
(pH*i*)	**pH*f*** [a]	**NH_3 (mM)**	**pH*f*** [a]	**NH_3 (mM)**	**pH*f*** [a]	**NH_3 (mM)**	**pH*f*** [a]	**NH_3 (mM)**
4	4.46[c]	0	5.18[b]	0.12 (0.03)	4.96[c]	0	5.87[c]	0
7	7.13[b]	0	8.05[c]	32.95 (3.09)	7.28[b]	0	7.20[b]	2.0 (0.08)
11	7.64[a]	0	8.49[a]	40.95 (2.12)	8.01[a]	0	8.3[a]	8.0 (0.05)

Legend:

Two day old mycelia from YPG were incubated in 1% crude crab chitin buffered medium supplemented with nutrients as indicated above at potential inducer pH*i* 4.0, 7.0 and 11. Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 3) were separated by using Duncan's method ($p < 0.05$). Values following same letters in each column are not significantly different.

[a] pH*f*: Final pH of the spent culture after 24 h of incubation.

Values in brackets represent S.D.

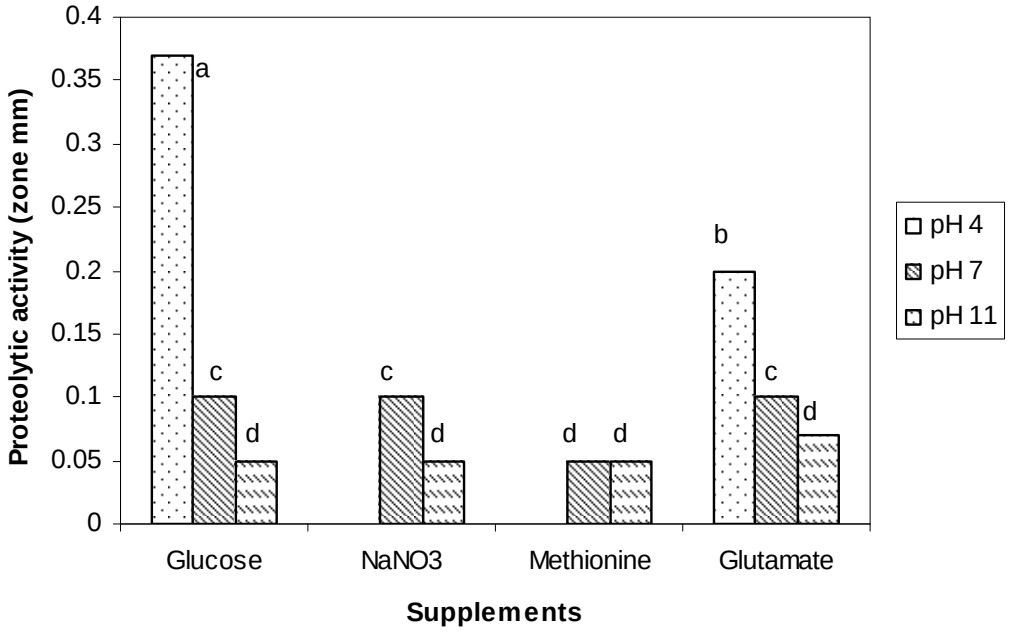


Figure 11.1: Proteolytic activity determined after incubation of mycelial inocula (*B. bassiana*) for 24 h in 1% crude crab chitin medium at pH 4, 7 and 11 supplemented with 0.5% nutrients as indicated above. Protease activity was determined against 1% casein. Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 3) were separated by using Duncan's methods ($p < 0.05$). Same alphabetical letters following the bars are not significantly different.

The PG activity/release (Fig. 11.2) was observed at higher pH (neutral to alkaline), indicating that pH is involved in the release of PG. This also reveals the importance of pH as signal in comparison food cues. Therefore, even in the presence of nitrogen sources like glutamate and $NaNO_3$ PG activity is high. Furthermore, there was no effect of glucose repression on PG activity at pH*i* 7 and 11, which was evidenced at pH*i* 4. In addition, reduction of PG activity at pH 4 could be attributed to the acidic pH which is not optimum for PG. The only exception is the significant reduction of the PG at pH*i* 7 when supplemented with glutamate due to the accumulation of high amounts of NH_3 and subsequent degradation of the protease.

In addition, results showed that that mycelia upon incubation (after 2 h) at pH*i* 4, 7, 11 have intracellular pH of 7.17 (±0.05), 6.67 (±0.07) and 6.14 (±0.04), respectively. Whereas, after 24 h of incubation of mycelia intracellular pH was 5.58 (±0.05), 6.07 (±0.05) and 6.17 (±0.06). These results reaffirm homeostatic mechanisms ensuring that the intracellular pH is within the physiological range.

11.5 Discussion

It is well recognized that EPF can use their molecular arsenals such as extracellular proteases and chitinases to infect a broad range of insects (Khachatourians, 1991; Khachatourians, 1996). The synthesis of proteases in two EPF, *B. bassiana* and *Metarhizium anisopliae* have been shown to be regulated under the influence of carbon/nitrogen ratios (St. Leger et al., 1986a; Bidochka and Khachatourians, 1988a; St. Leger, 1995, Khachatourians et al., 2007). There is strong evidence that in *A. nidulans* the PacC protein sets up the relationship with external pH.

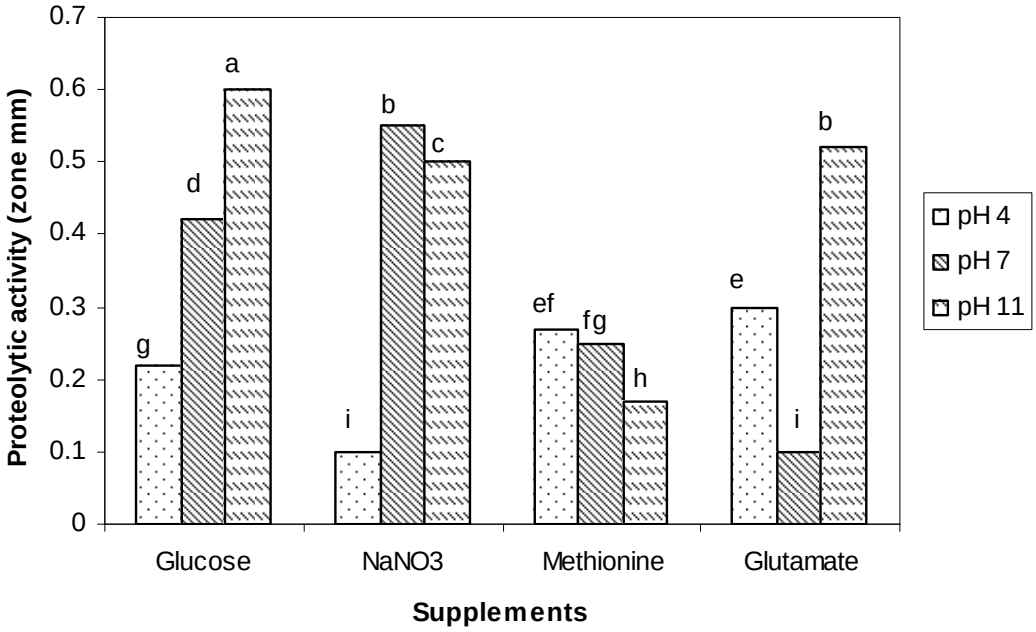


Figure 11.2: Proteolytic activity determined after incubation of mycelial inocula (*B. bassiana*) for 24 h in 1% crude crab chitin medium at pH 4, 7 and 11 supplemented with 0.5% nutrients as indicated above. Protease activity was determined against 1% gelatin. Level of significance was determined by using one way completely randomized design ANOVA, where means (n = 3) were separated by using Duncan's methods ($p < 0.05$). Same alphabetical letters following the bars are not significantly different.

PacC protein is a transcriptional factor which is processed under alkaline and acidic conditions to ensure that the metabolites, permeases and other enzymes are secreted into the habitat for optimal physiological functioning of fungi including several phytopathogenic fungi (Penalva and Arst, 2004).

St. Leger et al. (1998) showed that the ambient pH in *M. anisopliae* mycelia could regulate expression of pathogenicity determinant genes in growing cultures. In a subsequent study they reported that *M. anisopliae* can regulate the pH and protease activity by secreting ammonia (St. Leger et al., 1999). Among the phytopathogens, ammonia is considered as major virulence factor for causing tissue destruction and disease development (Prusky and Yakoby, 2003). Production of compounds contributing to alkalinity, such as ammonium, has not been extensively studied in fungi despite of its diverse role in fungal physiology. As a nutrient, ammonia regulates nitrogen metabolism (Arst and Cove, 1969). Reports also indicate that ammonia resulting from protein degradation in saprophytes can determine the choice of morphogenetic pathways in *Dictyostelium discoideum* (Schindler and Sussman, 1977; Schaap et al., 1995) and inhibits sporulation in *Coprinus cinereus* and *Saccharomyces cerevisiae* (Moore et al., 1987).

Results showed that *B. bassiana* secreted ammonia and regulated the protease activity at three pH*i* (4, 7 and 11). Ammonia in culture supernatant at all three pH*i* tested increased the pH of the medium. In the absence of cuticle ammonia formation at pH*i* 7 and 11 suggests its production under C/ N derepression conditions. This is a likely event due to the catabolic processes which begin within the mycelia and without consideration of exogenous C/ N sources. Similarly, St. Leger et al. (1999) reported the release of ammonia under C/ N derepression conditions with inducer level of pH 6.5 for saprophytic fungi (*A. nidulans* and *N. crassa*), but

obviously not for the entomopathogenic fungus, *M. anisopliae*. The ammonia production was significantly higher when 2% chitin was added to the buffers at all three pH*i* tested. This observation is congruent with the degradation of the cuticle by the action of the proteases synthesized/released. It also corroborates with the observation that ammonia formation during growth on proteinaceous substrates as sole carbon source will result in dissimilation of amino nitrogen produced in excess of that required for growth (Jennings, 1989). Incubation of mycelial inocula in the presence of amino acids may indicate two different catabolic routes that *B. bassiana* can use to metabolize amino nitrogen. Higher level of ammonia was produced when the basal medium was supplemented with glutamate, which during catabolism liberates two ammonia molecules per molecule (Griffin, 1994). This explains the elevated level of ammonia in comparison to methionine. Furthermore, higher levels of ammonia, evident at pH*i* 7, was indicative of the role of pH in the formation of ammonia, either because of the dissociation constant of the glutamate or stability of the catabolizing of excess amino nitrogen during amino acid catabolism (Jennings, 1989). St. Leger et al. (1999) observed the opposite effect that increasing the abundance of amino acids (100 mM) at pH*i* 6.5 decreased the formation of ammonia, suggesting that in nature, continual formation of ammonia requires release of low levels of amino acids from a protein source.

Interestingly, ammonia formation was completely inhibited when basal medium was supplemented with glucose and methionine at all the pH*i*'s. St. Leger et al (1999) suggested that methionine inhibit ammonia synthesis either through induction of catabolite repressible enzyme(s), presumably deaminases, or regulation of the enzyme activity via substrate inhibition. Regulation of amino acid oxidase in *N. crassa* is inducible by amino acids and would release ammonia upon induction (Sikora and Marzluf, 1982). Similarly higher concentrations of glucose

and amino acid have repressed the ammonia formation in *M. anisopliae, A. fumigatus and N. crassa* (St. Leger et al., 1999). This inhibition can be due to added glucose which would have switched metabolism from catabolizing amino acids to catabolizing sugars for biosynthesis and freeing unused ammonia.

Analysis of protease activity revealed that PC and PG are regulated either by C/ N sources or ambient pH. PC activity was higher with the addition of glucose and glutamate implying that the fungi was growing at its maximum capacity with both C and N sources and requiring more nitrogen to complement the fast growth rate. In case of PG release/activity was optimum at pH*i* 7 and 11, respectively even in the presence of preferred nitrogenous sources. This indicated that PG is under the influence of pH regulatory element presumably *pacC* rather then carbon and nitrogen regulatory elements (i.e., *areA* and *creC*). Methionine has repressed the PG and PC activity or release implying that there might be some regulatory role of sulfurs gene regulatory element, which as yet has not been described in EPF. Glutamate at pH*i* 7 has resulted in the repression of PG presumably due to high ammonia formation. At high concentration it becomes toxic as it raises intracellular pH, thus inhibiting the protein synthesis and enzyme activity (Doyle and Butler, 1990).

Regulation of protease activity/release due to ammonia formation is a poorly described phenomenon in EPF. However, St. Leger et al. (1999) reported increase in pH of the infected cuticle by *M. anisopliae* propagules/mycelia after 60 h post inoculation. In chapter 6 and 7 the release of ammonia and citrate from the conidia of *B. bassiana* and *M. anisopliae* upon hydration was reported. Taken together, these results suggest that ammonia release from mycelia or conidia reflect an adaptation of the parasitic fungi to modify microhabitat. Additionally, during insect mycosis EPF face gradient of nutrients as well as pH which may form upon fungal metabolite

secretion and degradation of the insect tissues, which could be detrimental if not counter balanced by pH-homeostatic mechanisms. Indeed the regulation of intracellular pH by *B. bassiana* and ammonia formation has functions related to regulation of the microenvironment and protease activity/release. It represents a previously unconsidered virulence factor in *B. bassiana* with the potential to devour insect cuticle/tissues and disturb the host's immune system.

12.0 Effect of pH on growth, morphology, conidiation and protease release/activity of entomopathogenic fungus, *Beauveria bassiana*

12.1 Abstract

Beauveria bassiana is an important broad range commercially important mycoinsecticide. The growth of five *B. bassiana* isolates was tested at pH*i* 4, 7 and 11. The Differences in growth rates were observed and correlated with pH*i* and incubation time. In viability assessment, the colonies appeared faster on the tested medium in the order as follow: pH 7 > 4 > 11. Measurement of growth pattern indicated a strong correlation with the average colony size (mm)/ day, implying the change in the pH to be a result of release of metabolic acid and base. Use of pH gradient plates (pH 4 to 7, 7 to 11 and 4 to 11) allowed examination of the effect of a broader pH range on the growth of *B. bassiana* isolates. To assess particularity of this effect, colony morphology, radial extension rate, conidiation, secretion of metabolites and protease release/activity were evaluated for *Metarhizium anisopliae,* and the saprophytic fungus, *Aspergillus nidulans*, at pH*i* 4 and 8. Significant differences were observed for all the measured characteristics at the selected pH*i* for *B. bassiana* and *M. anisopliae* in contrast to *A. nidulans*. Results revealed that pH has a significant role in the growth and development of EPF. In addition, EPF appear to be well adapted to the pathogenic mode of life style not only as reflected by their better ability for ambient pH regulation in contrast to saprophyte but also for key virulence factors, the secretion of metabolites and protease (Gelatinase-like) release/activity.

12.2 Introduction

Beauveria bassiana is a ubiquitous entomopathogenic fungus that lives a saprophytic life in the soil and case wide spread epizootics in insect pests when ever they cross a threshold level. This mechanism for the control of insect population ensures natural checks and balance of ecosystem. *B. bassiana* is an important mycoinsecticide widely being used world wide (Feng et al., 1994; Qazi and Khachatourians, 2005) that can infect more then 700 species of insects (Ferron, 1985). EPF have advantage over other biocontrol agents (BCA) because of their multiple modes of contact and action, e.g., hydrolytic enzymes that can help degrade insect cuticle.

To be effective as a mycoinsecticide, conidia are sprayed in water or invert emulsion in oil. As the spore attachment to the insect cuticle is a prerequisite for host infection, several factors could affect the survival and germination of conidia. Amongst well documented factors that may influence the persistence and efficacy of EPF includes UV damage, temperature, dryness and light (Wraight et al., 2001; Chelico et al., 2005, 2006; Khachatourians, 1996; Khachatourians et. al., 2007). However, role of pH and its regulation during growth and metabolism of EPF has been inadequately described.

The optimum pH for the growth of *B. bassiana* was reported from 5.0-6.0 (Sanzhimitupova, 1980) and 6.0-8.5 (Galani, 1988). Shimazu and Sato (1996) have showed that *B. bassiana* can grow well above pH 10. Similarly, Padmavathi et al. (2003) have described the pH range for growth of *B. bassiana* isolates to be from 5.0-13. In another EPF, *M anisopliae* optimum pH of growth was reported from 5.0-8.5 (Milner, 1989), 4.0-7.8 (Rath, 1992) and 3-10 (Dillon and Charnley, 1991). Ironically, none of the investigators have mentioned the innate ability of EPF to modulate their ambient pH during growth as per cellular requirement.

The successful infection of EPF primarily relies upon the release of proteases and chitinases (Khachatourians, 1996; Clarkson and Charnley, 1996), which are regulated by nutritional cues (C/ N ratios) (St. Leger, et al. 1988; Bidochka and Khachatourians, 1988a; St. Leger, 1995) and ambient pH (St. Leger et al., 1998).

Similarly, there is strong evidence of pH regulation in filamentous fungus, *A. nidulans,* which is regulated by *pacC.* PacC is a transcription factor which is processed under alkaline and acidic conditions to regulate the delicate balance of pH ensuring that the metabolites, permeases and enzymes are secreted in the habitat for optimal function. *pacC* homolog have been identified in filamentous fungi and several phytopathogenic fungi (Penalva and Arst, 2004). There are many other examples of genes and biological processes which are regulated by ambient pH including: (i) dimorphism in the human pathogen, *Candia albicans* (Ramon et al., 1999), (ii) metabolic acids/bases secretion in the phytopathogens, *Sclerotinia sclerotiorum, Colletotrichum* spp. and *Alternaria* spp. (Prusky and Yakoby, 2003), and (iii) pigmentation, growth, development and differentiation in *Ustilago hordei* (Lichter and Mills, 1998) and (iv) permeases, extracellular enzymes and secondary/exported metabolites in *Aspergillus* spp. (Arst and Penalva, 2003).

Beyond those which were centered on the pH tolerance of the growth medium there is a need to identify and further document the significance of pH in growth and metabolism of fungi. The main objectives of this study include the ability of *B. bassiana* to: (i) grow at the selected pH*i* (acid, neutral and alkaline), (ii) modify the ambient pH during growth for optimal protease release/secretion, and (iii) compare conidiation at the selected pH*i.*

12.3 Materials and methods

12.3.1 Fungal isolates and culture conditions

Beauveria bassiana isolates GK 2015 (a GK 2018 mutant) GK 2016 and GK 2051 (a GK 2016 mutant) was obtained from Department of Applied Microbiology and Food Science. *Metarhizium anisopliae* (MA 2038), *Beauveria bassiana* isolates 2833, ATCC 44860 and *A. nidulans* (AN 189) were generously provided by USDA, USA. Fungal strains were maintained on YPGA for 12 days at 27° C. Conidia were pooled with 0.03% tween 80 as previously described (Qazi and Khachatourians, 2007).

12.3.2 Variable pH plates and pH gradients plates

Plates with defined pH were made by using 1% yeast extract and 2% agar (w/v). pH of the medium was adjusted at 4, 7 and 11 prior to autoclaving with 0.2 N HCl and 0.2 N NaOH. After which time the media was autoclaved at 121° C for 15 min. Medium was tempered at 55° C before pouring plates. Petri plates of pH 4, 7 and 11 were made to test the growth of *B. bassiana*. In case of gradient Petri plates, 12 ml of the medium was dispensed by using sterile glass pipette (1% yeast extract and 2% agar) and plates were allowed to cool at slanting position with the help of wooden board (angled at approximately 60°). After 30 min approximately 12 ml of the media of different pH was poured in each plate. In this way gradient plates of pH 4 to 7, 7 to11 and 4 to 11 were made. Plates were cooled at room temperature before inoculation.

12.3.3 Colony morphology, radial extension rate and protease activity

Protease activity (Gelatinase-like) was determined during the growth of *B. bassiana* on gelatin based agar medium. Briefly, each plate contained 1% yeast extract, 0.5% glucose, 1% gelatin (Difco, carbohydrate free; BD Diagnostics, Sparks, USA), 0.005% phenol red (pH < 6

yellow colour and pH > 8 red colour) and 1.5% (w/v) agar. Essentially, 5 µl of the washed conidia (10^7) were point inoculated at the center of each plate. Plates were incubated for 7 days at 27°C after which time colony morphology, radial extension rate and protease activity (clearing zones) were measured. In addition, both sides of plates were scanned on HP 2200 (Hewlett-Packard Company, Palo Alto, USA) flat bed scanner at 300 dpi to reveal the color change caused by the secretion of metabolic acid(s) or base. Images were saved in TIFF format and 1-D lane densitometry was performed as described in the Appendix-1 for mycelial density profiles. Four replicates were performed in each case.

12.3.4 Statistical analysis

Analysis of variance (ANOVA) was performed by using one way completely randomized ANOVA design using CoStat version 6.204 (CoHort Software, Monterey, CA, USA). Level of significance was determined by using Duncan's methods of mean separation where $p < 0.05$. The whole study was repeated two times.

12.4 Results

12.4.1 Growth rate and colony size at selected pH*i*

Growth of the selected EPF were tested on three inducing pHs (pH*i*) 4, 7 and 11. It is evident from the data that the selected strains of *B. bassiana* grew at all the selected pH*i*'s (Fig. 12.1-12.4). However, the rate/speed of growth and radial extension rate at various time intervals were different. In all the strains tested, maximum growth (represented as percentage maximum growth) was observed at pH*i* 7. Furthermore, the growth observed was dependent on pH*i* of the medium and duration of incubation. The timings of colonies appearing on the agar medium were in the following order: pH*i* 7 > 4 > 11. In addition, the growth rate was also dependent upon the

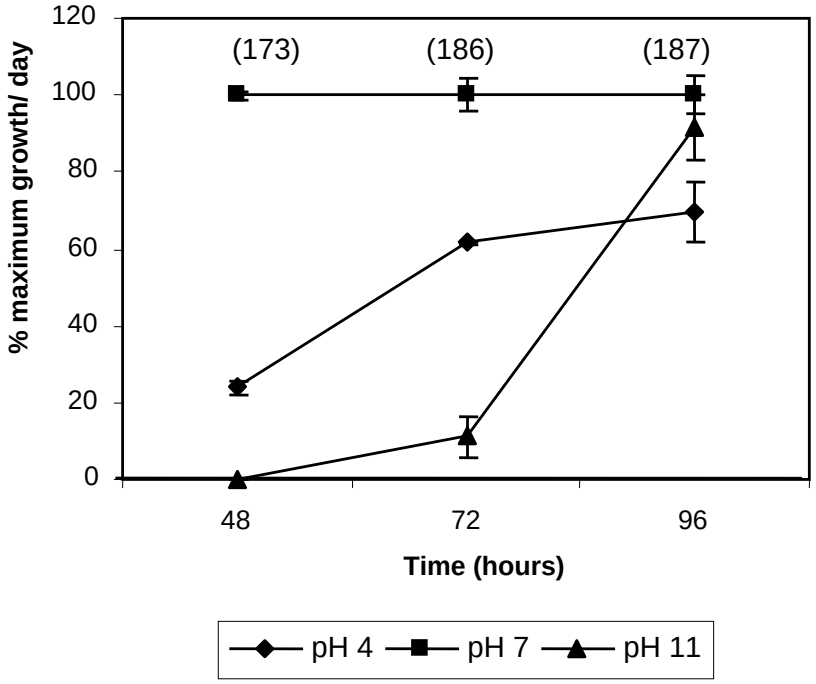
time of incubation. This may imply that isolates has secreted the organic acid(s) and base(s) in order to modify the habitat of the medium to a permissive level suitable for optimal growth.

It is evident from the Fig. 12.1-12.4 that no colonies were formed on medium with pH*i* 11 after 48 h. Similarly, even after 96 h of incubation the average size (mm) of the colonies at pH*i* 4 and 11 was smaller in comparison to those at pH*i* 7. This data show that the pH of the medium has a significant role in the growth of isolates and colony size.

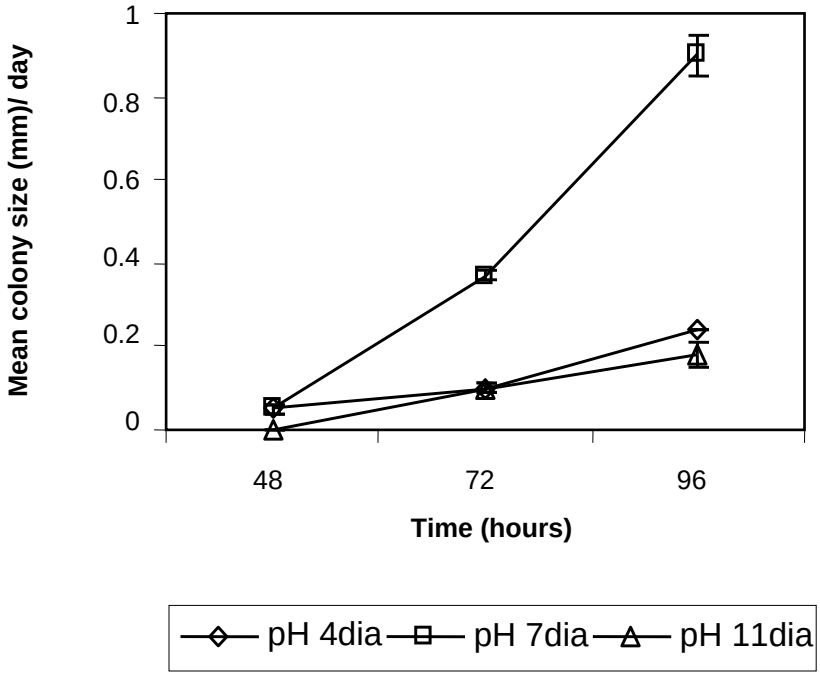
In order to cover a wider range of pH and its impact upon growth, pH gradient plates (pH 4/7, 4/11 and 7/11) were used. Results revealed that the isolates tested have different rate of growth (Fig. 12.5-12.6). The pH gradient effect apparent on growth and colony size was also evident from Fig. 12.7. All the isolates of *B. bassiana* except isolate GK 2016 showed maximum growth at pH 7/11. Conversely, isolate GK 2016 showed maximum growth at pH 4/11 after 48 h, indicating that this isolate has better ability to modify the broad pH gradient of the agar plate for optimal growth in comparison to other strains tested.

12.4.2 Colony morphology, radial extension rate and conidia production

Results indicated that the pH has a significant role in determining the colony morphology or shape, speed of growth and radial extension rate. These results showed the differential ability of the fungi to acquire nutrients under the influence of pH of the growth media. At pH*i* 4, colony produced by isolate GK 2016 was circular and pulvinate with entire margins (Fig. 12.8a, b). At pH*i* 8 colonies were also circular but slightly umbonate with entire margins (Fig. 12.8c, d). In the case of isolate, MA 2038, the colony at pH*i* 4 (Fig. 12.9a, b) was some what circular/slightly irregular, raised and slightly undulated where as at pH*i* 8 the colonies were slightly irregular, raised and undulated (Fig. 12.9c, d). Conversely, isolate AN 189 produced slightly convex,

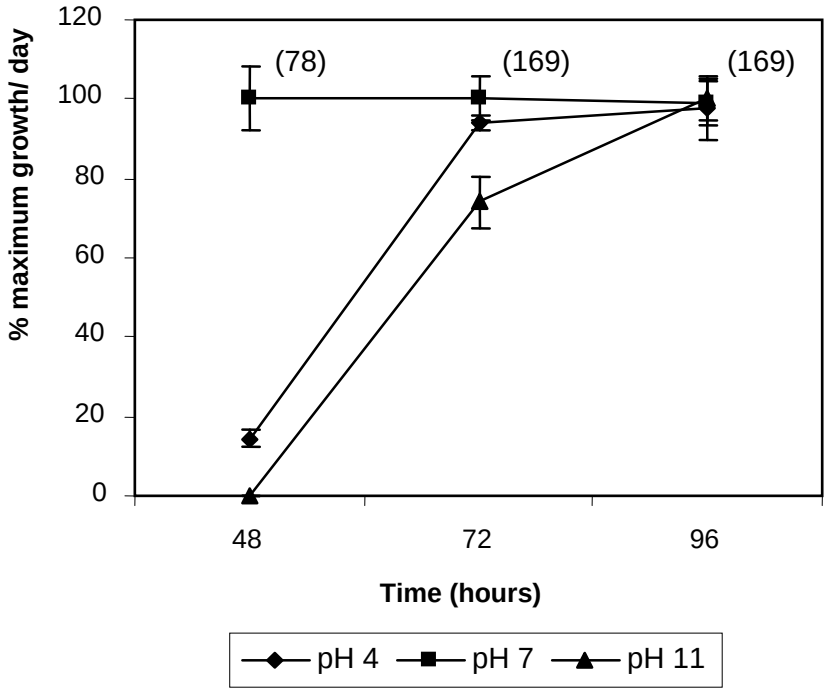


(a)

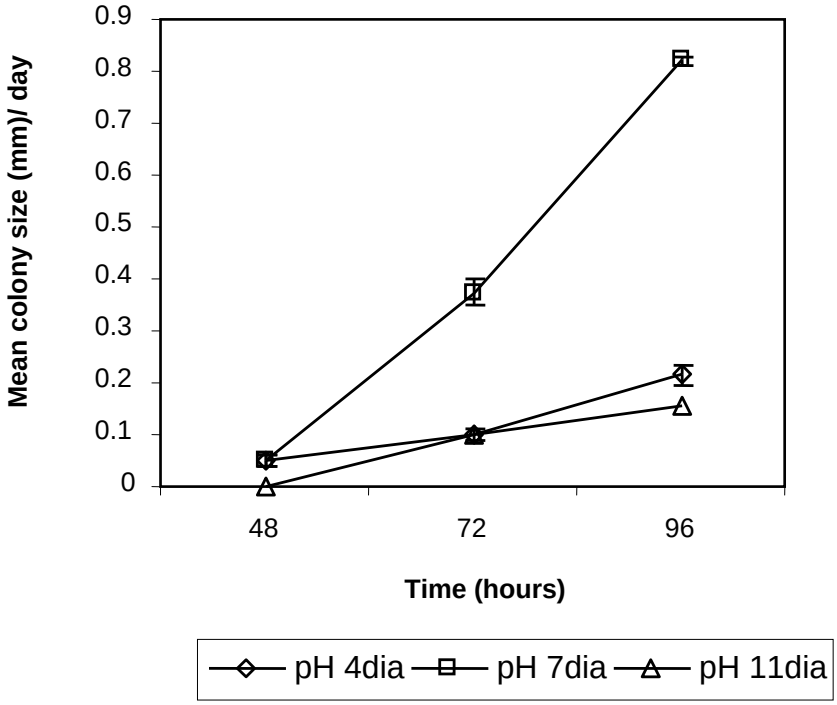


(b)

Figure 12.1a, b: Growth rate of *B. bassiana* (GK 2015) was estimated on pH*i* 4, 7 and 11 (a). In addition, mean colony size (mm)/ day was also measured (b). Error bars represent S.D., where n = 3. Mean colony size was measured for 25 randomly selected colonies on plates. Absolute values corresponding to 100% are indicated at the top of means.

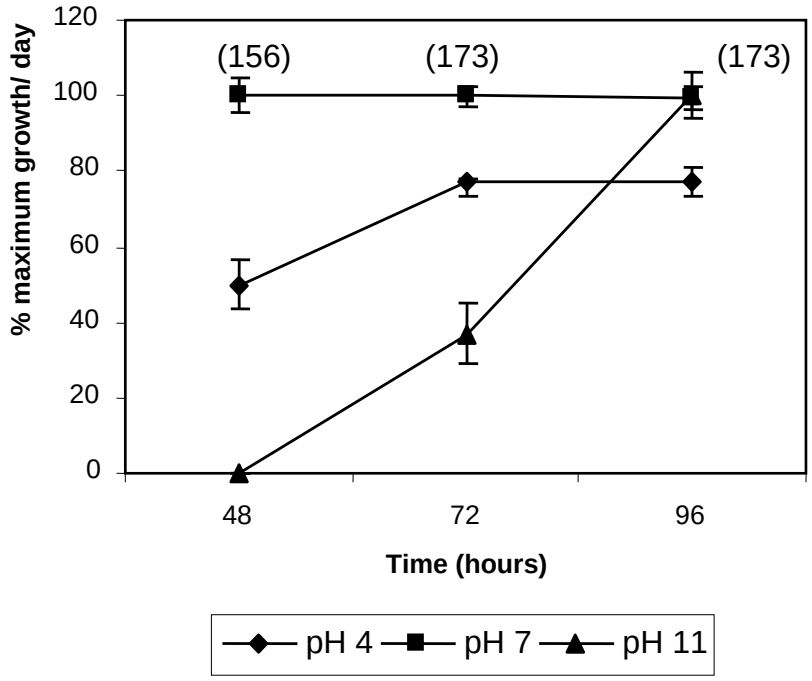


(a)

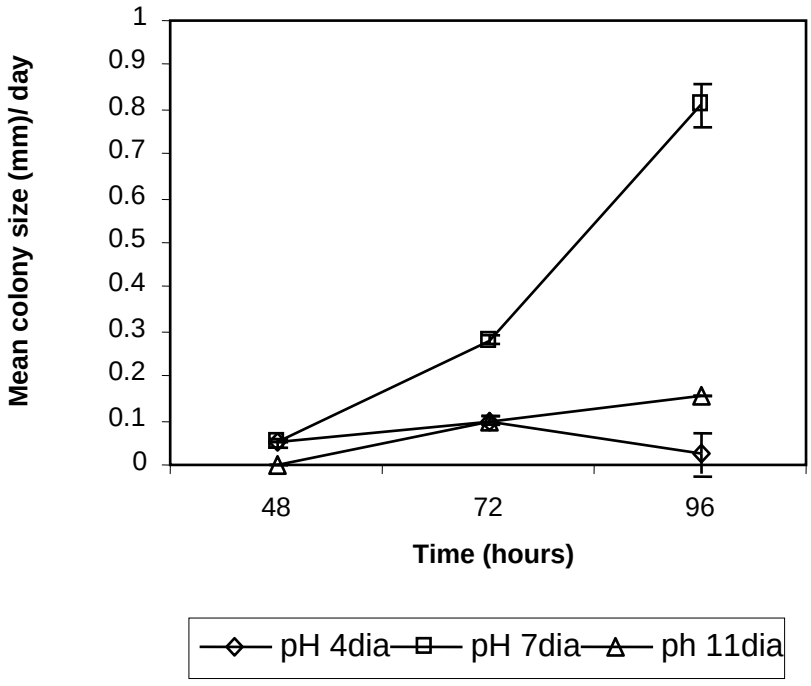


(b)

Figure 12.2a, b: Growth rate of *B. bassiana* (GK 2016) was estimated on pH*i* 4, 7 and 11(a). In addition, mean colony size (mm)/ day was also measured (b). Error bars represent S.D., where n = 3. Mean colony size was measured for 25 randomly selected colonies on plates. Absolute values corresponding to 100% are indicated adjacent to each mean.



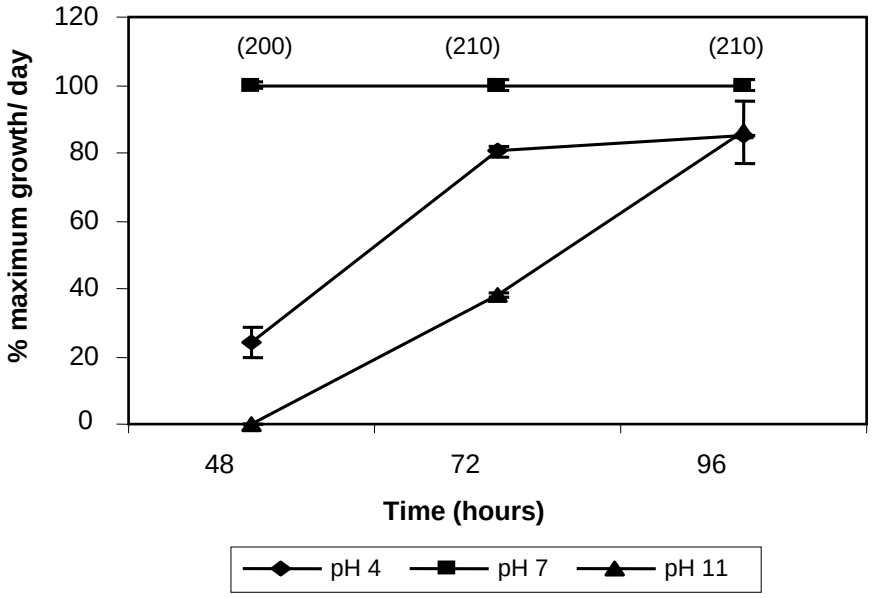
(a)



(b)

Figure 12.3a, b: Growth rate of different isolates of *B. bassiana* (GK 2051) was estimated on pH*i* 4, 7 and 11(a). In addition, mean colony size (mm)/ day was also measured (b). Error bars represent S.D., where n = 3. Mean colony size was measured for 25 randomly selected colonies on plates. Absolute values corresponding to 100% are indicated adjacent to each mean.

(a)



(b)

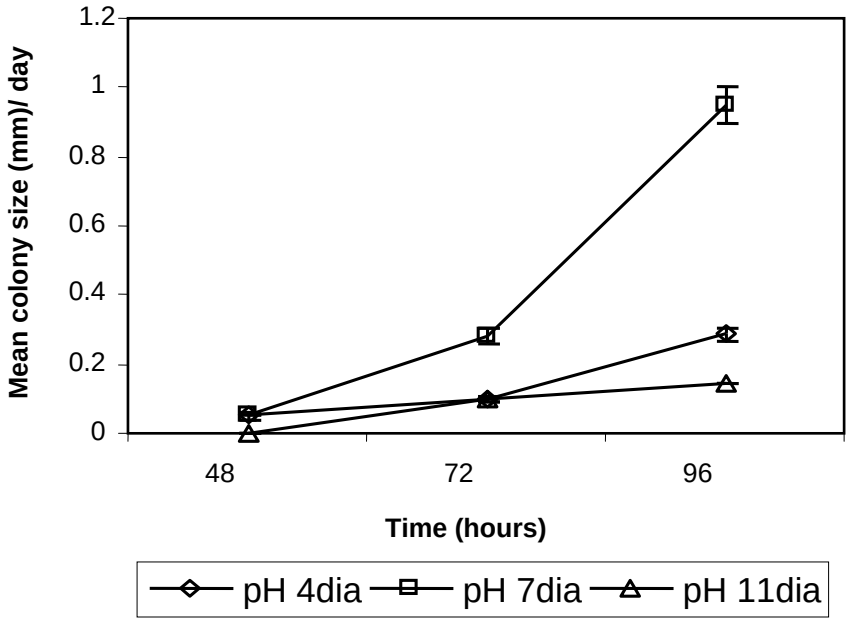
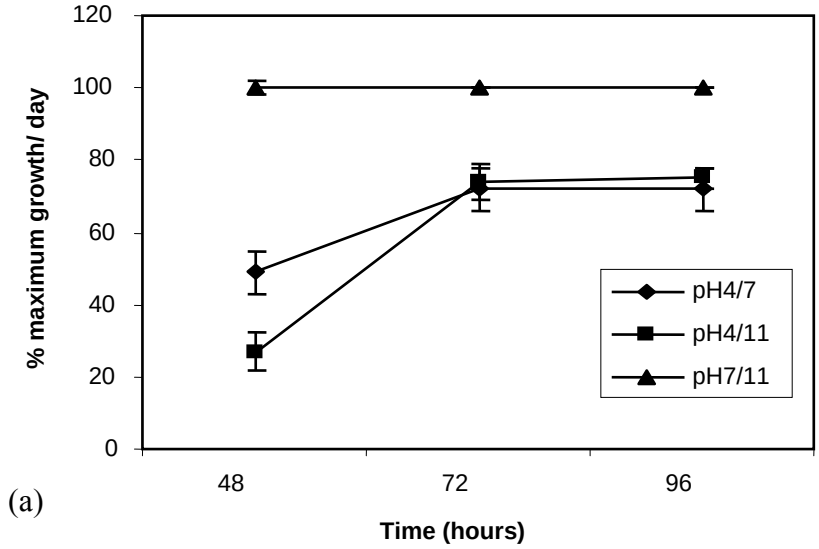
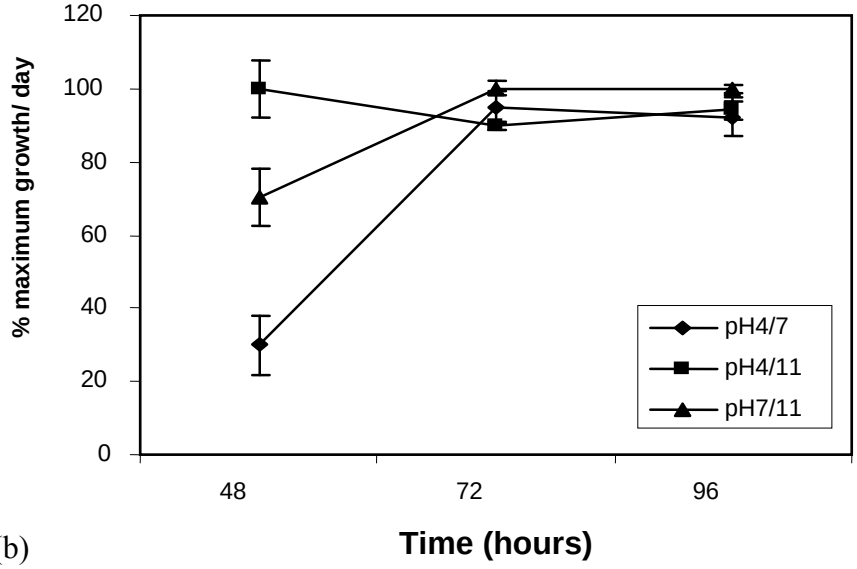


Figure 12.4a, b: Growth rate of *B. bassiana* (USA) was estimated on pH*i* 4, 7 and 11(a). In addition, mean colony size (mm)/ day was also measured (b). Error bars represent S.D., where n = 3. Mean colony size was measured for 25 randomly selected colonies on plates. Absolute values corresponding to 100% are indicated adjacent to each mean.

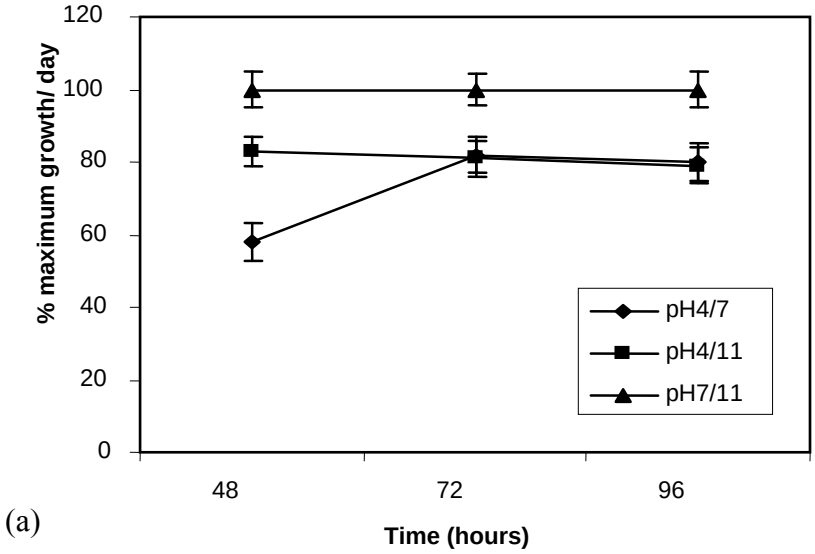


(a)

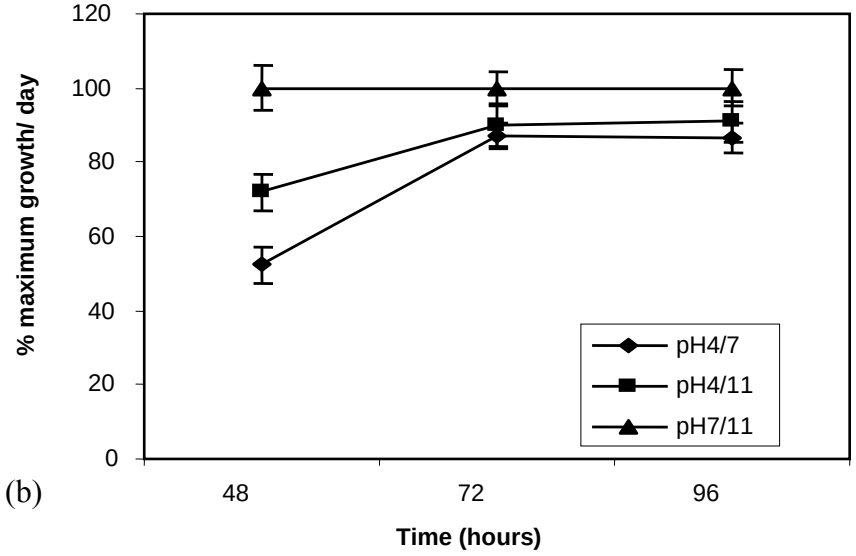


(b)

Figure 12.5a, b: Growth rate of different isolates of *B. bassiana* (a) GK 2015 and (b) GK 2016 on pH gradient plates. Percentage maximum growth was evaluated on pH gradient plates at various time intervals on 1% yeast extract and 2% agar. Error bars represent S.D., where n = 3.



(a)



(b)

Figure 12.6a, b: Growth rate of different isolates of *B. bassiana* (a) GK 2051 and (b) USA on pH gradient plates. % maximum growth was evaluated on pH gradient plates at various time intervals on 1% yeast extract and 2% agar. Error bars represent S.D., where n = 3.

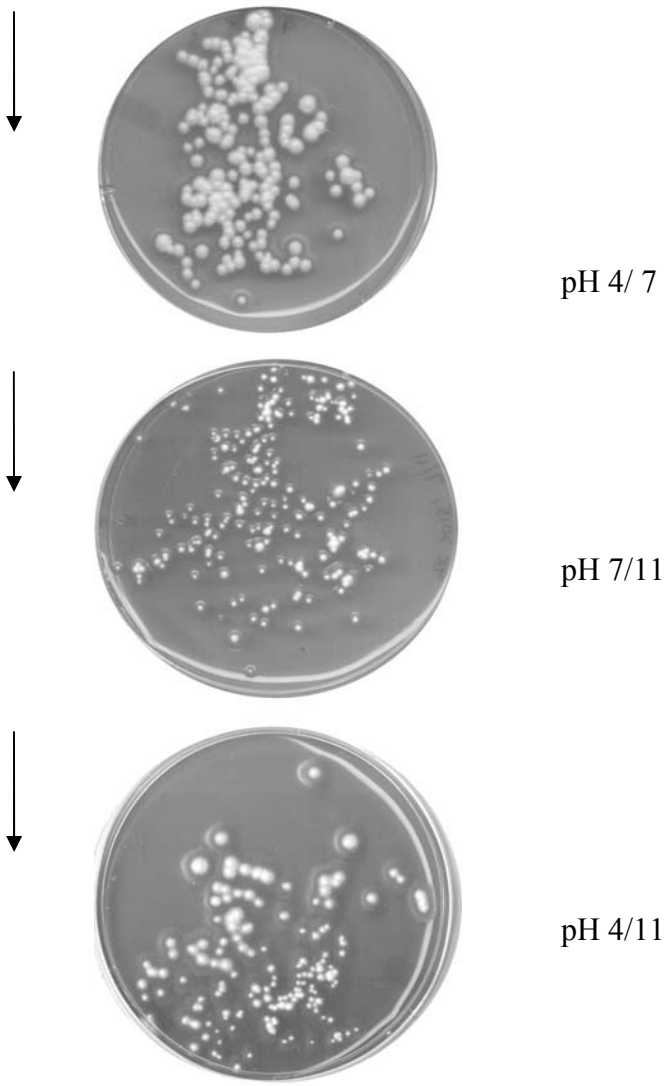


Figure 12.7: Growth of *B. bassiana* (GK 2015) on pH gradient plates. The pH gradient values are indicated on the right side of each plate, whereas the pH linear gradient is represented on left side by an arrow (⟶) showing low to high pH gradient, respectively.

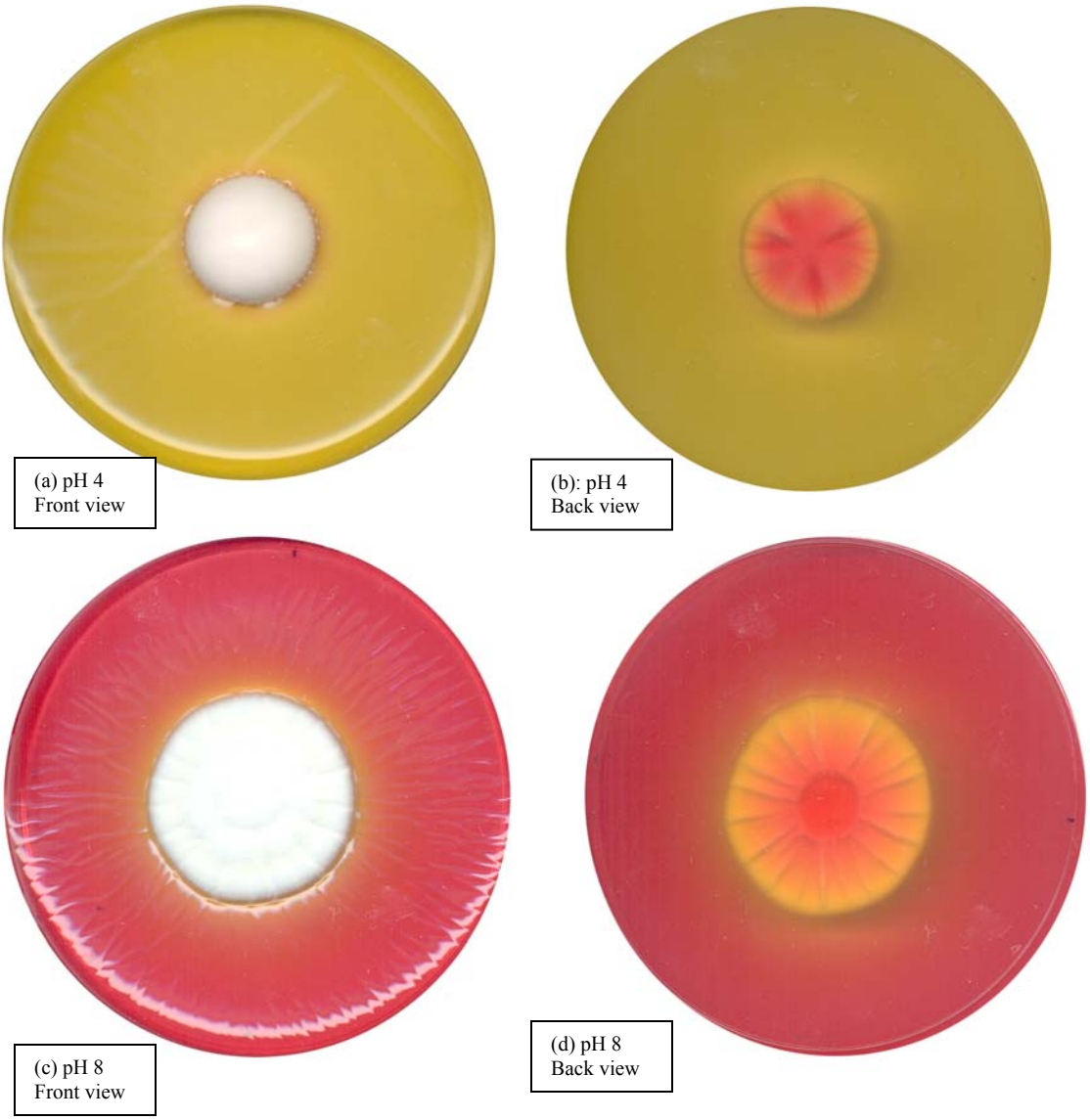


Figure 12.8a-d: Growth of *B. bassiana* (GK 2016) on 1% yeast extract, 0.5% glucose, 1% gelatin, 0.005% phenol red (Yellow colour pH < 6 and red colour pH > 8) and 1.5% agar (YGGPA) adjusted at pH 4 (a, b) and 8 (c, d), respectively..

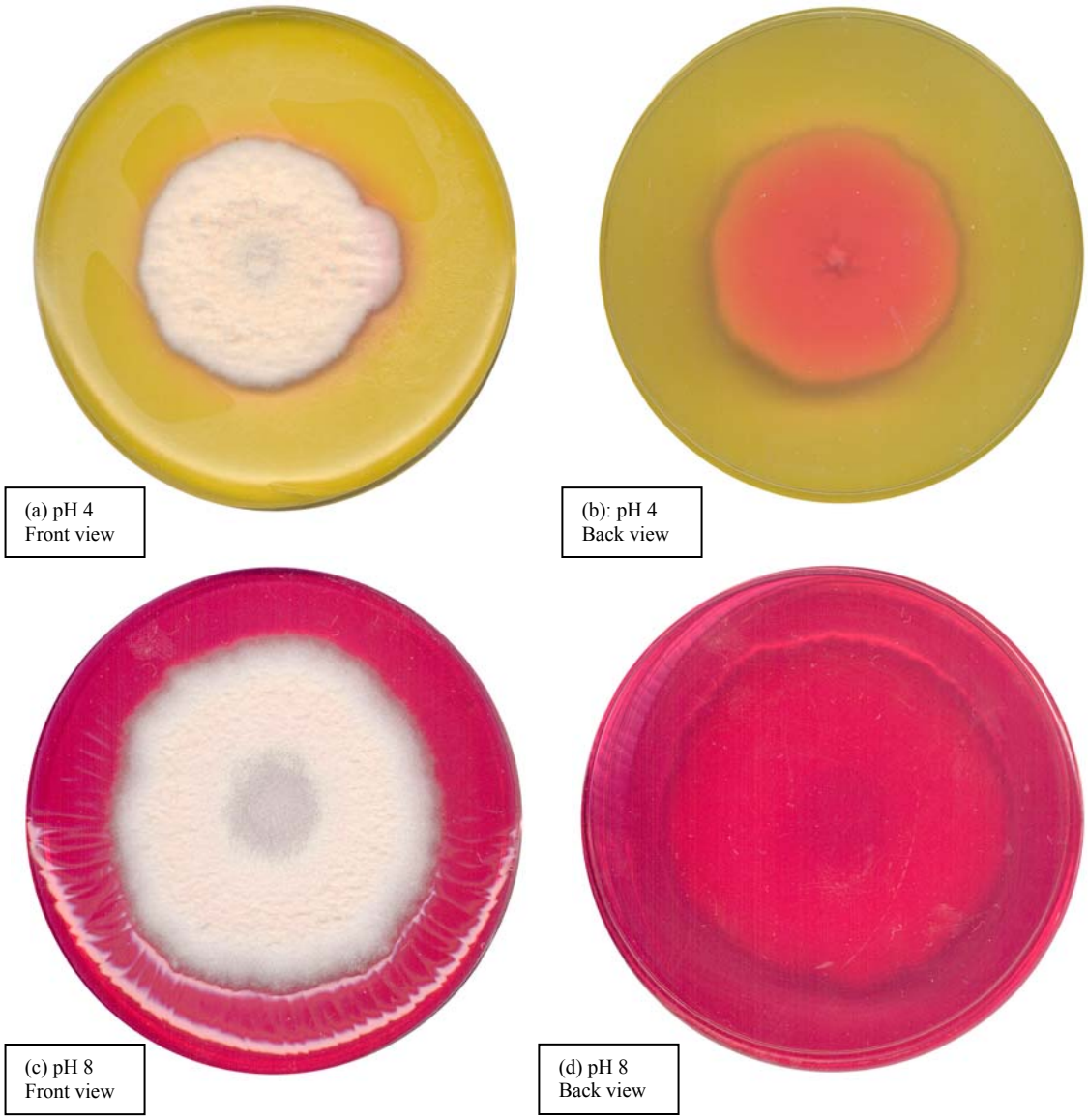


Figure 12.9a-d: Growth of *M. anisopliae* (MA 2038) on 1% yeast extract, 0.5% glucose, 1% gelatin, 0.005% phenol red (Yellow colour pH < 6 and red colour pH > 8) and 1.5% agar (YGGPA) adjusted at pH 4 (a, b) and 8 (c, d), respectively.

irregular and undulated colonies at pH*i* 4 (Fig. 12.10a, b). However, at pH*i* 8 the colonies forms had irregular margin and slightly convex shape with lobated margins (Fig. 12.10c, d).

Mycelial density profiles (as determined by densitometry and represented in pixels) obtained at pH*i* 4 and 8 for GK 2016 (Appendix 1; Fig. 1.0 and 1.1, respectively), MA 2038 (Appendix 1; Fig. 1.2 and 1.3, respectively) and AN 189 (Appendix 1; Fig. 1.4 and 1.5, respectively) were different. Significant differences existed between the biomass densities profiles obtained for the same strain at two selected pH*i*'s. The reverse side images of the fungal plates provided quantitative differences of the growth of the fungi inside the agar. This may represent the ability of the fungi to utilize the available nutrients at different rate, which in turn end up at different agar depths. Furthermore, the conidia production and colony diameter or radial extension of the two entomopathogens at the selected pH*i*'s were significantly different as appreciated from Table 12.1. On the other hand, no difference was observed for the radial extension rate for AN 189 grown at pH 4 and 8.

12.4.3 Proteolytic enzymes (Gelatinase) and secretion of organic acids and base

It can also be appreciated from the Fig. 12.8b (back side) that the isolate GK 2016 has secreted base, presumably ammonia (red colouration), at pH*i* 4 around the colony to modify the pH of the surroundings. This modification in pH helps release/activity of protease. Similarly, during growth at pH*i* 8 (Fig. 12.8d) the central portion of the colony (back side) have red colour indicative of pH > 8 where as colony margins have yellow colour qualitatively showing pH < 6, and hence the secretion of organic acids can be considered to cause such an effect. The yellow colour was diffusing away from the colony margins into the periphery indicating that the pH of the rest of the periphery of the colony was < 8 (yellow colouration). The release/activity of

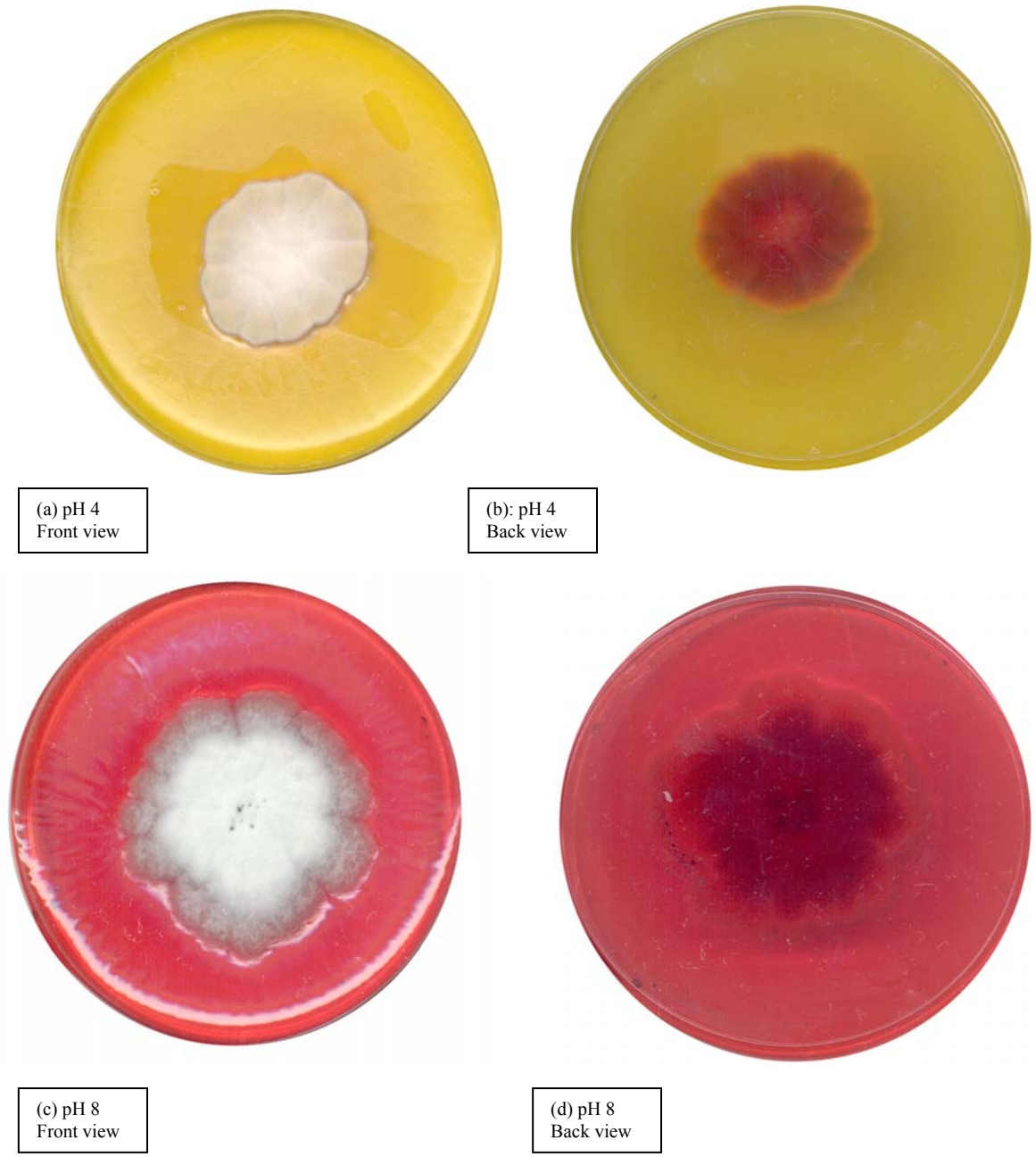


Figure 12.10a-d: Growth of *A. nidulans* (AN 189) on 1% yeast extract, 0.5% glucose, 1% gelatin 0.005% phenol red (Yellow colour pH < 6 and red colour pH > 8) and 1.5% agar (YGGPA) adjusted at pH 4 (a, b) and 8 (c, d), respectively.

Table 12.1 Radial extension rate, conidia count and protease activity for the fungi at two inducer pHs (pH*i*)

Isolates	pH*i* 4				pH*i* 8			
	Cd/ cm^2	Cd/ plate	PG	REX (cm)	Cd/ cm^2	Cd/ plate	PG	REX (cm)
GK 2016	0.01	0.24	0.23a	2.38c	2.8	10.67	0.48b	3.47c
MA 2038	0.47	2.03	0.20a	4.27a	1.26	7.35	0.6a	5.82a
AN 189	0.03	0.098	0b	3.1b	0.01	0.068	0c	4.95b

Legend:
: Cd/ cm^2 and Cd/ plate: Conidia production/cm^2 and per colony, respectively X 10^7.
: REX: radial extension rate (cm).
PG: proteolytic activity was measured on 1% gelatin agar plates.

protease (Gelatinase) was also significantly different at pH*i* 4 and 8 (Table 12.1). However, isolate Ma 2038 grown at pH*i* 4 (Fig. 12.9b) had red color at the base of colony which was indicative of pH > 8. Secretion of metabolites was not evident on pH*i* 8 plates (Fig. 12.9d). In addition, the protease secreted at pH*i* 4 was significantly lower than pH*i* 8 (Table 12.1).
In case of isolate AN 189 (saprophyte), no visible colour change was evident on either plates grown at pH*i* 4 (Fig. 12.10b) or 8 (Fig. 12.10d). Besides, for AN 189 no protease was detected on either plate grown at pH*i* 4 and 8, respectively. This may indicate the difference in the time of release or it could be linked to the non-permissive pH*i*, which resulted in the loss of the activity of the secreted protease.

These results suggest the different ability of the two groups, EPF versus saprophytic fungi to modify the ambient pH. In general, it appears that the entomopathogens are well adapted to modify the pH and release/activity of protease than the saprophyte, *A. nidulans*.

12.5 Discussion

pH plays a vital role in growth and metabolism of fungi. It can affect growth rates and growth limits in conidial fungi (Wolf and Wolf, 1947; Cochrane, 1958; Rosenberg, 1975), conidia formation (Hamsa and Ayres, 1977; Carels and Shepherd, 1978), germination (Cochrane, 1958), pigment production (Wolf and Wolf, 1947; Hamsa and Ayres, 1977; Carels and Shepherd, 1978) inhibition by antimycotics (Holmquist et al., 1983) and toxin formation (Joffe and Lisker 1969; Reddy et al., 1971; Holmquist et al., 1983).

Despite of the significance of pH in the growth and development of fungi, few investigators have attempted to study the role of pH on the physiology and growth of EPF. The effect of pH on the toxin production was studied in *B. bassiana* (Goral and Lappa, 1971; Sharma

et al., 1992). Shimazu and Sato (1996) studied the effect of media pH on the growth of *B. bassiana*. Similarly, Galani (1988) studied the influence of pH of the medium on biomass production in *B. bassiana, V. lecanii* and *P. farinosus.* On the other hand, Hallsworth and Magan (1996) have showed that culture age, pH and temperature affect the polyol and trehalose content of the fungal propagules.

Three pH*i* were selected to study the growth of the representative isolates of *B. bassiana*. The growth rate/ day for all the isolates were tested on the inducer pH*i*'s. The speed of growth and average colony size of *B. bassiana* isolates was dependent on two factors: (i) pH*i* of the medium, and (ii) time of incubation. This implies that *B. bassiana* during growth tend to modify the pH of the microenvironment optimal for the growth and enzyme synthesis/activity. In doing so, EPF secreted metabolites (acid or base depending on the pH*i*) for optimal growth conditions. Hence, the average colony size was different at the pH*i*'s tested. Furthermore, pH gradient plates also showed that the pH can affect the growth of the *B. bassiana* isolates.

In addition, colony morphology, conidiation/sporulation and radial extension rate were tested on pH*i* 4 and 8, which encompass the pH found in many insect guts (Bignell, 1984). It is evident from the visual inspection of the colonies and mycelial density profiles that the pH can affect all the aforementioned characteristics. Similarly, Lichter and Mills (1998) have found that pH of the medium can effect the pigmentation and hyphal growth in phytopathogen, *Ustilago hordie.* On the other hand, Ramon et al. (1999) have reported that change in pH of the growth medium can affect dimorphism in human fungal pathogen *C. albicans*. In addition, they indicated that the ability to respond to ambient pH is critical to the growth and virulence of the *C. albicans.*

The variability in the colony morphology and radial extension/growth rate reflects the difference in the translocation of the nutrients under the influence of pH. The role of pH in the acquisition of the nutrients by the fungal hyphae is well documented phenomenon (Jennings, 1989; Olsson, 2000; Griffin, 1994). In addition, pH also affects the cell permeability and this effect is particularly important for compounds that ionize. At low pH cell membrane becomes saturated with hydrogen ions so that passage of essential cations is limited, on the other hand at higher pH it become saturated with hydroxyl ions and limits the passage of essential anions (Moore-Landecker, 1996). Significant difference was observed between the pH*i* of the growth medium (evident from colour change of pH indicator) during growth of EPF, which suggests a difference in metabolic activity due to C/ N sources. In a similar context, Griffin (1994) reported that a difference of 0.3 units and 1.0 pH unit means two fold and 10 fold alteration in the hydrogen ion concentration of the liquid medium for fungal growth. It was evident that the change in pH is an important part of fungal growth because the transport of amino acids and ions, both cations and anions depends upon pH of the media.

It was also evident from data that EPF can regulate the ambient pH by releasing ammonia and organic acid(s). In case of phytopathogenic fungi, ambient pH regulation by releasing organic acid and ammonia is well documented phenomenon (Prusky and Yakoby, 2003). Amongst EPF, Bidochka and Khachatourians (1991) have reported that the organic acids, especially citric and oxalic acids could aid in solubilization of the cuticular proteins. St. Leger et al. (1998) showed that the ambient pH in *M. anisopliae* can regulate expression of pathogenicity determinant genes. In a subsequent study, they reported that *M. anisopliae* mycelia regulate the pH and protease activity by secreting ammonia (St. Leger et al. 1999). Similarly in *B. bassiana* Khachatourians et al. (2007) and in chapter 6 and 7, it has been shown that conidia and mycelia

can regulate the ambient there by releasing ammonia and citrate for the optimal release/activity of extracellular enzymes.

Taken together these results suggest that pH has an important role in the growth and development of EPF. Indeed the results suggest that EPF are better adapted to ambient pH regulation than the saprophyte, *A. nidulans*. This ability may have evolved in EPF to take advantage of host niche by releasing ammonia and organic acids that can solubilize cuticle, provide buffering action to the secreted enzymes and help circumvent the host immune response.

13.0 GENERAL DISCUSSION

An extensive body of literature has been accumulated over the years of research on the physiology, biochemistry, molecular biology of the entomopathogenic fungi (EPF). This has helped to provide the understanding of fungal pathogenesis at a mechanistic level. As a result, there are now recombinant DNA-based mycoinsecticides over-expressing protease and chitinase, respectively (St. Leger et al., 1996b; Fan et al., 2007). However, the environmental concerns associated with the release of these mycoinsecticides and their wider adoption or acceptance by the public needs to be addressed. Despite the research spanning over decades, there are still outstanding critical issues limiting the use of EPF because of slow speed of kill, consistency of performance, formulation and cost of production (Cherry et al., 1999; Lomer et al., 2001; Shah and Pell, 2003). Collectively, these issues suggest that there are still gaps in scientific knowledge and societal adoption of the use of EPF, which require careful reconsideration, such as steps involved in fungal-pathogenesis and growth/development.

A wide variety of fungal attributes such as extracellular enzymes, organic acids, toxins and pigments are postulated to be the "virulence factors" encoded by pathogenicity determinant genes (Khachatourians, 1991). However, the precise role of each of these attributes in causing insect mortality is not clear. One of the possible reasons is that these virulence factors were studied in isolation and reductionistic fashion. A holistic integrative approach is badly needed in order to draw appropriate correlations between host insects and growth/development cycle of fungi.

In this thesis I wanted to provide a comprehensive study of conidia as they go through successive transformative stages to become infectious agents. These stages involve hydration,

swelling and germination, all physical changes, which are associated with release of cuticle-degrading enzymes (CDE). Recently, Qazi and Khachatourians (2007) have provided the first report on the discovery of multiple metalloproteases during hydration. Metabolic activity of conidia upon hydration and its particular condition(s) provided rich data. We can now explain the plasticity or flexibility of conidia in adapting to many environments. In addition to conidial performance without *de novo* synthesis of gene products, growing propagules of these fungi behave uniquely when starved for C/ N or placed in variable pHs. Finally, this thesis produced an innovative methodology for isozyme separation, quantification and zymography, which now verifies the presence of functional proteins, only deduced from genomics (EST) data two years earlier.

The involvement of extracellular enzymes in cuticle degradation dates back three decades. Ultrastructural studies showed that the cuticle dissolution occurs in the vicinity of conidia (Zacharuk, 1970), mucus (Brey et al., 1986) and germ tubes (Persson et al., 1984). Conidial enzymes have received little or no serious consideration despite their vital role in the initial phases of infection. Nonetheless, St. Leger et al. (1991) provided the first evidence of the conidial Pr1, NAGase and esterase from conidia of *M. anisopliae* during washing treatment of conidia with detergent, reducing agents and buffer treatments. More than decade later, Shah et al. (2005) reported the presence of Pr1 from *M. anisopliae* and showed that the enzyme levels and pathogenicity were influenced by the growth media. In this author's opinion, the significant contribution in lieu of conidial enzymes came from the work of Bidochka's group (Small and Bidochka, 2005) who reported for the first time that Pr1 up-regulation occurs at the appressoria formation and during conidiation. However, previous investigators were unable to relate and

describe the most critical issue, the functional role of these enzymes to the initiation events of infection or for that matter in overall fungal pathogenesis.

In this thesis, proteomic/functional proteomic, biochemical and physiological evidences were obtained to show that hydrated conidia of *B. bassiana* and *M. anisopliae* are metabolically active and could release: (i) several (4-8) isozymes of protease (Pr1, Pr2, acidic and MPr), (ii) organic acid and ammonical base to drive optimal enzyme functions, and (iii) proteins that may have important functions in growth, development and pathogenesis.

During the hydration or soaking period, metabolic activation of conidia was observed under extreme starvation of nutrients. The release of the multiple proteases was observed during swelling phase of conidia in water. The metabolic activation of conidia of *Neurospora crassa* was described by Schmit and Brody (1976). Data presented here rectify an erroneous piece of literature that has been around for 22 years. Dillon and Charnley (1985, 1991) reported their study of metabolic activation of conidia of EPF and used Electrozone/Celloscope particle counter (Model II LTS, Particle Data Inc., UK) to conclude that conidia of EPF do not undergo a swelling phase in de-ionized water but would do so if provided with exogenous nutrients. In contrast, the present results revealed the swelling of conidia of EPF, which was measured by the most sensitive 7th generation electronic particle counting sizing Multisizer III (Coulter Counter^{TM} ; Beckman Coulter, Inc., California, USA). There was conidial swelling which parallels microscopic examinations. In addition, uptake of radioisotopes by the water soaked conidia indicated that they were active in DNA, RNA and protein synthesis. Results also revealed that the swelling rate was affected by the type of water (type I, type II and tap) which reflected the difference in ionic composition of the water.

Results indicated that the release of protease isozymes from hydrated conidia is regulated by the ambient pH. Upon incubation of conidia in water, both EPF have released citrate and ammonia thereby creating changes in pH. In addition, washing of conidia in different buffers (pH*i* 4-10) affected the release/activity of Pr1 and Pr2. There are several reasons to explain the effects of pH on the enzyme release/activity such as change in stability, level of activities, pH inhibition and the interfering effects of buffer ions in addition to interference with the spore permeability.

Isozymes of protease should provide spores with an expanded potential to obtain precursor nutrients and initial modification of the cuticle even before germination. The ease with which the enzymes were removed by incubation in water suggests that proteases are associated with the surface of spores. It is probable that these enzymes could be released in moist environmental conditions. It was found that the functional feature for some of the isoforms had long term (4 days) stability as revealed by 1-DE native gels combined with quantitative 2-D spot densitometry.

During separation of isozymes by using proteomics techniques it was observed that there is a need to either improve the existing technologies or to devise new tools for the biochemical analysis of isozymes for complete and better separation. Therefore, two new techniques i.e., 2-DE zymography and EOM were developed. These tools revealed a more economical analysis of protease isozymes from complex biological samples with sensitivity and in less time. The combination of CHAPS with carrier ampholytes have revealed the presence of clearer bands on IEF and after second dimension not revealed by the use of carrier ampholytes alone. Enzyme preparation using carrier ampholytes showed one and three isozymes with samples of *B. bassiana* and *M. anisopliae*, respectively, whereas four and eight isozymes appeared on the gel

when carrier ampholytes in combination with CHAPS was used. Further, 2-DE zymopheretogram of protease released from *B. bassiana* with copolymerized gelatin indicated one isozyme of Mr 70 kDa (pI 6.3). Conversely, 2-DE zymography with copolymerized gelatin for *M. anisopliae* showed six protease activities of pI 3.7-9 (Mr 115-129 kDa). These results show that the use of detergent for solubilization of biological sample is crucial for complete separation of proteins. A CHAPS is non-denaturing and hence can preserve the activity of the enzymes (Hjelmeland, 1980). In addition, detergents are ideal for breaking hydrophobic interactions, which can play an important role in the structural integrity of proteins and protein/protein interactions, and which are very much essential for the analytical isoelectric focusing (Rabilloud et al., 2007). Furthermore, during non-reducing 2-DE zymography, the extraction of proteinaceous material (spots) from parallel gel (lacking substrate) was performed after Park et al. (2002). The results of mass spectrometry confirmed elastase (serine protease)-like activities previously identified from *M. anisopliae* (Shimizu and Yazawa, 2001; unpublished sequence, accession numbers, AB073327.1). The technique described could be used to identify other enzymes by using different substrates, by combining with immunoblotting or for peptide sequence analysis by mass spectrometry.

The EOM analysis revealed three isoforms of Pr1 from *M. anisopliae* (pI 8.1-9.7) and *B. bassiana* (pI 8.4-9.7), respectively. Previous findings have been incremental and often failed to provide a general “big picture”. St. Leger et al. (1994) reported four isoforms of Pr1 (pI range 9.3-10.2) from growing mycelia of *M. anisopliae* and subsequently reported seven bands (pI range 6.8-9.6) of Pr1 from *Verticilium lecanii* strain 1335 (St. Leger et al., 1997). In a similar study on *Verticillium albo-atrum* and *Verticillium dahliae* low activity levels of neutral (pI 7) and basic (pI 9.5) subtilisin-like proteases were identified by EOM. In contrast, only one activity

band of Pr2 was identified from *M. anisopliae* and no activity was observed from *B. bassiana*. St. Leger et al. (1994) have found Pr2 (two major isoforms pI 4.4, 4.9 and two minor isoforms pI 5.2) from the growing mycelia of *M. anisopliae*. They further provided ultrastructural evidence that two isoforms (pI 4.4, 4.9) were secreted by appressoria and on the penetrating hyphae when grown the cockroach cuticle (St. Leger et al., 1996a). In addition, St. Leger et al. (1997) found high levels of Pr2-like activities (pI 4.5 and > 10) among PF, *V. albo-atrum* and *V. dahliae* with plant cell walls as inducers. Use of EOM in combination of metalloprotease inhibitors revealed that conidia from both fungal species had MPr released during tween treatment of conidia. Four isoforms (pI 4.4, 5.5, 6.5-7.0, 7.5) of thermolysin like MPr were identified from *M. anisopliae* and one from *B. bassiana* (pI of 5.5), which were insensitive to 1-10 phenanthroline (except pI 7.5) like *Penicillium* spp. proteases (Gripon et al., 1980). In addition, one isozyme of zinc-neutral MPr from *M. anisopliae* (pI 6.1) and *B. bassiana* (pI 6.5-7.6), respectively was observed for the first time. Only one isoform (pI 7.3) of thermolysin-like metalloprotease was reported from *M. anisopliae* mycelia grown in basal medium containing cockroach cuticle (St. Leger et al., 1994).

Apart from cuticle-degrading enzymes, several proteins were identified by using 2-DE and mass spectrometry. The release of these proteins during mild detergent wash revealed that they could be release in moist environmental conditions. Similarly, proteomic analysis of the tween wash has shown the presence of *ROD1,* which has been shown to be involved in the detoxification of xenobiotics in *S. cerevisiae*. Furthermore, extracellular enzymes like glucanses, elastase (serine protease) and lipase were identified that have several vital biological functions from growth, to cell wall remodeling and degradation of insect cuticle (substrate). Interestingly, galectin 7 was identified which is an animal lectin, and may have a role in the attachment of conidia to substrate/insect cuticle. The thesis therefore has provided the beginning of the

inventory of proteome of conidia. These results suggest that conidia are endowed with the variety of proteins and enzymes that may have diverse functions during fungal metabolism/pathogenesis.

The multiple proteases identified from hydrated conidia under starvation conditions warranted further investigation on their regulation during synthesis on insect cuticle under the supply of C/ N and ambient pH. Previously, cuticle-degrading enzyme synthesis from growing mycelia was shown to be regulated by the C/ N (Bidochka and Khachatourians, 1988a) and ambient pH (St. Leger et al., 1998). Much of the work has been done with the appressorial or mycelial stages, and therefore do not account for pre-germination events, that is, from spores to the pre-appressorial stage (St. Leger et al., 1986a; Bidochka and Khachatourians, 1987).

The regulation of the CDE from germinating conidia of *B. bassiana* show high activity of both Pr1 and Pr2 suggesting that exogenous carbon and nitrogen sources are essential for post-swelling stages of growth. Carbon catabolite repression (CCR) was not observed for either of Pr1 and Pr2 at 0.25 h when supplemented with glucose. Conversely, nitrogen metabolite repression (NMR) was observed for Pr2 and not for Pr1. Hence, the Pr1 and Pr2 are regulated differently by C/ N sources. Similar results were reported by St. Leger et al. (1988) for exponentially growing mycelia of *M. anisopliae* in minimal media after 72 h of growth. Pr2 synthesis in ExM supplemented with nitrogen (-C+N) and NAG (-CN+NAG) indicated that the repression occurs in spite of the presence of cuticle. Similarly, St. Leger et al. (1988) found that synthesis of Pr1 and Pr2 in exponentially growing mycelia of *M. anisopliae* was repressed from the 72 h upon supplementation with sucrose (1%), alanine (1%), *N*-acetyl-D-glucosamine (1%) or NH_4Cl (0.2%) in minimal media containing cuticle or cellulose.

During mycelial growth, CCR was observed at 24 h for Pr1 in ExM supplemented with both carbon and nitrogen sources (+CN), but not for Pr2. CCR was overruled by the lack of preferred nitrogen source for both Pr1 and Pr2 due to the addition of carbon source (+C-N) into ExM medium. Addition of preferred nitrogen source to the medium in absence of added carbon (-C+N) repressed the synthesis of Pr1 and Pr2, indicating NMR to be operative. In addition, Pr2 synthesis was inhibited by the addition of NAG with in one hour. In similar vein, Bidochka and Khachatourians (1988b) provided evidence that synthesis of serine protease (Pr1-like) in *B. bassiana* was repressed in gelatin medium containing GlcNAc at levels of > 1.07 μmol^{-1} mg of fungal dry $weight^{-1}$.

Ambient pH regulatory studies with conidia demonstrated that the buffered cuticle at selected pH*i* provided conditions for significantly high, but varying levels of expression of the CDE from both EPF at all pH*i* (pH 4-10) tested. In addition, expression of the CDE was evident in buffers without cuticle under de-repression conditions. High CDE activities in the buffered cuticle containing media corroborated previous findings that their synthesis by mycelia of EPF is induced by the degradation products of cuticle (St. Leger, 1995; St. Leger et al., 1998). In similar a context, Paterson et al. (1994a) showed that the locust cuticle enhanced production of mycelial Pr1 by *M. anisopliae* to a level approximately 10-fold that of the derepressed mycelium.

Contextual to ambient pH, results revealed a very complicated picture for the regulation of Pr1 and Pr2. The regulation of the production of both these enzymes was influenced mainly by two factors: (i) cuticle, and (ii) pH of the media. The relationship between enzyme activities and mycelial dry weight help elucidate the levels of Pr1, Pr2 and NAGase synthesis whether it is due to induction or increase in fungal dry weight. The significant increase in the Pr1 and Pr2 activities (in comparison to other pH*i* supplemented with cuticle) per mg dry weight at pH*i* 10

showed the influence of alkaline pH in the induction of both enzymes for *B. bassiana.* Similarly, significant increase in the Pr1 and Pr2 activities for *M. anisopliae* at pH*i* 10 and 6, respectively, indicated the role of induction to be due to pH rather than increase in fungal biomass. St. Leger et al. (1998) have reported the pH optima of *M. anisopliae* Pr1 and Pr2 as 8 and 6-8, respectively. In addition, St. Leger et al. (1986c) also reported the optimum pH for Pr1 activity on locust cuticle to be 9. In similar vein, a subtilisin-type serine protease (*pepC*) produced by *Aspergillus niger* is expressed equally high levels at pH 3 and 8, while the expression of aspartyl protease genes (*pepA* and *pepB*) is completely turned off under alkaline conditions (Jarai and Buxton 1993). Production of NAGase at alkaline pH*i* from both EPF could be relevant in terms of coordinated Pr1 and Pr2 release under alkaline conditions at infection sites. High levels of Pr1 and Pr2 production at pH*i* 10 by the two EPF will cause the degradation of proteinaceous components of cuticle that may induce high NAGase activity. These results are in agreement with those obtained for *M. anisopliae* (St. Leger, 1993). The significant role of cuticular proteins is in determining the pH optima for Pr1 and Pr2, as during infection they could equally raise the pH (St. Leger et al., 1986c; St. Leger et al., 1998).

This thesis delineated conidia specific activities. As a result apart from conidial enzymes, investigations were conducted on the enzymes synthesized by the growing mycelia of *B. bassiana*. Results showed that *B. bassiana* secretes ammonia and regulates the protease activity at three selected pH*i* (4, 7, and 11). Ammonia production in culture supernatant at tested pH*i* increase the pH of the medium. In the absence of cuticle ammonia was produced by growing mycelia of *B. bassiana* at pH*i* 7 and 11, which suggests its production under C/ N derepression conditions. Similarly, St. Leger et al. (1999) reported the release of ammonia under C/ N derepression conditions at pH*i* 6.5 for *A. nidulans* and *N. crassa*, but not for the *M. anisopliae*.

The ammonia production was significantly higher when 2% chitin was added to the buffers at all three pH*i* tested, implying that the ammonia formation during growth on proteinaceous substrates as sole carbon source results in dissimilation of amino nitrogen produced in excess of that required for growth (Jennings, 1989). Incubation of mycelial inocula in presence of amino acids show two different catabolic routes that *B. bassiana* can utilize to metabolize amino nitrogen. Higher levels of ammonia were produced when the basal medium was supplemented with glutamate. The break down of one molecule of glutamate could liberate two ammonia molecules (Griffin, 1994). Furthermore, a higher level of ammonia, evident at pH*i* 7, is indicative of the role of pH in the formation of ammonia, because of the stability of the excess amino nitrogen during amino acid catabolism (Jennings, 1989). In contrast, St. Leger et al. (1999) have reported that increasing the abundance of amino acids (100 mM) decreased the formation of ammonia, suggesting that in nature, continual formation of ammonia requires release of low levels of amino acids from a protein source. These results may reflect the unique specificity for the inducer pH (6.5) used by the investigators.

Interestingly, ammonia production was completely inhibited when basal salts medium was supplemented with glucose and methionine at all the pH*i*'s. St. Leger et al. (1999) has previously shown that 100 mM of methionine inhibit ammonia synthesis in *M. anisopliae* through induction of catabolite repressible enzyme(s), presumably deaminases, or regulation of the enzyme activity via substrate inhibition. Furthermore, methionine could supply amino nitrogen for the growing fungus thereby limiting its need to devour cuticle for the supply of amino nitrogen. This observation corroborates with repression of protease activity after the supplementation of methionine. Regulation of amino acid oxidase in *N. crassa* is inducible by amino acids and would release ammonia upon induction (Sikora and Marzluf, 1982). Similarly

higher concentrations of glucose and amino acid have repressed the ammonia formation in *M. anisopliae, A. fumigatus and N. crassa* (St. Leger et al., 1999). This inhibition can be due to added glucose, which would have switched metabolism from catabolism of amino acids to that of sugars for the biosynthesis and freeing unused ammonia.

Regulation of protease activity due to ammonia formation revealed that gelatinase-like protease activities (PC and PG) are regulated either by food cues or ambient pH. PC activity was higher with the addition of glucose and glutamate implying that the fungus was growing at its maximum capacity with both C and N sources, respectively, and requiring more nitrogen to complement the fast growth rate. In the case of PG release/activity was optimum at pH*i* 7 and 11, even in the presence of preferred nitrogenous sources. This suggests that PG is under the influence of pH regulatory element, presumably *pacC* rather then carbon and nitrogen regulatory elements (i.e., *areA* and *creA*). Methionine has repressed the PG and PC release/activity implying that there might be some regulatory role of sulfur metabolism gene regulatory element, which is poorly described in EPF. Glutamate at pH*i* 7 has resulted in the repression of PG presumably due to high ammonia formation. At high concentration it becomes toxic as it raises intracellular pH, thus inhibiting the protein synthesis and enzyme activity (Doyle and Butler 1990).

pH plays a vital role in growth and metabolism of fungi (Penalva and Arst, 2002). In order to study the role of pH in the growth and development of *B. bassiana*, three pH*i* were selected to study the growth of the representative isolates. The growth rate/ day for all the isolates tested revealed that the average colony size of *B. bassiana* isolates was dependent upon two factors: (i) pH*i* of the medium, and (ii) time of incubation. This implies that *B. bassisna* during growth tend to modify the pH of the microenvironment thereby secreting metabolites

(acid or base depend on the pH*i*) for optimal growth conditions. Hence, the average colony size on pH gradient plates as well as on different pH*i*'s tested was different.

In addition, colony morphology, conidiation/sporulation and radial extension rate were measured on pH*i* 4 and 8. These results indicated that the ambient pH has a significant effect on the above mentioned characteristics. Similarly, Lichter and Mills (1998) have found that pH of the medium can effect the pigmentation and hyphal growth in phytopathogen, *Ustilago hordie.* Conversely, Ramon et al. (1999) have showed that change in pH of the growth medium can affect dimorphism in *Candida albicans*. They also reported that the ability to respond to ambient pH is critical to its growth and virulence.

The variability in the colony morphology and radial extension/growth rate reflects the difference in the translocation of the nutrients under the influence of pH as previously documented for fungal hyphae (Martell and Calvin, 1952; Jennings, 1989; Olsson, 2000; Griffin, 1994). In addition, pH also affects the cell permeability and this effect is particularly important for compounds that ionize. It is known that at low pH cell membrane becomes saturated with hydrogen ions so that passage of essential cations is limited. On the other hand, at higher pH, it becomes saturated with hydroxyl ions and limits the passage of essential anions (Moore-Landecker, 1996). As evident from colour change of pH indicator, a significant difference was observed between the pH*i* of the growth medium during growth of EPF, which imply a difference in metabolic activity due to C/ N sources. In a similar context, Griffin (1994) reported that a difference of 0.3 units and 1.0 pH unit means two fold and 10 fold alteration in the hydrogen ion concentration of the liquid medium for fungal growth.

It was also evident from data that EPF conidia and mycelia can regulate the ambient pH by releasing ammonia and organic acid(s). In the case of phytopathogenic fungi, ambient pH

regulation by releasing organic acid and ammonia is a well documented phenomenon (Bateman and Beer, 1965; Godoy et al., 1990; Cunningham and Kuiack, 1992). Amongst EPF, Bidochka and Khachatourians (1991) have reported that the organic acids (citric and oxalic acids) could aid in solubilization of the cuticular proteins when compared with inorganic acids. St. Leger et al. (1998) showed that the ambient pH in *M. anisopliae* can regulate expression of pathogenicity determinant genes. In a subsequent study they reported that *M. anisopliae* mycelia regulate the pH and protease activity by secreting ammonia (St. Leger et al., 1999).

Taken together, these results suggest that pH has an important role in the synthesis/release of extracellular enzymes, growth and development of the two EPF. Results show novel observations regarding conidial pH adaptations and release of multiple proteases. These results suggest that ambient pH regulation for the synthesis/releases of proteases is higher in hierarchy than C/ N regulation. It also suggests that there is an involvement of complex gene regulatory network (*areA*, *creA* and *pacC*) in EPF, which has previously been shown to control C, N and pH in filamentous fungi such as *A. nidulans*. Furthermore, the different propagules (conidia, mycelia, etc.) because of their different nutritional requirements respond differently to the nutrient signals or ambient pH. Ironically, this phenomenon has not been previously investigated in EPF, however, homology of *areA* and *creA* have been identified from *M. anisopliae* (Screen et al., 1997, 1998).

It is tempting to speculate that conidial proteases (Pr1, Pr2 and MPr) are different from mycelial enzymes, which have been shown to be regulated in a different fashion. In a similar context, a very important contribution is that of White and Agabian (1995). They reported that the synthesis of aspartyl protease isozymes from *Candida albicans*. They further showed that the pattern of isozyme(s) secretion was determined by the cell type (W and O cells) of strain WO-1,

whereas the levels of isozymes released were determined by pH of the inducing medium. Contextual to EPF, Cho et al. (2006a) have provided the first evidence of stage specific gene expression in *B. bassiana.* Their EST analysis of cDNA libraries from aerial conidia, blastospores and submerged conidia has 20-30% unique sequences to each library. They found unique EST sequences to conidia as subtilisin-like protease, tripeptidyl-peptidase and a thermophlic serine protease. In a subsequent study, Cho et al. (2006b) showed a comparative analysis of EST from aerial conidia with growing mycelia on chitin and oosporein producing media. They reported eight subtilisins from *B. bassiana* with varying level of similarity to *M. anisopliae* enzymes (PR1A, PR1B, PR1C, PR1D, PR1G, PR1H, PR1J, PR1K), four of which (conidia_11-D01.e, conidia_22-C04.e, conidia_08-G10.e and conidia_12-H11.e) were unique to conidia. Collectively, these results raise a crucial question and warrant further investigations on the genes and proteins up-regulated during the development cycle of these EPF. The diversity of protease isozymes identified here may also be similar to other unidentified up-regulated protease genes during conidiation.

Results show that hydrated conidia are endowed with multiple proteases and proteins. It is probable that release of same occurs under natural humid conditions or during formulations. In addition, data reveals that citrate and ammonia released from hydrated conidia can provide buffering conditions for proteases isozymes. In a similar vein, Herbert Arst (personal communication) has recently observed the conidial pH adaptations on their plate assay medium. It is likely that EPF have evolved multiple genes to produce isoforms of protease and other protein for the subversion of the host immune response. In such an event, immediate release of conidial isozymes (Pr1, Pr2 and MPr) in the vicinity of insect integument provides an additive effect for the removal of cuticle monomers. It would be expected that most of the hydrophilic

(Arg-Y or Lys-Y) residues present in the cuticular proteins are attacked by isozymes of Pr2 where as hydrophobic residues (Phe-Y or Leu-Y) are degraded by Pr1. Conversely, MPr can help evasion of unique peptide bonds of protein moieties in the cuticle/epicuticle.

Indeed, the results suggest that conidia are endowed with isoforms of protease, which implies that they are pre-adapted for pathogenic mode of life. The release of multiple proteases from the conidia upon hydration implies their involvement in cuticle dissolution. It also suggests that theses isozymes may have some affinity to different insect taxa due to difference in the chemical composition of the cuticle. It could be possible that their role is to function in different climatic conditions. Moreover, results also show that the EPF are well adapted to environmental cues such as nutrients (C/ N) and ambient pH. This ability may have evolved in EPF to take advantage of host niche by devouring cuticle and acquiring nutrients, which may create gradients of pH. To counter balance such gradients EPF secretes metabolites to create pH to secretion permissive level for optimal function of these enzymes. The relative importance of these environmental cues for the regulation of CDE depends upon the nutritional status of the fungus. It is due to this reason conidia after hydration regulate the release of proteases under the influence of ambient pH and high expression activity was evident. Furthermore, supply of C-source to germinating conidia would not repress the synthesis of Pr1 and Pr2. In contrast, a supply of N-source would repress Pr2, but not Pr1. On the other hand, mycelia respond to the nutrients or pH in a different way, where CCR was observed for Pr1, but not for Pr2. These results clearly show that C/ N repression depends on three different factors: (i) enzyme in question, (ii) kind of nutrient, and (iii) infective structure of the fungi (conidia, appressoria, mycelia, etc). Therefore, repression occurs when it is necessary for the growing fungus to establish nutritional relationship with the host tissues/structures. The relevance of nutrient and

ambient pH during the growth of EPF is a very complex and important question to address. However, one fact which has become clear from this series of investigations is that the regulation of enzymes from conidia and mycelia are regulated in a different fashion. It has been shown for the first time that hydrated conidia have the potential to modify the cuticle under the influence of pH by using multiple proteases before appressoria or gem tube formation. This strategy of the pathogen likely evolved to maximize the chances of establishing a successful infection that can circumvent the protease inhibitors present in the insect integument and/or haemolymph.

14.0 CONCLUSIONS

Several conclusions have been reached during the undertaking of this thesis work regarding conidial hydration of *B. bassiana* and *M. anisopliae* and the role that ambient pH plays. A summary of conclusions is presented in the cartoon model of EPF infection cycle (Fig 14.1-14.3). In addition, these are listed below:

1. Hydrated conidia are endowed with the isoforms of protease, which were active up to day 4 and have the potential cuticle-degrading ability.
2. Metabolic activation and swelling of conidia of the two EPF was observed during incubation in water.
3. During the swelling phase of conidia or nutritionally starved conditions, conidia of two EPF secreted metabolites for the optimal function of proteases.
4. Washing of conidia in various buffers showed that pH can affect the enzymes activity or release from conidia.
5. pH regulation is higher in hierarchy than C/ N regulation for the release of enzymes during the initial events of germination.
6. C/ N regulation occur only when it is necessary for the infective structure of the fungi to establish nutritional status with host tissues/structures.
7. Conidia and mycelia regulate the synthesis of protease and chitinase under the influence of C/ N in a different fashion.
8. Pr1 and Pr2 have different regulatory mechanisms.
9. Growing mycelia of *B. bassiana* regulate the activity/release of protease (Gelatinase-like) by producing ammonia.

14.1 General model of the infection cycle of EPF

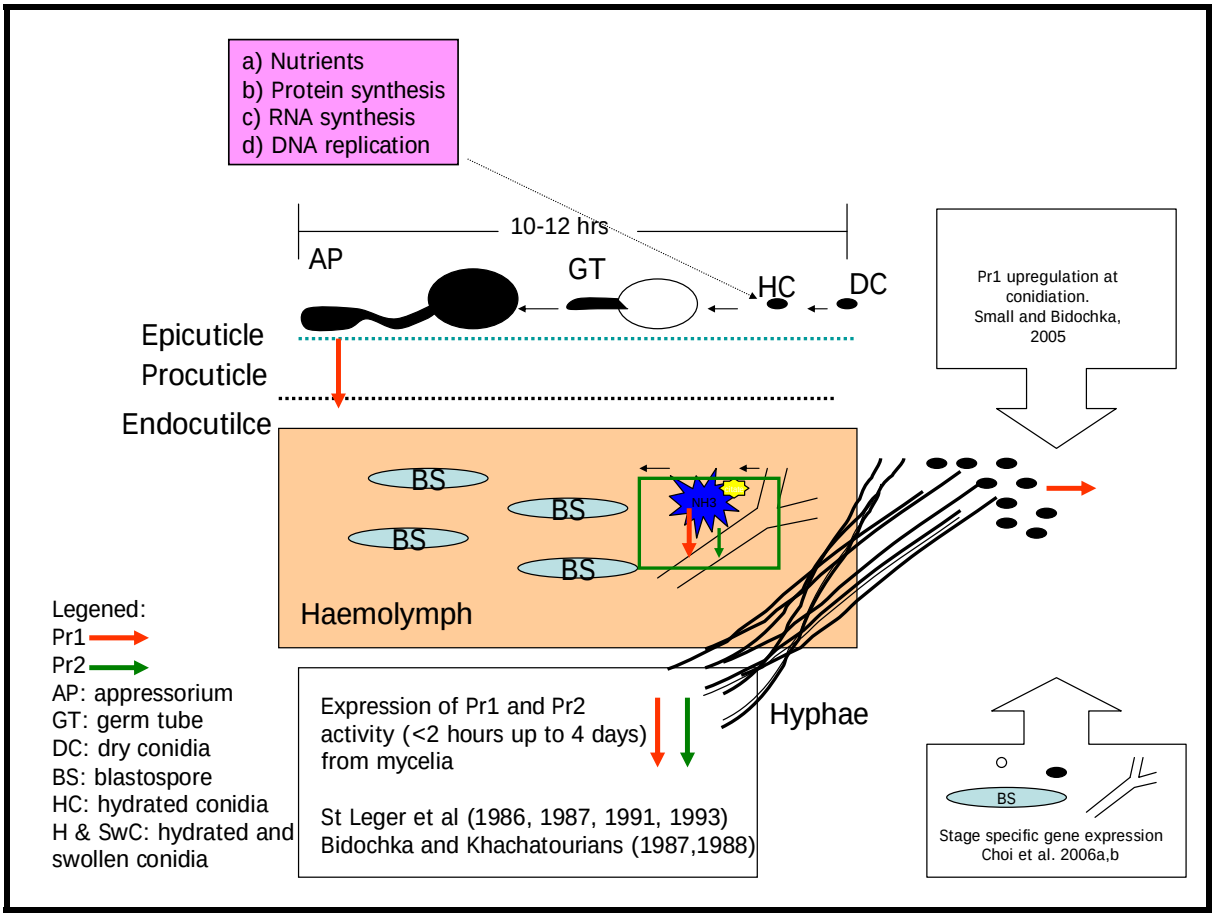


Figure 14.1: Current cartoon model of the infection cycle of EPF. The model shows the different growth stages of the fungus along with their involvement in fungal pathogenesis. In this model, grey block arrows show new findings revealing the significance of the stage specific gene expression of proteases.

14.2 Current cartoon model of the infection cycle of EPF

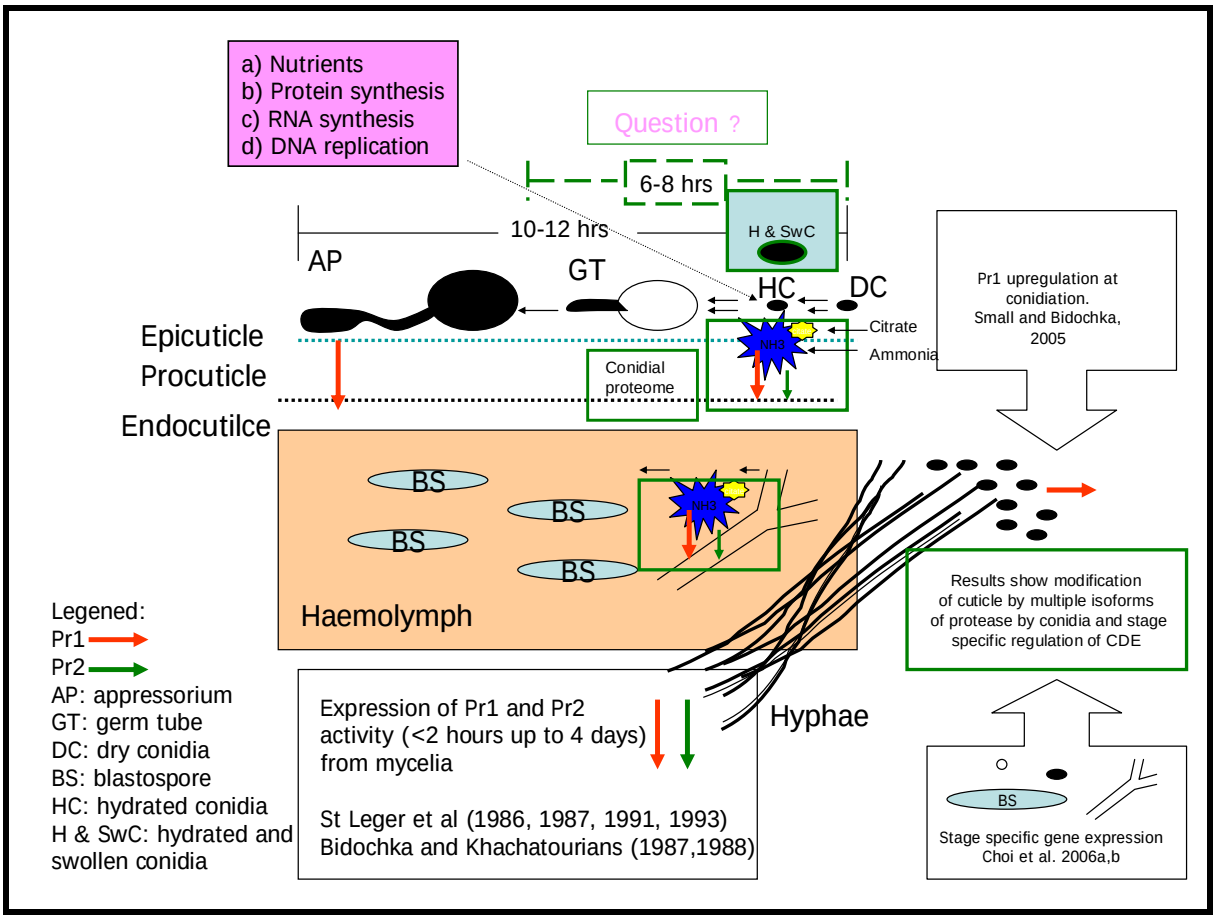


Figure 14.2: Current cartoon model of the infection cycle of EPF, which is based on the results of the thesis. The model shows that each morphogenetic phase of the fungus (conidia, appressoria and mycelia) has an important and different role in the fungal pathogenesis. In this model, the green rectangles/shapes represent the thesis findings and grey block arrows show the results from other investigators that supports the current work.

14.3 Proposed model of the mechanistic action of multiple proteases

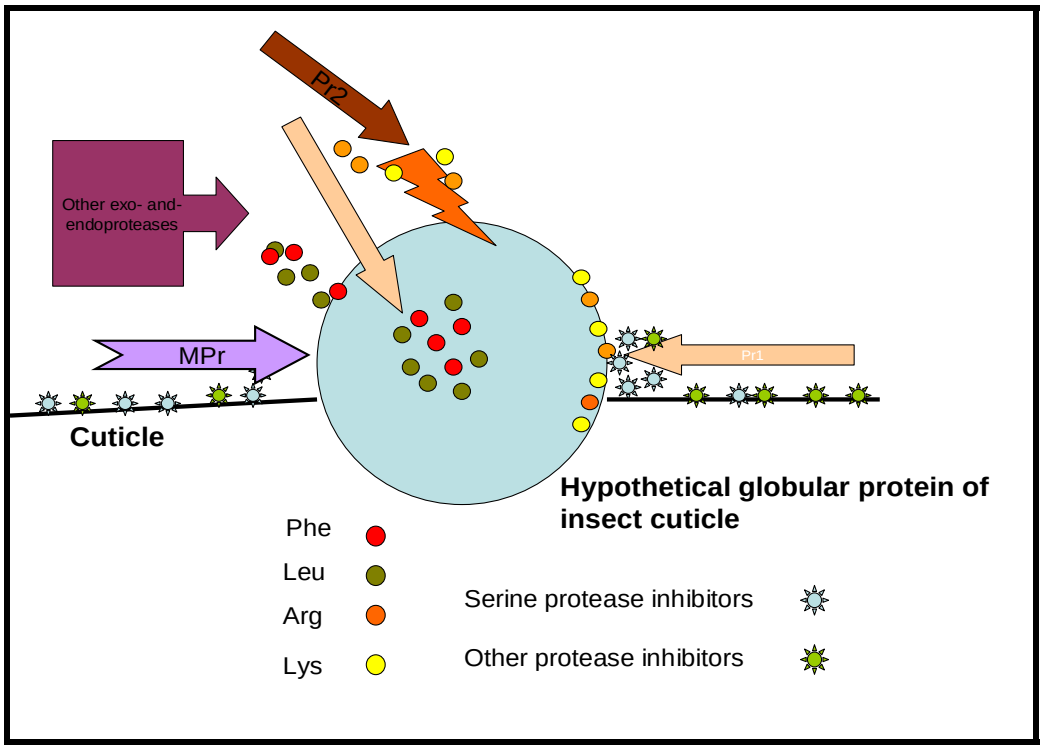


Figure 14.3: Proposed model of the mechanistic action of multiple proteases associated with conidia. The model shows the synergestic action of Pr1, Pr2 and MPr that can complement each other in the modification/degradation of the cuticle. The action of multiple proteases can help avoid protease inhibitor present in the cuticle and hemolymph. Furthermore, it can act on the hydrophobic and hyrdophylic peptides of the insect integument in an efficient manner.

10. Ammonia production by the *B. bassiana* could be considered as a virulence factor that can destroy insect tissues and or host immune response.
11. Proteomic technologies for the biochemical analysis of isozymes require improvements. The use of detergent in combination with ampholytes is useful for the complete separation of protease isozymes from the biological samples.
12. 2-DE zymography with copolymerized gelatin is more sensitive than overlay indictor gel zymograms. 2-DE zymography can be combined with mass spectrometry for protein/peptide identification.
13. Newly devised EOM technique for biochemical analysis of isozyme is cost effective and sensitive.
14. It is the first report that conidia of EPF can start degradation of the cuticle prior to the formation of appressoria or germ tube by multiple proteases (Pr1, Pr2, MPr and acidic) and not by Pr1 alone as previously believed.
15. Release of multiple protease during the initial events of infection process could avoid protease inhibitors present in the cuticle and or haemolymph
16. Presence of multiple protease isozymes could reflect that the ability of EPF to utilize different substrates or to infect insects belonging to different taxa.
17. Diversity of isozymes may offer selective advantage to EPF in exploring new habitats (substrates) either as pathogen or saprophyte.
18. pH can affect the colony morphology, conidia production and radial extension rate of the growing fungus.

15.0 Future directions

Molecular and biochemical studies of EPF are increasing our understanding of the mechanisms by which these pathogens invade and kill their insect. Ironically, last three decades of the research on EPF molecular biology has focused on increasing the speed of kill by using recombinant DNA-based technologies. In the author's opinion, an equally important, yet neglected aspect of research is to work in the context of physiology and ecology of EPF. In this vein, I have provided the first comprehensive study of conidia of EPF as they progress through germination phase. The results of this thesis have raised three important questions to be pursued. Firstly, a novel observation regarding ambient pH modulation during the swelling phase of conidia along with the release of multiple proteases reveals that ambient pH regulation occurs during pre-germination phase. Secondly, the regulation of the synthesis/production of CDE by the growing fungus depends on ambient pH, C and N sources as well as the growth stage of the fungal propagule. Lastly but not the least, conidia secrete a variety of other proteins in type II water that may have important functions for EPF. The metabolic activation of conidia thereby secreting metabolites and proteins in type II water raise many questions for the formulation and application research and development in EPF. In this context, further studies delineating the role of conidia during formulation in different solvents would be helpful in their use as mycoinsecticides.

Author strongly feels that this study has raised some critical aspects of research for those who do the actual research and others who just read about it in scientific literature. It is further hoped, that collaborative research from scientific community in the fields of genomics, proteomics and metabolomics will provide better insights in the ambient pH regulation of the EPF in order to improve their design and use.

16.0 LITERATURE CITED

Abbot, R.J. and Marzluf, G.A. 1984. Major extracellular protease of *Neurospora crassa*. J. Bacteriol. 159: 505-510.

Ai-Ling, Wu. A-L., Hallstrom, T.C. and Moye-Rowley W.S. 1996. *ROD1*, a novel gene conferring multiple resistance phenotypes in *Saccharomyces cerevisiae.* J. Biol. Chem. 271: 2914-2920.

Ait-Lahsen, H., Soler, A., Rey, M., De La Cruz, J., Monte, E. and Llobell, A. 2001. An antifungal exo-ά-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum.* Appl. Environ. Microbiol. 67: 5833–5839.

Alverson, J. 2003. Effects of mycotoxins, kojic acid and oxalic acid on biological fitness of *Lygus hesperus* (Heteroptera: Miridae). J. Invertebr. Pathol. 83: 60-62.

Anderson, S.O. 1980. Cuticle sclerotization. In: Miller, T.A. (Ed), Cuticle techniques in Arthropods. New York, Springer-Verlag. pp. 185-217.

Anonymous. 1977. IUPAC-IUB Commission on Biochemical Nomenclature, Nomenclature of multiple forms of enzymes: recommendations 1976. J. Biol. Chem. 252: 5939

Arst, H.N.Jr. 1995. Nitrogen metabolite repression in *Aspergillus nidulans*: An historical perspective. Can. J. Bot. (Suppl. I): S148-S152.

Arst, H.N.Jr. and Cove, D.J. 1969. Methylammonium resistance in *Aspergillus nidulans*. J. Bacteriol. 98: 1284-1296.

Arst, H.N.Jr. and Penalva, M.A. 2003. pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends Genet. 19: 224-231.

Asaff, A., Cerda-Garcia-Rojas, C. and de la Torre, M. 2005. Isolation of dipicolinic acid as an insecticidal toxin from *Paecilomyces fumosoroseus*. Appl. Microbiol. Biotechnol. 68: 542-547.

Bagga, S., Hu, G., Screen, S.E. and St. Leger, R.J., 2004. Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae.* Gene 324: 159-169.

Bailey, C. and Arst, H.N.Jr. 1975. Carbon catabolite repression in *Aspergillus nidulans* Eur. J. Biochem. 51: 573-577.

Bainbridge, B.W. 1971. Macromolecular composition and nuclear division during spore germination in *Aspergillus nidulans.* J. Gen. Microbiol. 66: 319-325.

Baratto, C.M., da Silva, M.V., Santi, L., Passaglia, L., Schrank, I.S., Vainstein, M.H. and Schrank, A. 2003. Expression and characterization of the 42 kDa chitinase of the biocontrol fungus *Metarhizium anisopliae* in *Escherichia coli*. Can. J. Microbiol. 49:723-726.

Barnes, M. and Parker, M.S. 1966. The increase in size of mould spores during germination. Trans. Brit. Mycol. Soc. 49: 487-494.

Barnes, M. and Parker, M.S. 1967. Use of the Coulter Counter to measure osmotic effects on the swelling of mould spores during germination. J. Gen. Microbiol. 49: 287-292.

Barnes, M. and Parker, M.S. 1968. Automatic sizing of mold spores. Trans. Brit. Mycol. Soc. 51: 33-39.

Barondes, S., Cooper, D., Gitt, M. and Leffler, H. 1994. Galectins. Structure and function of a large family of animal lectins. J. Biol. Chem. 269: 20807-20810.

Bateman, D.F. and Beer, S.V. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. J. Phytopathology. 58: 204-211.

Beynon, R.J. and Salvesen, G. 2001. Commercially available protease inhibitors. In: Beynon, R.J. and Bond, J.S. (Eds.), Proteolytic enzymes: A practical approach. Oxford University Press, New York, Oxford, pp. 317-331.

Bhagwat, A.S. and Mahadevan, P.R. 1970. Conserved mRNA from the conidia of *Neurospora crassa*. Mol. Gen. Genet. 109: 142-151.

Bidochka, M.J and Khachatourians, G.G. 1988b. *N*-acetyl-D-glucosamine-mediated regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. Appl. Environ. Microbiol. 54: 2699-2704.

Bidochka, M.J. and Khachatourians, G.G. 1987. Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana.* Appl. Environ. Microbiol. 53: 1679-1684.

Bidochka, M.J. and Khachatourians, G.G. 1988a. Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. Exp. Mycol. 12: 161-168.

Bidochka, M.J. and Khachatourians, G.G. 1991. The implication of metabolic acids produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper, *Melanoplus sanguinipes.* J. Invert. Pathol. 58: 106-117.

Bidochka, M.J. and Khachatourians, G.G. 1992. Growth of the entomopathogenic fungus *Beauveria bassiana* on cuticular components from the migratory grasshopper *Melanoplus sanguinipes.* J. Invertbr. Pathol. 59: 165-173.

Bidochka, M.J. and Khachatourians, G.G. 1993. Regulation of extracellular *N*-acetyl-D-glucosaminidase production in the entomopathogenic fungus *Beauveria bassiana.* Can. J. Microbiol. 39: 6-12.

Bidochka, M.J. and Khachatourians, G.G. 1994. Basic proteases of entomopathogenic fungi differ in their adsorption properties to insect cuticle. J. Invertebr. Pathol. 64: 26-32.

Bidochka, M.J. and Melzer, M.J. 2000. Genetic polymorphisms in three subtilisin-like protease isoforms (Pr1A, Pr1B, and Pr1C) from *Metarhizium* strains. Can. J. Microbiol. 46: 1138-1144.

Bidochka, M.J., Pfeifer, T.A. and Khachatourians, G.G. 1987. Development of the entomopathogenic fungus *Beauveria bassiana* in liquid cultures Mycopathologia 99: 77-83.

Bidochka, M.J., St. Leger, R.J., Stuart, A. and Gowanlock, K. 1999. Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and plant virulence, extracellular proteases and carbohydrases. Microbiology 145: 955-963.

Bidochka, M.J., Tong, K.I. and Khachatourians, G.G. 1993. Purification and properties of two extracellular *N*-acetyl-D-glucosaminidases produced by the entomopathogenic fungus *Beauveria bassiana.* Can. J. Microbiol. 39: 40-45.

Bieth, J., Spiess, B., and Wermuth, C.G. 1974. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem. Med. 11: 350.

Bignell, D.E. 1984. Direct potentiometric determination of redox potentials of the gut contents in the termites *Zootermopsis nevadensis* and *Cubitermes severus* and in three other arthropods. J. Insect Physiol. 30: 169–174.

Bignell, E., Negrete-Urtasun, S., Calcagno, A.M., Haynes, K., Arst, H.N.Jr. and Rogers, T. 2005. The *Aspergillus* pH-responsive transcription factor PacC regulates virulence. Mol. Microbiol. 55: 1072-1084.

Bing, L.A. and Lewis, L.C. 1991. Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. Environ. Entomol. 20: 1207-1211.

Bjellqvist, B., Sanchez, J.C., Pasquali, C., Ravier, F., Paquet, N., Frutiger, S., Hughes, G.J. and Hochstrasser, D.F. 1993. Micropreprative two-dimensional electrophoresis allowing the separation of samples containing milligram amounts of proteins. Electrophoresis 14: 1375-1378.

Bonnen, A. and Brambl, R. 1983. Germination physiology of *Neurospora crassa* conidia. Exp. Mycol. 7: 197-207.

Borgstrom, B. and Brockman, H. L. 1984. Lipases. Elsevier, Amsterdam.

Borooah, J., Leaback, D.H. and Walker, P.G. 1961. Studies on glucosaminidase. 2 substrates for *N*-acetyl-β-glucosaminidase. Biochem. J. 78: 106-110.

Boucias, D.G. and Pendland, J.C. 1991. Attachment of mycopathogens to cuticle: The initial event of mycoses in arthropod hosts. In: Cole, G.T. and Hoch, H.C. (Eds.), The fungal spore and disease initiation in plants and animals. Plenum press, New York, pp.101-124.

Boucias, D.G. and Pendland, J.C. 1993. Variability in the ability of galactose and mannose specific lectins to bind to cell wall surfaces during growth of the insect-pathogenic fungus *Paecilomyces farinosus.* Eur. J. Cell Biol. 60: 322-330.

Boucias, D.G., Pendland, J.C. and Latge, J.P. 1988. Nonspecific factors involved in attachment of entomopathogenic Deuteromycetes to host insect cuticle. Appl. Environ. Microbiol. 54: 1795-1805.

Bowen, D., Rocheleau, T., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R. and ffrench-Constant, R. H. 1998. Cloning of a novel insecticidal toxin from the bacteria *Photorhabdus luminescens.* Science 280: 2129-2132.

Bowers, Jr.G.N., McComb, R.B., Christensen, R.G.Jr. and Schaffer, R. 1980. High-Purity 4-Nitrophenol: Purification, characterization, and specifications for use as a spectrophotometric reference material. Clin. Chem. 26: 724-729.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brambl, R., Plesofsky-Vig, N., Hammett, J.R. and Russell, P.L. 1987. Preserved polyadenylated ribonucleic acid in dormant conidia of *Neurospora crassa* and new RNA synthesis during spore germination. Exp. Mycol. 11: 317–330.

Brey, P.T., Latge, J.P. and Prevost, M.C. 1986. Integumental penetration of the pea aphid, *Acyrthosiphon pisum,* by *Conidiobolous obscurus.* J. Invertebr. Pathol. 48: 34-41.

Brodie, H.J. and Neufeld, C.C. 1942. The development and structure of conidia of *Erysipe polygoni* DC and their germination at low humidity. Can. J. Res. 20: 41-61.

Bundy, H.F. 1962. A new spectrophotometric method for the determination of chymotrypsin activity. Anal. Biochem. 3: 431-435.

Butt, T.M. 2002. Use of entomogenous fungi for the control of insect pests In: Esser, K. and Bennett, J.W. (Eds.) Mycota. Springer, Berlin, pp. 111–134.

Butt, T.M., Segers, R., Leal, S.C., and Kerry, B.R. 1998. Variation in the subtilisins of fungal pathogens of insects and nematodes. In: Bridge, P., Couteaudier, Y. and Clarkson, J. (Eds.), Molecular variability of fungal pathogens. CABI-International, pp. 149-169.

Caddick, M.X., Brownlee, A.G. and Arst, H.N.Jr. 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans.* Mol. Gen. Genet. 203: 346-353.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi L. and Righetti, P.G. 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25: 1327-1333.

Carels, M. and Shepherd, D. 1978. The effect of pH and amino acids on conidiation and pigment production of *Monascus major* ATCC 16362 in submerged shaken culture. Can. J. Microbiol. 24: 1346-1357.

Chaney, A.L. and Marbach, E.P. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8: 130-132.

Charnley, A.K. 1997. Entomopathogenic fungi and their role in pest control. In: Wicklow, D. and Soderstrom, M. (Eds.), The Mycota IV, Environmental and microbial relationships. Springer-Verlag, Heidelberg, Germany, pp 185-201.

Charnley, A.K., St. Leger, R.J., 1991. The role of cuticle-degrading enzymes in fungal pathogenesis in insects. In: Cole, G.T. and Hoch, H.C. (Eds.), The fungal spore and disease initiation in plants and animals. Plenum press, New York, pp.267-286.

Chelico, L., Haughian, J.L. and Khachatourians, G.G. 2006. Nucleotide excision repair and photoreactivation in the entomopathogenic fungi *Beauveria bassiana, Beauveria brongniartii, Beauveria nivea, Metarhizium anisopliae, Paecilomyces farinosus* and *Verticillium lecanii.* J. Appl. Microbiol. 100: 964-972.

Chelico, L., Haughian, J.L., Woytowich, A.E. and Khachatourians, G.G. 2005. Quantification of ultraviolet-C irradiation induced cyclobutane pyrimidine dimers and their removal in *Beauveria bassiana* conidiospore DNA. Mycologia 97: 621-627.

Cherry, A.J., Jenkins, N.E., Heviefo, G., Bateman, R. and Lomer, C.J. 1999. Operational and economic analysis of a West African pilot-scale production plant for aerial conidia of *Metarhizium* spp. for use as a mycoinsecticide against locusts and grasshoppers. Biocont. Sci. Technol. 9: 35-51.

Chet, I. 1987. *Trichoderma*- applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi, In: Chet, I. (Ed), Innovative approaches to plant diseases. John Wiley and Sons, NewYork, pp. 137-160.

Chevallet, M., Santoni, V., Poinas, A., Rouquié, D., Fuchs, A., Kieffer, S., Rossignol, M., Lunardi, J., Garin, J. and Rabilloud, T. 1998. New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis. 19:1901-1909.

Cho, E.M., Liu, L., Farmerie, W. and Keyhani, N.O. 2006a. EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria* (Cordyceps) *bassiana.* I. Evidence for stage-specific gene expression in aerial conidia, in vitro blastospores and submerged conidia. Microbiology 152: 2843-2854.

Cho, E-M., Boucias, D. and Keyhani, N.O. 2006b. EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria* (Cordyceps) *bassiana*. II. Fungal cells sporulating on chitin and producing oosporein. Microbiology 152: 2855-2864.

Choi, N.S., Kim, S.H. 1999. Application of fibrin zymography for determining the optimum culture time for protease activity. Biotechnol. Lett. 13: 899-901.

Choi, N.S., Yoo, K.H., Yoon, K.S., Maeng, P.J. and Kim, S.H., 2004. Nano-scale proteomics approach using two-dimensional fibrin zymography combined with fluorescent SYPRO Ruby Dye. J. Biochem. Mol. Biol. 37: 298-303.

Christopher, H., Evans, C.H. and Ridella, J.D. 1985. Inhibition, by lanthanides, of neutral proteinases secreted by human, rheumatoid synovium. Eur. J. Biochem. 151: 29-32.

Chung, H.S. and Wilcoxson, R.D. 1969. Effect of conidial number and matrix on germination of conidia in *Phoma medicagins.* Phytopathology 59: 440-442.

Chung, J. 1998. Characterization of mutanase produced by *Microbispora rosea*. J. Dent. Res. 77: 1322.

Clarkson, J.M. and Charnley, A.K. 1996. New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol. 4: 197-203.

Cochrane, V.W. 1958. Physiology of fungi. New York: Wiley, pp. 19-29, 55-61, 403-404.

Cohen, B.L. 1973. Regulation of intracellular and extracellular neutral and alkaline proteases in *Aspergillus nidulans.* J. Gen. Microbiol. 79: 311-320.

Cole, G.T. and Hoch, H.C. (Eds.) 1991. The fungal spore and disease initiation in plants and animals. Plenum Press : New York and London.

Cooper, D.N.W., Boulianne, R.P., Charlton, S., Farrell, E.M., Sucher, A. and Lu, B.C. 1997. Fungal galectins, sequence and specificity of two isolectins from *Coprinus cinereus.* J. Biol. Chem. 272: 1514-1521.

Cooper, W,G. and Jordon, R.T. 1963. The effect of time in culture and antibody on the mean cell volume, size distribution and generation time of *in vitro* populations of an AKR lymphoma cell. Presented at the Tissue Culture Association Fourteenth Annual Meeting, Boston.

Cunningham, J.E. and Kuiack, C. 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl. Environ. Microbiol. 58: 1451-1458.

d' Enfert, C. 1997. Fungal spore germination: Insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa.* Fungal Gen. Biol. 21: 163-172.

David, W.A.L. 1967. The physiology of the insect integument in relation to the invasion of pathogens. In: Beament, S.W.L. and Treherne (Eds.), Insects and physiology, Edinburg. Oliver and Boyd, pp. 17-35.

De Bernardis, F., Muhlschlegel, F.A., Cassone, A. and Fonzi, W.A. 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. 66: 3317-3325.

Del Mar, E.G., Largman, C., Brodrick, J.W. and Geokas, M.C. 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem. 99: 316-320.

Del Mar, E.G., Largman, C., Brodrick, J.W., Fassett, M. and Geokas, M.C. 1980. Substrate specificity of human pancreatic elastase 2. Biochemistry 19: 468-472.

Dillon, R.J. and Charnley, A.K. 1985. A technique for accelerating and synchronizing germination of conidia of the entomopathogenic fungus *Metarhizium anisopliae*. Arch. Microbiol. 142: 204-206.

Dillon, R.J. and Charnley, A.K. 1990. Initiation of germination in conidia of the entomopathogenic fungus *Metarhizium anisopliae*. Mycol. Res. 94: 299-304.

Dillon, R.J. and Charnley, A.K. 1991.The fate of fungal spore in the insect gut. In: Cole, G.T. and Hoch, H.C. (Eds.), The fungal spore and disease initiation in plants and animals. Plenum press, New York, pp.129-156.

Doyle, C. and Butler, M. 1990. The effect of pH on the toxicity of ammonia to a murine hybridoma. J. Biotechnol. 15: 91-100.

Dreyfus, L.A. and Iglewski, B.H. 1986. Purification and characterization of an extracellular protease from *Aspergillus flavus.* Infect. Immun. 51: 736-743.

El-Sayed, G.N., Ignoffo, C.M., Leathers, T.D. and Gupta, S.C. 1992. Use of a colorimetric system to detect enzymes expressed by germinating conidia of entomopathogenic fungi. Mycopathologia 118: 29-36.

El-Sayed, G.N., Ignoffo, C.M., Leathers, T.D. and Gupta, S.C. 1993a. Cuticular and non-cuticular substrate influence on expression of cuticle-degrading enzymes from conidia of an entomopathogenic fungus *Nomurea rileyi.* Mycopathologia 122: 79-87.

El-Sayed, G.N., Ignoffo, C.M., Leathers, T.D. and Gupta, S.C. 1993b. Insect cuticle and yeast extract effects on germination growth and production of hydrolytic enzymes of *Nomurea rileyi*. Mycopathologia 122: 143-147.

Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., Li, D., Jin, K., Bidochka, M.J. and Pei, Y. 2007. Increased insect virulence in *Beauveria bassiana* overexpressing an engineered chitinase. Appl. Environ. Microbiol. 73: 295-302.

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., Feng, J., Yang, X., Zhang, Y. and Pei, Y. 2005. Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. Appl. Environ. Microbiol. 71: 363-370.

Fang, W.G., Zhang, Y.J., Yang, X.Y., Wang, Z.K. and Pei, Y. 2002. Cloning and characterization of cuticle-degrading enzyme CDEP-1 from *Beauveria bassiana* (Chinese) Yi Chuan Xue Bao 29: 278-282.

Fargues, J. Smits, N., Vidal, C., Vey, A., Vega, F., Mercadier, G. and Quimby, P. 2001. Effect of liquid culture media on morphology, growth, propagule production and pathogenic activity of the Hyphomycete, *Metarhizium flavoviride.* Mycopathologia 154: 127-138.

Fargues, J., Delmas, J.C. and Lebrun, R.A. 1994. Leaf consumption by larvae of the Colorado potato beetle (Coleoptera : Chrysomelidae) infected with the entomopathogen, *Beauveria bassiana*. J. Econ. Entomol. 87: 67-71.

Feng, M.G., Poprawski, T.J. and Khachatourians, G.G. 1994. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control. Biocont. Sci. Technol. 4: 3-34.

Ferron, P. 1985. Fungal control. In: Kerkut, G.A. and Gilbert, L.I. (Eds.), Comprehensive insect physiology, biochemistry and pharmacology. Vol. 4, Pergamon Press, Oxford, pp. 314-346.

Freimoser, F.M., Hu, G. and St. Leger, R.J. 2005. Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. Microbiology 151: 361-371.

Freimoser, F.M., Screen, S., Hu, G. and St. Leger, R. 2003. EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle. Microbiology 149: 1893-1900.

French, R.C. and Gallimore, M.D. 1972. Effect of pretreatment with stimulator and water vapors on subsequent germination and infectivity of uredospores of *Puccinia graminis.* Phytopathology. 62: 116-119.

Frobius, A.C., Kanost, M.R., Peter, G. and Vilcinskas, A. 2000. Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*. Eur. J. Biochem. 267: 2046-2053.

Fuglsang, C.C., Berka, R.M., Wahleithner, J.A., Kauppinen, S., Shuster, J.R., Rasmussen, G., Halkier, T., Dalboge, H. and Henrissat, B. 2000. Biochemical analysis of recombinant fungal mutanases. A new family of ά-1,3-glucanases with novel carbohydrate-binding domains. J. Biol. Chem. 275: 2009–2018.

Galani, G. 1988. Cultivation of some entomopathogenic fungi in liquid media with various initial pH values. Analel-Institutului-de-Cercetari- pentru-Protecta-Plantelor 21: 54.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotina sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37: 179-191.

Goettel, M., St. Leger, R.J., Rizzo, R.J., Staples, R.C. and Roberts, D.W. 1989. Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta)* cuticle. J. Gen. Microbiol. 135: 2233-2239.

Goguen, J.D., Hoe, N.P. and Subrahmanyam, Y.V. 1995. Proteases and bacterial virulence: a view from the trenches. Infect. Agents Dis. 4: 47-54.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S and Singh, R.M.M. 1966. Hydrogen ion buffers for biological research. Biochemistry 5: 467-477.

Goral, V.M. and Lappa, N.V. 1971. Toxins of the entomophagous fungus *Beauveria bassiana* II. Effect of nitrogen sources on formation of the toxic protease in submerged culture. J. Invertebr. Pathol. 17: 211–215.

Gorg, A. and Weiss, W. 2000. Two-dimensional electrophoresis with immobilized pH gradients. In: Rabilloud, T. (Ed), Proteome research: Two-dimensional electrophoresis and identification methods. Springer, Berlin, Heidelberg, New York, pp. 57-99.

Gorg, A., Postel, W. and Gunther, S. 1988. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9: 531-546.

Greenawalt, J.W., Beck, D.P. and Hawley, E.S. 1972. Chemical and biochemical changes in mitochondria during morphogenetic development of *Neurospora crassa.* In: Azzone, G.F., Carafoli, E., Lehninger, A.L., Quagliariello, E. and Siliprandi, W. (Eds.), Biochemistry and biophysics of mitochondrial membranes. Academic Press Inc., New York, p. 541-558.

Griffin, D.H. 1994. Chemical requirements for growth. In: Fungal physiology. Wiley-Liss, New York, United States of America. pp.130-158.

Gripon, J.C., Auberger, B. and Lenoir, J. 1980. Metalloproteases from *Penicillium caseicolum* and *Penicillium roqueforti*: Comparison of specificity and chemical characterization. Int. J. Biochem. 12: 451-455.

Grove, G. and Marzluf, G.A. 1981. Identification of the product of the major regulatory gene of the nitrogen control circuit of *Neurospora crassa* as a nuclear DNA-binding protein. J. Biol. Chem. 256: 463-470.

Gudmundsdottir, B.K., Hastings, T.S. and Ellis, A.E. 1990. Isolation of a new toxic protease from a strain of *Aeromonas salmonicida* subspecies *achromogenes.* Dis. aquat. Org. 9: 199-208.

Guerrini, V.H. 1997. Excretion of ammonia by *Lucilia cuprina* larvae suppresses immunity in sheep. Vet. Immunol. Immunopathol. 56: 311-317.

Hallsworth, J.E. and Magan, N. 1996. Culture age, temperature and pH after the polyol and trehalose contents of fungal propagules. Appl. Environ. Microbiol. 62: 2435-2442.

Hames, B.D. 1997. Gel electrophoresis of proteins: A practical approach. In: Hames, B.D., Rickwood, D. (Eds.), IRL Press, Oxford and Washington D.C., pp. 1-147.

Hamsa, T.A.P. and Ayres, J.E. 1977. A differential medium for the isolation of *Aspergillus flavus* from cotton seed. J. Food. Sci. 42: 449-453.

Hanson, M.A. and Marzluf, G.A. 1975. Control of the synthesis of a single enzyme by multiple regulatory circuits in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 72: 1240-1244.

Harold, R. L., and F. M. Harold. 1980. Oriented growth of *Blastocladiella emersonii* in gradients of ionophores and inhibitors. J. Bacteriol. 144: 1159-1167.

Hassan, A.E.M., Dillon, R.J. and Charnley, A.K. 1989. Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta*. J. Invertebr. Pathol. 54: 277-279.

Hastings, T.S., Ellis, A.E. 1985. Differences in the production of haemolytic and proteolytic activities by various isolates of *Aeromonas salmonicida*. In: Ellis, A.E. (Ed), Fish and shellfish pathology. Academic Press, London, pp. 69-77.

Haverback, B.J., Dyce, B., Bundy, H. and Edmonson, H.A. 1960. Trypsin, trypsinogen and trypsin inhibitor in human pancreatic juice. Am. J. Med. 29: 421-433.

Hawker, L.H. 1955. Hypogeous fungi. IV and V. Trans. Brit. Mycol. Soc. 38: 73-77.

Hegedus, D.D. and Khachatourians, G.G. 1988. Production of an extracellular lipase by *Beauveria bassiana*. Biotech. Lett. 10: 637-642.

Hegedus, D.D., Bidochka, M.J. and Khachatourians, G.G. 1990a. *Beauveria bassiana* submerged conidia production in a defined medium containing chitin, two hexosamines or glucose. Appl. Microbiol. Biotechnol. 33: 641-647.

Hegedus, D.D., Bidochka, M.J. and Khachatourians, G.G. 1990b. Chitin and chitin monomers for the production of *Beauveria bassiana* submerged conidia. Appl. Microbiol. Biotechnol. 30: 637-642.

Hegedus, D.D., Bidochka, M.J., Miranpuri, G.S. and Khachatourians, G.G. 1992. A comparison of the virulence, stability and cell-wall surface characteristics of three spore types produced by the entomopathogenic fungus *Beauveria bassiana*. Appl. Microbiol. Biotechnol. 36: 785-789.

Hirabayashi, J. and Kasai, K. 1993.The family of metazoan metal-independent ß-galactoside-binding lectins: structure, function and molecular evolution. Glycobiology 3: 297-304.

Hitchcock, S.E. 1967. RNA and protein synthesis in *Neurospora crassa* conidia. Neurospora Newsletter. 11: 15. (http://www.fgsc.net/fgn/nn11/11hitch.pdf).

Hjelmeland, L.M. 1980. A nondenaturing zwitterionic detergent for membrane biochemistry: Design and synthesis. Proc. Natl. Acad. Sci. USA. 77: 6368-6370.

Holmquist, G.U., Walker, H.W. and Stahr, H.M. 1983. Influence of temperature, pH, water activity and antifungal agents on growth of *Aspergillus flavus* and *Aspergillus parasiticus*. J. Food. Sci. 48: 778-782.

Hughes, R.C. 1994. Mac-2: A versatile galactose-binding protein of mammalian tissues. Glycobiology 4: 5-12

Inbar, J. and Chet, I. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii* : Purification, characterization and role in mycoparasitism. Microbiology 140: 651-657.

Irvin, R.T., Mac-Alister, T.J. and Costerton, J.W. 1981a. Tris (hydroxymethyl) aminomethane buffer modification of *Escherichia coli* outer membrane permeability. J. Bacteriol. 145: 1397-1403.

Irvin, R.T., Mac-Alister, T.J., Chan, R. and Costerton, J.W. 1981b. Citrate-Tris (hydroxymethyl) aminomethane-mediated release of outer membrane sections from the cell envelope of a deep-rough (heptose-deficient lipopolysaccharide) strain of *Escherichia coli* 08. J. Bacteriol. 145: 1386-1396.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M. and Misset, O. 1994. Bacterial lipases, FEMS Microbiol. Rev.15: 29-63.

James, A.M. 1991. Charge properties of microbial cell surfaces. In: Mozes, N., Handley, P.S., Busscher, H.J. and Rouxhet, P.G. (Eds.), Microbial cell surface analysis: Structural and physicochemical methods. VCH Publishers: New York, pp. 221-262.

James, L. and O' Shea, P. 1994. The electrostatic nature of the cell surface of *Candida albicans*: a role in adhesion. Exp. Mycol. 18: 111-120.

James, R.R., Buckner, J.S. and Freeman, T.P. 2003. Cuticular lipids and silverleaf whitefly stage affect conidial germination of *Beauveria bassiana* and *Paecilomyces fumosoroseus*. J. Invertebr. Pathol. 84: 67-74.

Jansson, H.B. 1993. Adhesion to nematodes of conidia from the nematophagous fungus *Drechmeria consiospora*. Gen. Microbiol. 139: 1899-1906.

Jarai, G. and Buxton, F. 1993. Nitrogen, carbon and pH regulation of extracellular acidic proteases of *Aspergillus niger*. Curr. Genet. 26: 238-244.

Jeffs, L.B., Xavier, I.J., Matai, R.E. and Khachatourians, G.G. 1999. Relationships between fungal spore morphologies and surface properties for entomopathogenic members of the genera *Beauveria, Metarhizium, Paecilomyces, Tolypocladium* and *Verticillium.* Can. J. Microbiol. 45: 936-948.

Jennings, D.H. 1989. Some perspectives on nitrogen and phosphorus metabolism in fungi. In: Boddy, L., Marchant, R. and Read, D.J. (Eds.), Nitrogen, phosphorus and sulphur utilization by fungi. Cambridge, UK: Cambridge University Press, pp. 1-31.

Jennings, D.H. 1995. The physiology of fungal nutrition. Cambridge University Press.

Jobin, M-C. and Grenier, D. 2003. Identification and characterization of four proteases produced by *Streptococcus suis*. FEMS Microbiol. Lett. 220: 113-119.

Joffe, A. Z. and Lisker, N. 1969. Effect of light, temperature, and pH value on aflatoxin production in vitro. Appl. Microbiol. 18: 517-518.

Jones, L., Hobden, C. and O' Shea, P. 1995. Use of a real-time fluorescent probe to study the electrostatic properties of the cell surface of *Candida albicans.* Mycol. Res. 99: 969-976.

Joshi, L., St. Leger, R.J. and Bidochka, M.J. 1995. Cloning of a cuticle-degrading protease from the entomopathogenic fungus, *Beauveria bassiana*. FEMS Microbiol. Lett. 125: 211-217.

Joshi, L., St. Leger, R.J. and Roberts, D.W. 1997. Isolation of a cDNA encoding a novel subtilisin-like protease (Pr1B) from the entomopathogenic fungus, *Metarhizium anisopliae* using differential display-RT-PCR. Gene 197: 1-8.

Kang, S.C., Park, S. and Lee, D.G. 1998. Isolation and characterization of a chitinase cDNA from the entomopathogenic fungus, *Metarhizium anisopliae*. FEMS Microbiol. Lett. 165: 267-271.

Kang, S.C., Park, S. and Lee, D.G. 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. J. Invertebr. Pathol. 73: 276-281.

Kenney, W.C. 1974. Molecular nature of isozymes. Horizons. Biochem. Biophysic. 1: 38.

Kerwin, J.L. 1984. Fatty acid regulation of the germination of *Erynia variabilis* conidia on adults and puparia of the lesser housefly, *Fannia canicularis.* Can. J. Microbiol. 38: 158-161.

Kerwin, J.L. and Washino, R.K. 1986. Oosporogenesis by *Lagenidium giganteum:* Induction and maturation are regulated by calcium and calmodulin. Can. J. Microbiol. 32: 663-672.

Khachatourians, G.G. 1986. Production and use of biological pest control agents. Trends Biotechnol. 4: 120-124.

Khachatourians, G.G. 1991. Physiology and genetics of entomopathogenic fungi. In: Arora, D.K., Ajello, L. and Mukerji, K.G. (Eds.), Handbook of applied mycology, Vol 2: Humans, animals, and insects. Marcel Dekker Inc, New York, pp. 613-661.

Khachatourians, G.G. 1996. Biochemistry and molecular biology of entomopathogenic fungi. In: Howard, D.H. and Miller, J.D. (Eds.), The Mycota VI: Human and animal relationships. Springer-Verlag, Berlin and Heidelberg, Germany, pp. 331-363.

Khachatourians, G.G. and Valencia, E. 1999. Integrated pest management and entomopathogenic fungal biotechnology in the Latin Americas II. Key Research and Development Prerequisites. Revista de la Academia Colombia de Ciencias Exactas Fisicas y Naturales 23: 489-496.

Khachatourians, G.G., Qazi, S.S. and Reid, W.R. 2007. Biochemistry and molecular biology of entomopathogenic fungi. In: Brakhage, A.A. and Zipfer, P.F. (Eds.), The Mycota VI: Human and animal relationships. Springer-Verlag Berlin & Heidelberg, Germany, pp. 1-42. (in press).

Kim, H-K., Hoe, H-S., Suh, D.S., Kang, S.C., Hwang, C. and Kwon, S-T. 1999. Gene structure and expression of the gene from *Beauveria bassiana* encoding bassiasin I, an insect cuticle-degrading serine protease. Biotechnol. Lett. 21: 777-783.

Kim, S.H., Choi, N.S., Lee, W.Y. 1998. Fibrin zymography: A direct analysis of fibrinolytic enzyme on gels. Anal. Biochem. 263: 115-116.

Kirkland, B.H., Eisa, A. and Keyhani, N.O. 2005. Oxalic acid as a fungal acaricidal virulence factor. J. Med. Entomol. 42: 346-351.

Klebl, F. and Tanner, W. 1989. Molecular cloning of a cell wall exo-β-1,3-glucanase from *Saccharomyces cerevisiae*. J. Bacteriol. 171: 6259-6264.

Kleiner, D.E. and Stetler-Stevenson, W.G. 1994. Quantitative zymography: Detection of picogram quantities of gelatinases. Anal. Biochem. 218: 325-329.

Klose, J. 1975. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissue. A novel approach to testing for induced point mutations in mammals. Humangenetik 26: 231-243.

Klose, J. 1999. Genotypes and phenotypes. Electrophoresis 20: 643–652.

Klotz, S.A., Drutz, D.J. and Zajic, J.E. 1985. Factors governing adherence of *Candida* species to plastic surfaces. Infect. Immun. 50: 97-101.

Kobayashi, T. Hakamada, Y. Hitomi, J., Koike, K. and Ito, S. 1996. Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. Appl. Microbiol. Biotechnol. 45: 63-71.

Kocholaty, W. and Weil, L. 1938. Enzymic adaptation in *Clostridium histolyticum*. Biochem. J. 32: 1696-1701.

Kothary, M.H. and Kreger, A.S. 1985. Production and partial characterization of an elastolytic protease of *Vibrio vulnificus.* Infect. Immun. 51: 736-743.

Kudoi, T. and Horikoshii, K. 1983. Effect of pH and sodium ion on germination of alkalophilic *Bacillus* Species. Agric. Biol. Chem. 47: 665-669.

Lane, B.S., Trinci, A.P. and Gillespie, A.T. 1991a. Endogenous reserves and survival of blastospores of *Beauveria bassiana* harvested from carbon and nitrogen limited batch cultures. Mycol. Res. 95: 821-828.

Lane, B.S., Trinci, A.P. and Gillespie, A.T. 1991b. Influence of cultural conditions on the virulence of conidia and of blastospores of *Beauveria bassiana* to the green leafhopper *Nephotettix virescens.* Mycol. Res. 95: 829-833.

Lantz, M.S. and Ciborowski, P. 1994. Zymographic techniques for detection and characterization of microbial proteases. Meth. Enzymol. 35: 563-593.

Lark, K.G. and Lark, C. 1960. Changes during the division cycle in bacterial cell wall synthesis, volume and ability to concentrate free amino-acids. Biochim. Biophys. Acta. 43: 520-530.

Latge, J, Monsigny, M. and Prevost, M. 1988. Visualization of exocellular lectins in the entomopathogenic fungus *Conidiobolus obscurus.* J. Histochem. Cytochem. 36: 1419-1424.

Le John, H.B. and Lovett, J.S. 1966. Ribonucleic acid and protein synthesis in *Rhizophlyctis rosea* zoospores. J. Bacteriol. 91: 709-717.

Lecuona, R., Riba, G., Cassier, P. and Clement, J.L. 1991. Alterations of insect epicuticular hydrocarbons during infection with *Beauveria bassiana* or *Beauveria brongniartii*. J. Invertebr. Pathol. 58: 10-18.

Lichter, A. and Mills, D. 1998. Control of pigmentation of *Ustilago hordei*: The effect of pH, thiamin and involvement of the cAMP cascade. Fungal Genet. Biol. 25: 63-74.

Lin, S.F. and Liu, S.D. 2004. The effect of four entomopathogenic fungi on aphid control in the greenhouse and enzymes synthesized by these fungi. Plant Protection Bulletin (Taipei). 46: 143-154.

Lomer, C.J., Bateman, R.P., Johnson, D.L., Langewald, J. and Thomas, M. 2001. Biological control of locusts and grasshoppers. Ann. Rev. Entomol. 46: 667-702.

Lord, J.C. and Howard, R.W. 2004. A proposed role for the cuticular fatty amides of *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) in preventing adhesion of entomopathogenic fungi with dry-conidia. Mycopathologia 158: 211-217.

Lord, J.C., Anderson, S. and Stanley, D.W. 2002. Eicosanoids mediate *Manduca sexta* cellular response to the fungal pathogen *Beauveria bassiana*: a role for the lipoxygenase pathway. Arch. Insect Biochem. Physiol. 51: 46-54.

Lorito, M., Hayes, C.K., di Pietro, A., Woo, S.L. and Harman, G.F. 1994. Purification, characterization, and synergistic activity of a glucan 1,3-β-glucosidase and an *N*-acetyl-β-glucosaminidase from *Trichoderma harzianum.* Phytopathology 84: 398–405.

Lotan, R., Belloni, P.N., Tressler, R.J., Lotan, D., Xu, X.C. and Nicolson, G.L. 1994. Expression of galectins on micro vessel endothelial cells and their involvement in tumor cell adhesion. Glycoconj. J. 11: 462–468.

Lovett, J.S. 1968. Reactivation of ribonucleic acid and protein synthesis during germination of *Blastocladiella* zoospores and the role of the ribosomal nuclear cap. J. Bacteriol. 96: 962-969.

Lyerly, D. and Kreger, A.S. 1979. Purification and characterization of a *Serratia marcescens* metalloprotease. Infect. Immun. 24: 411-421.

Macko, V. 1981. Inhibitors and stimulants of spore germination and infection structure formation in fungi. In: Turian, G. and Hohl, H.R. (Eds.), The fungal spore: Morphogenetic controls. New York, USA, Academic Press, pp. 565-585.

Manocha, M.S. and Sahai, A.S. 1993. Mechanisms of recognition in necrotrophic and biotrophic mycoparasites. Can. J. Microbiol. 39: 269-275.

Markert, C.L. and Moller, F. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. Proc. Natl. Acad. Sci. USA. 45: 753.

Marrier J.R. and Boulet, M. 1958. Direct determination of citric acid in milk with an improved, pyridine acetic anhydride method. J. Dairy Sci. 41: 1683-1687.

Martell, A.E. and Calvin, M. 1952. Chemistry of metal chelate compounds. New York, Prentics Hall, pp. 516-541.

Marzluf, G.A. 1977. Regulation of gene expression in fungi. In: Copeland, J.C. and Marzluf, G.A. (Eds.), Regulatory biology. The Ohio State University Press, Columbus, Ohio. pp. 196-242.

Marzluf, G.A. 2001. Metabolic regulation in fungi. In: Khachatourians, G.G. and Arora, D.K. (Eds.), Applied mycology and biotechnology Vol I. Agriculture and food production. Elsevier Science Amstrdam, pp. 55-72.

Marzluf, G.A. and Metzenberg, R.L. 1968. Positive control by the CYS-3 locus in regulation of sulfur metabolism in *Neurospora.* J. Mol. Bio. 33: 423-437.

Matsuda, S., Kawanami, Y., Takeda, H., Ooi, T. and Chung, J. 1998. Characterization of mutanase produced by *Microbispora rosea.* J. Dent. Res. 77: 1322.

Mattern, C.F.T., Brackett, F.S. and Olsen, B.J. 1957. Determination of number and size of particles by electrical garing: Blood cells. J. Appl. Physiol. 10: 56-70.

McKerrow, J.H., Sun, E., Rosenthal, P.J. and Bouvier, J. 1993. The proteases and pathogenicity of parasitic protozoa. Annu. Rev. Microbiol. 47: 821-53.

Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. 174: 1-7.

Mellado, E., Dubreucq, G., Mol, P., Sarfati, J., Paris, S., Diaquin, M., Holden, D.W., Rodriguez-Tudela, J.L. and Latge, J.P. 2003. Cell wall biogenesis in a double chitin synthase mutant (chsG-/chsE-) of *Aspergillus fumigatus*. Fungal Genet. Biol. 38: 98-109.

Mellon, J.E. and Cotty, P.J. 1996. Purification and partial characterization of an elastinolytic proteinase from *Aspergillus flavus* filtrates. Appl. Microbiol. Biotechnol. 46: 138-142.

Milner, R.J. 1989. Ecological considerations on the use of *Metarhizium* for control of soil-dwelling pests. In: Robertson, L.N. and Allsopp, P.G. (Eds.), Proceedings of a Soil-Invertebrate Workshop, Queensland Department of Primary Industries Conference and Workshop series QC 89004, Indoorpilly, Queensland, pp. 10-13.

Moore, D., Horner, J. and Liu, M. 1987. Co-ordinate control of ammonium-scavenging enzymes in the fruit body cap of *Coprinuscinereus* avoids inhibition of sporulation by ammonium. FEMS Microbiol. Lett. 44: 239-242.

Moore-Landecker, E. 1996. Growth. In: Fundamentals of the fungi. Prentice-Hall, Upper Saddle River, New Jersey, pp. 279-308.

Muller, R.H., Davis, S.S., Illum, L. and Mak, E. 1986. Particle charge and surface hydrophobicity of colloid drug carriers. In: Gregoriadis, G., Senior, J. and Posle, G. (Eds.), Targeting of drugs with synthetic systems, Plenum Press: New York, pp. 239-263.

Nahar, P., Ghormade, V. and Deshpande, M.V. 2004. The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: Possible edge to entomopathogenic fungi in the biological control of insect pests. J. Invertebr. Pathol. 85: 80-88.

Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. 1979. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase: Studies with peptide substrates related to the ά-protease inhibitor reactive site. J. Biol. Chem. 254: 4027-4032.

Napolitano, R. and Juarez, M.P. 1997. Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*. Arch. Biochem. Biophys. 344: 208-214.

Neihof, R. and Nichols, W.H. 1973. Physicochemical studies of microbial cell walls: Comparative electrophoretic behaviour of intact cell walls. Biochim. Biophys. Acta 318: 23-32.

Neville, A.C. 1975. Biology of the arthropod cuticle. Springer-Verlag, Berlin.

North, M.J. 1982. Comparative biochemistry of the proteinases of eukaryotic microorganisms. Microbiol. Rev. 46: 308-340.

O' Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Ohlsson, B.G., Westrom, B.R. and Karlsson, B.W. 1986. Enzymoblotting: A method for localizing proteinases and their zymogens using para-nitroanilide substrates after agarose gel electrophoresis and transfer to nitrocellulose. Anal. Biochem. 152: 239-244.

Ohtake, Y., Satou, A., and Yabuuchi, S. 1990. Isolation and characterization of glutathione biosynthesis-deficient mutants in *Saccharomyces cerevisiae.* Agric. Biol. Chem. 54: 3145-3150.

Olsson, S. 2000. Evidence of diffusion being the mechanism of translocation in the hyphae of three molds. Exp. Mycol. 15: 302-209.

Ong, K.L. and Chang, F.N. 1997. Analysis of proteins from different phase variants of the entomopathogenic bacteria *Photorhabdus luminescens* by two-dimensional zymography. Electrophoresis 18: 834-839.

Padmavathi, J., Devi, K.U. and Rao, C.U.M. 2003. The optimum and tolerance pH range is correlated to colonial morphology in isolates of the entomopathogenic fungus *Beauveria bassiana* – a potential biopesticide. World. J. Microbiol. Biotech. 19: 469-477.

Park, G.S., Kho, C.W., Cho, S., Lee, D.H., Kim, S.H. and Park, B.C. 2002. A functional proteomic analysis of secreted fibrinolytic enzymes from *Bacillus subtilis* 168 using a combined method of two-dimensional gel electrophoresis and zymography. Proteomics 2: 206-211.

Paterson, I.C., Charnley, A.K., Cooper, R.M., and Clarkson, J.M. 1994a. Partial characterization of specific inducers of a cuticle-degrading protease from the insect-pathogenic fungus *Metarhizium anisopliae.* Microbiology 140: 3153-3159.

Paterson, I.C., Charnley, A.K., Cooper, R.M., and Clarkson, J.M. 1994b. Specific induction of a cuticle-degrading protease of the insect-pathogenic fungus *Metarhizium anisopliae.* Microbiology 140: 185-189.

Peberdy, J.F. 1990. Fungal cell wall—a review, In: Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Goosey, W. and Copping, L.G. (Eds.), Biochemistry of cell walls and membranes in fungi. Springer-Verlag, Heidelberg, Germany, pp. 5-24.

Penalva, M.A. and Arst H.N.Jr. 2004. Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. Annu. Rev. Microbiol. 58: 425-51.

Penalva, M.A. and Arst, H.N.Jr. 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol. Mol. Biol. Rev. 66: 426-46.

Pendland, J.C. and Boucias, D.G. 1986. Lectin binding characteristics of several entomogenous hyphomycetes: Possible relationship to insect hemagglutinins. Mycologia 78: 818-824.

Pendland, J.C. and Boucias, D.G. 1992. Ultrastructural localization of carbohydrate in cell walls of the entomogenous hyphomycete *Nomuraea rileyi.* Can. J. Microbiol. 38: 377-386.

Pendland, J.C., Heath, M.A. and Boucias, D.G. 1987. Function of a galactose-binding lectin from *Spodoptera exigua* larval haemolymph: Opsonization of blastospores from entomogenous hyphomycetes. J. Insect Physiol. 34: 533-540.

Perillo, N.L., Pace, K.E., Seilhamer, J.J. and Baum, L.G. 1995. Apoptosis of T-cells mediated by galectin-1. Nature 378: 736–739.

Persson, M. Hall, L. and Soderhall, K. 1984. Comparison of peptidase activity in some fungi pathogenic to arthropods. J. Invertebr. Pathol. 44: 342-348.

Powers, J.C. and Harper, J.W. 1986. Protease inhibitors. Barrett, S.J. and Salvesen, C. (Eds.). Elsevier, Amsterdam, pp. 219-289.

Prusky, D. and Yakoby, N. 2003. Pathogenic fungi: Leading or led by ambient pH? Mol. Plant Pathol. 6: 509-516.

Prusky, D., McEvoy, J.L., Leverentz, B., and Conway, W.S. 2001. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol. Plant Micro. Intr. 14: 1105-1113.

Qazi, S.S. and Khachatourians, G.G. 2005. Insect pests of Pakistan and their management practices: Prospects for the use of entomopathogenic fungi. Biopest. Internat. 1: 13-24.

Qazi, S.S. and Khachatourians, G.G. 2007. Hydrated conidia of *Metarhizium anisopliae* release family of metalloproteases. J. Invertebr. Pathol. 95: 48-59.

Qazi, S.S. and Khachatourians, G.G. 2008. Addition of exogenous carbon and nitrogen sources to aphid exuviae modulates synthesis of proteases and chitinase by germinating conidia of *Beauveria bassiana.* (DOI 10.1007/s00203-008-0355-9)

Rabilloud, T. and Chevallet, M. 2000. Solubilization of proteins in 2-D electrophoresis. In: Rabilloud, T. (Ed) Proteome research: Two-dimensional electrophoresis and identification methods. Springer, Berlin, Heidelberg, New York, pp. 9-27.

Rabilloud, T., Adess, C., Giraudel, A. and Lunardi, J. 1997. Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients Electrophoresis 18: 307-316.

Rabilloud, T., Luche, S., Santoni, V. and Chevallet, M. 2007. Detergents and chaotropes for protein solubilization before two-dimensional electrophoresis. Meth. Mol. Biol. 355: 111-119.

Ramon A.M., Porta, A. and Fonzi, W.A. 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by PRR2. J. Bacteriol. 181: 7524-7530.

Rao, M.B., Tanksale, A.M. Ghatage, M.S., and Deshpande, V.V. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62: 597-635.

Rapp, P. 1992. Formation, separation and characterization of three β-1,3-glucanases from *Sclerotium glucanicum*. Biochim. Biophys. Acta 1117: 7-14.

Rath, A.C. 1992. *Metarhizium anisopliae* for control of the Tasmanian pasture scarab *Adoryphorus couloni.* In: Jackson, A.T. and Glare, R.T. (Eds.), The use of pathogens in scarab pest management. Andover: Intercept Publications, pp. 217-227.

Reddy, T.V., Viswanathan, L. and Venkitasubramanian, T.A. 1971. High aflatoxin production on a chemically defined medium. Appl. Microbiol. 22: 393-396.

Reese, E.T., and Mandels, M. 1959. β-D-1,3-glucanases in fungi. Can. J. Microbiol. 5: 173-185.

Reisfeld, R.A., Lewis, V.J. and Williams, D.E. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281.

Reynolds, S.E. 1975. The mechanism of plasticization of the abdominal cuticle of Rhodnius. J. Exp. Biol. 62: 81-89.

Rhodes, J.C., Amlung, T.W. and Miller, M.S. 1990. Isolation and characterization of an elastinolytic proteinase from *Aspergillus flavus.* Infect. Immun. 8: 2529-2534.

Righetti, P.G. 1990. Immobilized pH gradients: Theory and methodology, Elsevier Biomedical Press, Amsterdam.

Rosenberg, S.L. 1975. Temperature and pH optima for 21 species of thermophilic and thermotolerant fungi. Can. J. Microbiol. 21: 1535-1540.

Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast. Meth. Enzymol. 194: 281-301.

Samples, D.R., Richter, R.L. and Dill, C.W. 1984. Measuring proteolysis in cheddar cheese slurries: Comparison of Hull and trinitrobenzene sulfonic acid procedure. J. Dairy Sci. 67: 60-63.

Sanderink, G-J, Arthur, C.M., Galteau, M-M., Wellman-Bednawska, M. and Siest, G. 1986. Multiple forms of serum aminopeptidases separated by micro two-dimensional electrophoresis under non-denaturing conditions. Electrophoresis 7: 471-475.

Sanzhimitupova, R.D. 1980. Effect of the pH of the medium on the growth and development of the causal agent of mycosis of the seabuckthorn moth (*Gelecjia hippophaella* Schrk). Izvestiya-Sibirskogo-Otdeleniya-Akademii-Nauk-SSSR-Biology 15: 39-41.

Schaap, P., Brandt, R. and vanEs, S. 1995. Regulation of *Dictyostelium* adenylcyclases by morphogen-induced modulation of cytosolic pH or Ca^{2+} levels. Dev. Biol. 168: 179-188.

Schaeffer, H.J., Leykan, J. and Walton, J.D. 1994. Cloning and targeted gene disruption of EXG1, encoding exo-β-1,3-glucanase, in the phytopathogenic fungus *Cochliobolus carbonum.* Appl. Environ. Microbiol. 60: 594-598.

Schindler, J. and Sussman, M. 1977. Ammonia determines the choice of morphogenetic pathways in *Dictyostelium discoideum*. J. Mol. Biol. 116: 161-169.

Schmit, J.C. and Brody, S. 1976. Biochemical genetics of *Neurospora crassa* conidial germination. Bacteriol. Rev. 40: 2-41.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. 1997. Carbon regulation of the cuticle-degrading enzyme PR1 from *Metarhizium anisopliae* may involve a trans-acting DNA-binding protein CRR1, a functional equivalent of the *Aspergillus nidulans* CREA protein. Curr. Genet. 31: 511-518.

Screen, S., Bailey, A., Charnley, K., Cooper, R. and Clarkson, J. 1998. Isolation of a nitrogen response regulator gene (nrr1) from *Metarhizium anisopliae*. Gene 221: 17-24.

Screen, S.E. and St. Leger, R.J. 2000. Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. J. Biol. Chem. 275: 6689-6694.

Screen, S.E., Hu, G. and St. Leger, R.J. 2001. Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. J. Invertebr. Pathol. 78: 260-266.

Segers, R., Butt, T.M., Carder, J.H., Keen, J.N., Kerry, B.R. and Peberdy, J.F. 1999. The subtilisins of fungal pathogens of insects, nematodes and plants: distribution and variation. Mycol. Res. 103: 395-402.

Segers, R., Butt, T.M., Kerry, B.R. and Peberdy, J.F. 1994. The nematophagous fungus *Verticillium chlamydosporium* produce a chymoelastase-like protease that hydrolyses host nematode proteins in situ. Microbiology 140: 2715-2723.

Seifert, K.A. and Gams, W. 2001. The taxonomy of anamorphic fungi. In: McLaughlin, D.J., McLaughlin, E.G. and Lemke, P.A. (Eds). The Mycota VII Part A. Berlin Heidelberg: Springer-Verlag, pp. 307-347.

Shah, F.A., Wang, C.S. and Butt, T.M. 2005. Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae.* FEMS Microbiol. Lett. 251: 259-266.

Shah, P.A. and Pell, J.K. 2003. Entomopathogenic fungi as biological control agents. Appl. Microbiol. Biotechnol. 61: 413-423.

Shams, N.B., Hanninen, L.A., Kenyon, K.R. 1994. Increased gelatinolytic and caseinolytic activity in the thermally injured, nutritionally compromised rat cornea: detection of a 27-kDa lymphoreticular cell-associated caseinase. Curr. Eye Res. 13: 11-9.

Sharma, S., Agarwal, G.P. and Rajak, R.C. 1992. Effect of temperature, pH and light on toxin production by *Beauveria bassiana* (Bal) Vuill. Indian J. Exp. Biol. 30: 918-919.

Shimazu, M. and Sato, H. 1996. Media for selective isolation of an entomogenous fungus *Beauveria bassiana* Deuteromycotina, Hyphomycetes. Appl. Entomol. Zool. 31: 291-298.

Sikora, L. and Marzluf, G.A. 1982. Regulation of L-amino acid oxidase and D-amino acid oxidase in *Neurospora crassa*. Mol. Gen. Genet. 186: 33-39.

Sivan, A. and Chet, I. 1989. Degradation of fungal cell walls by lytic enzymes from *Trichoderma harzianum*. J. Gen. Microbiol. 135: 675-682.

Small, C-L.N. and Bidochka, M.J. 2005. Up-regulation of Pr1, a subtilisin-like protease, during conidiation in the insect pathogen *Metarhizium anisopliae*. Mycol. Res. 109: 307-313.

Smith, R.E. 1984. Identification of protease isozymes after analytical isoelectric focusing using fluorogeneic substrates impregnated into cellulose membranes. J. Histochem. Cytochem. 32: 1265-1274.

Smith, R.J., Pekrul, S. and Grula, E.A. 1981. Requirement for sequential enzymatic activities for penetration of the integument of the corn earworm (*Heliothis zea*). J Invert. Pathol. 38: 335-344.

Soil, D.R. and Sonneborn, D.R. 1972. Zoospore germination in *Blastocladiella emersonii,* IV, Ion control over cell differentiation. J. Cell Sci. 10: 315-333.

St. Leger, R.J. 1993. Biology and mechanisms of invasion of deuteromycete fungal pathogens. In: Beckage, N.C.,Thompson, S.N., Federici, B.A. (Eds.) Parasites and pathogens of insects, Vol 2. Academic Press, Inc., San Diego, California, pp. 211-229.

St. Leger, R.J. 1995. The role of cuticle-degrading proteases in fungal pathogens of insects. Can. J. Bot. 73 (Suppl. 1): S1119-S1125.

St. Leger, R.J., Bidochka, M.J. and Roberts, D.W. 1994. Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae.* Arch. Bioch. Biophys. 313: 1-7.

St. Leger, R.J., Butt, T.M., Staples, R.C. and Roberts, D.W. 1989. Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae.* Exp. Mycol. 13: 253-262.

St. Leger, R.J., Butt, T.M., Staples, R.C. and Roberts, D.W. 1990. Second messenger involvement in differentiation of the entomopathogenic fungus *Metarhizium anisopliae.* J. Gen. Microbiol. 136: 1779-1789.

St. Leger, R.J., Charnley, A.K., and Cooper, R.M. 1986a. Cuticle-degrading enzymes of entomopathogenic fungi: Synthesis in culture on cuticle. J. Invertebr. Pathol. 48: 85-95.

St. Leger, R.J., Charnley, A.K., and Cooper, R.M. 1986c. Cuticle-degrading enzymes of entomopathogenic fungi: Mechanisms of interaction between pathogen enzymes and insect cuticle. J. Invertebr. Pathol. 47: 295-302.

St. Leger, R.J., Cooper, R.M and Charnley, A.K. 1987. Production of cuticle- degrading enzymes by the entomopathogen *Metarhizium anisopliae* during the infection of cuticles from *Calliphora vomitoria* and *Manduca sexta.* J. Gen. Microbial. 133: 1371-1382.

St. Leger, R.J., Cooper, R.M. and Charnley, A.K. 1986b. Cuticle-degrading enzymes of entomopathogenic fungi: Regulation of production of chitinolytic enzymes. J. Gen. Microbiol. 132: 1509-1517.

St. Leger, R.J., Durrands, P.K., Cooper, R.M. and Charnley, A.K. 1988. Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch. Microbiol. 150: 413-416.

St. Leger, R.J., Frank, D.C., Roberts, D.W. and Staples, R.C. 1992. Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene for the entomopathogenic fungus *Metarhizium anisopliae*. Eur. J.Biochem. 204: 991-1001.

St. Leger, R.J., Goettel, M, Roberts, D.W. and Staples, R.C. 1991. Prepenetration events during the infection of host cuticle by *Metarhizium anisopliae.* J. Invertebr. Pathol. 58: 168-179.

St. Leger, R.J., Joshi, L. and Roberts, D.W. 1997. Adaptation of proteases and carbohydrases saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology 143: 1983-1992.

St. Leger, R.J., Joshi, L. and Roberts, D.W. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl. Environ. Microbiol. 64: 709-713.

St. Leger, R.J., Joshi, L., Bidochka, M.J. and Rizzo, N.W. and Roberts, D.W. 1996a. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl. Environ. Microbiol. 62: 1257-1264.

St. Leger, R.J., Joshi, L., Bidochka, M.J. and Roberts, D.W. 1996b. Construction of an improved mycoinsecticide over expressing a toxic protease Proc. Natl. Acad. Sci. USA 93: 6349-6354.

St. Leger, R.J., Nelson, J.O. and Screen, S.E. 1999. The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. Microbiology 145: 2691-2699.

Stahmann, K.P., Pielken, P., Schimz, K.L. and Sahm, H. 1992. Degradation of extracellular β-(1,3)-(1,6)-D-glucan by *Botrytis cinerea.* Appl. Environ. Microbiol. 58: 3347-3354.

Strasser, H., Abendstein, D., Stuppner, H. and Butt, T.M. 2000a. Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. Mycol. Res. 104: 1227-1233.

Strasser, H., Vey, A. and Butt, T.M. 2000b. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? Biocont. Sci. Technol. 10: 717-735.

Stryer, L. 1995. *Biochemistry*, 4th ed. W.H. Freeman, New York.

Sussman, A.S. and Halvorson, H.O. 1966. Spores – Their dormancy and germination, Harper and Row, New York, USA.

Takehara, T, Inoue, M., Morioka, T. and Yokogawa, K. 1981. Purification and properties of endo-ά-1,3-glucanase from a *Streptomyces chartreusis* strain. J. Bacteriol. 145: 729-735.

Teter, S.A., Eggerton, K.P., Scott, S.V., Kim, J., Fischer, A.M. and Klionsky, D.J. 2001. Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. J. Biol. Chem. 276: 2083–2087.

Thomas, K.C., Khachatourians, G.G. and Ingledew, W.M. 1987. Production and properties of *Beauveria bassiana* conidia cultivated in submerged culture. Can. J. Microbiol. 33: 12-20.

Titball, R.W., Bell, A. and Munn, C.B. 1985. Role of caseinase from *Aeromonas salmonicida* in activation of hemolysin. Infect. Immun. 49: 756-759.

Tomkins R.G. 1932. Measuring germination. Trans. Brit. Mycol. Soc. 17: 147-149.

Tong, Y. and Kanost, M.R. 2005. *Manduca sexta* Serpin-4 and Serpin-5 inhibit the prophenol oxidase activation pathway. J. Biol. Chem. 280: 14923-14931.

Towbin, H., Stalhelin, T., and Gordon, J. 1979. Expression of p^{21}ras in fresh primary and metastatic human colorectal tumors. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Twyman, R.M. 2004. Principles of proteomics. Garland Scientific/ BIOS Scientific Publishers, New York.

Uribe, V.D. 2004. Mitochondrial genomic diversity and respiratory physiology in the entomopathogenic fungus *Beauveria bassiana.* Ph.D thesis. University of Saskatchewan, Saskatoon, Canada.

Urtz, B.E. and Rice, W.C. 2000. Purification and characterization of a novel extracellular protease from *Beauveria bassiana.* Mycol. Res. 104: 180-186.

Valadares-Inglis, M.C. and Peberdy, J.F. 1997. Location of chitinolytic enzymes in protoplasts and whole cells of the entomopathogenic fungus *Metarhizium anisopliae*. Mycol. Res. 101: 1393-1396.

Valencia, E. and Khachatourians, G.G. 1998. Integrated pest management and entomopathogenic fungal biotechnology in the Latin Americas I. Opportunities in a global agriculture. Revista de la Academia Colombia de Ciencias Exactas Fisicas Y Naturales 22: 193-198.

Van den Boogert, P.H.J.F., Dijksterhuis, J., Velvis, H. and Veenhuis, M. 1992. Adhesive knob formation by conidia of the nematophagous fungus *Drechmeria coniospora.* Ant van Leeuwenhoek 61: 221-229.

Van Ettan, J.L., Dahlberg, K.A. and Russo, G. 1983. Fungal spore germination. In: Smith, J.E (Ed), Fungal differentiation. Mycology. Vol. 4. New York and Basel: Marcel Dekker Inc., pp. 235-266.

Van-Brunt, J. and Harold, F.M. 1980. Ionic control of germination of *Blastocladiella emersonii* zoospores. J. Bacteriol. 141:735-744.

Wang, C.S., Li, Z.Z. and Butt, T.M. 2002. Molecular studies of co-formulated strains of the entomopathogenic fungus, *Beauveria bassiana*. J. Invertebr. Pathol. 80: 29-34.

Warrior, P. 2006. Opportunities and challenges for microbial pesticides in the global marketplace. APS.CPS.MSA Joint Meeting. July 29th-Aug 2nd, 2006. Quebec city, Quebec, Canada.

Wei, H., Scherer, M., Singh, A., Liese, R. and Fischer, F. 2001. *Aspergillus nidulans* ά-1,3-glucanase (Mutanase), mutA, is expressed during sexual development and mobilizes mutan. Fungal Genet. Biol. 34: 217-227.

Weiland, J.J. 2004. Production of protease isozymes by *Aphanomyces cochlioides* and *Aphanomyces euteiches*. Physiological and Molecular Plant Pathology 65: 225-233

White, T.C. and Agabian, N. 1995. *Candida albicans* secreted aspartyl proteinases: Isozyme pattern is determined by cell type, and levels are determined by environmental factors. J. Bacteriol. 177: 5215-5221.

Wilkins, M.R., Sanchez, J.C., Gooley, A.A., Appel, R.D., Humphery-Smith, I., Hochstrasser, D.F. and Williams, K.L. 1996. Progress with proteome projects: Why all proteins

expressed by a genome should be identified and how to do it. Biotech. Gen. Eng. Rev. 13, 19-50.

Wisecup, W.G and Crouch, B.G. 1963. Evaluation and calibration of an electronic particle counter for enumeration of multispecies blood cells. Am. J. Clin. Path. 39: 349-354.

Wolf, F.A. and Wolf, F.T. 1947. The fungi. Vol. 2. London: Wiley, pp. 155-161.

Woolford, C.A., Daniels, L.B., Park, F.J., Jones, E.W., Van Arsdell, J.N. and Innis, M. A. 1986. The PEP4 gene encodes an aspartyl protease implicated in the post-translational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. Mol. Cell Biol. 6: 2500-2510.

Wraight, S.P., Jackson, M.A. and De Kock, S.L. 2001. Production, stabilization and formulation of fungal biocontrol agents. In: Butt, T.M., Jackson, C.W. and Magan, N. (Eds.), Fungi as biocontrol agents: Progress, problems and potential. CAB International, Wallingford, UK. pp. 253-287.

Yang, R.Y., Hsu, D.K. and Liu, F.T. 1996. Expression of galectin-3 modulates T-cell growth and apoptosis. Proc. Natl. Acad. Sci. USA. 93: 6737–6742.

Yangaita, T. 1957. Biochemical aspects of germination of conidiospores of *Aspergillus niger*. Archiv. Fur Mikrobiologie 26: 329-344.

Yarwood C.E. 1936. The tolerance of *Erysiphe polygoni* and certain other powdery mildews to low humidity. Phytopathology 26: 845-859.

Yarwood, C.E. 1950. Water content of spores. Am. J. Bot. 37: 636–639.

Yoshida, S., Yamashita, M., Yonehara, S. and Eguchi, M. 1990. Properties of fungal protease inhibitors from the integument and haemolymph of the silkworm and effect of an

inhibitor on the fungal growth. Comp. Biochem. Physiol. (Part B-Biochem and Mol. Biol.) 95: 559-564.

Zacharuk, R.Y. 1970. Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae (Coleoptera). III. Penetration of the host integument. J. Invertebr. Pathol. 15: 372-396.

Zellner, S.R., Gustin, D.F., Buck, J.D. and Meyers, S.P. 1963. Growth and multiplication of *Rhoclotoda glutinis* as determined by viable and total cell counts. Ant. Van Leeuwenhock, J. Microbial. Scrol. 29: 203-210.

Zhao, Z. and Russel, P.J. 2003. Trypsin activity assay in substrate-specific one-and-two-dimensional gels: A powerful method to separate and characterize novel proteases in active form in biological samples. Electrophoresis 24: 3284-3288.

Zonneveld, B.J. 1972. Morphogenesis in *Aspergillus nidulans.* The significance of a ά-1,3-glucan of the cell wall and alpha-1,3-glucanase for cleistothecium development. Biochim. Biophys. Acta 273: 174-187.

(A) APPENDIX 1

(i) Mycelial density profiles as quantified by image analysis and densitometry

(ii) Dry Weight Biomass

Mycelial density profiles were measured and represented in terms of pixel density. Plates grown till day 7 were scanned on flat bed scanner (HP 2200) at the depth of 300dpi. Both sides of the plates were scanned in order to quantify the growth above and in the agar. Image was imported in Adobe Photoshop (Version, 6) and changed to grey scale. All images were adjusted at the auto-contrasts and auto-levels. The brightness was adjusted at the level of 50 for all the images before saving images in TIFF format at auto settings of the program. For 1-D lane densitometry TIFF images were analyzed by Alpha Imager^{TM}, version (5.5). In order to perform the 1-D lane densitometry a square was drawn around each colony margins and 1-D lane densitometry option of Alpha Imager^{TM} was used to select one lane in the centre of the colony in such a way that the lane would have 100 unit (as per Alpha Imager^{TM} scale). This selection would have 50 units on either side of the colony. For pixel density value auto grid option in the menu was used and image obtained for the pixel map was saved with v-line to score pixel values in the image.

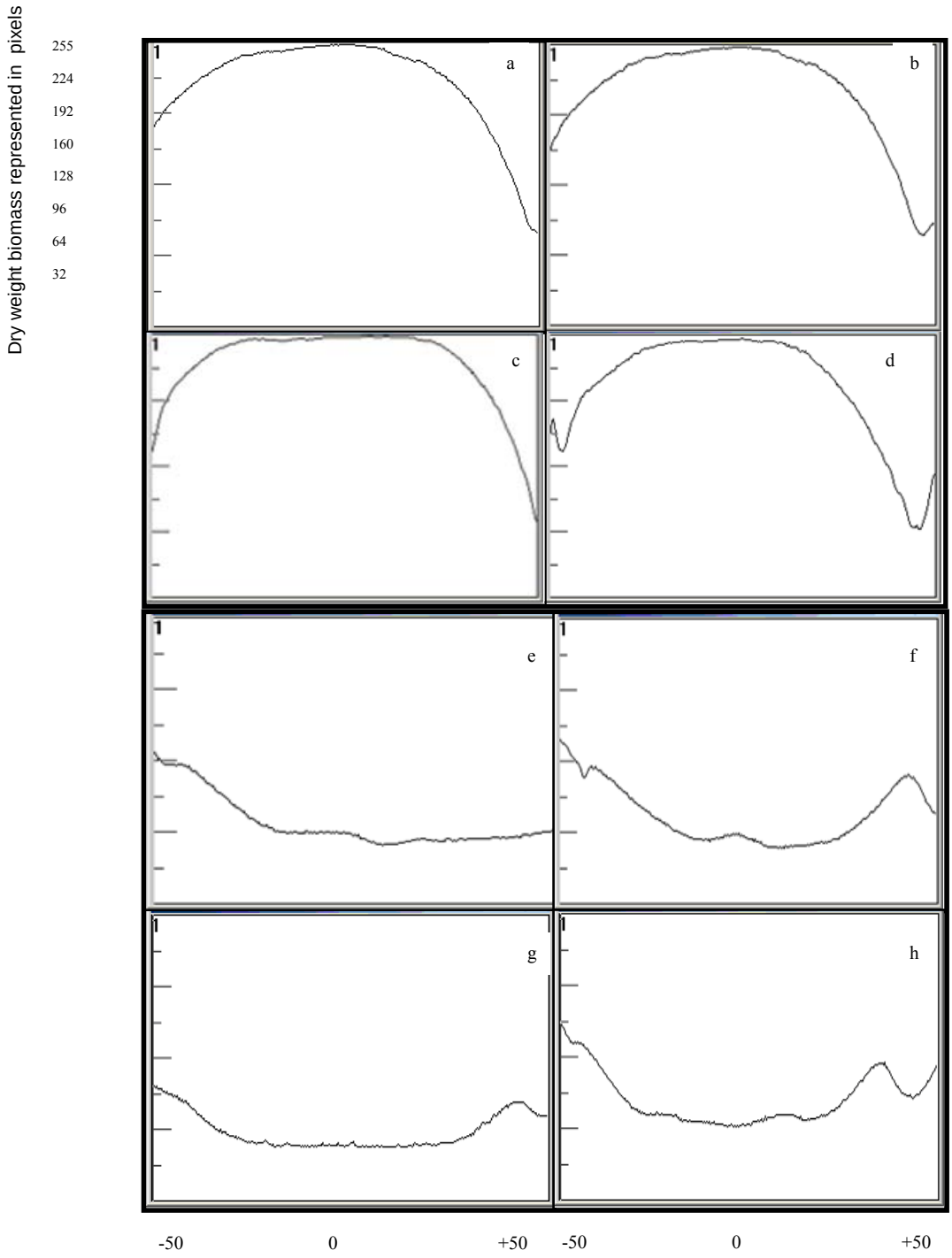


Figure 1.0: Dry weight biomass density profiles as determined by the 1-D lane densitometry of GK 2016 at pH*i* 4. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plate depicting the growth of the fungi inside the agar.

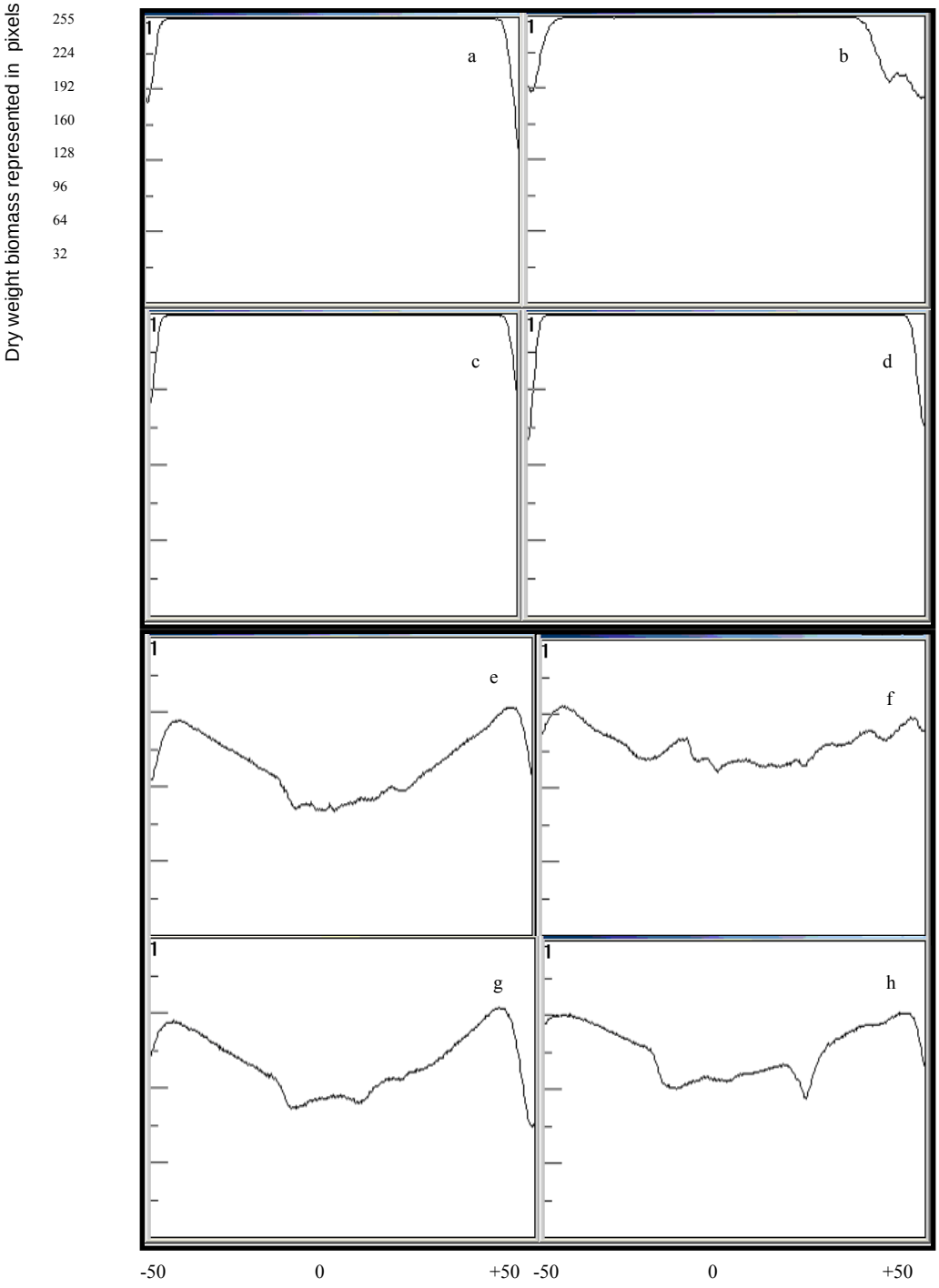


Figure 1.1: Dry weight biomass density profiles as determined by the 1-D lane densitometry of GK 2016 at pH 8. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plate depicting the growth of the fungi inside the agar.

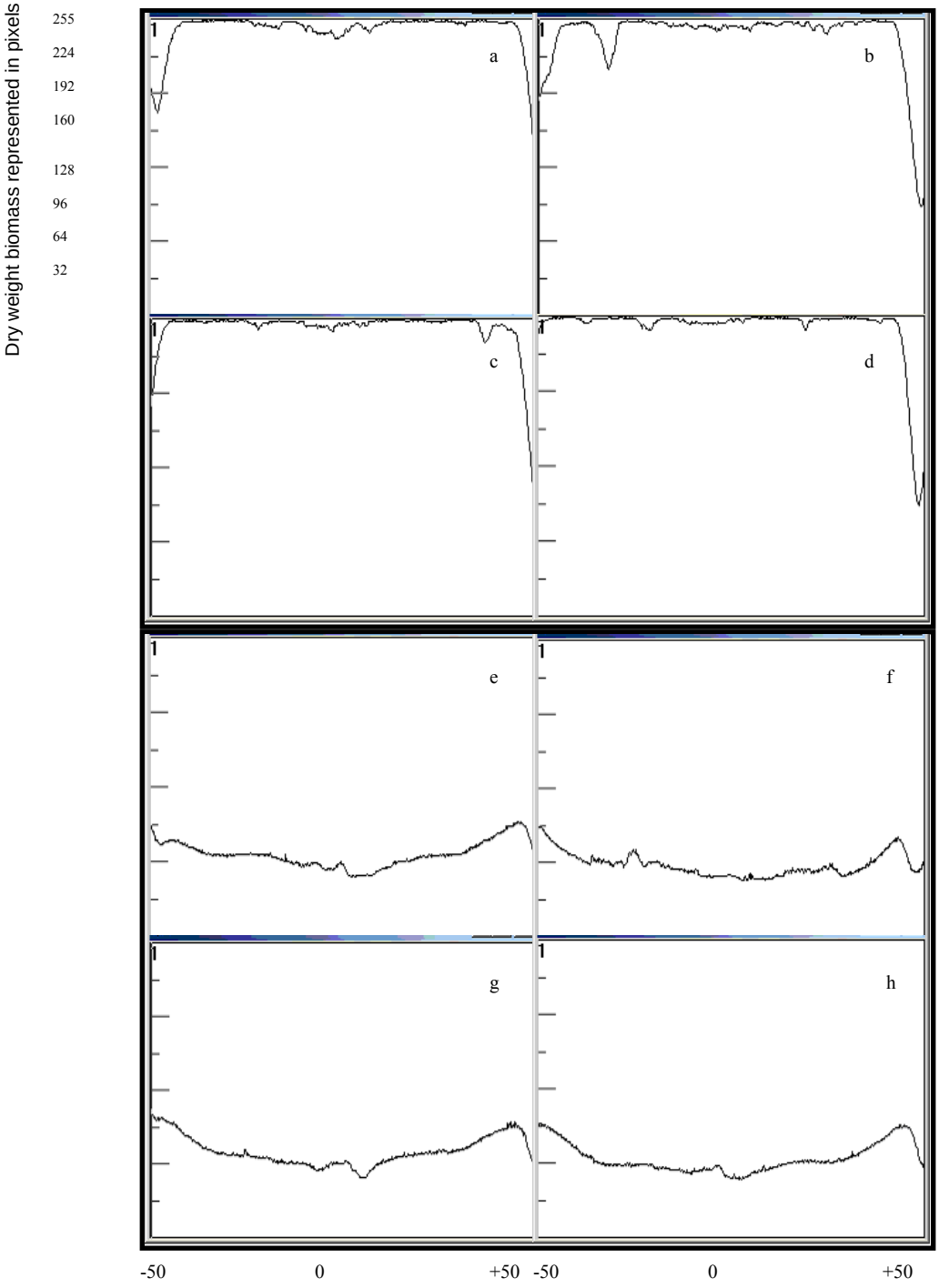


Figure 1.2: Dry weight biomass density profiles as determined by the 1-D lane densitometry of MA 2038 at pH 4. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plates depicting the growth of the fungi inside the agar.

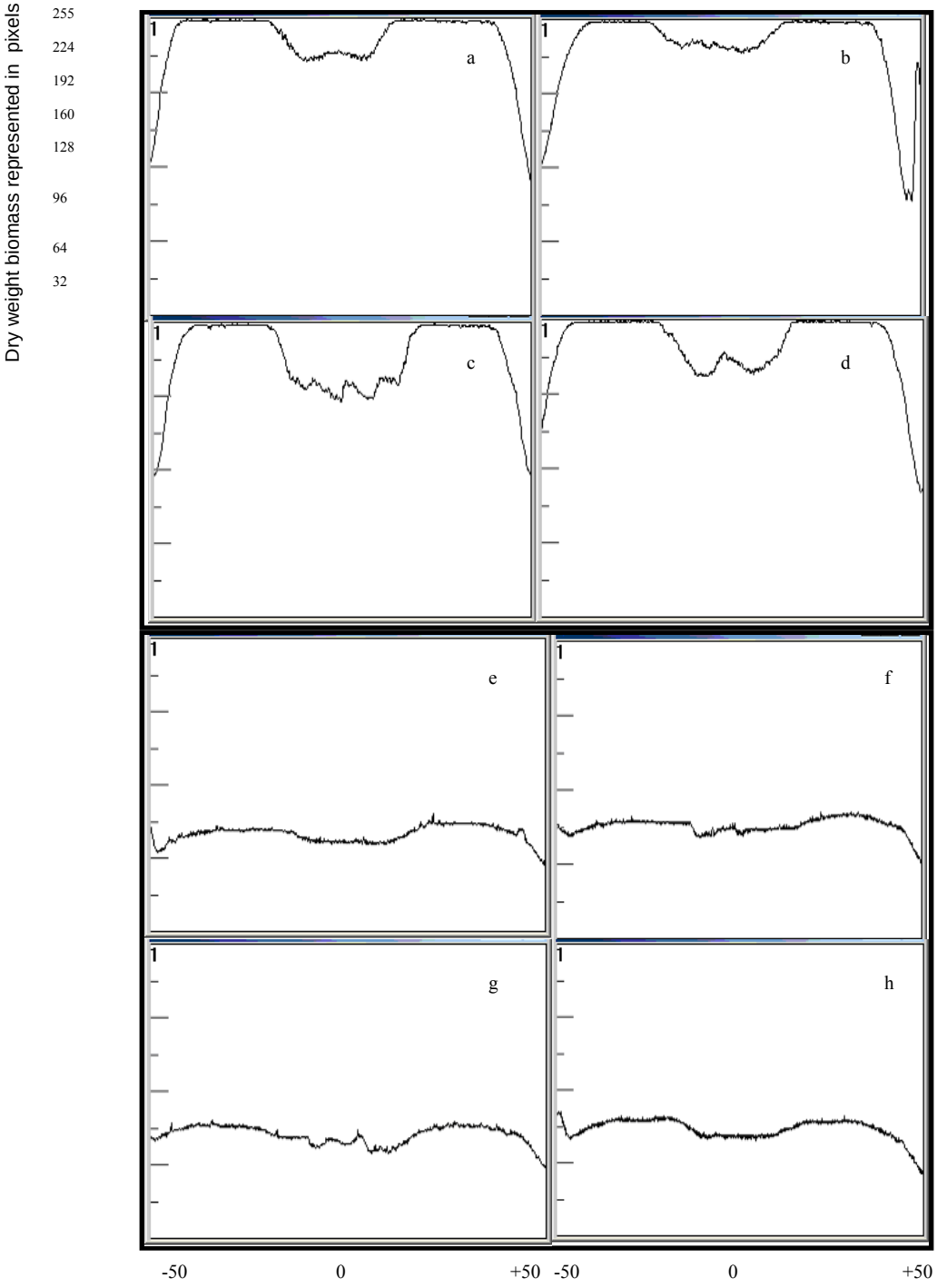


Figure 1.3: Dry weight biomass density profiles as determined by the 1-D lane densitometry of MA 2038 at pH 8. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plates depicting the growth of the fungi inside the agar.

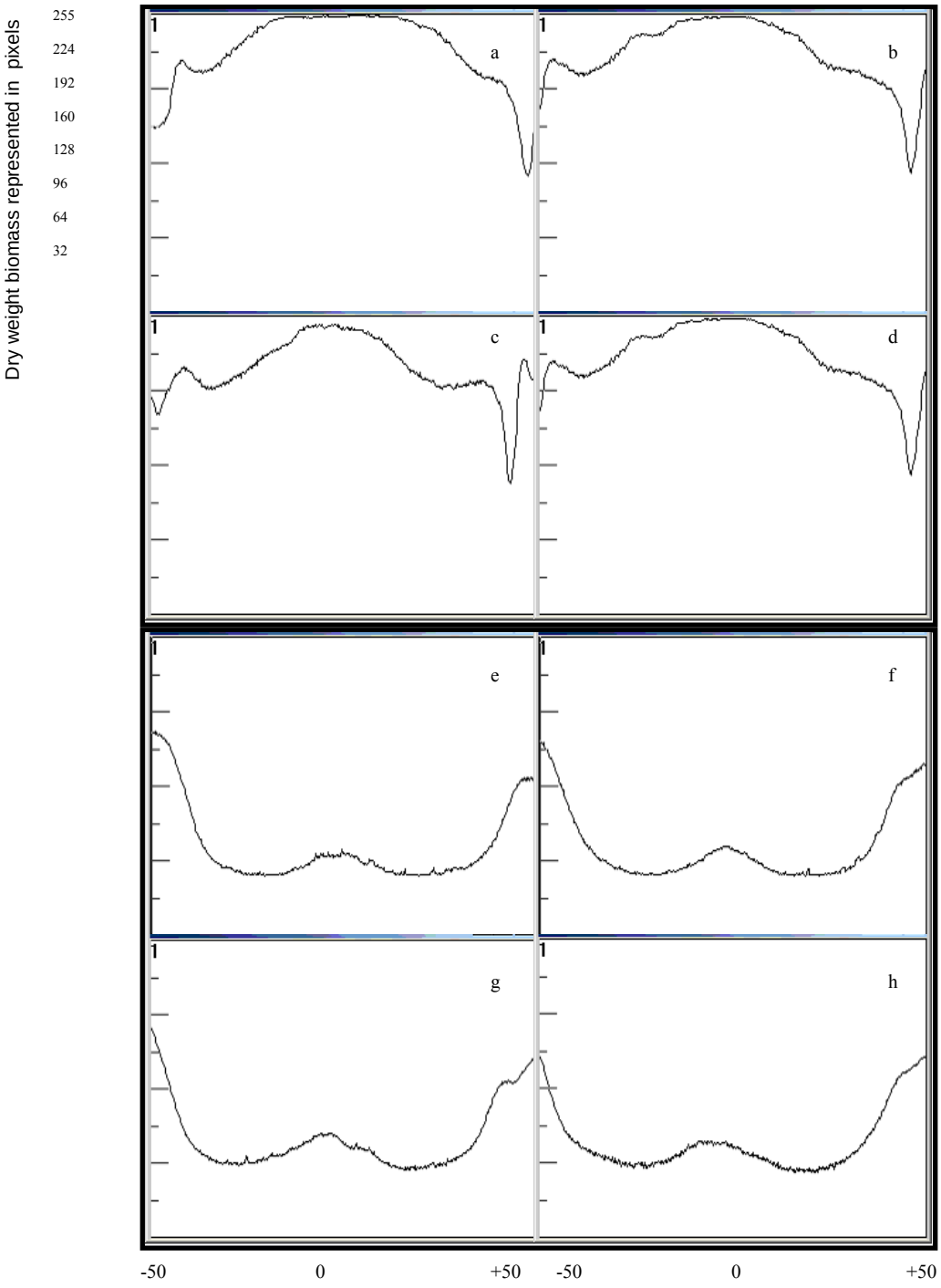


Figure 1.4: Dry weight biomass density profiles as determined by the 1-D lane densitometry of AN 189 at pH 4. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plates depicting the growth of the fungi inside the agar.

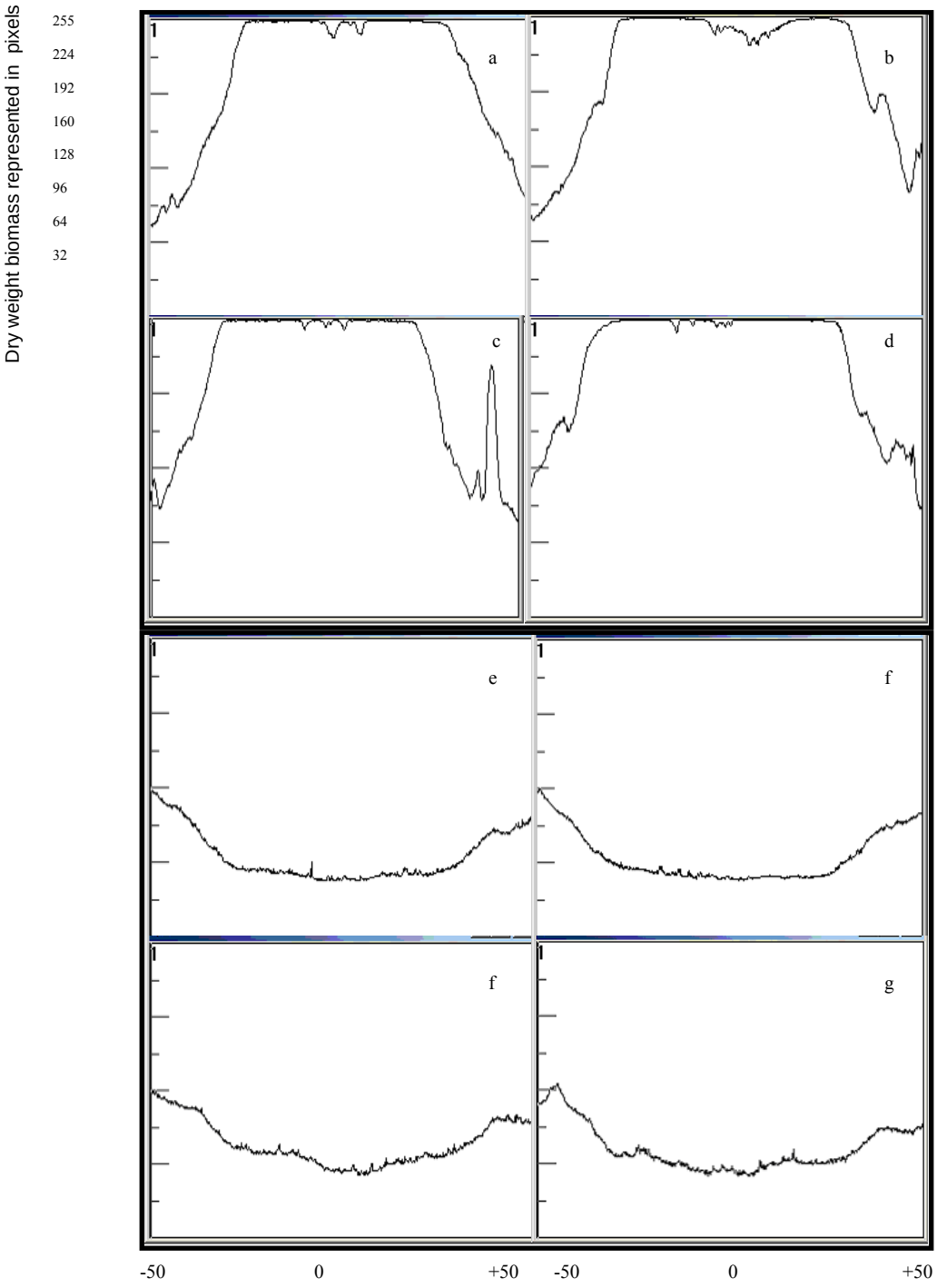


Figure 1.5: Dry weight biomass density profiles as determined by the 1-D lane densitometry of AN 189 at pH 8. The upper panel (a-d) shows the front side of the colony where as the bottom half (e-h) shows the inverted plates depicting the growth of the fungi inside the agar.

(A) APPENDIX 2

(i) Changes in pH of the liquid media by EPF

The representative isolates of entompathogenic fungi listed in chapter 12 (section 12.3.1) were used in this experiment. Furthermore, *P. fumosoroseus* (PF 1360) and *V. lecani* (V1463) used in this study were generously provided by USDA, USA. Isolates were tested for their ability to grow and modify the three selected pH*i*'s. All the strains have showed growth on pH 4, 7 and 11, respectively. The visual inspection of growth ranged from highest to lowest in the order as follows: pH 7 > pH 4 > pH 11 (+++, ++, +). The results also revealed that EPF have the ability to modify the pH due to the secretion of ammonia and organic acids (citric, malic, oxalic etc) of TCA cycle intermediate (Fig. 17.1).

Similarly, the ability of EPF to modify the pH of the complex media (Fig. 17.2) was also investigated. Results from a variety of EPF indicated that the change in pH of the growth medium is a characteristic of all the fungal species. There is also a role of the influence of the medium composition. For example, the yeast extract peptone (0.05X YP) medium resulted in the alkalinity of the medium for most of the strains tested. Moreover, it is the characteristic ability of the fungi in the utilization of the nutrients in the media.

One thing which was evident from this screening of fungi that change in pH of the liquid medium is a characteristic ability of each specific strain. The chemical composition of the media is also important in driving the pH of the medium.

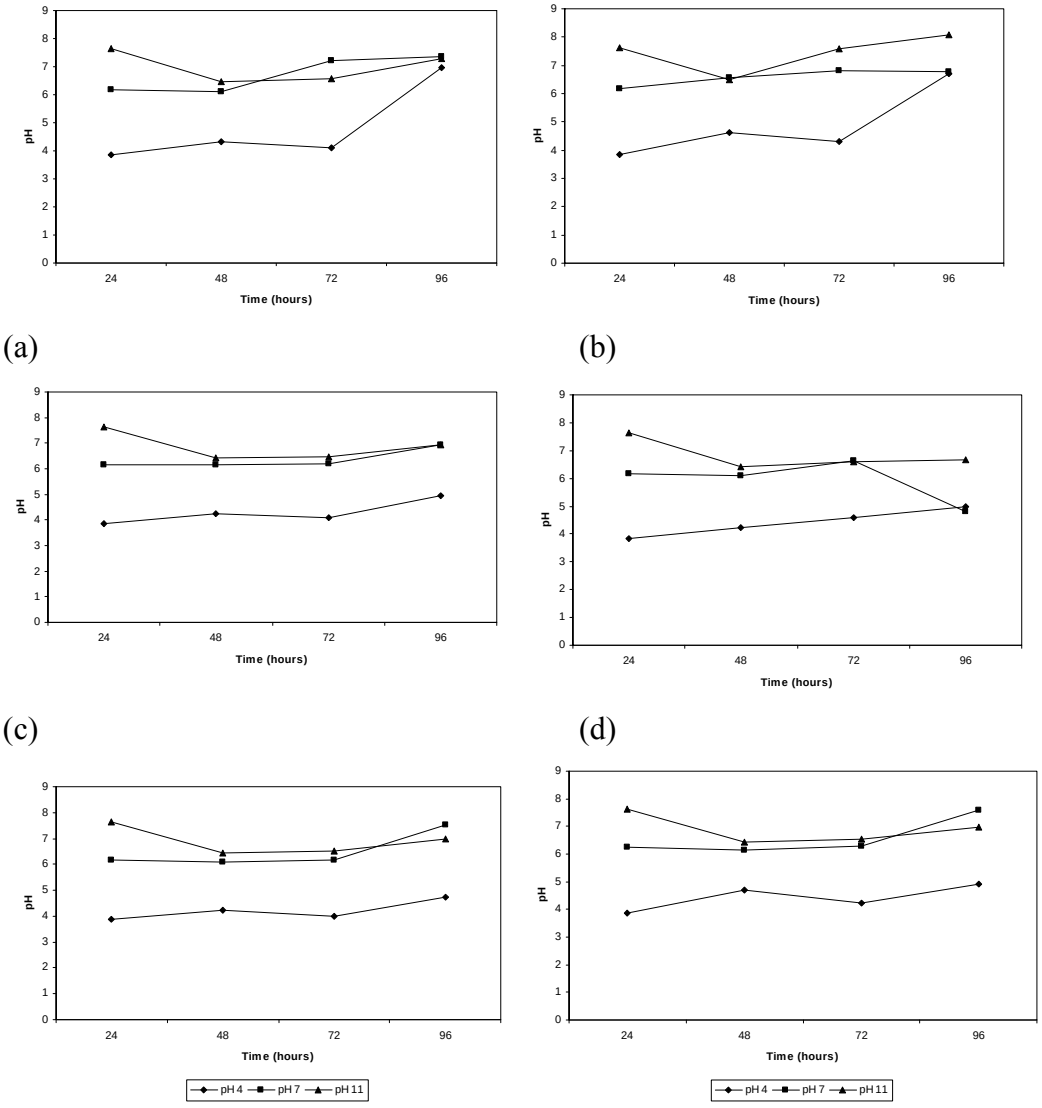


Figure 2.1: Change in pH of the liquid medium (YPG) during growth of the entompathogenic fungi at pH 4, 7 and 11. Representative isolates of EPF, GK 2016 (a), GK 2015 (b), ARSEF 3113 (c), *P. fumosoroseus* PF 1360 (d), *M. anisopliae* (e) and *V. lecani* V1463(f).

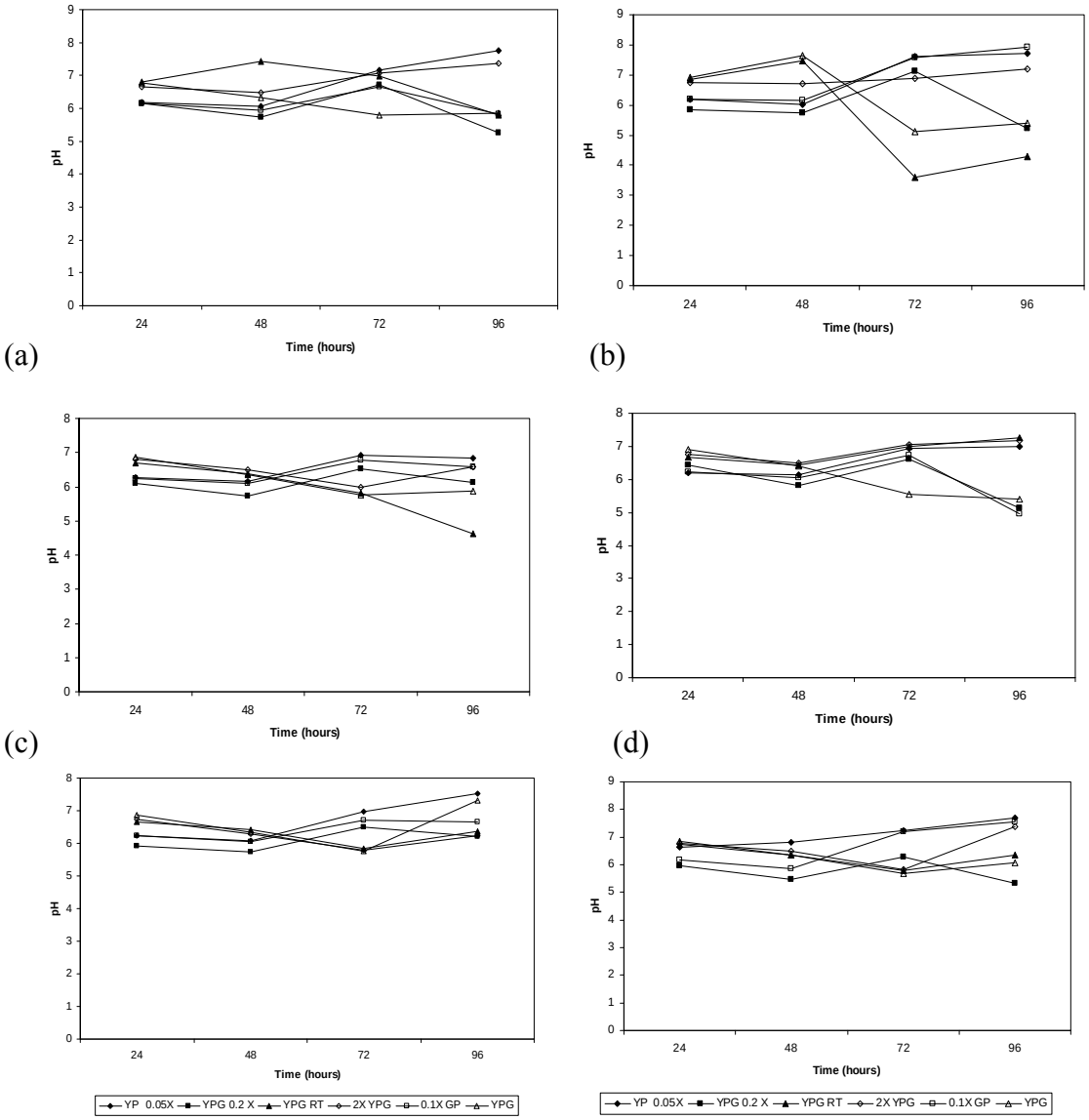


Figure 2.2: Change in pH of the liquid medium during growth of isolates GK 2016 (a), GK 2015 (b), ARSEF 3113 (c), *P. fumosoroseus* PF 1360 (d), *M. anisopliae* (e) and *V. lecani* V1463 (f). The growth and pH was estimated on different concentration of media at 27° C as follows: yeast extract (0.05 X YP), yeast extract peptone glucose (0.02 X YPG, YPG RT, 2 X YPG and YPG) and glucose peptone (0.1 X GP). [*YPG RT = growth/pH estimated at room temperature]

(C) APPENDIX 3

(i) Tap water quality details

Characteristic	Reported As	Tap Water
Color (apparent)	APHA	1.8
Conductivity at 25°C	µmhos/cm	344
pH		8.6
Temperature	C	12
Turbidity	NTU	<0.1
Aluminum	mg Al/ L	0.031
Arsenic	mg As/ L	0.0001
Barium	mg Ba/ L	0.032
Boron	mg B/ L	0.026
Cadmium	mg Cd/ L	<0.001
Calcium	mg Ca/ L	26
M-Alkalinity ¶	mg $CaCO_3$/ L	79
P-Alkalinity	mg $CaCO_3$/ L	5
Carbonate	mg $CaCO_3$/ L	7
Bicarbonate	mg $CaCO_3$/ L	83
Total Hardness	mg $CaCO_3$/ L	126
Chloride	mg Cl/ L	10
Chlorine Residual	mg Cl_2/ L	1.7
Chromium	mg Cr/ L	<0.001
Copper	mg Cu/ L	<0.001
Cyanide	mg CN/ L	<0.001
Fluoride	mg F/ L	0.49
Iron	mg Fe/ L	0.004
Lead	mg Pb/ L	<0.002
Magnesium	mg Mg/ L	15
Manganese	mg Mn/ L	0.0015
Mercury	mg Hg/ L	<0.00005

Potassium	mg K/ L	3.1
Selenium	mg Se/ L	0.0003
Silver	mg Ag/ L	<0.001
Sodium	mg Na/ L	21
ulfate	mg SO_4/ L	82
Uranium	mg U/ L	0.0010
Zinc	mg Zn/ L	<0.005
Ammonia	mg N/ L	0.34
Nitrate (& Nitrite)	mg N/ L	0.74
Total Kjeldahl Nitrogen	mg N/ L	0.50
Soluble Ortho Phosphate	mg P/ L	<0.02
Total Phosphate	mg P/ L	<0.01
BOD 5 day	mg/ L	NR*
Phenolics	mg Phenol/ L	0.00007
Soluble Organic Carbon	mg C/ L	2.6
Total Dissolved Solids	mg/ L	246
Total Suspended Solids	mg/ L	1
Volatile Suspended Solids	mg/ L	1
Chlorophyll-*a*	mg/ L	NR*
Fecal Coliform	CFU/ 100 ml	0
Fecal Streptococcus	CFU/ 100 ml	NR*
HPC	CFU/ mL	4
Total Coliform	CFU/ 100 ml	0
F. Total Trihalomethanes	mg/ L THM	0.037
G. Pesticide Scan, H. Organic Scan, I. Radiochemicals		--[2]

Legend:
1 Tested in distribution system
2 Concentrations has less than detection limits for all constituents tested as required by Provincial Permit to operate
3 Quarterly data/ typical finds
4 Yearly averages

Free aminonitrogen (FAN) mM ml^{-1}

¶ M-Alkalinity (Total Alkalinity) measures the amount of carbonate, bicarbonate and hydroxide present in terms of "ppm as calcium carbonate". The M-Alkalinity measurement is based on a sulfuric acid titration using a Methyl orange.

P-Alkalinity measures the amount of carbonate and hydroxyl alkalinity present in terms of "ppm as calcium carbonate". The P-Alkalinity measurement is based on a sulfuric acid titration using a Phenolphthalein indicator.

NR* not required or not applicable by Provincial Permit to operate.

Source: City of Saskatoon, Saskatchewan, Canada.