CLONING, EXPRESSION, AND CHARACTERIZATION OF A PUTATIVE CHITINASE GENE FROM *XENORHABDUS NEMATOPHILA*

Dissertation submitted to the

Jawaharlal Nehru University

for the award of the degree of

MASTER OF PHILOSOPHY

MUKESH KUMAR

SCHOOL OF LIFE SCIENCES

JAWAHARLAL NEHRU UNIVERSITY

NEW DELHI - 110067

INDIA

JULY 2011

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067
INDIA

CERTIFICATE

The research work embodied in this thesis entitled "**Cloning, expression and characterization of a putative chitinase gene from *Xenorhabdus nematophila***" has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full for any other degree or diploma of any University.

Mukesh kumar
(Candidate)

Neera Sarin
Prof. Neera Bhalla-Sarin
(Supervisor)

Neera Sarin
Prof. Neera Bhalla-Sarin
(Dean)

Dedicated

To

My Loving Family

ACKNOWLEDGEMENTS

At the outset, I would like to express my sincere gratitude to my Babuji and Maa who gave me enough strength, love, care, patience, consolation, and encouragement to accomplish this journey. I thank Almighty for all the blessings he has bestowed upon me and having made me meet me so many good and loving people to whom I would like to thank heartedly.

I have had the privilege of being supervised by Prof. Neera Bhalla-Sarin, Dean, SLS, JNU. I thank her for giving me the opportunity to work in her laboratory and providing me valuable guidance throughout my M. Phil. She has provided endless ideas, enthusiasm, and support, alongside outstanding mentorship and freedom which has helped me to comprehend how our own lives have changed since the beginning of this work. It is her guidance and meticulous efforts that has shaped this wonderful work of mine.

I owe sincere gratitude to Dr. Nirupama Banerjee (ICGEB, New Delhi) for providing me invaluable suggestions and introducing me to the chitinase gene. I have greatly benefited from her immensely helpful and practical advice. I wish to thank her for discussing with me and correcting my infinite scientific and stylistic errors.

I ought not to forget the timely help provided by Dr. A. K. Saxena for teaching me the basics of bioinformatics, protein purification and the discussions on site directed mutagenesis. Thanks a lot to Dr. Ashis Kumar Nandi and Dr. Supriya Chakraborty for providing me suggestions related to my research work.

My sincere thanks go out to my senior Dr. Mohd. Aslam Yusuf for stimulation to carry out quality research work. His enthusiastic passion to carry out biological experiments was extremely inspiring. It was impossible to compile my whole work without his absorbing discussions and immense mental support.

I greatly appreciate the important assistance from Jogendra, Faiz, and other CIF staff for their help. Thanks a lot to Dr. Gajender Saini and other AIRF staff who helped to perform my experiments timely. I am thankful to the SLS office staff, especially Meenu ma'am for her student friendly nature.

I would like to acknowledge the guidance and help provided by my senior and friend Shashi Kant. I found him all along in every experiment during my research work. The scientific and non-scientific interactions with him will always be cherished.

Working together in the lab with seniors and colleagues stimulated me to finish my study. I would like to acknowledge the guidance and help provided by my seniors Deepak, Nisha Kant, Arun, Manju, Prerna, Preeti, Punam, and Neetu for their constant encouragement and moral support throughout my research work. I acknowledge my senior Bushra for her encouraging guidance.

I thank Ramesh and Subhash ji for their help in performing my experiments timely by providing clean glasswares and other necessary things.

My sincere and heartful thanks to my fellow labmate Saquib. My thanks also go out to other colleagues, Shashi, Ashish, Sameena, Ajit and Neelam for their friendship, help and nice discussions. They not only helped me at different stages of this work but also made my association with lab a wonderful experience. I would also like to thank Bincy, Bhavya, Swati, Sonia, Arushi and Ankit for their valuable support.

This acknowledgement can never be completed without the mention of my dear friends Raghvendra, Nidhi, Omkar, Sampurna, Mudassir, Naresh, Vasudha, Abha and Sarojini. Their constant presence has helped a great deal in the ups and downs of my journey. I am deeply indebted to Ketki Patne (Kitty) for her affection, invaluable help, and support.

I would like to thank all my friends and colleagues at JNU for their ever warm and welcoming attitude. I spent many happy sessions with Shivendra, Abhishek, Shailesh, Praveen, Pragya, Santosh, Shashank and Sumit.

I am sincerely grateful to my uncles and aunts for their constant encouragement. I owe my deep sense of gratitude to my sisters Rekha, Seema, Aarti and Deepawali for their love care and support. I gratefully acknowledge my cousin Dilip Bhaiya, Harendra Bhaiya, Manjesh, Chandan and Narendra for their affection.

Finally, I gratefully acknowledge the Council of Scientific and Industrial Research (CSIR) for providing me the Fellowship (JRF) during the tenure of my M. Phil program.

Mukesh Kumar

List of abbreviations

%	Percentage
~	Approximately
°C	Degree centigrade
β-ME	β-mercaptoethanol
µg	Micrograms
µl	Microlitre
µM	Micromolar
4MU	4-Methylumbelliferone
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CD	Circular Dichroism
CIAP	Calf Intestinal Alkaline Phosphatase
cm	Centimeter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside tri-phosphates
DTT	Dithiothretiol
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
g	Grams
HCl	Hydrochloric acid
hr	Hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilo bases
KDa	Kilo Dalton
L	Litre
LB	Luria broth

M	Molar
mg	Milligrams
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Milliliter
mM	Millimolar
MW	Molecular weight
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
nM	Nanomolar
O.D.	Optical Density
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion concentration
PMSF	Phenyl methyl sulphonyl chloride
RNA	Ribonucleic acid
rpm	Rotation per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
sec	Second
TEMED	N N N' N'-Tetramethylethylenediamine
Tris	Tris [hydroxymethyl] amino methane
UV	Ultraviolet
V	Volt
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

CONTENTS

Table of Contents

CHAPTER 1:
INTRODUCTION

Crop plants growing in natural environment undergo continuous exposure to various stresses like biotic and abiotic stresses. These stresses are unfavorable to the growth of plants and could be caused by drought, flooding, high or low temperature, disease and insect infestation (Rosenzweig, 2001). Biotic stresses affect the crops badly and lead to huge losses in productivity. Several attempts have been made to adopt novel means to develop crops with improved yield through genetic engineering approaches. Transgenic plants have been developed with improved tolerance to biotic stress with the help of genetic engineering tools. Amongst various stresses, insects are one of the major factors which affect crop yields worldwide (Matson, 1997). Insects are arthropods that have a chitinous exoskeleton. Chitin is the main component forming the exoskeleton of insects and crustaceans. It is also present in the micro-filarial sheath of parasitic nematodes (Lee, 2011). Chitin is the second-most-abundant polysaccharide in nature after cellulose. β-(1–4)-poly-*N*-acetyl-D-glucosamine constitute the repeating units of chitin, which are linked by β-1,4 glycosidic bonds. Chitin does not accumulate in the environment because chitinolytic bacteria and saprophytes efficiently recycle most of the chitin in nature. Due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development, they are receiving attention with regard to their development as biopesticides or chemical defense proteins in transgenic plants and microbial biocontrol agents.

Chitinases are produced by many organisms. The biological functions of chitinases in various organisms are diverse (Fan, 2007). Chitinase was used as a natural defence protein by plants to resist pathogen attack (Kumar, 2004). Overproduction of the chitinase in different crop plants makes them resistant to pathogens. There is a need for

further characterization and investigation of chitinases from different species which could be used for commercial purposes in industries or for use as a transgene in making transgenic plants resistant to different pests. The present work was undertaken with the objective of cloning and characterization of the chitinase gene and protein from *Xenorhabdus nematophila*, a bacterium that lives in a symbiotic relationship with *Steinernema carpocapsae,* a nematode, which can be used for raising insect and fungal resistant plants in subsequent research.

The objectives of the present work are outlined below:

1) Isolation of putative chitinase gene from the genomic DNA of *Xenorhabdus nematophila.*
2) Molecular cloning of the chitinase gene.
3) Study the expression of putative chitinase gene in *E. coli.*
4) His-Tag fusion protein purification by Ni-NTA affinity chromatography.
5) Characterization of the putative chitinase protein.

CHAPTER 2:
REVIEW OF LITERATURE

2.1 CHITIN

Chitin is the most widespread amino polysaccharide found in nature. It is mainly found in arthropod exoskeletons, fungal cell walls and microfilarial sheath of helminths. It is the second most abundant biopolymer next to cellulose. Chitin is a β-(1,4)-linked GlcNAc polymer. Since hydrolysis of chitin by chitinase treatment leads to release of glucosamine in addition to *N*- acetylglucosamine so it was concluded that glucosamine might be a significant portion of the polymer. Chitin comprises 16% of the dry weight of the organism in filamentous fungi and basidiomycetes (Gooday, 1979). Cell walls of Mucoraceae contain chitosan in addition to chitin. In yeasts, the amount of chitin in the cell wall is much lower, but bud scars are largely composed of chitin (Kuranda, 1991).

2.2 CHITIN CHARACTERISTICS FOUND IN INSECTS

Insect cuticles form an exoskeleton which is more or less rigid structure due to the presence of chitin and sclerotized proteins. Insects periodically replace their old cuticle with a new one during molting to allow growth and development (Merzendorfer, 2003). This is called ecdysis. Ecdysis is initiated by a process that separates epidermal cells from the old cuticle by molting fluid secretion and ecdysial membrane formation which is called apolysis. The molting fluid contains proteases and chitinases (Reynolds and Samuels, 1996). Chitin is found in the endo- and exocuticle but not in the epicuticle, the outermost part of the integument (Andersen, 1979). Chitin is also an integral part of insect peritrophic matrices. It works as a permeability barrier between the food bolus and the midgut epithelium which enhances digestive processes and protects the brush border

from mechanical disruption and also from attack by toxins and pathogens (Tellam, 1996). Insect growth and development is dependent on the capability to remodel chitinous structures. Hence, insects regularly synthesize and degrade chitin in a highly regulated manner to allow ecdysis and regeneration of the peritrophic matrices. Diflubenzuron, a chemical that interferes with chitin metabolism can be used for the control of agricultural pests. Due to its unique character, chitin is attracting more and more interest as a basic material for the chemical and pharmaceutical industry (Merzendorfer, 2003).

2.3 STRUCTURE OF CHITIN

Chitin is a polymer of N-acetylglucosamine (NAG). NAG is a monosaccharide derivative from glucose (Fig 1). It is an amide between glucosamine and acetic acid.

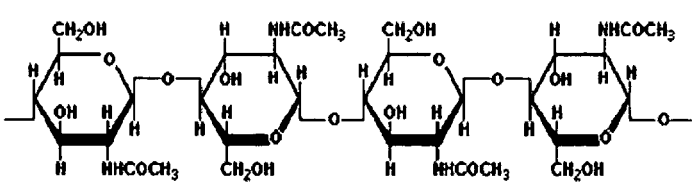


Fig 1: Chitin structure

The X-ray diffraction analysis shows that chitin occurs in three polymorphic forms: α-, β, and γ-chitins (Kurita, 2001). All these forms differ in the arrangement of molecular chains within the crystal cell. Chains are arranged in an antiparallel fashion in α-chitin, parallel in β-chitin and mixed in γ-chitin (Peberdy 1985). The most abundant form of chitin is α-chitin.

2.4 BIOSYNTHESIS AND DEGRADATION OF CHITIN

The synthesis of chitin takes place by the catalysis of precursor uridine diphosphate N-acetyl-D-glucosamine by the enzyme chitin synthase (Kobayashi, 2001). Because chitin is very important in the organisms it is present, its synthesis and breakdown is very much regulated. Chitin synthases and chitinolytic enzymes work together in remodeling chitinous structures (Merzendorfer, 2003). Chitin-degrading enzymes can be used to convert chitin-containing material into biotechnologically utilizable components. Hence, it is very important for the chemical and pharmaceutical industry. Chitinases and their inhibitors may be utilized as insecticides to control pests or as fungicides for the treatment of microbial infections (Kramer, 1997).

Mechanism of chitin hydrolysis by chitinases

Acid catalyzed glycosyl hydrolysis has two pathways:-

1. Retention of the stereochemistry of the anomeric oxygen at C-1 relative to the initial configuration (Scheme 1; Brameld, 1998)

2. Inversion of the stereochemistry (Scheme 2; Tews, 1997)

Hen egg white lysozyme is an example of retaining mechanism (Scheme 1; Iseli, 1996). This mechanism proceeds as follows. The β-(1,4) glycosidic oxygen is first protonated and then stabilized by a second carboxylate through electrostatic or covalent interaction (Brameld, 1998). Water yields the hydrolysis products through nucleophilic attack which retains the initial anomeric configuration. This is commonly known as the double-

displacement mechanism of hydrolysis. Family 19 chitinases have a lysozyme-like fold as deciphered through X-ray crystal analysis which suggests a double-displacement mechanism. The hydrolysis products of family 19 chitinases show inversion of anomeric configuration. Family 18 chitinases yield hydrolysis products that retain the anomeric configuration at C-1. But the X-ray crystal structure shows absence of a second acidic residue in the active site, which stabilizes the oxocarbenium ion. So, none of the mechanism is consistent with the observed structure and hydrolysis products of chitinases (Tews, 1997).

2.5 CHITINASES

Chitinolytic enzymes are essential for maintaining normal life cycle functions in many organisms, like morphogenesis of arthropods or cell division and sporulation of fungi (Kuranda, 1991). They are also found in some organisms that do not contain chitin but can utilize chitin as a nutrient source. Many bacterial genera such as *Streptomyces* sp., convert insoluble chitin into soluble compounds by the action of different chitinolytic enzymes (Charpentier, 1983; Schrempf, 2001). Chitinase genes have been identified in *Autographa californica* which is a member of the family Baculoviridae, which is restricted to arthropod hosts (Hawtin, 1995). This chitinase plays a very important role in viral infectivity (Thomas, 2000; Saville, 2002). Plant and vertebrate chitinases act in defense against chitin-containing pathogens or pests (Boot, 1995; Gooday, 1999). Chitin-degrading enzymes play a crucial role in postembryonic development in insects mainly during larval molting and pupation. Chitinolytic enzymes are also found in the digestive

fluid of spiders, where they help the entry of harmful ingredients through the cuticle of the prey (Krishnan, 1994). Chitin is hard to break due to its physicochemical properties. Hence, its degradation usually requires the action of more than one enzyme type. As a result of these properties, the overall rate of chitin hydrolysis is limited by the action of chitinase.

2.6 CLASSIFICATION AND TYPES OF CHITINASES

Chitinases are classified mainly into two major groups: Endochitinase and Exoochitinase. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites (Shoresh, 2008). It generates low molecular mass multimers of N-acetylglucosamine, like chitotetraose, chitotriose, and diacetylchitobiose (Songsiriritthigul, 2010). Exochitinases can be divided into two subgroups: chitobiosidases (EC 3.2.1.29) and β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30). Chitobiosidase causes release of diacetylchitobiose starting at the nonreducing end of chitin microfibril (Gummadi, 2009). Cleavage of the oligomeric products of endochitinases and chitobiosidases by β-(1,4) N-acetyl glucosaminidases generates monomers of N-acetylglucosamine (Sahai and Manocha 1993). By the action of chitosanase (EC 3.2.1.132) deacetylation of chitin to chitosan takes place which is finally converted to glucosamine residues (Tsigos, 1999).

On the basis of amino acid sequence similarity, chitinases are categorized into families 18, 19, and 20 of glycosyl hydrolases (Henrissat and Bairoch 1993). Family 18 contains chitinases from viruses, bacteria, fungi, animals, and some plants. A characteristic feature of the family 18 chitinases is their multi-domain structure, which is consistently found in

all primary structures. Family 19 contains plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases (Hart, 1995). Family 20 contains the β-N-acetylhexosaminidases from bacteria, *Streptomycetes*, and humans. The families 18 and 19 do not share amino acid sequence similarity. Both have completely different 3-D structures and molecular mechanisms. Therefore, they are likely to have evolved from different species (Suzuki, 1999). Based on the amino acid sequence of individual catalytic domains, bacterial chitinases are separated into three major subfamilies, A, B, and C (Watanabe, 1993). Only chitinases of subfamily A have a third domain insertion of an α+β fold region between the 7^{th} and 8^{th} (α/β)8 barrel. Chitinases in subfamilies B and C do not have this insertion. Several bacterial species like *Serratia marcescens* (Suzuki, 1999), *Bacillus circulans* WL-12 (Alam et al. 1995), and *Streptomyces coelicolor* A3 (Saito, 1999) are reported that possess chitinases belonging to different subfamilies.

Insect chitinases belong to family 18 of the glycohydrolase superfamily (Fitches, 2004). These chitinases have molecular masses ranging from 40 kDa to 85 kDa. They also vary with respect to their pH optima (pH 4–8) and isoelectric points (pH 5–7). The basic structure of insect chitinase consists of three domains (1) the catalytic region (2) a PEST-like region, rich in some amino acids such as proline, glutamate, serine and threonine, and (3) a cysteine-rich region (Kramer, 1997). The last two domains are not necessary for chitinase activity because other naturally occuring chitinases that lack these two regions are still enzymatically active (Girard, 1999; Feix, 2000; Yan, 2002). Different combinations of these three basic domains are found in different chitinases.

The N-terminal half of the enzyme comprises catalytic domain of family 18 chitinases. The N-terminal part of the domain affects the binding or the hydrolysis of the substrate (Perrakis, 1996). Sequence alignments show two highly conserved regions within the catalytic domain (Henrissat, 1991; Coutinho, 2003). The catalytic domain of family 18 chitinases consists of a TIM-barrel structure that forms a groove on the enzyme's surface (Lasters, 1988). This groove is considered as the active center, which binds chitin (Armand, 1994; Drouillard, 1997).

Insect chitinases bind to their substrate through the C-terminal chitin-binding domain, which contains a six-cysteine motif that is also found in nematode chitinases which facilitates catalysis (Venegas, 1996).

2.7 MULTIPLE FORMS OF CHITINASES

Many organisms produce multiple isomeric forms of chitinases from posttranslational processing of a single-gene product or the products of multiple genes. The posttranslational modifications are differential glycosylation and/or proteolysis. Multiple chitinolytic enzymes are known in several microorganisms such as *Bacillus licheniformis* X-74 (Takayanagi, 1991), *Streptomyces* sp. J. 13-3 (Okazaki, 1995), *Pseudomonas aeruginosa* K-187 (Wang, 1997), *Bacillus circulans* WL-12 (Mitsutomi, 1998), *Serratia marcescens* (Suzuki, 2002), and *Streptomyces griseus* HUT 6037 (Itoh, 2002). The synergistic action of chitinases Chi A, Chi B, and Chi C1 of *S. marcescens* 2170 on chitin degradation was also reported (Suzuki, 2002). It was proposed that Chi A and Chi B digest chitin chains in the opposite direction. Chi A degraded the chitin chain from the

reducing end and Chi B from the nonreducing end. Addition of Chi A with Chi B improved chitin degradation efficiency. Four chitinases, I, II, III, and IV were reported in a thermophilic bacterium, *Bacillus licheniformis* X-74. Chitinase I predominantly produced $(GlcNAc)_2$ whereas chitinases II, III, and IV produced $(GlcNAc)_2$ and GlcNAc. Chitinases II, III, and IV converted $(GlcNAc)_4$ into $(GlcNAc)_6$ through transglycosylation reaction (Takayanagi, 1991).

2.8 ACTIVITY ASSAYS FOR CHITINASES

If chitin is degraded by chitinases then chitooligomers, chitobiose, and GlcNAc, are released. This increase in reducing activity can be detected through many methods. Many commercial kits are available for monitoring reducing sugar levels (Howard, 2003). Garcia (1993) describes an easy and inexpensive method using bicinchoninic acid. Culture supernatants isolated from chitin grown cultures can be tested for chitinase activity with this method. A method of fluorescent labeling and then high performance liquid chromatography (HPLC) of the products can be used for the detection of reducing sugars (Kim, 1995).

To measure chitinase activity in solution, a chromogenic assay was developed which uses chromogenic substrates (p-nitrophenyl-labeled substrates) such as pNP-GlcNAc, pNP-$(GlcNAc)_2$, and pNP-$(GlcNAc)_3$. Glucosaminidase, chitobiosidases and endochitinase activities can be calculated by measuring the release of p-nitrophenyl from pNP-GlcNAc, pNP-$(GlcNAc)_2$ and pNP-$(Glc\text{-}NAc)_3$, respectively (Edwards, 2000; Felt, 1998). Chitinase activity can be determined by measuring the amount of reducing sugars.

Reducing sugars are liberated from colloidal chitin by the chitinase activity (Garcia, 1993).

Chitinase activity can also be detected on gels by using fluorescent substrates (Fung, 2002). First the proteins are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Casein–EDTA procedure is used to reactivate enzymes in the gels by removing SDS (Hedrick, 1968). The activity of chitinase enzymes is proportional to the intensity of fluorescent bands on the gel observed under UV illumination, obtained because of the enzymatic hydrolysis of fluorescent 4-methylumbelliferone from the GlcNAc mono- and oligosaccharides, such as 4-MU-GlcNAc, 4-MU-$(GlcNAc)_2$ and 4-MU- $(GlcNAc)_3$ impregnated in the gel. Three different chitinase activities are detected by acting as dimeric, trimeric, and tetrameric substrates, respectively. GlcNAcases release fluorescent product from the dimeric substrate. The trimeric substrate is preferred by the chitobiosidases. Endochitinases are identified by degradation of the tetrameric substrate.

2.9 CHITIN-BINDING ASSAYS AND ISOLATION OF CHITIN-BINDING PROTEINS AND ENZYMES

A significant number of proteins associated with chitin degradation contain chitin-binding domains. These binding domains could conceivably be used to purify chitinolytic enzymes from protein preparations. In fact, a chitin-binding domain from a chitinase of *B. circulans* is employed as a purification tag in the Impact Protein Expression Systems (New England Biolabs, Beverly, Mass.). Secreted chitin-binding proteins or enzymes can

be easily isolated from bacterial cultures by their affinity for chitin. This is especially useful in the event that promoters from an organism of interest are not active in *E. coli*, or if screening of genomic libraries is not possible due to gene toxicity. Chitin incorporated into liquid cultures can be isolated and washed to remove nonspecifically adsorbed proteins. Many chitin-binding domains appear to bind the chitin polymer so tightly that conventional methods for eluting proteins from a matrix are ineffective. The *B. circulans* chitin-binding domain is not eluted from chitin when treated with N-acetyl-glucosamine, chitooligosaccharides, or high concentrations of sodium chloride. SDS-PAGE loading dye can be used to elute bound proteins for use in zymograms. Alternatively, addition of 1% SDS or 6 M guanidinium hydrochloride followed by incubation at 50–80 °C can be used to denature and remove bound proteins.

2.10 CHITINASE PRODUCTION

Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation, and fed-batch fermentation. In addition to these, solid-state fermentation and biphasic cell systems have also been used for the production of chitinase. Generally, chitinase produced from microorganisms is inducible in nature. Extracellular chitinase production is reported to be influenced by media components such as carbon sources, nitrogen sources, and agricultural residues such as rice bran, wheat bran, etc. (Bhushan 1998; Dahiya, 2005). An enhancing effect of glucose on chitinase production was reported by Bhushan (1998) when glucose was used with chitin in production medium. However, a suppressing effect of glucose on chitinase production was reported by

Miyashita (1991). Several other physical factors such as aeration, pH, and incubation temperature also affect chitinase production. The addition of amino acids and their analogs such as tryptophan, tyrosine, glutamine, and arginine (0.1 mM) in the growth medium stimulated chitinase production from *Bacillus* sp. BG-11 (Bhushan, 1998). Some other methods, such as cell immobilization (Bhushan, 1998), biphasic cell systems (Chen, 1995), solid-state fermentations, etc., have been used for improving chitinase production from different microorganisms (Bhushan, 1998). In an immobilized system, whole cell immobilization of an organism to a solid support such as polyurethane foam was applied. Chitinase production was enhanced up to 4.8-fold over a period of 72 h in submerged fermentation. Enhanced production of extracellular chitinase by *S. marcescens* in an aqueous two-phase system (ATP) of PEG and dextran was reported by Chen, (1995). They reported a maximum chitinase activity of 41.5 units in ATPs [2% (w/w) PEG 20,000 and 5.0% (w/w) dextran T 500] compared with 13.6 units in a polymer-free system. In solid substrate fermentation using flake chitin as the solid substrate, the maximum chitinase yield obtained from *Enterobacter* sp. NRG4 was 616 U/g solid substrate after 168 h of growth at 30°C and 75% moisture level. When wheat bran was used in combination with chitinous substrates, the chitinase yield increased. Maximum enzyme yield was 1475 U/g at wheat bran to flake chitin ratio, 1; moisture, 80%; and inoculum, 2.6 ml after 168 h (Dahiya, 2005).

2.11 *XENORHABDUS NEMATOPHILA*

Xenorhabdus nematophila is a symbiotic bacterium present in the entomopathogenic nematode *Steinernema carpocapsae.* This bacetria is fatal for a wide range of agriculturally important insect pests (Poinar, 1979). Both the partners, the bacterium and the nematode, can survive in the absence of the other. But *X. nematophila* is required for *S. carpocapsae* nematodes to reproduce efficiently during their life cycle (Mitani, 2004) (Fig 2). This bacterium has symbiotic and pathogenic stages. The symbiotic phase is completed in the nematode gut, whereas pathogenic stage in the insect larval body (Joshi, 2008). *X. nematophila* is a gram-negative facultatively anaerobic bacterium. There are numerous peritrichous flagella found on the bacteria (Akhurst, 1990).

Classification of *X. neamtophila*

Bacteria

Proteobacteria

Gammaproteobacteria

Enterobacteriales

Enterobacteriaceae

Xenorhabdus

Nematophila

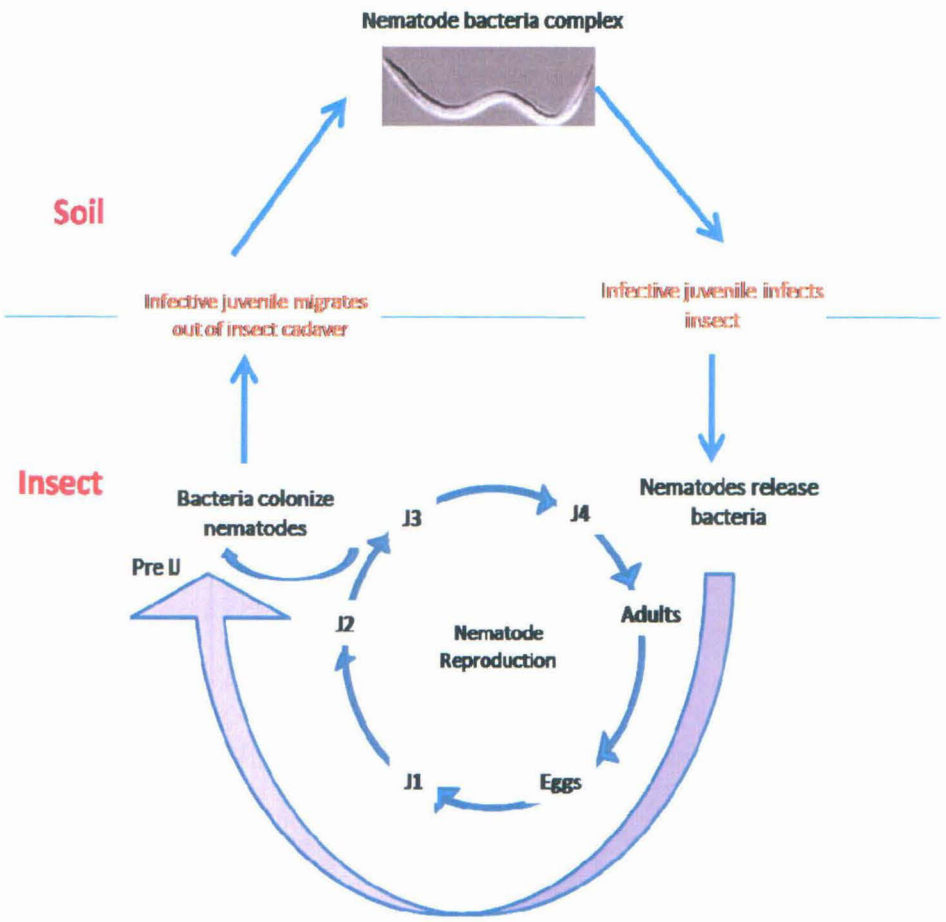


Fig 2: Life cycle of *Xenorhabdus nematophila*.

The *X. nematophila* and *Steinernema carpocapsae* association is highly toxic to many insect species, causing rapid larval death (Joshi, 2008). The chitinase present in the bacteria may be utilized to degrade chitin present in the insects that cause damages to various crops. The *X. nematophila* chitinase had not been characterized until the present study.

CHAPTER 3:
MATERIALS AND METHODS

3.1 SOURCES OF MATERIALS AND CHEMICALS

The genomic DNA of *Xenorhabdus nematophila* 19061 was received as a kind gift from Prof. Nirupama Banerjee, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi.

Most of the chemicals like Tris-HCl, potassium acetate, sodium acetate, EDTA, sodium dodecyl sulphate (SDS), sodium chloride, agarose, etc. were of molecular biology grade from Sigma Chemical Company, St. Louis, USA. Antibiotics like Ampicillin, Kanamycin, dyes like Bromophenol blue, Ethidium Bromide and biochemicals like lysozyme were of regular grade and purchased from Sigma Chemical Company, USA. Luria broth and agar powder were obtained from Hi-Media laboratories, Mumbai. Organic solvents like chloroform, isopropyl alcohol, acetone, etc. were procured from Fisher Scientific, Mumbai.

Plasmid spin miniprep kit, QIAquick PCR purification kit and QIAquick gel extraction kit were purchased from Qiagen Inc., Valencia, USA. Pgemt Easy cloning kit was obtained from Promega Corporation, Madison, USA. All Restriction Endonucleases, T4 DNA ligase and Taq DNA Polymerase were purchased from MBI, Fermentas.

3.2 ORGANISMS AND GROWTH CONDITIONS

The genotype of *E. coli* DH5α used was F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1. The genotype of *E. coli* BL21 (DE3) was F- *ompT* *hsd*SB(rB-, mB-) *gal dcm* (DE3). The fresh standard liquid media of Luria Bertani (LB) Broth was used for microbial

growth in selective liquid cultures. LB medium was used for selective plate cultures. The medium was used in techniques like preparation of competent cells, transformation of *E. coli*, plasmid isolation and also for preparing frozen glycerol stocks.

Clone details:

Clone name- **Chitinase-pGEMT easy**

Insert name- Putative chitinase

Vector name- pGEMT-easy

Restriction sites- TA cloning

Size of insert- 2 kb

Size of vector- 3 kb

Size of clone- 5 kb

Clone name- **Chitinase-pET28b**

Insert name- Chitinase

Vector name- pET28b

Restriction sites- Insert- *XhoI* and *NotI*

Vector- *SalI* and *NotI*

Size of insert- 2 kb

Size of vector- 5.3 kb

Size of clone- 7.3 kb

Size of expressed protein- 76 KDa

Clone name-	**Chitinase-pRT101**
Insert name-	Chitinase
Vector name-	pRT101
Restriction sites-	*XhoI* and *BamHI.*
Size of insert-	2 kb
Size of vector-	3.3 kb
Size of clone-	5.3 kb

Clone name-	**Chitinase-pCAMBIA1301**
Insert name-	CaMV35S-chitinase-polyA
Vector name-	pCAMBIA1301
Restriction sites-	*HindIII*
Size of insert-	3 kb
Size of vector-	11.8 kb
Size of clone-	14.8 kb

3.3 MAINTENANCE OF BACTERIAL CULTURE

All *E. coli* strains were maintained as stocks in 50% glycerol frozen at -80°C. To revive these cultures, the frozen stocks were streaked on LB agar with appropriate antibiotic selection to obtain a working plate.

All media and solutions were made in milli Q water. All solutions and media were sterilized by autoclaving at 15 lb/sq. inch for 15 min or filter sterilized by passing through a 0.22 µM Millipore filter.

3.4 PREPARATION OF PLASMID DNA FROM *E. COLI* TRANSFORMANTS

3.4.1 Mini-preparation of plasmid DNA (Alkaline lysis method)

E. coli culture having the plasmid was inoculated in (10 µl) LB broth containing 100 µg/ml ampicillin. This was allowed to grow overnight at 37°C. 2 ml of overnight grown culture was added in an eppendorf and centrifuged at 12,000 rpm for 1 min at room temperature. The pellet was resuspended in 100 µl of ice cold solution I by vortexing. 200 µl of solution-II (SDS: NaOH) was added and mixed gently by inverting the tube 3-4 times. 150 µl of ice cold solution-III was added, mixed thoroughly and incubated on ice for 10 min and then centrifugation was done for 15 min. The supernatant was taken out carefully and 0.3 ml of isopropanol was added, incubated at room temperature for 15 min and then centrifuged at room temperature for 10 min at 12,000 rpm. The pellet obtained was of plasmid DNA. The pellet was washed once with 1 ml of 70% ethanol, dried under vacuum and dissolved in 50 µl of TAE containing 20 µg/ml of DNase free RNase by tapping the tube gently.

Solution I:

25 mM Tris-HCl, pH 8.0

10 mM EDTA

50 mM Dextrose

Solution II:

1% SDS

0.2N NaOH

Solution III:

60 ml of 5M potassium acetate

11.5 ml of glacial acetic acid

28.5 ml of H_2O

10X TAE:

0.4 M Tris

0.01 M EDTA

0.2 M Acetic acid

pH 8.5

3.4.2 Plasmid isolation kit

Qiagen Plasmid Purification kit was also used for the plasmid isolation from *E. coli* cells. A single colony was picked from a freshly streaked selective plate, inoculated in a culture of 10 ml LB medium containing the appropriate selective antibiotic and incubated for 12–16 h at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 13000 rpm for 3 min at room temperature. The pelted cells were resuspended in 250 µl of Buffer P1 and transferred to a microcentrifuge tube. Then 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times. This was followed by neutralization with 350 µl of Buffer N3, immediate and thorough mixing by inverting the tube 4–6 times and centrifugation at 13,000 rpm for 10 min. The supernatant was applied to the QIAprep spin column followed by centrifugation for 30–60 s. The flow-through was discarded and the column was washed with 0.75 ml of Buffer PE and centrifuged for 30–60 s. The

flow-through was again discarded and the column was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of each QIAprep spin column, let stand for 1 min, and centrifuged for 1 min.

3.4.3 Agarose gel electrophoresis

The percentage of agarose in the gel was adjusted to meet the requirement of the experiment to obtain maximum resolution of the DNA fragments. Generally 0.8% agarose was used.

Solutions:

Gel loading solution

0.5% Bromophenol blue

50% glycerol in Sterile H_2O

Ethidium bromide stock:

0.5 mg /ml in H_2O

In 1X TAE, appropriate amount of agarose was suspended and heated in a microwave oven to obtain a clear gel. Ethidium bromide was added after cooling the gel to 55°C so that the final concentration became 0.5µg/ml. The gel was run in 1X TAE buffer at 80V and the band was visualized under UV illumination.

3.4.4 Elution of DNA from agarose gel

DNA was digested with appropriate restriction enzymes, as required, in a buffer optimal for the enzyme activity. The DNA fragments were separated by agarose gel electrophoresis. The gel slice containing the desired fragment was excised carefully under UV light. The gel slice was placed in an eppendorf tube and the DNA was eluted by using QIAquick Gel Extraction Kit (Qiagen, Germany).

3.5 DNA MANIPULATION FOR CLONING PURPOSES

3.5.1 Polymerase Chain Reaction (PCR)

For amplification of chitinase gene the genomic DNA of *Xenorhabdus nematophila* was used as a template. A 50 µl PCR reaction was set up containing 1X *Pfu* buffer (with $MgSO_4$), 0.2 µM of each of primers, 0.2 mM dNTPs mixture, 1.5 mM $MgCl_2$, and 2.5 units of *Pfu* polymerase.

Primers used for the amplication of the chitinase gene were:

Forward Primer: 5'-CATCTCGAGATGTCTCAAAATGTTTATCG-3'

Reverse Primer: 5'-TAGGGATCCCTACGATTTACGACG-3'

PCR conditions for amplification of putative chitinase using *Pfu polymerase*:

Step No.	Step	Temperature (^{0}C)	Time
1.	Initial denaturation	95	3 min
2.	Denaturation	95	30 sec
3.	Annealing	62	30 sec
4.	Elongation	72	4 min
5.	Final elongation	72	10 min
6.	Hold	4	

Step No. 2, 3, and 4 were repeated for 30 cycles.

For colony PCR, 25 µl PCR reaction was set up containing 1X *Taq* buffer (with $MgCl_2$), 0.2 µM of each of primers, 0.2 mM dNTPs mixture, 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase.

PCR conditions for colony PCR:

Step No.	Step	Temperature (^{0}C)	Time
1.	Initial denaturation	94	5 min.
2.	Denaturation	94	30 sec
3.	Annealing	62	30 sec
4.	Elongation	72	2 min
5.	Final elongation	72	10 min
6.	Hold	4	

Step No. 2, 3, and 4 were repeated for 30 cycles.

For construction of site-directed mutant of chitinase, the pET28b-chitinase plasmid was used as a template. A 50 µl PCR reaction was set up containing 1X Phusion HF buffer, 0.5 µM of each of primers, 0.2 mM dNTPs mixture, 3.5 mM $MgCl_2$, 2.5 units of Phusion DNA polymerase, 3% (v/v) DMSO.

3.5.2 Restriction enzyme digestion of DNA

Digestion of DNA using restriction endonucleases like *Bam*HI, *NotI, XhoI,* etc. was done for cloning and confirmation of the different vector constructs. All the conditions for the reaction like buffers, temperature, and time were followed as recommended by the manufacturers (MBI Fermentas, USA).

The different components were added in a micro centrifuge tube on ice as follows:

Nuclease-free water	: 17 µL
10X buffer (NEB USA)	: 2 µL
Plasmid DNA	: (0.2-1µg μL^{-1})
Restriction enzymes	: 1 µL
Final volume	: 20 µL

The contents were mixed gently and incubated at appropriate temperatures (usually 37°C) for 90 min to 3 hrs. The samples were spun down and subjected to heat inactivation of the enzymes at 65°C for 10-20 min. The complete digestion of plasmid and the insert was visualized on 1% agarose gel containing ethidium bromide (10 µg/ml) under UV illumination.

3.5.3 Dephosphorylation of DNA termini

To prevent the possibility of self ligation of the vector itself, dephosphorylation of the 5' overhang was done using Calf Intestinal Alkaline Phosphatase (CIAP). For the CIAP treatment, the vector DNA digested with the desired restriction endonuclease was eluted from the agarose gel slice and then treated with CIAP in appropriate buffer. The reaction was stopped by heating at 80^0C for 10 minutes. The dephosphorylated DNA was purified using a PCR purification kit (Qiagen, Germany).

3.5.4 Ligation of DNA termini

The insert and vector DNA were digested with appropriate restriction endonucleases to generate compatible ends. The concentration of the insert and vector DNA was estimated spectrophotometrically and by observing the band intensity on an agarose gel. The molar ratio of the insert and vector DNA in the ligation mixture was 3:1 and 5:1. The ligation mixture contained 1X T4 ligation buffer, 1U T4 DNA ligase and insert and vector DNA as per the calculation for the 3:1 or 5:1 molar ratio in a final volume of 20 µl. The ligation mixture was incubated at 16^0C for 16 hours. This ligation mixture was used directly for transformation in the competent *E. coli* cells.

3.5.5 Preparation of competent *E. coli* cells

E. coli DH5α competent cells were prepared by $CaCl_2$ treatment according to Sambrook et al (1998) with slight modifications. A flask having 50 ml of LB

medium was inoculated with 500 µl of overnight grown culture of DH5α. It was allowed to grow at 37^{0}C at 220 rpm untill OD_{600} reached 0.5-0.6. The culture was allowed to cool on ice for 15-20 min and centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was discarded and pellet was resuspended in 10ml of ice-cold 100 mM $CaCl_2$ by gently swirling the tube. The resuspended pellet was incubated on ice for 2 h. The cells were again centrifuged at 3000 rpm for 10 min at 4^{0}C and the pellet obtained was again resuspended in 1ml of ice-cold 100 mM $CaCl_2$ by gently swirling the tube. It was stored on ice for 12h. After 12h, 1ml 50% sterile glycerol made in 100 mM $CaCl_2$ was added gently. The competent cells were stored in aliquots of 200 µl in sterilized tubes. The competent cells were stored at –80^{0}C freezer after snap freezing into liquid nitrogen.

3.5.6 Transformation of competent *E. coli* cells

For transformation of DNA, *E. coli* strain DH5α competent cells were taken out from -80^{0}C and kept on ice for 5 min to thaw the cells. An appropriate volume of recombinant DNA (~100 ng) was mixed with the cells and incubated on ice for 30 min. Heat-shock was given for 90 sec at 42^{0}C. The tubes were immediately transferred on ice for 10 min. Thereafter, 800 µl of LB medium was added to it. These tubes were incubated for 1h at 37^{0}C with shaking at 220 rpm. Cells were then centrifuged at 5000 rpm for 10 min, the pellet was resuspended in 100 µl of LB, and plated on LB agar plates containing suitable antibiotics. The LB agar plates were incubated overnight at 37^{0}C. The observed colonies on the plates were screened for

the presence of insert using colony PCR. Further confirmation of the insert was done by plasmid isolation and restriction digestion.

3.5.7 Screening of transformants by colony PCR

The transformed *E. coli* colonies obtained on the selection medium were analyzed for the presence of the recombinant plamid by using colony PCR. The colonies were streaked again on LB agar plate containing appropriate antibiotic to get secondary colonies. The secondary colonies were lysed by boiling in 15 µl water. The lysate, thus obtained, was directly used as the template in PCR reaction for confirming the amplification of the cloned insert as per the steps outlined in an earlier section.

3.5.8 DNA Sequencing

The DNA sequencing was got done through commercial DNA sequencing facility at Eurofins Genomics India Pvt Ltd, Bangalore, India using universal primers or gene specific primers, as per the requirement.

3.6 RECOMBINANT PROTEIN PURIFICATION

E. coli cells harbouring the pET28b-chitinase construct were grown overnight in LB medium containing 50 µg/ml kanamycin, sub-cultured in fresh 50 ml LB medium and grown till OD_{600} reached 0.5-0.6. The culture was induced with 1 mM IPTG and incubated for another 4-5 h. The cells were washed, resuspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl) and disrupted by sonication. The lysate was

centrifuged at 10,000 rpm for 20 min. The supernatant, thus obtained, was the soluble fraction. The pellet was resuspended again in 10 ml lysis buffer and sonicated and centrifuged as before and finally washed with 1 M NaCl followed by centrifugation. The pellet was resuspended in lysis buffer and kept at 37°C for 1 hour followed by centrifugation to get the insoluble fraction. The two fractions were loaded onto Ni-NTA agarose (Qiagen, Germany) affinity column pre-equilibrited with the 50 mM Tris, pH 8.0, 300 mM NaCl. The column was washed with 5 column volumes of the 50 mM Tris, pH 8.0, 300 mM NaCl buffer followed by 5 column volumes of 50 mM Tris, pH 8.0, 300 mM NaCl with 20 mM Imidazole to remove non-specifically bound proteins. The recombinant protein was eluted with buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl and 100 mM Imidazole. The different fractions were resolved on a 12% SDS-PAGE. Fractions containing single band protein were pooled and dialyzed extensively against 50 mM phosphate buffer, pH 7.6, at 4°C. The purity of the final fractions was determined by SDS–PAGE.

3.7 PROTEIN GEL ELECTROPHORESIS (SDS-PAGE)

The proteins were resolved by Sodium Dodecyl – Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The proteins were boiled for 5 min after adding 1X gel loading sample buffer and electrophoresed on a 12% SDS-polyacrylamide gel. The recipe for preparation of separating and stacking gel and buffers used is given below. After the run, the gel was stained with Coomassie Brilliant blue and photographed.

Separating Gel (20 ml):

Components	12%
Water	7.95 ml
Acryl/bisacrylamide (30 %)	6.65 ml
Lower Tris 1.5M, pH 8.8	5 ml
SDS (10 %)	0.2 ml
APS (1.5 %)	0.2 ml
TEMED	8 µl

Stacking Gel (3.75 %): 20 ml

Water	11.3 ml
Acryl/bisacrylamide (30 %)	2.5 ml
Upper Tris 0.5M, pH 6.8	5.0 ml
SDS (10 %)	0.2 ml
APS (1.5 %)	1.0 ml
TEMED	15 µl

Tris-Glycine Running Buffer (10 X):

Tris Base	30 g
Glycine	144 g
SDS	10 g

Water to make final volume 1000 ml.

pH should be 8.5-8.8

Acryl amide stock (30 %):

Acrylamide (29.2 %)	58.4 g
N-N'-Methylene bisacrylamide (0.8%)	1.6 g

Dissolved in 125 ml volume made up to 200 ml and filtered through Whatman 1 paper.

Staining Solution:

Methanol	400 ml
Glacial acetic acid	200 ml
Coomassie Brilliant Blue	2 g
Water	400 ml

Destaining Solution

Methanol	1000 ml
Glacial acetic acid	200 ml
Water	800 ml

3.8 BIOCHEMICAL STUDIES

3.8.1 Protein estimation

Protein concentration was determined by the Bradford assay. Bradford's reagent was purchased from Sigma Aldrich. Assay was performed by adding 1 ml of reagent to 5-50 µl of protein sample. The absorbance was taken at 595 nm. BSA was used as a test protein for plotting a standard curve.

3.8.2 Spectrophotometric Estimation of Nucleic acids

The quantity and quality of the nucleic acid was determined spectrophotometrically by measuring the OD at 260 nm and 280 nm using a nanodrop spectrophotometer. The amount of DNA was calculated by taking A_{260} and A_{280}. The purity of the nucleic acid was determined by calculating the ratio of the A_{260}/A_{280}.

3.8.3 Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectroscopy plays an important role in the study of protein folding as it allows the characterisation of secondary and tertiary structures of proteins in native, unfolded, and partially folded states. Secondary structure can be determined by "far-UV" spectral region (190-250 nm).

The concentration of the protein used for the CD was 0.4 mg/ml. It was diluted five times with the buffer used (50 mM phosphate buffer, pH 7.6). First, base reading of the buffer was taken. Then sample of the protein was used for the CD spectrum. The reading was taken at the wavelength range 200 nm-260nm. This reading was used for the calculation of percentage of different secondary structures.

3.8.4 Chitinase assay

The chitinase assay kit of Sigma Aldrich, USA was used for determining the activity of the purified chitinase. This enzyme assay depends upon the hydrolysis of chitinase substrates. The hydrolysis of the substrate releases 4-Methylumbelliferone (4MU) which is a fluorescent molecule that can be measured fluorimetrically at an excitation wavelength of 360 nm and an emission wavelength of 450 nm in the basic pH environment.

Three different substrates were used for the detection of the various types of the chitinolytic activities. First one was 4-Methylumbelliferyl N,N'-diacetyl-β-D-chitobioside which is used for the detection of chitobiosidase activity of the enzyme. Chitobiosidase activity is a type of exochitinase activity. Second substrate is 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide which is also used for the

exochitinase activity (β-N-acetylglucosaminidase activity). The third substrate 4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotriose was used for the endochitinase activity of the enzyme.

The reaction mixtures were prepared according to the manufacture's recommendations as follows:

S.N.	Assay	Substrate Working Solution	Sample or Standard Solution	Assay Buffer
1	Blank	150 µl	-	-
2	Positive Control	143 µl	7 µl of Chitinase Control Enzyme	-
3	Sample	143 µl	7µl of chitinase (purified)	-
4	Standard Blank	-	-	150µl
5	350ng/assay Standard	-	7µl of 50µg/ml	143µl

The mixture was incubated at 37^0C for 40 minutes. Reaction was stopped by adding 300µl of stop solution. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

The chitinase activity was calculated using only a single standard concentration. The fluorescence of 350 ng (6.65µmole/ml) standard was measured and then the following equation was used:

$$\text{Units/ml} = \frac{(FLU_{sample} - FLU_{blank}) \times 6.65 \times 0.45 \times DF}{FLUs_{standard} \times time \times V_{enz}}$$

FLU_{sample} = Fluorescence of the sample

FLU_{blank} = Fluorescence of the Blank (containing only substrate working solution)

0.45 = Final reaction volume in milliliters after addition of the stop solution

DF = Enzyme dilution factor

$FLU_{standard}$ = Fluorescence of the standard solution minus the fluorescence of the standard Blank.

time = minutes

V_{enz} = Volume of the sample in milliliter

CHAPTER 4:
RESULTS

4.1 ISOLATION OF PUTATIVE CHITINASE GENE FROM THE GENOMIC DNA OF *XENORHABDUS NEMATOPHILA*

PCR based approach was used to isolate the putative chitinase gene *of Xenorhabdus nematophila*. The putative chitinase gene sequence of *X. nematophila* is present in the GenBank database. Forward and Reverse primers were designed based on this sequence and used for the PCR amplification of the gene. The annealing temperature was estimated by doing a gradient PCR and using a gradient from 58^0C to 64^0C. The amplified product was checked on a 0.8% agarose gel (Fig. 3). The putative chitinase gene was found to amplify at the annealing temperature from 58^0C to 62^0C but a single band without any spurious annealing appeared only at 62^0C. This annealing temperature (62 ^{0}C) was used for further amplification of the putative chitinase gene. The amplified PCR product of the gene was used for the cloning into the pGEMT-easy vector.

4.2 CLONING AND SEQUENCE ANALYSIS OF THE PUTATIVE CHITINASE GENE

T-vector based cloning is a popular method of cloning the amplified PCR product using *Taq* and other polymerases. *Taq* polymerase lacks 5' to 3' proofreading activity and is able to add adenosine triphosphate residue to the 3' ends of the double stranded PCR product. These PCR products can be cloned in a linearized vector with complementary 3' T-overhangs. But due to the lack of proofreading activity of the *Taq* polymerase, it cannot be used for the amplification of a long gene targets. *Pfu* DNA polymerase is a thermo-stable enzyme which is also having proofreading activity, but

the generated PCR fragments have blunt ends. It is not possible to clone blunt ended PCR amplified product into pGEMT-easy vector. So it is needed to generate or add extra Adenine at the 3' end in order to clone it in a T-A cloning vector like pGEMT-easy (Fig. 4). Therefore, the putative chitinase gene amplified using the *Pfu* polymerase, as above, was incubated with *Taq* DNA polymerase for 10 minutes to add the adenine at the 3' ends of the amplicon. Thereafter, the putative chitinase gene was ligated to the pGEMT-easy vector. The ligated product was transformed in the *E. coli*. The recombinant colonies were screened using colony PCR. The confirmation of the clone was done by restriction digestion. After the double digestion with *XhoI* and *BamHI*, a ~2 kb fallout was observed (Fig. 5). This chitinase-pGEMT easy plasmid construct was sequenced. The sequence was compared with the putative gene sequence present in the database. The BLAST results showed 100 % identity.

The sequence of the cloned chitinase gene was submitted to the GenBank database (Accession No: JN222361). The nucleotide sequence and the amino acid sequence for the chitinase gene are shown in Fig. 5.

4.3 EXPRESSION OF PUTATIVE CHITINASE PROTEIN IN *E. COLI*

The putative chitinase gene was cloned in the expression vector pET28b which has a provision for introducing a hexa-histidine tag at the N-terminal of the expressed protein (Fig. 6). The chitinase gene was released from the pGEMT easy-chitinase clone obtained as described above by digestion with *XhoI* and *NotI*. The pET28b vector was digested with *SalI* and *NotI*. The restriction enymes *XhoI* and *SalI* generate compatible ends. The chitinase gene fragment was ligated with the linearized

pET28b vector using T4 DNA ligase. After the ligation the *XhoI/SalI I* site was lost. The pET28b vector having the chitinase gene ligated was transformed into *E. coli* BL21 (DE3) cells and the recombinant clones were identified through colony PCR (Fig. 7). Further confirmation of the clone was done by restriction digestion with *BamHI*. After the double digestion with *SalI* and *NotI* no fall-out was obtained, as expected, because of the loss of *SalI* site (Fig. 8). The correct sequence of the chitinase gene into the pET28b vector was confirmed by sequencing (Fig. 9).

The expression of the chitinase fusion protein in *E. coli* BL-21 cells was induced by adding different concentrations of IPTG (0.1mM, 0.2mM, 0.5mM, 0.7mM and 1.0 mM) at 37^{0}C. The expression of the fusion protein was observed after 5 h of induction. All the tested concentrations of IPTG caused induction of recombinant protein expression after 5 hours of IPTG addition to the culture (Fig. 10).

The recombinant protein was found in the cell pellets as well as in the supernatant after sonication. It indicates that the expression of the fusion protein at high levels in *E. coli* resulted in the formation of a mass of inclusion bodies. A protein band of approximately 76 kDa was observed in the supernatant and pellet fractions after the lysis of the induced *E. coli* clones (Fig. 10).

4.4 PURIFICATION OF THE FUSION PROTEIN

In order to study the function of the putative chitinase the gene was expressed as a fusion protein with a His tag at the N-terminus as described above. Since the expressed protein had a His tag at its N-terminus, purification of the protein was

done using Ni-NTA agarose. The fusion protein present in the supernatant fraction was bound to the Ni-NTA agarose resin as described in the materials and methods section. The resin was further washed with buffer containing NaCl and the purified fusion protein was eluted from the resin using imidazole containing buffer. Protein fractions at different stages of purification were analysed on 12% SDS-PAGE. A partially purified band (~76 KDa) was detected on a Commassie stained gel. Fig. 11 shows the protein profile of the purified recombinant protein of the putative chitinase. The pooled fractions containing the purified protein were dialysed against 50 mM phosphate buffer (pH 7.6), the protein was aliquoted and stored at – 20 C for further use.

The purified protein band was processed for MALDI-TOF analysis. Fig. 12 shows the mass spectrophotometric analysis of the purified protein. It shows the spectrum of the trypsin digested protein band. The peaks matched with the putative chitinase in the database (Mascot search). The peptide ions gave intensity coverage of 64.8 % and sequence coverage MS 34.9 %. Fig. 13 shows the Mascot search results of the trypsin digested peptide ions.

4.5 CHARACTERIZATION OF THE *XENORHABDUS NEMATOPHILA* CHITINASE

4.5.1 Chitinase Assay

Three substrates were used for the detection of the various types of the chitinolytic activities. 4-Methylumbelliferyl N,N'-diacetyl-β-D-chitobioside is used for the

detection of chitobiosidase activity, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide is used for the chitobiosidase activity and 4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotriose was used for the endochitinase activity of the enzyme. The chitobiosidase activity of the *X. nematophila* chitinase was very low (0.43 units/mg protein) while the β-N-acetylglucosaminidase activity and endochitinase activity were 23.1 units/mg and 15.36 units/mg protein, respectively (Fig. 14).

4.5.2 In-silico characterization of *X. nematophila* chitinase

4.5.2.1 Multiple Sequence Alignment

The homology of the *X. nematophila* chitinase gene with the chitinase genes reported from other organisms was evaluated using blastp and blastn program. The chitinase gene from *X. nematophila* showed 56 % identity at the protein level with the sequence of an exochitinase ChiB2 from *Photorhabdus temperata* (Fig. 15). At the nucleotide level, it was 64 % identical to the MH-1 insecticidal toxin island of *Yersinia* sp. It also showed 64 % identity with *Xenorhabdus bovienii* at the nucleotide level and 56 % Identity at protein level. The identity of the chitinase with other species was: *Salmonella enterica* (40 %), *Trichoderma viride* (28 %), *Metarhizium anisopliae* (30 %), *Arabidopsis thaliana* (25 %), *Nicotiana tabacum* (24 %), and *Bombyx mori* (32 %).

4.5.2.2 Structural characteristics of the *X. nematophila* chitinase

The conserved domains in the *X. nematophila* chitinase were searched using the tool at NCBI. Two highly conserved domains identified were GH18 and ChiA. The GH18 (glycosyl hydrolases, family 18) type II chitinases hydrolyze chitin, an abundant polymer of N-acetylglucosamine and have been identified in bacteria, fungi, insects, plants, viruses, and protozoan parasites. The structure of this domain is an eight-stranded alpha/beta barrel with a pronounced active-site cleft at the C-terminal end of the beta-barrel. The ChiA domain is involved in the carbohydrate transport and metabolism. It is classified as a model that may span more than one domain and is not assigned to any domain superfamily.

The percentage of different secondary structures like α-Helix and β-sheet are calculated online through SOMCD (http://geneura.ugr.es/cgi-bin/somcd/index.cgi) from the CD data (Fig. 16). The putative chitinase protein from *Xenorhabdus nematophila* has 48 % α-Helix, 9.5 % β-sheet, 16.9 % Turn and 25.6 % random coils.

4.5.2.3 Cladogram

The chitinase sequences from different organisms compared by the Multiple Sequence Alignment were used to generate a Cladogram for deciphering the evolutionary relationship amongst them. The Cladogram generated using the tool at the EBI website (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) showed *X. nematophila* to be evolutionarily most close to *S. enterica* (Fig. 17).

4.5.2.4 Protein structure prediction

Protein Homology/analogY Recognition Engine (PHYRE) web server was used for the structure prediction of the chitinase protein. The PHYRE server uses the database of structural classification of protein (SCOP) and protein data bank (PDB). The accuracy of protein structure prediction depends mainly on the sequence similarity between the query and the template. If a template has >30% sequence identity to the query, then usually most of the alignment will be accurate. In this condition the relative positions of structural elements in the model will be reliable. Below 30% sequence identity, confident matches are routinely made by PHYRE.

After submission of the chitinase sequence on the PHYRE web server (http://www.sbg.bio.ic.ac.uk/phyre/), many model structures were found. The most accurate model structure uses the template family 18 chitinaseA (ChiA) from *Serratia marcescens* (Fig. 18). The model structure was further verified by the structural superposition of the model structure and the template structure (Fig. 19). The root mean square deviation (RMSD) value which is important because it indicates the divergence of the model and template structures was calculated to be 3.1 A^{o}. On the basis of the model structure and template structure, we can easily determine the key amino acids residues involved in catalysis of chitin which is a substrate for chitinase.

4.6 CLONING OF *X. NEMATOPHILA* CHITINASE GENE IN BINARY VECTOR pCAMBIA 1301 FOR PLANT TRANSFORMATION

The *X. nematophila* chitinase gene was cloned in a subcloning vector pRT101 at *XhoI* and *BamHI* sites in between CaMV35S promoter and polyA terminator sequences. The CaMV35S-chitinase-polyA cassette was excised from this vector by digestion with *HindIII* endonuclease and then cloned in the binary vector pCAMBIA1301 at the *HindIII* site (Fig. 20). The confirmation of the clone was done by restriction digestion (Fig. 21). This recombinant plasmid was then mobilized into the *Agrobacterium* strain LBA4404 for future use in plant transformation experiments.

FIGURES

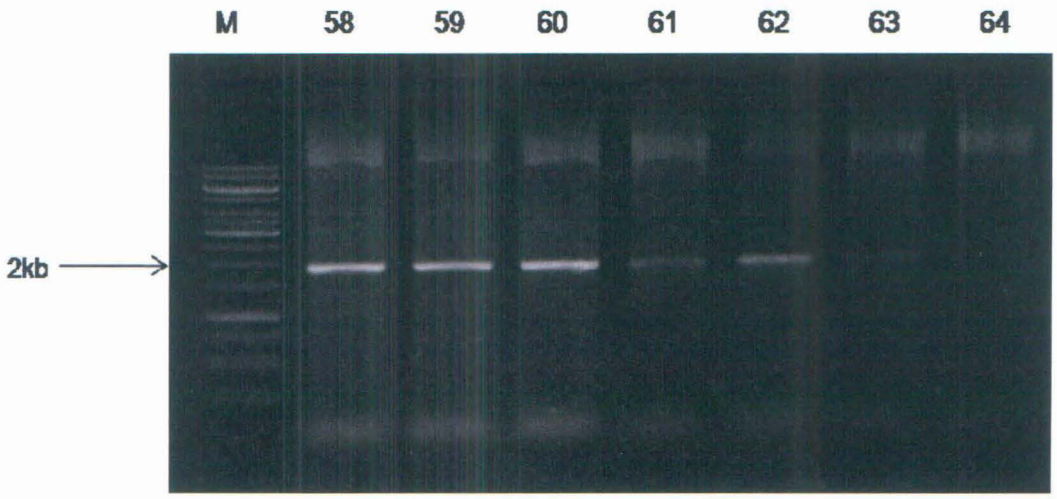


Fig 3: Gradient PCR amplification of the chitinase gene to find out the annealing temperature. The genomic DNA of *X. nematophila* was taken for the gradient PCR amplification. Amplification at different annealing temperatures (58^0C – 64^0C) was checked. At 62^0C annealing temperature, the amplification was found to be good.

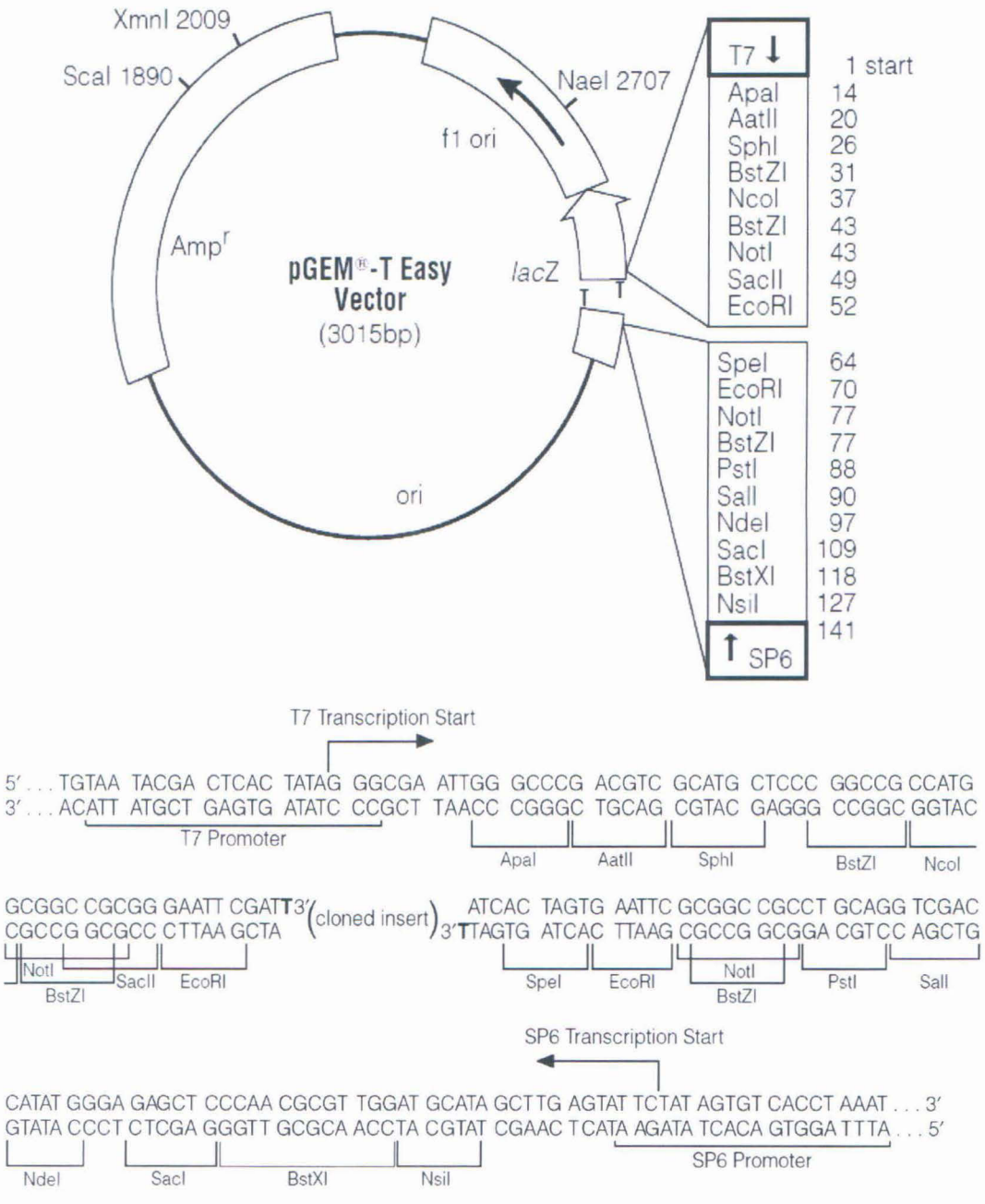


Fig 4: pGEMT Easy vector map and multiple cloning sites.

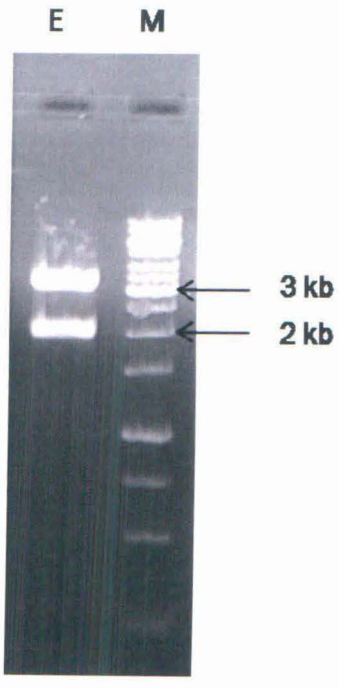


Fig 5: Restriction digestion of pGEMT easy vector to release chitinase gene. The chitinase-pGEMT easy vector was digested with *XhoI* and *BamHI* to check the correct cloning of the chitinase gene in the pGEMT easy vector. A band of ~2kb was observed in the lane E. 1.0 kb DNA ladder was loaded in the lane M.

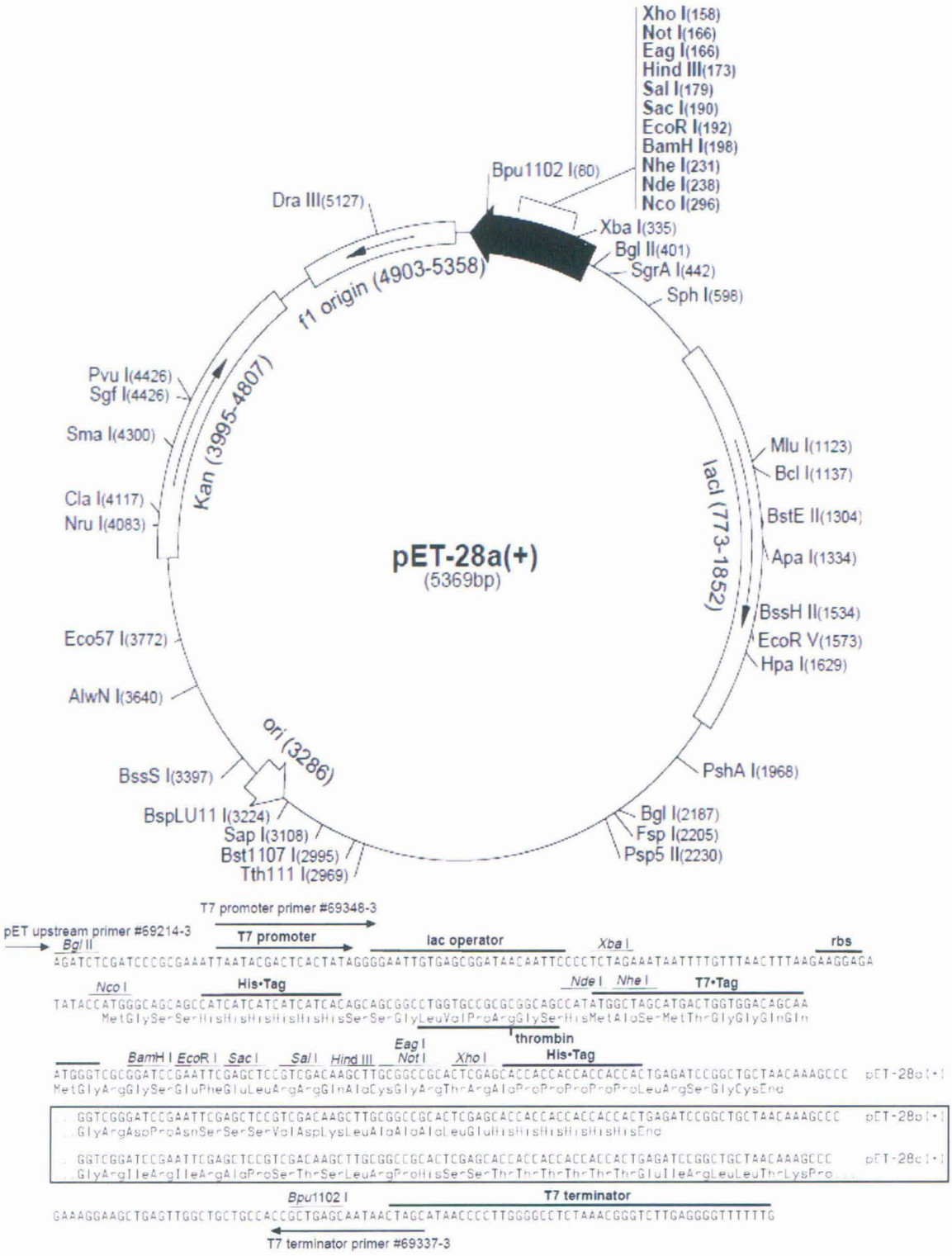


Fig 6: pET28 Easy vector map and multiple cloning sites.

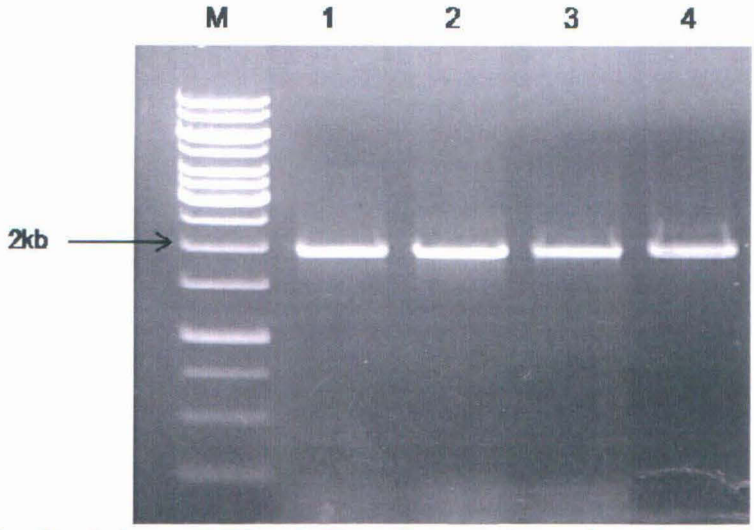


Fig 7: Colony PCR of *E.* coli transformed with chitinase-pET28b construct. Competent cells of *E. coli* were transformed with pET28b. Colony PCR was done to select positive colonies. An amplicon of ~2 kb in lane 1, 2, 3 and 4 corresponding to colonies 1, 2, 3 and 4 was obtained respectively.

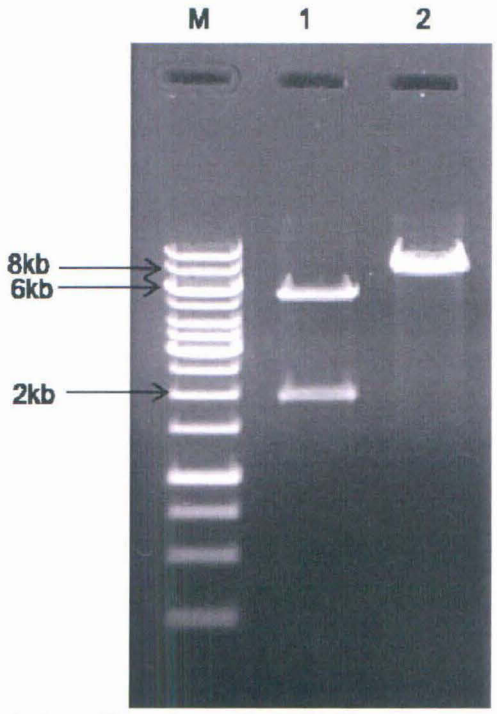


Fig 8: Restriction digestion of pET28b vector to release chitinase gene. The chitinase-pETvector was digested with *BamHI* to check the cloning of the chitinase gene in the pET28b vector. A band of ~2kb was observed in the lane 1. Lane 2 shows only linearization of the vector when chitinase-pET28b construct was digested with *SalI* and *NotI*. Not*I* cut the plasmid at its restriction site but *SalI* was not. It shows the loss of the *SalI* restriction recognition site.

```
atg tct caa aat gtt tat cga tac cct tca att aaa gcg atg tct gac gcc agc agc gaa
 M   S   Q   N   V   Y   R   Y   P   S   I   K   A   M   S   D   A   S   S   E

gta ggc gca tct ctg gtt gcc tgg cag aat caa tct ggt ggt caa acc tgg tat gtc att
 V   G   A   S   L   V   A   W   Q   N   Q   S   G   G   Q   T   W   Y   V   I

tat gat agc gcg gtt ttt aaa aac atc ggc tgg gtt gaa cgc tgg cat att ccc gac cgc
 Y   D   S   A   V   F   K   N   I   G   W   V   E   R   W   H   I   P   D   R

aat att tca cct gat tta ccg gtt tat gag aat gcc tgg caa tat gtc cgt gag gcg aca
 N   I   S   P   D   L   P   V   Y   E   N   A   W   Q   Y   V   R   E   A   T

ccg gaa gaa att gcc gat cac ggt aac ccc aat acg cct gat gta ccg ccg gga gaa aaa
 P   E   E   I   A   D   H   G   N   P   N   T   P   D   V   P   P   G   E   K

acc gag gta ttg caa tat gat gca ctc aca gaa gaa acc tat cag aag gtg gga tat aaa
 T   E   V   L   Q   Y   D   A   L   T   E   E   T   Y   Q   K   V   G   Y   K

cct gac ggc agc gga act cct ttg agt tat tct tca gca cgt gtt gcc aag tcc ctg tac
 P   D   G   S   G   T   P   L   S   Y   S   S   A   R   V   A   K   S   L   Y

aac gaa tat gaa gtt gat ccg gaa aat aca gaa ccg ctg cct aaa gtc tct gcc tat att
 N   E   Y   E   V   D   P   E   N   T   E   P   L   P   K   V   S   A   Y   I

act gac tgg tgc cag tat gat gcg cgt ttg tcg cca gaa acc cag gat aac act gcg ctg
 T   D   W   C   Q   Y   D   A   R   L   S   P   E   T   Q   D   N   T   A   L

acc agc gac gat gcc ccc ggc cgt ggt ttt gat ctg gaa aaa atc ccg cct acc gcc tac
 T   S   D   D   A   P   G   R   G   F   D   L   E   K   I   P   P   T   A   Y

gac cgc ctg att ttc agt ttt atg gcc gtc aac ggt gat aaa ggc aag tta tcc gaa cgg
 D   R   L   I   F   S   F   M   A   V   N   G   D   K   G   K   L   S   E   R

att aat gag gtt gtt gac ggg tgg aac cgg caa gca gaa gct agc agt ggc cag att gcc
 I   N   E   V   V   D   G   W   N   R   Q   A   E   A   S   S   G   Q   I   A

cct att aca tta ggc cat att gta ccc gtt gat cct tat ggt gat tta ggc acc aca cgc
 P   I   T   L   G   H   I   V   P   V   D   P   Y   G   D   L   G   T   T   R

aat gtc ggt ctg gac gcg gat cag cgc cgt gat gcc agc ccg aag aat ttc ttg caa tat
 N   V   G   L   D   A   D   Q   R   R   D   A   S   P   K   N   F   L   Q   Y

tac aat cag gat gca gcc tcc ggt tta ctg ggg gga ttg cgt aat ctg aaa gcg cga gca
 Y   N   Q   D   A   A   S   G   L   L   G   G   L   R   N   L   K   A   R   A

aaa cag gca ggg cac aag ctg gaa ctc gca ttc agt atc ggt ggc tgg agt atg tca ggg
 K   Q   A   G   H   K   L   E   L   A   F   S   I   G   G   W   S   M   S   G

tat ttc tct gtg atg gcc aaa gat cct gag caa cgt gct aca ttt gtg agt agc atc gtc
 Y   F   S   V   M   A   K   D   P   E   Q   R   A   T   F   V   S   S   I   V

gac ttc ttc cgg cgt ttt ccc atg ttt act gcg gtg gat atc gac tgg gaa tac ccc ggc
 D   F   F   R   R   F   P   M   F   T   A   V   D   I   D   W   E   Y   P   G

gcc aca ggt gaa gaa ggt aat gaa ttc gac ccg gaa cat gat ggc cca aac tat gtt ttg
 A   T   G   E   E   G   N   E   F   D   P   E   H   D   G   P   N   Y   V   L

tta gtg aaa gag ctg cgt gaa gca ctg aac atc gcc ttt gga acc cgg gcc cgt aaa gaa
 L   V   K   E   L   R   E   A   L   N   I   A   F   G   T   R   A   R   K   E

atc acg ata gcc tgt agc gcc gtc gtt gcc aaa atg gag aag tcc agc ttc aaa gaa atc
 I   T   I   A   C   S   A   V   V   A   K   M   E   K   S   S   F   K   E   I

gca cct tat tta gac aat atc ttt gtg atg acc tac gac ttc ttt ggt acc ggt tgg gca
 A   P   Y   L   D   N   I   F   V   M   T   Y   D   F   F   G   T   G   W   A
```

Contd.

```
gaa tac atc ggt cac cat act aac ctg tat ccc ccc aga tat gaa tat gac ggc gat aac
 E   Y   I   G   H   H   T   N   L   Y   P   P   R   Y   E   Y   D   G   D   N

cct cct ccg ccc aat cct gat cgg gac atg gat tac tcg gca gat gag gcg atc cgc ttt
 P   P   P   P   N   P   D   R   D   M   D   Y   S   A   D   E   A   I   R   F

tta ctg tca caa ggt gta caa ccg gag aaa att cac ctc gga ttt gct aac tat gga cgt
 L   L   S   Q   G   V   Q   P   E   K   I   H   L   G   F   A   N   Y   G   R

tca tgt ctg ggt gct gat ctg aca act cgc cgc tat aac aga aca gga gag cca ctg ggc
 S   C   L   G   A   D   L   T   T   R   R   Y   N   R   T   G   E   P   L   G

acg atg gaa aaa ggt gct ccg gaa ttc ttc tgt ctg ctg aat aac caa tac gat gcg gaa
 T   M   E   K   G   A   P   E   F   F   C   L   L   N   N   Q   Y   D   A   E

tat gaa att gca cgc ggg aaa aat cag ttt gaa ctg gtg aca gac acg gaa acc gac gct
 Y   E   I   A   R   G   K   N   Q   F   E   L   V   T   D   T   E   T   D   A

gac gca ctc ttt aat gct gac ggt ggt cac tgg att tca ctg gat acg ccc cgc act gtg
 D   A   L   F   N   A   D   G   G   H   W   I   S   L   D   T   P   R   T   V

ctg cat aag gga att tat gca acc aaa atg aaa ttg ggc ggg atc ttc tct tgg tca ggc
 L   H   K   G   I   Y   A   T   K   M   K   L   G   G   I   F   S   W   S   G

gat cag gat gat ggc ctg ttg gca aat gct gct cac gaa ggt ttg ggt tac tta cct gta
 D   Q   D   D   G   L   L   A   N   A   A   H   E   G   L   G   Y   L   P   V

cgc gga aaa gag aag att gat atg gga ccg tta tat aac aaa gga cgt ctc att cag ctt
 R   G   K   E   K   I   D   M   G   P   L   Y   N   K   G   R   L   I   Q   L

cct aaa gta acc cgt cgt aaa tcg tag
 P   K   V   T   R   R   K   S   *
```

Fig 9: Nucleotide and corresponding amino acid sequence of the putative chitinase gene.

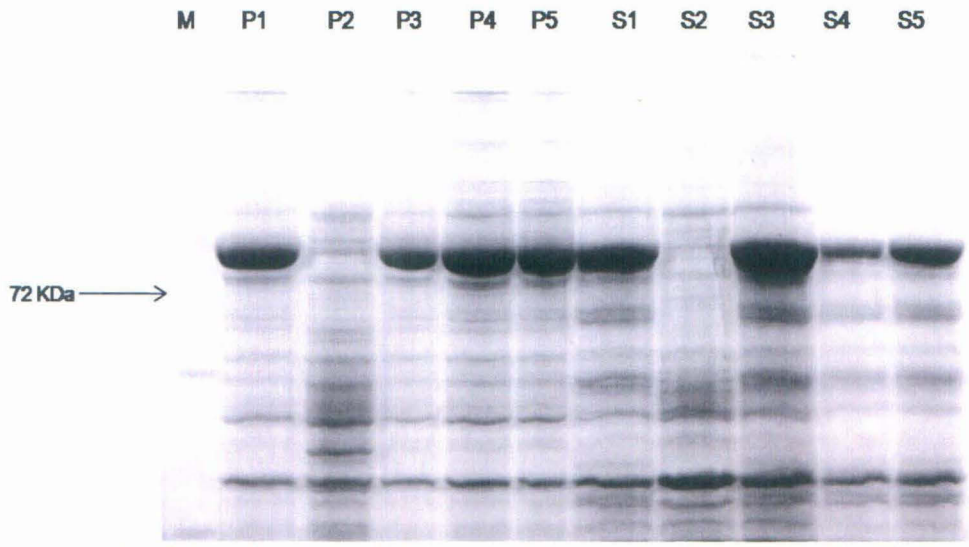


Fig 10: Induction of the recombinant protein in *E. coli* cells by IPTG. Both pellet and supernatant fractions were analyzed on a 12% SDS-PAGE. Lane M: protein marker, P1: 0.2 mM IPTG (pellet), P2: uninduced pellet fraction, P3: 0.5 mM IPTG (pellet), P4: 0.7 mM IPTG (pellet), P5: 1.0 mM IPTG (pellet), S1: 0.2 mM IPTG (supernatant), S2: uninduced supernatant fraction, S3: 0.5 mM IPTG (supernatant), S4: 0.7 mM IPTG (supernatant) and S5: 1.0 mM IPTG (supernatant).

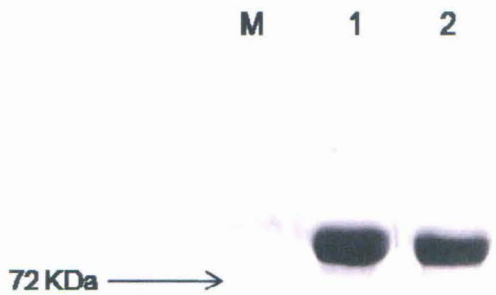


Fig 11: Ni-NTA purified recombinant chitinase protein from *E. coli* cells. The recombinant protein was purified on a Ni-NTA affinity column as described in materials and methods. The purified protein was checked on a 12% SDS-PAGE.

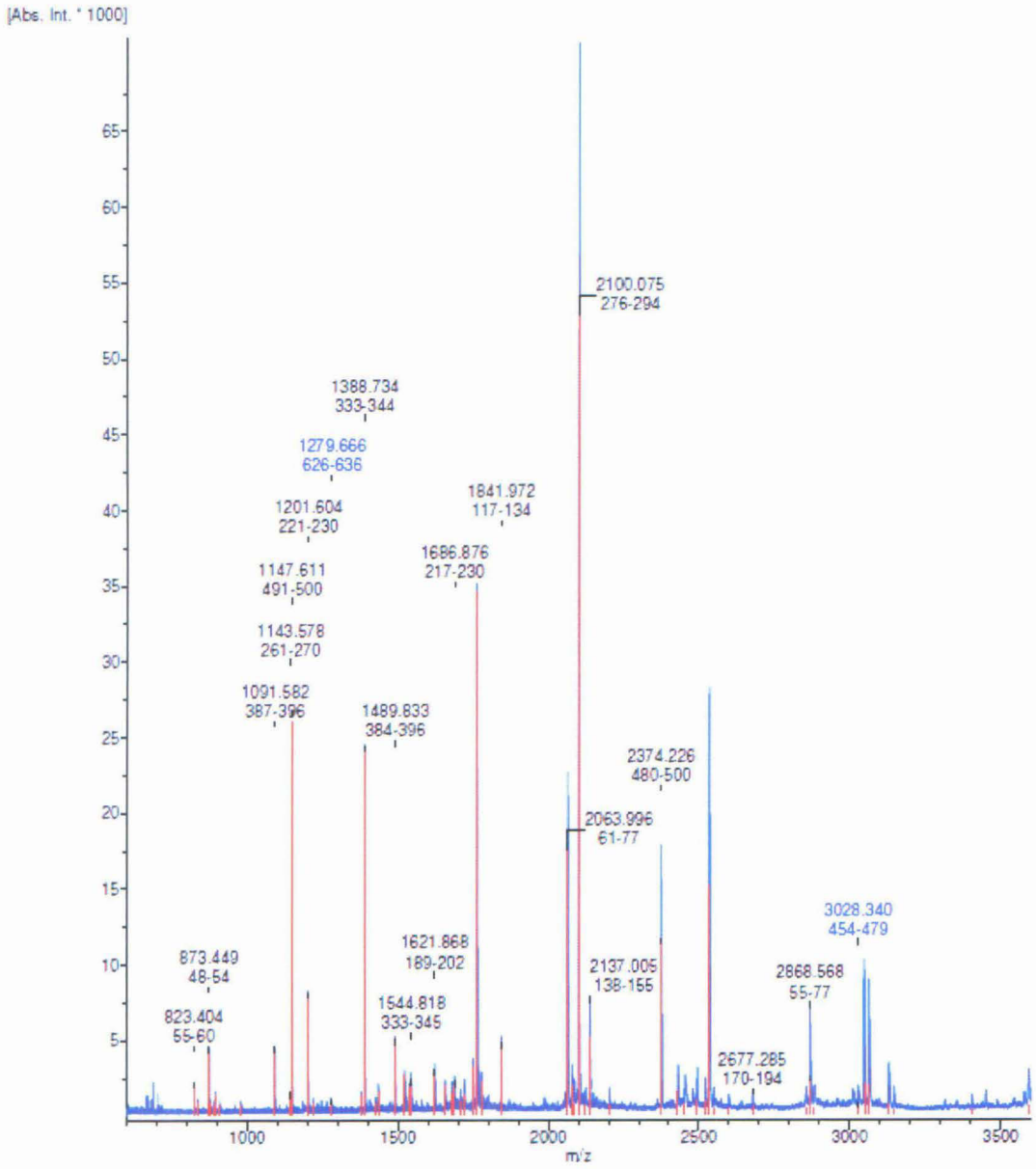


Fig 12: Mass spectrum of the trypsin digested chitinase protein using MALDI-TOF.

	Accession	Mass	Score	Description
1.	gi|300723469	72587	182	putative chitinase [Xenorhabdus nematophila ATCC 1
2.	gi|119908962	106408	80	PREDICTED: TBC1 domain family, member 9 (with GRAM
3.	gi|73983892	134610	69	PREDICTED: similar to Y45F10A.6b isoform 4 [Canis
4.	gi|325661540	30250	66	hypothetical protein HMPREF0490_00898 [Lachnospira
5.	gi|332217289	144334	66	PREDICTED: TBC1 domain family member 9 [Nomascus l
6.	gi|73983894	144103	65	PREDICTED: similar to Y45F10A.6b isoform 1 [Canis
7.	gi|139394668	144356	65	TBC1 domain family member 9 [Homo sapiens]
8.	gi|297674388	144427	65	PREDICTED: TBC1 domain family member 9-like [Pongo
9.	gi|332820386	144352	65	PREDICTED: TBC1 domain family member 9 [Pan troglo
10.	gi|226363953	22396	65	TetR family transcriptional regulator [Rhodococcus
11.	gi|221059015	52884	64	RNA 3'-Terminal Phosphate Cyclase-like protein [Pl
12.	gi|34533741	116887	63	unnamed protein product [Homo sapiens]
13.	gi|251792813	83458	63	DNA-dependent helicase II [Aggregatibacter aphroph
14.	gi|315634405	83397	63	excision endonuclease subunit UvrD [Aggregatibacte
15.	gi|222823892	32099	63	NAD-dependent protein deacetylase (SIR2 family), [
16.	gi|16552274	26664	62	unnamed protein product [Homo sapiens]
17.	gi|291414205	42440	62	PREDICTED: F-box only protein 25 isoform 4 [Orycto
18.	gi|73983890	116487	62	PREDICTED: similar to Y45F10A.6b isoform 3 [Canis
19.	gi|256840378	34484	62	DNA primase [Parabacteroides sp. D13]
20.	gi|20521682	147264	62	KIAA0882 protein [Homo sapiens]
21.	gi|225447719	115858	62	PREDICTED: hypothetical protein [Vitis vinifera]
22.	gi|313222316	62087	61	unnamed protein product [Oikopleura dioica]
23.	gi|258591698	37040	60	GHMP kinase [NC10 bacterium 'Dutch sediment']
24.	gi|197118616	95804	60	glucan phosphorylase [Geobacter bemidjiensis Bem]
25.	gi|194208431	148061	60	PREDICTED: TBC1 domain family, member 9 (with GRAM
26.	gi|238498772	42264	60	hypothetical protein AFLA_070620 [Aspergillus flav
27.	gi|255099022	39243	60	recombination activating protein 1 [Hydrelaps darw
28.	gi|255099024	39371	60	recombination activating protein 1 [Hydrelaps darw
29.	gi|281342601	130279	60	hypothetical protein PANDA_016736 [Ailuropoda mela
30.	gi|76157574	26841	59	SJCHGC07886 protein [Schistosoma japonicum]
31.	gi|187924032	28282	59	extracellular solute-binding protein family 3 [Bur
32.	gi|194399677	41136	59	recombination activating protein 1 [Hydrelaps darw
33.	gi|221039550	45516	58	unnamed protein product [Homo sapiens]
34.	gi|219128897	64649	58	predicted protein [Phaeodactylum tricornutum CCAP
35.	gi|49614366	13698	58	structural glycoprotein E2 [Bovine viral diarrhea
36.	gi|26330496	40972	58	unnamed protein product [Mus musculus]
37.	gi|50510677	146169	58	mKIAA0870 protein [Mus musculus]
38.	gi|187478217	47600	58	flagellar hook-associated protein 2 [Bordetella av
39.	gi|330826402	34640	57	NADPH:quinone reductase [Alicycliphilus denitrific
40.	gi|124486710	145710	57	DENN domain-containing protein 3 [Mus musculus]
41.	gi|301783243	142634	57	PREDICTED: TBC1 domain family member 9-like [Ailur
42.	gi|123454653	29926	57	acetyltransferase, GNAT family-related protein [Tr
43.	gi|296203583	78386	57	PREDICTED: RING finger protein 6 isoform 1 [Callit
44.	gi|283788323	54258	57	uronate isomerase [Citrobacter rodentium ICC168]
45.	gi|297484553	142010	57	PREDICTED: TBC1 domain family, member 9 (with GRAM
46.	gi|319944751	178039	56	glutamate synthase alpha subunit [Lautropia mirabi
47.	gi|147677857	112619	56	hypothetical protein PTH_1522 [Pelotomaculum therm
48.	gi|291401196	143177	56	PREDICTED: TBC1 domain family, member 9B (with GRA
49.	gi|15240812	38821	56	NADP-dependent oxidoreductase, putative [Arabidops
50.	gi|297293420	141534	56	PREDICTED: TBC1 domain family member 9, partial [M

Fig 13: Mascot search results confirming the purified protein to be the *Xenorhabdus nematophila* chitinase

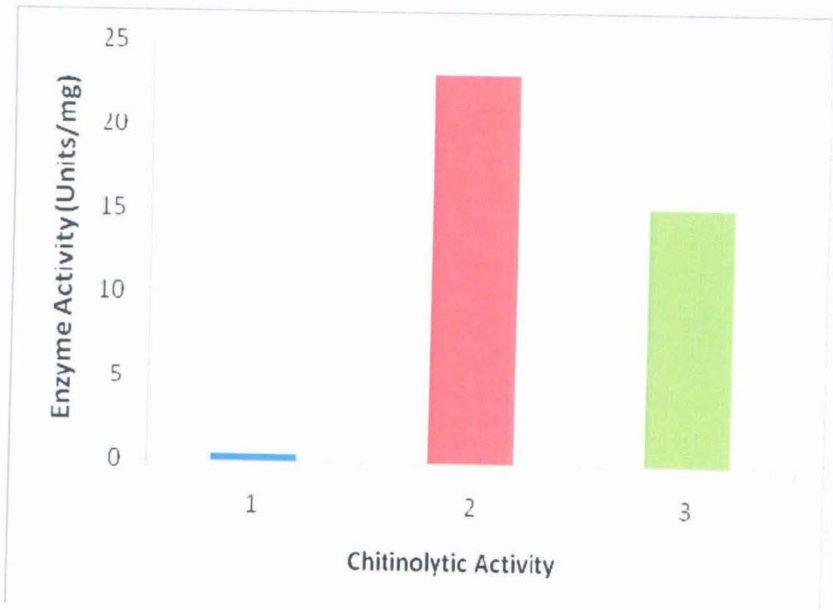


Fig 14: Different chitinolytic activities of *Xenorhabdus nematophila* chitinase. 1 - chitobiosidase activity, 2 - β-N-acetylglucosaminidase activity, 3 - endochitinase activity.

```
T.viride          ------------------------------------------------------------
M.anisopliae      ------------------------------------------------------------
A.thaliana        ------------------------------------------------------------
N.tabacum         ------------------------------------------------------------
B.mori            -MGHRMIALVFAVAAFAVGSCGPPGKPNLGWGERTFAIVEVNQAATAYNQLVTKKDAADV 59
X.nematophila     MSQNVYRYPSIKAMSDASSEVGASLVAWQNQSGGQTWYVIYDSAVFKNIGWVERWHIPDR 60
S.enterica        ------------------------------------------------------------
D.persimilis      ------------------------------------------------------------

T.viride          ------------------------------------------------------MLGFL 5
M.anisopliae      ------------------------------------------------------------
A.thaliana        ------------------------------------------------------------
N.tabacum         ------------------------------------------------------------
B.mori            SVNWNVWTGDAADKSRVLLDKKEVWSGAGSATSAAFKVKKGGRYQMQVELCNSDGCSSSE 119
X.nematophila     NISPDLPVYENAWQYVREATPEEIADHGNPNTPDVPPGEKTEVLQYDALTEETYQKVGYK 120
S.enterica        ----------------------------------MKMATSKLIQGDTITETTHAANGFD 25
D.persimilis      ------------------------------------------------------MWI 3

T.viride          GKSMALLAALQATLTSATPVSTNDVSVEKRASGYTNAVYFTNWGIYGRNFQPQDLVASDI 65
M.anisopliae      --------------------MSND--------GYRTVAYFVNWAIYARKHRPQDLPVENL 32
A.thaliana        --------------------MSS---------TKLISLIVS------------------ 12
N.tabacum         --------------------MANS---------VTLFAIIFSCFLLQQLVCTNSQNVKGG 31
B.mori            GVEIVVADTDGSHLRPLDYSIGEKNKPFKQTSGKVVGAYFVEWGVYPRKFPVDRVPVPNL 179
X.nematophila     PDGSGTPLSYSSARVAKSLYNEYEVDPENTEPLPKVSAYITDWCQYDARLSPETQDNTAL 180
S.enterica        PATSDDKISYTSARVAKPVYNKYKN----STTKPKVFGYYTDWSQYDSRLQGNMSQPGRG 81
D.persimilis      RSSEILVLLLGAVLMAASSVAQGN-------VSKHVVCYQGTWSVYRPGTGQFGVEDIDP 56
                                                .

T.viride          T--------------------HVIYPFMNFQAD--------------------------- 78
M.anisopliae      T--------------------HVLYAFANVRTDT-------------------------- 46
A.thaliana        ------------------------------------------------------------
N.tabacum         Y--------------------WFKDSGLALNNID-------------------------- 45
B.mori            T--------------------HLLYGFIPICGGDGINDSLK------------------- 200
X.nematophila     TSDDAPGRGFDLEKIPPTAYDRLIFSFMAVNGDKGKLSERINEVVDGWNRQAEASSGQIA 240
S.enterica        YDLTKVS---------PTAYDKLIFGFVGITGFRKIDTEDRD-------------VVAEA 119
D.persimilis      FLCT-----------------HLIYAFLGIEESG--------------------------- 73

T.viride          -----------------GTVVSGDAYADYQKHYSDDSWNDVGNNAY-----GCVNQLFKL 116
M.anisopliae      -----------------GEVHLTDTWADTDIHWEGDSWNDTGNNIY-----GCLKQLNLL 84
A.thaliana        -----------------ITFF---------LNSQTNQVTVSSANQP-----KFSTFTQTV 41
N.tabacum         -----------------STLFTHLFCAFADLNPQLNQLIISPENQD-----SFRQFTSTV 83
B.mori            -EIEGSFQALQRSCSGREDFKVSIHDPWAALQKPQKGLSSWNEPYK-----GNFGQLMQL 254
X.nematophila     PITLGHIVPVDPYGDLGTTRNVGLDADQRRDASPKNFLQYYNQDAASGLLGGLRNLKARA 300
S.enterica        AALCGKVKYEPTFLDPWGDFQSYINLGFDVSGWDVDPKTVTQSNAKG-LLGALRDMQAKA 178
D.persimilis      ----------------------------QLRVIDSYLDLEDNSGR-----GNIKSFNAL 99
                                                        .

T.viride          KKANRNLKVMLSIGG-WTWSTNFPSAASTDANRKNFAKTAITFMKDWG-FDGIDVDWEYP 174
M.anisopliae      KKRNRNLKVLLSIGG-WTYSANFKNPASTPQGRETFAKSCVELLKNLG-FDGIDIDWEYP 142
A.thaliana        QRRNPSVKTLLSIGGGIADKTAYASMASNPTSRKSFIDSSIRVARSYG-FHGLDLDWEYP 100
N.tabacum         QRKNPSVKTFLSIAGGRANSTAYGIMARQPNSRKSFIDSSIRLARQLG-FHGLDLDWEYP 142
B.mori            KQANTGLKVLPSIGG-WTLADPFFFFTDKTK-RDRFVASVKDFLQTWKFFDGVDIDWEFP 312
X.nematophila     KQAGHKLELAFSIGG-WSMSGYFSVMAKDPEQRATFVSSIVDFFRRFPMFTAVDIDWEYP 359
S.enterica        KAAGHTLALSMSIGG-WSMSNGFHETAASDSSRKIFAKGVVKLFKQFPMFSEVDIDWEYP 237
D.persimilis      KLKNPTLKTLAAIGGWNEGSKKFSIVAADAEKRARFVNDVVHFLQRHG-FDGLDLDWEYP 158
                  :  .  :    :*.*       :   :     *  *      . :    *  :*:***:*

T.viride          A----------DDTQATNMVLLLKEIRSQLDAYAAQYAPGYHFLLSIAAPAGPEHYSALH 224
M.anisopliae      Q----------NPEEARNYVELLAAVRQELDAYAAKCVDNYHFELTVACPAGPNNYEKFE 192
A.thaliana        S----------SATEMTNFGTLLREWRSAVVAEASSSGKPRLLLAAAVFYSNNYYSVLYP 150
N.tabacum         L----------SAADMTNLGTLLNEWRTAINTEARNSGRAALLLTAAVSNSPRVNGLNYP 192
B.mori            GGKGAN-PDLGSPKDGDVYVQLMKELRQMLDELAAETGRTYELTSAIS--AGWDKIQVVN 369
X.nematophila     GATGEEGNEFDPEHDGPNYVLLVKELREALNIAFGTRAR---KEITIACSAVVAKMEKSS 416
S.enterica        NNEGAG--NPFGPEDGANYALLIAELRKQLDSAGLSNVKISIAASAVTTIFDYAKVK--- 292
D.persimilis      GQR-----HSLDNEDRTNYITLLKELKEGLEPFS-------YILSAAVGSAQFSAETSYE 206
                              :      *:   : :              :

T.viride          MADLGQVLDYVNLMAYDYAGS-WSSYSGHDANLFAN----------PSNPNSSPYNTDQ 272
M.anisopliae      IAEMDKYLDFWNLMAYDYAGS-WDSVSGHQANIY----------PGNNASTPFSTNA 238
A.thaliana        VSAVASSLDWVNLMAYDFYG---PGWS-RVTGPPAA----------LFDPSNAGPSGDA 195
N.tabacum         VESLARNLDWINLMAYDFYG---PNWSPSQTNSHAQ----------LFDPVNH-VSGSD 237
B.mori            YKEAQKYMDHIFVMSYDFKGA-WSNDTLGHQTALYA----------PSWSPKETYTTDF 417
X.nematophila     FKEIAPYLDNIFVMTYDFFGTGWAEYIGHHTNLYPPRYEYDGDNPPPPNPDRDMDYSADE 476
S.enterica        -DLMAAGLYGINLMTYDFFGTPWAETLGHHTNRKALE--------------EGGWAVET 336
D.persimilis      IPAIVPYLDLINVMAYDLHGP-WDEVVGINAPLYPAAS---------DKSERQKQLNVDA 256
                         :    :*:**  *                                .     .
```

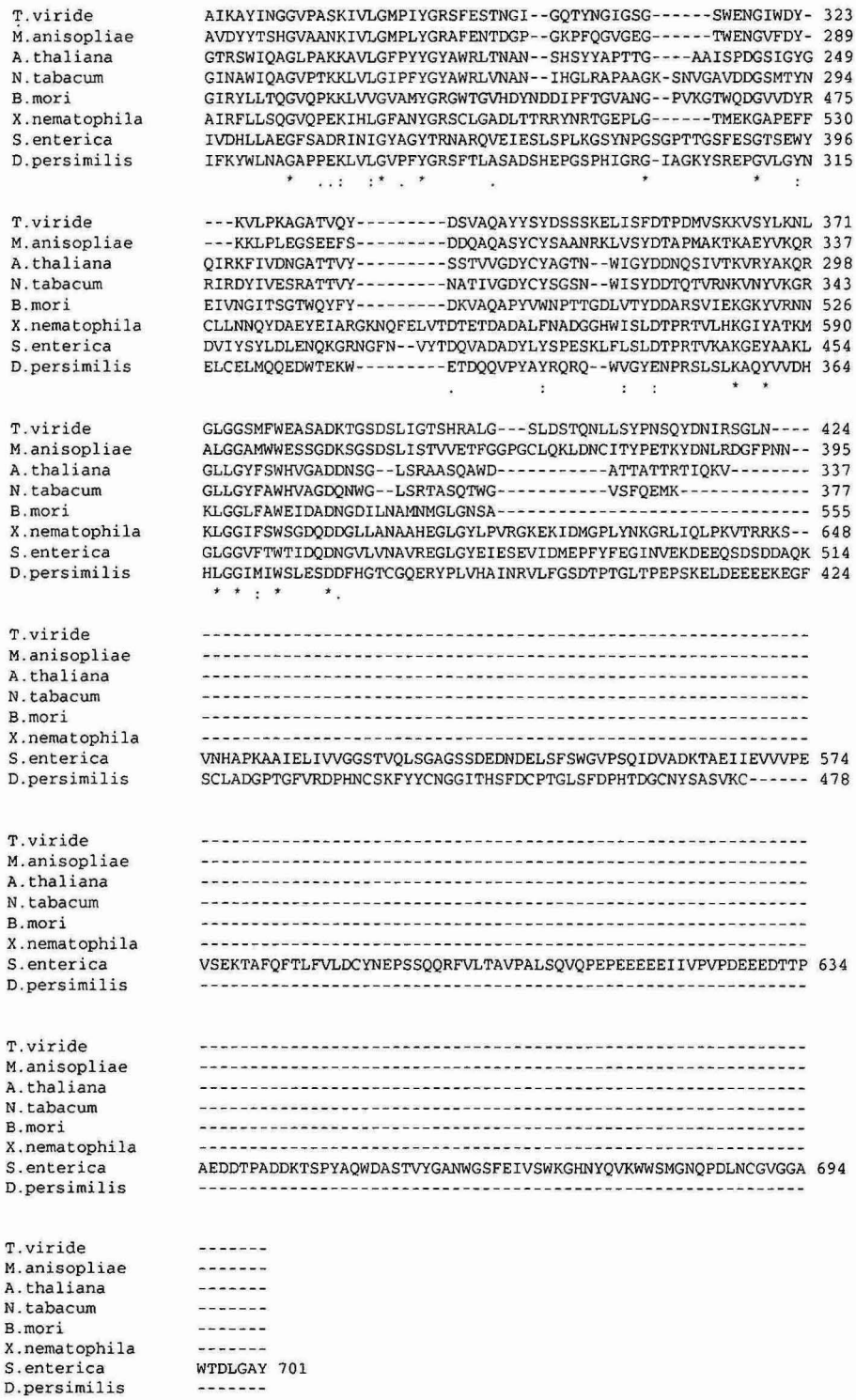


Fig 15: Multiple sequence alignment of the chitinase gene. Clustal 2.1 was used for multiple sequence alignment. An * (asterisk) indicates positions which have a single, fully conserved residue, A : (colon) indicates conservation between groups of strongly similar properties while A . (period) indicates conservation between groups of weakly similar properties.

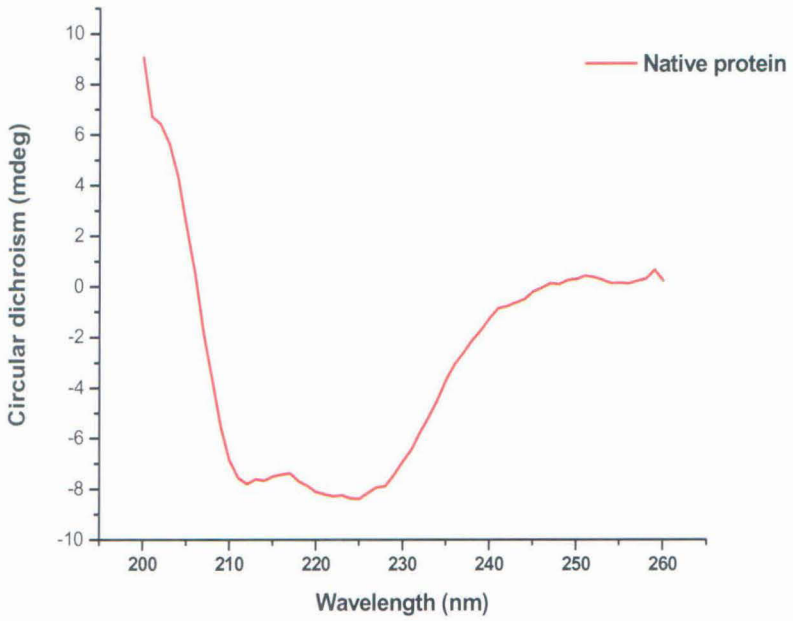


Fig 16: Far UV circular dichroism spectrum of the native chitinase.

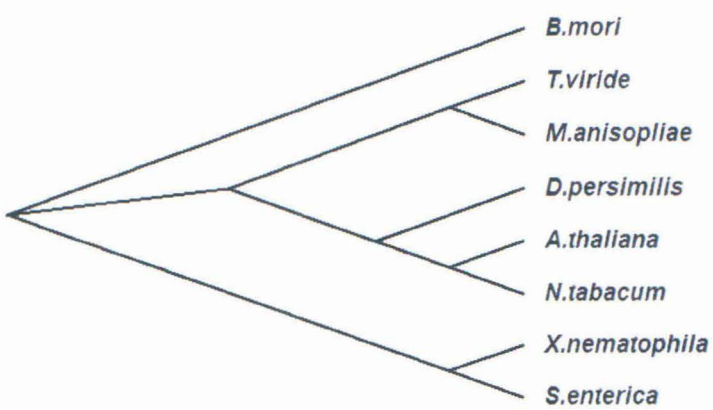
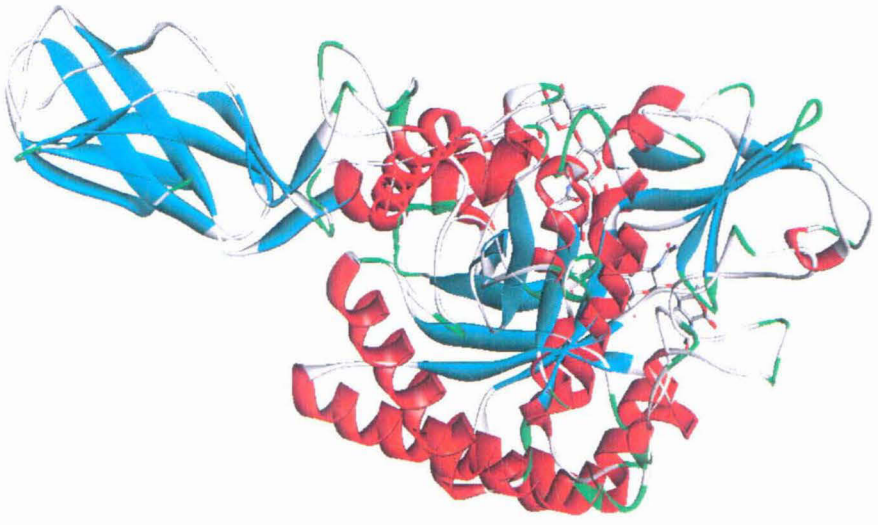
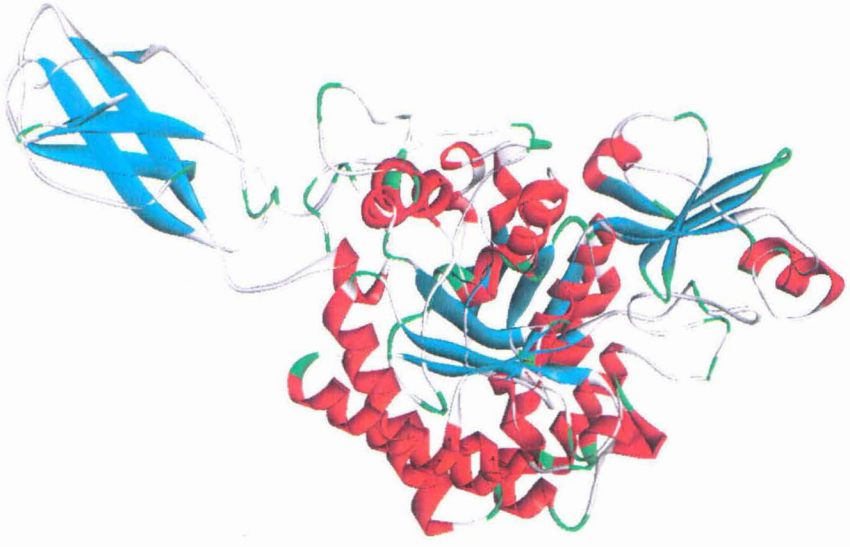


Fig 17: Slanted Cladogram tree showing the evolutionary relationship of *X. nematophila* chitinase with chitinases from different organisms.



(a) Template



(b) Model generated by Phyre web server

Fig 18: Structure of template and model generated by the PHYRE software. (a) Template protein structure for the model generation of chitinase protein of the *X. nematophila*, (b) Model structure of the chitinase protein generated by PHYRE program.

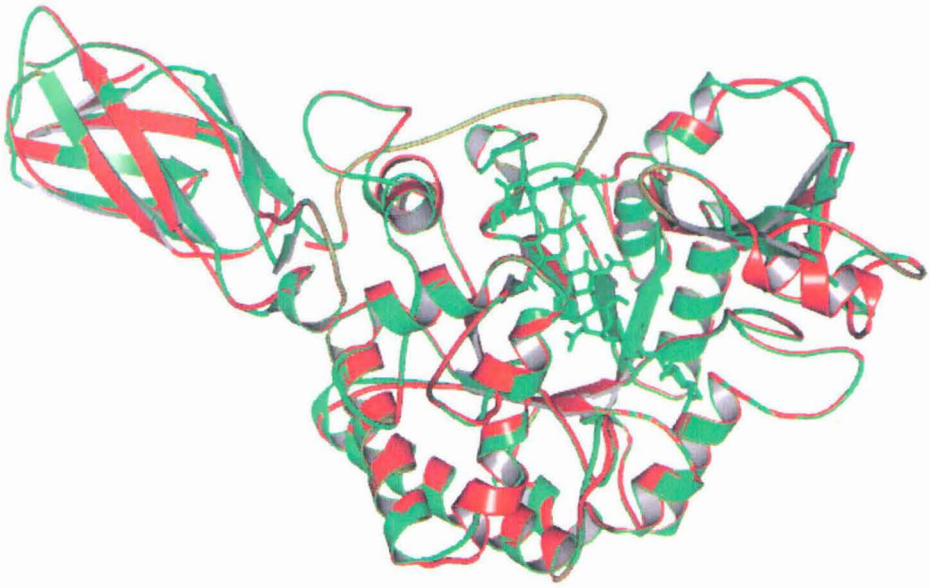


Fig 19: Superimposition of the model structure of chitinase with the template. The model structure of the chitinase protein was checked by the superposition with the template by using Pymol.

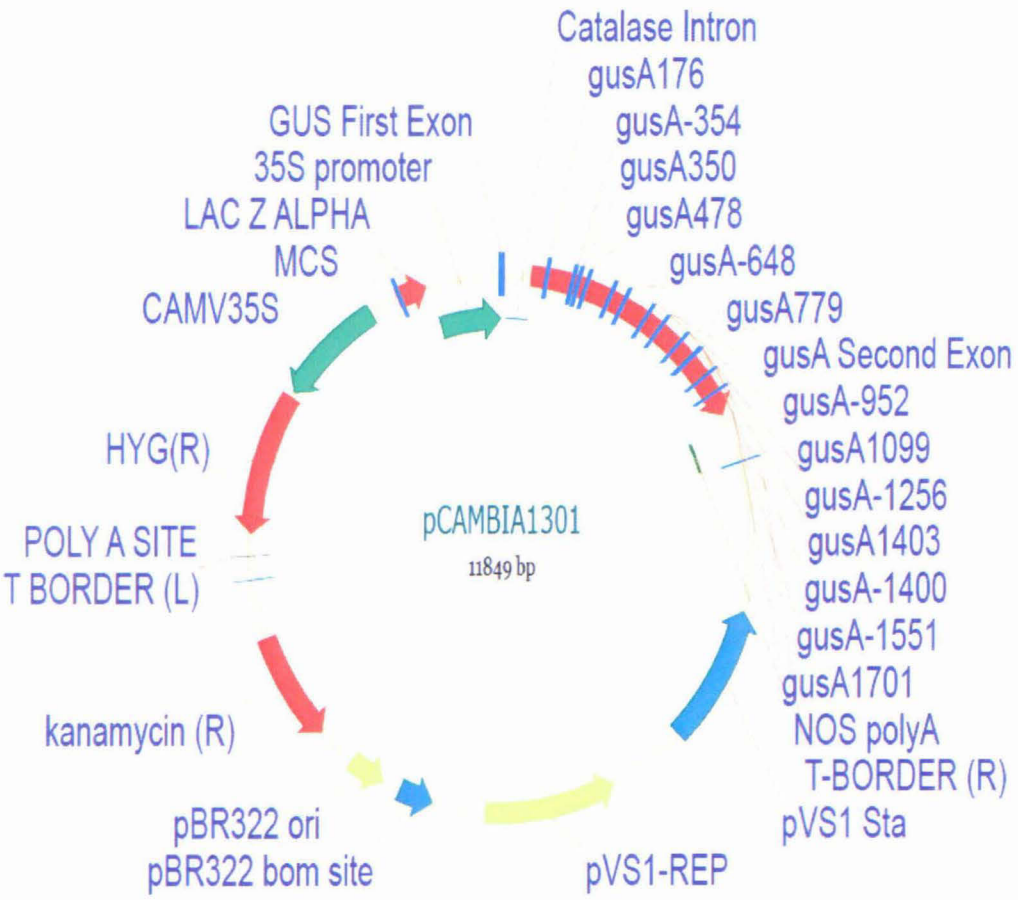


Fig 20: pCAMBIA1301 vector map

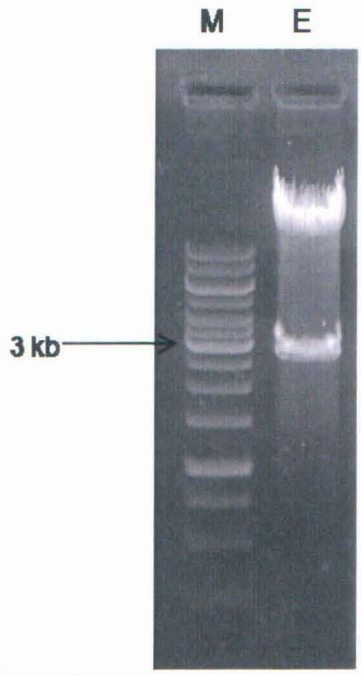


Fig 21: Restriction digestion of chitinase-pCAMBIA 1301 vector to release chitinase gene. The pCAMBIA 1301 vector was digested with *HindIII* to check the correct cloning of the chitinase gene in the pCAMBIA 1301 vector. A band (CaMV35S-chitinase-polyA) of ~3kb was observed in the lane E. 1.0 kb DNA ladder was loaded in the lane M.

CHAPTER 5:
DISCUSSION

Chitin is produced by a wide range of organisms which is catalysed by chitinases (Ike, 2006). On the basis of enzyme activity, chitinase enzymes are classified into endochitinases, exochitinases and N-acetylglucosaminases (Lee, 2007). Till now, chitinases has been functionally characterized from various bacteria: *Bacillus circurans* (Alam, 1995), *Aeromonas sp.* (Shiro, 1996), *Alteromonas sp.* (Tsujibo, 1992), *Janthiobacterium lividum* (Gleave, 1995), *Serratia marcescens* (Burberg, 1996) etc.

In this study, we cloned and analysed the putative chitinase gene from *X. nematophila*. This is the first step towards the molecular characterization of this chitinase. Evaluation of the deduced amino acid sequence of the cloned chitinase gene and determination of the chitinase activity in the purified protein confirmed it to be the *X. nematophila* chitinase.

5.1 Expression and purification of recombinant *X. neamtophila* chitinase from bacterial system

A high level of expression of chitinases in the soluble fraction is important for its purification. Different vectors have previously been used for the expression of chitinases from other organisms. The pNSC2-2 plasmid was used to express *Clostridium paraputrificum* ChiA in *E. coli* (Morimoto, 1997), pET32a was used for the expression of chitinase gene from *Sanguibacter* sp. (Yong, 2006). A truncated chitinase was purified as a His-tagged fusion protein using pET46 Ek/LIC expression vector (Larsen, 2011). In this investigation, the *X. nematophila* chitinase gene was amplified from the genomic DNA of *X. nematophila*. The gene amplified through PCR from the genomic DNA was first cloned into the pGEMT-easy vector and then into the expression vector, pET28b vector. This vector adds an N-terminal hexa-histidine tag to the expressed recombinant chitinase

protein. The fusion protein was successfully expressed in *E. coli* cells. The ability to produce the chitinase in *E. coli* would be useful for large-scale protein production and structural and functional studies on the chitinase.

The induction of recombinant protein standardised at different concentrations of IPTG and temperatures. A significant amount sufficient for purification was found in the soluble fraction, though some protein was also present in the insoluble fraction. The recombinant protein expressed from the *E. coli* host was purified by His tag affinity chromatography. Ni-NTA column was used for the purification. This column allows the preparation of biologically active protein molecules. Upto 100 mM imidazole in the elution buffer was sufficient to elute six His-tag proteins. This small His-tag does not interfere with the enzyme activity. The molecular weight of the protein was similar to that calculated from the deduced amino acid sequence. The purified protein was also analysed by mass spectrometry. Coupling two stages of mass analysis (MS/MS) is a very useful in identifying proteins and compounds. The MS/MS search identified the purified protein to be a chitinase.

5.2 Characterization of *X. nematophila* chitinase

The recombinant chitinase purified from *E. coli* harbouring chitinase-pET28b was used for several biochemical analyses. The enzyme activity was calculated by using different substrates. The chitobiosidase activity was found to be very low compared to the β-N-acetylglucosaminidase and endochitinase activity. Both the chitobiosidase and β-N-acetylglucosaminidase are exochitinase activities in nature. Chitobiosidase activity was also reported in *Trichoderma harzianum* (Harman, 1993) and *Pseudomonas fluorescens*

(Nielsen, 1999). This chitobiosidase requires the trimer form, β-N-acetylglucosaminidase requires at least dimer while endochitinase requires the tetramer of the N-acetylglucosamine for the activity. The β-N-acetylglucosaminidase from *Arhrobacter protophormiae* was well characterized (Takegawa, 1991). It was shown that the enzyme was activated by the addition of disaccharides such as cellobiose, gentiobiose, and di-acetylchitobiose. Chitobiosidase releases acetylchitobiose from the non-reducing end of chitin while β-N-acetylglucosaminidase releases N-acetylglucosamine. The activity assays show that the *X. nematophila* chitinase protein from *X. nematophila* has both exochitinase and endochitinase activities. The endochitinases and exochitinases have multiple industrial applications. The chitinase enzyme can be used for the biodegradation of chitin waste (Songsiriritthigul, 2010).

The analysis of CD spectrum from the SOMCD showed that the most of the secondary structure of the chitinase protein is in the form of α-Helix. The percentage of random coil is also more than the β-sheet and turn.

The 3D structure was determined by the PHYRE web server. The superposition of the model with the template showed a significant result. From this modelling, we identified some key residues predicted to be important for the chitin binding acitivity and catalysis. The structure and function can be further characterised by mutating these key residues in future studies.

CHAPTER 6:
SUMMARY AND CONCLUSIONS

Chitinases are produced by a large number of organisms. To exploit the commercial potential of chitinases and to make use of them in generating insect and fungus resistant plants by transgenic approach, isolation of chitinase genes and the purification and characterization of its protein from different sources is necessary. In the present study we cloned the chitinase gene from *Xenorhabdus nematophila* and characterized the recombinant protein purified from a bacterial expression system.

The highlights of the investigation are as follows:

- The putative chitinase gene from *X. nematophila* 19061 was isolated and cloned into the pGEM-easy vector. The construct was named as chitinase-pGEMT easy vector.

- The chitinase gene was cloned in the expression vector pET28b for expression and purification of hexa-histidine tagged chitinase protein.

- The pET28b-chitinase construct was transformed in competent cells of *E. coli* BL21 (DE3) bacterium.

- The conditions for optimal expression of His-tagged chitinase protein in *E. coli* were standardized. IPTG (0.5 mM) was used for the induction of protein expression at 37 C and 5 h incubation. Most of the expressed protein was present in the soluble fraction.

- The recombinant His-tagged chitinase protein was purified in native form by Ni-NTA chromatography. The protein was eluted from the column by applying 100 mM of imidazole in the elution buffer. The eluted protein was dialyzed against 50 mM of phosphate buffer (pH 7.6).

- The activity of the purified *X. nematophila* chitinase protein was assayed using three different substrates. The chitinase enzyme activities obtained were:

  i. Chitobiosidase activity - 0.43 units/mg

  ii. β-N-acetylglucosaminidase activity - 23.1 units/mg

  iii. Endochitinase activity - 15.36 units/mg

- The secondary structure of the protein was determined through CD spectroscopy. The protein was found to contain 48 % α-Helix, 9.5 % β-sheet, 16.9 % Turn and 25.6 % random coil structures.

- The structure of the protein was predicted through PHYRE web server. On the basis of the structure some key residues was identified which could be important in chitin binding and catalysis. These were Trp^{316}, Glu^{357}, Gly^{438}, Tyr^{472} and Trp^{598}.

Conclusions

In the present study *X. nematophila* chitinase gene was isolated and the protein was successfully purified and characterized. It was found to have exochitinase, as well as, endochitinase activity.

Future Prospective

The chitinase gene of the *X. nematophila* can be used against pathogens like insects, fungi etc. It can be overexpressed in plant systems to alleviate biotic stress. The mutant of the chitinase gene made in this study could be used in experiments aimed at understanding the mechanisms of chitin binding and degradation.

CHAPTER 7:
REFERENCES

- Andersen, S.O. (1979). Biochemistry of the insect cuticle. Annu. Rev. Entomol. 24, 29-61.
- Armand, S., Tomita, H., Heyraud, A., Gey, C., Watanabe, T. and Henrissat, B. (1994). Stereochemical course of the hydrolysis reaction catalyzed by chitinases A1 and D from *Bacillus circulans* WL-12. FEBS Lett. 343, 177-180.
- Bhushan, B. and Hoondal, G.S. (1998). Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. Biotechnol. Lett. 20, 157–159.
- Boot, R.G., Renkema, G.H., Strijland, A., van Zonneveld, A.J. and Aerts, J.M. (1995) Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. J. Biol. Chem. 270, 26252-26256.
- Brameld, K.A. and Goddard, W.A., III (1998). The role of enzyme distortion in the single displacement mechanism of family 19 chitinases. Proc. Natl. Acad. Sci. U.S.A. 95, 4276-4281.
- Charpentier, M. and Percheron, F. (1983). The chitin-degrading enzyme system of a *Streptomyces* species. Int. J. Biochem. 15, 289-292.
- Chen, J.P. and Lee, M.S. (1995). Enhanced production of *Serratia marcescens* chitinase in PEG/dextran aqueous two-phase system. Enzyme Microb. Technol. 17, 1021–1027.
- Coutinho, P.M., Deleury, E., Davies, G.J. and Henrissat, B. (2003). An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. 328, 307-317.

- Dahiya, N., Tewari, R., and Hoondal, G.S. (2006). Biotechnological aspects of chitinolytic enzymes: a review. Appl. Microbiol. Biotechnol. 71, 773-782.
- Dahiya, N., Tewari, R., Tiwari, R.P. and Hoondal, G.S. (2005) Chitinase production in solid-state fermentation by *Enterobacter* sp. NRG4 using statistical experimental design. Curr. Microbiol. 51, 222-228.
- Drouillard, S., Armand, S., Davies, G.J., Vorgias, C.E. and Henrissat, B. (1997). *Serratia marcescens* chitobiase is a retaining glycosidase utilizing substrate acetamido group participation. Biochem. J. 328, 945-949.
- 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* strains overexpressing an engineered chitinase. Appl. Environ. Microbiol. 73, 295-302.
- Feix, M., Gloggler, S., Londershausen, M., Weidemann, W., Spindler, K.D. and Spindler-Barth, M. (2000) A cDNA encoding a chitinase from the epithelial cell line of chironomus tentans (Insecta, diptera) and its functional expression. Arch. Insect Biochem. Physiol 45, 24-36.
- Felt, O., Buri, P. and Gurny, R. (1998) Chitosan: a unique polysaccharide for drug delivery. Drug Dev. Ind. Pharm. 24, 979-993.
- Fitches, E., Wilkinson, H., Bell, H., Bown, D.P., Gatehouse, J.A. and Edwards, J.P. (2004) Cloning, expression and functional characterisation of chitinase from larvae of tomato moth (*Lacanobia oleracea*): a demonstration of the insecticidal activity of insect chitinase. Insect Biochem. Mol. Biol. 34, 1037-1050.

- Fung, K.L., Zhao, K.J., He, Z.M. and Chye, M.L. (2002) Tobacco-expressed *Brassica juncea* chitinase BjCHI1 shows antifungal activity in vitro. Plant Mol. Biol. 50, 283-294.
- Garcia, E. (1993). Assessment of endo-1,4-beta-D-glucanase activity by a rapid colorimetric assay using disodium-2–2'-bicinchoninate. J. Food Biochem. 17, 135–145.
- Gooday, G.W. (1979). Chitin synthesis and differentiation in *Coprinus cinereus*. In: J.H. Burnett and A.P.J. Trinci, Editors, Fungal Walls and Hyphal Growth. Cambridge University Press, Cambridge. 203–223.
- Gooday, G.W. (1999). Aggressive and defensive roles for chitinases. EXS. 87, 157-169.
- Gummadi, S.N., Kumar, D.S., Dash, S.S. and Sahu, S.K. (2009) Industrially Important Carbohydrate Degrading Enzymes from Yeasts: Pectinases, Chitinases, and β-1,3-Glucanases. Yeast biotechnology: Diversity and application. 673-691.
- Harman, G.E., Hayes, C.K., Lorito, M., Broadway, R.M., Di Pietro, A., Peterbauer, C. and Tronsmo, A. (1993) Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology. 83, 313–318.
- Hart, P.J., Pfluger, H.D., Monzingo, A.F., Hollis, T. and Robertus, J.D. (1995). The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 A resolution. J. Mol. Biol. 248, 402-413.
- Hawtin, R.E., Arnold, K., Ayres, M.D., Zanotto, P.M., Howard, S.C., Gooday, G.W., Chappell, L.H., Kitts, P.A., King, L.A. and Possee, R.D. (1995).

Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. Virology. 212, 673-685.

- Hedrick, J.L. and Smith, A.J. (1968). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. 126, 154–164.
- Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280, 309-316.
- Henrissat, B. and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 293, 781–788.
- Howard, M.B., Ekborg, N.A., Weiner, R.M. and Hutcheson, S.W. (2003) Detection and characterization of chitinases and other chitin-modifying enzymes. J. Ind. Microbiol. Biotechnol. 30, 627-635.
- Iseli, B., Armand, S., Boller, T., Neuhaus, J.M. and Henrissat, B. (1996) Plant chitinases use two different hydrolytic mechanisms. FEBS Lett. 382, 186–188.
- Itoh, Y., Kawase, T., Nikaidou, N., Fukada, H., Mitsutomi, M., Watanabe, T. and Itoh, Y. (2002) Functional analysis of the chitin-binding domain of a family 19 chitinase from *Streptomyces griseus* HUT6037: substrate-binding affinity and cis-dominant increase of antifungal function. Biosci. Biotechnol. Biochem. 66, 1084-1092.

- Joshi, M.C., Sharma, A., Kant, S., Birah, A., Gupta, G.P., Khan, S.R., Bhatnagar, R. and Banerjee, N. (2008) An insecticidal GroEL protein with chitin binding activity from *Xenorhabdus nematophila*. J. Biol. Chem. 283, 28287-28296.
- Kim, Y., Liu, J., Han, X., Pervin, A. and Lindhardt, R. (1995). Analysis of fluorescently labeled sugars by reverse phase ion pairing HPLC. J. Chrom. Sci. 33, 162–167.
- Kramer, K.J. and Muthukrishnan, S. (1997) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem. Mol. Biol. 27, 887-900.
- Krishnan, A., Nair, P.N., and Jones, D. (1994). Isolation, cloning, and characterization of new chitinase stored in active form in chitin-lined venom reservoir. J. Biol. Chem. 269, 20971-20976.
- Kumar, S.M., Kumar, B.K., Sharma, K.K. and Devi, P. (2004). Genetic transformation of pigeonpea with the rice chitinase gene. Plant Breed. 123, 485–489.
- Kuranda, M.J. and Robbins, P.W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J. Biol. Chem. 266, 19758-19767.
- Kurita, K. (2001) Controlled functionalization of the polysaccharide chitin. Progress in Polymer Science 26, 1921-1971.
- Larsen, T., Petersen, B.O., Storgaard, B.G., Duus, J., Patcic, M.M. and Leisner, J.J. (2011) Characterization of a novel *Salmonella typhimurium* chitinase which hydrolyzes chitin, chitooligosaccharides and an N-acetyllactosamine conjugate. 44, 426-436.

- Lasters, I., Wodak, S.J., Alard, P. and van Cutsem, E. (1988). Structural principles of parallel beta-barrels in proteins. Proc. Natl. Acad. Sci. USA 85, 3338-3342.
- Lee, C.G., Da Silva, C.A., Dela Cruz, C.S., Ahangari, F., Ma, B., Kang, M.J., He, C.H., Takyar, S. and Elias, J.A. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu. Rev. Physiol 73, 479-501.
- Matson, P.A., Parton, W.J., Power, A.G. and Swift, M.J. (1997). Agricultural intensification and ecosystem properties. Science. 277, 504–509.
- Merzendorfer, H. and Zimoch, L. (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393-4412.
- Mitsutomi, M., Isono, M., Uchiyama, A., Nikaidou, N., Ikegami, T. and Watanabe, T. (1998). Chitosanase activity of the enzyme previously reported as β-1,3-1,4-glucanase from *Bacillus circulans* WL-12. Biosci. Biotechnol. Biochem. 62, 2107-2114.
- Miyashita, K., Fujii, T. and Sawada, Y. (1991). Molecular cloning and characterization of chitinase gene from *Streptomyces lividans*. J. Microbiol. 137, 2065–2072.
- Morimoto, K., Karita, S., Kimura, T., Sakka, K. and Ohmiya, K. (1997) Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. J. Bacteriol. 179, 7306-7314.

- Nielsen, M.N. and Sorensen, J. (1999) Chitinolytic activity of *Pseudomonas fluorescens* isolates from barley and sugar beet rhizosphere. FEMS Microbiol. Ecol. 30, 217-227.
- Okazaki, K., Kato, F., Watanabe, N., Yasuda, S., Masui, Y. and Hayakawa, S. (1995) Purification and properties of two chitinases from *Streptomyces* sp. J-13-3. Biosci. Biotechnol. Biochem. 59, 1586-1587.
- Peberdy, J.F., (1985) Mycolytic enzymes. Fungal protoplasts—applications in biochemistry and genetics. Marcel Dekker, New York, 31–44.
- Perrakis, A., Ouzounis, C., Wilson, K.S. and Vorgias, C. (1996). Implications of the tertiary structure determination of chitinase A. Similarities with Fibronectin III domains, oviductal proteins and narbonin. In Advances in Chitin Science, vol. 1 (ed. A. Domard C. Jeuniaux, R. A.A. Muzzarelli and G. Roberts). 34-41.
- Poinar, G.O., Jr. (1979). Nematodes for biological control of insects. CRC Press, Boca Raton, FL.
- Reynolds, S.E. and Samuels, R.I. (1996). Physiology and biochemistry of insect moulting fluid. Adv. Insect Physiol. 26, 157-232.
- Rosenzweig, C., Iglesias, A., Yang, X.B., Epstein, P.R. and Chivian. E. (2001). Climate change and extreme weather events: implications for food production, plant diseases, and pests. Global Change and Human Health. 2, 90–104.
- Sahai, A.S., and Manocha, M.S. (1993). Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol. Rev. 11, 317-338.

- Saito, A., Fujii, T., Yoneyama, T., Redenbach, M., Ohno, T., Watanabe, T. and Miyashita, K. (1999) High-multiplicity of chitinase genes in *Streptomyces coelicolor* A3(2). Biosci. Biotechnol. Biochem. 63, 710-718.
- Saville, G.P., Thomas, C.J., Possee, R.D. and King, L.A. (2002). Partial redistribution of the *Autographa californica* nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL ER-retention motif. J. Gen. Virol. 83, 685-694.
- Schrempf, H. (2001). Recognition and degradation of chitin by *Streptomycetes*. Antonie Van Leeuwenhoek 79, 285-289.
- Shaikh, S.A. and Desphande, M.V. (1993) Chitinolytic enzymes their contribution to basic and applied research. World J. Microbiol. Biotechnol. 9, 468–475.
- Shoresh, M. and Harman, G.E. (2008) Genome-wide identification, expression and chromosomal location of the genes encoding chitinolytic enzymes in Zea mays. Mol. Genet. Genomics. 280, 173-185.
- Songsiriritthigul, C., Lapboonrueng, S., Pechsrichuang, P., Pesatcha, P. and Yamabhai, M. (2010) Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste. Bioresour. Technol. 101, 4096-4103.
- Suzuki, K., Sugawara, N., Suzuki, M., Uchiyama, T., Katouno, F., Nikaidou, N. and Watanabe, T. (2002) Chitinases A, B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. Biosci. Biotechnol. Biochem. 66, 1075-1083.

- Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B. and Watanabe, T. (1999) The third chitinase gene (chiC) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. Biochem. J. 343, 587-596.
- Takayanagi, T., Ajisaka, K., Takiguchi, Y. and Shimahara, K. (1991) Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochim. Biophys. Acta 1078, 404-410.
- Takegawa, K., Yamagucbi, S., Kondo, A., Iwamoto, H., Nakosbi, M., Kato, I. and Iwahara, S. (1991) Transglycosylation activity of endo-P-N-acetylglucosaminidase from *Arthrobacter protophormiae*. Biochem. Int. 24, 849-855.
- Tellam, R.L. (1996). The peritrophic matrix. In Biology of the Insect Midgut (ed. M. J. Lehane and P. F. Billingsley), pp. 86-114. Cambridge: Chapman and Hall.
- Tews, I., Scheltinga, A.C., Terwisscha, V., Perrakis, A., Wilson, K.S. and Dijkstra, B.W. (1997) Substrate assisted catalysis unifies two families of chitinolytic enzymes. J. Am. Chem. Soc. 119, 7954–7959.
- Thomas, C.J., Gooday, G.W., King, L.A. and Possee, R.D. (2000). Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus chiA gene. J. Gen. Virol. 81, 1403-1411.
- Tsigos, I., Zydowicz, N., Martinou, A., Domard, A. and Bouriotis, V. (1999) Mode of action of chitin deacetylase from *Mucor rouxii* on N-acetylchitooligosaccharides. Eur. J. Biochem. 261, 698-705.

- Venegas, A., Goldstein, J. C., Beauregard, K., Oles, A., Abdulhayoglu, N. and Fuhrman, J.A. (1996) Expression of recombinant microfilarial chitinase and analysis of domain function. Mol. Biochem. Parasitol. 78, 149-159.
- Wang, S.L., Chiou, S.H. and Chang, W.T. (1997) Production of chitinase from shellfish waste by *Pseudomonas aeruginosa* K-187. Proc. Natl. Sci. Counc. Repub. China B 21, 71-78.
- Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J. Biol. Chem. 268, 18567-18572.
- Yan, J., Cheng, Q., Narashimhan, S., Li, C.B. and Aksoy, S. (2002) Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans*. Insect Biochem. Mol. Biol. 32, 979-989.
- Yong, T., Hong, J., Zhang-Fu, L., Li, Z., Qiong, D. X., Ke, T. and Shi-Gui, L. (2006) Cloning and expression of a chitinase gene from *Sanguibacter* sp. C4. Acta Genetica Sinica. 33, 1037-1046.