Extracellular alkaline protease production and efficacy studies of endotoxin from *Bacillus thuringiensis* subsp. *kurstaki*

Thesis submitted to the University of Calicut
in partial fulfillment of the requirement for the award of the degree of

Doctor of Philosophy
in
Botany

By
Jisha, V. N.

Research Supervisor
Dr. Sailas Benjamin

Enzyme Technology Laboratory
Biotechnology Division
Department of Botany
University of Calicut
KERALA
November, 2013

Dr. Sailas Benjamin
Director of Research &
Associate Professor of Biotechnology
Enzyme Technology Laboratory
Biotechnology Division
Department of Botany
University of Calicut
Kerala - 673 635
INDIA

Phone: +91-494-2401114
Extn. 406, 407
(M): +91-94955-48315
Fax: +91-494-2400269
Email: benjamin@uoc.ac.in;
Web: www.universityofcalicut.info

CERTIFICATE

This is to certify that the thesis entitled **"Extracellular alkaline protease production and efficacy studies of endotoxin from *Bacillus thuringiensis* subsp. *kurstaki*"** is an authentic record of the research work accomplished by **Mrs. Jisha, V. N.** at the Enzyme Technology Laboratory, Biotechnology Division in the Department of Botany, University of Calicut under my supervision and that no part thereof has been presented earlier for the award of any other degree or diploma.

Dr. Sailas Benjamin
(Research Supervisor)

Res: Eden Gardens, H.No.19/402 A, Chalappuram P.O., Kozhikode - 673 002, Kerala, India. Phone: +91-495-2303232

DECLARATION

I, **Jisha, V. N.** do hereby declare that this thesis entitled **"Extracellular alkaline protease production and efficacy studies of endotoxin from *Bacillus thuringiensis* subsp. *kurstaki*"** is the summary of the research work carried out by me under the supervision of **Dr. Sailas Benjamin**, Associate Professor, Department of Botany, University of Calicut in partial fulfillment of the requirement for the award of Doctor of Philosophy in Botany of the University of Calicut, and also declare that no part of this thesis has been submitted by me for the award of any other degree or diploma.

Calicut University **JISHA, V. N.**

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to **Dr. Sailas Benjamin**, Associate Professor, Enzyme Technology Laboratory, Biotechnology Division, Department of Botany, for the guidance, support and supervision during the study.

I take this opportunity to sincerely acknowledge the **University Grant Commission** for granting me Rajiv Gandhi National Research Fellowship.

I express my indebtedness to **Prof. M. Sabu**, Head, **Prof. P. V. Madhusoodanan, Prof. M. Sivadasan, Prof. Nabeesa Salim**, **Prof. K. V. Mohanan,** former Heads, Department of Botany, University of Calicut, for providing all the necessary facilities to carry out this work comfortably.

I also express my sincere thanks to **Prof. D. Girija** Centre for Plant Biotechnology and Molecular Biology, Kerala agricultural University, **Prof. S. Nandakumar** (Retd), **Prof. P. Manimohan**, **Prof. John E. Thoppil**, **Dr. A. Yoosuf** (Department of Botany), **Prof. M. A. Haq** (Retd), **Prof. K.V. Lazer**, **Prof. B. Ramani**, **Prof. M. Gokul Das, Dr. Y. Shibuvardhanan** (Department of Zoology), **Prof. Mohammad Shafi** (Retd) (Department of Chemistry), **Dr. E. Sreekumaran** (Department of Life Sciences), **Prof. M. V. Joseph**, **Prof. K. K. Elyas** and **Dr. S. Thara** (Department of Biotechnology), University of Calicut, for their help in completing my work.

I am also grateful to **Prof. Ichiro Yamato**, Department of Biological Science and Technology, Japan, **Prof. M. Mohanasundaram**, Tamil Nadu Agricultural University, Coimbatore, **Dr. R. Sreekumar,** Scientist, Sree Chitra Institute of Medical Science and Technology, Poojappura, Thiruvananthapuram, **Dr. Joseph Job**, and **Dr. P. K. George**, Common Instrumentation facility, St. Bazalios College Changanassery, Kottayam, **Dr. S. Krishnan Nair**, Department of Microbiology, Veterinary College, Thrissur, for the timely help to ensure the smooth progress of the work.

I extent my sincere gratitude to **Mrs. Geetha Shankar**, **Mrs. Jocelyn Jose**, and **Mr. P. M. Prakash**, Librarians, Department of Botany, **Librarians**, Indian Institute of Spices Research Institute, Calicut, and Kerala Agricultural University, Thrissur, for the help rendered during literature collection.

I extend my sincere and heartfelt gratitude to all **Teaching** and **Non-teaching Staff** of this Department.

I express my special thanks to **Dr. R. B. Smitha, Mr. S. Pradeep**, **Mrs. S. Sreedevi**, **Mr. K. N. Unni, Mr. S. Sajith, Mr. M. K. Sarath Josh, Mrs. Priji Prakasan, Mrs. T. P. Princy, Ms. Neethu Kannan, Mr. P. Mashhoor**, **Mr. Rajan I, Dr. Asha Ramachandran, Mr. Anil Raj, Dr. K. Sujithra, Dr. M. A. Dollymol, Mr. K. M. Prabhukumar**, **Mr. C. Shameer, Dr. Ratheesh Chandra, Dr. A. K. Abdussalam, Dr. V. P. Thomas, Mr. E. A. Babu, Dr. E. Sanoj, Dr. K. M. Vijayalakshmi, Dr. E. Juli, Dr. S. Sangeetha** and **Dr. U. M. Sheeja, Ms. V. Reshma**, for their constant encouragement and support throughout this course.

I would like to thank **Mr. K. Ajayakumar, Mr. B. Shaji** and **Mr. T.M. Santhosh**, Art and Photography unit, for taking photographs during the study, and also to **Bina** Photostat for their timely help.

I am much indebted to my beloved teacher **Dr. K. Azeez,** Head of the Department of Botany, Unity Women's College, Manjeri, for the support and motivation.

Words are not adequate to express my gratitude to my parents **Dr. V. V. Narayanan** and Smt. **N. N. Gouri**; my husband **Dr. V. K. Sreenivas**, my daughter **Athena**; my brother **Mr. Jithesh V. N.,** his wife **Vidya V**; my father-in-law **Mr. V. K. Raveendran** and mother-in-law **Soumini**, **Relatives** and **friends** for their love and support and above all to God, the Ultimate.

JISHA, V. N.

Dedicated to My Parents and Teachers.....

EQUIPMENTS USED

Item	Brand	Country
Compound microscope	Magnus	India
Binocular microscope with ocular meter	Olympus CX 21	India
Chromatography column	MAGNUM	India
Digital pH meter MK-VI	Systronics	India
Distillation Unit	Borosil	India
Electrophoresis Unit	Biotech	India
Environmental shaker	Orbitek	India
Gel-documentation system	BioRad	Italy
Flourescent Microscope	Leica	Germany
Heating Mantle	Kemi	India
Image analyser	Towa Opticals	Japan
Incubator	Technico	India
Laboratory centrifuge	Remi	India
Laboratory Oven	Labline	India
Laminar air flow cabinet	Kemi	India
Magnetic Stirrer (KMS – 400)	Kemi	India
Micropipettes (0.5 -1000 µL)	Accupipete, Biosystems and Microlit	India
Phase contrast microscope	Leica	Germany
Refrigerated centrifuge	Plastocrafts/Remi	India
Refrigerator	Godrej	India
Scanning electron microscope	JEOL, JWS 3000 Hitachi SU, 6000	Japan
UV-Visible spectrophtometer	Shimadzu	Japan
Vortex mixture	Kemi	India
Water bath	Scigenics Biotech	India
Web cam Companion 4.0	MEM 1300	Japan
Weighing balance	Shimadzu	Japan

ABBREVIATIONS

Bt	:	*Bacillus thuringiensis*
Btk	:	*Bacillus thuringiensis* subsp. *kurstaki*
ddH_2O	:	double distilled water
EDTA	:	Ethylene Diamine Tetraacetic Acid
g	:	gram
g/L	:	gram per litre
L	:	Litre
mg	:	milligram
mg/mL	:	milligram per millilitre
mL	:	milli Litre
MW	:	molecular weight
PEG	:	Polyethylene glycol
SmF	:	Submerged Fermentation
SSF	:	Solid State Fermentation
SDS-PAGE	:	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
TEMED	:	N,N,N',N'-tetra methyl ethylene diamine
TCA	:	Trichloroacetic acid
U/mL	:	Units per milliliter
U/mL_{eqv}	:	Units per milliliter equivalent
w/v	:	Weight per Volume
δ	:	delta
β	:	beta
µg	:	microgram
µL	:	microLitre

CONTENTS

Chapter I

Introduction

Chapter 1
INTRODUCTION

This study addresses three novel aspects of highly demanding *Bacillus thuringiensis* subspecies *kurstaki* Dulmage (*Btk*), *viz*., establishment of non-conventional strategies for the overproduction of insecticidal crystal toxins, isolation of protease as a by product from the effluent and its characterisation, and evaluation of the entomotoxicity potentials of the *δ*-endotoxin produced by biphasic solid-state fermentation.

Bacillus thuringiensis Berliner (*Bt*) is a ubiquitous Gram-positive and sporulating bacterium producing insecticidal crystal proteins (the *δ*-endotoxin) juxtaposed to the endospores in the bacterium (sporangium) during the stationary phase of its growth cycle (Smitha *et al*., 2013a). The nascent *δ*-endotoxin is an inactive protoxin (molecular weight (MW) ranges from 28 to 140 kDa) complex of [crystal (Cry) alone or in combination with cytolytic (Cyt) toxins] high molecular mass, which upon ingestion is cleaved off into smaller active components at the high alkaline environments in the digestive tract of certain agricultural pests (Nakamura *et al.*, 1990; Jisha *et al*., 2013a).

The principal Cry toxins are encoded by different strain-specific *Cry* genes, thus different types of *Bt* are classified (de Maagd *et al*., 2003); but Cyt toxins augment the Cry toxins, *i.e*., enhance the effectiveness of insect control (Bravo *et al*., 2007). The *δ*-endotoxins are shown to cause mortality of insects belonging to different orders, *viz*., Coleoptera, Diptera and Lepidoptera (Schnepf *et al*., 1998) and mites (Payne *et al*., 1993). The predominant subspecies and strains of *Bt* are: *Bt aizawai* strain fz (Hoffmann and Frodsham, 1993); *Bt* strain BRL 43 (Chandi *et al*., 2007); *Bt finitimus* strain B-1166 VKPM (Wojciechowska *et al*., 1999); *Bt israelensis*

(Tilquin *et al*., 2008); *Bt* strain IPS 78/11 (Tigue *et al*., 2001); *Bt kurstaki* (Kati *et al*., 2007); *Bt japonensis* (Brown *et al*., 1997); *Bt sandiego* and *Bt tenebrionis* (Cranshaw, 2008); *Bt tolworthi* (Lambert *et al*., 1996); *Bt sotto* (Iizuka *et al*., 1994); *Bt* (Wellman and Cote, 2005); and *Bt kenya* (Vasquez *et al*., 1995); and *Bt galleriae* (Dubovskii *et al*., 2005).

Commercial *Bt* toxin is a mixture of endospores (which can give rise to new vegetative cells if the conditions are favorable to grow) and δ-endotoxin, which is the active ingredient in most (about 90%) of the microbial insecticides produced worldwide. Biopesticides are relatively selective in their action, compared to synthetic pesticides that may have undesirable side effects. *Btk* is commercially available as a biological insecticide under different trade names (Biobit, Dipel, Javelin, *etc.*), being used for the pest or insect control programmes in forestry, agricultural and urban settings around the world.

Commercial *Bt* products account for 90-95 % share of the total biopesticide market (Feitelson, 1993). Due to this economic interest, numerous approaches have been developed to enhance the production of *Bt* bioinsecticides (Jisha *et al*., 2013a). Conventionally, commercial production of *Bt* toxin has been achieved by submerged or liquid fermentation (SmF), or by batch or fed-batch process (Vu *et al*., 2010), but advantages of solid-state fermentation (SSF) for the production of both primary and secondary metabolites of microbial origin have well been appreciated by many investigators (Benjamin and Pandey, 1998; Jisha *et al*., 2013b). Compared to SmF, SSF received more attention recently, as it uses simpler fermentation medium, requires a smaller space, easier to aerate, higher productivity, lower waste water output, lower energy requirement, and contamination (Benjamin *et al.*, 2013). The product so obtained can be recovered in highly concentrated form, as against the dilute form obtained by SmF. However, application of SSF strategy for the production of *Bt* toxin seems to be ignored.

Bacteria capable of producing extracellular proteases are the focus of many investigators, especially with an industrial perspective (Jisha *et al*., 2013b). *Btk* is shown to produce an extracellular, metal chelator-sensitive protease during the early stages of sporulation (Li and Yousten, 1975), while Hotha and Banik (1997) showed that *Bt* strain H14 produced alkaline protease in an aqueous two-phase system comprising polyethylene glycol and potassium phosphate. In fact, because of overwhelming focus on *Bt* - toxin, exploitation of the potentials of *Bt* for the production of extracellular protease with an industrial perspective was found totally neglected (Jisha *et al*., 2013c).

The insecticidal activities of the Cry 1A (a, b and c) toxins produced by *Btk* strain HD1 were found much efficient against the larvae of *Choristoneura fumigferana, C. occidentalis*, *C. pinus*, *Lymantria dispar, Orgyia leucostigma, Malacosoma disstria* and *Actebia fennica* (Rosas-García, 2009). However, efficacy of *Bt* toxin for the control of acarid mites, especially *Eutetranychus orientalis* Klein is not addressed yet.

Thus, to address the aforesaid three lacunae, the following specific objectives were set for this study:

- To establish a novel fermentation strategy for the overproduction of *Btk* toxin (endospore + δ-endotoxin).
- To purify and physically demonstrate the δ-endotoxin produced by solid-state fermentation.
- To examine whether extracellular protease is produced in the effluent; and if yes, to purify and characterize the protease produced.
- To demonstrate the entomotoxicity potentials of the *Btk* toxin produced by novel strategy, using a locally available pest, *E. orientalis.*
- To release a protocol to be used by the public.

Chapter I

Review of literature

Chapter 2
REVIEW OF LITERATURE

[Jisha VN, Smitha RB, Pradeep S, Sreedevi S, Unni KN, Sajith S, Priji P, Sarath Josh MK and Sailas Benjamin (2013). Versatility of microbial proteases. *Advances in Enzyme Research*, 1: 39-51. doi:10.4236/aer.]

[Jisha VN, Smitha RB and Sailas Benjamin (2013). An overview on the crystal toxins from *Bacillus thuringiensis*. *Advances in Microbiology*, 3: 462-472. doi:10.4236/aim.2013]

2.1. Aim

This chapter reviewed various aspects of *Bacillus thuringiensis.* The biochemical studies on alkaline proteases from *Bacillus thuringiensis* subspecies *kurstaki* encompasses the diverse aspects such as a biphasic fermentation strategy for the overproduction of δ-endotoxin, its purification strategy; and production, purification, characterisation of alkaline protease; and efficacy study of δ-endotoxin against a devastating mite *Eutetranychus orientalis* Klein. Thus, this review makes a glimpse into the microbial proteases and δ-endotoxin produced by *Btk* with production conditions; coupled with *E. orientalis*, the test organism used for entomotoxicity assay.

2.2. *Bacillus thuringiensis* Berliner

Biological pesticide is one of the most promising alternatives over conventional chemical pesticides, which offers less or no harm to the environments and biota. *Bacillus thuringiensis* (commonly known as *Bt*) is an insecticidal Gram-positive spore-forming bacterium producing crystalline proteins called delta-endotoxins (δ-endotoxin) during its stationary phase or senescence of its growth. *Bt* was originally discovered from diseased

silkworm (*Bombyx mori*) by Shigetane Ishiwatari in 1902. But it was formally characterised by Ernst Berliner from diseased flour moth caterpillars (*Ephestia kuhniella*) in 1915 (Milner, 1994). The first record of its application to control insects was in Hungary at the end of 1920, and in Yugoslavia at the beginning of 1930s, it was applied to control the European corn borer (Lord, 2005).

Bt, the leading biorational pesticide was initially characterised as an insect pathogen, and its insecticidal activity was ascribed largely or completely to the parasporal crystals. It is active against more than 150 species of insect pests. *Bt* is normally marketed (as a mixture of dried spores and toxin crystals) under various trade names worldwide for controlling many plant pests, mainly caterpillars belonging to Lepidoptera (represented by butterflies and moths), mosquito larvae and a few others including unconventional targets like mites. The share of *Bt* products in agrochemical (fungicide, herbicide and insecticide) market is about only 1%. The first commercial *Bt* product was produced in 1938 by Libec in France, but the product was used only for a very short time due to World War II, and then in the USA in the 1950s (Nester *et al*., 2002). The toxicity of *Bt* culture lies in its ability to produce the crystalline protein, this observation led to the development of bioinsecticides based on *Bt* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera (Lacey *et al*., 2001; de Maagd *et al*., 2001; Nester *et al*., 2002). Now-a-days, *Bt* isolates are reported also active against certain nematodes, mites and protozoa (Marvier *et al*., 2007). It is already a useful alternative or supplement to synthetic chemical pesticide for applications in commercial agriculture, forest management, and mosquito control, and also a key source of genes for transgenic expression to transfer pest resistance in plants.

Due to this economic interest, numerous approaches have been developed to enhance the production of *Bt* bioinsecticides. The insecticidal activity of *Bt* is known to depend not only on the activity of the bacterial culture itself, but also on abiotic factors, such as the medium composition and cultivation strategy.

2.2.1. Bt toxins

Bt produces one or more types of parasporal crystalline proteins (called *δ*-endotoxins) concomitantly with sporulation. Cryastal (Cry) or cytolytic (Cyt) proteins singly or in their combination constitute the *δ*-endotoxins (Smitha *et al*. 2013a). Cry proteins are parasporal crystalline inclusions produced by *Bt* that exhibit experimentally verifiable toxic effect to a target organism or have significant sequence similarity to a known Cry protein. Cyt proteins are also parasporal inclusions exhibiting hemolytic (cytolitic) activity with obvious sequence similarity to a known Cyt protein. These toxins are highly specific to their target insect, but innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo *et al*., 2007). These crystalline proteins are mainly encoded by extra-chromosomal genes located on the plasmids. The parasporal crystalline proteins produced during the stationary phase of its growth cycle account for 20-30% of the dry weight of the cells of this phase (Agaisse and Lereclus, 1995). Expression of most *Cry* genes (*e.g.*, *cry* 1Aa, *cry* 2A, *cry* 4A, *etc*.) are well regulated in the sporulation phase of growth. Studies have shown that several Cry proteins - when expressed in either *E. coli* or *B. subtilis* - expressed as 130 to 140 kDa protoxin complex molecules that retain their biological activity. More than 200 types of endotoxin gene have been cloned from various strains of *Bt,* and sequenced so far. The plasmid profiles of most *Bt* strains are rather complex, with molecular weight varying from 2 to 200 kb and the number of plasmids ranging from 1 to 10 in most strains (McDowell and Mann, 1991). The self-

assembly of these 130 kDa proteins is spontaneous, mediated primarily by the C-terminus of the protein. Their cysteine-rich carboxyl terminus is highly conserved among lepidopteran-specific Cry proteins, which generates a number of disulfide bridges that allow good crystal packing and also protects the toxin from the attack of various proteases.

Commercial insecticides derived from *Bt* have a long history of successful use in the biocontrol of insect pests (Beegle and Yamamoto, 1992; Bravo *et al*., 2011). Many studies examined the composition and methods of preparation of nutrient media for entomopathogenic bacteria (Bti, 2011; Sierpinska, 1997). Chromsomal insertion of *Cry* gene may enhance the production of *δ*-endotoxins in *Bt* strains (Kalman *et al*., 1995). Erythromycin resistance may affect the sporulation processes in *Bt* and *B. subtilis* (Fargette and Grelet, 1975; Mahler and Halvorson, 1980). Most *Bacillus* strains produce a mixture of structurally different insecticidal crystal proteins (Cry proteins), which are encoded by different *Cry* genes. Each of these proteins may contribute to the insecticidal spectrum of a strain that makes it selectively toxic to a wide variety of insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera and Mallophaga, as well as to other invertebrates (**Table 1**) (Beegle and Yamamoto, 1992; de Maagd *et al*., 2003; Feitelson *et al*., 1992; Schnepf *et al*., 1998).

Bt strains are able to produce exoenzymes, such as proteases and α-amylases (Smitha, 2010). Apart from *δ*-endotoxin, some isolates of *Bt* produce another class of insecticidal small molecules called *β*-exotoxin, the common name for which is thuringiensis (Guo *et al*., 2008). *Beta*-exotoxin and the other *Bacillus* toxins (*δ* -endotoxins) may contribute to the general insecticidal toxicity of the bacterium to lepidopteran, dipteran, and coleopteran insects. *Beta*-exotoxin is known to be toxic to humans and almost all other forms of life and, in fact, its presence is prohibited in *Bt* products (Berlitz *et al*., 2012).

Engineering of plants to contain and express only the genes for δ-endotoxins avoids the problem of assessing the risks posed by these other toxins that may be produced in microbial preparations (Toledo *et al*., 1999).

Table 1. Endotoxin producing Bacilli and target organisms

***Bacillus* spp.**	**Target pest**	**Reference**
B. laterosporus & *Brevibacillus laterosporus*	*Musca domestica* and *Aedes aegypti*	Ruiu *et al*., 2007
B. sphaericus & *Bt israelensis*	*Culex quinquefasciatus, C. pipiens, C. tarsalis*	Boisvert and Boisvert 2000; Cohen *et al*., 2008; Park *et al*., 2005
Bt	Manduca sexta, Cabbage butterfly (*Pieris brassicae*) larvae, Colorado potato beetle larvae, *Aedes aegypti* gypsy moth (*Lymantria dispar*). (*Epinotia aporema*), *Cacyreus marshalli* (Lycaenidae), *Lobesia botrana* (Tortricidae), *Manduca sexta* (Sphingidae), *Pectinophora gossypiella* (Gelechiidae), *P. xylostella* (Plutellidae), *Spodoptera exigua*(Noctuidae) *Autographa californica, Spodoptera frugiperda Acyrthosiphon pisum,* Diamondback Moth (*Plutella xylostella*), acarids, bulb mite (*Rhizoglypus robini*); *Plutellax ylostella*	Dean *et al*., 2004; Herrero *et al*., 2001; Jiménez-Juárez *et al*., 2007; Juhasz and Skarka, 1990; Lambert *et al*., 1992; Lima *et al*., 2008; Payne *et al*., 1998; Peng *et al*., 2003; Porcar *et al*., 2009; Rajamohan *et al*., 1996; Sauka *et al*., 2007; Tang *et al*., 1996; Zouari Nabil *et al*., 2002
Bt berliner	*Lygus hesperus* (Hemiptera: Miridae)	Wellman and Cote, 2005
Bt BRL 43	First instar larvae of cotton leaf worm, cotton boll worm and black cut worm	Chandi *et al*., 2007
Bt galleriae	*Galleria mellonella* L. (Lepidoptera, Pyralidae)	Dubovskii *et al*., 2005
Bt H14	*Leishmania. Major*	Hanan *et al*., 2008
Bt IPS 78/11	Manduca sexta	Tigue, 2001

Bt israelensis IPS78/11	Sheep blowfly species (*Lucilia cuprina*, *L. sericata*, and *Calliphora stygia*).	Chilcott *et al*., 1998
Bt sotto	Cabbage butterfly	Iizuka *et al*., 1994
Bt kurstaki	*Helicoverpa zea; Scrobipalpula absoluta: Malacosoma neustria* and *Lymantria dispar* larvae	Kati *et al*., 2007; Vasquez *et al*., 1995
Bt kurstaki (serotype H3a, 3b, 3c) strain BNS3	*Prays oleae*	Rouis *et al*., 2007
Bt kurstaki HD1 & *Bt kurstaki* HD73	*Manduca sexta* larvae *Heliothis virescens*	Aronson *et al*., 1999
Bt tolworthi	*Spodoptera frugiperda*, *S.exigua*, Cutworms, *Ostrinia nubilalis* and *Plutella xylostella*, and *P. xylostella*	Capalbo *et al*., 2001
Bt tenebrionis	synanthropic mites	Erban *et al*., 2009

2.2.2. General structure of Cry toxin

The major component of crystals toxic to lepidopteron larvae is a 130 kDa protein (protoxin), which upon cleavage in the insect yields the functional (insecticidal) proteins of lower molecular weight; very often the crystal formed is an assemblage of many proteins (Crickmore *et al*., 1998). A *Bt* isolate (Soil-47) showed distinct bands of 32.1 and 34.6 kDa. The band corresponding to 32.1 kDa protein could arise from the type *Cry* 1 and/or *Cry* 4 gene, while the other (34.6 kDa) protein is possibly encoded by the type *Cyt* gene (Hasan *et al*., 2010). An unexpected finding was that a 20 kb heterologous DNA fragment was found intimately associated with the crystals from *Btk* HD73, the DNA is not susceptible to nuclease attack unless the protoxin is removed or proteolyzed to toxin. The active toxin is not associated with DNA; however, evidence was obtained which indicated that the DNA was involved in the generation of toxin from the crystal protein (Clairmont *et al*., 1998).

Structure determination of *Bt* toxins remains one of the most important tools in understanding and improving the utility of these proteins. Crystal structure of Cry III A has been published first (Li *et al*., 1991) and several others are now available. Xia *et al*. (2008) predicted the first theoretical model of the three dimensional (3D) structure of a Cry (Cry 5 Ba) toxin by homology modeling on the structure of the Cry 1Aa toxin, which is specific to Lepidopteran insects. The three-domain structure of Cry IIIA consisted of the following: an α-helical barrel (domain I) which shows some resemblance to membrane-active or spore-forming domains of other toxins (Ojcius and Young, 1991) a triangular prism of 'Greek key' beta sheets (domain II); and a *β*-sheet jelly-roll fold (domain III) (Li *et al*., 1991). Members of this 3-domain Cry family are used worldwide for insect control, and their mode of action has been characterised in some details (Bravo *et al*., 2007).

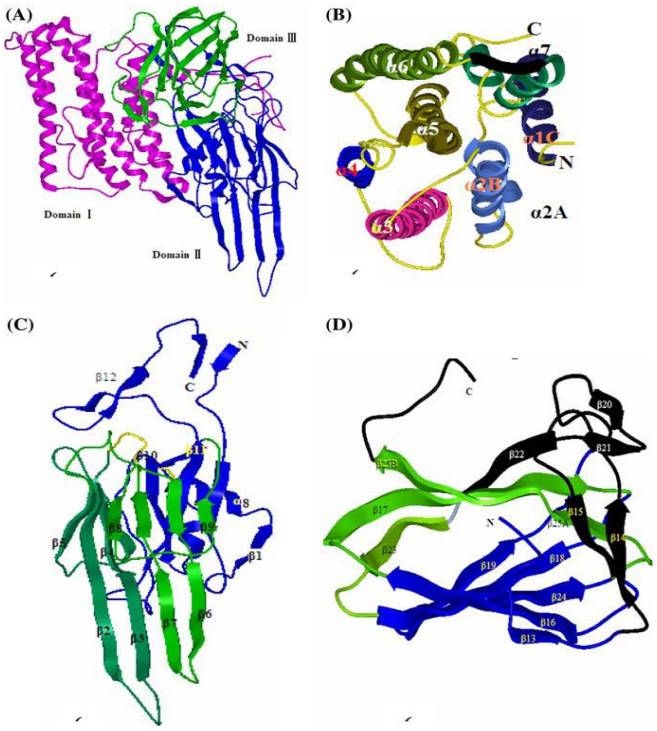


Figure 1. 3-D structure of Cry 2Ab1 (Lin *et al*., 2008): **A**. Cry 2A 3 domain structure; **B**. Vip 2A; **C**. Cry 23/Cry 37; **D**. Cyt 2A

2.2.3. Mode of action

Mode of action of *δ*-endotoxin involves several events that must be completed several hours after the ingestion in order to lead to insect death. Following ingestion of the inactive protoxin, the crystals are solubilized by the alkaline conditions in the insect midgut and are subsequently proteolytically converted into a toxic core fragment (Höfte and Whiteley, 1989), this activated toxin binds to receptors located on the apical microvillus membranes of epithelial midgut cells. For Cry1A toxins, at least four different binding sites have been described in different lepidopteran insects: a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Rosas-García, 2009). Cry toxins interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding, which would result is in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast, Cyt toxins directly interact with membrane lipids and insert into the membrane. Recent evidence suggests that Cyt synergises or overcomes resistance (for instance, to mosquitocidal-Cry proteins) by functioning as a Cry-membrane bound receptor (Bravo *et al*., 2007).

Once activated, the endotoxin binds to the gut epithelium and causes cell lysis by the formation of cation- selective channels, which leads to death. The activated region of the *δ*-endotoxin is composed of three distinct structural domains: an N-terminal helical bundle domain involved in membrane insertion and pore formation; a *beta*-sheet central domain involved in receptor binding; and a C-terminal *β*-sandwich domain that interacts with the N-terminal domain to form a channel. After binding, toxin adopts a conformation suitable for allowing its insertion into the cell membrane. Subsequently, oligomerisation occurs, and this oligomer forms a pore or ion

channel induced by an increase in cationic permeability within the functional receptors contained on the brush borders membranes. This allows the free flux of ions and liquids, causing disruption of membrane transport and cell lysis leading to insect death (Höfte and Whiteley, 1989; Aronson *et al*., 1999). The complete nature of this process is still elusive.

Differences in the extent of solubilisation sometimes explain differences in the degree of toxicity among Cry proteins (Aronson *et al*., 1999; Du *et al*., 1994). A reduction in solubility is speculated to be one potential mechanism for insect resistance (McGaughey and Whalon, 1992). Cry3A protein may be necessary for the solubilisation of toxins in the midgut of insects (Carroll *et al*., 1997). Most recently, two models were proposed for the action of crystal proteins *i.e*, the sequential binding model and signaling pathway model (Vachon *et al*., 2012).

2.2.4. Target pests

It is well documented that many insects are susceptible to the toxic activity of *Bt* of them, lepidopterans have exceptionally been well studied, and many toxins have shown activity against them (Rosas-García, 2009). The order Lepidoptera encompasses majority of susceptible species belonging to agriculturally important families such as Cossidae, Gelechiidae, Lymantriidae, Noctuidae, Pieridae, Pyralidae, Thaumetopoetidae, Tortricidae, and Yponomeutidae (Iriarte and Caballero, 2001). A novel crystal proteins exhibiting insecticidal activity against lepidopterans has been reported from *Bt* strains (Baum *et al*., 2007).

Dipterans are also important target pests, and many of them are highly susceptible to *Bt* Discovery of novel strains of *Bt* containing parasporal crystal proteins having pesticidal properties against white flies, aphids, leaf hoopers, and possibly other sucking insects of agronomic importance

extended the potential applications of this bacterium. However, the novel toxic activities found in these novel strains are not limited only to insects, as some of them produce crystals with activity against nematodes, protozoans, flukes, collembolans, mites and worms, among others (Rosas-García, 2009).

2.2.5. Production media and media formulations

Indeed, large quantities of spores with high insecticidal activity are required for practical applications. This means that while handling *Bt* as bioinsecticide, a high spore count is not sufficient to ensure toxicity, but it is necessary to reach high *δ*-endotoxin titers. One of the most underreported aspects of *Bt* is that of the production and formulation, although there are certain work existed in connection with *Bt* growth on several synthetic or complex media (Zouari *et al*., 1998). There are several formulations of media proposed by different authors. Our group explored the efficacy of various raw agricultural products as supplement to Luria-Bertani (LB) for enhancing the toxin production, and found potato flour as an efficient supplement to commercial LB medium (Smitha *et al*., 2013a). To develop a cost-effective process for the production of *Bt*-based insecticide, it is imperative to cultivate the bacterial strain in a nutrient rich medium to obtain the highest yields of spore-crystal complexes. Conventionally, *Bt*- crystals are being produced employing submerged or liquid fermentation (SmF) techniques, but recently many workers use nutrient-rich waste water or sludge from various treatment plants as the medium for the production of *Bt*-toxin (Yezza *et al*., 2006).

Solid-state fermentation: Solid-state fermentation (SSF) has been developed in eastern countries over many centuries, and has enjoyed broad application in these regions to date (Benjamin *et al*., 2013). The term SSF denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase (water). There are several advantages for SSF; for example, high productivities, extended stability of products and low

production costs, which say much about such an intensive biotechnological application. With increasing progress and application of rational methods in engineering, SSF will reach higher levels regarding standardization and reproducibility in future. This can make SSF as the preferred technique in the special fields of application such as the productions of enzymes and secondary metabolites, especially foods and pharmaceuticals (Hölker and Lenz, 2005).

Different production media and media compositions can change either the relative toxicity against several target insects or the insecticidal potency of products obtained from the same *Bt* strains (Tokcaer *et al*., 2006). According to Farrera *et al*. (1998) media with different composition showed changes in crystal production, *i.e.* different amounts of Cry proteins produced per spore would vary. The ingredients in the media affect the rate and synthesis of the different *δ*-endotoxins and also the size of the crystals produced. Using barley as the carbon source, Amin (2008) developed a cost-effectively protocol for the mass production of *Bt.*

Several media based on complex substrates such as corn steep liquor (Goldberg *et al*., 1980), peptones (Sikdar *et al*., 1991) blackstrap molasses and Great Northern White Bean concentrate (Morries *et al*., 1996) or LB supplemented with agricultural products (Smitha *et al*., 2013a) have been found efficient for *Bt* bioinsecticide production. Various investigators modified such commercial media by supplementing it with mineral nutrients or various salts, *i.e.,* enriched medium. Zouari *et al*. (1998) showed that *Bt* subspecies *kurstaki* (*Btk*) produced 1 g/L of *δ*-endotoxin in 4.5 g /L total dry biomass in a complex liquid medium, in which the sugar was replaced by gruel hydrolysate. A mixture of extracts from potato and Bengal gram or bird feather and de-oiled rice bran or wheat bran, chickpea husk and corncob was used to cultivate *Bt israelensis* and found that the mosquitocidal activity of the crude toxin was higher than that produced in the conventional medium

(Poopathy and Archana, 2011). Valicente *et al*. (2010) used LB medium supplemented with various salts, and agricultural by-products like soybean flour (0.5%) and liquid swine manure (4%) to increase *Bt* biopesticide production by SmF, which resulted in 1.18g/L dry cell mass. Zhuang *et al*., (2011) also claimed that they have purified *δ*-endotoxin (up to 7.14 mg/g medium) by one step centrifugation from wastewater sludge-based medium, however they did not provide any physical evidence for the purified crystals. From these reports, it seems that maximum yield of *Bt* toxin could be attained is 3.6 g/L (Valicente *et al*., 2010) in SmF or 7.14 g/Kg medium in SSF (Zhuang *et al*., 2011), where they did not provide the actual cost effect.

2.3. Bioassay

Well-designed studies under confined conditions required to understand the effect of *Bt* toxins on different organisms. It is considered that *Bt* toxins also to be toxic to lepidopeterous, coleopeterous and dipterous insects in addition to mites, nematodes, protozoa and flukes (Feitelson *et al*., 1992; Payne *et al*.,1998; Schnepf *et al*., 1998). These proteins are usually thought to act only on the actively feeding larvae of susceptible species by a mechanism involving consumption and proteolytic processing of the protein followed by binding to, and the lysis of midgut epithelial cells. It was found that proteolytically activated insecticidal crystal proteins significantly reduced the lifespan of adult *Heliothis virescens* and *Spodoptera exigua* at concentrations of 500 μg/mL, but not 167 or 25 μg/mL at their assay conditions (Grove *et al*., 2001). *Bt* crystal proteins showed *in vitro* cytotoxicity against human cancer cells and leukemic T cells (Mizuki *et al*., 1999). Interestingly, Xu *et al.* (2004) demonstrated that the *Bt* crystal proteins can protect plasmodium-infected mice from malaria. Moreover, non-conventional targets such as *Caenorhabditis elegans* (nematode) has been demonstrated for the first time (Marroquin *et al.*, 2000).

Toxins of *Btk* strain HD1 have widely been used to control the forest pests such as gypsy moth, spruce bud worm, the pine procesionary moth, the European pine shoot moth and the nun moth (Gui-ming *et al*., 2001). Direct feeding of crude pellet containing *Bt*-toxin (Fadel and Sabour, 2002), sprays (Mulligan *et al*., 1980), pollen diet formulation (Buchholz *et al*., 2006) are the normal mode of applications being practised in entomotoxicity assays. A different feeding strategy was successfully used for the bioassay of *A. guerreronis*, in which the dried solid fermented powder directly brushed on the infested coconut buttons (Smitha, 2010). Many authors used surfactants like BIT (1, 2-benzisothiazolin-3-one), one of the inert ingredients in Foray 48B (a *Btk* formulation), the siloxane (organosili cone) Triton-X-100, Tween 20 and Latron CS-7 - some surfactants for *Btk* formulations (Helassa *et al*., 2009).

The mortality rate of *Thaumetopoea solitaria* on the application of *Btk* toxin has been demonstrated by Er *et al*. (2007). Purified *Btk* toxin inhibited the growth of monarch larvae, but did not cause mortality (Hellmich *et al*., 2001). The LC_{50} value of *Btk* was found to be 398.1 µg/mL against caterpillars of *Arctornis submarginata* (Khewa and Mukhopadhyay, 2010). Toxicity of several formulations of *Btk* to beet armyworm (*Spodoptera exigua*) was determined using neonate larvae in a diet incorporation bioassay.

Probit analysis (LC_{50}) has been used by many authors for ascertaining the efficacy of various *Bt* formulations. For instance, Yashodha and Kuppusamy, (2008) successfully used dipping method for testing the efficacy of *Btk* formulation in Tween 20 on Brinjal. Gobatto *et al*. (2010) used various concentrations of spore suspension of *Bt* for estimating the probit value on mosquito and a moth. Payne *et al*. (1998) employed artificial feeding assay for Two-spotted spider mite (*T. urticae*), a related mite to *E. orientalis* with different feeding regime. They fed the mite with 5 mg spray-dried powder of

Bt broth (a mixture of pores, crystals, cellular debris) in 1 ml sucrose (10%) containing preservatives and surfactant.

Possible use of *Bt* preparation (Dipel 2X) as a substitute for chemical insecticides (Lannate and Hostathion) was evaluated against two major pests of potato crop, *Agrotis sp.* and *Spodoptera exigua.* The toxicity studies of *Bt* to four instars larvae of diamondback moth, *Plutella xylostella* (L.) suggested that *Bt* could be an important agent for the control of larval instars of *Plutella xylostella* (Ranjbari *et al*., 2011). The *Bt* diet suppressed the growth of the four mite species such as *Acarus siro* L., *Tyrophagus putrescentiae* (Schrank), *Dermatophagoides farinae* Hughes, and *Lepidoglyphus destructor* (Schrank) *via* feeding tests (Erban *et al*., 2009).

2.3.1. Resistance to Bt toxins

***Laboratory-selected strains*:** In the past, it was believed that insects would not develop resistance to *Bt* toxins, since *Bt* and insects have coevolved. Starting in the mid-1980s, however, a number of insect populations of several different species with different levels of resistance to *Bt* crystal proteins were obtained by laboratory selection experiments, using either laboratory-adapted insects or insects collected from wild populations (Ferre *et al.,* 1995; Tabashnik, 1994). Examples of laboratory-selected insects resistant to individual Cry toxins include the Indian mealmoth (*Plodia interpunctella*), the almond moth (*Cadra cautella*), the Colorado potato beetle (*Leptinotarsa decemlineata*), the cotton leafworm (*Spodoptera littoralis*), the beet armyworm (*S. exigua*), *etc*., (Schnepf *et al*., 1998).

Given the multiple steps in processing the crystal to an active toxin, it is not surprising that insect populations might develop various means of resisting intoxication. It is important, however, to keep in mind that selection in the laboratory may be very different from selection that occurs in the field. Insect

populations maintained in the laboratory presumably have a considerably lower level of genetic diversity than field populations. Several laboratory experiments to select for *Bt* resistance in diamondback moths failed, although the diamondback moth is the only known insect reported so far to have developed resistance to *Bt* in the field (Schnepf *et al*., 1998). It is possible that the genetic diversity of the starting populations was too narrow and thus did not include resistance alleles. In the laboratory, insect populations are genetically isolated; dilution of resistance by mating with susceptible insects - as observed in field populations - is excluded (Schnepf *et al*., 1998).

In addition, the natural environment may contain factors affecting the viability or fecundity of resistant insects, *i.e*., factors excluded from the controlled environment of the laboratory. Resistance mechanisms can be associated with certain fitness costs that can be deleterious under natural conditions (Trisyono and Whalon, 1997). Natural enemies, such as predators and parasites can influence the development of resistance to *Bt* by preferring either the intoxicated, susceptible or the healthy resistant insects. In the former case, one would expect an increase in resistance development, while in the latter, natural enemies can help to retard resistance development to *Bt*. Nevertheless, selection experiments in the laboratory are valuable because they reveal possible resistance mechanisms and make genetic studies of resistance possible.

Field-selected strains*:* The first case of field-selected resistance to *Bt* was reported from Hawaii, where populations of diamondback moth showed different levels of susceptibility to a formulated *Bt* product (Dipel). Populations from heavily treated areas proved more resistant than those populations treated at lower levels, with the highest level of resistance at 30-fold (Tabashnik *et al*., 1994).

The resistance trait is conferred largely by a single autosomal recessive locus (Tabashnik *et al*., 1997). This "Hawaii" resistance allele simultaneously confers cross-resistance to *Cry*1Aa, *Cry*1Ab, *Cry*1Ac, *Cry*1Fa, and *Cry*1Ja but not to *Cry*1Ba, *Cry*1Bb, *Cry*1Ca, *Cry*1Da, *Cry*1Ia, or *Cry*2Aa (369). At least one *Cry*1A-resistant diamondback moth strain has been shown to be very susceptible to *Cry* 9C (Lambert *et al*., 1992).

Resistance to *Btk* products and resulting failure in diamondback moth control has resulted in the extensive use of *Bt* subsp. *aizawai*-based insecticides in certain locations (Schnepf *et al*., 1998)**.** Insects in two colonies from Hawaii showed up to a 20-fold resistance to *Cry*1Ca, compared to several other colonies, including one obtained earlier from the same location, as well as moderately high resistance to *Cry*1Ab and *Btk*-based formulations (Schnepf *et al*., 1998).

A Malaysian strain highly resistant to the *kurstaki* and the *aizawai* subspecies was apparently mutated in several loci (Lambert *et al*., 1992). A *Cry*1Ab resistance allele associated with reduced binding to brush border membrane vesicles receptors was partially responsible for resistance to both subspecies. Genetic determinants responsible for subspecies *kurstaki*-specific and subspecies *aizawai*-specific resistance segregated separately from each other and from the *Cry*1Ab resistance allele in genetic experiments (Wright *et al*., 1997).

After less than 2 decades of intensive use of *Btk* in crucifer agriculture, resistant insects have evolved in numerous geographically isolated regions of the world, and subspecies *aizawai* resistance is beginning to appear even more rapidly.

Defying the expectations of scientists monitoring transgenic crops such as corn and cotton that produce insecticidal proteins derived from *Bt*, target

insect pests have developed little or no resistance to *Bt* crops thus far, according to US Department of Agriculture-funded scientists. These findings suggest that transgenic *Bt* crops could enjoy more extended, more profitable commercial life cycles and that the measures established to mitigate resistance before the crops were introduced are paying off (Fox, 2003).

Evolution of resistance in pests can reduce the effectiveness of insecticidal proteins from *Bt* produced by transgenic crops. Field outcomes support theoretical predictions that factors delaying resistance include recessive inheritance of resistance, low initial frequency of resistance alleles, abundant refuges of non-*Bt* host plants and two-toxin *Bt* crops deployed separately from one-toxin *Bt* crops. The results imply that proactive evaluation of the inheritance and initial frequency of resistance are useful for predicting the risk of resistance and improving strategies to sustain the effectiveness of *Bt* crops (Tabashnik *et al*., 2013).

2.3.2. Resistance management

Resistance management strategies try to prevent or diminish the selection of the rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently low for insect control (Alstad and Andow, 1995; Gould, 1994).

Proposed strategies include: the use of multiple toxins (stacking or pyramiding), crop rotation, high or ultrahigh dosages, and spatial or temporal refugia (toxin-free areas). Retrospective analysis of resistance development does support the use of refugia (Tabashnik**,** 1994). Experience with transgenic crops expressing *Cry* genes grown under different agronomic conditions is essential to define the requirements of resistance management. In transgenic plants, selection pressure could be reduced by restricting the expression of the crystal protein genes to certain tissues of the crop (those most susceptible to

pest damage) so that only certain parts of the plant are fully protected, the remainder providing a form of spatial refuge. It has been proposed that cotton lines in which *Cry* gene expression is limited to the young bolls may not suffer dramatic yield loss from *Heliothis* larvae feeding on other plant structures, since cotton plants can compensate for a high degree of pest damage (Gould, 1988).

Another management option is the rotation of plants or sprays of a particular *Bt* toxin with those having another toxin type that binds to a different receptor. A very attractive resistance management tactics is the combination of a high-dose strategy with the use of refugia (Schnepf *et al*., 1998).

2.4. Proteases

Recent years have witnessed a phenomenal increase in the use of enzymes as industrial catalysts. Proteases (synonymous as peptidase or proteinase) constitute a very large and complex group of enzymes, widely utilised in a host of industries. They differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima, and stability profile. Studies relating to such properties are imperative for the successful application of these enzymes in their respective industry (Sumantha *et al*., 2005). The main sources of the enzymes were from animals (*e.g.* calf stomach), plants (*e.g.* pineapple, fig, and papaya), microbes (*e.g*. *Bacillus* spp., *Pseudomonas* spp.) (Rao *et al*., 1998; Shafee *et al*., 2005), *etc*. But the production of enzymes from plant and animal sources is limited due to climatic reasons and ethical issues, respectively (Rao *et al*., 1998; Shafee *et al*., 2005).

Microbial sources have occupied an invincible domain in the production of all the three - acidic, neutral, and alkaline - major types of proteases. The alkaline proteases, an important group of industrial enzymes are produced by a wide

range of organisms including animals, fungi and bacteria. *Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Halomonas, Pseudomonas* and *Serratia* are the major bacterial genera which contribute to proteases (Shafee *et al*., 2005). *Bacillus* derived alkaline proteases are of immense utility in other industrial sectors too, *viz*., leather, food, textile, organic synthesis, and waste water treatment. *Bacillus* derived alkaline proteases are stable at elevated temperatures and pH, but majority of them are incompatible with detergent matrices (Deng *et al*., 2010). Therefore, alkaline proteases with superior performance for commercial exploitations, especially for detergents, are being focused.

2.4.1. General classification of proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified under the subgroup 4 of group 3 (hydrolases) (**Table 2**). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. On the basis of their site of action on protein substrates, proteases are broadly classified as endo- or exo-enzymes (Rao *et al*., 1998). They are further categorised as serine proteases, aspartic proteases, cysteine proteases or metallo proteases- depending on their catalytic mechanism (**Table 2**). Proteases are also classified into different clans and families depending on their amino acid sequences and evolutionary relationships. Based on the pH optima, they are referred to as acidic, neutral, or alkaline proteases (Rao *et al*., 1998).

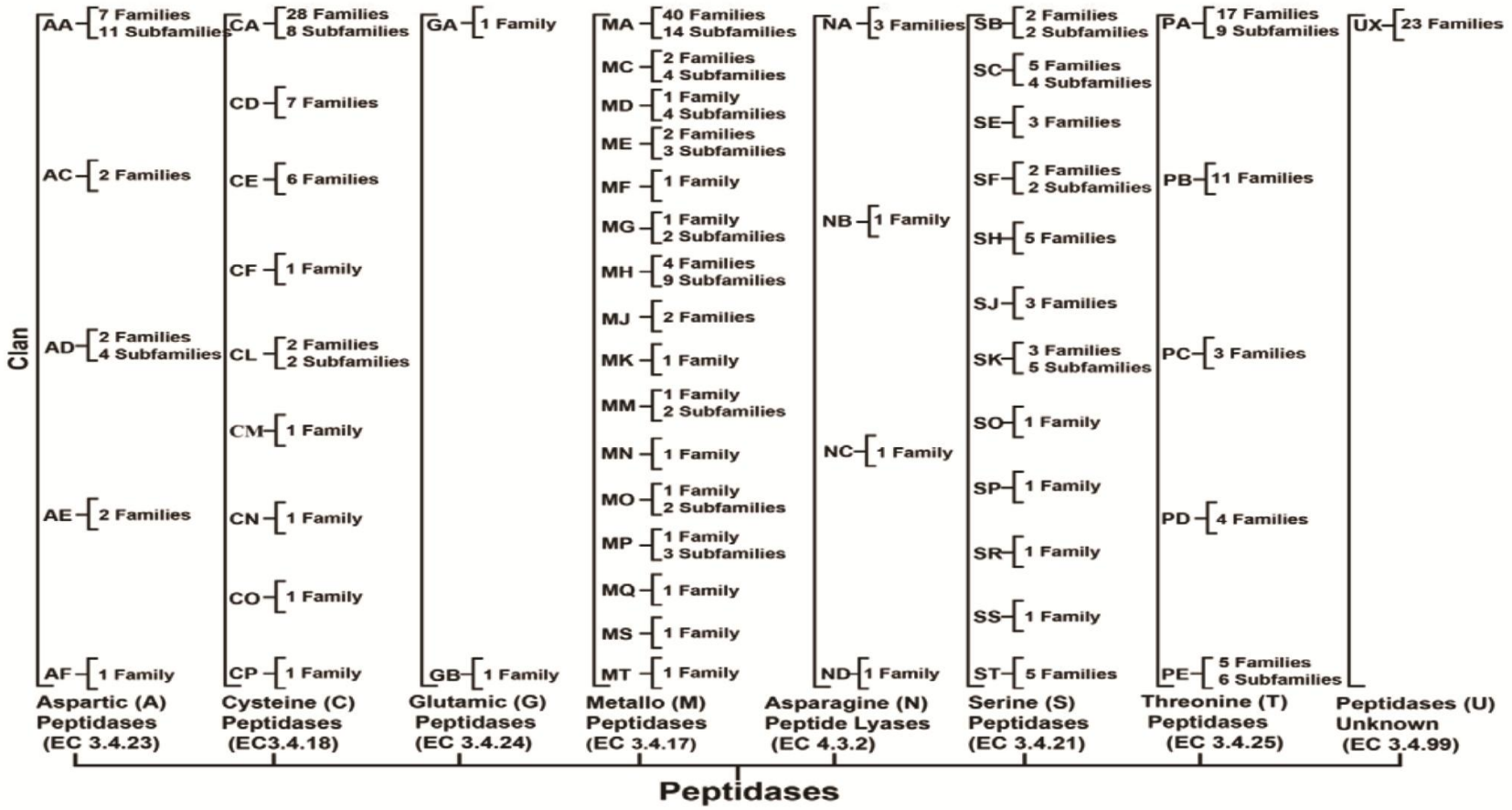
2.4.3. Phylogenetic tree

Based on amino acid sequences, proteases (peptidases) are classified into different clans and families, which have diverged from a common ancestor (Rawlings and Barrett 1993). Each peptidases has been assigned a code letter denoting the type of catalysis, *i.e*., S, C, A, M, or U for serine, cysteine, aspartic, metallo, or unknown type, respectively (**Figure 2**).

Table 2. General classification of proteases with their enzyme commission (EC) code, coupled with specific mechanism of action of each subgroup.

Protease	EC code	Mechanism
Exopeptidases	3.4.11-19	Cleave the peptide bond proximal to the amino or carboxy termini of the substrate
Aminopeptidases	3.4.11	Those acting at a free N-terminus liberate a single amino acid residue
Dipeptidases	3.4.13	Exopeptidases specific for dipeptides
Dipeptidyl peptidase	3.4.14	Release of an N-terminal dipeptide from a polypeptide
Tripeptidyl peptidase	3.4.14	Release of an N-terminal tripeptide from a polypeptide
Peptidyl dipeptidase	3.4.15	Release of free C-terminus liberate a dipeptide
Carboxypeptidase	3.4.16-18	Release of a single residue C-terminal from a polypeptide
Serine type protease	3.4.16	Carboxypeptidase have an active centre serine involved in the catalytic process
Metalloprotease	3.4.17	Carboxypeptidase use a metal ion in the catalytic mechanism
Cysteine type protease	3.4.18	Carboxypeptidase have a cysteine in the active centre
Omega peptidases	3.4.19	Remove terminal residues that are linked by isopeptide bonds
Endopeptidases	3.4.21-24	Cleave internal bonds in polypeptide chains
Serine protease	3.4.21	Endopeptidases have an active centre serine involved in the catalytic process
Cysteine protease	3.4.22	Possesses a cysteine in the active centre
Aspartic protease	3.4.23	An aspartic acid residue for their catalytic activity
Metalloprotease	3.4.24	Use a metal ion (often, but not always, Zn^{2+}) in the catalytic mechanism.
Endopeptidases of unknown catalytic mechanism	3.4.99	Acting on peptide bonds

Figure 2. Classification of proteases (peptidases), depending on their amino acid sequences and evolutionary relationships



2.4.4. Sources of major protease

Animal proteases: The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. Chymotrypsin is found prepared from the pancreatic extracts of animals. Pure chymotrypsin is an expensive enzyme, which is used only in diagnostic and analytical applications. Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates (Rao *et al*., 1998; Ward, 1985). Pepsin had been used in laundry detergents as early as 1913, which is now being replaced by a mixture of serine and metal microbial proteases, which appears to be less degradable by detergents, alkaline conditions and high temperatures (Adinarayana and Ellaiah, 2002). Rennet is a pepsin-like protease that is produced as an inactive precursor in the stomach of all nursing mammals. It is converted to active rennin by the action of pepsin. It is being used extensively in the dairy industry to produce stable curd with good flavor (Rao *et al*., 1998).

Plant proteases: Papain, bromelin, keratinases, and ficin are some of the well-known proteases of plant origin, however, their production from plant sources is a time consuming process. Papain is a traditional plant protease with a long history of use especially in tonics, which is active between pH 5 and 9 (Schechler and Berger, 1967). It is extracted from the latex of *Carica papaya* fruits. Bromelain is prepared from the stem and juice of pineapples (Secor, 2005). But the problem associated with the production of plant proteases lies in the selection of suitable climatic areas for cultivation. As the concentration of enzyme in plant tissue is generally low, processing of large amounts of plant material is necessary.

Microbial proteases: Although protease-producing microorganisms, plants and animals have cosmopolitan distribution in nature; microbial community is preferred over the others for the large scale production of proteases due to

their fast growth and simplicity of life for the generation of new recombinant enzymes with desired properties. Microorganisms account for a two-third share of commercial protease production in the enzyme market across the world (Kumar and Takagi, 1999). Proteases play a decisive role in detergent, pharmaceutical, leather, food and agricultural industries. Currently, the estimated value of the global sales of industrial enzymes is over 3 billion USD (Deng *et al*., 2010), of which proteases account for about 60% of the total sales (Godfrey and West, 1996; Rao *et al*.,1998).

Proteins are degraded by microorganisms, and they utilize the degradation products as nutrients for their subsistence. Degradation is initiated by proteinases (endopeptidases) secreted by microorganisms followed by further hydrolysis by peptidases (exopeptidases) at the extra- or intra-cellular locations. A variety of proteases are produced by microorganisms depending on the species of the producer or the strains, even belonging to the same species. Neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment-friendly technologies (Rao *et al*., 1998). Alkaline proteases have numerous applications in the food industries, silver recovery from X-ray films and several bioremediation processes. There are two types of secreted proteases - intracellular and extracellular. Intracellular proteases are vital to sustain various cellular and metabolic processes, such as, sporulation and cell differentiation, protein turn over, enzyme maturation and hormones and also in protoxin activation of *Bt*-based biopesticides. Extracellular proteases carry out protein hydrolysis in fermented media and enable the cell to absorb and utilize hydrolytic products (Hartley, 1960). Alkaline serine proteases are the most dominant group of proteases produced by bacteria, fungi, yeast and actinomycetes.

Fungal proteases: Fungal proteases magnetised the interest of researches due to high diversity, broad substrate specificity, and stability under extreme conditions; it offers an advantage of separation of mycelium by simple filtration. Fungal proteases can conveniently be produced in solid-state fermentation process. Fungal proteases are also used in for modifying food proteins. The different alkaline proteases producing fungal species are included in **Table 3**.

Bacterial proteases: Bacterial alkaline proteases have more commercial importance in laundry, food, leather and silk industries due to their high production capacity and catalytic activity. Bacterial alkaline proteases are characterised by their high activity at alkaline pH (8-12), with optimal temperature between 50 and 70 °C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry. Prominent bacteria producing proteases are displayed in the **Table 4**.

Table 3. Major fungi producing alkaline proteases.

Fungus	**References**
Aspergillus candidus	Nasuno and Ohara, 1971
A. flavus	Malathi and Chakraborty, 1991
A. fumigatus	Larcher *et al*., 1992; Monod *et al*., 1991
A. melleus	Luisetti *et al*., 1991
A. niger	Barthomeuf *et al*., 1992; Dubey *et al*., 2010
A. oryzae	Murakami *et al*., 1991; Murthy and Lonsane, 1993; Nakadai *et al*., 1973
A. sojae	Hayashi *et al*., 1967
A. sydowi	Danno and Yoshimura, 1967
Cephalosporium sp. KSM 388	Tsuchiya *et al*., 1987
Chrysosporium keratinophilum	Dozie *et al*., 1994
Conidiobolus coronatus	Sutar *et al*., 1991
Entomophthora coronata	Jonsson, 1968
Fusarium eumartii	Olivieri *et al*., 2002
Paecilomyces lilacinus	Den Belder *et al*., 1994
Scedosporium apiospermum	Larcher *et a*l., 1992
Tritirachium album Limber	Ebeling *et al*., 1974; Jany and Mayer, 1985; Samal *et al*., 1990
Rhizopus oligosporus	Devi *et al*., 2011

Table 4. Major bacteria producing proteases.

Organisms	References
Alteromonas sp.	Yeo *et al*., 1995
Arthrobacter protophormiae	Takegawa *et al*., 1993
Brevibacterium linens	Juhasz and Skarka, 1990; Rattray *et al*., 1995
Hyphomonas jannaschiana VP 3	Shi *et al*., 1997
Lactobacillus helveticus	Valasaki *et al*., 2008
Malbranchea pulchella var. *sulfurea*	Ong and Gaucher, 1972
Microbacterium sp.	Gessesse *et al*., 2003
Nocardiopsis dassonvillei	Tsujibo, 1990
Oerskovia xanthineolytica TK-1	Saeki *et al*., 1994
Pimelobacter sp. 2483	Oyama *et al*., 1997
Pseudomonas aeruginosa	Tang *et al*., 2010
Pseudomonas maltophilia	Kobayashi *et al*., 1985
Pseudomonas sp. SJ 320	Cheong *et al*., 1993
Salinivibrio sp. Strain AF-2004	Heidari, 2007
Staphylothermus marinus	Antranikian and Klingeberg, 1991
Streptomyces isolate EGS- 5	Ahmad, 2011
Streptomyces microflavus	Rifaat *et al*., 2006
Streptomyces moderatus	Chandrasekaran and Dhar, 1983
Streptomyces rectus	Peter and Campbell, 1974
Streptomyces rectus var. *proteolythicus*	Mizusawa *et al*., 1969
Streptomyces rimosus	Yang and Wang, 1999
Streptomyces sp. YSA-*130*	Yun *et al*. 1989; Yum *et al*., 1994
Thermoactinomyces sp.	Tsuchiya *et al*., 1992; Lee *et al*., 1996
Thermoactinomyces thalpophilus THM1	Anderson *et al*.,1997
Thermobacteroides proteolyticus	
Thermococcus celer, *T. stetteri*, *T. litoralis*	Antranikian and Klingeberg, 1991
Thermomonospora fusca	Desai and Dhalla, 1969; Gusek and Kinsella, 1987
Thermus aquaticus YT-1	Matsuzawa *et al*., 1988.
Thermus sp. strain Rt41A	Peek *et al*., 1992
Torula thermophila	Zakirov *et al.*, 1975
vibrio alginolyticus	Deane *et al*., 1987; Long *et al*., 1981; Deane *et al*.,1986
Vibrio metschnikovii RH 530	Kwon *et al*., 1994
Xanthomonas maltophila	Debette, 1991

***Alkaline proteases from Bacillus* spp.**: Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH. Of all the alkalophilic microorganisms, members of the genus *Bacillus* were found to be predominant and a prolific source of alkaline proteases (**Table 5**). Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. They play a specific catalytic role in the hydrolysis of proteins.

Alkaline protease from *Bacillus* species RGR-14 shows silk degumming efficiency *B. firmus* MTCC 7728 produces extracellular alkaline protease, with great potential in various industries, and several processes like silver recovery, bioremediation and protein hydrolysate production (Rao and Narasu, 2007). Three intracellular proteases were identified from sporulated culture of *Bacillus thuringiensis* subsp. *tenebrionis* by fractionation with ammonium sulfate; of these, one with 81 kDa was identified as metallo protease having major proteolytic activity at 60 ^{o}C. *B. thuringiensis* H14 in aqueous two phase system - composing of PEG X (X= 9000, 6000, 4000) and potassium phosphate - was able to produce an alkaline protease (Hotha and Banik, 1997). The behaviour of the synthesis of intracellular protease was studied by gelatin zymography in *B. thuringiensis* (*Btk*) strains HD1, *Btk* HD73 (Reddy and Venkateswaeralu, 2002). Alkaline protease was purified and characterised from a mutant of *B. polymyxa* (Madan *et al*., 2002). Several proteases may be produced by the same strain under various culture conditions.

Alkaline proteases are generally produced by submerged fermentation (SmF). In addition, solid-state fermentation (SSF) processes have been exploited to a lesser extent for the production of these enzymes (Chakraborty and Srinivasan, 1993; George *et al*., 1995). Research efforts have been directed mainly toward the evaluation of the effects of various carbon and nitrogenous

nutrients as cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimisation of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources.

Each organism or strain has its own special conditions for maximum enzyme yield. Production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. The synthesis of protease in *Bacillus* species is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phases (Strauch and Hoch, 1993). The extracellular enzyme production pattern is varied with the *Bacillus* strains. There is a little or no enzyme production occurs during the exponential growth phase (Frankena, 1985). However, in the case of *B. subtilis* ATCC strain 14416 and *B. sphaericus* strain BSE 18 (Dumusois and Priest, 1993), enzyme production was growth-associated and it occurs at the mid-exponential phase, and often a rapid auto deactivation process was observed after the culture reached the maximum enzyme activity. During alkaline protease production, it was also observed that the pH of the fermented medium dropped from alkaline to acidic; for instance, from pH 10.1 to 8.5 in the case of an alkalophilic *Bacillus* strain YaB (Tsai *et al*., 1998).

***Proteases from Bacillus thuringiensis*:** *Bacillus* is a gram +ve bacterium and is widely distributed in nature. *Bacillus* spp. are important industrial tools for a variety of reasons, including their capacity to secrete proteins in to the extra-cellular media and their GRAS (generally regarded as safe) status with the food and drug administration (Schallmey *et al*., 2004). The genus includes a variety of commercially important species, responsible for the production of a range of products including enzymes, fine biochemicals like antibodies and insecticides. Most species are harmless to humans and animals and only a few

pathogens are known. *Bt* one of the most widely experimenting bacteria producing a potent insecticidal protein, which makes it a successful biopesticide. *Bt* is also an excellent source of proteases. *Israelensis, kurstaki* and *tenebrionis* are the major sub-species of *Bt* (with many strains) capable of producing different proteases (Hotha and Banik, 1997; Andrews *et al.,* 1985; Reddy and Venkateswaeralu, 2002; Reddy *et al*., 2000; Zouari and Jaoua, 1999; Reddy, 2001; Zouari *et al*., 2002; Tyagi *et al*., 2002).

Table 5. Alkaline protease producing *Bacillus* species

***Bacillus* spp. and their strains**	**References**
Bacillus alcalophilus ATCC 21522	Horikoshi, 1971
B. alcalophilus subsp. *halodurans*	Takii *et al*., 1990
B. amyloliquefaciens	George *et al*., 1995; El-Beih *et al*., 1991
B. amyloliquefaciens S94	Son and Kim, 2002
B.cereus strain CA15	Uyar *et al*., 2011
B.circulans	Chislett and Kushner, 1961
B. coagulans PB-77	Gajju *et al*., 1996
B.firmus	Landau *et al*., 1992; Moon and Parulekar, 1991
B.intermedius	Itskovich *et al*., 1995
B.lentus	Bettel *et al*., 1992
B. licheniformis	El Enshasy *et al*., 2008; van Putten *et al*., 1996; Ageitos *et al.*, 2007
B. licheniformis UV-9 Mutant	Nadeem *et al*.,2009
B. megaterium	Yossana *et al*., 2006
B.proteolyticus	Boyer and Byng, 1996
B. pumilus	Xiubao *et al*., 1990; Vetter *et al*., 1993
B. pumilus CBS	Jaouadi *et al*., 2008
B. sphaericus	Dumusois and Priest, 1993
B. subt.ilis	Chu *et al*., 1992.
B.subtilis var. a*mylosacchariticus*	Tsuru *et al*., 1966
B. subtilis DKMNR	Keiza *et al*., 2011
Bacillus sp. Ya-B	Tsai *et al*., 1983.
Bacillus sp. NKS-21	Takagi *et al*., 1992
Bacillus sp. B21-2 [42]	Fujiwara and Yamamoto, 1987
Bacillus sp. Y	Shimogaki *et al*., 1991
Bacillus sp. CW-1121	Lee *et al*., 1991
Bacillus sp. KSM-K16	Kobayashi *et al*., 1995.
B. thermoruber B. T.2T	Manachini *et al*., 1988
B. stearothermophilus	Rahman *et al*., 1994
Bacillus sp. B001	Deng, 2010

2.4.5. General properties of alkaline proteases

Alkaline proteases useful for detergent applications were mostly active in the pH range 8-12 and at temperatures between 50-70 ^{o}C (Al- Shehri *et al*., 2004). The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions exhibiting higher pH optima, up to pH 12-13. The optimum temperature of alkaline proteases ranges from 50 to 70° C. Interestingly, the enzyme from an alkalophilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85 ^{o}C. Alkaline proteases from *Bacillus* sp., *Streptomyces* sp. and *Thermus* sp. are quite stable at high temperatures, and the addition of $CaCl_2$ further enhances enzyme thermostability (Nilegaonkar, 1998). In general, alkaline proteases require metal ions for their maximum activity. The most commonly used metal ions are Ca^{2+}, Mg^{2+} and Mn^{2+}. Ca^{2+} ion is also known to play a major role in enzyme stabilisation by increasing the activity and thermal stability of alkaline proteases at higher temperatures (Lee *et al*., 1996; Kumar, 2002). Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} are also used for stabilising proteases (Rattray *et al*., 1995). These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures. The presence of Ca^{2+} is known to activate proteases by increasing thermostability (Kotlova *et al*., 2007). However, Li et al., (2005) have reported slight inhibition of protease by Ca^{2+}, Hg^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Mg^{2+} and Mn^{2+}. Metal ions like Hg^{2+}, Cu^{2+}, Ag^{2+}, Fe^{2+} and Zn^{2+} are inhibitory to majority of proteases (Moallaei *et al*., 2006; Pena-Montes *et al*., 2008).

2.4.6. Molecular masses of proteases from Bacillus spp.

Alkaline proteases have different ranges of molecular masses such as 45 kDa, 36 kDa for the proteases from the wild strains and 40 kDa for the standard *B. subtilis* ATCC 6633 strain (Dias *et al*., 2008), 30-33 kDa (Olivieri *et al*.,

2002), 40 kDa (Niu *et al*., 2006). In general molecular mass of protease is ranged between 15 and 45 kDa (Kumar and Takagi, 1999; Gupta *et al*., 2002a). In some *Bacillus* sp., multiple electrophoretic forms of alkaline proteases were observed. The multiple forms of these enzymes may be due to the non enzymatic, irreversible deamination of glutamine or asparagine residues in the protein molecules, or of auto proteolysis. **Table 6** gives a summary of the molecular masses characterised from *Bacillus* spp.

Table 6. Molecular masses of proteases characterised from *Bacillus* spp.

Source	Molecular Weight (kDa)	Reference
Bacillus sp. No. AH-101	30	Takami *et al*., 1989
B. pumilus MK6-5	28	Kumar, 2002
B. pumilus UN-31-C-42	32	Huang *et al*., 2006
B. stearothermophilus F1	33.5	Rahman *et al*.,1994
Bacillus licheniformis MIR29	25/40	Ferrero *et al*., 1996
Bacillus sp. NKS-21	30	Gupta and Beg, 2003
Bacillus sp. SSR1	29,35	Singh *et al*., 2002
Bacillus sp. GX6638	36	Durham *et al*., 1987
Bacillus pseudofirmus AL-89	24	Gessesse *et al*., 2003
Bacillus sp. B18	28, 30	Fujiwara *et al*., 1993

2.4.7. Overview on the industrial applications of proteases

Proteases have a large variety of applications, mainly in the detergents, leather processing, metal recovery, medical purposes, food processing, feeds, and chemical industries, as well as in waste treatment (**Table 7**).

Detergent additives: The history of detergent enzymes dates back to 1914, when two German scientists, Rohm and Haas used pancreatic proteases and sodium carbonate in washing detergents. The product was named Burnus. The first detergent containing the bacterial enzyme was introduced into the market in 1956 under the trade name Bio-40. An alkaline protease, alcalase, was effectively incorporated in detergent powder and was marketed by Novo

Industry, Denmark under the trade name Biotex in 1963. Today, detergent enzymes account for 89% of the total protease sales in the world; and a significant share of the market is captured by subtilisins and alkaline proteases from many *Bacillus* species (Luisetti *et al*., 1991; Jany and Mayer, 1985; Known *et al*., 1994).

Leather tanning: Leather processing involves several steps such as soaking, dehairing, bating, and tanning. The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and for removal of non-fibrillar proteins such as albumins and globulins; at present, alkaline proteases with hydrated lime and sodium chloride are used for de-hairing, which resulted in a significant reduction in the amount of wastewater generated. In addition, studies carried out by different workers have demonstrated the successful use of alkaline proteases in leather tanning from *Aspergillus flavus, Streptomyces* sp., *B. amyloliquefaciens* and *B. subtilis* (Schechler and Berger, 1967).

Silver recovery: Alkaline proteases are used in silver recovery from used X-ray films. Used X-ray film contains approximately 1.5 to 2.0 % (by weight) silver in its gelatin layers. The silver recovery by burning film causes a major environmental pollution problem hence the enzymatic hydrolysis of the gelatin layers on the X-ray film enables the recycling of both silver and polyester film base (Debette, 1991).

Food industry: Alkaline proteases have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates,

and meat tenderisation (Ferrero *et al*., 1996). Proteases are invariably used in tonics, especially for indigestion.

Waste treatment: Dalev in 1994 reported an enzymatic process using a *B. subtilis* alkaline protease in the processing of waste feathers from poultry slaughterhouses. Feathers constitute approximately 5% of the body weight of poultry and can be considered a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis resulted in total solubilisation of the feathers. The end product was a heavy, grayish powder with a very high protein content which could be used as a feed additive. Similarly, many other keratinolytic alkaline proteases were used in feed technology for the production of amino acids.

Table 7. Common protease products from *Bacillus* spp. available in the market.

Organism	**Trade names**	**Manufacturer**
Bacillus licheniformis	Alcalase	Novo Nordisk , Denmark
Alkalophilic *Bacillus* sp.	Savinase, Esperase	Novo, Nordisk , Denmark
Alkalophilic *Bacillus* sp.	Maxacal, Maxatase,	Gist-brocades, The Netherlands
Alkalophilic *Bacillus* sp.	Opticlean, Optimase	Solvay Enzymes GmbH, Germany
Alkalophilic *Bacillus* sp.	Proleather	Amano Pharmaceuticals Ltd., Japan
Aspergillus sp.	Protease P	Amano Pharmaceuticals Ltd., Japan
B.amyloliquefaciens (savinase)	Durazym	Novo Nordisk, Denmark
Alkalophilic *Bacillus* sp.	Maxapem	Solvay Enzymes GmbH, Germany
Variant of *B. lentus*	Purafect	Genencor International, Inc

Other uses: Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship, in the synthesis of peptides, and in the sequencing of proteins. *Bt* is used for the integrated pest management in forestry. The derivatives of *Bt* strain HD1 subsp. *kurstaki* have widely been used to control the forest pests such as the gypsy moth (*Lymantria dispar*), spruce budworm (*Choristoneura fumiferana*), the pine processionary moth (*Thaumetopoea pityocampa*), the European pine shoot moth (*Rhyacionia buoliana*) and the nun moth (*Lymantria monacha*) (Ming *et al*., 2001).

2.5. *Eutetrunychus orientalis* Klein

E. orientalis (Acarina: Acariformes: Tetranychidae), commonly known as the oriental red spider mite is principally a pest of citrus; but has also been reported to cause severe damage to a wide variety of agricultural crops and medicinal plants such as banana, cassava, castor bean, cotton, fig, frangipani, maize, mulberry, oleander, peach, plum, rose, squash,grape, pawpaw, pear, quince and walnut (Walter *et al*., 1995). In Kerala (where this work conducted), *E. orientulis* has been shown to cause feeding damage to many plants including cultivated plants like *Manihot esculenta*, (Lal and Pillai, 1981) water hyacinth (Haq and Sumangala, 2003) or forest trees like *Azadirachta indica, Albizia lebbeck, Moringa oleifera, Ailanthus excelsa* and *Zizyphus jujube* (Yousuf *et al*., 2006) or *Phaseolus vulgaris* (common bean) *Citrus lemon, Cassia occidentalis Carica papaya, Musa* sp. (Sangeetha, 2009).

It is shown that *E. orientalis* could be controlled by employing some biological control agents like protozoans; (Hanula and Andreadis, 1988) entomopathogenic nematodes like *Steinernema* spp. and *Heterorhabditis bacteriophora*, entomopathogenic fungi like *Metarizium anisopliae* (or some

dipteran predators like *Cophinopoda chinensis, Philonicus albiceps Promachus yesonicus, Hymenopteran parasitoids*, *Scolia manilae, Tiphia vernalis* and *Tiphia popilliavora* (Yeh and Alm, 1995; Koppenhöfer *et al*., 1999; Choo *et al*., 2002; Lee *et al*., 2002; Yokoyama *et al*., 1998; Tashiro, 1987). Some workers used plant extracts and essential oils to control this mite, *e.g*., extracts of *Ocimum basilicum, Lavandula officinails Majorana hortensis Rosma- rinus officinalis* ((Refaat *et al*., 2002; Momen and Amer, 2003). Various pesticide (acaricides) are also being used to control this mite, which include flubenzimine omethoate, dicofol and sulfur (Sharaf, 1989; Potter, 1998). Payne *et al*. (1993) showed that strains of *Bacillus thuringiensis* (*Bt*) could be used to control various acarid pests, specifically *Tetranychus urticae*. To the best of our knowledge, no report is seen in literature which describes the use of *Bt* formulations for controlling *E. orientalis.*

Chapter III

Materials and Methods

Chapter 3
MATERIALS AND METHODS

3.1. Aim

The objective is to summarise all the materials and methods used in this study. No separate materials and methods will be given for succeeding chapters.

3.2. Organisms used

3.2.1. Bacterium

The standard strain of *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*) (**Figure 3**) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh (Strain designation: BA 83B, MTCC number: 868) and maintained in Luria-Bertani (LB) medium.

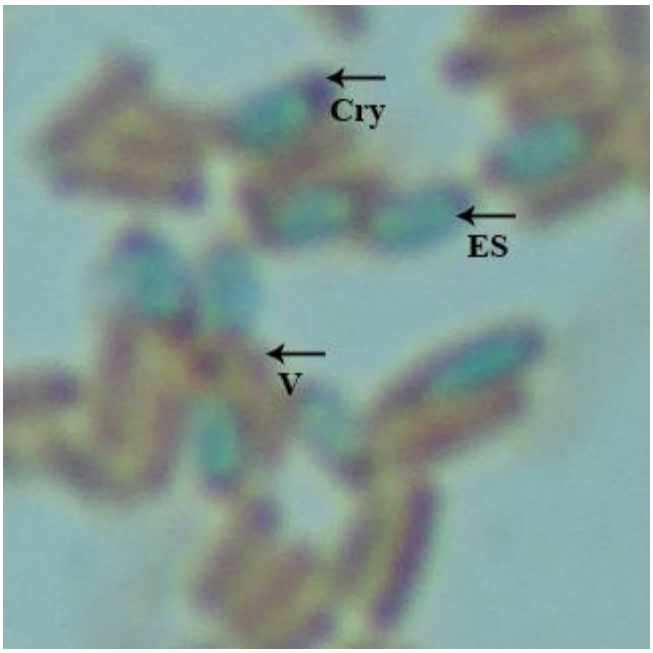


Figure 3. Vegetative cells and spores of 48 h old culture of *Btk* stained with malachite green and counter stained with safranin.

3.2.1.1. Subculturing

Frozen stocks on agar slants were activated periodically (fortnightly) and maintained on LB-agar slants.

3.2.2. Mite and its collection

Eutetranychus orientalis Klein (**Figure 4**) was collected from the leaves of severely infested *Vigna unguiculata* (L.) Walp (cow pea) and *Moringa oleifera* L. (drum-stick tree), from cultivated fields near Villunniyal (geographic coordinates: 11°7'54"N; 75°53'20"E), Malappuaram District, which was identified by Prof. M. Mohanasundaram, Tamil Nadu Agricultural University, Coimbatore.

Figure 4. *E. orientalis* on *Moringa oleifera* leaf.

3.2.3. Plant field trial

Vigna unguiculata (L.) Walp (cow pea) were used for field trial experiments (**Figure 5**).

Figure 5. *V. unguiculata* (L.) Walp infected with *E. orientalis.*

3.3. Chemicals

Analytical and bacteriological-grade chemicals from Chromous (India), Genei (India), Himedia (India), Merck India Ltd., Qualigens (India) and Sigma-Aldrich (USA) were used for the study.

3.4. Glassware

Glassware made by Borosil and Riviera were used for the study. Conical flasks (100, 250 and 500 mL), Petri dishes (100 × 15 mm, 75 × 15 mm), Measuring jars (10, 100, 200, 500 and 1000 mL), pipettes (1, 5, 10 mL), beakers (10, 50, 100, 250, 500 and 1000 mL); and glass column (50 × 3 cm) was used for the purification of protein.

3.5. Culture Media

3.5.1. LB medium

LB was the basal medium used for the whole study. *Btk* was cultured in LB medium, enriched with different flours supplements for the overproduction of extracellular alkaline protease, endospore and δ- endotoxin.

3.5.2 Nutrient broth

Nutrient broth was used as control for counting viable bacterial cells and spores only.

Table 8. Compositions of media used.

Designation	**Media used**	**Ingredients (g/L)**	**Mode of cultivation**	**Cultivation strategy**
NB (control)	Nutrient Broth (NB) Medium	Peptone 5g, NaCl 5g and beef extract 3 g (NB) in dd H_2O, initial pH 7.0.	Submerged fermentation (SmF)	Incubated at 37 ^oC, 150 rpm (up to 72 h).
LB (control)	Luria-Bertani (LB) medium	Tryptone 10 g, yeast extract 5 g and NaCl 10g (LB) in dd H_2O, initial pH 7.0.	SmF	Incubated at 37 ^oC, 150 rpm (up to 72 h).
Medium 1 (M1)	LB medium + 30% (w/v) soybean flour	Tryptone 10 g, yeast extract 5 g and NaCl 10g (LB) + 300g soybean flour in dd H_2O, initial pH 7.0.	Semi-solid fermentation	Incubated at 37 ^oC, 150 rpm, (up to 72 h).
Medium 2 (M2)	LB medium + 30% (w/v) soybean flour	Tryptone 10 g, yeast extract 5 g and NaCl 10g + 300g soybean flour in dd H_2O, initial pH 7.0.	Solid-state fermentation (SSF) after initial 12 h semi-solid fermentation	Incubated at 37 ^oC, 150 rpm. After initial fermentation for 12 h (Phase I), centrifuged (1000 × *g*, 10 min); solid and wet pellet so obtained incubated further (Phase II), *i.e.*, second phase was SSF. Thus, the strategy was biphasic.

3.5.3. Natural supplements used

Five agricultural products were used to supplement LB medium for enhancing the nutrient supply. Various proportions of the flours of Banana (BF), Bengal gram (BgF), Jack seed (JF), Soybean (SF) and Tapioca (TF) were used to supplement LB medium. Of these raw supplements, Banana flour was purchased locally (Bana-tone), and flours of Bengal gram, Jack seed, Soybean and Tapioca were prepared freshly from the naturally available sources using a mixer grinder. Source of raw supplements was maintained constant through out the experiments.

3.6. Inoculum preparation

Prior to inoculation, all the media were autoclaved at 15ψ, 121 ^{o}C and 20 min (1 atmos). Culture from the 2 weeks old frozen stock was streaked on the LB-agar medium in the petri-dishes. For the preparation of overnight seed culture (12 h), single colony from LB-agar plate was inoculated in to the LB broth.

Five µL seed culture was used to inoculate 1 mL sterilized medium, which was equivalent to [6.5×10^7 colony forming units (CFU)] per mL medium. Spread-plate method was used to count the CFU from the culture obtained after serial dilution.

3.7. Incubation

For submerged or liquid and semi-solid fermentations, the medium was incubated at 37 ^{o}C with constant shaking (150 rpm, and initial pH 7.0) in a temperature-controlled shaker (Orbitek, India); while for solid-state fermentation, the samples were incubated in an incubator (Technicho, India) at 37 ^{o}C.

3.8. Fermentation

3.8.1. Submerged fermentation (*SmF*)

3.8.1.1. SmF in the basal LB medium

Basal LB medium was used to monitor the extracellular alkaline protease production by SmF. Samples were withdrawn for assays at 3 or 6 h intervals.

3.8.1.2. SmF with defatted casein

For SmF, LB medium was supplemented with 1% (w/v) defatted and purified casein (Himedia, India). Basal LB medium was used as the control. Samples were withdrawn for assays at 6 h intervals.

3.8.1.3. SmF with natural raw substrates (1%)

In order to assess the efficacy of natural supplements, the LB medium was modified with 1% (w/v) of five natural substrates, *viz.*, flours of banana, Bengal gram, jack seed, soybean and tapioca.

3.8.2. Semisolid or Solid-State Fermentation (SSF)

The LB medium was modified with different proportions (10, 20, 30, 40, 50, 60, 80 and 100%, all w/v) of the flours of banana, Bengal gram, jack seed, soybean, tapioca, and LB medium (without flour) was used as control.

3.9. Enzyme harvest

Protease was harvested from the fermented liquid broth (SmF). To obtain crude supernatant for protease assay, the broth (whole flask harvest) was centrifuged at 1000 × *g* for 5 min at 4 ^{o}C in a refrigerated centrifuge (Plastocrafts/Remi, India). One mL equivalent fermented matter was used for the extraction of enzyme in the case of semi-solid fermentation or SSF. The supernatant obtained was centrifuged again (9440 × *g* for 10 min at 4 ^{o}C), the

clear solution obtained so was used for the crude protease assay and its purification further.

3.9.1. Enzyme assay

Extracellular protease was assayed according to the method of Anson (1938), with slight modification (Yang and Huang, 1994). Briefly, the reaction mixture contained 1 mL of 1% (10 mg/mL) defatted casein in 0.1 M phosphate buffer (pH 7.6) and 1 mL of enzyme solution (after proper dilution, wherever required), which was incubated for 20 min, at 37 ^{o}C. The reaction was stopped by adding 3 mL of 10% trichloroacetic acid (TCA). The absorbance of the liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the quantity of protease required to liberate 1µM of tyrosine in 1 minute under the assay conditions.

3.9.2. Calculation of protease activity

The following formula was used for calculating protease activity, Units/mL or Units/mL_{eqv} (Jisha *et al*., 2013c):

$$\text{Protease activity (U/mL or U/mL}_{eqv}) = \frac{\Delta E \times Vf \times Vd}{\Delta t \times \epsilon \times Vs \times d}$$

Where: ΔE = Absorbance at 280 nm; Vf = Final reaction volume including TCA; Vs = Volume (mL) of enzyme solution used; Vd = dilution factor; Δt = Reaction time; ε = Extinction coefficient of tryrosine (the product assayed); and d = Diameter of cuvette (1 cm for standard cuvette).

In the cultivation strategy, wherein above 10% (w/v) flours were supplemented to the LB, the activity was expressed in U/mLequivalent (U/mL_{eqv}), *i.e*., protease activity in the supernatant obtained from an equivalent mass of 1 mL LB + % flour supplemented initially, prior to autoclaving the medium. Thus, the protease activity expressed in this study is made uniform irrespective of LB alone or LB with various supplements.

For instance, if 3 g flour was initially added to 10 mL LB (30%, w/v),

1 mL_{eqv} = 10 mL + 3g ÷ 10 [*i.e*., protease activity from the volume of supernatant obtained from $1/10^{th}$ portion (weighed quantity) of the above matter, after required period of fermentation].

3.10. Protein estimation

Protein content was estimated using the method of Lowry *et al*. (1951), with Bovine serum albumin (BSA) as the standard.

3.10.1. Reagents

- Reagent A: 2% Na_2CO_3 in 0.1 N NaOH
- Reagent B: 500 mg $CuSO_4$ in 1% Rochelle salt solution
- Reagent C: 50 mL of Reagent A + 1 mL of Reagent B
- Folin's-Phenol reagent

3.10.2. Procedure

- Pipette out 0.5 mL of the enzyme solution in test tube and make up to 1 mL with 0.1 N NaOH. Add 5.0 mL of reagent C. Mix well and allow standing for 10 min.
- Add 0.5 mL of Folin's reagent, mix well and incubate at room temperature for 30 min.
- Read the absorbance at 660 nm using a UV-VIS spectrophotometer.
- Calculations were performed based on the standard graph constructed for BSA.

3.10.3. BSA - Standard graph

- 1 mg/mL stock solution was prepared with BSA
- Pipette out different aliquots of stock solution in test tubes and make upto 1 mL with 0.1 N NaOH. Add 5.0 mL of reagent C. Mix well and allow standing for 10 min.
- Add 0.5 mL of Folin's reagent, mix well and incubate at 25 ^{o}C for 30 min.
- Read the absorbance at 660 nm using UV- VIS spectrophotometer.
- The values were plotted concentration *vs.* absorbance at 660 nm.

3.11. Protease purification

Extracellular alkaline protease was purified by the method of Kunitate *et al*. (1989) method. The procedure consists of fractionation by ammonium sulphate followed by dialysis and gel permeation chromatography. The purified enzyme was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.11.1. Ammonium sulphate fractionation

Molecular biology grade ammonium sulphate was added slowly into the crude enzyme up to 80% saturation (0-20, 20-40, 40-60 and 60-80%). A magnetic stirrer was used for the continuous stirring, and the procedure was carried out at 4 ^{o}C. The precipitated protein was removed by centrifugation at 9440 × *g* for 10 min, 4 ^{o}C. The pellets were resuspended in a minimum volume of 0.1M phosphate buffer (pH 7.6).

3.11.2. Dialysis

The precipitate obtained after ammonium sulphate fractionation was dialysed against 0.1M phosphate buffer (pH 7.6) for 24 h at 4 ^{o}C with continuous

stirring and two buffer changes. Cellulose membrane dialysis tubes were used for dialysis. Protease activity and protein content of the dialysate were determined at every step.

3.11.3. Spin column purification

Partially purified enzyme was further purified using a spin column (Vivaspin 6, Sweden), which contained polyethersulfone (PES) semi-permeable membrane for the molecular weight cut off below (45 kDa) (**Figure 6**), this fraction was used for further purification.

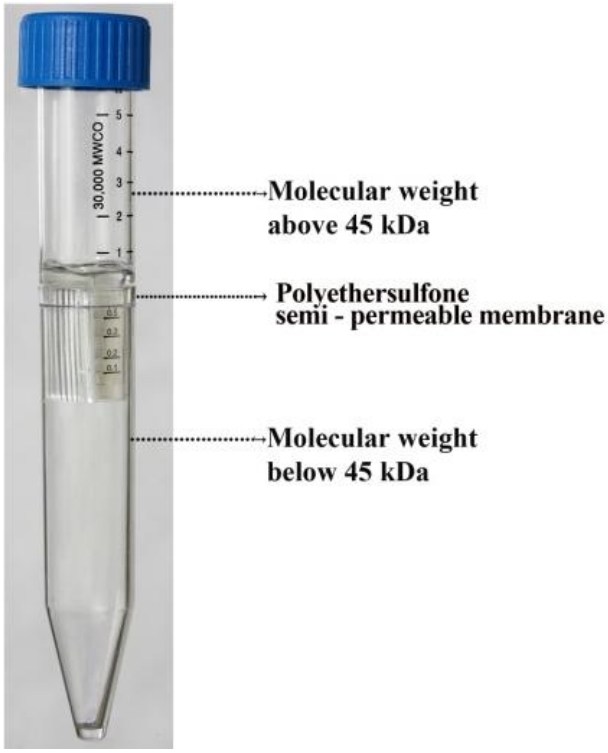


Figure 6. Vivaspin 6 spin column

3.11.4. Gel permeation chromatography

The above dialysate was used for gel permeation chromatography. Gel permeation chromatography was performed in a column packed with Sephadex G-100 (Sigma Aldrich, USA) using a peristaltic pump (Riviera, India) in a cold chamber (**Figure 7**).

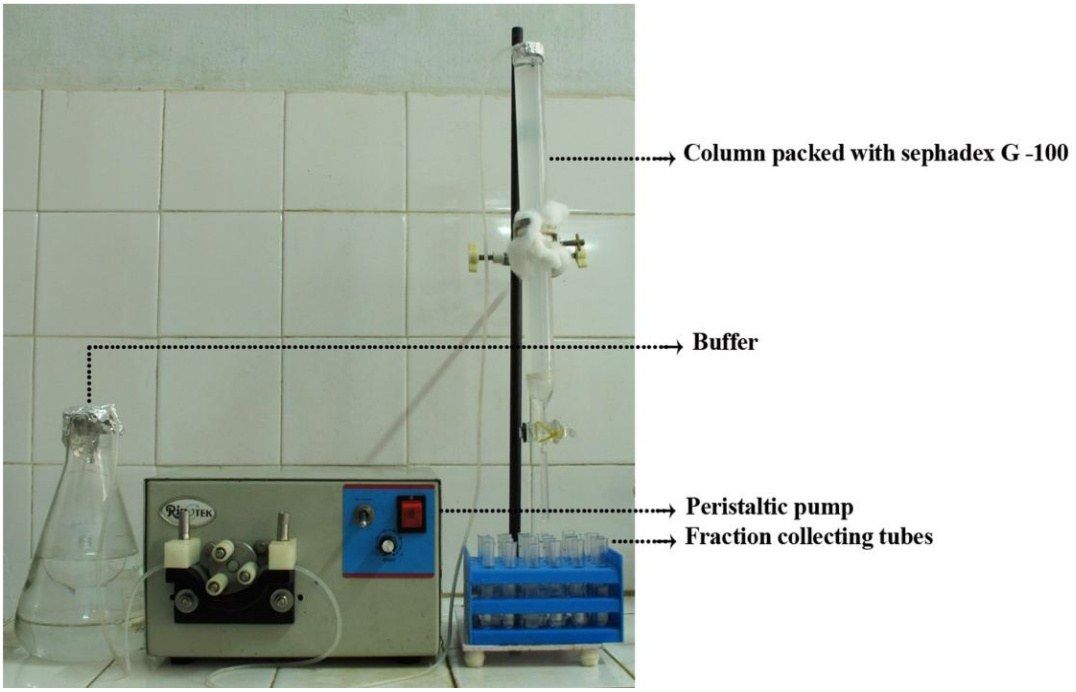


Figure 7. Gel permeation chromatography column packed with sephadex G – 100.

3.11.5. Electrophoresis

3.11.5.1. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (*SDS–PAGE*)

The purified protease was subjected to SDS-PAGE (reducing PAGE) to confirm the purity and to determine the approximate molecular weight (MW) of the purified protein. SDS-PAGE was conducted using a vertical mini gel (8 × 7 cm) slab system (BioTech, India). Electrophoresis was performed on 12 % gel and voltage of 50 V for stacking gel and 70 V for resolving gel till the sample dye reached the bottom (Laemmli, 1970). Gel was stained using 0.1% Coomassie Brilliant Blue (CBB) R-250 in 50% methanol and 10% glacial acetic acid. The destaining solvent system contained 10% glacial acetic acid and 45% methanol.

3.11.5.1.1. Sample preparation

Enzyme solution and sample buffer were mixed in the 1:1 ratio. The contents were mixed well in a clean tube (Eppendorf) and heated in a boiling water bath for 5 min. Broad range protein MW markers (Genei, Banglore) containing myosin (205 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalboumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) aprotinin (6.5 kDa) and insulin (3.5 kDa) were used for MW determination.

3.11.5.2. Native gel electrophoresis

Nondenaturing (native) polyacrylamide gel electrophoresis or native PAGE was carried out according to the modified method of Kazan *et al*. (2005). The electrophoresis was performed on 10% (w/v) gel containing 1% skim milk, and the voltage for stacking gel and resolving gel were 50 V, 100 V respectively. Gel was incubated for 3 h at 37 ^{o}C in 0.1 M Glycine-NaOH buffer (pH 10.0) for proteolysis of skim milk proteins. Afterwards, the gel was stained with 0.2% Coomassie Brilliant Blue G 250 solution, and destained with 50% methanol and 10% glacial acetic acid. A clear zone on the gel indicated the presence of alkaline protease activity.

3.12. Optimisation and characterisation

The protease active fraction obtained from gel permeation chromatography was characterised and its various properties were studied. Effects of pH, temperature, substrate concentration, different metal ions inhibitors, surfactants and detergents on protease activity were studied.

3.12.1. Effect of pH on the protease activity and stability

Effect of pH on protease activity was tested using buffers of different pH ranging from pH 7 to 12. Stability of the enzyme at various pH values was

studied by pre-incubating the enzyme in buffers of different pH (7-12) for 1h. After incubation, the enzyme activity was measured. Buffers used were 0.2 M sodium phosphate (pH 7-8), 0.2 M glycine-NaOH (pH 9-10) and 0.2 M NaOH-Na_2HPO_4 (pH 1-12). (20 min incubation at 37 ^oC using 10 mg/mL casein as substrate).

3.12.2. Effect of temperature on the enzyme activity and stability

Effect of temperature on the enzyme activity was determined by incubating the reaction mixture (enzyme + substrate) at different temperatures (60-80 ^oC). To determine the temperature stability, the enzyme was pre-incubated at different temperatures (60-80 ^oC), for 1 h and activity was assayed under standard reaction conditions (20 min incubation at pH 9 using 10 mg/mL casein as substrate).

3.12.3. Effect of substrate concentration

Protease was treated with different concentrations (0.5, 1, 5, 10, 15, 20 mg/ mL) of casein. The reaction mixture was incubated from 5 min to 60 min under the reaction conditions (pH 9 and 70 ^oC).

3.12.4. Effect of metallic salts

Protease was pre-incubated for 1 h with various concentrations (1, 2 and 2.5 mM) of different metallic salts (Hg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{2+} and K^+), then the activity was measured under the reaction conditions (30 min incubation at pH 9, 70 ^oC and 15 mg/mL casein as substrate).

3.12.5. Inhibitors on protease activity

The enzyme was pre-incubated for 1 h with varying concentrations (1-30 mM) of different protease inhibitors like ethylene-diamenetetraacetic acid (EDTA), β-mercaptoethanol and then assayed the protease activity, under the

reaction conditions: (30 min incubation at 70 ^{o}C, pH 9 and 15 mg/mL casein as substrate).

3.12.6. Effect of surfactants and commercial detergents

Efficacy of *Btk* protease as an additive in detergent was determined by testing its stability in surfactants and commercial detergents. The protease was incubated with different concentrations of surfactants like SDS (0.1, 0.4, 0.8, and 1%), Triton X-100 (0.1, 0.4, 0.8, and 1%) for 30 min, and the activity of the protease was measured by the standard assay procedure. Stability of the protease in commercial detergents was also tested by incubating (1h) measured quantity (100 µL) of the protease with the solutions of the different commercial detergents at a detergent concentration at 7 mg/mL (to simulate washing conditions) (Phadatare *et al.*, 1993). The commercial detergents tested were: Ariel, Tide, Surf Excel and Sunlight, which are widely used in India. The activity was measured by standard assay procedure and compared with the control. The reaction conditions were: (20 min incubation at 37 ^{o}C using 10 mg/mL casein as substrate).

3.12.7. Calculation of Km and Vmax

The *Km* and *Vmax* values were calculated using the effect of casein on enzyme activity using the software Hyper 32 and Graph pad prism.

3.12.8. MALDI-TOF/TOF

MALDI-TOF/TOF analysis of the purified enzyme was carried out in Bioinformatics Department, BGI, Beijing.

3.12.8.1. Protein in gel digestion

- The gel points were washed once with 500 µL of H_2O followed by three times 500 µL of 25 mM ammonium bicarbonate in 50% acetonitrile for 60 min each on a mixer.

- Supernatants were discarded after each washing step.
- The gel points were dehydrated by the addition of 500 µL of acetonitrile.
- Disulfide bonds were cleaved by incubating the samples for 60 min at 56 °C with 200 µL of 10 mM DTT in 25 mM ammonium bicarbonate buffer.
- Alkylation of cysteines was performed by the addition of 200 µL of 55 mM iodoacetamide in 25 mM ammonium bicarbonate buffer and incubation of the samples for 45 min at room temperature in darkness. Gel bands were washed twice with 25 mM ammonium bicarbonate buffer and dehydrated with 500 µL of acetonitrile.
- Gel points were covered with trypsin solution (10 µg/ µL in 25 mM ammonium bicarbonate buffer).
- After a 30-min incubation of ice, the remaining trypsin solution was removed, and 25 µL of 25 mM ammonium bicarbonate were added.
- Proteolysis was performed overnight at 37 °C and stopped by adjusting the samples to 5% formic acid.

3.12.8.2. MALDI-TOF/TOF Analysis by ultrafleXtreme (Bruker)

- Drip 1 µL peptide solution after digesting the sample gel point onto the Anchor chip target plate.
- After the droplet were dried in room temperature, drip 0.1 µL matrix (CHCA) onto the plate at the same place.
- Load the plate into spectrometer and set the instrument's parameter for reflect mode, the mass range was from 500 to 3500 Da, and the scan resolution is 50,000.
- Select about three to five most abundant MS peaks for MS/MS scan.

3.12.8.2.3. Bioinformatics Analysis

First, marking peaks from the original MS files, and then searching protein based on the database, simultaneously filtering and making quality control the search results, finally making standard statistical analysis for reliable result.
Software used: Mascot 2.3.02.
Data Base used: Thuringiensis (95989 sequences).

3.12.9. Calculations

Relative activity = Present activity / Initial (crude) activity × 100

Specific activity = Enzyme activity /Protein

Fold purification = Specific activity /Initial specific activity

% Yield = Present activity /Initial activity × 100

3.13. Endospore Production

3.13.1. Endospore production by SmF and SSF

Spore production was also done with the three natural substrates having highest alkaline protease activity, *viz*., flours of soybean, Bengal gram and jack seed. The medium prepared with these substrates at different concentrations (1, 10, and 20, 30, 40, 50, 60, 80 and 100%, w/v) and 6 h time interval ranging from 6 to 72h.

3.13.2. Working spore solution preparation

Culture medium was centrifuged at 9440 × *g* for 10 min. The collected pellets were re-suspended in sterile ddH_2O (4 ^oC) and made up to 10 mL and centrifuged at 9440 × *g* for 10 min., this procedure repeated 3 more times. Then the pellet collected and stored at 4 ^oC for overnight, *i.e*., allowing the sporangia to burst for releasing the endospore and δ-endotoxin. Spore

suspension was centrifuged and the pellet was collected and re-suspended in ddH_2O (4 ^oC) and centrifuged at 9440 × *g* for 10 min. Final pellet was diluted to 50% concentration with ddH_2O, *i.e*., to make the working spore solution.

3.13.3. Preparation of slides

Smears were prepared using 10 µL working spore solution on a clean glass slide. The slide was placed on a heating block for 3 min, so as to dry completely. The slides were washed with ice-cold absolute alcohol (50 mL) in a Coplin jar for 1 min. The slide was removed from the jar and allowed to dry for 3 min on a heating block.

3.13.4. Endospore Staining

3.13.4.1. Malachite Green Staining

Malachite-green staining technique was used for spore staining (Bartholomew and Mittwer, 1950). Bacterial smears were prepared in the usual manner using standard sterile techniques. The smear was allowed to air dry and heat-fixed at 60 ^oC in a hot air oven. Smears were flooded with malachite-green and placed on a warm hot plate allowing the preparation to steam for 10 min, cool, and wash under running tap water; then counter stained with safranin for 1 min; washed with running tap water and air-dried. The slides were observed under the binocular microscope (100 X magnification). The photographs were taken.

3.13.4.2. Acridine Orange Staining

Acridine orange staining technique was used as demonstrated by Laflamme *et al*. (2004). Ten µL of 0.1 µg/mL acridine orange staining solution was applied to the area of smear and covered with a cover-slip.

3.13.4.3. Coomassie Brilliant Blue (CBB) Staining

Purified *Btk* toxin was stained with CBB solution (0.25% CBB, 50% ethanol, and 7% acetic acid) for 3 min, washed in tap water, and observed using Phase-contrast microscope (Leica DM 2500, Germany) fitted with Leica DFC 295 digital camera.

3.13.5. Measurement of spore size

The length and breadth of the endopores were measured using an ocular and stage micrometer attached to Olympus CX 21 binocular microscope.

3.13.6. Visualisation of endospres

The spores stained by malachite-green were visualized by the Image Analyser fitted with Nikon digital camera. The spores stained with acridine orange (Laflamme *et al.*, 2004) were visualised using Olympus fluorescent microscope.

3.13.7. Spore count

One mL culture (of LB and NB) or 1 mL equivalent M1 or M2 (**Table 8**) after appropriate period of fermentation was withdrawn from the whole flask and made up to 10 mL with sterile ddH_2O. M1 and M2 were sonicated using a sonicator with probe of 5 output wattage (1 × 5 min with 1 min interruption) at 4 ^oC, so as to get the embedded cells and spores detached from the Soybean flour matrix. These samples were subjected to appropriate serial dilution; 10 µL of this serially diluted sample was taken for sporangia/spore counting. A thin smear was prepared on a clean glass slide; heat-fixed and stained with Coomassie Brilliant blue (CBB) R-250. Using phase contrast microscope, sporangia and spores were counted from different fields of this preparation. Sporangia or spores were counted directly from the fields in the microscope and their number per mL or mL_{eqv} was arrived at, *i.e.*, number of sporangia or

spores on slide × 100 (for converting 10 µL sample used for making smear into 1 mL) × dilution factor.

3.13.8. Viable cell counts

Hundred µL of the serially diluted (as described in section **3.13.7** above) sample was spread on nutrient agar plate and incubated at 37 ^{o}C for 12 h in an incubator. Viable cell (equal to number of colonies) were manually counted from the colonies, and thus the colony forming units (CFU)/mL or mL_{eqv} was calculated directly using the formula, CFU = number of colonies on the agar plate × 10 (for converting 100 µL used for plating into 1 mL) × dilution factor.

3.14. *Btk* δ-endotoxin (crystal protein) production and purification

3.14.1. Crystal production during SSF

Crystal production was monitored by supplementing 3 natural substrates (one each, not in combination) with LB. The LB medium was modified with flours of 10% (w/v) jack seed or 30% (w/v) soybean or Bengal gram and crystal production was monitored up to 72 h at 6 h intervals. For increasing the production of crystal protein, the 12 h culture was centrifuged at 1000 × *g*, 10 min for removing the supernatant, so as to make the medium ready for SSF. The pellets were collected and incubated at 37 ^{o}C.

3.14.2. Crystal protein isolation

Crystal protein isolation was done by the biphasic separation method, with some deviation from the methods of Pendleton and Morrison (1966), and Neema (2007).

3.14.2.1. Solutions

- Tris-EDTA (100 mM Tris HCl, pH 7.2, and 10 mM EDTA, pH 8)
- 0.5 M NaCl
- 1% Sodium sulphate
- Carbon tetrachloride

3.14.2.2. Protocol

The step-wise protocol employed for the purification of δ-endotoxin was:

- 10 g solid fermented matter was re-suspended in 10 mL ddH_2O (or 10 mL LB control (broth) directly taken without further dilution) and centrifuged;
- The resultant pellet was re-suspended in 10 mL Tris-EDTA buffer (pH 7.0) and centrifuged;
- The resultant pellet was re-suspended in 5 mL 0.5 M NaCl and centrifuged immediately;
- The resultant pellet was washed in 10 mL Tris-EDTA buffer (pH 7.0) and centrifuged;
- The resultant pellet was mixed with 35 mL sterile ddH_2O, then 30 mL 1% sodium sulphate and 35 mL CCl_4 were added;
- Sonicate the mixture, subsequently transferred in to a separating funnel set-up and allowed to stand for 30 min. The upper aqueous phase containing crystals and spores (*Btk* δ-endotoxin) was separated from the lower organic phase (debris with vegetative cells) by draining;

- Aqueous phase centrifuged to settle spores as pellet and crystals (δ-endotoxin) remained in the solution.
- All the above centrifugation steps were performed at 9440 × *g* for 10 min at 5 ^{o}C, for long term storage, the *Btk* toxin was dehydrated, re-suspended in Phenylmethylsulfonyl fluoride (PMSF) and stored at 4 ^{o}C.

3.14.2.3. Malachite Green - Safranin (MG-S) staining for δ-endotoxin

Using standard protocols (Bartholomew and Mittwer 1950), the raw fermented matter after SSF or pellets after SmF was stained with malachite green, and counter stained with safranin. The slides were observed under the binocular microscope. The photographs were taken with the aid of the Image Analyser equipped with Nikon digital camera.

3.14.2.4. Coomassie Brilliant Blue (CBB) staining for δ-endotoxin

Purified *Btk* δ-endotoxin was stained with CBB solution (0.25% CBB, 50% ethanol, and 7% acetic acid) for 3 min, washed in tap water, and observed using Phase-contrast microscope (Leica DM 2500, Germany) fitted with Leica DFC 295 digital camera (Sharif and Alaeddinoĝlu, 1988).

3.14.3. Scanning Electron Microscopy (SEM)

Purified crystals were treated with Sorensen phosphate buffer (pH 7.2) and stored at 4 ^{o}C overnight. Fixed in gluteraldehyde phosphate buffer for 10 min and then washed with phosphate buffer 15 min and dehydrated. Purified crystals were dried on a metal support, at RT, and covered with gold for 60 Sec. at 40 mA. Samples were observed and photographed by Scanning Electron Microscope (SEM) (JEOL, JWS 3000) and Hitachi SU, 6000.

3.15. Efficacy studies of *Btk δ*-endotoxin against *Eutetranychus orientalis*

3.15.1. Preparation of raw Btk δ-endotoxin, after SmF

LB was used for conventional submerged fermentation (SmF) of *Btk*, which was the control medium. LB was autoclaved and inoculated with *Btk* seed (overnight) culture. Seed culture contained about 6.5×10^7 colony forming units (CFU) per mL; 5 µL of this seed culture was used to inoculate 1 mL LB for SmF. It was incubated in an environmental shaker (37 ^{o}C, 150 rpm, and initial pH 7.0) in Erlenmeyer flasks plugged with non-absorbent cotton. The whole pellet was harvested (9440 × *g* for 10 min at 5 ^{o}C) and used as *Btk* *δ*-endotoxin (control).

3.15.2. Preparation of raw Btk δ-endotoxin, by SSF

For solid-state fermentation (SSF), LB was supplemented with 30% (w/v) raw soybean four (SF); this LB- soybean flour mixture was designated in this study as medium 1 (M1). For making soybean flour, well dried soybean seeds were purchased from local market, which were ground into flour using a mixer-grinder. Five µL *Btk* seed culture (containing ~6.5×10^7 CFU per mL) was used to inoculate 1 mL LB in M1 (this was to make the seed culture uniform as in control, irrespective of the soybean flour added in the medium) and incubated (150 rpm, 37 ^{o}C) in an environmental shaker (Scigenics Biotech, India). After 12 h incubation, the partially fermented matter was centrifuged aseptically (1000 × *g* for 10 min, 4 ^{o}C) to collect the supernatant for harvesting extracellular enzymes and the pellet further incubated (37 ^{o}C) under static condition up to 48h. The pellet so obtained was used directly as the raw toxin for bioassay, *i.e*., solid-fermented matter with *δ*-endotoxin and endospores.

3.15.3. Culturing of E. orientalis

Leaf disc method (Payne *et al*., 1993) was employed to culture *E. orientalis* in petri-dishes containing healthy and fresh whole leaves (for juice feeding) of *Vigna unguiculata* (L.) Walp, providing suitable temperature using tungsten bulb (37 ^{o}C) and humidity (inserting moist cotton in the petri-dish) in a specially designed hood. Heavily infested *V. unguiculata* (L.) Walp leaves from the field were carefully transported to the laboratory and transferred them on to the leaf in the petri-dish set as above using a brush. The brush was made single-bristled for this purpose. The leaves in the petri-dishes were changed after every 24 h. Various growth stages of the mite obtained from this culture set-up were used to depict its life cycle. These mites were used for bioassay (feeding assay) *in vitro*.

3.15.4. Bioassay or in vitro toxicity assay to determine the entomotoxicity (Tx)

Entomotoxicity (Tx) of *Btk* toxin was evaluated through bioassay using oriental red mites (*E. orientalis*). Ten, 20 or 40 (all in mg) of *Btk* pellet (obtained from LB control after 72 h SmF) was mixed with 1 mL of 10% sucrose solution to form diet or the solid-fermented matter obtained after 48 h SSF (including crystalline δ-endotoxins, spores, cellular debris and debris of soybean flour) was directly used as the feed without adding sucrose solution. The feeding set-up was as follows: (a) a sterile glass disc was taken; (b) a sterile glass ring was placed above this, the bottom of this set up was sealed thoroughly using parafilm (this was essential to prevent leakage of the feed and escaping of mites); (c) then the artificial feed inside; (d) the diet prepared as above was poured on the thick pile of sterile tissue paper in the feeding chamber or regarding solid-fermented matter, the fermented matter was directly placed in the chamber and covered with a sterile tissue paper; (e) 20 healthy standardized adult female mites were carefully transferred on

the tissue paper (feed), and finally (f) the set-up was covered (top) with another glass disc without restricting aeration. **Figures 31 a** and **b** give a clear view on the feeding set-up with solid-fermented matter.

Control tests were also performed to assess exact net effect of *Btk* toxin obtained by either SmF or SSF on entomotoxicity. To compare the effect of *Btk* toxin obtained by SmF, uninoculated LB medium was mixed with sucrose solution and poured on the tissue paper in the *in vitro* feeding chamber. Similarly, to compare the effect of *Btk* toxin obtained by SSF, standardized mature females mites were fed with uninoculated sterile soybean powder in LB medium (SSF control). To check the normal survival, mature females were transferred to another petri-dish and leaf disc feeding continued. Mortality was assessed on 3^{rd} and 7^{th} days. Mortality rate on the 3^{rd} day of feeding was taken for probit analyses.

3.15.5. Monitoring the growth and mortality of mites

The life cycle of the mite and mortality rates were observed constantly through a Magnus compound microscope. The photographs were taken using a digital camera attached to the microscope (Webcam companion 4.0 MEM 1300, Japan).

3.16. Field trial

3.16.1. Preparation of the toxin

Btk-fermented matter (spores, crystalline δ-endotoxins, cellular debris and growth media including raw soybean substrates) was mixed with desirable quantity of sterile distilled water (10 mg/mL) and directly sprayed on the infested plants using a hand sprayer.

3.16.2. Application of the toxin

Field trials were performed on *V. unguiculata* (L.) Walp during summer (April-May), when the atmospheric temperature was around 37 ^{o}C. Two weeks old healthy seedlings were transferred to the pots; 20 such potted plants were chosen and divided in to 2 (experimental and control) groups, and maintained in open air where ample sunlight was available. The pots mixture contained garden soil, sand and cow dung in the ratio 6:3:1, respectively. The potted plants were watered in the morning and evening. Both the group of plants were maintained as distinct groups in the field, separated about 50 meters apart in sunny areas. After 10 days of acclimatisation of the plantlets in the pots, they were infested with the mites; *i.e*., leaves of heavily infested *V. unguiculata* (L.) Walp plants from the field were collected and these leaves were carefully placed on the potted plants, so as to get approximately equal number of mites transferred to each potted plant (experimental group). After 20 days of the transfer of the mites to the potted plants, *Btk* toxin (48 h raw solid-fermented matter with embedded δ-endotoxin and spores) was applied to the experimental group of plants.

Photographs were taken using a digital camera.

3.16. 3. Statistical analysis

SPSS (Statistical Package for the Social Sciences) version 20 software was used for the probit analyses (Finney, 1971). LC_{50} (50% lethal concentrations) value of *Btk* formulation against *E. orientalis* was estimated from the regression equation of the probit. The antilog value of the corresponding log value was the $LC_{50.}$

3.17. Statistics and software used

Microsoft Excel 2007 was used for calculations and ploting graphs, SPSS (Statistical Package for the Social Sciences) version 20 was used for the graphical expression of bioassay (probit analysis), Adobe Photoshop CS5 was used to set the photographs, Hyper 32 was used to plot *Lineweaver-Burk plot* and Graph pad prism was used to plot non-linear fit of Michaelis-Menten data.

Chapter 4

PROTEASE FROM *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI*

4.1. Part I: Production conditions and Partial purification

[Jisha VN, Smitha RB and Sailas Benjamin (2013). Production and partial purification of protease from *Bacillus thuringiensis* subsp. *kurstaki* by submerged and solid-state fermentations. ***Letters in Applied Microbiology***. (under review)]

4.1.1 Aim

This study evaluates how efficiently the valuable protease could be recovered from the fermentation medium in which *Btk* is grown, without affecting the yield of *δ*-endotoxin. Thus, the specific aims are: (a) to examine the efficacy of *Btk* in producing extracellular protease as a by-product without affecting its unique role for producing *δ*-endotoxin; (b) to examine the efficacy of natural agricultural products as supplements to the LB medium for the dual production of protease and *δ*-endotoxin; (c) to establish a cultivation strategy for the dual production of protease and *δ*-endotoxin; and (d) to partially purify the extracellular protease from the supernatant.

4.1.2. Materials and Methods

Materials and materials pertaining to this chapter are described under **chapter 3**, under sections: **3.8, 3.9, 3.10** and **3.11.**

4.1.3. Results

4.1.3.1. Alkaline protease activity in LB or LB + casein

In commercial LB basal medium, the maximum extracellular protease activity was obtained (131 U/mL) at 18h, subsequently it declined (**Figure 8A**). Maximum protease activity in conventional LB medium supplemented with 1% (w/v) defatted casein was 139 U/mL (at 18h). It indicated that there was no significant increase over the control, which contained only LB (**Figure 8A**).

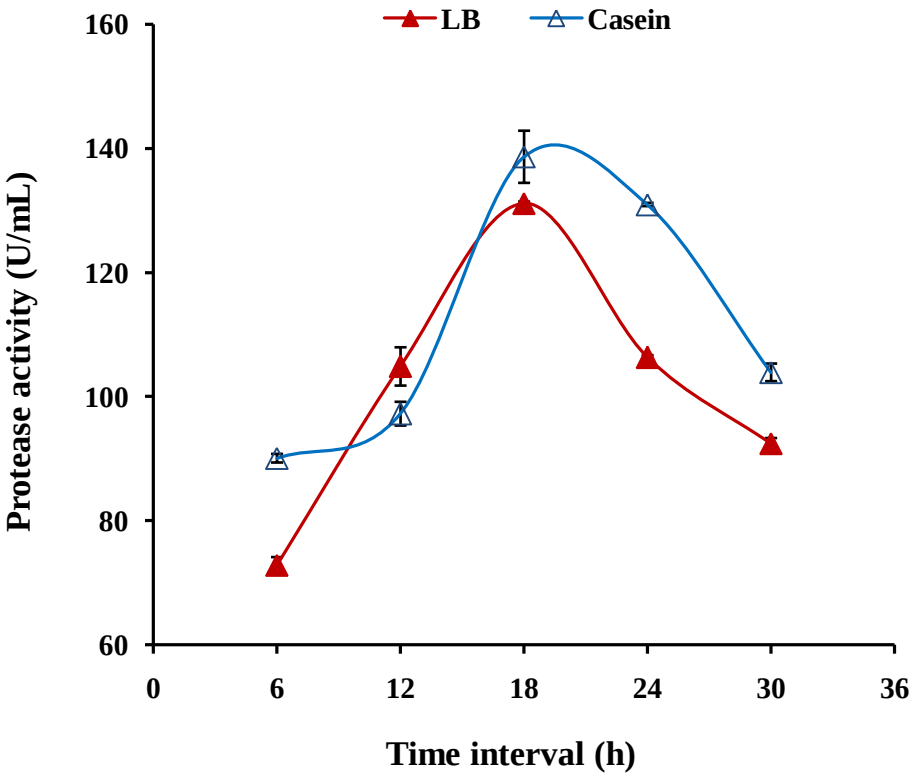


Figure 8A. Comparative profile of protease (crude) activity in the supernatant obtained (9440 × *g* for 10 min at 4 ^{o}C) from LB (control) and 1% casein supplemented LB media.

4.1.3.2. Protease activity in LB medium supplemented with different flours at 1% concentration (SmF)

The flours of banana, Bengal gram, jack seed, soybean and tapioca at 1% (w/v) concentration were used for SmF. Addition of these supplements to LB did not affect the texture of the medium, *i.e.*, it remained in the liquid state throughout the study, hence the cultivation strategy was treated as SmF and protease activity was calculated as U/mL. Comparable protease activities were obtained at 12h or 18h harvest, depending on the supplement in the LB medium (**Figure 8B**). The maximum protease activity (291 U/mL, at 12h) was in the soybean flour supplemented medium, which was 2.2 folds increase over the LB control. Bengal gram flour supplemented medium showed the second highest protease activity (232 U/mL, 12h), which was 1.7 folds increase over the LB control. However, the protease activity in the banana flour supplemented medium was the least (89 U/mL, 12h), which was about 0.3 fold less than that of the LB control. Contrary to the above maximum activity at 12h, jack seed flour (209 U/mL) and tapioca flour (127 U/mL) supplemented media showed the maximum activity at 18h fermentation (**Figure 8B**).

4.1.3.3. Protease production by SSF with different supplements

It is customary that activity of enzyme produced during SSF would be expressed in units per gram dry fermented substance (U/gds). Unlike this study, expression of activity in U/gds is effective only if SSF is involved in a study. Thus, we wanted to compare the actual protease yield obtained during SSF (in flour supplemented LB medium) with that obtained during SmF. In order to avoid ambiguity and false positive results, we presented the protease activity obtained during SSF as U/mL_{eqv} *i.e.*, activity equivalent to the actual liquid LB medium used to prepare the flour-supplemented medium; this would further enable direct comparison of cost effectiveness.

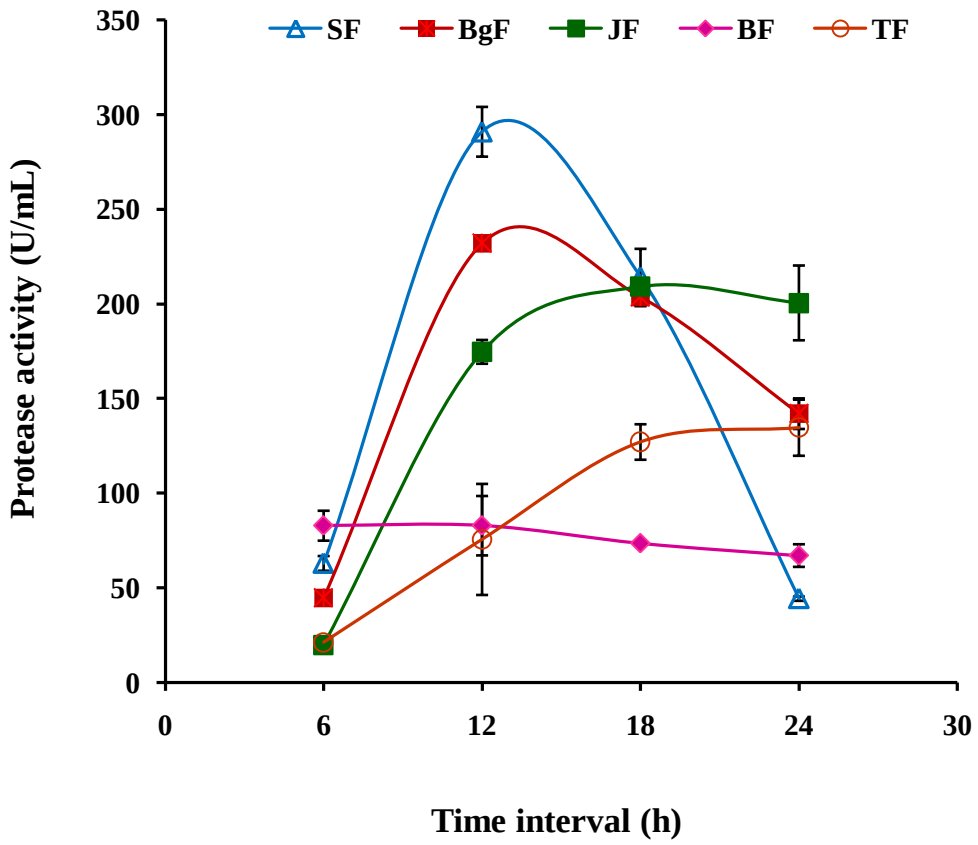


Figure 8B. Comparative profile of crude protease activity in the supernatant obtained (9440 × *g* for 10 min at 4 ^{o}C) from LB supplemented with 1% banana flour (BF), Bengal gram flour (BgF), jack seed flour (JF), tapioca flour (TF) and soybean flour (SF).

Protease activity in LB supplemented with 10 % (w/v) or more (20, 30, 40, 50, 60, 80 and 100 %) flours of banana, Bengal gram, jack seed, soybean and tapioca is presented under this session. It was noticed that at the time (12 or 18h) of enzyme harvest from the flour-supplemented medium, the texture of the medium was totally changed as demonstrated in **Figure 9a** and **b** for soybean flour. The pellet (**Figure 9c**) obtained after removing the supernatant was incubated further for monitoring the concomitant production of endospore and *δ*-enotoxin, as we described for potato flour supplemented medium (Smitha *et al*., 2013a). Up to 50% (w/v), the medium was almost a viscous and slimy semi-solid mass, *i.e*., prior to the removal of supernatant. Only the representative data from banana, Bengal gram, jack seed, soybean and tapioca flour supplemented media against respective time, which showed the maximum protease activity in each preparation was presented in **Figure 10A and B; Table 9.** The maximum protease activity (2097 U/mL_{eqv}) was obtained with soybean flour supplemented medium (30%, w/v concentration) at 12h, which was about 16 folds increase over the LB control. Maximum protease activity in Bengal gram flour (30 %) supplemented medium was 1865 U/mL_{eqv}, which was about 14 folds increase over the LB control. As shown in **Figure 10A,** 20% banana flour (1139 U/mL_{eqv}), 10% jack seed flour (659 U/mL_{eqv}), and 10% tapioca flour (172 U/mL_{eqv}) supplemented LB media showed the maximum protease activity at 18h fermentation. Of these five natural supplements, soybean flour supplemented medium showed the highest protease activity, and hence this combination was used for further studies including partial purification of protease.

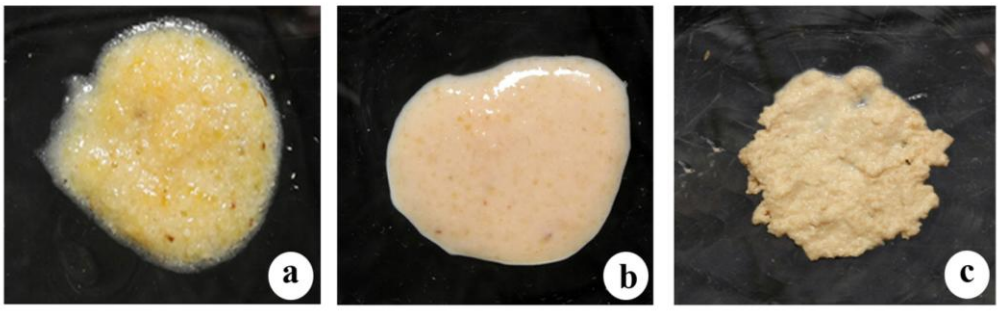


Figure 9. Texture of 30% soybean flour supplemented LB medium. **a**. texture of autoclaved (15 ψ, 121 °C, 20 min) medium prior to inoculation; **b**. at 12 h fermentation (150 rpm, 37 °C), *i.e.*, prior to separation of supernatant by brief spinning (1000 × *g* for 10 min at 4 °C) for the harvest of protease; and **c**. pellet obtained after separation of supernatant (1000 × *g* for 10 min at 4 °C). This pellet was incubated further for the concomitant production of endospore and *δ*-endotoxin.

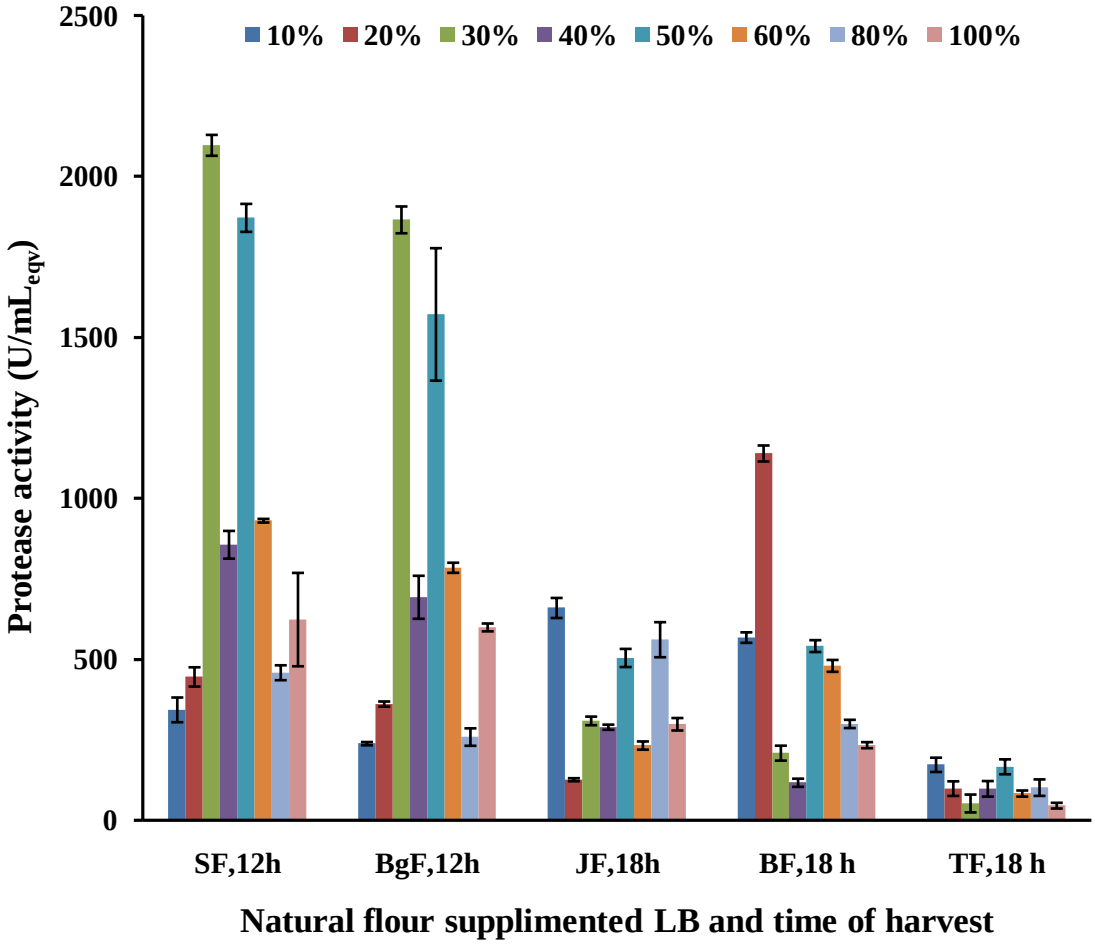


Figure 10A. Comparative profile of crude protease activity in the supernatant (1000 × *g* for 10 min at 4 ^{o}C) obtained from LB supplemented with 10, 20, 30, 40, 50, 60, 80 and 100% (w/v) banana flour (BF), Bengal gram flour (BgF), jack seed flour (JF), tapioca flour (TF) or soybean flour (SF). Only respective time (12 or 18 h) in which the maximum activities obtained was shown here. To obtain clear solution for protease assay, the above supernatant was centrifuged again at high speed (9440 × *g* for 10 min at 4 ^{o}C). Here, the protease activity is expressed in U/mL$_{eqv}$, *i.e.*, per mL protease activity is equated to the activity in the supernatant obtained from 1 mL + % flour initially added in the medium.

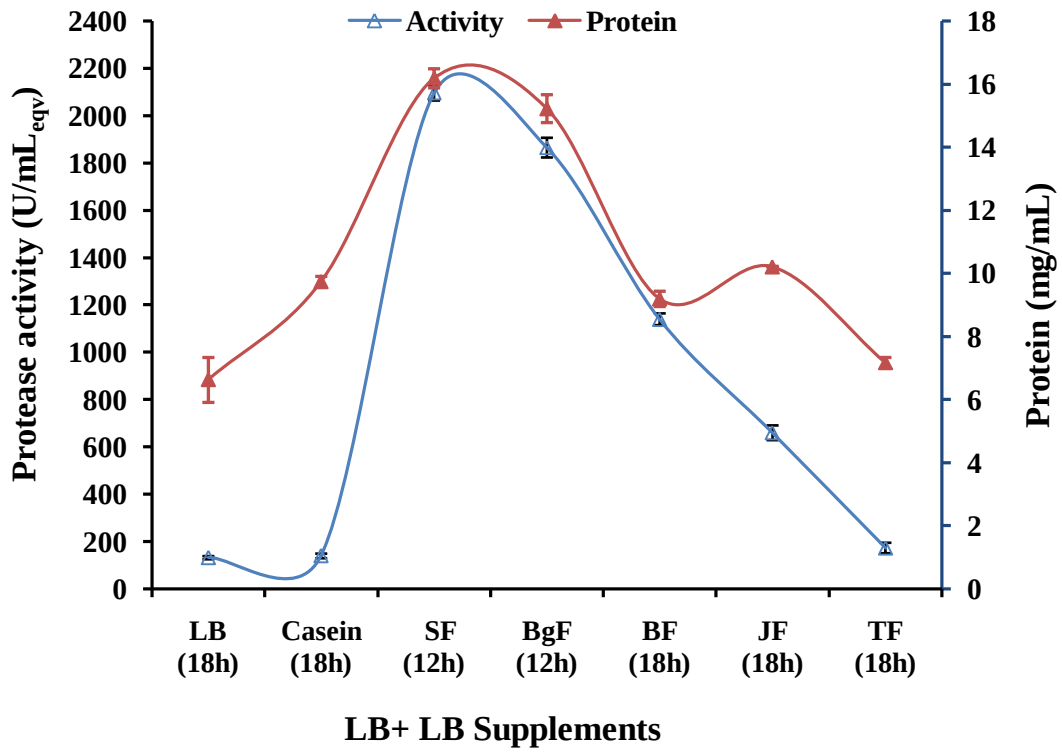


Figure 10B. Comparative profile of crude protease activity and protein yield in the supernatant (1000 × *g* for 10 min at 4 ^{o}C) obtained from LB and LB supplemented with 1% casein, 10, 20, 30, 40, 50, 60, 80 and 100% (w/v) banana flour (BF), Bengal gram flour (BgF), jack seed flour (JF), tapioca flour (TF) or soybean flour (SF). Only respective time (12 or 18 h) in which the maximum activities obtained was shown. To obtain clear solution for protease assay, the above supernatant was centrifuged again at high speed (9440 × *g* for 10 min at 4 ^{o}C). Here, the protease activity is expressed in U/mL$_{eqv}$, *i.e.*, per mL protease activity is equated to the activity in the supernatant obtained from 1 mL + % flour initially added in the medium and protein in mg/mL.

Table 9. Alkaline protease activity of *Btk* on LB medium supplemented with flours of banana flour, Bengal gram flour, jack seed flour, tapioca flour or soybean flour at different time intervals.

Natural supplements	Time interval	Concentration of flours (%)							
		10	20	30	40	50	60	80	100
Banana flour	6	65.57 ± 9.39	68.85 ± 11.73	169.06 ± 63.52	176.43 ± 12.55	237.7 ±12.79	340.16 ± 88.94	694.88 ± 10.76	131.76 ± 19.69
	12	362.7 ± 40.311	690.57 ± 61.58	116.8 ± 6.15	163.93 ±24.85	604.51 ± 21.585	250 ± 31.55	94.26 ± 29.05	352.66 ± 34.06
	18	567.42 ± 16.29	**1139.34** ± 24.84	208.4 ± 23.19	116.39 ± 12.66	540.98 ± 18.44	479.51 ± 18.44	299.181 ± 12.79	233.191 ± 9.41
	24	118.851 ± 4.36	875 ± 33.86	110.65 ± 38.39	75.81 ± 25.59	187.09 ± 5.002	348.56 ± 9.46	149.59 ± 15.63	96.11 ± 19.96
Tapioca flour	6	74.59 ± 4.69	132.99 ± 2.15	124.79 ± 3.84	142.95 ± 14.40	121.11 ± 64.23	45.90 ± 3.75	49.79 ± 1.62	56.97 ± 4.65
	12	24.59 ± 5.25	74.59 ± 2.48	92.83 ± 27.98	168.64 ± 18.38	38.19 ± 2.20	47.34 ± 2.10	72.13 ± 8.37	40.98 ± 5.81
	18	**172.1** ± 22.16	98.15 ± 22.72	110.25 ± 27.72	97.54 ± 24.13	165.98 ± 23.19	82.87 ± 1.89	101.22 ± 5.12	45.29 ± 8.89
	24	43.65 ± 2.46	104.5 ± 18.44	98.97 ± 10.16	41.19 ± 3.74	35.66 ± 9.28	56.47 ± 5.58	66.93 ± 5.30	60.94 ±1.70
Jack seed flour	6	117.21 ± 6.45	186.47 ± 9.99	312.29 ± 17.13	102.45 ± 3.75	129.91 ± 6.15	202.8 6 ± 3.07	350.4 ± 34.22	210.25 ± 15.51
	12	79.71 ± 3.03	42.82 ± 6.51	530.12 ± 17.34	256.96 ± 14.53	103.27 ± 29.97	542.82 ± 30.57	504.50 ± 31.59	245.498 ± 11.73
	18	**659.42** ± 31.13	125.81 ± 4.77	308.60 ± 13.34	288.93 ± 8.06	504.09 ± 28.17	231.96 ± 2.55	560.86 ± 54.41	298.15 ± 19.41
	24	146.92 ± 21.84	108.60 ± 3.03	154.09 ± 18.44	532.17 ± 37.99	653.68 ± 31.54	378.68 ± 3.25	301.84 ± 6.47	313.15 ± 11.34
Soybean flour	6	65.61 ± 1.60	118.60 ± 4.07	128.68 ± 15.18	287.29 ± 7.41	481.35± 17.57	457.78 ± 8.30	1517.82 ± 38.24	1593.85 ± 10.93
	12	342.86 ±38.42	445.24 ± 29.87	**2096.72** ± 32.44	855.73 ± 43.07	1871.31 ± 43.41	930.53 ± 5.71	458.19 ± 23.16	623.36 ± 145.05
	18	223.72 ± 13.03	591.68 ± 14.67	354.30 ± 37.45	767.41 ± 53.44	990.98 ± 22.13	1714.34 ± 182.45	713.52 ± 257.57	512.09 ± 18.83
	24	229.63 ± 26.87	221.80 ± 6.77	50.00 ± 2.15	92.62 ±1.56	420.49 ± 31.98	595.90 ± 10.54	1190.77 ± 163.61	481.96 ± 26.49
Bengal gram flour	6	57.04 ± 2.54	70.90 ± 3.09	480.61 ± 309.03	327.66 ± 9.76	420.69 ± 14.86	379.09 ± 24.04	696.51 ± 9.30	116.80 ± 1.22
	12	237.90 ± 5.049	360.86 ± 8.08	**1865.1** ± 41.64	692.62 ± 66.77	1571.31 ± 205.83	784.22 ± 15.70	258.40 ± 27.10	598.97 ± 12.20
	18	220.12 ± 0.92	265.94 ± 5.10	617.00 ± 63.74	471.10 ± 10.15	280.73 ± 13.04	1729.30 ± 30.42	1125.20 ± 82.00	583.4 ± 37.44
	24	129.91 ± 15.37	163.89 ± 2.77	40.77± 3.09	75.82 ± 1.98	379.50± 7.33	452.86 ± 19.60	1084.22± 24.66	411.88± 6.05

4.1.3.4. Partial purification of Protease

Two extracellular proteases were partially purified from the culture filtrate of *Btk*, obtained by both SmF and SSF (**Figure 11A-C**). The proteases were purified by ammonium sulphate fractionation (60%-80% fraction), molecular weight (MW) cut-off for obtaining protein fraction below 45 kDa using spin column (Vivaspin 6), and finally by sephadex G-100 gel filtration. Sephadex G-100 gel profile showed a clear peak for protease active fraction.

Zymogram profile showed a clear proteolytic zone on acrylamide gel impregnated with 1% skim milk (**Figure 11A**). The approximate MW of purified proteases was estimated to be 43 and 32 kDa, as judged by SDS-PAGE (**Figure 11B**).

4.1.4. Discussion

Our group already demonstrated that *Btk* is an efficient producer of extracellular amylase (Smitha *et al*., 2013b). In continuation to this, in this study, we explored whether *Btk* could produce extacellular protease too. The most significant aspect of the present study was the production of protease from *Btk* employing the natural and easily available agricultural products. It was observed that the production of alkaline protease was significantly enhanced by the enrichment of the basal medium (LB) with the addition of naturally available raw products. SSF holds tremendous potentials for the production of enzymes, and therefore, it is of special interest in those processes where the crude fermented product may be used directly as enzyme source (Benjamin and Pandey, 1998). The SSF process was observed to be less sensitive to contamination than SmF (Frankena *et al*., 1986). In SSF, the selection of a suitable supplement for fermentation is a critical factor, which involves the screening of a few naturally available products for microbial growth and enzyme production. All the supplements used in this study at different concentrations (1, 10, 20, 30, 40, 50, 60, 80 and 100%, w/v) supported the growth and enzyme secretion, while soybean flour was proven superior to other supplements.

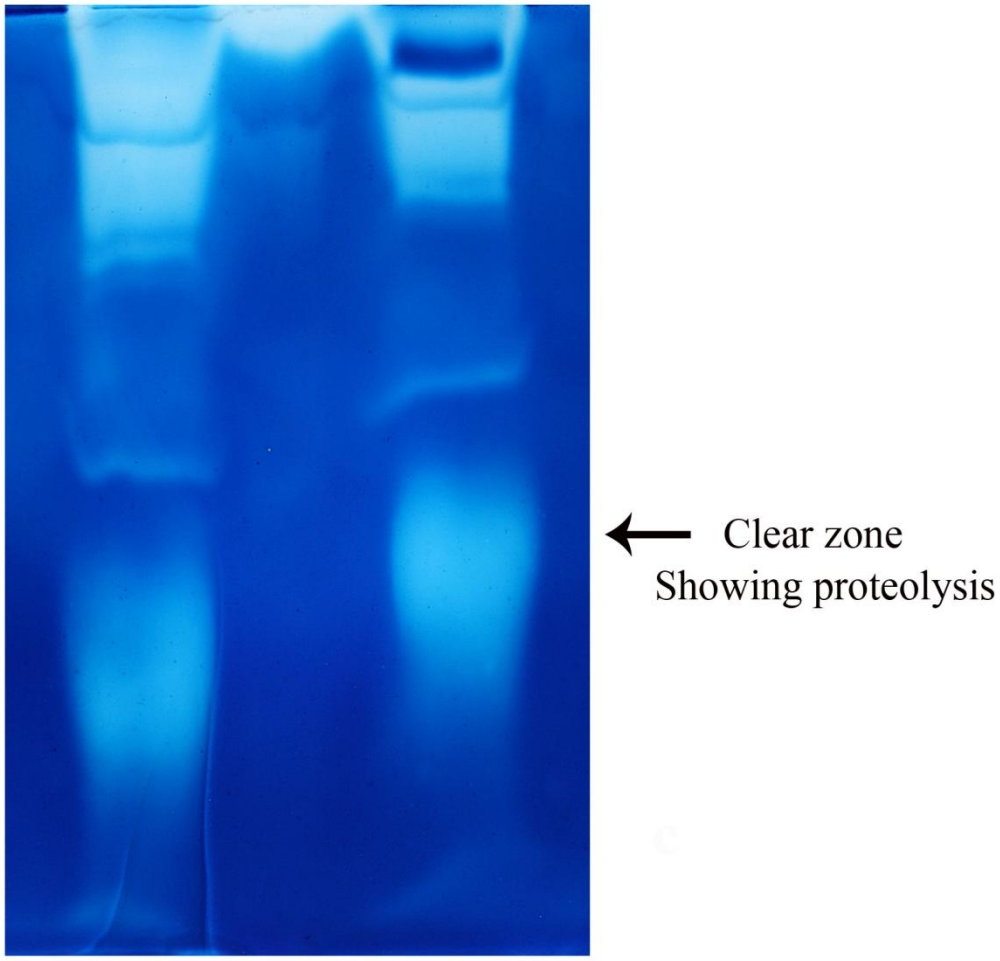


Figure 11A. Native page profile showing clear zone of proteolysis against blue background. $(NH_4)_2SO_4$ fraction (60-80%) of crude protein (lane 2) harvested from soybean flour supplemented (30%, w/v) LB at 12 h fermentation, and lane 1: corresponding protease obtained from LB basal medium after 18 h fermentation.

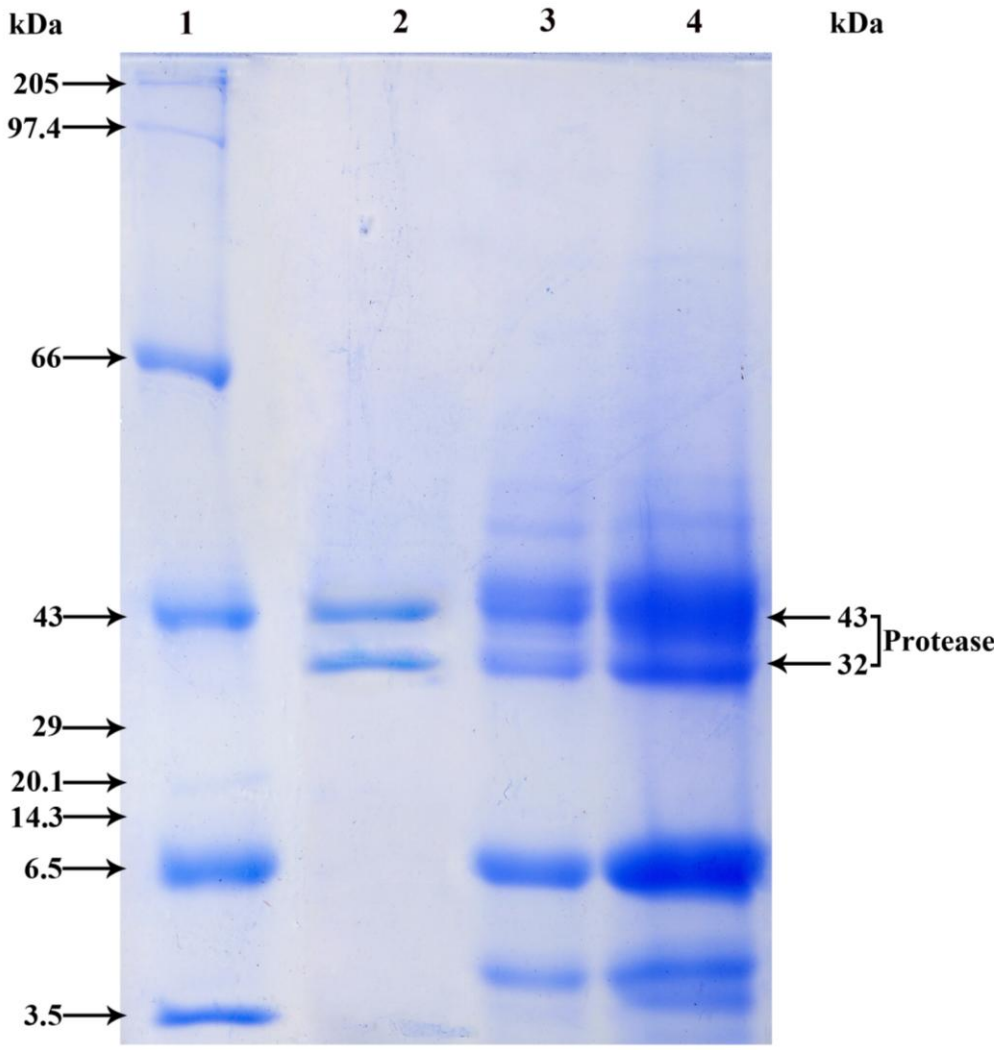


Figure 11B. SDS-PAGE profiles showing protease active bands with approximate MWs of 43 and 32 kDa. $(NH_4)_2SO_4$ fraction (60-80%) of crude protein harvested from soybean flour supplemented (30%, w/v) LB at 12 h fermentation was subjected to spin column MW cut-off (Vivaspin 6 column, Sweden); subsequently, the lower fraction (lane 3) containing proteins below 45 kDa was subjected to sephadex G-100 gel filtration so as to obtain clear protease active fraction (lane 2), and lane 1 is the profile of standard protein MW marker. Lane 4 is the profile of 40-60% $(NH_4)_2SO_4$ fraction from soybean flour supplemented (30%, w/v) LB after spin column cut-off.

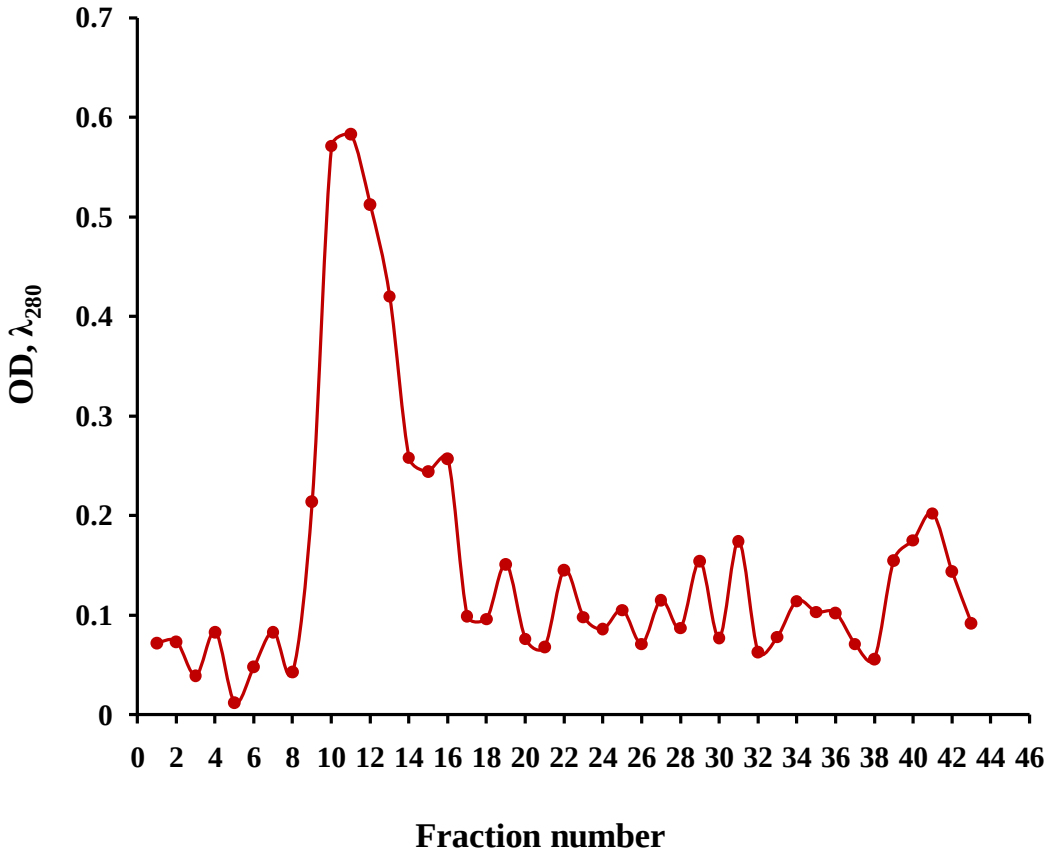


Figure 11C. Sephadex G-100 elusion profile of the partially purified protease. $(NH_4)_2SO_4$ fraction (60-80%) of crude protein harvested from soybean flour supplemented (30%, w/v) LB at 12h fermentation was subjected to MW cut-off using Vivaspin 6 column, and the lower fraction was loaded on sephadex G-100, which was eluted using phosphate buffer (pH 7.6) with a flow rate of 1.5 mL per 10 min.

In general, the protease produced in soybean flour supplemented medium by *Btk* showed the highest activity (2097 U/mL_{eqv}). There are reports, wherein soybean meal was used as natural supplement for protease production from various bacteria, for instance a *Bacillus* sp. produced 3208 U/mL protease (18h) (Saurabh *et al*., 2007). Nitrogen sources have regulatory effect on the enzyme synthesis. Production of protease is highly dependent on the both carbon and nitrogen sources available in the medium (Moon and Parulekar 1991; Chu *et al*., 1992; Patel *et al*., 2005). Thus, the protein-rich soybean flour supplemented medium enhanced the availability of carbon and nitrogen in the medium. Nilegaonkar *et al.* (2007) reported that 124 U/ mL protease was produced by *Bacillus cereus* in a medium containing soybean meal. In another study, soybean meal was used as an inducer for protease production from *Conidiobolus coronatus* (Deshpande *et al*., 2007). Joo *et al.* (2002) showed that the supply of soybean meal (1.5% concentration) in the medium resulted in the increased production of protease from *B. horikoshii*. A recombinant *B. subtilis*, one of the best known producers of protease, showed 3050 U/mL protease activity (about 30% increase over the present report) in a medium containing soybean meal and glucose, in the absence of inorganic nitrogen source (Calik *et al*., 2003). Some other researchers also reported that the presence of soybean meal in the growth medium substantially improved the production of alkaline protease. All these findings suggest that protease production is highly responsive to the nature of nitrogen sources. Therefore, LB medium enriched with soybean flour was used as the production medium in this study.

From the SDS-PAGE profile of this study, it is evident that the approximate MW of the proteases produced by *Btk* was between 43 and 32 kDa. Literature shows that protease from *Bacillus* spp. generally varies between 43 and 29 kDa. For instance, Kezia *et al*. (2011a) reported a 43 kDa protease from *B. subtilis* and Monod *et al*. (1991) reported a 33 kDa protease from

A. fumigates, while *B. licheniformis* produced a 34 kDa protease (Ageitos *et al*., 2007). A protease of MW between 26 and 29 kDa was reported from *Bacillus* sp. by Sinha *et al*. (2007), and a 39.5 kDa protease from *B. circulans* was reported by Rao *et al*. (2009). All these results show that the MW of most of the proteases from the genus *Bacillus* generally fall around 40 and 30 kDa (Sousa *et al*., 2007), thus the MWs of the two proteases reported herein corroborate with the published data.

This study demonstrated the production and partial purification of a protease from a strain of *Btk* as a by-product during the production of *δ*- endotoxin. The crude protease produced by *Btk* in soybean flour supplemented medium showed about 2097 U/mL_{eqv} activity, which were 16 folds higher than its activity found in commercial LB medium. In industry, the supernatant obtained after the harvest of *Bt δ*-endotoxin is normally discarded as effluent. We have already demonstrated the purification and quantification of *δ*-endotoxin from potato flour supplemented LB medium, using similar strategy as demonstrated herein (Smitha *et al*., 2013a). Thus, if the solid-state fermentation strategy reported herein is used for the production of *Bt δ*-endotoxin, the protease to be obtained as a by-product would fetch more profit to the producer; which would, in turn reduce the overall production cost.

Chapter 4

PROTEASE FROM *BACILLUS THURIENGIENSIS* SUBSP. *KURSTAKI*

4.2. Part II: Purification and Characterisation of Protease

[Jisha VN, Smitha RB and Sailas Benjamin (2013). Purification and characterisation of protease from *Bacillus thuringiensis* subsp. *kurstaki*. ***Enzyme and Microbial Technology*** (under review)]

4.2.1. Aim

This section on protease from *Btk* addresses its purification and characterisation.

4.2.2 Materials and methods

Materials and methods pertaining to this chapter are described under **Chapter 3**; sections: **3.8.2, 3.9, 3.10, 3.11** and **3.12.**

4.2.3 Results

4.2.3.1. Source of protease used for purification

As shown in previous section, 30% (w/v) soybean flour supplemented LB medium supported the maximum protease production at 12 h incubation, hence supernatant obtained from this fermentation state was used for further purification and characterisation of protease.

4.2.3.2. Partial purification of protease by ammonium sulphate fractionation

Two extracellular proteases were partially purified from the supernatant obtained by the cultivation of *Btk* in LB basal medium (SmF) and 30% soybean supplemented LB medium (SSF) (**Figure 11B**). Of various ammonium sulphate fractions, 60-80% fraction showed the maximum protease activity (1.96 fold purified fraction with 0.97 % yield) (**Table 10**).

4.2.3.3. Purification by spin column gel permeation chromatography

The 60-80 ammonium sulphate fraction (as described above) was subsequently subjected to spin column (Vivaspin 6) purification with MW cut-off of 45 kDa. This partially purified fraction (2.7 fold purified fraction with 0.90 % yield) with MW (less than) 45 kDa cut off was used for the studies on protease (**Table 10**).

4.2.3.4. Purification by sephadex G-100 gel permeation chromatography

Forty two fractions (1.5 mL/10 min) were collected, and the OD measured at 280 nm showed a major peak, corresponding to 10-13 fractions (**Figure 11C**). Purified protein showed that the apparent MWs of purified proteases were estimated to be 43 and 32 kDa, as that of the crude and partially purified ammonium sulphate fractions (**Figure 11B**). The purification fold of sepahadex G-100 fraction of protease was 12.79 with 0.3 % yield (**Table 10**).

4.2.3.5. Protease used for characterisation

The '*initial activity*' (2097 U/mL_{eqv}) denoted in this study to express fold increase or decrease is of the 60-80% ammonium sulphate fraction of crude protease obtained from 30% soybean supplemented LB medium (assay conditions: 10 mg/mL casein as substrate, pH 7.6, 37 ^{o}C, 20 min incubation).

The fraction obtained by spin column purification was used for further characterisation studies.

Table 10. Summary of purification of extracellular *Btk* protease from the supernatant in LB medium supplemented with 30% (w/v) raw soybean flour.

Purification	Total protein (mg)	Total activity (U/mL_{eqv})	Specific activity (U/ mg protein)	Yield (%)	Fold Purification
Crude extract	17.21 ± 0.92	2465.16 ± 4.35	138.027	100	1
60 - 80 % $(NH_4)2\ SO_4$ Fraction	9.06 ± 0.40	2387.3 ± 7.24	271.90	0.968	1.97
Spin column Fraction	5.29 ± 0.93	2213.11 ± 28.97	371.95	0.897	2.7
Sephadex G-100 column Fraction	0.46 ± 0.05	741.8 ± 11.59	1766.2	0.3	12.8

4.2.3.6. Characteristics of Protease

4.2.3.6.1. Effect of pH on protease activity and stability

Effect of pH on protease activity was measured under the standard assay conditions; *i.e*., 10 mg/mL casein as substrate, 37 ^{o}C and 20 min incubation with varying pH.

Purified protease was active in the 8.5 to 11.5 pH range, with the optimum being at pH 9 (activity was 7684.43 U/mL_{eqv}), which was 3.66 folds increase over the initial activity. At higher pH, the activity decreased considerably. But, another peak of activity obtained at pH 11 with 3.07 folds increase (6455 U/mL_{eqv}) over the initial activity (**Figure 12A**), it indicates the presence of two proteases corresponding to two bands as seen on the SDS-PAGE (**Figure 11B**). Briefly, the protease showed a broad range of pH stability, *i.e.*, between

8.8 and 9.6 (**Figure 12B**). From this, it is evident that the protease is better active at alkaline pH.

4.2.3.6.2. Effect of higher temperature on protease activity and stability

Though protease assay was performed at 37 ^{o}C in the previous sections, in order to find out temperature stability of the protease, assays were performed at higher temperature. Effect of temperature on protease activity was measured under the assay conditions: 10 mg/mL casein, pH 9 and 20 min incubation with varying temperature.

Results indicated that the better stability of protease produced by *Btk* was at 70 ^{o}C with an activity of 7923 U/mL$_{eqv}$ (**Figure 13A**); which was 3.78 folds increase over the initial activity, with comparable activities at 65 ^{o}C and 75 ^{o}C. The enzyme was more or less stable up to 80 ^{o}C (**Figure 13B**). At pH 11, the protease showed the maximum activity (7633 U/mL$_{eqv}$) at 75 ^{o}C (**Figure 14**); thus, the existence of two temperature optima further confirms that there exist two proteases.

4.2.3.6.3. Effect of substrate concentration

Effect of substrate concentration was measured under the assay conditions: pH 9, 70 ^{o}C and 20 min incubation at varying substrate (casein) concentration (0.5, 1, 5, 10, 15, 20 mg/mL). Here, effect of substrate concentration was tested with 0.05 to 2% (0.5-20 mg/mL) casein for 5 min interval up to 60 min incubation. The maximum activity was about 7992 U/mL$_{eqv}$ for 30 min at 15 mg/mL casein concentration, which was 3.8 folds increase over initial activity.

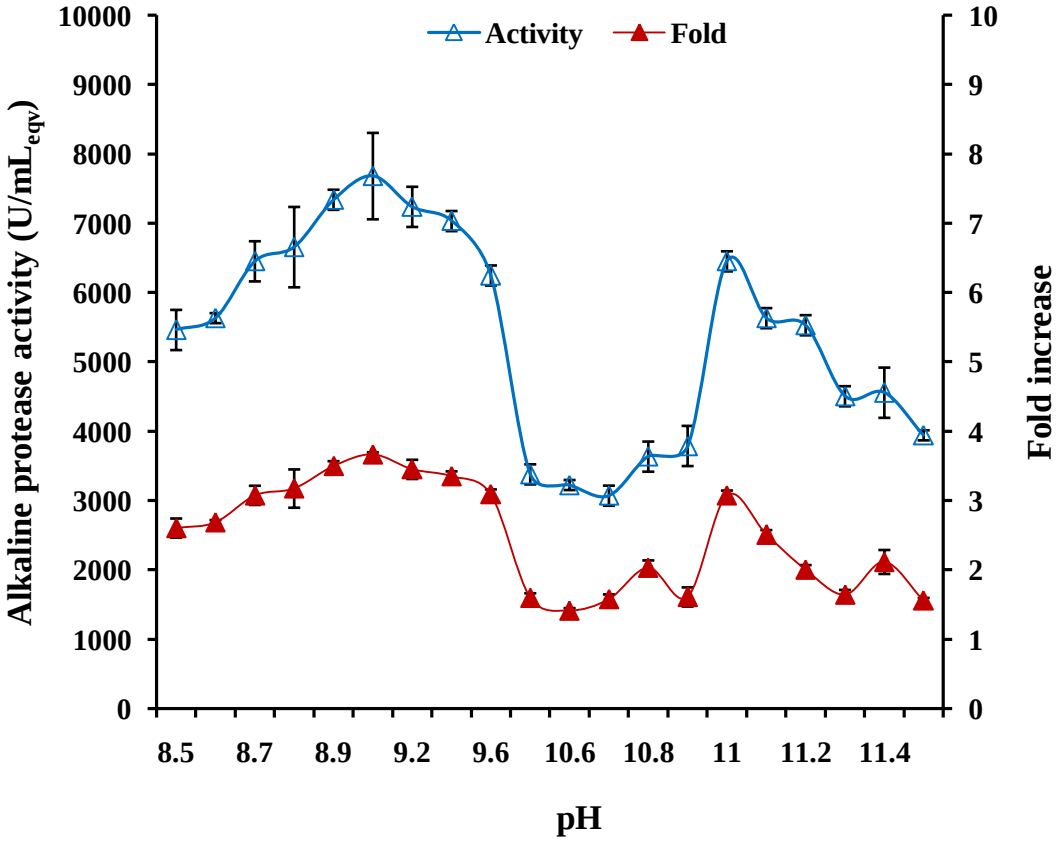


Figure 12A. Optimisation of pH and fold increase over initial activity. Effect of pH on protease activity was studied at 37 ^{o}C, varying pH, 10 mg casein as substrate for 20 min incubation (30% Soybean flour supplemented LB after 12 h fermentation).

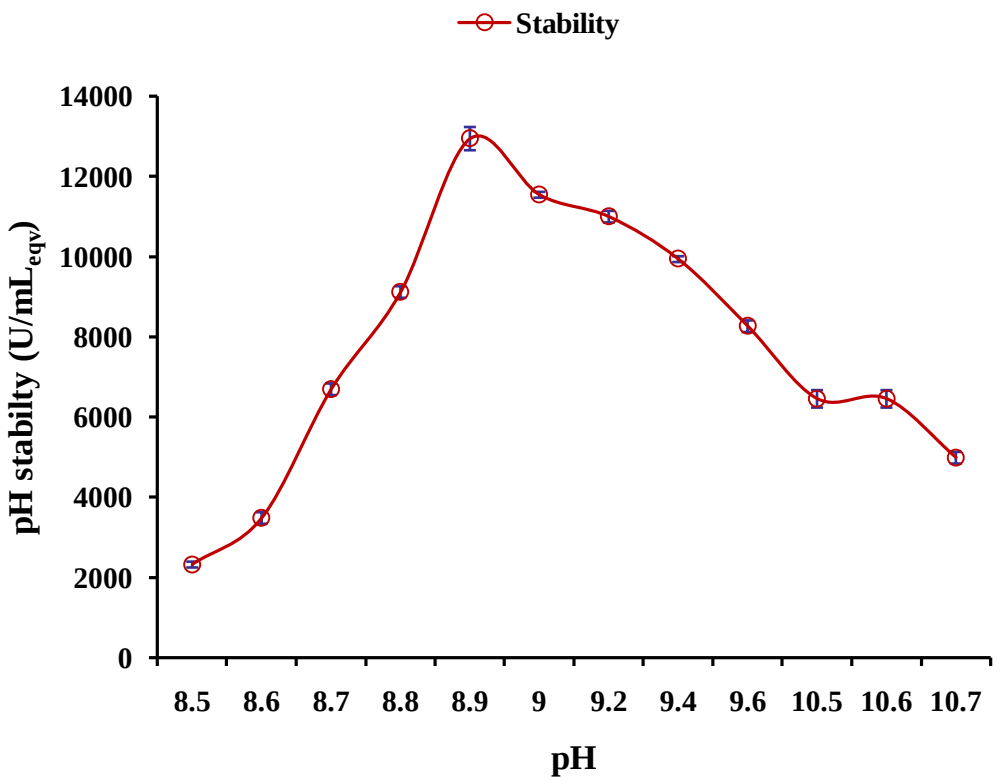


Figure 12B. pH stability of alkaline protease. Stability of the enzyme at various pH values was assayed by pre-incubating the enzyme in buffers of different pH for 1h.

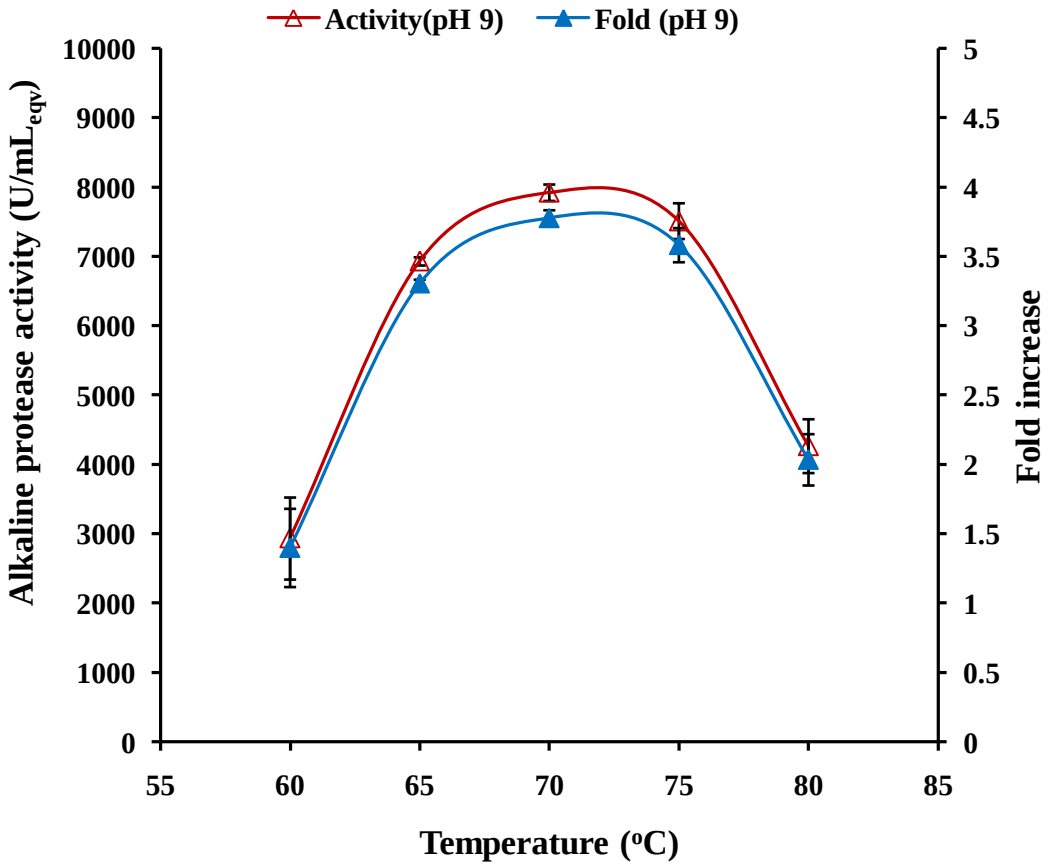


Figure 13A. Optimisation of temperature. Effect of temperature on the protease activity was determined by incubating the reaction mixture at different temperatures (60-80 ^{o}C) with pH 9 and 20 min incubation using 10 mg/mL casein.

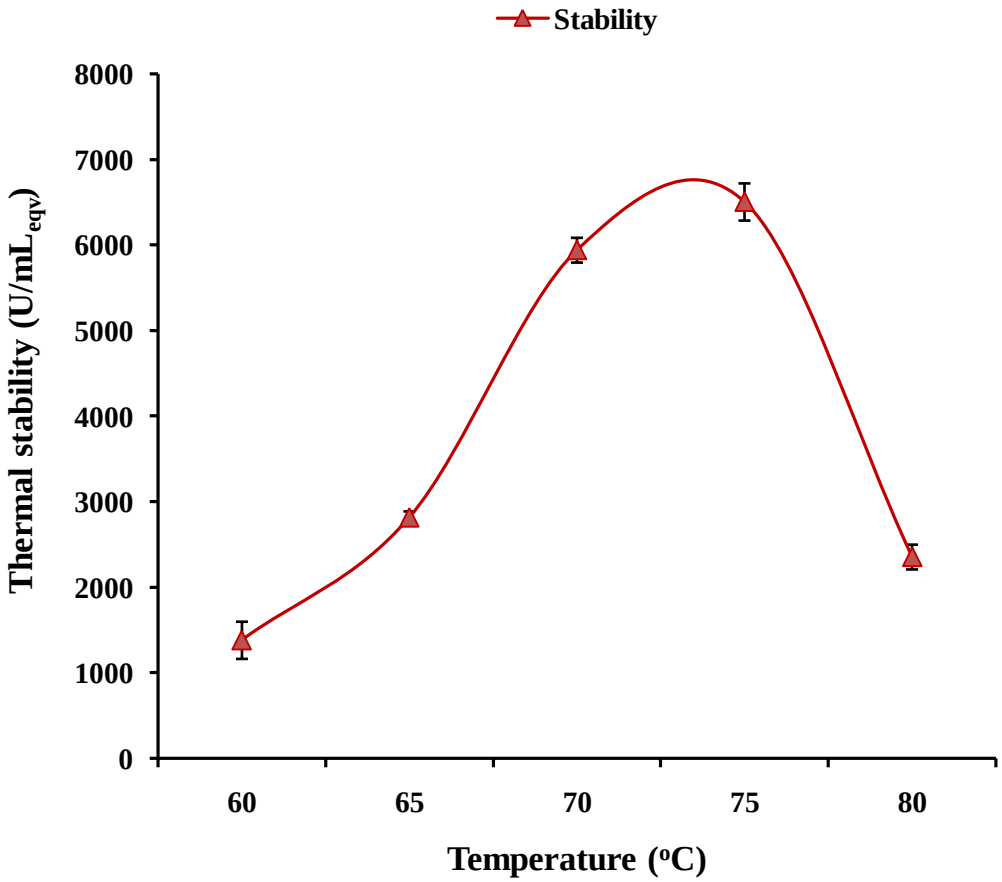


Figure 13B. Thermal stability of alkaline protease. For thermal stability the protease was pre-incubated at different temperatures (60-80^{o} C), for 1h and then activity was assayed.

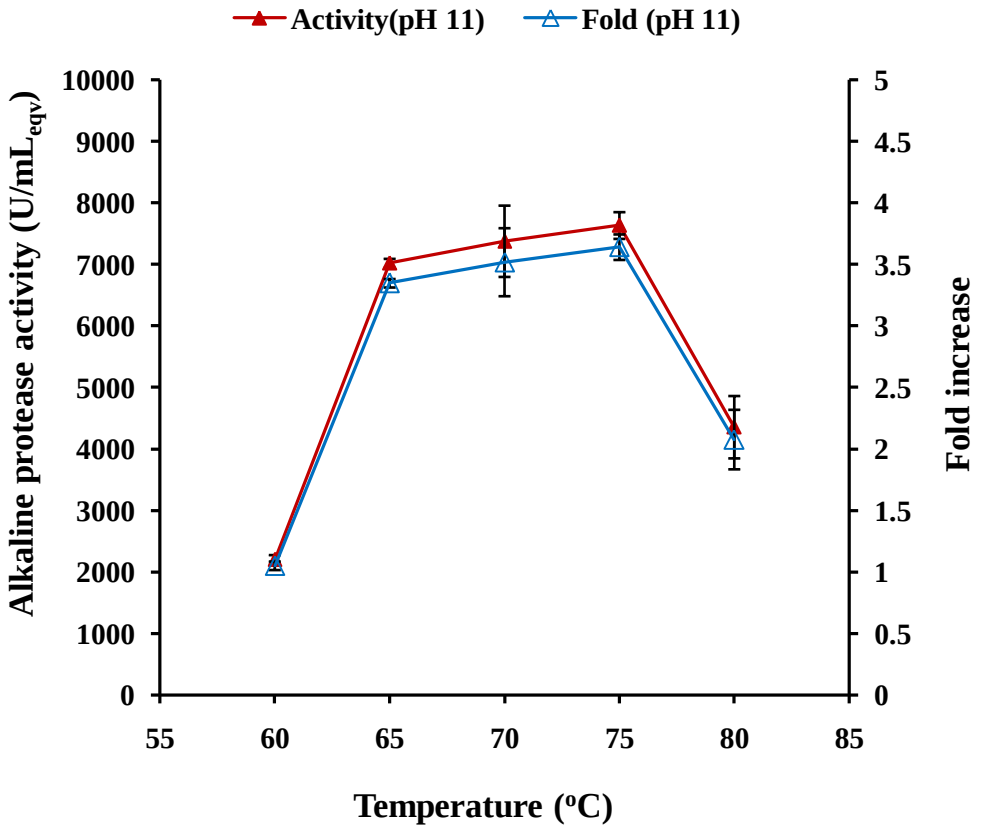


Figure 14. Optimisation of temperature. Effect of temperature on the protease activity was determined by incubating the reaction mixture at different temperatures (60-80 ^{o}C) with pH 11 and 20 min incubation using 10 mg/mL casein.

4.2.3.6.4. Effect of metal salts on protease activity

Effect of metal ions (salts) on protease activity was studied under the assay conditions: pH 9, 70 ^{o}C, 30 min incubation, and 15 mg/mL (casein) substrate with varying concentration (mM) of salt concentrations.

The protease activity was enhanced with the addition of Mn^{2+}, Ca^{2+} and Mg^{2+}, and the maximum activity (11732 U/mL$_{eqv}$) was obtained in the presence of 2 mM Mn^{2+}, which was 5.6 folds increase over the initial activity (**Figure 15A** and **B**).

Optimised condition:

The optimised condition for *Btk* protease activity was: 15 mg/mL (1.5%) casein as substrate, pH 9.0 and 70 ^{o}C temperature for 30 min incubation in the presence of 2 mM Mn^{2+}. At this condition, the activity was 11732 U/mL$_{eqv,}$ equivalent to 5.6 folds increase over the initial activity (**Figure 16**).

4.2.3.6.5. Effect of EDTA and β-mercaptoethanol on protease activity

Reaction conditions applied for this study were: 15 mg/mL casein, 9.0 pH and temperature 70 ^{o}C for 30 min incubation.

Of the complex compounds, presence of 1mM EDTA or *β*-mercaptoethanol in the reaction mixture decreased the protease activity by 0.94 (activity 1972 U/mL$_{eqv}$) and 0.85 (1741 U/mL$_{eqv}$) fold, respectively (**Figure 17**).

4.2.3.6.6. Compatibility with Detergents

Normal protease assay conditions were adopted for this study; *i.e*., 20 min incubation at 37 ^{o}C using 10 mg/mL casein as substrate at pH 7.6.

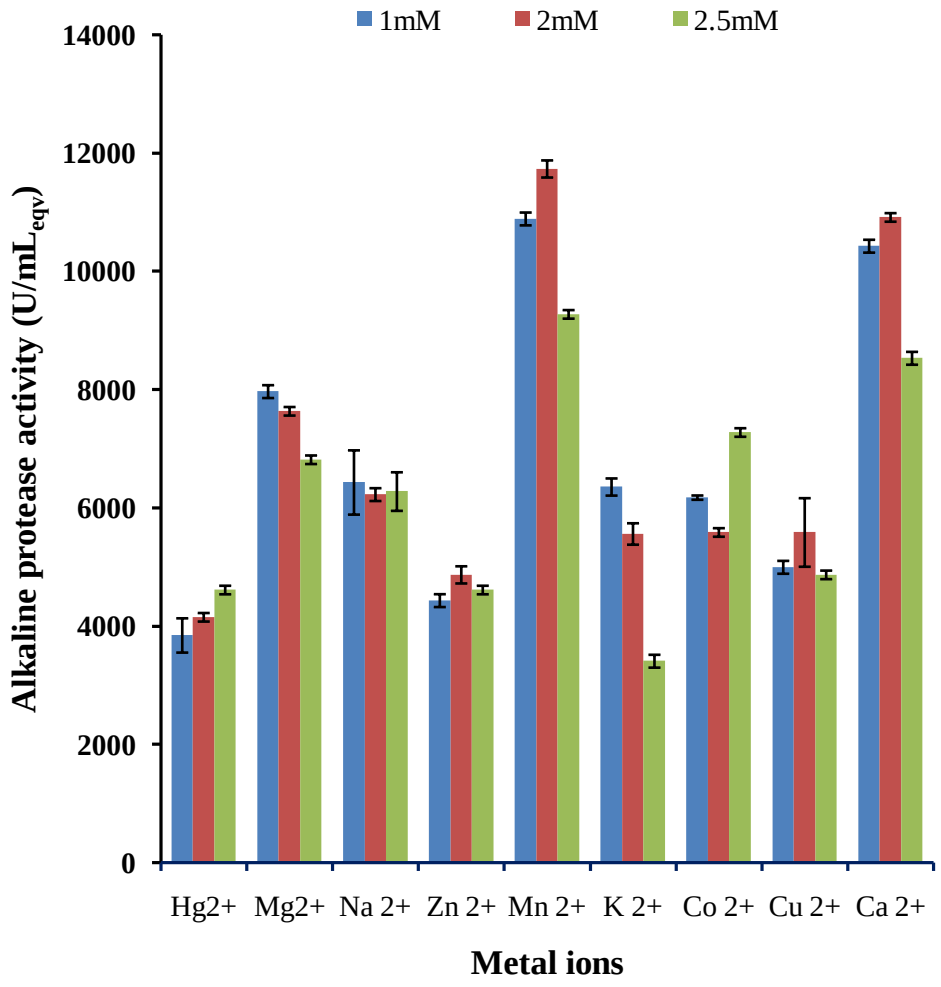


Figure 15A. Effect of metal ions (mM) on protease activity. This activity was at 70 ^{o}C, 9.0 pH with 15 mg/mL casein for 30 min incubation.

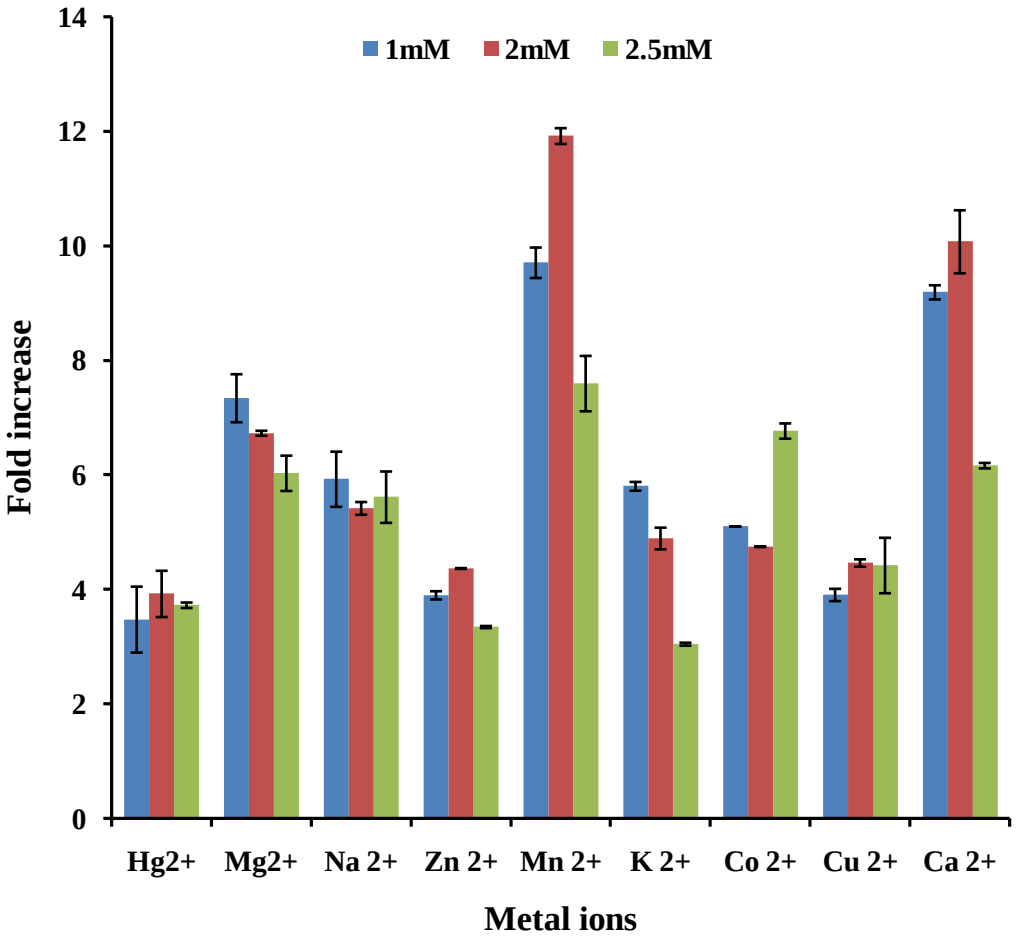


Figure 15B. Effect of metal ions (mM) on protease activity showed increase over initial activity.

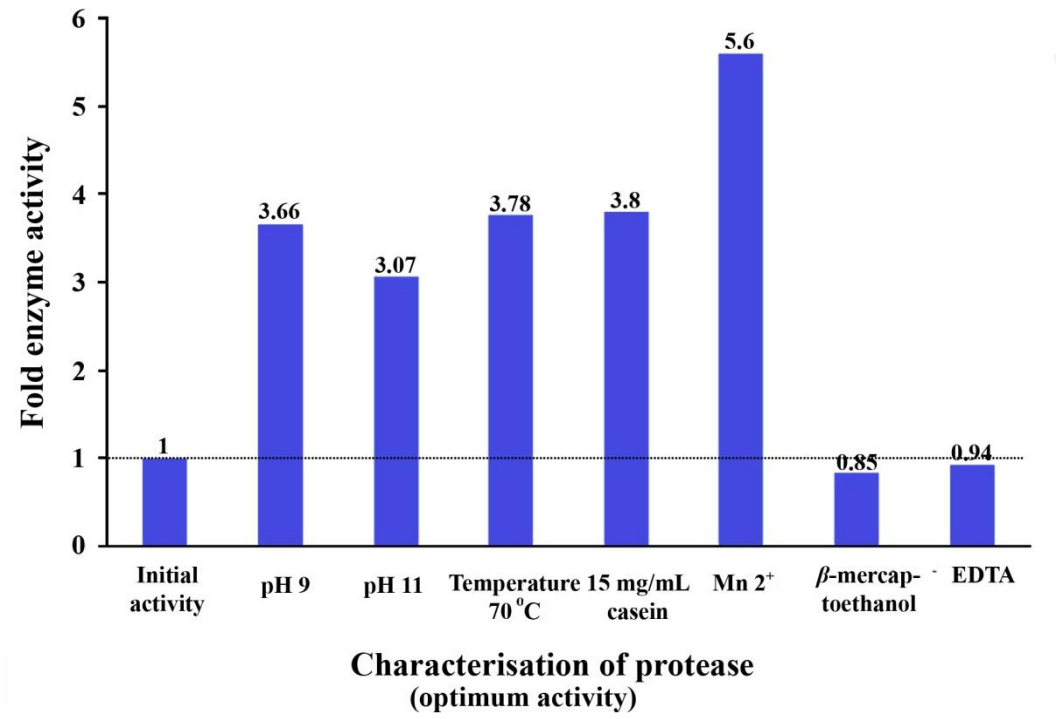


Figure 16. Consolidated data showing the maximum protease activity under optimised reaction conditions.

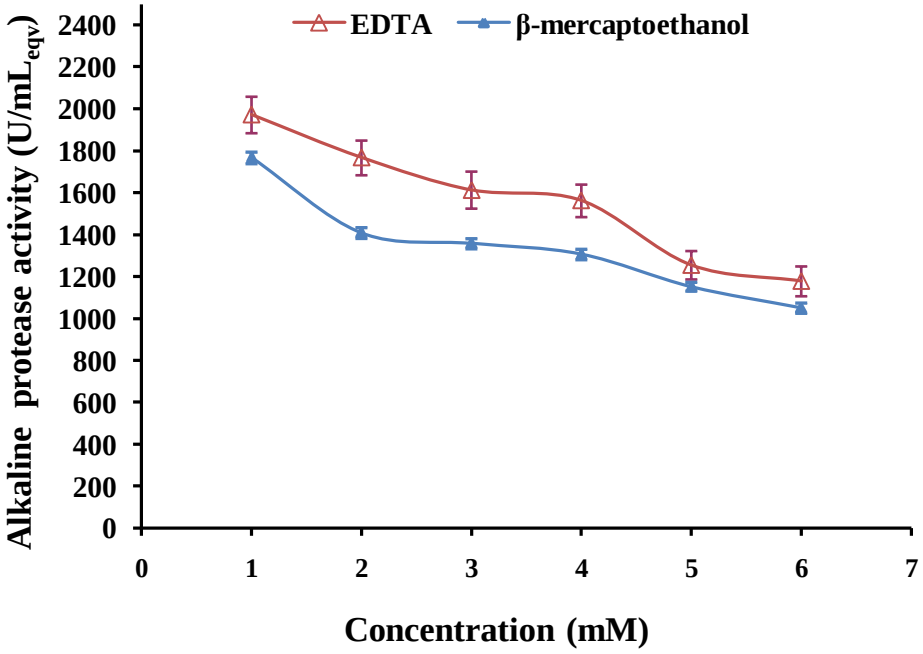


Figure 17. Effect of EDTA and *β*- mercaptoethanol on protease activity.

Stability of the protease in commercial detergents was tested by incubating (1h) measured quantity (100 µL) of the protease with the solutions of the different commercial detergents at a detergent concentration of 7 mg/mL (to simulate washing conditions).

Presence of detergents in the reaction system variously affected the protease activity. Protease activity was not disturbed when incubated with different concentrations of detergents/ surfactants like SDS (0.1, 0.4, 0.8, and 1%) and Triton X-100 (0.1, 0.4, 0.8, and 1%) for 30 min. Maximum activity obtained in the presence of 0.2% SDS was 6199 U/mL_{eqv} with 4.9 folds increase over initial activity (2097 U/mL_{eqv}), and 0.2% Triton X-100 with activity 6019 U/mL_{eqv}, which was 4.86 folds increase over initial activity (**Figure 18**). Unlike SDS and Triton X-100, protease showed good stability in the presence of commercial detergents tested (Ariel, Tide, Surf Excel and Sunlight); *i.e.*, the maximum stability showed in the presence of Ariel with activity 4867 U/mL_{eqv} and was over 2.3 folds increase over the initial activity (**Figure 19**).

4.2.3.6.7. Enzyme kinetics

The *Km* and *Vmax* values for *Btk* protease (of sephadex G-100 fraction) were calculated using the data obtained for different substrate concentration. The *Km* and *Vmax* values were found to be 0.90 mg/mL and 879.3 U/mg, respectively (**Figure 20** and **21**).

4.2.3.6.8. MALDI-TOF/TOF

From the SDS-PAGE Profile, the approximate MW of the lower band was calculated as 32 kDa and the calculated MW of the intact protease, *i.e.*, from the amino acid sequence deduced by the MALDI-TOF/TOF technique was 32.5 kDa. Thus the profile on the SDS-PAGE and the calculated MW of the aminoacid sequence are more or less the same, *i.e.*, 32.5 kDa. The **Table 11** summarises the first 10 BLAST hits of protein sequence showing matched peptides (**Figure 22**).

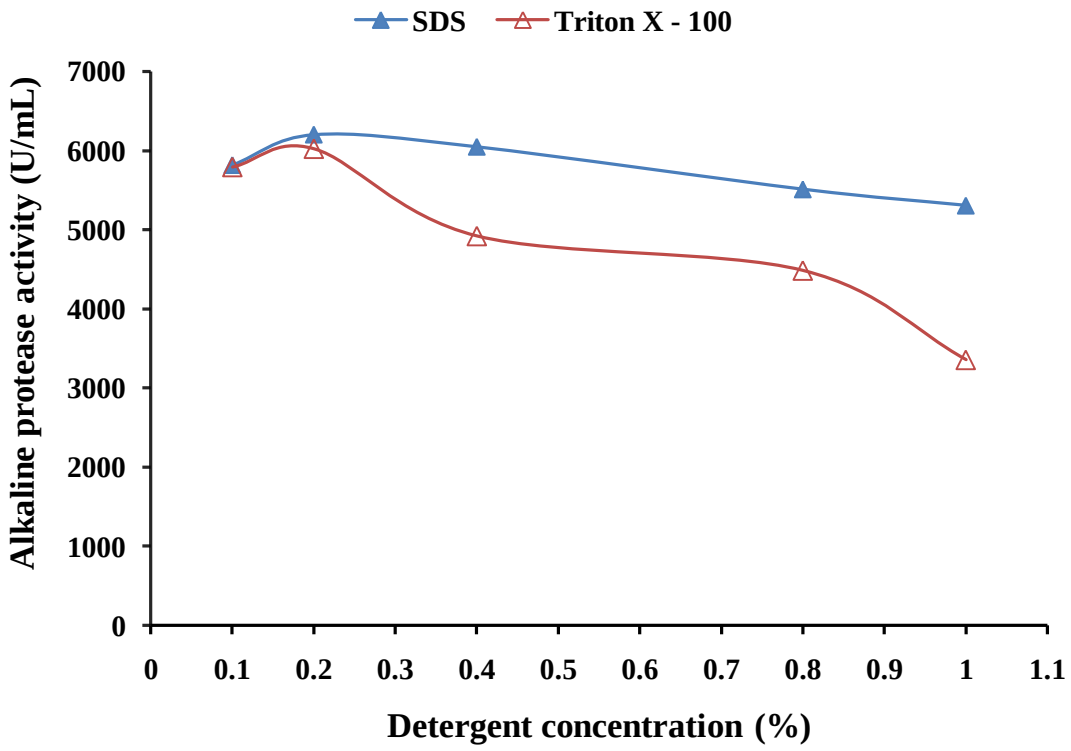


Figure 18. Effect of surfactants on protease stability. The protease was incubated with different concentrations of surfactants like SDS (0.1, 0.4, 0.8, and 1%), Triton X-100 (0.1, 0.4, 0.8, and 1%) for 30 min.

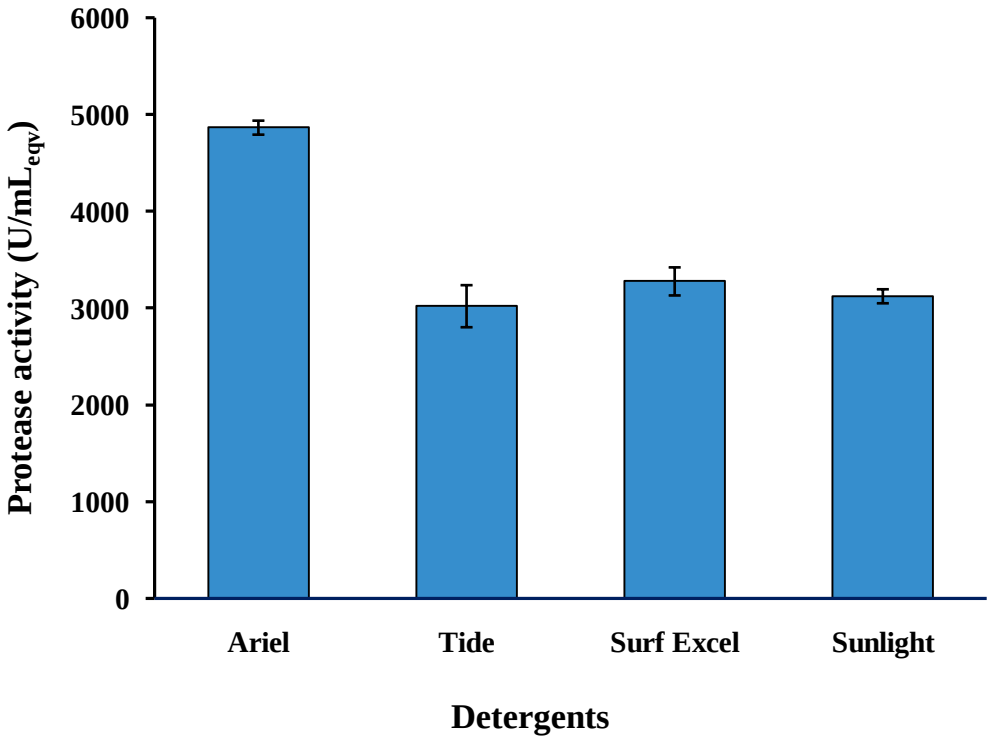


Figure 19. Effect of commercial detergents on alkaline protease stability.

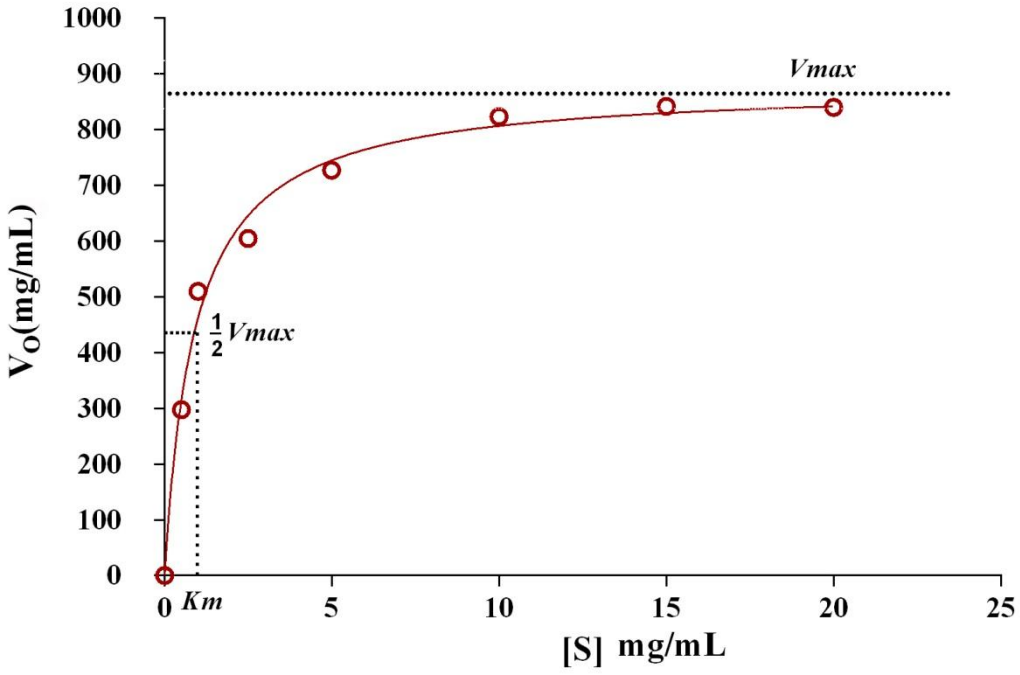


Figure 20. Nonlinear fit of Michaelis-Menten data. (Effect of substrate concentration on alkaline protease activity)

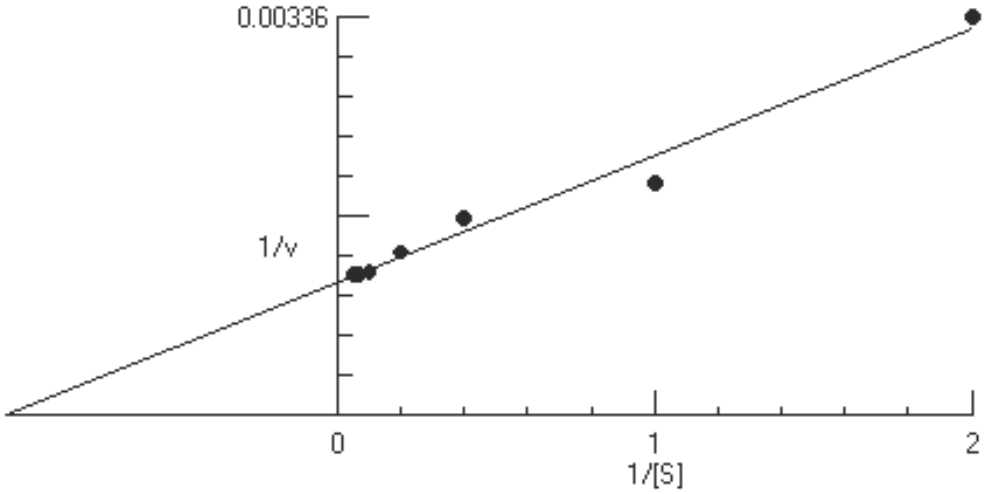


Figure 21. Lineweaver-Burk plot

MFDYNNLSPN	EFEILCSDIL	SRELGVELRH	FSPGRDGGVD	LTESPTNKDI
VVQVKHYTKS	SFSSLKASLE	KELIKLQQMN	PRPRQYYVCT	SKDITVENVR
DIYNIFKDYM	SSDKNIFTKN	EIDAILSRDI	NRDILRKNFK	LWLVADQILT
QLINRDVFID	GEVLLSNLEV	DFKYFVQTNL	FDQCIDILKR	SRSILICGDP
GVGKSLTSKM	LAFYYVKKGY	QIRYTTNGII	SDLKKSIQDN	RDLKEVILLD
DCLGQYYLKL	KDWQDQELV	LMNYIAISEN	KVLILNSRVT	VLNEAQNNSG
VLRRYLEDEN	VKIKTINMND	ISEKEKAYIF		

Figure 22. MALDI-TOF/TOF analysis: Sequences showing matched peptides (32.5 kDa).

Table 11. First 10 BLAST hit of Protein sequence showing matched peptides

Sl. No.	Description	Max score	Query cover	E value	Accession
1	RecName: Full=Uncharacterised protein y4cD	82.0	60%	6e-16	P55386.1
2	RecName: Full=Lon protease homolog 2, peroxisomal	43.9	66%	0.001	Q4WVD9.1
3	RecName: Full=Lon protease homolog 2, peroxisomal	42.0	66%	0.006	A2RAF6.1
4	RecName: Full=Transitional endoplasmic reticulum ATPase homolog 1; AltName: Full=Cell division cycle-related protein 48.1; AltName: Full=p97/CDC48 homolog 1	39.3	35%	0.038	P54811.1
5	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd >sp|Q2FJD8.1|MFD_STAA3 RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q2G0R8.1
6	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q5HIH2.1
7	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q6GJG8.1
8	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd >sp|Q99WA0.1|MFD_STAAM RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q7A7B2.1
9	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd >sp|Q8NXZ6.1|MFD_STAAW RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q6GBY5.1
10	RecName: Full=Transcription-repair-coupling factor; Short=TRCF; AltName: Full=ATP-dependent helicase mfd	38.1	17%	0.11	Q2YVY2.1

4.2.4. Discussion

Characterisation of enzyme is an important step toward developing a better understanding on the functioning of the enzyme (Yadav *et al*., 2010). Alkaline protease in the culture supernatant could be purified by conventional procedures involving fractionation by ammonium sulphate, molecular weight cut-off membrane filter and molecular sieving on sephadex G-100. Precipitation by ammonium sulphate is the most commonly used method for the purification of protein from the crude extract (Bell *et al.*, 1983). Specific activity of the protease increased from 138 U/mg (crude protease) to 1766 U/mg, after final step of purification (sephadex G-100) with 12.79 folds purification and 0.3% overall yield. These results indicate the effectiveness of purification method. Various percentage yields and folds were reported for proteases from: *Bacillus* spp., *viz*., *Bacillus* sp. K25 with 40% yield and 10.08 folds purification (Mathew 1999), *Bacillus* sp. PS719 with 39% yield and 18.5 folds purification (Hutadilok-Towatana *et al*., 1999), *B. subtilis* with 7.5% yield and 21 folds purification (Adinarayana *et al*., 2003), and *Bacillus* strain HS08 with 5.1% yield and 4.25 folds purification (Guangrong *et al*., 2006).

Sephadex G-100 gel filtration gives rise to comparatively pure fractions of the enzyme with a significant increase in its specific activity. Even though, SDS-PAGE profile of purified protease showed two bands; only the lower band (32 kDa) was used for the determination of the amino acid sequence by MALDI-TOF/MS analysis (**Figure 22**). The calculated MW from the amino acid sequence data based on MALDI-TOF/TOF analysis and that of the profile on the SDS-PAGE were more or less the same *i.e*., 32.5 kDa. A varieties of MWs for proteases from other *Bacillus* species were reported; *i.e*., 30.9 kDa protease from a thermophilic *Bacillus* strain HS08 (Guangrong *et al*., 2006), 27.0 kDa from *B. megaterium* (Yossan *et al*., 2006), 75.0 kDa from

Bacillus sp. S17110 (Seong and Choi, 2007), 34.0 kDa from *B. thuringiensis* (Kunitate *et al*., 1989), 38.0 kDa from *B. cereus* KCTC 3674 (Kim *et al*., 2001), 15.0 kDa from *B. subtilis* PE-11 (Adinarayana *et al*., 2003), 34.0 kDa from *B. cereus* BG1 (Ghorbel-Frikha *et al*., 2005), 66.2 kDa, 31.0 kDa and 20.1 kDa from *B. licheniformis* strains BLP1, BLP2 and BLP3, respectively (Cheng *et al*., 2006).

Most of the commercially available proteases are also active in the pH range of 8 and 12 (Gupta *et al*., 2002a). The results obtained in this study indicated that the protease from *Btk* is fit for use in detergents and was commercially significant, because the pH optimum in the present study was pH 9, similar results were obtained with protease from *B. stearothermophilus* (Dhandapani and Vijayaragavan, 1994), *Bacillus* sp. MPTK 712 (Kumar *et al*., 2012), *Bacillus* sp. (Agrawal *et al*., 2012), and *Bt* (Kunitate *et al*., 1989). Alkaline proteases of the genus *Bacillus* showed an optimum activity and a good stability at high alkaline pH values (Margesin *et al*., 1992). The optimum pH range of *Bacillus* alkaline proteases lies generally between pH 9 and 11, with a few exceptions of higher pH optima like 11.5 (Fujiwara and Yamamoto, 1987), 11 and 12 (Kumar and Takagi, 1999), 12 and 13 (Takami *et al*., 1989; Fujiwara *et al*., 1991; Ferrero *et al*., 1996). In general, all currently used detergent-compatible proteases are alkaline in nature optimally acting at high pH; therefore they fit to the pH of laundry detergents; which is generally in the range of 8 to 12.

The optimum temperatures of alkaline proteases range from 50 to 70 ^{o}C (Jaouadi *et al*., 2008; Zhang *et al*., 2010). *Bt* exhibited optimum activity at broad temperature range with maximum (70 ^{o}C) at alkaline pH (Kunitate *et al*., 1989; Rao *et al*., 2009). In addition, their thermal stability is the most important parameter regarding their utility in different sectors like detergent industry.

In addition, protease required divalent cations like Ca^{2+} and Mn^{2+} or combination of these cations for maximum activity (Kumar and Takagi, 1999; Beg and Gupta 2003). Most of the previous studies showed that the effects of sodium, calcium and manganese ions in increasing the activity, which was similar to the present results (Adinarayana *et al*., 2003; Beg and Gupta, 2003; Nascimento and Martins, 2004). In addition, these cations would enhance the stability of protease from *Bacillus* spp. (Durham *et al*., 1987). These metal ions may protect the enzyme from thermal denaturation and maintain its active conformation at high temperature by enabling proper folding.

The effect of inhibitors on protease activity was examined after the protease was pre-incubated with the inhibitor for 1 h at 37 ^{o}C. The presence of the chelating agent EDTA and *β*-mercaptoethanol in the reaction mixture decreased the protease activity, which may be due to the change in the protein conformation induced by these agents. Some proteases are inhibited by metal chelating agents like EDTA, indicating their metal ion dependency for activity (Steele *et al*., 1992).

Stability patterns of protease in the presence of surfactants and commercial detergents also disclose its promising commercial utility in detergent formulations. Detergent-stable proteases have been studied by several groups with varying levels of activity in the presence of different detergents (Joo and Chang, 2005; Kuddus and Ramteke, 2009). Most of the manufacturers recommend the use of detergents in the range 0.1 to 0.2% (w/v) for washing purposes. Detergent stability of an alkaline protease is an important property for its industrial use, as they are currently supplemented in detergent formulations for better washing efficiency.

The protease from *Btk* was characterised further for its *Km* and *Vmax* using casein as a substrate, which were 0.9 mg/mL and 879.3 U/mg, respectively. The *Km* value represents the dissociation constant (affinity for substrate) of

the enzyme-substrate (ES) complex. Low values of *Km* indicate that the ES complex is held together tightly and dissociates rarely before the substrate is converted to product, and *Vmax* indicates the speed of its catalysis. Using casein as a substrate, Kaur *et al.* (1998) reported a *Km* of 3.7 mg/mL for *B. polymyxa* protease, while Thangam and Rajkumar (2002) reported a *Km* and *Vmax* of 1.66 mg/mL and 526 U/mg/min, respectively for alkaline the protease from *Alcaligenes faecalis.* A *Km* values of 0.4 towards casein (mg/mL) was reported for the alkaline proteases from *B. alcalophilus* var. *halodurans* (Rao *et al*., 1998). The *Km* and *Vmax* were determined to be 0.5 mg/mL and 230 U/mg/min, respectively for protease from *B. sphaericus* strain, in which the reaction condition at pH 10 and 55 ^{o}C (Aboul-Soud *et al*., 2011). The *Km* and *Vmax* of protease produced by the haloalkaliphilic *Bacillus* sp. were found to be 2 mg/mL and 289.8 μg/min, respectively (Guptha *et al*., 2005). In another haloalkaliphilic *Bacillus* sp. AH-6, the respective *Km* and *Vmax* were 2.5 mg/mL and 625 U/min (Dodia *et al*., 2008). The *Km* (0.7614 mg/mL) and *Vmax* (2582 U/min) of *B. subtilis* DKMNR was determined at 70 ^{o}C (Kezia *et al.*, 2011b).

Briefly, the protease characterised from *Btk* (both fractions) is best active at alkaline pH and higher temperatures, which makes it suitable to be used in detergents and treatment of effluent rich in protease.

Chapter V

Endospore production patterns of Btk

Chapter 5

ENDOSPORE PRODUCTION PATTERNS OF *Btk*

[Jisha VN and Sailas Benjamin (2013). Concomitant production of delta-endotoxin and endospore from *Bacillus* thuringiensis subsp. *kurstaki*. ***Journal of Basic Microbiology*** (Under review)]

5.1. Aim

The purpose of this study was to evaluate the concomitant production patterns of endospores and *δ*-endotoxin from *Btk* in altered Luria-Bertani (LB) medium, using LB basal medium as control. The LB medium was supplemented with various concentrations (w/v) of naturally available agricultural products (flours) such as Bengal gram, jack seed and soybean.

5.2. Materials and methods

Materials and methods pertaining to this chapter are described under **Chapter 3**, under section: **3.13.**

5.3. Results

This study makes a comparison of the concomitant production of endospore and *δ*-endotoxin by *Btk* in LB (control) against 30 % (w/v) soybean flour supplemented LB, 30% (w/v) Bengal gram flour supplemented LB and 10% (w/v) jack seed flour supplemented LB. These specific media combinations were chosen based on the best performance of the flour-supplemented media in terms of the production of endospore and *δ*-endotoxin. Microscope-aided spore counting and staining techniques were used to demonstrate the results.

As shown in **Figure 23**, concomitant production of endospore and *δ*-endotoxin is presented in 4 vertical panels with 3 rows indicating harvest time (24, 48 and 72 h). Many trials were made for monitoring the growth patterns of *Btk* in different media combinations comprising different percentages (1, 10, 20, 30, 40, 50, 60, 80 and 100%) of flours in LB. For simplicity of data presentation, only the best results out of various percentages of soybean flour, jack seed flour and Bengal gram flour supplements are shown in the **Figure 23**. First panel represents LB (control), second panel represents 30% soybean flour supplemented LB, third panel represents 10% jack seed flour supplemented LB, and the fourth panel represents Bengal gram flour supplemented LB media. Horizontal panels represent harvest time. Three staining techniques were used, *viz*., malachite green-safranin, acridine orange and coomassie brilliant blue, the former two were specific for visualising endospores and the latter was for *δ*-endotoxin.

For cross-comparison, the length and diameter of endospores (only LB control and 30% soybean flour supplemented media were also measured (**Table 12**).

5.3.1. Size of endospores

The size of endospores harvested at 24 h time intervals from the soybean flour-supplemented LB medium was compared with that of LB control. The control sample showed that the endospores achieved the maximum length (1.10 ± 0.13 µm) and diameter (0.63 ± 0.07 µm) at 72 h. However, in soybean supplemented medium, the maximum length (2.10 ± 0.16 µm) and diameter (1.63 ± 0.16 µm) were achieved at 48 h and 72 h. It further showed that the length and diameter of endospores formed in soybean flour supplemented medium were almost double the size of endospores produced in the control. Moreover, the endopsores achieved maximum size in about 48 h in soybean supplemented medium, as against 72 h in LB control. It clearly indicates the

advantage of solid-state fermentation, this was also true in the case of the number of endospores and vegetative cells (**Table 13**), *i.e*., as seen in soybean flour supplemented media.

Table 12. Time-dependent variations in the average length and diameter of endospores (µm) produced in LB (control) and 30% soybean flour supplemented LB.

Sample	**Harvest Time**					
	24 h		**48 h**		**72 h**	
	Diameter	Length	Diameter	Length	Diameter	Length
LB	0.25 ± 0.05	0.75 ± 0.15	0.55 ± 0.09	0.75 ± 0.11	0.63 ± 0.07	1.10 ± 0.13
30% soybean flour + LB	0.87 ± 0.07	1.25 ± 0.13	1.34 ± 0.25	2.10 ± 0.16	1.63 ± 0.16	2.10 ± 0.28

5.3.2. Endospore and δ-endotoxin production pattern at 24 h:

At 24 h, almost all cells in soybean supplemented medium contained endospores. A few cells started rupturing to release the endospores. Size of the spore was much bigger in samples with higher concentration of soybean flour (20, 30, 40, 50, 60, 80 and 100%), and number of spores was higher in 30% soybean flour supplemented; and the number of parasporal bodies (crystals) was equal to that of the endospores, because both are produced concomitantly (**Figure 23**). The results obtained from Bengal gram (30%) supplemented LB medium was comparable to those data obtained from soybean flour supplemented medium, but number of endospore production was less than that of the pattern in the latter. Compare to the soybean flour and Bengal gram flour supplemented media, jack seed flour supplemented medium showed different pattern of results; *i.e*., endospore was not that much bigger in size and number of ruptured endospores are less in number.

However, in control, most of the cells were intact with endospores and size of endospore was smaller than that in flours supplemented media (**Figure 23**).

5.3.3. Endospore and δ-endotoxin production pattern at 48 h:

The 48 h samples contained higher number of endospores in all the samples, except control. At this stage, most of the sporangia were lysed and the endospores were exposed, highest numbers of endospores were noticed in soybean flour supplemented medium, parasporal bodies or crystals were clearly observed in this stage as darkly stained bodies with safranin, (**Figure 23**) dark blue stained with CBB staining (**Figure 23**). The number and size of endospores and crystals obtained from Bengal gram flour supplemented medium were similar to that of soybean flour supplemented medium, and in Jack seed flour supplemented medium, the size and number of endospores and crystals were smaller and lesser, respectively than the other two supplements. In soybean supplemented medium, the maximum size and exposed endospore and crystals were seen at 48 h and 72 h, respectively, which were much higher in size and number than those obtained in the LB medium.

5.3.4. Endospore and δ-endotoxin production pattern at 72 h:

At 72 h, releases of spores and crystals were more or less complete in all the natural flour supplemented media. In fact, the release pattern of endospores and crystals in LB medium at 72 h was similar to those obtained at 48 h cultivation in soybean flour supplemented medium as clearly seen in both malachite green-safranin and coomassie brilliant blue stained samples (**Figure 23**). At this stage release of endospores was almost complete in all the flour supplemented media, while in LB medium many intact sporangia could be seen.

5.4. Discussion

Bacillus spp. are known to produce endospores (Jisha *et al*., 2013a). Sporulation process is induced by starvation or adverse environmental conditions indicating the end of their life cycle, it is controlled by many factors - such as unfavorable environmental conditions like desiccation, heat, and ultraviolet radiations and unavailability of carbon and nitrogen sources (Tortora *et al*., 2004). These spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals (Setlow, 2000). Many important by-products (solvents, antibiotics, enzymes, insecticides, *etc*.) are produced by spore-formering bacteria. Typically, the maximum synthesis rate of these products occurs at the onset and during the sporulation process (Liu and Bajpai, 1995).

Malachite-green, a low cost stain is commonly used for staining spores, which specifically stains endospores greenish-blue, thus fast visual screening for endospores and crystals. In the present study, malachite-green was used as the primary stain for staining the endospores; the vegetative cells were pinkish upon counter staining with safranin and crystals also darkly stained with safranin. Hamouda *et al*. (2002) stained endospores of *Bt* at different stages of germination with malachite green and safranin, and found that with malachite-green, spores were stained greenish-blue and vegetative cells took pink colouration by safranin. Chilcott *et al*. (1998) studied endospore production pattern of *Bt* serotypes and found that cells and spores of all *Bt* serotypes appeared phase light under phase-contrast microscopy, while the crystals appeared phase dark.

Schichnes *et al*. (2006) developed a quick and simple fluorescent staining (acridine orange) technique for endospores produced by *B. subtilis*, and this stain was used to differentiate viable and non-viable spores (Sharma and Prasad, 1992). Fadel and Sabour (2002) cultivated four strains of *Bt* on locally

available sugar cane molasses for monitoring the production profile of bioinsecticide.

Bacteria produce endospores normally at the senescence of their growth phase (Prescott *et al*., 1996; Jisha *et al*., 2013a), mainly due to the unavailability of carbon and nitrogen sources (Tortora *et al*., 2004). Sporulation time and size of endospore and the rupturing of sporangial wall are important aspects to be considered, because δ- endotoxin production is concomitant with sporulation. In our study development of sporangia was seen even at 12 h (especially in flour supplemented media), and rupturing of sporangia started in the natural flour supplemented samples from 24 h cultivation onwards. In natural flour supplemented media, most of the spores were released at 48 h cultivation, but in LB control, similar trend was observed after 72 h cultivation. General trend was that *Bt* strains take 3-5 days for the maximum production of toxins under the normal culture conditions by SmF. However, Chestukhina *et al.* (1980) showed that *Bt* produced copious amounts of spores and crystals at 48 h. The mature spores were eventually liberated by lysis of the mother cell (sporangia). The entire process of lysis takes place over a period of 6-7 h and requires the temporal regulation of more than 50 unique genes (Chestukhina *et al.*, 1980).

Since sporulation and germination in Bacilli are dependent on the nutritional status of the organism (Rajalakshmi and Shethna, 1980), a study on the nutritional requirement of *Bt* is important for delineating the control mechanisms which regulates the formations of spore and parasporal crystal. Certain amino acids support growth, sporulation and crystal formations in *Bt* (Rajalakshmi and Shethna, 1980). In our study, soybean and Bengal gram flour supplemented medium showed highest number of spores with much enhanced size and shape. In this enriched media, sporulation starts at 12 h onwards, similar result was noticed in the industrial medium TSB

(Tryptocase Soy Broth) and in the sludge, the sludge achieving the highest number of spores was ISWWTP (Industrial sludge wastewater treatment plant) with 2.16×10^8 CFU/mL (Teixeira, 2012). Variables like physical and chemical constitution, total solids concentration, available oxygen, initial volume inoculum, or temperature are common to limit bacterial growth. In this batch fermentation, ideal parameters for *Bt* growth were used (Lachhab, 2001; Yezza *et al*., 2005; Chang, 2007; Zhuang *et al*., 2011).

In the present study we proposed solid-state fermentation for the maximum yield of endospore and endotoxin without agitation; we suppose this is the reason for the decrease in production time. The highest yield obtained should be due to the anaerobic condition and water stresses developed in the solid medium during fermentation. Similar results were previously reported with other strains of *Bacillus* and other subspecies of *Bt* (da Silva *et al*., 2011). The toxicity was about four times higher under non-aerated conditions (da Silva *et al*., 2011). Sarrafzadeh and Navarro (2006) observed the highest concentration of spores being 100% mature under anaerobic conditions for *Bt* strain H14. Das and Danker (2008) showed that anoxic and water stresses favoured production of spores and crystals at an early stage of growth.

5.4. Conclusions

Water stress and non-aeration imparted through solid-state fermentation were found as crucial factors for the enhancement of endospore production and thus, δ-endotoxin concomitantly with reduced time. Thus, maximum yield of endospores during solid-state fermentation was observed in 24 h less gestation period, this is advantageous as it directly reduces production cost. Normal gestation period or harvest time for *Btk* is 72 h, which could be reduced to 48 h, if solid-state fermentation is employed as we demonstrated in this study.

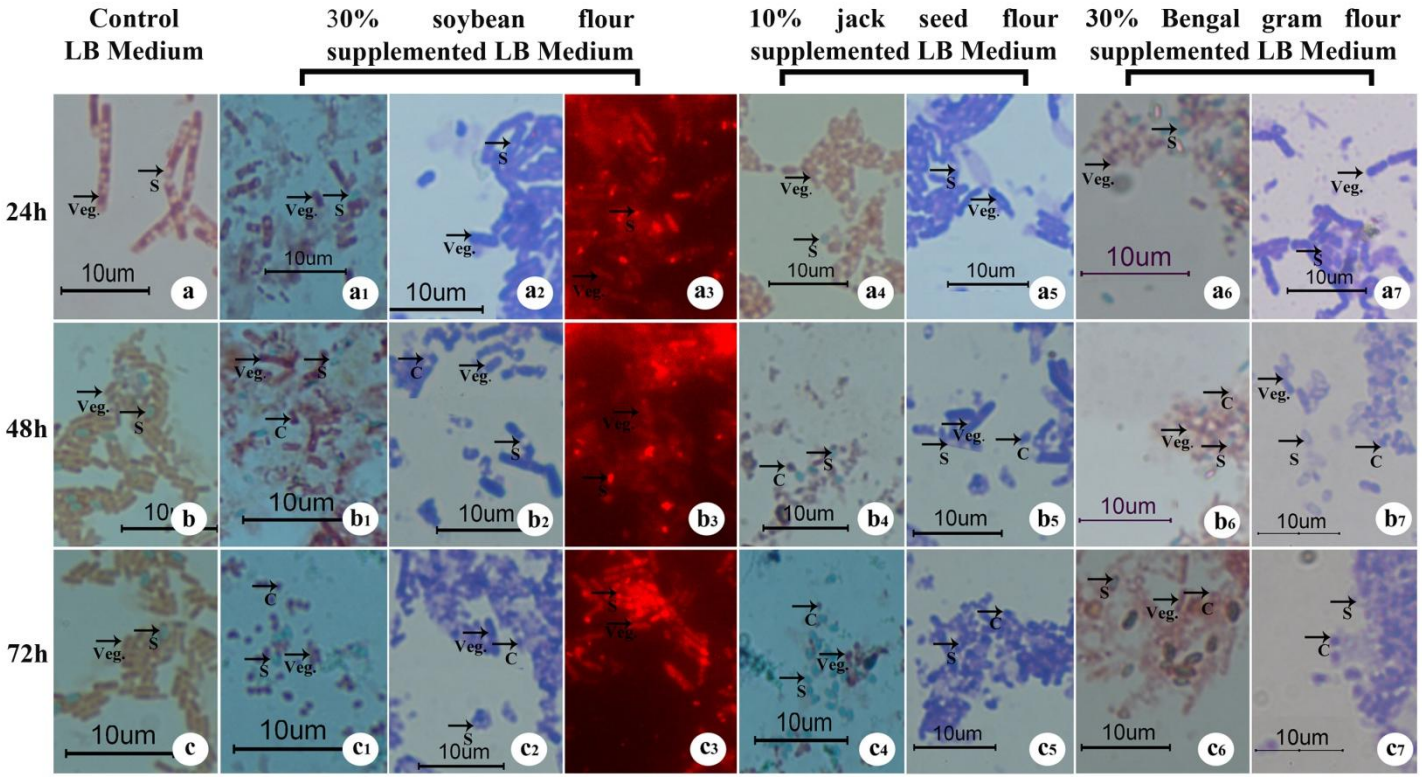


Figure 23. Patters comparing frequency of vegetative cells, endospores and *δ*-endotoxin. Time- dependent (24, 48 and 72 h) vegetative cells, endospores and *δ*-endotoxin production patterns of *Btk* in LB control against 30% soybean flour supplemented LB or 10% jack seed flour supplemented LB or 30% Bengal gram flour supplemented LB. The patterns of *Btk* growth and concomitant production of endospore and *δ*-endotoxin are presented in 4 vertical panels (marked on the top of figures): first panel shows LB control, second panel shows 30% soybean flour supplemented LB, third panel shows 10% jack seed flour supplemented LB and forth panel shows 30% Bengal gram flour supplemented LB. The staining pattern also marked on the top of each column, below the panels as malachite green-saffranin (MG-S), coomassie-brilliant blue (CBB) and fluorescent acridine orange (AO). MG-S and AO specifically stain endospores and CBB is specific for *δ*-endotoxin (the crystal protein). Production patterns of vegetative cell (Veg), spores (S) and crystals (C) are evident from the figures.

Chapter VI

Jisha V. N. “Extracellular alkaline protease production and efficacy studies of endotoxin from Bacillus thuringiensis subsp. kurstak i” . Department of Botany, University of Calicut. 2013 .

Chapter 6

BIPHASIC FERMENTATION FOR THE PRODUCTION OF *δ*-ENDOTOXIN

[Jisha VN, Smitha RB and Sailas Benjamin (2013). Biphasic solid-state fermentation is an efficient strategy for the over production of *δ*-endotoxin by *Bacillus thuringiensis*. ***BMC Biotechnology*** (in Press)]

6.1. Aim

This study evaluates the purification of *δ*-endotoxin from SSF matter by biphasic SSF strategy in which the first phase is with free water and a subsequent phase without free water in the medium, especially for the cultivation of *Bt* strains. This unique study, address (a) the biphasic fermentation strategy for the enhanced production of *δ*-endotoxin from *Btk*; (b) the demonstration of purified *δ*-endotoxin from the solid fermented matter, and (c) quantification of the purified *δ*-endotoxin.

6.2. Materials and Methods

Materials and methods pertaining to this chapter are described under **Chapter 3,** under sections: **3.13.7, 3.13.8** and **3.14**.

6.3. Results

As described under 'materials and methods', for the clarity of presentation, three designations were given for the media used in this study (**Table 13**). The first (NB) and second (LB) media were controls, which contained only the ingredients as in commercial formula; the third one was designated as M1, which contained LB fortified with 30% (w/v) soybean flour (SF); while the fourth one was M2, which was nothing but M1 with no free water, embedded with 12 h old *Btk*. Various combinations of LB with soybean flour (1, 5, 10,

20, 30, 40, 50, 60, 70, 80, 90 and 100% (w/v) were investigated to odd out the best among them for further elaborate studies. From this, it was found that 30% (w/v) soybean flour in LB was optimum for SSF. Regarding biphasic SSF, after 12 h initial incubation of M1 (first phase), the supernatant containing extracellular enzymes was harvested from briefly fermented semi-solid mass by centrifugation. The pellet (with embedded *Btk*) so obtained (M2) was incubated further in the second phase under static condition for enhancing *δ*-endotoxin yield.

The *δ*-endotoxin production profiles in LB control, M1 and M2 were monitored up to 72 h at 12 h intervals. However, only data from LB-control and M2 are presented in **Figure 24-28**, and in **Figure 29**, data from M1 also included.

Initially we faced the problem of inconsistency in *δ*-endotoxin yields. This problem was solved when the seed culture was prepared afresh every time from the 1 month old frozen stock in LB.

6.3.1. Malachite Green- Safranin (MG-S) staining

Upon MG-S staining, the spores were seen greenish-blue due to the absorption of malachite green stain, the primary stain and crystals were pink in color by absorbing safranin, the contrasting counter stain (**Figure 24a-d**). **Figure 24** depicts the images of raw sample, which was directly subjected to staining without any pretreatment. Crystals, spores and comparatively much smaller vegetative cells are seen scattered in LB-control harvested at 72h incubation (**Figure 24a**); while much bigger *Btk* cells are seen mixed with crystals and spores embedded in the Soybean flour solid matter with increasing proportions of crystals (**Figure 24-d**).

6.3.2. Viable cell and spore counts

A general observation showed that maximum number of viable cells was found in M2 at 24 h, but almost 4 times fewer than in M1 (its maximum at 36h) (**Table 13**). On the contrary, the maximum number in M2 and both controls (LB and NB) were at 36 h; at this time, the viable cell count in M1 was highest (about three times more than LB) and NB (about 2 times more that LB). Another striking observation was that the viable cell count was drastically declined in M2 with almost no viable cells at 72 h, while in M1 and controls viable cell count was gradually declined from 36 h onwards, their higher proportion (about one-third in M1 and LB, and over half in nutrient broth) was found even at 72 h. From this, it is evident that size of endospores and *δ*-endotoxin (also refer **Figures 24, 26** and **27**) is the crucial factor which decides the quantity of *Bt δ*-endotoxin produced. This data further indicated that though presence of rich nutrients and free water in the medium favored vegetative life of *Btk*, water-restricted solid-state environment as in M2 much favored sporulation and concomitant production of *δ*-endotoxin at an early stage, thereby reducing gestation time.

6.3.3. Purification of δ-endotoxin crystals from solid substrate

Since we faced initial difficulty for the purification of *δ*-endotoxin from solid fermented matter, we adopted a modified phase separation methods proposed by Pendleton and Morrison (1966), and Neema (2007). From Pendleton and Morrison (1966), we adopted the phase separation strategy employed at the end of our protocol. From Neema (2007), we adopted the strategies of brief alkaline treatment (to avoid solubility of crystal proteins at high pH) and usage of TE buffer. In steps 2-5 of our protocol, the supernatant was completely discarded to avoid any soluble proteins.

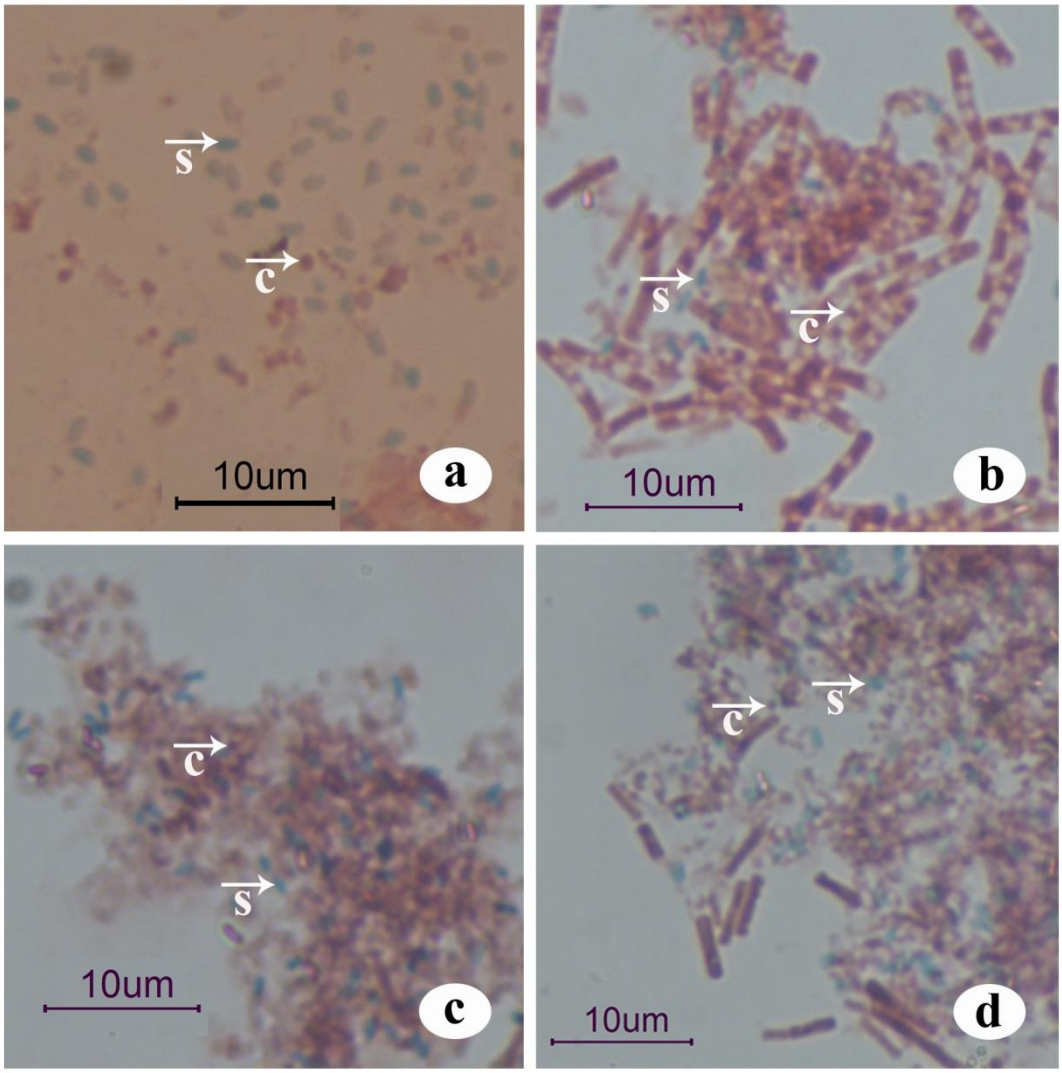


Figure 24. Images (by Image Analyser) of crystals (c) and spores (s) upon staining with malachite green-safranin: **a.** 72 h LB control after SmF; and **b-d** are unpurified fermented matter after SSF showing a mixture crystals, spores and vegetative cells embedded M2, 24h (including initial 12h fermentation), 48h and 72h, respectively. Spores are seen greenish-blue and crystals are pink in colour. Cells and spores are much larger in samples after SSF (b-d).

Period (h)	Nutrient Broth (NB control)			Luria-Bertani (LB Control)			Medium 1 (M1)			Medium 2 (M2)		
	Vegetative cells	Sporangia	Spores	Vegetative cells	Sporangia	Spores	Vegetative cells	Sporangia	Spores	Vegetative cells	Sporangia	Spores
0	$3.214 \pm 0.162 \times 10^5$	77 ± 11	6 ± 2	$3.330 \pm 0.145 \times 10^5$	82 ± 13	7 ± 2	$3.021 \pm 0.172 \times 10^5$	91 ± 16	5 ± 3	$3.213 \pm 0.142 \times 10^5$	88 ±21	6 ± 3
12	$6.432 \pm 0.286 \times 10^7$	$0.021 \pm 0.005 \times 10^3$	86 ± 11	$4.432 \pm 0.286 \times 10^7$	$0.019 \pm 0.004 \times 10^3$	97 ± 9	$3.326 \pm 0.525 \times 10^8$	$0.113 \pm 0.034 \times 10^3$	297 ± 54	$3.167 \pm 0.582 \times 10^8$	$0.118 \pm 0.036 \times 10^3$	286 ± 61
24	$6.635 \pm 0.532 \times 10^9$	$0.602 \pm 0.172 \times 10^3$	316 ± 85	$1.443 \pm 0.436 \times 10^9$	$0.388 \pm 0.011 \times 10^3$	124 ± 14	$2.663 \pm 0.432 \times 10^{10}$	$2.652 \pm 0.331 \times 10^4$	$0.810 \pm 0.473 \times 10^3$	$1.425 \pm 0.653 \times 10^{10}$	$9.251 \pm 0.437 \times 10^6$	$1.210 \pm 0.592 \times 10^3$
36	$1.007 \pm 0.452 \times 10^{10}$	$6.627 \pm 0.342\ 10^8$	$1.103 \pm 70 \times 10^3$	$5.432 \pm 0.542 \times 10^9$	$4.231 \pm 0.019 \times 10^4$	$0.011 \pm 0.006 \times 10^3$	$6.528 \pm 0.643 \times 10^{10}$	$1.437 \pm 0.853 \times 10^7$	$1.023 \pm 0.463 \times 10^4$	$5.003 \pm 0.428 \times 10^9$	$9.288 \pm 0.853 \times 10^9$	$8.255 \pm 0.463 \times 10^7$
48	$9.126 \pm 0.427 \times 10^9$	$1.909 \pm 0.122 \times 10^9$	$2.732 \pm 0.042 \times 10^5$	$5.415 \pm 0.332 \times 10^9$	$2.019 \pm 0.012 \times 10^8$	$6.215 \pm 0.012 \times 10^3$	$5.512 \pm 0.510 \times 10^{10}$	$9.461 \pm 0.624 \times 10^9$	$2.121 \pm 0.564 \times 10^7$	$2.202 \pm 0.348 \times 10^9$	$1.061 \pm 0.624 \times 10^{10}$	$2.121 \pm 0.564 \times 10^9$
60	$3.641 \pm 0.563 \times 10^9$	$7.953 \pm 0.194 \times 10^9$	$6.436 \pm 0.102 \times 10^8$	$5.053 \pm 0.347 \times 10^9$	$1.072 \pm 0.031 \times 10^9$	$7.126 \pm 0.071 \times 10^8$	$3.742 \pm 0.532 \times 10^{10}$	$1.472 \pm 0.162 \times 10^{10}$	$1.321 \pm 0.325 \times 10^9$	$2.202 \pm 0.348 \times 10^7$	$1.472 \pm 0.525 \times 10^{10}$	$0.202 \pm 0.632 \times 10^{10}$
72	$7.635 \pm 0.532 \times 10^9$	$3.105 \pm 0.123 \times 10^9$	$1.523 \pm 0.098 \times 10^9$	$2.074 \pm 0.343 \times 10^9$	$2.239 \pm 0.064 \times 10^9$	$0.923 \pm 0.100 \times 10^9$	$2.227 \pm 0.252 \times 10^{10}$	$2.355 \pm 0.423 \times 10^{10}$	$8.014 \pm 0.562 \times 10^9$	$1.441 \pm 0.592 \times 10^4$	$1.129 \pm 0.373 \times 10^9$	$1.453 \pm 0.432 \times 10^{10}$

Table 13. A profile showing variations in the number of vegetative cells (viable cells), intact endospores (sporangia) and free endospores of *Btk* during 72 h fermentation (37 ^{o}C) in various media. NB and LB are positive controls. Medium 1 (M1) is the LB liquid + 30% soybean flour (throughout in semi-solid state) and medium 2 (M2) is the LB liquid + 30% soybean flour [after 12 h short fermentation, free liquid (supernatant) in it was removed by centrifugation (1000 × *g*, 10 min) and further incubation of M2 was in solid-state]. Total endospores were shown as sporangia (*i.e.*, *Btk* cells did not burst to release the endospores) and free endospores (*i.e.*, *Btk* cells bursted and endospores released along with *δ*-endotoxin). The figures given below are the respective total number per ml liquid broth or per ml equivalent (*i.e.*, total number for M1, it is equivalent to 1ml LB + 30% Soybean flour and for M2 it is 1 ml LB + 30% Soybean flour without free liquid). Experiments were conducted in 100 ml conical flasks containing 25 ml LB or NB; or 25 ml LB + 30 % Soybean flour. Samples from whole flask were withdrawn for each analysis. Values given here are the average of 3 independent experiments ± SE.

Figure 25 shows the separation of aqueous (aq) and organic (o) phases in a separating flask adopted for the purification of *δ*-endotoxin from the debris. Before sonication, the aq phase was unclear, though some heavier particles already settled (**Figure 25a**), while after sonication, a clear upper aq phase was obtained which exclusively contained endospore and *δ*-endotoxin (**Figure 25b**) approximately in the ratio 1:3 (as seen through microscope), respectively. Upon further centrifugation (9440 × *g*), the *δ*-endotoxin crystals were remained in solution leaving the heavier endospores in the settled pellet. By this method, the recovery percentage of *δ*-endotoxin from solid-fermented matter was between 55 to 59% from the medium containing soybean flour, however, about 95% was recovered from LB control by the modified bi-phasic purification method.

6.3.4. CBB staining for visualizing δ-endotoxin

CBB specifically stained the *δ*-endotoxin, which could easily be distinguished from the unstained spores by phase-contrast microscopy (**Figure 26a-e**). **Figure 26a** is the image of the aq phase (as in **Figure 25b**) of 72 h LB-control showing a mixture of deeply stained *δ*-endotoxins and unstained endospores. From the **Figure 25a**, the purity of crystals in other preparations (**Figure 26b-e**) could easily be compared visually. **Figure 26b** shows the images of crystals (obtained after the centrifugation of the mixture as in **Figure 25a**) with negligible number of spores, which shows the efficiency of our purification protocol to over 95%. This aspect is further evident from the **Figures 26c-e**, *i.e.*, the images of pure *δ*-endotoxins from solid-fermented matter (M2). Evidently, **Figures 26b-e** were presented as the visual data for the purity of crystals produced by *Btk* obtained after conventional SmF (**Figure 26b**) and SSF (**Figure 26c-e**); which we described for the first time for SSF. It has to be noted that magnification of crystals produced by SmF (in LB control, **Figure 26b**) was 10 times higher than that of SSF (**Figure 26c-e**). Moreover, though equal volumes of purified crystals proteins were taken for slide preparation, the frequency of crystal count also higher in samples obtained from SSF.

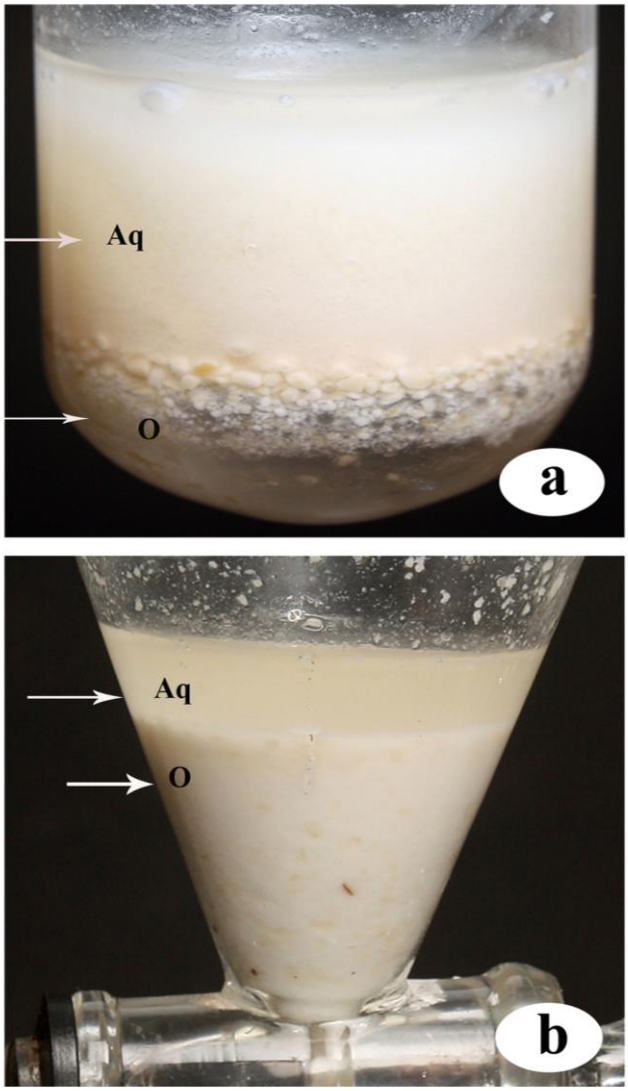


Figure 25. Separation of aqueous (Aq) and organic (O) phases for the purification of δ-endotoxin form the solid-fermented matter (M2): **a**. before sonication showing unclear upper Aq and lower O phases; and **b**. after sonication clear Aq and O phases are evident. This Aq phase was collected and centrifuged (9440 × g for 10 min) to settle down the spores as pellet and δ-endotoxin (crystals) in solution.

6.3.5. Scanning Electron Microscopy (SEM)

Further, we wanted to view the magnified version of the raw and purified crystals (**Figure 26**) produced both by LB-control (SmF) (**Figure 27a** and **c**) and M2 (SSF) (**Figure 27b** and **d**), respectively. **Figure 27a** is the image of LB control showing a mixture of spores, crystals and vegetative cells of LB control (72 h), while **Figure 27b** shows the picture of large and variously shaped crystals embedded in the raw solid matter (M2) at 48 h. **Figures 27c** and **d** are the corresponding images of purified crystals after SmF and SSF.

6.3.6. SDS-PAGE resolution of δ-endotoxin

Our next objective was to resolve the purified crystal proteins (as shown in **Figure 27**) on SDS-PAGE (**Figure 28**), which showed prominent bands with an apparent MW of ~45, 35 and 6 kDa in both control and M2 with a series of weak bands between 45 and 35 kDa in both.

6.3.7. Quantification of purified δ-endotoxin

Finally, we wanted to quantify the purified *δ*-endotoxin crystals from the LB-control, M1 and M2 (**Figure 29**). For easy comparison, the quantity of purified crystal protein was represented in 'per mL LB' or 'per mL LB equivalent'. The maximum yield in M2 was ~36.7 fold more at 48 h (including initial 12 h) than that obtained in the LB-control at 72h (at this point maximum yield obtained in LB-control). Moreover, even after 12h incubation of M2 (total 24h including prior 12h incubation), the yield difference was 20 fold more in the M2. Thus, it is evident that the gestation period is highly decreased (by two-third) even in the initial stage, surprisingly the production in M2 doubled in another 24h with no further significant increase in LB control. For comparison, yield in M1 (without removal of supernatant) was also monitored up to 72h, which showed maximum yield (32 fold) at 72h, as control. However, the yield in M2 at 48h was not significantly changed from 48h, upon further incubation.

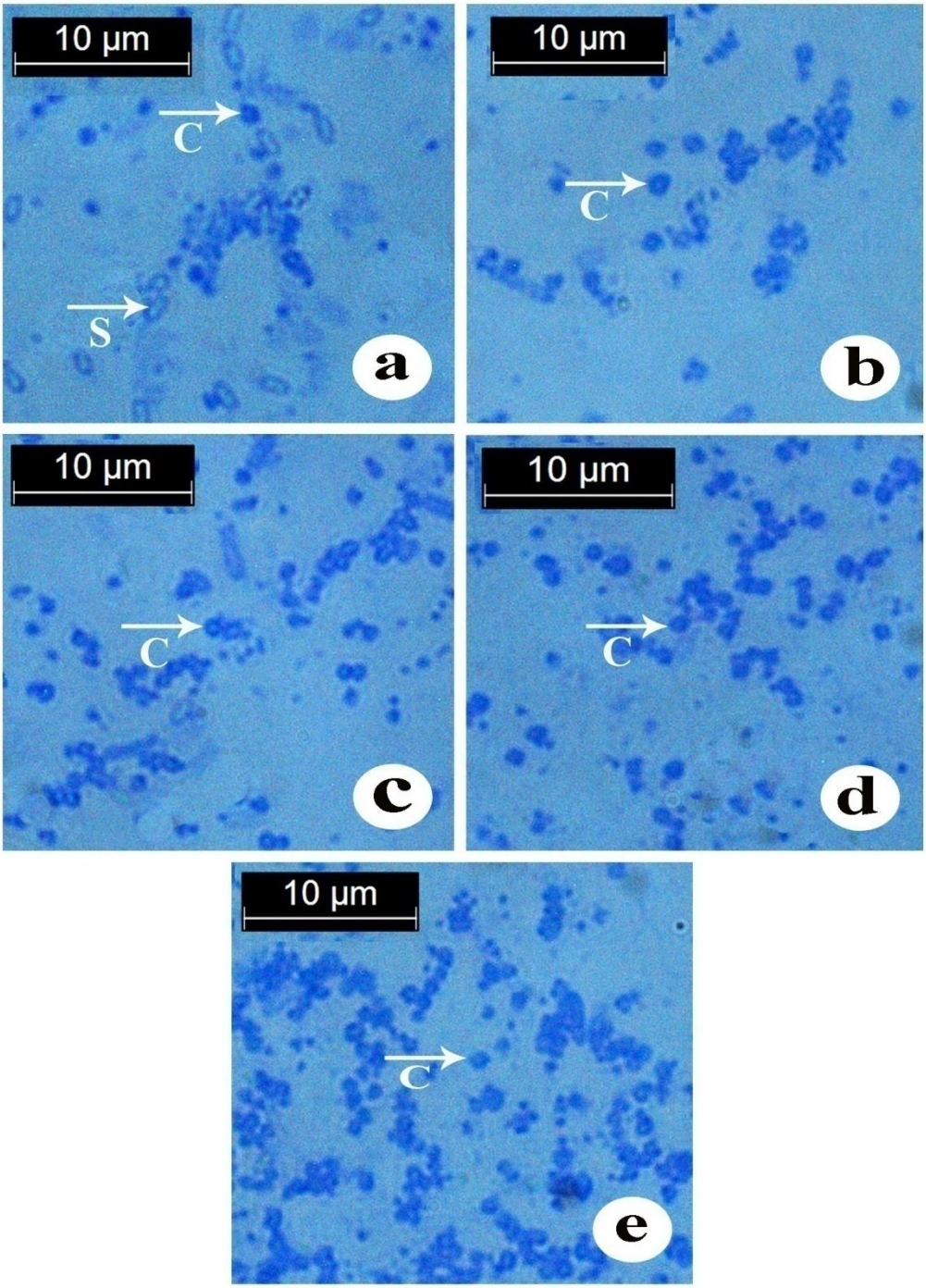


Figure 26. Phase-contrast images (CBB staining): **a**. mixture of crystals and spores of the aqueous phase of LB control (72 h) obtained in biphasic separation, upon CBB staining. Crystals were deeply stained with CBB, while spores did not take stain; **b**. crystals purified from LB control (72 h) as above, crystals (supernatant) were separated from spores (pellet) by the centrifugation (9440 × *g* for 10 min) of aqueous phase obtained in biphasic separation; **c**. crystals purified from M2 (24 h, including initial 12 h) with a few spores, **d**. crystals purified from M2 (48 h); and **e**. crystals purified from M2 (72 h).

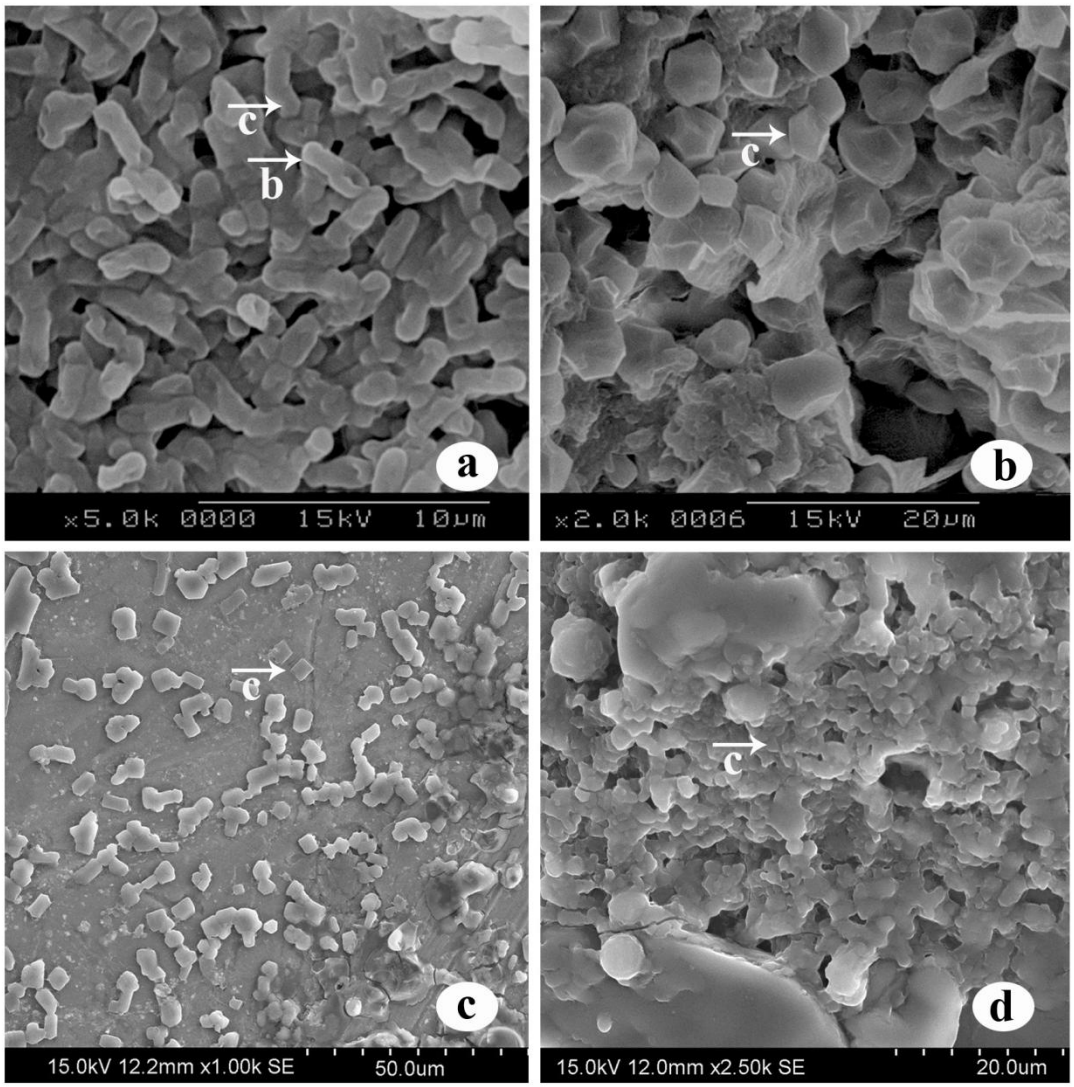


Figure 27. SEM images of crude and purified *δ*-endotoxin: **a**. crude pellet of LB control after SmF (72h) showing a mixture of crystals, spores and vegetative cells; **b**. crude pellet of solid-fermented matter, M2 (48 h) showing big crystals embedded in the solid fermented matter; **c**. purified *δ*-endotoxin crystals from LB control (72 h); and **d**. purified *δ*-endotoxin crystals from M2 (48 h) respectively. The shape variations of crystals are evident in Figure 27c and d. It is evident that the magnification of Figure 27 c (of LB control) was 2.5 times higher than that of SSF (Figure 27b, d), *i.e.*, former is 50 µm and the latter is 20 µm. Briefly, enhancement in yield is directly related to the enhanced size of crystals produced.

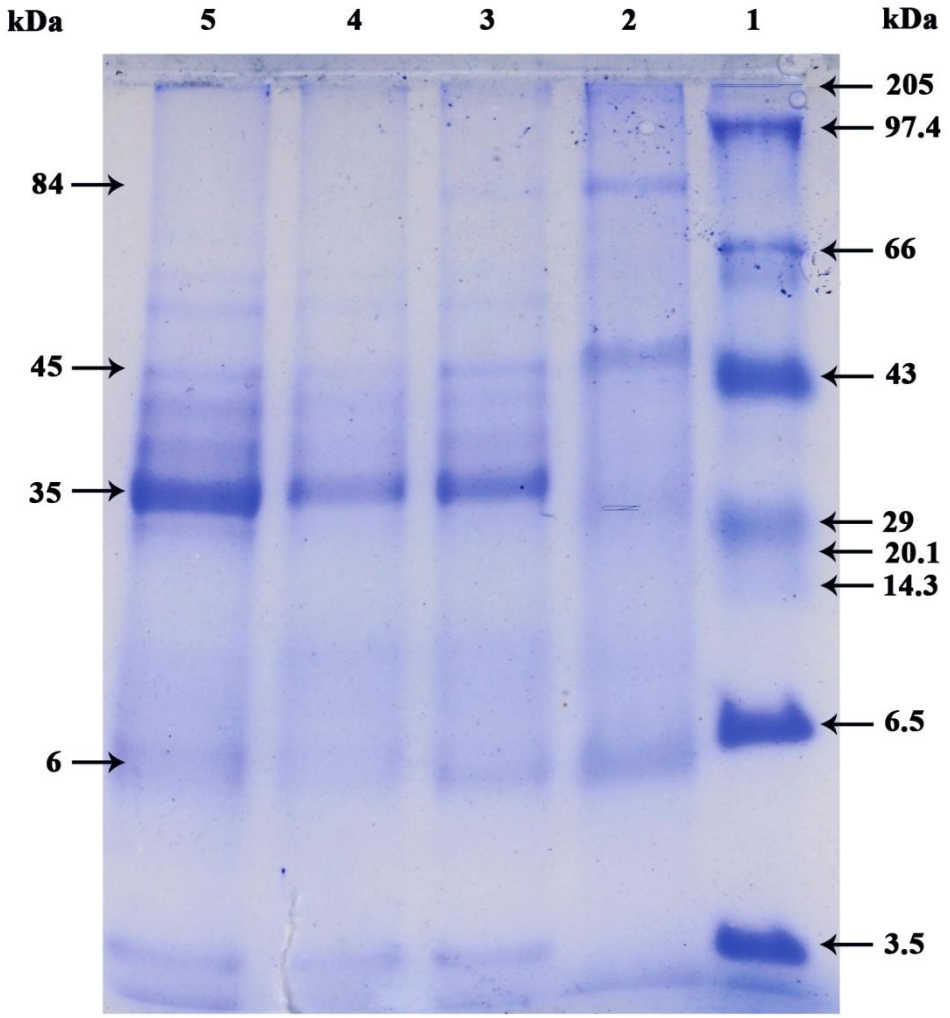


Figure 28. SDS-PAGE profile of purified δ-endotoxin crystal proteins: lane 1, the mlecular marker; lane 2, LB-control after SmF (72 h); lane 3, M2 (24 h, including initial 12 h); lane 4, M2 (48 h); and lane 5, M2 (72 h). Molecular marker; lane 2, LB-control after SmF (72 h); lane 3, M2 (24 h, including initial 12 h); lane 4, M2 (48 h); and lane 5, M2 (72 h).

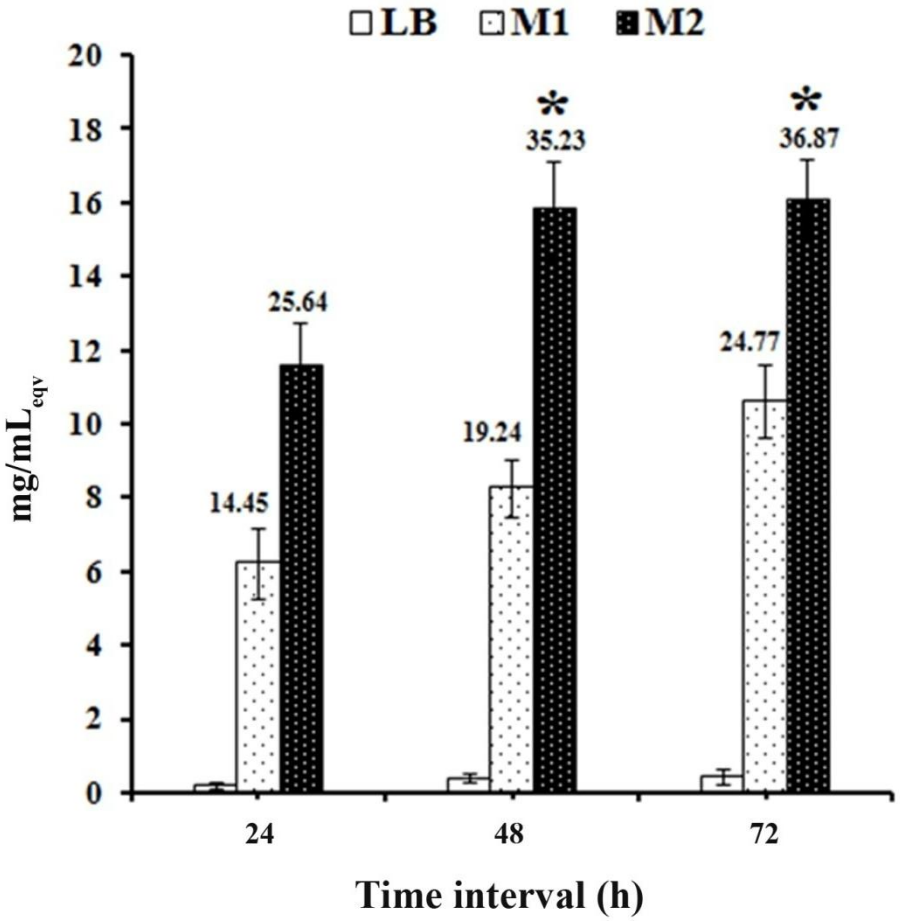


Figure 29. Quantification summary of the purified *δ*-endotoxin crystal proteins. For easy comparison, the quantity of purified crystal protein was given as 'LB equivalents', *i.e.*, corresponding to the initial volume of LB actually used for preparing M2, even if it was made into solid masses after 12 initial fermentation. In other words, higher or lower yields of *δ*-endotoxin from 1mL LB-control = 1mL LB + 30% soybean flour (M1) = M2 (corresponding mass of M1 after the removal of supernatant). Fold increase in yield (in comparison to LB control at 72h) of *δ*-endotoxin on M1 and M2 are given at the top of corresponding bars. Fold increase was calculated in comparison to LB-control at 72 h, whose value was 0.43 mg/mL under our experimental conditions. The endotoxin shown here is the sum total of them normally released along with the rupturing of sporangia and those released from intact sporangia upon treatment during purification. The unit "mL per equivalent" used in this figure is defined as *δ*-endotoxin produced equivalent to 30 mg (30 %, w/v) soybean flour (soybean flour) + 1 ml LB (of M1) initially added prior to autoclaving to initiate fermentation or 30 mg soybean flour without free liquid in the medium (of M2), in comparison to per ml LB control.

6.4. Discussion

Our first objective was to pin-point the best natural substrate as supplement to LB medium for maximizing *Bt*- *δ*-endotoxin production employing *Btk*. Among various natural substrates tested, raw soybean flour was found as the best supplement to LB. Subsequently, we explored whether the stress imparted by solid-state environment with no free water would enhance *δ*-endotoxin production upon SSF. Hence in the first phase, the supernatant (free moisture) in the partially fermented semi-solid medium was eliminated (after 12h initial incubation) by centrifugation to impart water stress, so as to influence the *Btk* cells in switching over to excessive sporulation swiftly with concomitant production of crystal proteins in the second phase of SSF. Aeration may play a crucial role in sporulation. Sarrafzadeh *et al*. (2005) showed highest rate of sporulation in the absence of oxygen and the mature spores were the only population present under this condition at the end of culture; sporulation in a large portion of cells failed under saturated oxygenation and either mature spores or vegetative cells were present at the end of culture. Thus, second phase of SSF environment adopted in our study restricted not only the availability of free water but also the access of oxygen to the cells tangled amidst the solid particles, and the stress induced due to this oxygen starvation might have compelled the *Btk* cells to finish their growth cycle at an early stage after producing spores and crystals concomitantly, hence reduced gestation time (Smitha *et al*., 2013a and b). Other crucial factor contributed to enhanced yield should be nutritional status of the medium, especially more nitrogen, *i.e*., free proteins in soybean flour (M1 and M2) or in beef extract (NB). This is evident from **Table 13**, where in comparison to LB, total cell count was much higher in M1, M2 and NB. We presented the data equivalent to the LB initially added while preparing the medium; additional effects in yield are due to supplement or fermentation strategy.

A very simple, but pioneering SSF strategy we adopted in this study is unparallel in terms of *δ*-endotoxin yield from *Bt*, *i.e*., manyfold increase in the yield with proven purity. In fact, addition of different medium supplements and optimisation of production conditions would have further increased the yield. Reports show that conventional commercial formulae like LB or a medium with yeast extract was the most favoured liquid medium for *δ*-endotoxin production by SmF employing a *Bt* strain. Various investigators modified such commercial media by supplementing it with mineral nutrients or various salts, *i.e*., enriched medium. Zouari *et al*. (1998) showed that *Btk* produced 1 g/L of *δ*-endotoxin in 4.5 g/L total dry biomass in a complex liquid medium in which the sugar was replaced by gruel hydrolysate. In another report by the same group (Khedher *et al*., 2011), a *Bt* strain produced up to 3.6 g/L toxin when heat and NaCl stress were applied to the liquid medium. Fed-batch fermentation with two intermittent feeds (at 10 and 20 h) yielded the maximum *δ*-endotoxin concentration at 72 h, *i.e*., 1.7 g/L (Vu *et al*., 2010). Poopathi's group used a mixture of extracts of potato and Bengal gram or bird feather and deoiled rice bran or wheat bran, chickpea husk and corncob to cultivate *Bt* serovar *israelensis* and found that the mosquitocidal activity of crude toxin was higher than that produced in the conventional medium (Poopathi and Archana, 2011). Valicente *et al*. (2010) used LB medium supplemented with various salts, and agricultural by-products like soybean four (0.5%) and liquid swine manure (4%) to increase *Bt* biopesticide production by SmF, which resulted in 1.18 g/L dry cell mass. Zhuang *et al*. (2011) also claimed that they have purified *δ*-endotoxin (up to 7.14 mg/g medium) by one step centrifugation from wastewater sludge-based medium, however they did not provide any physical evidence for the purified crystals. From these reports, it seems that maximum yield of the toxin could be attained is 3.6 g/L (Khedher *et al*., 2011) in SmF or 7.14 g/Kg medium in SSF (Zhuang *et al*., 2011), where they did not provide the actual cost effect.

In fact, many studies did not mention the exact quantity of crystals in the raw mixture. Our biphasic SSF strategy clearly showed the endotoxin yield up to 15.8 g/L LB_{eqv} (48 h) or 37 fold higher yield than LB control (72 h). In fact, we could purify about 55% of the total crystals from the solid fermented mass.

Secondly, focusing on the known protocols (Neema, 2007; Pendleton and Morrison, 1966), we made a pioneering effort for the purification of crystal proteins to homogeneity from the solid matter after SSF, and succeeded in the endeavor. Since both the protocols (Neema, 2007; Pendleton and Morrison, 1966) were found independently unsuccessful to purify *δ*-endotoxin from solid-fermented matter, we combined some steps of both the protocols with slight modification as described under 'materials and methods'. It enabled us to purify the crystal proteins successfully from the fermented matter to over 95% purity, which to our knowledge is the first ever report describing the purification of *δ*-endotoxin from SSF matter.

The aqueous fraction obtained after phase separation contained a mixture of crystals and spores as evidenced by the phase-contrast images in which crystals differentially absorbed CBB stain deeply; from this mixture the *δ*-endotoxin was differentially separated from spores by ordinary centrifugation. The SEM images of our crystal preparations revealed it as a mixture of variously shaped crystals, as described by Schnepf *et al*. (1998), which may be directly correlated to the MW variations of *δ*-endotoxins. According to Schnepf *et al*. (1998), depending on the protoxin composition, the crystals would assume various forms such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B) or rhomboidal (Cry11A) shapes. Employing response surface methodology, Adjalle *et al*. (2011) demonstrated a spray drying process for the raw solid fermented matter from starch industry waste water and waste

water sludge (after proper dilution) and showed that optimum solid particles in these media were 15 and 25 g/L, respectively for maximum efficiency of drying. Since raw product (crystals, spores and vegetative cells) is sufficient for field applications, without going through purification protocols, the filtrate of the fermented matter (from the medium we demonstrated) could directly be dried employing the method of Adjalle *et al*. (2011). This would benefit the farmers, especially from third world countries as envisaged by Anilkumar *et al*. (2008).

Data revealed that overproduction is directly related to the size of the crystals produced, rather than total number of cells or spores. From **Figure 26** crystals produced by SSF (**Figure 26c-e**) were 10 times bigger than those produced in LB control (**Figure 26b**). This fact further confirmed by **Figure 27** too, where the crystals purified from SSF strategy (**Figure 27d**) were much bigger, though their magnification was 2.5 times lesser than LB control (**Figure 27c**), *i.e*., this differences are physically evident from size as well as scale bar.

Thirdly, we wanted to resolve the purified crystal proteins by SDS-PAGE, which showed prominent bands with an apparent MW ~45, 35 and 6 kDa in both the control and PM. Interestingly, 84 kDa band found in control was weak in solid-fermented matter and the 6 kDa band found common in all samples may represent the *Cyt* protein, which needs further confirmation. Wei *et al*. (2003) observed that the nematocidal Cry 6A contains an unusually small active toxin core with a predicted molecular mass of 43 kDa. We showed predominant crystal fractions with ~ 45 kDa and ~35 kDa, which are close to the findings of Wei *et al*. (2003) and Hasan (2010). The larger Cry proteins would generally form crystalline inclusions in the mother cell compartment by their self-assembly in to the characteristic crystal due to the cysteine-rich C-terminal half of the Cry protoxins contributes to crystal structure through the formation of disulfide bonds (Bietlot *et al*., 1990).

However smaller proteins need the help of accessory proteins to form crystals (Baum and Malvar, 1995). The crystal toxin of *Btk* is composed primarily of the glycoprotein protoxin (Kumar and Venkateswerlu, 1998), when whole crystals are solubilised by either reducing or denaturing agents or by mild alkali, several smaller proteins would be formed. These smaller components apparently are the products of proteolytic activity on the bigger and composite protoxin molecules. Knowles *et al*. (1992) also found that *Bt kyushuensis* synthesized a mosquitocidal crystalline inclusion containing several proteins ranging from 140 to 14 kDa.

Finally, for the comparison of yield, we wanted to quantify the purified *δ*-endotoxins from the LB-control, M1 and M2. Many workers developed several purification strategies such as ultracentrifugation (Nakamura *et al*., 1990), column chromatography (Zouari *et al*., 1998), zonal gradient centrifugation (Ang and Nickerson, 1978) phase separation (Neema, 2007; Pendleton and Morrison, 1966), *etc*., for the purification of crystal proteins produced by SmF. Nevertheless, no protocol is available yet for the purification of *δ*-endotoxins until we described it. It seems that various investigators (Khedher *et al*., 2011; Poopathi and Archana, 2011; Valicente *et al*., 2010; Vu *et al*., 2010; Yezza *et al*., 2005) reported the yield of *Bt* crystals as a raw mixture of crystals and spores (very often the total pellet) in a range of 1 to 5 g/L yield. In comparison, we showed the yield of purified *δ*-endotoxins up to 16 mg/mL LB_{eqv}, though only ~60% was recovered from the fermented matter.

As far as cost of the production medium is concerned, the calculated cost of ingredients for 1 L LB medium (Himedia, India) is 1.18 US dollar (excluding water) at our laboratory and that of 1L LB with 30% (w/v) soybean flour supplement is 1. 50 US dollar, *i.e*., only 21.5 % increase in cost of medium. By this approximately mere one-fifth of increase in production cost, 37 fold

more *δ*-endotoxin was produced in one-third decreased gestation period. Thus, the 21.5 % in production cost for the sake of 37 fold increase in yield is negligible. However, complete crystals purified from the fermented matter, the total yield would have doubled from 37 fold. Nevertheless, as far as agricultural purpose is concerned, a raw mixture of *Bt δ*-endotoxin comprising *δ*-endotoxin, endospores and less vegetative cells embedded in the sticky (as of soybean protein in the medium) solid fermented medium would be sufficient for expected results, as we found in our entomotoxicity assay (Smitha *et al*., 2013a).

6.5. Conclusion

Briefly, we showed in this study that - in comparison to SSF - the main disadvantages of cultivation in LB (SmF) are low yield of *δ*-endotoxin and longer gestation time, *i.e*., by 24 h. We successfully demonstrated the enhanced production by biphasic fermentation strategy on bench scale. For scale-up studies, we already designed a 5 L fermenter for SSF, in which special attention has been given for the maintenance of optimum temperature and aeration. Preliminary data from this study shows that *Bt δ*-endotoxin yield by SSF at this culture conditions is much better (data not shown) than as reported herein for the bench level studies. Thus, the demonstrated biphasic fermentation strategy can be used for better yield on large scale.

Chapter VII

Jisha V. N. “Extracellular alkaline protease production and efficacy studies of endotoxin from Bacillus thuringiensis subsp. kurstak i” . Department of Botany, University of Calicut. 2013 .

Chapter 7

ENTOMOTOXICITY ASSAY

[Jisha VN, Smitha RB, Ramani N and Sailas Benjamin (2013). Direct application of *Bacillus thuringiensis* toxin efficiently combats *Eutetranychus orientalis,* a lab-to-field study. ***Acarologia*** (Submitted)]

7.1. Aim

This lab-to-field study evaluates the efficacy of a raw preparation of *Btk* obtained by solid-state fermentation to combat *Eutetranychus orientalis* Klein, the oriental red spider mite. The specific aims are (a) whether *Btk* δ-endotoxin is effective to combat *E. orientalis, (*b) whether *Btk* δ-endotoxin in solid-fermented matter is as good as that obtained by SmF, (c) to demonstrate the bioassay on *E. orientalis,* (d) to find out the LC_{50} dose of the *Btk*; and finally to demonstrate the field trial of *Btk* δ-endotoxin.

7.2. Materials and methods

Materials and materials pertaining to this chapter are described in the **Chapter 3**, under sections: **3.15** and **3.16**.

7.3. Results

7.3.1. Collection of mites

E. orientalis was collected from the infested plants and cultured at the lab conditions (by leaf disc method) providing suitable temperature and humidity. Though *E. orientalis* infestation was found in many plants, we focused on *Moringa oleifera* L. and *V. unguiculata* (L.) Walp (**Figure 30a** and **b**). Unlike other related mites, *E. orientalis* lives on the adaxial (towards axis) surface of the leaves and often forms webbing. The infested leaves show injuries as white or yellowish dots. The mites suck the juice from the leaves by piercing

their stylets in to the leaves, as marked by the characteristic light coloured patches on the leaves. Feeding on juice from the leaves results in water loss and drying of the damaged leaves with characteristic yellowing around the midrib, it ultimately led to the formation of necrotic lesions. Severe infestation leads to the total loss of yield and even death of the plant.

7.3.2. Standardised mites

For bioassays, the mites were standardised at the laboratory conditions as described above; leaf disc method (Sangeetha, 2009). Only adult healthy females from this artificial set-up were selected for feeding assays, *i.e.*, actively moving large females were carefully chosen as evidenced by their activity and morphology. This was to ensure that formation of new generation with all stages.

7.3.3. E. orientalis and its life cycle

Egg-to-adult developmental stages of *E. orientalis* are depicted in **Figure 32**. There are five definite stages in the life cycle of this mite, *viz.*, egg, larva, protonymph, deutonymph and adult; all stages are visible with the aid of a lens. Translucent eggs are oval or circular, six-legged larva is pale-orange; protonymphs and deutonymphs are pale-brown. Both these nymph are eight-legged like adults; adult female are brownish, oval with flattened body and only 0.3-0.5 mm, and the males are still smaller than females which could be distinguished visually. The mites are often red or brown in color, hence the name.

During summer, when rapid multiplication occurs, the lifespan of this mite is about 22-30 days. Female mites start laying eggs along the midrib on the adaxial surface of the leaf in 1-2 days after attaining maturity. Egg-to-adult developmental period spans 10-12 days (**Figure 32**); larvae hatch out from the eggs in 4 or 5 days; protonymphal and deutonymphal stages each lasts for 1or 2 days. Deutonymph transformed into adult in 1 or 2 days.

Figure 30. *E. orientalis* infested plants in the field. **a**. *V. unguiculata*. **b**. *M. oliefera* showing injuries as white or yellowish dots. The mites suck the juice from the leaves by piercing their stylets in to the leaves, as marked by the characteristic light colored punctures on the leaves. On the inset of figure **a** and **b** shows the single mite from the infested plant.

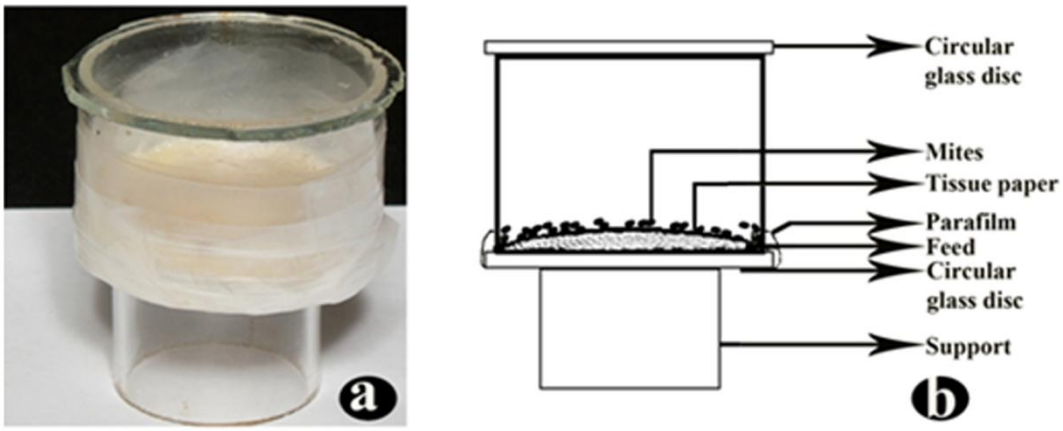


Figure 31. Experimental set-up for *E. orientalis* feeding assay. **a**. this set-up consists of a sterile glass disc with a sterile glass ring was placed above this, the bottom of this set-up was sealed thoroughly using parafilm, calculated quantity of the slurry (feed) prepared was placed on it; the slurry was covered with a sterile tissue paper; 20 healthy standardized adult female mites were carefully transferred on to the feed, and finally the set-up was covered (top) with another glass disc without restricting aeration; and **b**. Schematic diagram of experimental set-up.

7.3.4. Bioassays

Bioassay or *in vitro* toxicity assay for the endotoxin from *Btk* was assessed for submerged as well as solid fermented matter. Thus, LD_{50} values were assessed separately to compare the efficiency of the *Btk* toxin obtained in conventional submerged and solid-fermented matter obtained by our biphasic fermentation strategy.

7.3.5. Btk δ- endotoxin

The raw toxin obtained from submerged fermentation (spore, crystal and cell debris) after 72 h fermentation was used to feed the mites (**Figure 33a**). SSF (48 h) spore, crystal, cell debris and soybean fermented matter (**Figure 33b**).

7.3.6. In vitro toxicity of LB control

Figure 34 (**a-e**) shows the effect of toxin; that (a) only control mites passed through all succeeding stages for next generation. (b) uninoculated LB medium has no negative effect on the growth of mites it passed through all stages of growth cycle. (c) feeding with 10 mg/mL toxin concentration shows 58.34% mortality on 3^{rd} day and 68.34% mortality after 7^{th} day. (d) 20 mg/mL toxin concentration shows 63.34% mortality on 3^{rd} day and 73.34% mortality after 7^{th} day. (e) 40 mg/mL toxin concentration shows 70% mortality on 3^{rd} day and 78.34 % mortality after 7^{th} day. **Figure 35** is the graph showing mortality of mites. 50% lethal concentrations (LC_{50}) for LB control toxin was log 1.3424 = 22 mg/mL (**Figure 36**).

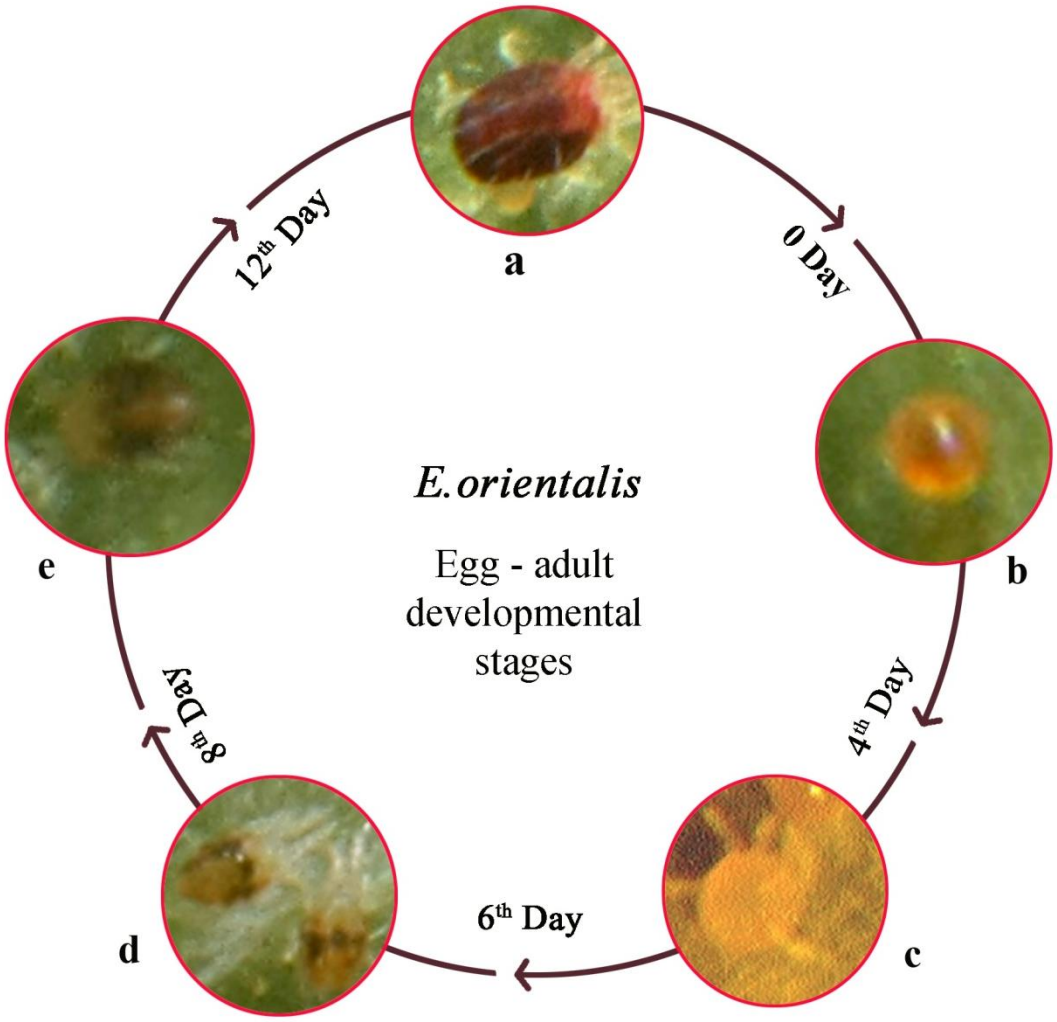


Figure 32. Egg-to-adult developmental stages of *E. orientalis*. **a**. adult mite, adult female are brownish, oval and flattened and only 0.3-0.5 mm long; **b**. translucent eggs are oval or circular; **c**. larvae, six-legged larva is pale-orange; **d**. protonymph; and **e**. deutonymph, protonymphs and deutonymphs are pale-brown. Both the nymphs are eight-legged like adults.

7.3.7. In vitro toxicity of SSF fermented matter

In vitro toxicity of SSF fermented matter showed significant increase in the mortality rate of mite in different concentration of toxin (**Figure 36a-e**). First observation on third day and mortality rate was determined after 7 days. **Figure 36** shows the effect of raw toxin application on *E. orientalis.* (a) only control mites passed through all succeeding stages for next generation. (b) uninoculated solid fermented matter has no negative effect on the growth of mites it passed through all stages of growth cycle. (c) feeding with 10 mg/mL toxin concentration shows 66.67% mortality on 3rd day and 93.34% mortality after 7th day. (d) 20 mg/mL toxin concentration shows 71.67% mortality on 3rd day and 96.67% mortality after 7th day. (e) 40 mg/mL toxin concentration shows 78.34% mortality on 3rd day and 98.34% mortality after 7th day. From the figure it is clear that soybean flour based *Bt* formulation is very effective to control this drastic mite (**Figure 37**). Mortality rate of mites was high in soybean supplemented fermented matter than in LB. LC_{50} value of soybean + LB + crystal formulations was log 0.9784 = 9.51 mg/mL (**Figure 38**).

7.3.8. Field trial

The bioassay of *Btk* crystal formulations against *E. orientalis* were conducted under field conditions (**Figure 40**). *Btk* fermented matter (spores, crystalline delta endotoxins, cellular debris and growth media including raw soybean substrates), mixed with desirable quantity of distilled water, was applied in the infested plants (**Figure 40b**) (infestations introduced in potted plants for experiments). We observed significant reduction in the population growth and infestation, after 10 days new leaves developed without any infection (**Figure 40c)**.

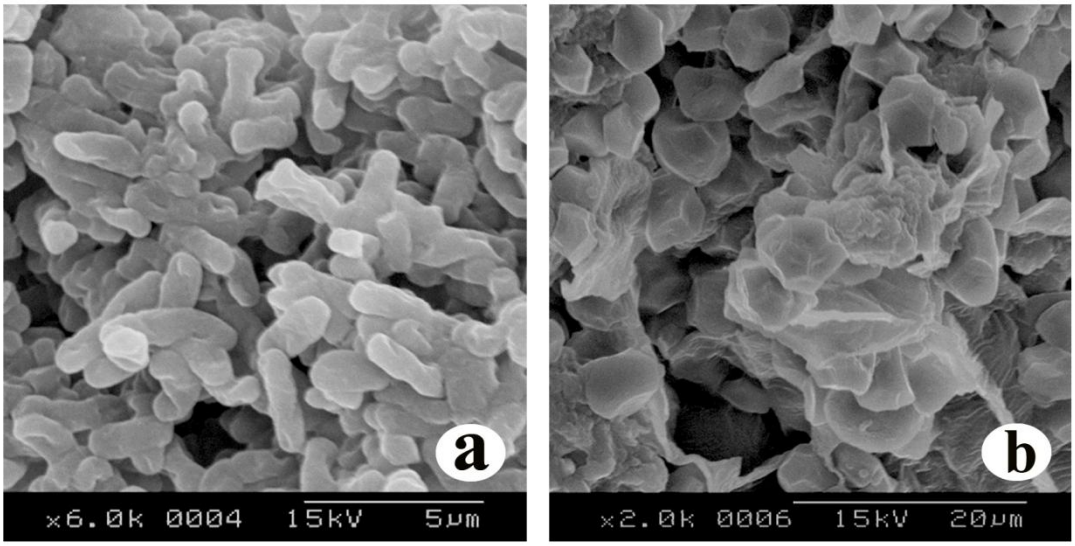


Figure 33. Toxin used for treatment. **a**. Fermented culture of LB control (SEM view); and **b**. SSF fermented matter (SEM view); this raw toxin (spore, crystal and cell debris) obtained after 72 h fermentation in LB (control) or solid-fermented matter without wetting agent (spore, crystal, cell debris and fermented SF) after 48 h was used to feed the mites.

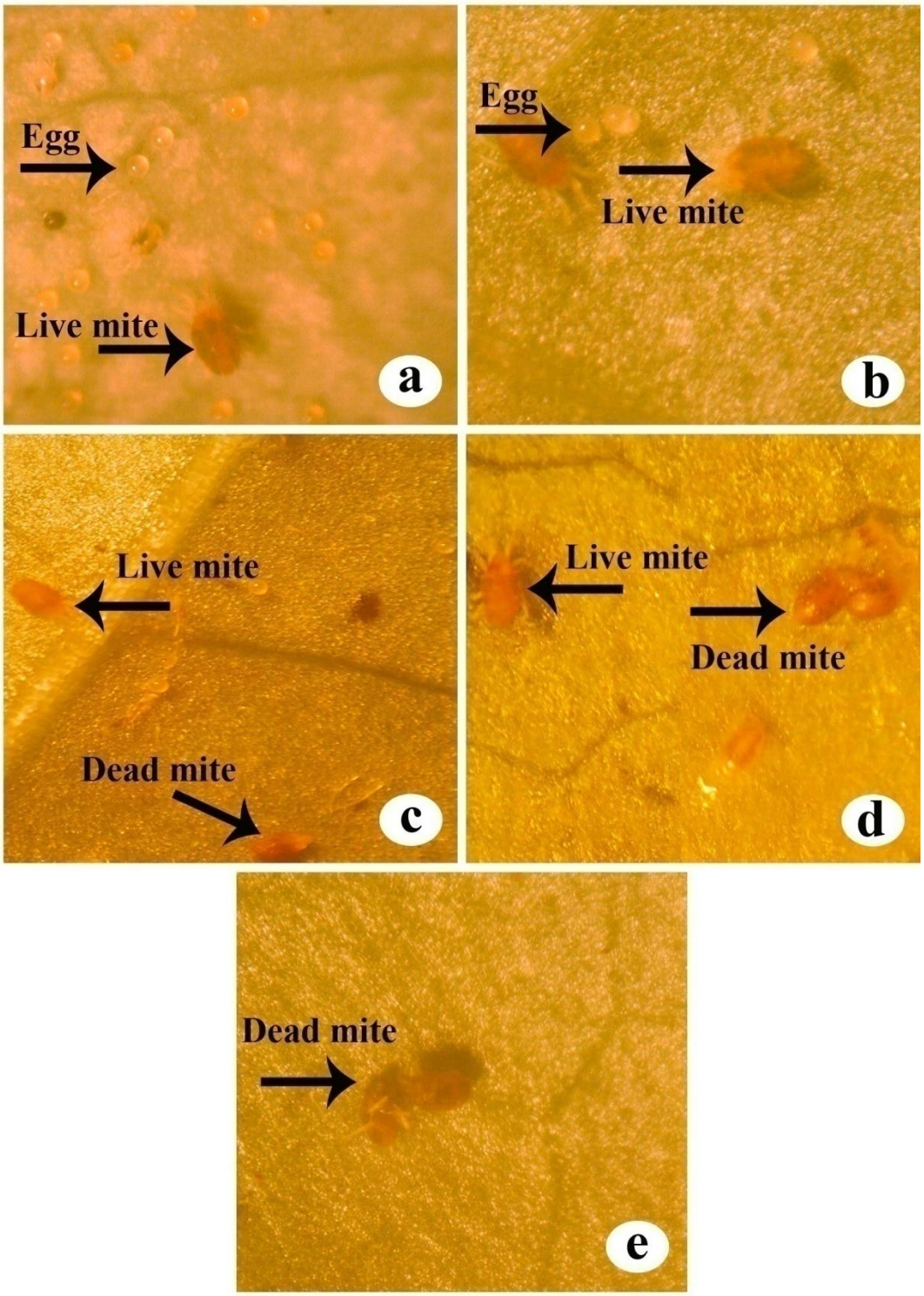


Figure 34. Effect of raw pellet obtained from SmF (72 h) on the mortality of *E. orientalis*. **a**. control (untreated mites on leaf) showing next generation; **b**. uninoculated LB medium treated mites; **c**. 10 mg/mL 10% sucrose treated mites after 7 days; **d**. 20 mg/mL 10% sucrose treated mites after 7 days; and **e**. 40 mg/mL 10% sucrose treated mites after 7 days.

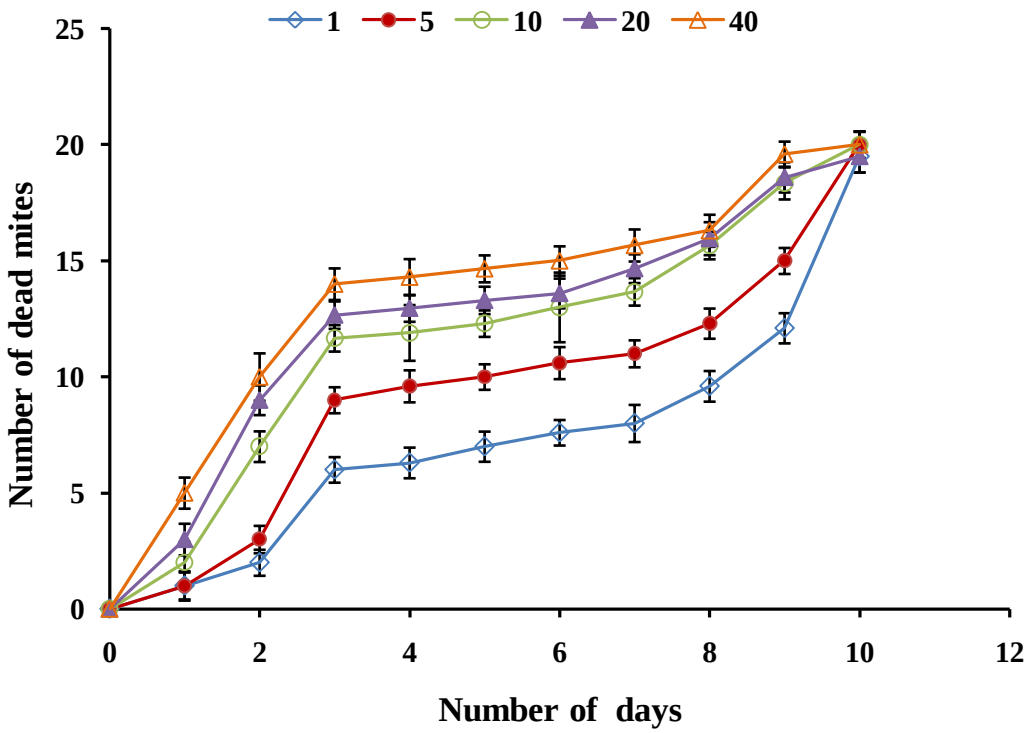


Figure 35. Graph showing mortality of mites-LB control

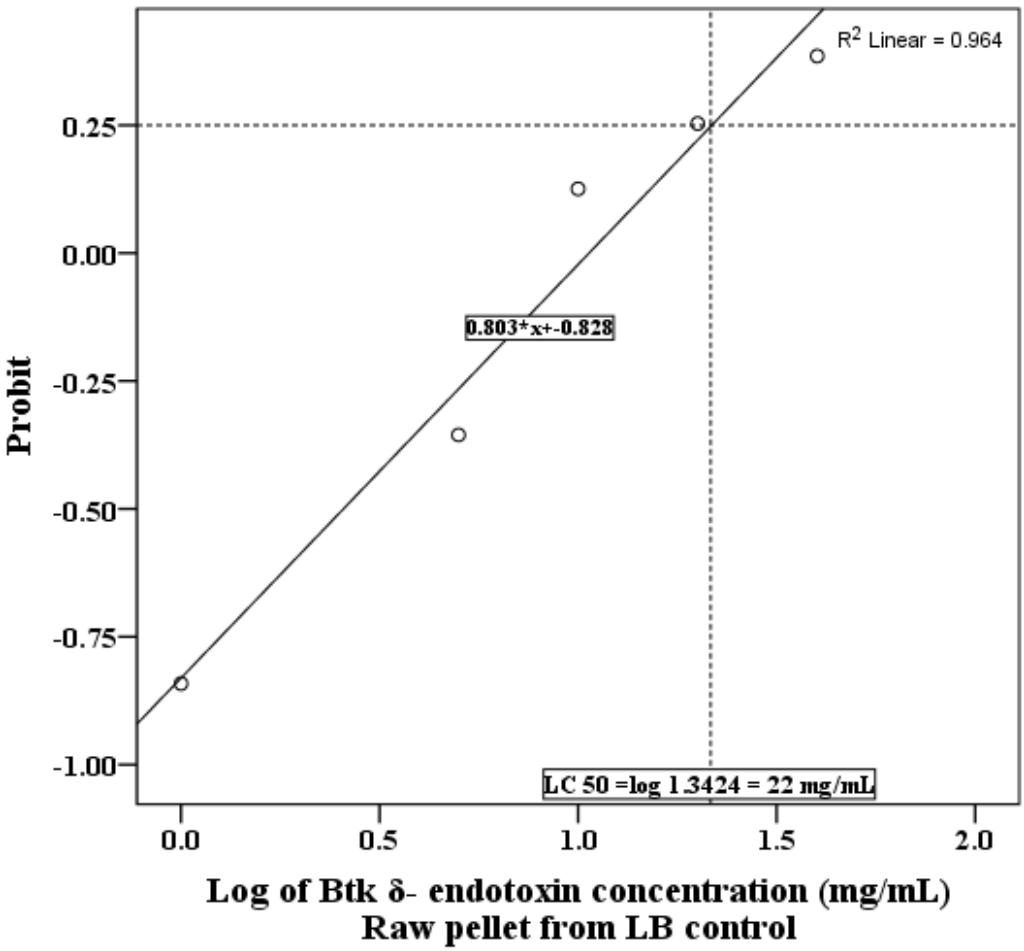


Figure 36. Probit graph showing LC_{50} value of *δ*-endotoxin (LB control). Mortality on 3rd day of treatment was taken for the probit analysis.

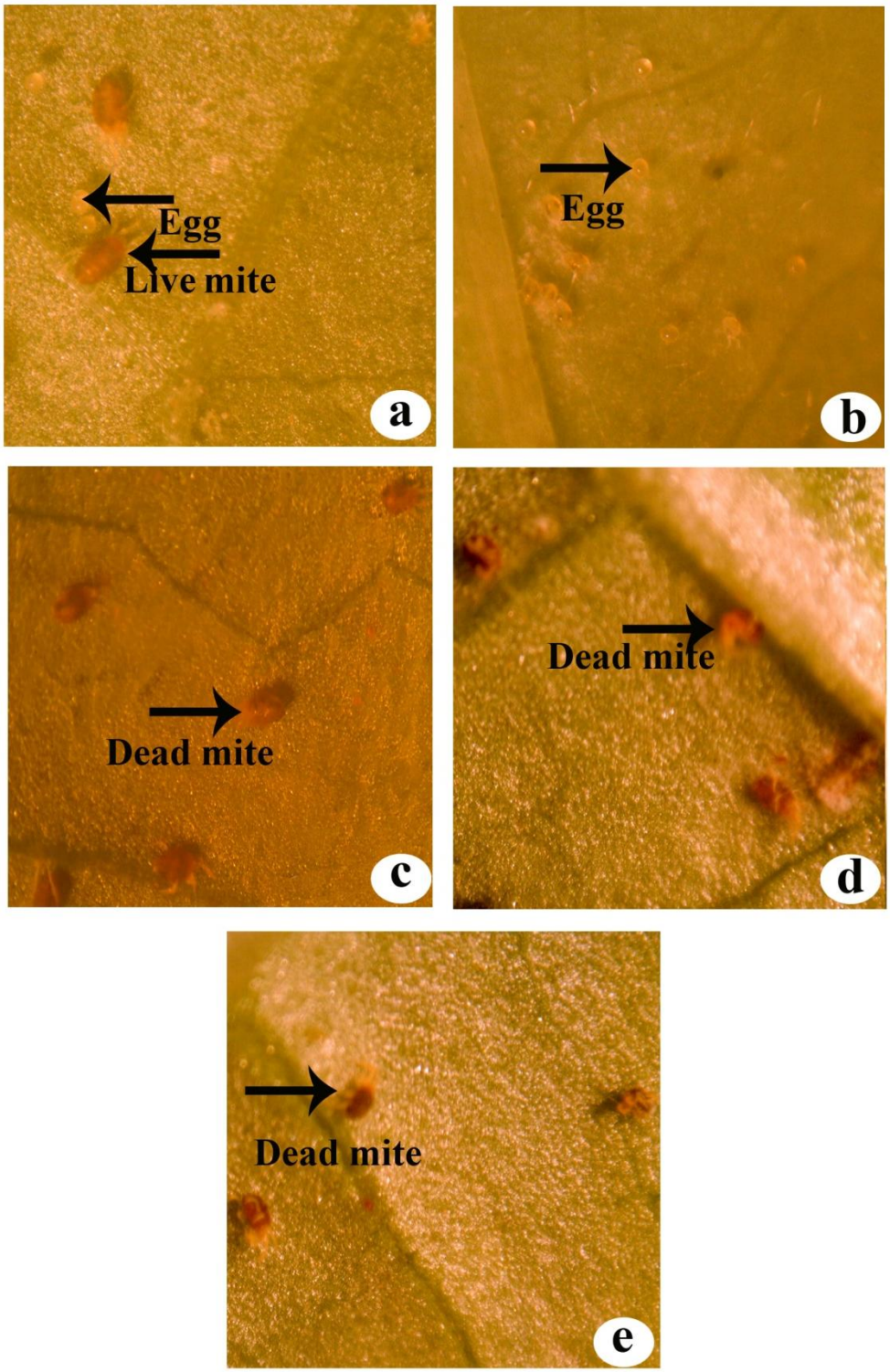


Figure 37. Effect of raw solid-fermented matter obtained from SSF (48 h) on the mortality of *E. orientalis*. **a**. control (untreated mites on leaf) showing next generation; **b**. uninoculated fermented matter (LB+30% soybean flour without free water) treated mites; **c**. 10 mg raw pellet treated mites after 7 days; **d**. 20 mg raw pellet treated mites after 7 days; and **e**. 40 mg raw pellet treated mites after 7 days.

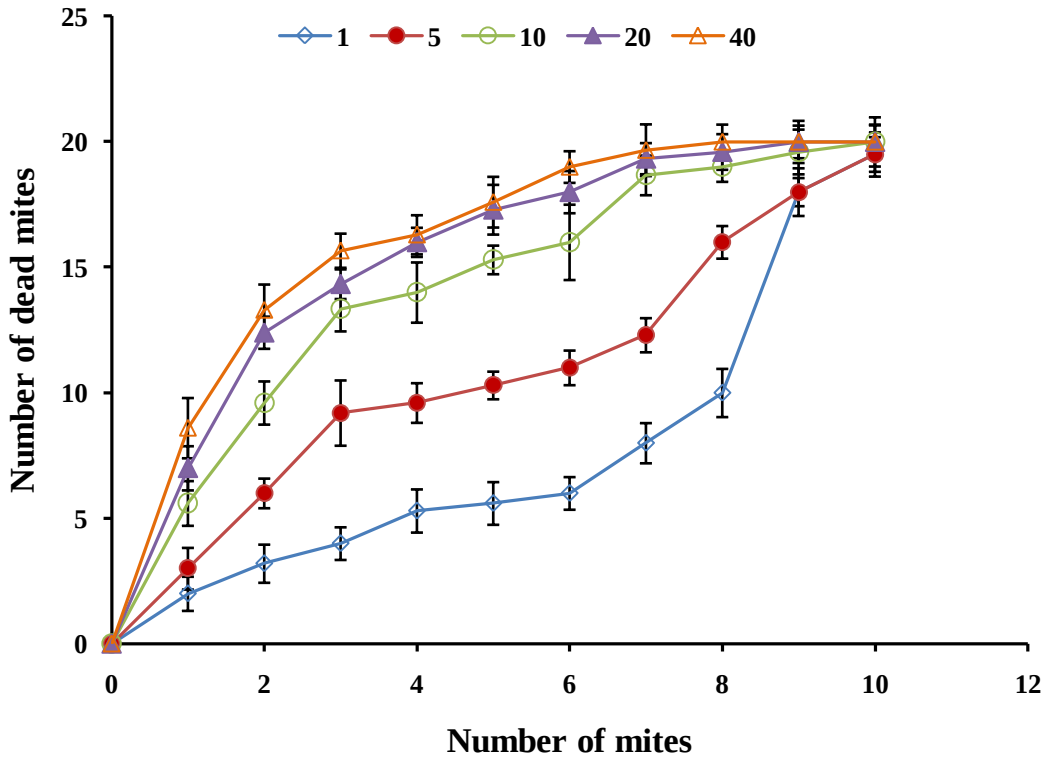


Figure 38. Graph showing mortality of mites-SSF

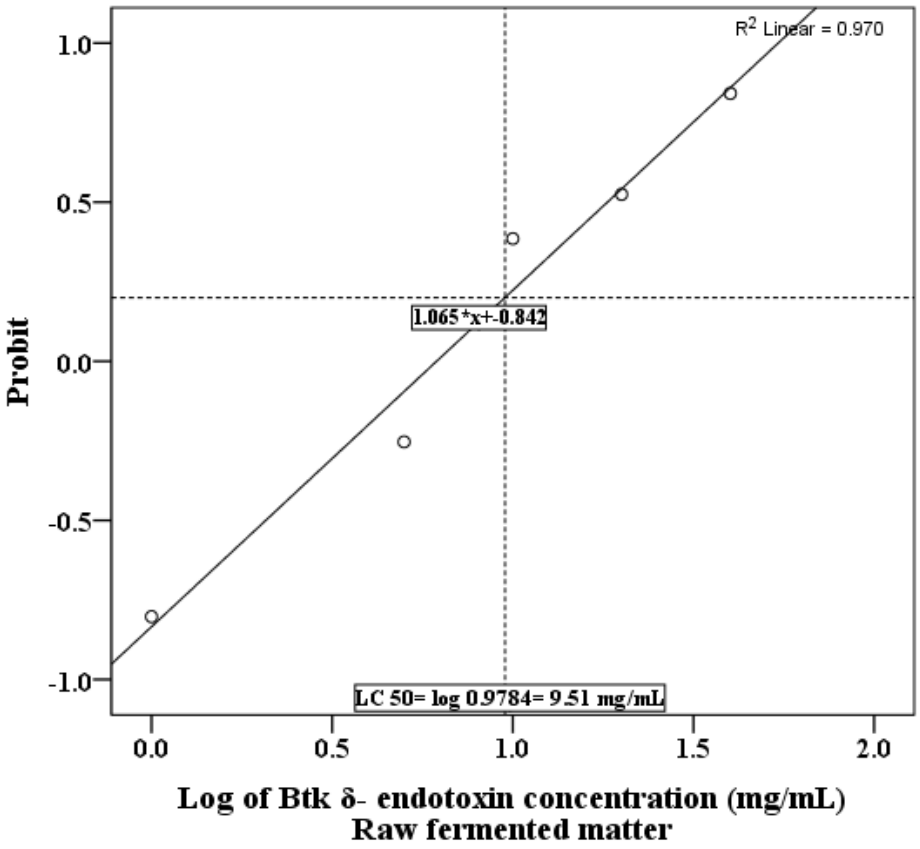


Figure 39. Probit graph showing LC_{50} value of *δ*-endotoxin (SSF). Mortality on 3rd day of treatment was taken for the probit analysis.

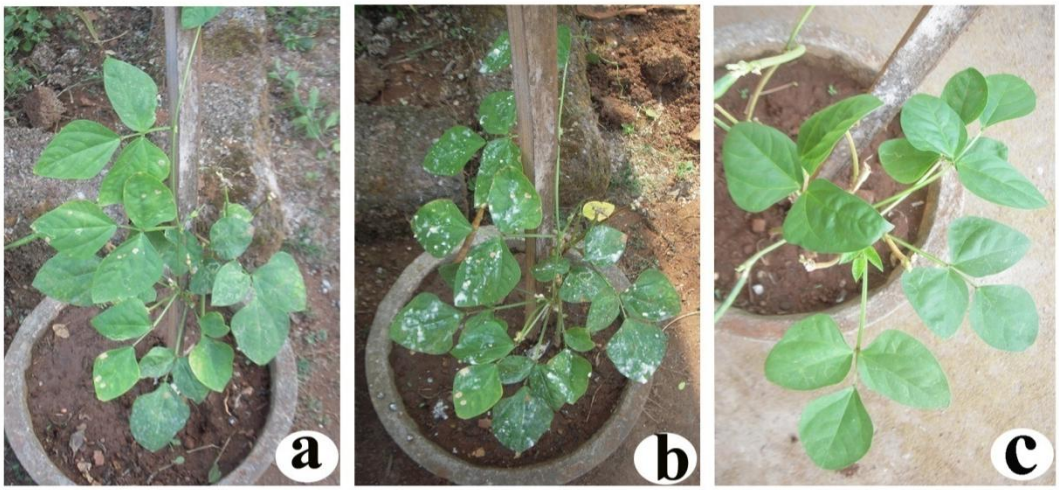


Figure 40. Field trial experiments. **a**. *Vigna unguiculata* mite infected plant before the application of *δ*-endotoxin; **b**. *δ*-endotoxin applied *V. unguiculata* plants; **c**. *V. unguiculata* plant showing fresh uninfected leaves. *Btk* fermented solid matter (spores, crystalline *δ*-endotoxins, cellular debris and growth media including unspent soybean substrates) mixed with desirable quantity of distilled water (10 mg/mL) was applied to the infested plants using a hand sprayer.

7.4. Discussion

The focus of this study was to check the suitability of *Btk* δ-endotoxin to combat *E.orientalis* an acarid mite, which causes severe damage to orchards, vegetable crops and medicinal plants. The life cycle of *E. orientalis* (oriental red mite, one of the spider mites) spans about 22 to 30 days, with slight seasonal variations (Sangeetha, 2009). Most species of spider mites prefer to colonize and lay eggs on the underside of leaves, but some spider mites - such as *E. orientalis* - are often found on the upper side of the leaf. The eggs of spider mites are deposited singly and directly on the leaf surfaces of the host. Female adults can lay more than 100 eggs during their 30 day life cycle. High temperature favours the rapid multiplication of this mite. Development from egg to adult takes about 10 days in the tropics (in our study, it was 10-12 days, where average temperature during the study was ~ 32 ^{o}C), hence many overlapping generations will appear in a single season. Their populations can increase rapidly and cause extensive plant damage in a very short time when conditions (temperature, humidity and food) are suitable (Zhang, 2008).

We found in our locality that *E. orientalis* infestation was very severe in *M. oleifera* and *V. unguiculata*, hence we focused on these host plants. *M. oleifera* (moringa or drum-stick tree) is the most widely cultivated species in the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan for vegetable fruit (drum-stick), leafy vegetable and medicinal purpose. This rapidly-growing tree was advocated for traditional medicinal and industrial uses. *V. unguiculata* is one of the most important food legume crops. Attack of mites on these plants causes heavy yield loss, so controlling of these mites has become essential.

Strains or subspecies of *Bt* show specificity towards different pests (Smitha, 2010). Toxins of *Btk* strain HD1 have widely been used to control the forest pests such as the gypsy moth, spruce bud worm, the pine procesionary moth, the European pine shoot moth and the nun moth (Gui-ming *et al*., 2001). Direct feeding of crude pellet containing *Bt* δ-endotoxin (Fadel and Sabour, 2002), sprays (Mulligan *et al*., 1980), pollen diet formulation (Buchholz *et al*., 2006) are the normal mode of applications being practiced in bioassays. Artificial feeding assay has been used for the bioassay of *E.orientalis* (Payne *et al*., 1993; Hubert *et al*., 2007; Sobotnik *et al*., 2008). A different feeding strategy was successfully used for the bioassay of *A. guerreronis,* in which the dried solid fermented powder directly brushed on the infested coconut buttons (Smitha, 2010). Many authors used surfactants like BIT (1,2-enzisothiazolin-3-one), one of the inert ingredients in Foray 48B (a *Btk* formulation). The siloxane (organosilicone) Triton-X-100, Tween 20 and Latron CS-7 are some surfactants for *Btk* formulations (Helassa *et al*., 2009). In our study we completely avoided such surfactants, because the unutilized soy protein remained in the solid fermented matter behaved as an efficient adhesive, which also made the raw fermented substrate stick to the host surface upon application in the field, it remained attached for many days even after watering thereby affecting the mite.

The quantity of *Bt* δ-endotoxin required for 50% mortality (LC_{50}) of the pests would vary from pest to pest and type of preparation. We showed that LC_{50} value was different for LB control and solid-fermented matter; normally the raw toxin as demonstrated in our LB control was used by various authors (Moar *et al*., 1986). We (for the first time) applied solid-fermented matter directly, in our study crystal protein concentration was very high which was evidenced from the figure. Many authors showed that the mortality rate of many insects up on the application of *Btk* δ-endotoxin (Heckel *et al*., 1999; Aranda *et al*., 1996; Smitha, 2010). Er *et al*. (2007) determined the effect of

Btk on the larvae of *Thaumetopoea solitaria* in the search for an alternative control method with minimal undesirable side effects. Purified *Btk* toxin inhibits the growth of monarch larvae that did not cause mortality (Hellmich *et al*., 2001). The LC_{50} value of *Btk* was found to be 398.1 μg/mL against caterpillars of *Arctornis submarginata* (Khewa and Mukhopadhyay, 2010). Toxicity of several formulations of *Btk* to beet armyworm, *Spodoptera exigua* (Hübner), was determined using neonate larvae in a diet incorporation bioassay. LC_{50} s for formulations of the spore/crystal complex of two isolates, Dipel 2X and Javelin, were 299 μg/mL and 81.2 μg/mL of diet (Moar *et al*., 1986). In our assay system, the LC_{50} value of the raw toxin obtained after 72 h liquid fermentation (unpurified crystal and spores) was 2.2 fold higher (22 mg/mL) than that of the raw solid-fermented matter (9.51 mg/mL). It indicated that solid fermented matted (obtained after 48 h fermentation, *i.e*., initial 12 semi-liquid state and reaming 36 h SSF) contained higher proportion of *δ*-endotoxin, though mixed with unutilized soybean debris as the medium ingredient.

Probit analysis (LC_{50}) has been used by many authors for ascertaining the efficacy of various *Bt* formulations. For instance, Yashodha and Kuppusamy (2008) successfully used dipping method for testing the efficacy of *Btk* formulation in Tween 20 on brinjal. Gobatto *et al*. (2010) used various concentrations of spore suspension of *Bt* for estimating the probit value on mosquito and a moth. We employed artificial feeding assay for the probit analysis on *E. orientalis*, a first report. Payne *et al*. (1993) employed artificial feeding assay for Two-spotted spider mite (*T. urticae*), a related mite to *E. orientalis* with different feeding regime. They fed the mite with 5 mg spray-dried powder of *Bt* broth (a mixture of pores, crystals, cellular debris) in 1 mL sucrose (10%) containing preservatives and surfactant. We did two probit analyses; one with raw pellet (a mixture of crystals, spores, cell debris and a few vegetative cells) obtained from SmF in LB (control) and the other

was raw solid fermented matter in soybean flour (a mixture of crystals, spores, cell debris of cells and soy four). The LC_{50} (probit) value for the raw toxin obtained after 72h SmF was 2.2 fold higher (22 mg/mL) than that of the raw solid-fermented matter (9.51 mg/mL). It clearly shows that solid fermented matter was more efficient though it contained high proportion of soybean debris. Moreover, we added 10% sucrose to the pellet obtained after SmF, while neither sucrose nor any other ingredient was supplemented in the solid-fermented matter. Thus, the solid-fermented matter acted as a best formulation, which completely replaces a carrier (sucrose), surfactant, *etc*. In an artificial feeding assay in which 0.001 to 100 mg spore suspension was incorporated per 1g fish food, Erban *et al*. (2009) showed that 50% suppression of population growth (RC_{50}) was 25 to 38 mg/g feed against mites: *Acarus siro, Tyrophagus putrescentiae, Dermatophagoides farinae* and *Lepidoglyphus destructor*. All these evidences from published *Bt* toxicity on different species uphold the success of our toxin production strategy and its efficacy.

Chapter VIII

Jisha V. N. “Extracellular alkaline protease production and efficacy studies of endotoxin from Bacillus thuringiensis subsp. kurstak i” . Department of Botany, University of Calicut. 2013 .

SUMMARY AND CONCLUSIONS

Title

Extracellular alkaline protease production and efficacy studies of endotoxin from *Bacillus thuringiensis* subsp. *kurstaki*.

Principal Goal

This unique study addresses the importance of biphasic fermentation strategy for the dual production of alkaline proteases and *δ*-endotoxin by *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*), coupled with the purification of *δ*-endotoxin, purification and characterisation of protease, and entomotoxicity assay of raw *δ*-endotoxin produced by solid-state fermentation against an unconventional agricultural pest, *Eutetrunychus orientalis* Klein.

Introduction

Bacillus thuringiensis (*Bt*) is a ubiquitous Gram-positive and sporulating bacterium, known to produce a parasporal crystal proteins (*δ*-endotoxin) juxtaposed to the endospore during the stationary or senescence phase of its growth cycle. The crystal toxins produced by many subspecies of *Bt* (*aizawai, dendrolimus, jegathesan, israelensis, kondukian, kurstaki, medellin, morrisoni, pondicheriensis, tolworthi, etc.*) and their varieties-upon ingestion-are shown to cause mortality of insects belonging to different orders, *viz.*, Coleoptera, Diptera and Lepidoptera and certain mites. *Bt* subspecies *kurstaki* (*Btk*) is a major subtype of *Bt*, being used to combat lepidopteran insects including gypsy moth, spruce budworm and hemlock looper.

Bt mainly intended for the production of *Bt*-toxin (a mixture of endospores and *δ*-endotoxin), which is marketed under various trade names like Dipel 2X, Biobit, Javelin, *etc*. However, a few reports described extracellular hydrolytic

enzymes like proteases and amylases produced by various strains of *Bt* during their early phase of growth, *i.e.*, during the process of the production of *δ*-endotoxin during late phase. Nevertheless, the following gap areas are seen left unnoticed: (1). No report is available in literature describing a cultivation strategy for *Bt* strains wherein a biphasic solid-state fermentation (SSF) strategy was employed, of which the first phase was with sufficient free water and a subsequent phase without it in the growth medium, *i.e.*, first phase semi-solid state and the second phase being SSF; (2). No strategy is seen demonstrated for the dual production of extracellular protease and *δ*-endotoxin, wherein extracellular protease was purified and isolated as a by-product; (3). No protocol is available yet for the purification of *δ*-endotoxin (produced by *Bt*) from solid-fermented matter; and (4) Entomotoxicity potentials of the *δ*-endotoxin produced during SSF was not evaluated against *E. orientalis* - neither in the laboratory nor in the field.

Thus, specific aims of this study were*:*

- To develop a strategy for the production of extracellular alkaline protease as a by-product from *Btk* employing submerged fermentation (SmF) and solid-state fermentation (SSF), and purification and characterisation of extracellular alkaline protease produced.
- To evaluate the dual production of extracellular protease and *δ*-endotoxin during *Btk* fermentation, and establish a biphasic fermentation strategy for the enhanced production of endospore and *δ*-endotoxin.
- To develop a protocol for the purification of *δ*-endotoxin from the solid-fermented matter.

- To establish a lab-to-field entomotoxicity assay for the *δ*-endotoxin produced by *Btk* against *E. orientalis*, a devastating mite attacking on many agriculturally important plants.

The data obtained were presented in different chapters and they are summarised as below:

A. Production and Purification of Alkaline Protease

Bacillus thuringiensis subsp. *kurstaki* (Strain designation: BA 83B, MTCC number: 868), IMTECH, Chandigarh was used in this study. In order to enrich the growth medium, the basal Luria-Bertani (LB) medium was supplemented with 1 to 100% (w/v) banana flour, Bengal gram flour, jack seed flour, soybean flour or tapioca flour. The medium was incubated at 37 ^{o}C with constant shaking (150 rpm, initial pH 7.0) in a temperature-controlled shaker (in SmF), and for SSF, the samples were incubated in an incubator at 37 ^{o}C. Alkaline protease was harvested from the supernatant obtained after SmF or SSF, and activity was expressed in U/mL (SmF) or U/mL$_{eqv}$ (SSF), *i.e*., equivalent to the actual LB added to the medium. Purification of protease was carried out by the techniques such as ammonium sulfate fractionation, spin column purification (Vivaspin 6) and gel filtration chromatography. The following parameters were optimised for the protease produced by *Btk*: *pH* (7 to12), *temperature* (30 to 100 ^{o}C), *substrate (casein) concentration* (0.5, 1, 5, 10, 15, and 20 mg/mL), *metal ions* (Hg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{+} and K^{+}), *inhibitors* (EDTA, *β*-mercaptoethanol), *surfactants* (SDS, Triton X-100) and *commercial detergents* (Ariel, Tide, Surf Excel and Sunlight). The *Km* and *Vmax* values were calculated using the software Hyper 32 and GraphPad Prism. MALDI-TOF/TOF technique was used for the amino acid sequence determination of the alkaline protease.

The crude protease produced by *Btk* in 30% (w/v) soybean flour supplemented LB medium showed about 2097 U/mL_{eqv} activity, which was 16 folds higher than its activity found in commercial LB basal medium (control). Supernatant obtained from 30% (w/v) soybean flour supplemented LB medium at 12 h fermentation was used for the purification of protease, and 60-80% ammonium sulphate fraction showed the maximum protease activity with 1.96 purification folds and 0.97 % yield. It was subjected to spin column (Vivaspin 6) purification with molecular weight cut-off of 45 kDa (2.7 folds purified fraction with 0.90 % yield), followed by sephadex G-100 column chromatography (12.79 folds purified with 0.3 % yield). SDS-PAGE profile of the crude and partially purified proteases showed two active bands with apparent MWs of 43 and 32 kDa. Zymogram profile of impregnated protease also revealed a clear zone of proteolytic activity against the blue background upon coomassie brilliant blue staining.

Partially purified protease (spin column fraction of 60-80% $(NH_4)_2SO_4$ fraction) was used for the optimisation/characterisation studies, which was active in the pH range between 8.5 and 11.5, with the optimum being at pH 9 [7684 U/mL_{eqv}, it was about 3.66 folds increase over initial (30 % soybean flour + LB) activity]. A second peak for pH optimum was obtained at pH 11 [(6455 U/mL_{eqv}) with 3.07 folds increase over initial activity]. Occurrence of these two peaks for pH optima indicates the presence of two proteases. The protease was stable upto 80 ^oC, and the maximum was at 70 ^oC with an activity of 7923 U/mL_{eqv} (3.78 folds increase over initial activity). Regarding substrate (casein) optimum, the maximum activity 7992 U/mL_{eqv} (3.8 folds) was obtained at 15 mg/mL concentration (pH 9, temperature 70 ^oC and 30 min incubation). The protease activity was enhanced with the addition of Mn^{2+}, Ca^{2+} or Mg^{2+}. Maximum activity (11732 U/mL_{eqv}) was obtained in the presence of 2 mM Mn^{2+}, which was 5.6 folds increase over the initial activity. The optimised conditions for *Btk* protease activity were: 15 mg casein at 9.0 pH and 70 ^oC incubation for 30 min in the presence of 2 μM Mn^{2+} (11732

U/mL_{eqv}). Of the complex compounds, presence of the chelating agent EDTA and *β*-mercaptoethanol in the reaction medium decreased the protease activity, *i.e.*, 0.94 and 0.85 folds, respectively. Presence of detergents in the reaction system variously affected the protease activity. Maximum activity was obtained in the presence of 0.2% SDS (6199 U/mL_{eqv}) and 0.2% Triton X-100 (6019 U/mL_{eqv}). Unlike SDS and Triton X-100, protease produced by *Btk* showed good stability in the presence of commercial detergents tested (Ariel, Tide, Surf Excel and Sunlight); *i.e.*, the maximum stability showed in the presence of 'Ariel' (4867 U/mL_{eqv}).

The *Km* and *Vmax* values were found to be 0.90 mg/mL and 879 U/mg, respectively. MALDI-TOF/TOF profile of the purified protease contained 330 amino acids, and the calculated MW of the intact protein was 32.5 kDa, which was in conformity with the protease active band on the SDS-PAGE gel.

The advantage of this study was that it demonstrated the production, purification and characterisation of highly active alkaline protease as a valuable by-product. In industry, the supernatant obtained after the harvest of *Btk δ*-endotoxin is normally discarded as effluent. Thus, if the fermentation strategy reported herein is used for the production of *Btk δ*-endotoxin, the protease to be obtained as a by-product during the initial stage of growth would fetch more profit to the producer, which would, in turn reduce the overall production cost.

B. Concomitant production of endospore and δ-endotoxin

Endospore production was carried out in the LB medium with the 3 agricultural supplements which supported better alkaline protease production *viz*., soybean flour, Bengal gram flour and jack seed flour. The endospores were stained with malachite-green, acridine orange and coomassie brilliant blue. Visualisation and measurement of endospores were done with the help of image analyser attached with Nikon digital camera and Olympus

fluorescent microscope, equipped with a BP 480/20 excitation filter and Nikon digital camera.

Delta-endotoxin from *Btk* is conventionally produced by SmF; but successful application of biphasic fermentation strategy for the overproduction of *δ*-endotoxin and subsequent purification from fermented solid matter is not addressed yet. In the first phase (semi-solid fermentation) of the biphasic fermentation strategy, *Btk* was grown in LB medium supplemented with 30% (w/v) raw soybean flour (medium 1, M1) for 12 h; after harvesting the supernatant from M1 (for the purification of protease as by-product), the pellet or solid matter (medium 2, M2) with only bound water was incubated (solid-sate fermentation, SSF) further for the concomitant production of endospore and *δ*-endotoxin (phase 2), and thus the term biphasic fermentation strategy.

The Maximum number and size of spores were obtained in 30 % soybean flour supplemented medium at 48 h. It was observed that parasporal crystal bodies (*δ*-endotoxin) and endospores were stained darkly and lightly, respectively with coomassie brilliant blue; while with fluorescent acridine orange stain, the spores were much brighter and clearer. Endospores were stained greenish-blue with malachite-green. The maximum yield of endospores during SSF was observed in 24 h less gestation period, *i.e*., normal gestation period or harvest time for *Btk* in SmF is 72 h, which could be reduced to 48 h, if SSF strategy is employed.

Delta-endotoxin crystal production was carried out in LB medium modified with flours of 10% (w/v) jack seed, 30% (w/v) soybean and Bengal gram. The crystal production was monitored up to 72 h at 6 h intervals. For increasing the production of crystal protein, the 12 h culture was centrifuged at 1000 × *g* for 10 min for removing the supernatant, so as to make the medium ready for SSF. The pellets were collected and incubated at 37 ^{o}C. *Btk δ*-endotoxin was isolated and purified. Purified *δ*-endotoxin was observed and photographed using scanning electron and phase contrast microscopes.

Delta-endotoxin from the fermented solid matter was purified for the first time by the modified phase separation method from M1, M2 and LB control, and its purity was physically confirmed by both staining and microscopic techniques. The maximum *δ*-endotoxin yield in M2 (48 h) was 15.8 mg/mL_{eqv} (recovery from the fermented matter was 55-59%); while that of LB control was only 0.43 mg/mL at 72h (recovery from the normal LB medium was 95%), *i.e.*, though recovery percentage was less in SSF than in control, 36.74 folds more yield in M2 obtained by 24 h less gestation period. The purified crystal proteins showed apparent MWs of 45, 35 and 6 kDa on SDS-PAGE.

Briefly, this unique study - both physically quantitatively - demonstrated how *Btk δ*-endotoxin is purified (95-99% purity) from solid-fermented matter for the first time, coupled with its overproduction (15.8 mg/mL_{eqv}) at the expense of only 21.5% higher production cost (due to soyflour) in one-third less gestation period, and that higher *δ*-endotoxin yield was directly related to their increased size of the cells and crystals due to solid-state fermentation.

C. Entomotoxicity Assay

For entomotoxicity assay, the raw pellets obtained after SmF (72 h) or SSF [30% (w/v) raw soybean flour supplemented medium (48 h)] was used as control or treatment, respectively. Leaf disc method was employed to culture *E. orientalis* in petri-dishes containing healthy and fresh whole leaves (for juice feeding) of *Vigna unguiculata* (L.) Walp, providing suitable temperature and humidity in a specially designed hood. These mites were used for bioassay *in vitro*, and 10, 20 and 40 (mg) of *Btk* pellets (obtained from LB control after 72 h SmF) mixed with 1 mL of 10% sucrose solution or the solid-fermented matter obtained after 48h SSF without sucrose solution directly used as the feed to the *E. orientalis* for entomotoxicity studies. Mortality was assessed every day. Mortality rate on the 3rd day of feeding was taken for probit analyses. In the field trail experiments, *Btk* fermented matter mixed with desirable quantity of sterile distilled water (10 mg/mL) was

directly sprayed on the infested plants (*V. unguiculata*) using a hand sprayer during the summer season.

For the bioassay, an artificial feeding system was developed and leaf disc method was used for further experiments in the laboratory. In our assay system, the LC_{50} (probit) concentration of the raw toxin (unpurified mixture of crystals, spores, cell debris and few vegetative cells) obtained after 72h liquid fermentation (LB control) was 2.2 times higher (22 mg/mL of 10% sucrose) than that of the raw solid-fermented matter (9.51 mg/mL) obtained after 48h fermentation (unpurified mixture of crystals, spores, cell debris and debris of soy flour), *i.e*., the efficacy of the solid-fermented matter was more than twice the effect of the toxin obtained from SmF (LB control), which further confirms the efficacy of the solid-fermented matter. Field level experiments showed that 10 mg/mL raw toxin (SSF matter) completely eliminated the *E. orientalis* from the infested plants.

Briefly, this first ever lab-to-field study demonstrated that the application of raw solid-fermented matter without an addition of any adhesive or surfactant efficiently controls the devastating mite, *E. orientalis.*

Conclusions

- Demonstrated that *Btk* is a potential organism for the production of highly active alkaline protease as by-product during the process of *δ*-endotoxin production employing biphasic SSF strategy, and that soybean flour (30%, w/v) supplemented LB was better for the dual production of extracellular protease and *Btk*-toxin.

- The optimised conditions for *Btk* protease activity were: 15 mg/mL casein at 9.0 pH and 70 ^{o}C incubation for 30 min in the presence of 2 μM Mn^{2+}. Its sephadex-G100 purification fold was 12.79, and *Km* and *Vmax* values were determined as 0.90 mg/mL and 879.3 U/mg. The 32 kDa protease possessed 330 amino acids.

- Modified biphasic separation was efficient for the purification of *δ*-endotoxin from the solid-fermented medium. This unique study physically and quantitatively demonstrated how *Btk δ*-endotoxin was purified (95-99% purity) from solid-fermented matter for the first time, coupled with its over-production (15.8 mg/mL_{eqv}) at the expense of only 21.5% higher production cost in one-third less gestation period, and that higher *δ*-endotoxin yield was directly related to their increased size due to SSF.

- Raw *Btk*-toxin from SSF matter proved as an effective acaricide to control

  E. orientalis. It was shown that the raw solid-fermented matter containing unutilized soybean flour embedded with *δ*-endotoxin and endospores produced by *Btk* was highly efficient to combat *E. orientalis*.

Deliverables

- A protocol for the production of alkaline protease from *Btk*.
- Kinetic and molecular data for the alkaline protease produced by *Btk*.
- A SSF strategy for the overproduction of *δ*-endotoxin from *Btk* by 24h less gestation time.
- A protocol for the purification of *δ*-endotoxin produced by *Btk* from SSF matter.
- Successful application of raw fermented matter against *E. orientalis*.

BIBLIOGRAPHY

Abdullah MAF and Dean DH (2004). Enhancement of Cry19Aa Mosquitocidal activity against *Aedes aegypti* by mutations in the putative loop regions of Domain II. ***Applied and Environmental Microbiology***, **70**: 3769-3771. doi:10.1128/AEM.70.6.3769-3771.2004

Aboul-Soud MAM, Foda MS, Kahil T, Asar AR, El-Desoky GE, Al-Othman AM, Al-Othman ZA and Giesy JP (2011). Purification and biochemical characterisation of alkaline protease from an Egyptian biopesticide-producing *Bacillus sphaericus* strain. ***African Journal of Microbiology Research***, **5**: 5076-5084. doi: 10.5897/ajmr11.939.

Adinarayana K and Ellaiah P (2002). Response surface optimisation of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. ***Journal of Pharmaceutical Sciences***, **5**: 272-278.

Adinarayana K, Ellaiah P and Prasad DS (2003). Purification and partial characterisation of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. ***AAPS Pharmaceutical Science and Technology***, **4**: 440-448.

Adjalle KD, Vu DK, Tyagi RD, Brar SK, Valéro JR and Surampalli RY (2011). Optimisation of spray drying process for *Bacillus thuringiensis* fermented wastewater and wastewater sludge. ***Bioprocess Biosystem Engineering***, **34**: 237-246.

Agaisse H and Lereclus D (1995). How does *Bacillus thuringiensis* produce so much insecticidal Crystal protein? ***Journal of Bacteriology***, 177: 6027-6032.

Ageitos JM, Vallejo JA, Sestelo ABF, Poza M and Villa T (2007). Purification and characterisation of a milk-clotting protease from *Bacillus licheniformis* strain USC13. ***Journal of Applied Microbiology***, **103**: 2205-2213. doi:10.1111/j.1365-2672.2007.03460.

Agrawal R, Singh R, Verma A, Panwar P and Verma AK (2012). Partial Purification and Characterisation of Alkaline Protease from *Bacillus* sp. isolated from Soil. ***World Journal of Agricultural Sciences***, **8**: 129-133.

Ahmad SM (2011). Production of thermostable alkaline protease from an alkaline-resistant *Streptomyces* isolate EGS-5. ***International Journal of Academic Research***, **3**: 393- 404.

Al-Shehri L, Abdul-Rahman M and Yassar S (2004). Production and some properties of protease produced by *Bacillus licheniformis* isolated from *Tihametaseer*, Saudi Arabia. ***Pakistan Journal of Biological Sciences***, **7:** 1631-1635. doi: 10. 3923 /pjbs .2004 .16 31.1635

Alstad DN and Andow DA (1995). Managing the evolution of insect resistance to transgenic plants. ***Science***, **268**: 1894-1896. doi:10.1126/science.268.5219.1894

Amin G, Alotaibi S, Narmen YA and Saleh W (2008). Bioinsecticide production by the bacterium *Bacillus thuringiensis*. 1. Pattern of cell growth, toxin production and by-product synthesis. ***Archives of Agronomy and Soil Science***, **54**: 387-394. doi:10.1080/03650340802158201

Anderson JK, Grimble GK and Cowan DA (1997). A process for producing a thermostable proteolytic enzyme from *Thermoactinomyces thalpophilus* THM1. PCT Patent Application, WO 23605.

Andrews Jr RE, Bibilos MM and Bulla Jr LA (1985). Protease activation of the entemocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. ***Applied Environmental Microbiology***, **50**: 737-742.

Ang BJ and Nickerson KW (1978). Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. ***Applied Environmental Microbiology***, **36**: 625-626.

Anilkumar KJ, Rodrigo-Simón A, Ferré J, Pusztai-Carey JM, Sivasupramaniam SM and Moar WJ (2008). Production and

Characterisation of *Bacillus thuringiensis Cry*1Ac-Resistant Cotton Bollworm *Helicoverpa zea* (Boddie). ***Applied Environmental Microbiology***, **74**: 462-469.

Anson ML (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. ***Journal of General Physiology,*** **22**: 79-89.

Antranikian G and Klingeberg M (1991). Thermostable protease from *Staphylothermus.* PCT Patent Application, WO 9119791.

Antranikian G and Klingeberg M (1991). Thermostable protease from *Thermobacteroides.* PCT Patent Application, WO 9119790.

Antranikian G and Klingeberg M (1991). Thermostable protease from *Thermococcus.* PCT Patent Application, WO 9119792.

Aqel H (2012). Phenotypic and protease purification of two different thermophilic *Bacillus* strains HUTBS71 and HUTBS62. ***Annals of Biological Research***, **3**: 1747-1756.

Aranda E, Sanchez J, Peferoen M, Güereca L and Bravo A (1996). Interactions of *Bacillus thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). ***Journal of Invertebrate Pathology***, **68**: 203-212.

Aronson AI, Geng C and Wu L (1999). Aggregation of *Bacillus thuringiensis Cry*1A toxins upon binding to target insect larval midgut vesicles. ***Applied and Environmental Microbiology***, **65**: 2503-2507.

Bartholomew JW and Mittwer T (1950). A simplified bacterial spore stain. ***Biotechnic and Histochemistry***, **25**: 153-156.

Barthomeuf C, Pourrat H and Pourrat A (1992). Collagenolytic activity of a new semi-alkaline protease from *Aspergillus niger*. ***Journal of Fermentation and Bioengineering***, **73**: 233-236. doi:10.1016/0922-338X(92)90168-T.

Baum JA and Malvar T (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis.* ***Molecular Microbiology***, **18**: 1-12.

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T and Pleau M (2007). Control of coleopteran insect pests through RNA interference. ***Nature Biotechnology***, **25**: 1322-1326. doi:10.1038/nbt1359

Beegle CC and Yamamoto T (1992). History of *Bacillus thuringiensis* Berliner Research and Development. ***Canadian Entomologist***, **124**: 587-616. doi:10.4039/Ent124587-4.

Beg QK and Gupta R (2003). Purification and characterisation of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. ***Enzyme and Microbial Technology***, **32**: 294-304.

Bell DJ, Hoare M and Dunnill P (1983). The formation of protein precipitates and their centrifugal recovery. ***Advances in Biochemical Engineering/ Biotechnology***, **26**: 1-72.

Benjamin S and Pandey A (1996). Optimisation of liquid media for lipase production by *Candida rugosa.* ***Bioresource Technology***, **55**: 167-170.

Benjamin S and Pandey A (1998*). Candida rugosa* lipases: molecular biology and versatility in biotechnology. ***Yeast***, **14**: 1069-1087. doi: 10.1002/(SICI)1097-0061(19980915)14.

Benjamin S, Smitha R, Jisha VN, Pradeep S, Sajith S, Sreedevi S, Priji P, Unni KN and Sarath Josh MK (2013). A monograph on amylases from *Bacillus* spp. ***Advances in Bioscience and Biotechnology,*** **4**: 227-241. doi: 10.4236/abb.2013.42032.

Berlitz DL, Azambuja AOD, Sebben A, Oliveira JVD and Fiuza LM (2012). Mortality of *Oryzophagus oryzae* (Costa Lima, 1936) (Coleoptera: Curculionidae) and *Spodoptera frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae) Larvae exposed to *Bacillus thuringiensis* and

extracts of *Melia azedarach*. ***Brazilian Archives of Biology and Technology***, **55**: 725-731.

Bettel C, Klupsch S, Papendorf G, Hastrup S, Branner S and Wilson KS (1992). Crystal structure of the alkaline protease from *Bacillus lentus* at 1.4 angstrom resolution. ***Journal of Molecular Biology***, **223**: 427-445. doi:10.1016/0022-2836(92)90662-4

Bietlot HP, Vishnubhatla I, Carey PR, Pozsgay M and Kaplan H (1990). Characterisation of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis.* ***Biochemical Journal***, **267**: 309-315.

Boisvert M and Boisvert J (2000). Effects of *Bacillus thuringiensis* var. *israelensis* on target and non-target organisms: A review of laboratory and field experiments. ***Biocontrol Science and Technology***, **10**: 517-561. doi:10.1080/095831500750016361

Boyer EW and Byng GS (1996). *Bacillus proteolyticus* species which produce an alkaline protease. US Patent 5518917.

Bravo A, Gill SS and Soberón M (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. ***Toxicon***, **49**: 423-435.
doi: http://dx.doi.org/10.1016/j.toxicon.2006.11.022.

Bravo A, Likitvivatanavong S, Gill S and Soberon M (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. ***Insect Biochemistry and Molecular Biology***, **41**: 423-443. doi:10.1016/j.ibmb.2011.02.006

Brown D, Wilkinson H and Gatehouse J (1997). Diffentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. ***Insect Biochemistry and Molecular Biology***, **27**: 625-638. doi: http://dx.doi.org/10.1016/S0965–1748(97)00043-X.

Bti H (2011). Production of *Bacillus thuringiensis* subsp. *israelensis* from Agro-based product (corncob). ***Current Science***, **101**: 1009-1010.

Buchholz S, Neumann P, Merkel K and Hepburn RR (2006). Evaluation of *Bacillus thuringiensis* Berliner as an alternative control of small hive beetles, *Aethina tumida* Murray (Coleoptera; Nitidulidae). ***Journal of Pest Science***, **79**: 251-254. doi:10.1007/s10340-006-0141-x

Calık P, Celik E, Telli IlE, Oktar C and Özdemir E (2003). Protein-based complex medium design for recombinant serine alkaline protease production. ***Enzyme and Microbial Technology***, **33**: 975-986.

Capalbo FD, Valicente FH, OMI, Pelizer LH (2001). Solid-State Fermentation of *Bacillus thuringiensis* tolworthi to control fall armyworm in Maize. ***Electronic Journal of Biotechnology***, **4**: 9-10.

Carroll J, Convents D, Van Damme J, Boets A, Van Rie J and Ellar D (1997). Intramolecular proteolytic cleavage of *Bacillus thuringiensis* Cry3A *δ*-endotoxin may facilitate its coleopteran toxicity. ***Journal of Invertebrate Pathology***, **70**: 41-49. doi: 10.1006/ jipa.1997.4656.

Carter ME, Villani MG, Allee LL and Losey and Berlin JE (2004). Absence of non-target effects of two *Bacillus thuringiensis* Coleopteran Active *δ*-Endotoxins on the bulb mite, *Rhizoglypus robini* (Claparede) (Acari, Acaridae). ***Journal of Applied Entomology***, **128**: 56-63. doi:10.1046/j.1439-0418.2003.00788.x

Chakraborty R and Srinivasan M (1993). Production of a thermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation. ***Journal of Microbiology and Biotechnology***, **8**: 7-16.

Chandi CM, Srimonta G, Asitava B, Kalyan SG, Swagata D, Mrinal KM and Soumitra KS (2007). Prediction-based protein engineering of domain I of cry2A entomocidal toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects. ***Protein Engineering Design and Selection***, **20**: 599-606. doi: 10.1093/protein/gzm058.

Chandrasekaran S and Dhar SC (1983). A low-cost method for the production of extracellular alkaline proteinase using tapioca starch. ***Journal of Fermentation Technology***, **61**: 511-514.

Chang M, Zhou S, Lu N and Ni J (2007). Enhanced *Bacillus thuringiensis* production from sewage sludge with alkaline and ultrasonic pretreatments. ***Water Air and Soil Pollution***, **186**: 75-84.

Cheng CZL, Zaohe W and Juan F (2006). Purification and characterisation of extracellular protease of *Bacillus licheniformis*. CAB abstract. Available via Dialog.

Cheong C, Chun SS and Kim YH (1993). Production and properties of an alkaline protease from *Pseudomonas* sp. SJ-320. ***Korean Bidern Journal***, **26**: 479-484.

Chestukhina GG, Zalunin IA, Kostina LI, Kotova TS, Kattrukha SP and Stepanov V (1980). Crystal forming proteins of *Bacillus thuringiensis*. ***Journal of Biochemisty***, **187**: 457-465.

Chilcott C N and Wigley P J (1993). Isolation and toxicity of *Bacillus thuringiensis* from soil and insect habitats in New Zealand. ***Journal of Invertebrate Pathology,*** **61**: 244-247.

Chilcott CN, Wigley PJ, Broadwell AH, Park DJ and Ellar DJ (1998). Activities of *Bacillus thuringiensis* insecticidal crystal proteins Cyt1Aa and Cyt2Aa against three species of sheep blowfly. ***Applied Environmental Microbiology***, **64:** 4060-4061.

Chislett ME and Kushner DJ (1961). A strain of *Bacillus circulans* capable of growing under highly alkaline conditions. ***Journal of General Microbiology***, **24**: 187-190. doi: 10.1099/00221287-24-2-187

Chu I, Lee C and Li TS (1992). Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. ***Enzyme and Microbial Technology***, **14**: 755-761. doi:10.1016/0141-0229(92)90116-6

Clairmont FR, Milne RE, Carrière MB and Kaplan H (1998). Role of DNA in the activation of the Cry1A insecticidal crystal protein from *Bacillus thuringiensis*. ***Journal of Biological Chemistry***, **273**: 9292-9296. doi:10.1074/jbc.273.15.9292

Cohen S, Dym O, Albeck S, Ben-Dov E, Cahan R, Firer M and Zaritsky A (2008). High-Resolution Crystal Structure of Activated Cyt2Ba Monomer from *Bacillus thuringiensis* subsp. *israelensis*. ***Journal of Molecular Biology***, **380**: 820-827. doi: 10. 1016/j.jmb.2008.05.010

Cranshaw WS (2008). Home and Garden**.** ***Journal of Insects' Series***, **5**: 1-3.

Crickmore N, Zeigler D, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J and Dean D (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. ***Microbiology and Molecular Biology Reviews***, **62**: 807-813.

da Silva M, Furijo Jr A, Furlan AS and Souza O (2011). Production of bio-inseticide *Bacillus thuringiensis* var. *israelensis* in semi continuous processes combined with batch processes for sporulation. ***Brazilian Archives of Biology and Technology***, **54:** 45-52**.** http://dx.doi.org/10.1590 /S1516-89132011000100006.

Dalev PG (1994). Utilisation of waste feathers from poultry slaughter for production of a protein concentrate. ***Bioresource Technology***, **48**: 265-267. doi:10.1016/0960-8524(94)90156-2

Danno G and Yoshimura S (1967). Studies on an alkaline proteinase of *Aspergillus sydowi.* Part I. Purification and some properties of the proteinase. ***Agricultural and Biological Chemistry***, **31**: 1151-1158. doi:10.1271/bbb1961.31.1151

Das J and Danker T (2008). Microbial population dynamics, especially stress tolerant *Bacillus thuringiensis,* in partially anaerobic rice field soils during post-harvest period of the Himalayan, island, brackish water and coastal habitats of India. ***World journal of Microbiology and Biotechnology***, **24**: 1403-1410.

de Maagd RA, Bravo A and Crickmore N (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. ***Trends in Genetics***, **17**: 193-199. doi:10.1016/S0168-9525(01)02237-5

de Maagd RA, Bravo A, Berry C, Crickmore N and Schnepf HE (2003). Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. ***Annual Review of Genetics***, **37**: 409-433. doi: 10.1146/annurev.genet.37.110801.143042.

Deane SM, Robb FT and Woods DR (1986). Isolation and characterisation of a *Vibrio alginolytius* mutant that overproduces extracellular proteases. ***Journal of General Microbiology***, **132:** 893-898.

Deane SM, Robb FT and Woods DR (1987). Production and activation of a SDS-resistant alkaline serine exo-protease of *Vibrio alginolyticus.* ***Journal of General Microbiology***, **133**: 391-398.

Debette J (1991). Isolation and characterisation of an extracellular proteinase produced by a soil strain of *Xanthomonas maltophila.* ***Current Microbiology***, **22**: 85-90. doi:10.1007/ BF02105381

Den Belder E, Bonants PJM, Fitters PFL and Waal-Wijk C (1994). New alkaline serine protease of *Paecilo myceslilacinus.* European Patent Application, No. EP 0623672. Beck CM.

Deng AH, Wu J, ZhangY, Zhang GQ and Wen TY (2010). Purification and characterisation of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. ***Bioresource Technology***, **101**: 7100-7106. doi:10.1016/j.biortech.2010.03.130

Desai AJ and Dhalla SA (1969). Purification and properties of a proteolytic enzyme from *Thermophilic actinomycetes*. ***Journal of Bacteriology***, **100**: 149-155.

Deshpande VV, Jogdand VV, Kanagaraj J, Kumar DA, Laxman RS, Manikandan P, More SV, Naidu RB, Rao BSR, Rao MB, Rele MV, Rengarajulu P, Samayavaram R and Samivelu N (2007). Process for the preparation of alkaline protease. US Patent No. 7186546 B2

Devi PR, VijayaRaghavan P, Vasudheven I, Joshua L and VijayaKumar M (2011). Purification and Characterisation of Protease from *Rhizopus oligosporus*. ***International Journal of Biological Technology***, **2**: 46-49.

Dhandapani R and Vijayaragavan R (1994). Production of a thermophilic, extracellular alkaline protease by *Bacillus stearothermophilus* AP-4. ***World Journal of Microbiology and Biotechnology*****, 10**: 33-35.

Disney DR, Vilela DM, Silvestre MPC and Schwan RF (2008). Alkaline protease from *Bacillus* sp. isolated from coffee bean grown on cheese whey. ***World Journal of Microbiology and Biotechnology***, **24**: 2027-2034. doi:10.1007/s11274-008-9706-6

Dodia MS, Rawal CM, Bhimani HG, Joshi RH, Khare SK and Singh SP (2008). Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp. AH-6. ***Journal of Industrial Microbiology and Biotechnology*** **35**: 121–31.

Dozie INS, Okeke CN and Unaeze NC (1994). A thermostable, alkaline-active, keratinolytic proteinase from *Chrysosporium keratinophilum.* ***World Journal of Microbiology and Biotechnology***, **10**: 563-567.

Du C, Martin PA and Nickerson KW (1994). Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. ***Applied and Environmental Microbiology***, **60**: 3847-3853.

Dubey R, Adhikary S, Kumar J and Sinha N (2010). Isolation, production, purification, assay and characterisation of alkaline protease enzyme from *Aspergillus niger* and its compatibility with commercial detergents. ***Developmental Microbiology and Molecular Biology***, **1**: 75-94.

Dubovskii I, Olifirenko O and Glupov V (2005). Level and activities of antioxidants in intestine of larvae *Galleria mellonella* L. (Lepidoptera, Pyralidae) at peroral infestation by bacteria *Bacillus thuringiensis*

subsp. *galleriae*. ***Journal of Evolutionary Biochemistry and Physiology***, **41**: 20-25. doi:10.1007/s10893-005-0030-6

Dumusois C and Priest FG (1993). Extracellular serine protease synthesis by mosquito-pathogenic strains of *Bacillus sphaericus*. ***Journal of Applied Bacteriology***, **75:** 416-419. doi:10.1111/j.1365-2672.1993.tb02796.x

Durham DR, Stewart DB and Stellwag EJ (1987). Novel alkaline and heat stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. ***Journal of Bacteriology***, **169**: 2762-2768.

Ebeling W, Hennrich N, Klockow M, Metz H, Orth HD and Lang H (1974). Proteinase K from *Tritirachium album* Limber. ***European Journal of Biochemistry***, **47**: 91-97. doi:10.1111/j.1432-1033.1974.tb03671.x

El-Beih FM, Abu-Shady MR, Gamal RF and Abd El-Rahim MKI (1991). Factors affecting the production of extracellular alkaline proteinase by two local isolates of *B. amyloliquefaciens.* ***Annals of Agricultural Sciences***, **36**: 363-376.

El Enshasy H, Abuoul-Enein A, Helmy S and El Azaly Y (2008). Optimisation of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. ***Australian Journal of Applied Science***, **2**: 583-593.

Er MK, Karada S and Mart C (2007). Effectiveness of *Bacillus thuringiensis* var. *kurstaki* on *Thaumetopoea solitaria* Frey. (Lepidoptera: Thaumetopoeidae) larvae in laboratory conditions. ***Turkish Journal of Agriculture and Forestry***, **31**: 255-261.

Erban T, Nesvorna M, Erbanova M and Hubert J (2009). *Bacillus thuringiensis* var. *tenebrionis* control of synanthropic mites (Acari: Acaridida) under laboratory conditions. ***Experimental and Applied Acarology***, **49**: 339-346. doi:10.1007/s10493-009-9265-z

Fadel M and Sabour M (2002). Utilisation of diary byproduct in the production of bioinsecticide. ***Online Journal of Biological Science***, **2**: 116-120.

Fargette F and Grelet N (1975). *Bacillus thuringiensis* Berliner mutants resistant to various antibiotics and showing alterations of sporulation. ***Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D: Sciences naturelles***, 280: 499.

Farrera R, Pérez-Guevara F and De La Torre M (1998). Carbon: nitrogen ratio interacts with initial concentration of total solids on insecticidal crystal protein and spore production in *Bacillus thuringiensis* HD-73. ***Applied Microbiology and Biotechnology***, **49**: 758-765. doi:10.1007/s002530051243

Feitelson JS (1993). The *Bacillus thuringiensis* family tree. in ***Advanced Engineered Pesticides*** (Kim, L., ed.), Marcel Dekker, New York, pp 63–71.

Feitelson JS, Payne J and Kim J (1992). *Bacillus thuringiensis* insects and beyond. ***Nature Biotechnology***, **10**: 271-275. doi:10.1038/nbt0392-271

Ferre J, Escriche B, Bel Y and Van Rie J (1995). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal crystal proteins. ***FEMS Microbiology Letters***, **132**: 1-7. doi:10.1016/0378-1097(95)00271-6

Ferrero M, Castro G, Abate C, Baigori M and Sineriz F (1996). Thermostable alkaline protease of *Bacillus licheniformis* MIR 29: Isolation, production and characterisation. ***Applied Microbiology and Biotechnology***, **45**: 327-332. doi:10.1007/s002530050691

Finney DJ (1971). Probit Analysis: 3d Ed: Cambridge University Press.

Fox JL (2003). Resistance to *Bt* toxin surprisingly absent from pests. ***Nature Biotechnology***, **21**: 958-959. doi:10.1038/nbt0903-958b

Frankena J, Koningstein GM, van Verseveld HW and Stouthamer AH (1986). Effect of different limitations in chemostat cultures on growth

and production of exocellular protease by *Bacillus licheniformis*. ***Applied Microbiology and Biotechnology***, **24**: 106-112.

Frankena J, Van Verseveld HW and Stouthamer AH (1985). A continuous culture study of the exocellular protease in *Bacillus licheniformis*. ***Applied Microbiology and Biotechnology***, **22**: 169-176. doi:10.1007/BF00253604

Fujiwara N and Yamamoto K (1987). Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. ***Journal of Fermentation Technology***, **65**: 345-348. doi:10.1016/0385-6380(87)90098-7

Fujiwara N, Masui A and Imanaka T (1993). Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. ***Journal of Biotechnology***, **30**: 245-256. doi:10.1016/0168-1656(93)90117-6

Fujiwara N, Yamamoto K and Masui A (1991). Utilisation of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. ***Journal of Fermentation and Bioengineering*, 72**: 306-308.

Gajju H, Bhalla TC and Agarwal HO (1996). Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77. ***Indian Journal of Microbiology***, **36**: 153-155.

George S, Raju V, Krishnan MRV, Subramanian TV and Jayaraman K (1995). Production of protease by *Bacillus amyloliquefaciens* in solid-state fermentation and its application in the unhairing of hides and skins. ***Process Biochemistry***, **30**: 457-462.

Gessesse A and Gashe BA (1997). Production of alkaline protease by an alkalophilic bacteria isolated from an alkaline soda lake. ***Biotechnology Letters***, **19**: 479-481. doi:10.1023/A:1018308513853

Gessesse A, Rajni HK, and Gashe BA (2003). Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. ***Enzyme and Microbial Technology***, **32**: 519-524. doi:10.1016/S0141-0229(02)00324-1

Ghorbel-Frikha B, Sellami-Kamoun A, Fakhfakh N, Haddar A, Manni L and Nasri M (2005). Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. ***Journal of Industrial Microbiology and Biotechnology***, **32**: 186-194.

Gobatto V, Giani SG, Camassola M, Dillon AJP, Specht A and Barros NM (2010). *Bacillus thuringiensis* isolates entomopathogenic for *Culex quinquefasciatus* (Diptera: *Culicidae) and Anticarsia gemmatalis (Lepidoptera: Noctuidae).* ***Brazilian Journal of Biology***, **70**: 1039-1046. doi:10.1590/S1519-69842010000500018

Gobatto V, Giani SG, Camassola M, Dillon AJP, Specht A and Barros NM (2010). *Bacillus thuringiensis* isolates entomopathogenic for *Culex quinquefasciatus* (Diptera: Culicidae) and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). ***Brazilian Journal of Biology***, **70**: 1039-1046.

Godfrey T and West S (1996). Introduction to industrial enzymology. Industrial enzymology, Mac. Millan Press, London, 1-8.

Goldberg I, Sneh B, Battat E and Klein D (1980). Optimisation of a medium for a high yield production of spore-crystal preparation of *Bacillus thuringiensis* effective against the Egyptian cotton leaf worm *Spodoptora littoralis* Boisd. ***Biotechnology Letters***, **2**: 419-426. doi:10.1007/BF00162348

Gould F (1988). Genetic engineering integrated pest management and the evolution of pests. ***Trends in Biotechnolology***, **6**: 15-18.

Gould F (1994). Potential and problems with high-dose strategies for pesticidal engineered crops. ***Biocontrol Science and Technology***, **4**: 451-461. doi :10.1080/09583159409355 357

Grove M, Kimble W and McCarthy WJ (2001). Effects of individual *Bacillus thuringiensis* insecticidalcrystal proteins on adult *Heliothis virescens* (F.) and *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). ***Biocontrol***, **46**: 321-335. doi: 10.1023/A:10114 24400297

Guangrong H, Tiejing Y, Po H and Jiaxing J (2006). Purification and characterisation of a protease from Thermophilic *Bacillus* strain HS08. ***African Journal of Biotechnology***, **5**: 2433-2438.

Gui-ming L, Xiang-yue Z and Lu-Quan W (2001). The Use of *Bacillus thuringiensis* on Forest Integrated Pest Management. ***Journal of Forestry Research***, **12**: 51-54. doi:10.1007/BF02856801

Guo S, Liu M, Peng D, Ji S, Wang P, Yu Z and Sun M (2008). New strategy for isolating novel nematicidal crystal protein genes from *Bacillus thuringiensis* strain YBT-1518. ***Applied Environmental Microbiology***, **74**: 6997-7001. doi:10.1128/AEM.01346-08

Gupta A, Roy I, Patel RK, Singh SP, Khare SK and Gupta MN (2005). One-step purification and characterisation of an alkaline protease from haloalkaliphilic *Bacillus sp.* ***Journal Chromatography A***, **20**: 103-108.

Gupta R and Beg Q (2003). Purification and characterisation of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. ***Enzyme and Microbial Technology***, **32**: 294-304. doi:10.1016/S0141-0229(02)00293-4

Gupta R, Beg Q and Lorenz P (2002a). Bacterial alkaline proteases: molecular approaches and industrial applications. ***Applied Microbiology and Biotechnology***, **59**: 15-32.

Gupta R, Beg Q, Khan S and Chauhan B (2002b). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. ***Applied Microbiology and Biotechnology***, **60**: 381-395. doi:10.1007/s00253-002-1142-1

Gusek TW and Kinsella JE (1987). Purification and characterisation of the heat-stable serine proteinase from *Thermomonospora fusca* YX. ***Biochemistry Journal***, **246**: 511-517.

Hamouda T, Shih A Y and Baker J R (2002). A rapid staining technique for the detection of the initiation of germination of bacterial spores. ***Letters in Applied Microbiology***, **34**: 86-90.

Hanan A, El-Sadawy HAA, Georgy JM, Shaaban S, Hossary EI and Kassem HA (2008). Fusion of *Bacillus stearothermophilus* leucine aminopeptidase II with the raw-starch-binding domain of *Bacillus* sp. strain TS-23 a-amylase generates a chimeric enzyme with enhanced thermostability and catalytic activity. ***Journal of Indian Microbiology Technology***, **3**: 23-29.

Hartley BS (1960). Proteolytic enzymes. ***Annual Review of Biochemistry***, **29**: 45-72. doi:10.1146/annurev.bi.29.070160.000401

Hasan MH, Akter A, Ilias M, Khan SN and Hoq MM (2010). Growth, sporulation and toxin production by *Bacillus thuringiensis* isolates in media based on mustard-seed meal. ***Bangladesh Journal Microbiology***, **27**: 51-55.

Hayashi K, Fukushima D and Mogi K (1967). Isolation of alkaline proteinase from *Aspergillus sojae* in homogeneous form. ***Agricultural and Biological Chemistry***, **3**: 1237-1241. doi:10.1271/bbb1961.31.1237

Heckel DG, Gahan LJ, Liu Y and Tabashnik BE (1999). Genetic mapping of resistance to *Bacillus thuringiensis* toxins in diamondback moth using biphasic linkage analysis. ***Proceedings of National Academy of Science USA***, **96**: 8373-8377.

Heidari HRK, Ziaee AA, Schaller J and Amoozegar MA (2007). Purification and characterisation of an extra- cellular haloalkalineprotese produced by the moderately halophylic bacterium, *Salinivibrio* sp. strain AF-2004. ***Enzyme and Microbial Technology***, **40:** 266-272.

doi:10.1016/j.enzmictec.2006.04.006

Helassa N, Quiquampoix H, Noinville S, Szponarski W and Staunton S (2009). Adsorption and desorption of monomeric *Bt* (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite. ***Soil Biology and Biochemistry***, **41**: 498-504. doi: 10.1016/ j.soilbio.2008.12.008

Hellmich RL, Siegfried BD, Sears MK, Stanley-Horn DE, Daniels MJ, Mattila HR, Spencer T, Bidne KG and Lewis LC (2001). Monarch larvae sensitivity to *Bacillus thuringiensis* purified proteins and pollen. ***Proceedings of the National Academy of Sciences of the United States of America***, **98**: 11925-11930.

Herrero S, González-Cabrera J, Tabashnik BE and Ferré J (2001). Shared Binding Sites in Lepidoptera for *Bacillus thuringiensis* Cry1Ja and Cry1A Toxins. ***Applied and Environmental Microbiology***, **67**: 5729-5734. doi:10.1128/AEM.67.12.5729-5734.2001

Hoffmann M and Frodsham A (1993). Natural Enemies of Vegetable Insect Pests. Cooperative Extension, Cornell University, Ithaca, NY., pp. 63.

Höfte H and Whiteley H (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. ***Microbiological Reviews***, **53**: 242-255.

Hölker U and Lenz J (2005). Solid-state fermentation-are there any biotechnological advantages? ***Current Opinion in Microbiology***, **8**: 301-306. doi:10.1016/j.mib.2005.04.006

Horikoshi K (1971). Production of alkaline enzymes by alkalophilic microorganisms. Part I. Alkaline protease produced by *Bacillus* No. 221. *Agricultural* ***and Biological Chemistry***, **35**: 1407-1414. doi:10.1271/bbb1961.35.1407

Hotha S and Banik RM (1997). Production of Alkaline Protease by *Bacillus thuringiensis* H 14 in Aqueous Two Phase Systems. ***Journal of Chemical Technology and Biotechnology***, **69**: 5-10. doi: 10.1002/(SICI)1097-660(199705)69:1<5:AID-CTB661>3.0.CO;2-I.

Huang G, Ying T, Huo P and Jiang YZ (2006). Purifi- cation and characterisation of a protease from thermo- philic *Bacillus strain* HS08. ***African Journal of Biotechnology***, **5**: 2433-2438.

Hubert J, Stejskal V, Munzbergova Z, Hajslova J and Arthur FH (2007). Toxicity and efficacy of selected pesticides and new acaricides to stored product mites (Acari: Acaridida). ***Experimental and Applied Acarology***, **42**: 283-290.

Hutadilok-Towatana N, Painupong A and Suntinanalert P (1999). Purification and characterisation of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS719. ***Journal of Bioscience and Bioengineering***, **87**: 581-587.

Iizuka T, Ishikawa S, Asano S, Bando H, Zheng Z and Murai N (1994). Insecticidal activity of the CryIA(a) and Cry IB delta - endotoxin of *Bacillus thuringiensis* was retained after the coding region of the gene was truncated and expressed in *Escherichia coli*. ***Journal of Sericultural Science of Japan***, **63**: 303-309.

Iriarte J and Caballero P (2001). Biología ecología de *Bacillus thuringiensis*: Phytoma. España**,** pp. 15-44.

Itskovich E L, Znamenskaya LV, Balaban NP, Ershova TA and Leshchinskaya IB (1995). Biosynthesis of alkaline proteinase by *Bacillus intermedius*. ***Microbiology***, **64**: 530-536.

Jany KD and Mayer B (1985). Proteinase K from *Tritirachium album* Limber I. Molecular mass and sequence around the active site serine residue. ***Biological chemistry Hoppe-Seyler***, **366**: 485-492. doi:10.1515/bchm3.1985.366.1.485

Jaouadi B, Ellouz-Chaabouni S, Rhimi M and Bejar S (2008). Biochemical and molecular characterisation of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. ***Biochimie***, **90:** 1291-1305. doi:10.1016/j.biochi.2008.03.004

Jiménez-Juárez N, Muñoz-Garay C, Gómez I, Saab-Rincon G, Damian-Almazo JY, Gill SS, Soberón M and Bravo A (2007). *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non-toxic to Manduca sexta larvae. ***Journal of Biological Chemistry,*** **282**: 21222-21229. doi:10.1074/jbc.M701314200

Jisha V N, Smitha RB and Benjamin S (2013a). An Overview on the Crystal Toxins *from Bacillus thuringiensis*. ***Advances in Microbiology***, **3**: 462-472. doi: http://dx.doi.org/10.4236/aim.2013.35062.

Jisha VN, Smitha RB, Pradeep S, Sreedevi S, Unni K.N, Sajith S, Priji P, Sarath Josh MK and Benjamin S (2013b). Versatility of microbial proteases. ***Advances in Enzyme Research***, **1**: 39-51. doi: http://dx.doi.org/10.4236/aer.2013.13005

Jisha VN, Smitha RB and Sailas Benjamin (2013c). Production and partial purification of protease from *Bacillus thuringiensis* subsp. *kurstaki* by submerged and solid-state fermentations. ***Letters in Applied Microbiology,*** (in press).

Jonsson AG (1968). Protease production by species of *Entomophthora*. ***Applied Microbiology***, **16**: 450-457.

Joo HS and Chang CS (2005). Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimisation and some properties. ***Process Biochemistry***, **40**: 1263-1270.

Joo HS, Kumar CG, Park GC, Kim KT, Paik SR and Chang CS (2002). Optimisation of the production of an extracellular alkaline protease from *Bacillus horikoshii*. ***Process Biochemistry***, **38**: 155-159.

Juhasz O and Skarka B (1990). Purification and characterisation of an extracellular proteinase from *Brevibacteriurn linens.* ***Canadian Journal of Microbiology***, **36**: 510-512. doi:10.1139/m90-089

Kalman S, Kiehne KL, Cooper N, Reynoso MS and Yamamoto T (1995). Enhanced production of insecticidal proteins in *Bacillus thuringiensis*

strains carrying an additional crystal protein gene in their chromosomes. ***Applied Environmental Microbiology***, **61**: 3063-3068.

Kati H, Sezen K, Nalcacioglu R and Demirbag Z (2007). A highly pathogenic strain of *Bacillus thuringiensis* Serovar *kurstaki* in Lepidopteran pests. ***The Journal of Microbiology***, **45**: 553-557.

Kaur M, Dhillo S, Chaudhary K and Singh R (1998). Production, purification and characterisation of a thermostable alkaline protease from *Bacillus polymyxa*. ***Indian Journal of Microbiology***, **38**: 63-67.

Kazan D, Denizci AA, Öner MNK and Erarslan A (2005). Purification and characterisation of a serine alkaline protease from *Bacillus clausii* GMBAE 42. ***Journal of Industrial Microbiology and Biotechnology,*** **32**: 335-344.

Kezia D, Chandrakala G, Prasanthi V, Naidu SV and Rao MN (2011a). Influence of different factors on production of purified protease by *Bacillus subtilis* DKMNR. ***International Journal of Pharma and Bio Sciences***, **2**: 73-85.

Kezia D, Rao MN and Naidu S (2011b). Influence of various environmental parameters on protease secretion from *Bacillus subtilis* DKMNR. ***International Research Journal of Pharmacy***, **2**: 178-182.

Khedher SB, Kamoun A, Jaoua S and Zouari N (2011). Improvement of *Bacillus thuringiensis* bioinsecticide production by sporeless and sporulating strains using response surface methodology. ***New Biotechnology***, **28**: 705-712.

Khewa S and Mukhopadhyay A (2010). Biocontrol potential of a newly isolated bacterial agent against *Arctornis submarginata* (Walker) (Lepidoptera: Lymantriidae) occurring in Darjeeling Terai region. ***Journal of Biopesticides,*** **3**: 114-116.

Kim MJ, Chung HS and Park SJ (1993). Properties of alkaline protease isolated from *Nocardiopsis dassonvilei.* ***Korean Biochemical Journal***, **26**: 81-85.

Kim S, Kim Y and Rhee IK (2001). Purification and characterisation of a novel extracellular protease from *Bacillus cereus* KCTC 3674. ***Archives of Microbiology***, **175**: 458-461.

Knowles BH, White PJ, Nicholls CN and Ellar DJ (1992). A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. ***Proceedings of Biological Science***, **248**: 1-7.

Kobayashi T, Hakamada Y, Adachi S, Hitomi J, Yoshimatsu T, Koike K, Kawa S and Ito S (1995). Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. ***Applied Microbiology and Biotechnology***, **43**: 473-481. doi:10.1007/BF00218452

Kobayashi T, Ogasawara A, Ito S and Saitoh M (1985). Purification and some properties of alkaline proteinase produced by *Pseudomonas maltophila*. ***Agricultural and Biological Chemistry,*** **49**: 693-698. doi:10.1271/bbb1961.49.693

Kotlova EK, Ivanova NM, Yusupova MP, Voyushina TL, Ivanushkina NE and Chestukhina GG (2007). Thiol-dependent Serine proteinase from *Paecilo myceslilacinus*: Purification and catalytic properties. ***Biochemistry* (*Moscow*)**, **72**: 117-123. doi:10.1134/S0006297907010142

Kuddus M and Ramteke PW (2009). Cold-active extracellular alkaline protease from an alkaliphilic *Stenotrophomonas maltophilia*: production of enzyme and its industrial applications. ***Canadian Journal of Microbiology***, **55**: 1294-1301.

Kumar CG (2002). Purification and characterisation of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. ***Letters in Applied Microbiology***, **34**: 13-17. doi:10.1046/j.1472-765x.2002.01044.x

Kumar CG and Takagi H (1999). Microbial alkaline proteases: From a bioindustrial viewpoint. ***Biotechnology Advances***, **17:** 561-594. doi:10.1016/S0734-9750(99)00027-0

Kumar D, Venkatachalam P, Govindarajan N, Balakumaran M and Kalaichelvan P (2012). Production and purification of alkaline protease from *Bacillus* sp. MPTK 712 Isolated from Dairy Sludge. ***Global Veterinaria***, **8**: 433-439.

Kumar NS and Venkateswerlu G (1998). Analysis of 66 kDa toxin from *Bacillus thuringiensis* subsp. *kurstaki* reveals differential amino terminal processing of protoxin by endogenous protease(s). ***IUBMB Life***, **45**: 769-774.

Kunitate A, Okamoto M and Ohmori I (1989). Purification and characterisation of a thermostable serine protease from *Bacillus thuringiensis*. ***Agricultural and biological chemistry***, **53**: 3251-3256.

Kwon YT, Kim JO, Moon SY, Lee HH and Rho HM (1994). Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. ***Biotechnology Letters***, **16**: 413-418. doi:10.1007/BF00245062

Lacey L, Frutos R, Kaya H and Vail P (2001). Insect pathogens as biological control agents: Do they have a future? ***Biological Control***, **21**: 230-248. doi:10.1006/bcon.2001.0938

Lachhab (2001). Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids. ***Process Biochemistry,*** **37**: 197-208.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. ***Nature***, **227**: 680-685.

Laflamme C, Lavigne S, Ho J and Duchaine C (2004). Assessment of bacterial endospore viability with fluorescent dyes. ***Journal of Applied Microbiology***, **96**: 684-692.

Lambert B, Buysse L, Decock C, Jansens S, Piens C, Saey B, Seurinck J, Van Audenhove K, Van Rie J, Van Vliet A and Peferoen M (1996). A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. ***Appliled Environmental Microbiology***, **62**: 80-86.

Lambert B, Theunis W, Agouda R, Van Audenhove K, Decock C, Jansens S, Seurinck J and Peferoen M (1992). Nucleotide Sequence of Gene *Cry* IIID Encoding a Novel Coleopteran-Active Crystal Protein from Strain BTI109P of *Bacillus thuringiensis* subsp *kurstaki*. ***Gene***, **110**: 131-132. doi:10.1016/0378-1119(92)90457-Z

Landau NS, Egorov NS, Gornova B, Krasovskaya SB and Virnik AD (1992). Immobilisation of *Bacillus firmus* cells in cellulose triacetate fibres and films and their use in proteinase biosynthesis. ***Applied Biochemistry and Microbiology***, **28**: 84-88.

Larcher G, Bouchara JP, Annaix V, Symoens F, Chabasse D and Tronchin G (1992). Purification and characterisation of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate. ***FEBS Letters***, **308**: 65-69. doi:10.1016/0014-5793(92)81052-N

Larcher G, Cimon B, Symoens F, Tronchin G, Chabasse D and Bouchara JP (1996). A 33 kDa serine proteinase from *Scedosporiumapiospermum.* ***Biochemical Journal***, **315**: 119-126.

Lee JK, Kim YO, Kim HK, Park YS and Oh TK (1996). Purification and characterisation of a thermostable alkaline protease from *Thermoactinomyces* sp. E79 and the DNA sequence of the encoding gene. ***Bioscience, Biotechnology, and Biochemistry***, **60**: 840-846. doi:10.1271/bbb.60.840

Lee WJ, Son GM and Choi C (1991). Production and purification of alkaline protease from *Bacillus* sp. CW- 1121. ***Journal of the Korean Society of Food Science and Nutrition***, **20**: 388-394.

Li E and Yousten AA (1975). Metalloprotease from *Bacillus thuringiensis*. ***Applied microbiology,*** **30**: 354–361.

Li J, Carroll J and Ellar DJ (1991). Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. ***Nature***, **353**: 815-821. doi:10.1038/353815a0

Li X, Hummer A, Han J, Xie M, Melnik-Martinez K, Moreno RL, Buck M, Mark MD and Herlitze S (2005). G protein β2 subunit-derived peptides for inhibition and induction of G protein pathways. Examination of voltage-gated Ca2+ and G protein inwardly rectifying K+ channels. ***Journal of Biological Chemistry***, **280**: 23945-23959. doi:10.1074/jbc.M414078200

Lima G, Aguiar R, Corrêa R, Martins E, Gomes A, Nagata T, De-Souza M, Monnerat R and Ribeiro B (2008). Cry2A toxins from *Bacillus thuringiensis* expressed in insect cells are toxic to two lepidopteran insects. ***World Journal Microbiology and Biotechnology***, **24**: 2941-2948. doi:10.1007/s11274-008-9836-x

Liu WM and Bajpai RK (1995). A modified growth medium for *Bacillus thuringiensis.* ***Biotechnolology***, **11**: 589-591.

Long S, Mothibelli MA, Robb FT and Woods DR (1981) Regulation of extracellular alkaline protease activity by histidine in a collagenolytic *Vibrio alginolytcus* strain. ***Journal of General Microbiology***, **127**: 193-199.

Lord JC (2005). From Metchnikoff to Monsanto and beyond the path of microbial control. ***Journal of Invertebrate Pathology***, **l89**: 19-29. doi:10.1016/j.jip.2005.04.006

Lowry O, Rosenbrough N, Farr AL and Randall RJ (1951). Protein measurement with Folin phenol reagent. ***Journal of Biological Chemistry,*** **193**: 265-75.

Luisetti M, Piccioni PO, Dyne K, Donnini M, Bulgheroni A, Pasturenzi L, Donnetta AM and Peona V (1991). Some properties of the alkaline proteinase from *Aspergillus melleus.* ***International Journal of Tissue Reaction***, **13**: 187-192.

Madan M, Dhillon S and Singh R (2002). Purification and characterisation of alkaline protease from a mutant of *Bacillus polymyxa.* ***Indian Journal of Microbiology***, **42**: 155-159.

Mahler I and Halvorson HO (1980). Two erythromy-cin-resistance plasmids of diverse origin and their effect on sporulation in *Bacillus subtilis.* ***Journal of General Microbiology***, **120**: 259-263.

Malathi S and Chakraborty R (1991). Production of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. ***Applied Environmental Microbiology***, **57**: 712-716.

Manachini PL, Fortina MG and Parini C (1988). Thermostable alkaline protease produced by *Bacillus thermoruber*, a new species of *Bacillus.* ***Applied Microbiology and Biotechnology***, **28**: 409-413. doi:10.1007/BF00268205

Margesin R, Palma N, Knauseder F and Schinner F (1992). Purification and characterisation of an alkaline serine protease produced by a psychrotrophic *Bacillus* sp. ***Journal of Biotechnology***, **24**: 203-206.

Marroquin LD, Elyassnia D, Griffitts JS, Feitelson JS and Aroian RV (2000). *Bacillus thuringiensis* toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. ***Genetics***, **155**: 1693-1699.

Marvier M, Creedy CM, Regetz J and Karieva P (2007). A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. ***Science***, **316**: 1475-1477. doi:10.1126/science.1139208

Mathew J (1999). Microbial Protease: Isolation, Purification and Characterization. Ph.D. Thesis, Mahatma Gandhi University, Kerala.

Matsuzawa H, Tokugawa K, Hamaoki M, Mioguchi M, Taguchi H, Terada I, Kwon, ST and Ohta T (1988). Purification and characterisation of aqualysin I (a thetmophilic alkaline serine protease) produced by *Themus aquaticus* YT-I. ***European Journal of Biochemistry***, **171**: 441-447. doi:10.1111/j.1432-1033.1988.tb13809.x

McDowell DG and Mann NH (1991). Characterisation and sequence analysis of a small plasmid from *Bacillus thuringiensis* var. *kurstaki* strain

HD1-DIPEL. ***Plasmid***, **25**: 113-120. doi:10.1016/0147-619X(91)90022-O

McGaughey WH and Whalon ME (1992). Managing insect resistance to *Bacillus thuringiensis* toxins. ***Science***, **258**: 1451-1455. doi:10.1126/science.258.5087.1451

Milner RJ (1994). History of *Bacillus thuringiensis*. ***Agriculture Ecosystems and Environment***, **49**: 9-13. doi:10.1016/0167-8809(94)90014-0

Ming LG, Xiang-yue Z and Lu-Quan W (2001). The use of *Bacillus thuringiensis* on forest integrated pest management. ***Journal of Forestry Research***, **12**: 51-54. doi:10.1007/BF02856801

Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H and Park YS (1999). Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. ***Journal of Applied Microbiology***, **86**: 477-486. doi:10.1046/j.1365-2672.1999.00692.x

Mizusawa K, Ichishima E and Yoshida F (1969). Production of thermostable alkaline protease by thermophilic *Streptomyces*. ***Applied Microbiology***, **3**: 366-371.

Moallaei H, Zaini F, Larcher G, Beucher B and Bouchara JP (2006). Partial purification and characterisation of a 37 kDa extracellular proteinase from *Trichophyton vanbreuseghemii*. ***Mycopathologia***, **161**: 369-375.

Moar WJ, Osbrink WLA, and Trumble JT (1986). Potentiation of *Bacillus thuringiensis* var. *kurstaki* with Thuringiensin on Beet Armyworm (Lepidoptera: Noctuidae). ***Journal Economic Entomology***, **79**: 1443-1446.

Monod M, Togni G, Rahalison L and Frenk E (1991). Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. ***Journal of Medical Microbiology***, **35**: 23-28. doi:10.1099/00222615-35-1-23

Moon SH and Parulekar SJ (1991). A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus.* ***Biotechnology and Bioengineering***, **37**: 467-483. doi:10.1002/bit.260370509

Morries ON, Converse V, Kanagaratnam P and Davies JS (1996). Effect of cultural conditions on spore-crystal yield and toxicity of *Bacillus thuringiensis* subsp. *aizawai* HD133. ***Journal of Invertebrate Pathology***, **67**: 129-136. doi:10.1006/jipa.1996.0020

Mulligan FI, Schaefer C and Wildner W (1980). Efficacy and persistance of *Bacillus sphaericus* and *Bacillus thuringieinsis* H. 14 against mosquitoes under laboratory and field conditions. ***Journal Economic Entomology***, **73**: 684-688.

Murakami K, Ishida Y, Masaki A and Tatsumi H (1991). Isolation and characterisation of the alkaline protease gene of *Aspergillus oryzae.* ***Agricultural and Biological Chemistry***, **55**, 2807-2711. doi:10.1271/bbb1961.55.2807

Murthy MVR and Lonsane BK (1993). Effect of the composition of conidial inoculum development agar me-dia on promoting production of proteinase by *Aspergillus oryzae* CFTRI 1480 in solid state fermentation system. ***Chemie, Mikrobiologie, Technologie der Lebensmittel***, **15**: 179-184.

Nadeem M, Qazi JI, Iqbal J and Baig S (2009). Effect of aeration and agitation rates on alkaline protease production by *Bacillus licheniformis* UV-9 mutant. ***Turkish Journal of Biochemistry***, **34**: 89-96.

Naimov S, Weemen-Hendriks M, Dukiandjiev S and Maagd RA (2001). *Bacillus thuringiensis* delta-endotoxin Cry1 hybrid proteins with increased activity against the Colorado potato beetle. ***Applied and Environmental Microbiology***, **67**: 5328-5330. doi:10.1128/AEM.67.11.5328-5330.2001

Nakadai T, Nasuno S and Iguchi N (1973). Purification and properties of alkaline proteinase from *Aspergillus oryzae*. ***Agricultural and Biological Chemistry***, **37**:2685-2694.

Nakamura K, Oshie K, Shimizu M, Takada Y, Oeda K and Ohkawa H (1990). Construction of chimeric insecticidal proteins between the 130-kDa and 135-kDa proteins of *Bacillus thuringiensis* subsp *aizawai* for analysis of structure-function relationship. ***Agricultural and Biological Chemistry***, **54**: 715-724.

Nascimento WCAD and Martins MLL (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp. ***Brazilian Journal of Microbiology***, **35**: 91-96.

Nasuno S and Ohara T (1971). Hyperproduction of proteinase and some hydrolytic enzymes by mutants of *Aspergillus sojae.* ***Agricultural and Biological Chemistry***, **35**: 829-835. doi:10.1271/bbb1961.35.829

Neema PM (2007). Cloning of genes encoding insecticidal proteins (*cry*/*vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala, India. M.Sc. Thesis, Kerala Agricultural University, Kerala, India.

Nester E, Thomashow L, Metz M and Gordon M (2002). 100 Years of *Bacillus thuringiensis*: *A critical scientific assessment*. Washington, DC: American Academy of Microbiology.

Nilegaonkar S, ZambareV, Kanekar P, Dhakephalkar P and Sarnaik S (2007). Production and partial characterisation of dehairing protease from *Bacillus cereus* MCM B-326. ***Bioresource Technology***, **98**: 1238-1245.

Nilegaonkar SS, Zambare VP, Kanekar PP, Dhakephalkar PK, Sarnaik SS, Babu NKC, Ramaniah B, Rajaram R, Ramasami T and Saikumari YK (1998). Novel protease for industrial applications. Patent Application No. 20080220499, 2008-09-11.

Niu Q, Huang X, Zhang L, Li Y, Li J, Yang J and Zhang K (2006). A neutral protease from *Bacillus nematocida*, another potential virulence factor

in the infection against nematodes. ***Archives of Microbiology***, **185**: 439-448. doi:10.1007/s00203-006-0112

Ojcius DM and Young JDE (1991). Cytolytic pore-forming proteins and peptides: is there a common structural motif? ***Trends in Biochemical Sciences***, **16**: 225-229. doi:10.1016/0968-0004(91)90090-I

Olivieri F, Zanetti ME, Oliva CR, Covarrubias AA and Casalongu´e CA (2002). Characterisation of an extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins. ***European Journal of Plant Pathology***, **108**: 63-72. doi:10.1023/A:1013920929965

Ong PS and Gaucher M (1972). Production, purification and characterisation of thermomycolase, the extra-cellular serine protease of the thermophilic fungus *Mal- branchea pulchella* var. *sulfurea.* ***Canadian Journal of Microbiology***, **22**: 165-176. doi:10.1139/m76-023

Oyama H, Kinjoh M, Watari M and Murao S (1997). Purification and characterisation of an alkaline protease produced by *Pimelobacter* sp. Z-483. ***Journal of Fermentation and Bioengineering***, **84**: 351-353. doi:10.1016/S0922-338X(97)89258-6

Park HW, Bideshi DK, Wirth MC, Johnson JJ, Walton WE and Federici BA (2005). Recombinant larvicidal bacteria with markedly improved efficacy against Culex vectors of west Nile virus. ***American Journal of Tropical Medicine and Hygiene***, **72**: 732-738.

Patel R, Dodia M and Singh SP (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. Production and optimisation. ***Process Biochemistry***, **40**: 3569-3575.

Payne J, Narva KE and Fu J (1998). *Bacillus thuringiensis* genes encoding nematode active toxins. US Patent No. 5831011.

Payne JM, Bagley AL and Raymond JC (1993). *Bacillus thuringiensis* isolates for controlling acarides. CUS Patent 5211946. Publishing Associates, Ithaca, NY, USA.

Peek K, Daniel RM, Monk C, Parker L and Coolbear T (1992) Purification and characterisation of a thermostable proteinase isolated from *Thermus* sp. strain Rt41A. ***European Journal of Biochemistry***, **207**: 1035-1044. doi:10.1111/j.1432-1033.1992.tb17140.x

Pena-Montes C, González A, Castro-Ochoa D and Farres A (2008). Purification and biochemical characterisation of a broad substrate specificity thermostable alkaline protease from *Aspergillus nidulans.* ***Applied Microbiology and Biotechnology***, **78**: 603-612. doi:10.1007/s00253-007-1324-y

Pendleton I and Morrison R (1966). Separation of the spores and crystals of *Bacillus thuringiensis*. ***Nature Biotechnology***, **212**: 728-729.

Peng R, Xiong A, Li X, Fuan H and Yao Q (2003). A delta-endotoxin encoded in *Pseudomonas fluorescens* displays a high degree of insecticidal activity. ***Applied Microbiology and Biotechnology***, **63**: 300-306. doi:10.1007/s00253-003-1343-2

Peter B and Campbell LL (1974). Properties of two homologous alkaline proteases from *Streptomyces rectus*. ***Journal of Bacteriology***, **120**: 1109-1115.

Phadatare SU, Deshpande VV and Srinivasan MC (1993). High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8. 20): Enzyme production and compatibility with commercial detergents. ***Enzyme and Microbial Technology***, **15**: 72-76.

Poopathi S and Archana B (2011). Production of *Bacillus thuringiensis* subsp *israelensis* from agro-based product (corncob). ***Current Science***, **101**: 1009-1010.

Porcar M, Grenier A-M, Federici B and Rahbé Y (2009). Effects of *Bacillus thuringiensis* δ-endotoxins on the pea Aphid (*Acyrthosiphon pisum*). ***Applied and Environ- mental Microbiology,*** **75**: 4897-4900. doi:10.1128/AEM.00686-09

Prescott L, Harley J and Klein D (1996). Microbiology, Third Edition. Wm.C. Brown Publishers.

Puri S (2001). An alkaline protease from a *Bacillus* sp.: Production and potential applications in detergent formulation and degumming of silk. M.Sc. Thesis, University of Delhi, New Delhi.

Rahman RNZA, Razak CN, Ampon K, Basri M, Yunus WMZ, and Salleh AB (1994). Purification and characterisation of a heat-stable alkaline protease from *Bacillus stearothermophilus* Fl. ***Applied Microbiology and Biotechnology***, **40:** 822-827. doi:10.1007/BF00173982

Rajalakshmi S and Shethna YI (1980). Spore and crystal formation in *Bacillus thuringiensis* var. *thuringiensis* during growth in cystine and cysteine. ***Journal of Bioscience***, **2**: 321-328.

Rajamohan F, Alzate O, Cotrill JA, Curtiss A and Dean DH (1996). Protein engineering of *Bacillus thuringiensis* δ-endotoxin: mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. ***Proceedings of the National Academy of Sciences of the United States of America***, **93**: 14338-14343. doi:10.1073/pnas.93.25.14338

Ranjbari S, Safaralizadeh MH and Aramideh S (2011). Insecticidal effect of *Bacillus thuringiensis* var. *kurstaki* on the various instars larvae of *Plutellax ylostella* L. (Lep.: plutellidae) under laboratory condition. ***Egyptian Academic Journal of Biological Sciences,*** **3**: 27- 32.

Rao K and Narasu L (2007). Alkaline protease from *Bacillus firmus* 7728. ***African Journal of Biotechnology***, **6**: 2493-2496.

Rao MB, Tanksale AM, Ghatge MS and Deshpande VV (1998). Molecular and biotechnological aspects of microbial proteases. ***Microbiology and Molecular Biology Reviews***, **62**: 597-635.

Rao SC, Sathish T, Ravichandra P and Prakasham R (2009). Characterisation of thermo-and detergent stable serine protease from isolated *Bacillus*

circulans and evaluation of eco-friendly applications. ***Process Biochemistry***, **44**: 262-268.

Rattray FP, Bockelmann W and Fox PF (1995). Purification and characterisation of an extracellular proteinase from *Brevibacterium linens* ATCC 9174. ***Applied Environmental Microbiology***, **61**: 3454-345.

Rawlings ND and Barrett AJ (1993). Evolutionary families of peptidases. ***Biochemical Journal***, **290**: 205-218.

Reddy CY and Venkateswaeralu G (2002). Intracellular protease of *Bacillus thuringiensis* subsp. *Kurstaki.* and protease deficient mutant *Btk-q*. ***Current Microbiology***, **45**: 405-409. doi:10.1007/s00284-002-3767-9

Reddy ST, Kumar SK and Venkateswералu G (2000). Identification and purification of the 69kDa intracellular protease involved in the proteolytic processing of the crystal *δ*-endotoxin of *Bacillus thuringiensis* subsp *tenebrionis.* ***FEMS Microbiology Letters***, **1837**: 63-66.

Reddy YC (2001). Studies on *Bacillus thuringiensis* proteases involved in the production of insecticidal toxins from protoxins. Ph.D. Thesis, Osmania University.

Rifaat HM, Hassanein SM, El-Said OH, Saleh SM and Selim MSM (2006). Purification and characterisation of extracellular neutral protease from *Streptomyces microflavus*. ***Arab Journal of Biotechnology***, **9**: 51-60.

Rosas-García NM (2009). Biopesticide production from *Bacillus thuringiensis*: an environmentally friendly alternative. ***Recent Patents in Biotechnology***, **3**: 28-36. doi:10.2174/187220809787172632

Rossa CA and Mignone C (1993). *δ*-endotoxin activity and spore production in batch and fed-batch cultures of *Bacillus thuringiensis*. ***Biotechnology letters***, **15**: 295-300.

Rouis S, Chakroun M, Saadaoui I and Jaoua S (2007). Proteolysis, histopathological effects, and immunohistopathological localisation of

δ-endotoxins of *Bacillus thuringiensis* subsp. *kurstaki* in the midgut of lepidopteran olive tree pathogenic insect Prays oleae. ***Molecular Biotechnology***, **35**: 141-148. doi:10.1007/BF02686109

Ruiu L, Floris I, Satta A and Ellar DJ (2007). Toxicity of a *Brevibacillus laterosporus* strain lacking parasporal crystals against *Musca domestica* and *Aedes aegypti*. ***Biological Control***, **43**: 136-143. doi:10.1016/j.biocontrol.2007.07.002

Saeki K, Iwata J, Watanabe Y and Tamai Y (1994). Purification and characterisation of an alkaline protease from *Oerskovia xanthineolytiw* TK-I. ***Journal of Fermentation and Bioengineering***, **77**: 554-556. doi:10.1016/0922-338X(94)90128-7

Samal BB, Karan B and Stabinsky Y (1990). Stability of two novel serine proteinases in commercial laundry detergent formulations. ***Biotechnology and Bioengineering***, **35**: 650-652. doi:10.1002/bit.260350611

Sangeetha GK (2009). Biological studies of spider mites (acari: Tetranychidae) infesting vegetable crops. Ph.D. Thesis. University of Calicut, Kerala, India.

Sarrafzadeh MH and Navarro JM (2006). The effect of oxygen on the sporulation, delta-endotoxin synthesis and toxicity of *Bacillus thuringiensis* H-14. ***World Journal of Microbiology and Biotechnology*, 22**: 305-310.

Sarrafzadeh MH, Guiraud JP, Lagneau C, Gaven B, Carron A and Navarro J (2005). Growth, sporulation, δ-endotoxins synthesis, and toxicity during culture of *Bacillus thuringiensis* H14. ***Current Microbiology***, **51**:75-81.

Sauka DH, Sánchez J, Bravo A and Benintende GB (2007). Toxicity of *Bacillus thuringiensis* δ-endotoxins against bean shoot borer (*Epinotia aporema* Wals.) larvae, a major soybean pest in Argentina. ***Journal of Invertebrate Pathology***, 94: 125-129. doi:10.1016/j.jip.2006.09.002

Saurabh S, Jasmine I, Pritesh G and Kumar SR (2007). Enhanced productivity of serine alkaline proteaseby *Bacillus* sp. using soybean as substrate. ***Malaysian Journal of Microbiology***, **3**: 1-6.

Schallmey M, Sing A and Ward OP (2004). Developments in the use of *Bacillus* species for industrial production. ***Canadian Journal of Microbiology***, **50**: 1-17. doi:10.1139/w03-076

Schechler I and Berger A **(**1967). On the size of the active site in proteases I papain. ***Biochemical and Biophysical Research Communications***, **27:** 157-162. doi:10.1016/S0006-291X(67)80055-X

Schichnes D, Nemson JA and Ruzin SE (2006). Fluorescent staining method for bacterial endospores. ***Microscope***, **54**: 91-93.

Schnepf E, Crickmore N, van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR and Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. ***Microbiology Molecular Biology Review***, **62**: 775-806.

Secor ER, Carson WF, Cloutier MM, Guernsey LA, Schramm CM, Wu CA and Thrall RS (2005). Bromelain exerts anti-inflammatory effects in an ovalbum ininduced murine model of allergic airway disease. ***Cellular Immunology***, **237**: 68-75. doi:10.1016/j.cellimm.2005.10.002

Seong CN and Choi SK (2007). InhA-like protease secreted by *Bacillus* sp. S17110 inhabited in turban shell. ***The Journal of Microbiology***, **45**: 402-408.

Setlow P (2000). Resistance of bacterial spores. In bacterial stress responses (Storz G and Hengge-Aronis R ed.), American Society for Microbiology, Washington, DC. Pp. 217-230.

Shafee N, Aris SN, Rahman R, Basri M and Salleh AB (2005). Optimisation of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. ***Journal of Applied Sciences Research***, **1**: 1-8.

Sharif FA and Alaeddinoğlu NG (1988). A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. ***Journal of Industrial Microbiology***, **3**: 227-229.

Sharma DK and Prasad DN (1992). Rapid identification of viable bacterial spores using a fluorescence method. ***Biotechnic and Histochemistry***, **67**: 27-29.

Shi J, Coyne VE and Weiner RM (1997). Identification of an alkaline metalloprotease produced by the hydrothermal vent bacterium *Hyphomonas jannaschiana* VP3. ***Microbios***, **91**: 15-26.

Shimogaki H, Takeuchi K, Nishino T, Ohdera M, Kudo T, Ohba K, Iwama M and Irie M (1991). Purification and properties of a novel surface active agent and alkaline-resistant protease from *Bacillus* sp. ***Agricultural and Biological Chemistry***, **55**: 2251-2258. doi:10.1271/bbb1961.55.2251

Sierpinska A (1997). Toxicity of *Bacillus thuringiensis* to some defoliating forest Lepidoptera. ***Folia Forestalia Polonica***, **39**: 79-92.

Sikdar DP, Majundar MK and Majundar SK (1991). Effect of minerals on the production of the delta-endotoxin by *Bacillus thuringiensis*. ***Biotechnology Letters***, **13**: 511-514. doi:10.1007/BF01049209

Singh J, Batra N and Sobti RC (2001). Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. ***Process Biochemistry***, **36**: 781-785. doi:10.1016/S0032-9592(00)00275-2

Sinha R, Joshi R, Dodia M and Singh S (2007). Production, purification and characterisation of an alkaline protease from an alkaliphilic *Bacillus* sp. ***Cell and Tissue Research***, **7**: 1031-1037.

Smitha RB (2010). Dual Production of Endotoxin and Amylase from *Bacillus thuringiensis* subsp. *kurstaki* by fermentation and efficacy studies of endotoxin against Eriophyid mite. Ph. D. Thesis. University of Calicut, Kerala, India

Smitha RB, Jisha VN, Pradeep S, Sarath Josh MK and Benjamin S (2013a). Potato flour mediated solid-state fermentation for the enhanced production of *Bt*-toxin. ***Journal of Bioscience and Bioengineering***, **116**: 595-601. http://dx.doi.org/10.1016/j.jbiosc.2013.05.008.

Smitha RB, Jisha VN, Sajith S and Benjamin S (2013b). Dual production of amylase and *δ*-endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* during biphasic fermentation. ***Microbiology* (*Moscow*), 82 (6)**: 00-00.

Sobotnik J, Krizkova IK, Vancova M, Munzbergova Z and Hubert J (2008). Chitin in the peritrophicmembrane of *Acarus siro* (Acari: Acaridae) as a target for novel acaricides. ***Journal Economic Entomology***, **101**: 1028-1033.

Son ES and Kim JI (2002). Purification and Charac-terisation of Caseinolytic Extracellular protease from *Bacillus amyloliquefaciens* S94. ***The Journal of Microbiology***, **40**: 26-32.

Sousa F, Jus S, Erbel A, Kokol V, Cavaco-Paulo A and Gubitz G (2007). A novel metalloprotease from *Bacillus cereus* for protein fibre processing. ***Enzyme and Microbial Technology***, **40**: 1772-1781.

Steele DB, Fiske MJ, Steele BP and Kelley VC (1992). Production of a low molecular weight, alkaline active, thermostable protease by a novel, spiral shaped bacterium, *Kurthia spiroforme*, sp. nov. ***Enzyme and Microbial Technology***, **14**: 358-360.

Strauch MA and Hoch JA (1993). Transition-state regulators: Sentinels of *Bacillus subtilis* post-exponential phase gene expression. ***Molecular Microbiology***, **7**: 337-342. doi:10.1111/j.1365-2958.1993.tb01125.x

Sumantha A, Sandhya C, Szakacs G, Soccol CR and Pandey A (2005). Production and partial purification of a neutral metalloprotease by fungal mixed substrate fermentation. ***Food Technology and Biotechnology***, **43**: 313-319.

Sutar II, Srinivasan MC and Vartak HG (1991). A low molecular weight alkaline proteinase from *Conidiobolus coronatus*. ***Biotechnology Letters***, **13**: 119-124. doi:10.1007/BF01030462

Tabashnik BE (1994). Evolution of resistance to *Bacillus thuringiensis*. ***Annual Review of Entomology***, **39**: 47-79. doi:10.1146/annurev.en.39.010194.000403

Tabashnik BE, Brevault T and Carriere Y (2013). Insect Resistance to *Bt* crops: Lessons from the first billion acres. ***Nature Biotechnology***, **31**: 510-521.

Tabashnik BE, Liu YB, Finson N, Masson L and Heckel DG (1997). One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. ***Proceedings of the National Academy of Sciences***, **94**: 1640-1644. doi:10.1073/pnas.94.5.1640

Takagi H, Kondou M, Hisatsuka T, Nakamori S, Tsai YC and Yamasaki M (1992). Effects of an alkaline elastase from an alkalophilic *Bacillus strain* on the tenderisation of beef meat. ***Journal of Agricultural and Food Chemistry***, **40**: 2364-2368. doi:10.1021/jf00024a008

Takami H, Akiba T and Horikoshi K (1989). Production of extremely thermostable alkaline protease from *Bacillus* sp. AH-101. ***Applied Microbiology and Biotechnology***, **30**: 120-124. doi:10.1007/BF00263997.

Takegawa K, Mai LH, Miyauchi C and Iwahara S (1993). Purification and characterisation of alkaline proteinase from *Arthrobader protophormiae*. ***Technical Bulletin of Faculty of Agriculture, Kagawa University***, **45**: 115-120.

Takii Y, Kuriyama N and Suzuki Y (1990). Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP1239. ***Applied Microbiology and Biotechnology***, **34**: 57-62. doi:10.1007/BF00170924

Tang JD, Shelton AM, Van Rie J, De Roeck S, Moar WJ, Roush RT and Peferoen M (1996). Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (Plutella xylostella). ***Applied and Environmental Microbiology***, **62**: 564-569.

Tang XY, Wu B, Ying HJ and He BF (2010). Biochemical properties and potential application of a solvent-stable protease from the high-yield protease producer *Pseudomonas aeruginosa* PT 121. ***Applied Biochemistry and Biotechnology***, **160**: 1017-1031. doi:10.1007/s12010-009-8665-1

Teixeira MB (2012). Using sewage sludge from municipal and industrial solid wastes to produce a *Bacillus thuringiensis* biopesticide.

Thangam EB and Rajkumar GS (2002). Purification and characterisation of alkaline protease from *Alcaligenes faecalis*. ***Biotechnology and Applied Biochemistry***, **35**: 149-154.

Tigue NJ, Jacoby J and Ellar DJ (2001). The α-helix 4 residue, Asn135, is involved in the oligomerisation of Cry1Ac1 and Cry1Ab5 *Bacillus thuringiensis* toxins. ***Applied Environmental Microbiology,*** **67**: 5715-5720. doi: 10.1128/AEM.67.12.5715-5720.2001

Tilquin M, Paris M, Reynaud S, Despres L, Ravanel P, Geremia RA and Gury J (2008). Long Lasting Persistence of *Bacillus thuringiensis* subsp. *israelensis* (*Bt israelensis*) in Mosquito Natural Habitats. ***Plos one***, **3**: 3432.

Tokcaer Z, Bayraktar E, Mehmetoğlu Ü, Özcengiz G and Alaeddinoğlu NG (2006). Response surface optimisation of antidipteran delta-endotoxin production by *Bacillus thuringiensis* subsp. *israelensis* HD 500. ***Process Biochemistry***, **41**: 350-355. doi:10.1016/j.procbio.2005.02.030

Toledo J, Liedo P, Williams T and Ibarra J (1999). Toxicity of *Bacillus thuringiensis*-exotoxin to three species of fruit flies (Diptera: Tephritidae). ***Journal of Economic Entomology***, **92**: 1052-1056.

Tortora GJ, Funke BR and Christine L (2004). Microbiology, An introduction, Eighth Edition. Pearson Education, Inc. and Dorling Kindersley Publishing Inc.

Trisyono A and Whalon ME (1997). Fitness costs of resistance to *Bacillus thuringiensis* in Colorado potato beetle (Coleoptera: Chrysomelidae). ***Journal of Economic Entomology***, **90**: 267-271.

Tsai YC, Juang RY, Lin SF, Chen SW, Yamasaki M and Tamura G (1988). Production and characterisation of an alkaline elastase produced by alkalophilic *Bacillus* Ya-B. ***Applied Environmental Microbiology***, **54**: 3156-3161.

Tsai YC, Yamasaki M, Yamamoto-Suzuki Y and Tamura G (1987). A new alkaline elastase of an alkalophilic *Bacillus.* ***Biochemistry International***, **7**: 577-583.

Tsuchiya K, Arai T, Seki K and Kimura T (1987). Purification and some properties of alkaline proteinases from *Cephalosporium* sp. KSM388. ***Agricultural and Biological Chemistry***, **51**: 2959-2965. doi:10.1271/bbb1961.51.2959

Tsuchiya K, Nakamura Y, Sakashita H and Kimura T (1992). Purification and characterisation of a thermostable alkaline protease from alkalophilic *Thermoactinomyces* sp. HS682. ***Bioscience, Biotechnology, and Biochemistry***, **56:** 246-250. doi:10.1271/bbb.56.246

Tsujibo H, Miyamoto K, Hasegawa T and Inamori Y (1990). Purification and characterisation of two types of alkaline serine proteases produced by an alkalophilic *Actinomycete*. ***Journal of Applied Bacteriology***, **4**: 520-529.

Tsuru D, Kira, H, Yamamoto T and Fukumoto J (1966). Studies on bacterial protease. Part XVI. Purifica- tion, crystallisation and some properties of alkaline protease of *Bacillus subtilis* var. *amylosacchariticus.* ***Agricultural and Biological Chemistry***, **30**: 1261-1268. doi:10.1271/bbb1961.30.1261

Tyagi RD, Sikati FV, Barnabe S, Vidyarthi A and Valero JR (2002). Simultaneous production of biopesticide and alkaline protease by *Bacillus thuringiensis* using waste water as a raw material. ***Water Science and Technology***, **46**: 247-254.

Uyar F, Porsuk I, Kizil G and Yilmaz EI (2011). Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15. ***Eurasian Journal of BioSciences***, **5**: 1-9.

Vachon V, Laprade R and Schwartz JL (2012). Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. ***Journal of Invertebrate Pathology***, **111**: 1-12. doi:10.1016/j.jip.2012.05.001

Valasaki K, Staikou A, Theodorou LG, Charamopoulou V, Zacharaki P and Papamichael EM (2008). Purification and kinetics of two novel thermophilic extracellular proteases from *Lactobacillus helveticus*, from kefir with possible biotechnological interest. ***Bioresource Technology***, **99**: 5804-5813. doi:10.1016/j.biortech.2007.10.018

Valicente FH, de Souza TE, Leite MIS, Freire FL and Vieira CM (2010). Production of *Bacillus thuringiensis* biopesticide using commercial lab medium and agricultural by-products as nutrient sources. ***Review Brasilian Milho Sorgo*, 9**: 1-11.

van Putten AB, Spitzenberger F, Krezmer G, Hitzmann B, Dors M, Simutis R and Schügerl K (1996). Improvement of the production of subtilisin Carlsberg alkaline protease by *Bacillus licheniformis* by online process monitoring and control in a stirred tank reactor. ***Journal of Biotechnology***, **49**: 83-93. doi:10.1016/0168-1656(96)01524-6

Vasquez M, Parra C, Hubert E, Espinoza P, Theoduloz C and Meza-Basso L (1995). Specificity and insecticidal activity of Chilean strains of *Bacillus thuringiensis*. ***Journal of Invertebrate Pathology***, **66**: 143-148. doi: http://dx.doi.org/10.1006/jipa.1995.1077.

Vetter R, Wilke D, Moeller B, Lerch M and Muecke C (1993). Alkaline protease from *Bacillus pumilus.* European Patent Application, EP 0572992.

Vu KD, Tyagi RD, Valéro JR and Surampalli RY (2010). Batch and fed-batch fermentation of *Bacillus thuringiensis* using starch industry wastewater as fermentation substrate**.** ***Bioprocess and Biosystems Engineering***, **33**: 691-700.

Ward OP (1985). Proteolytic enzymes. In: Comprehensive biotechnology, the practice of biotechnology current commodity products (Moo-Young, M. Ed.), Pergamon Press, Oxford. Pp. 789-818.

Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC and Aroian RV (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. ***Proceedings of National Academy of Science USA***, **100**: 2760-2765.

Wellman DE and Cote JC (2005). Development of a *Bacillus thuringiensis*-based assay on *Lygus hesperus*. ***Journal of Economic Entomology***, **98**: 1469-1479. doi: http://dx.doi.org/10.1603/0022-0493-98.5.1469

Wojciechowska J, Lewitin E, Revina L, Zalunin I and Chestukhina G (1999). Two novel delta-endotoxin gene families *cry*26 and *cry*28 from *Bacillus thuringiensis* ssp. *finitimus*. ***FEBS letters,*** **453**: 46-48. doi:10.1016/S0014-5793(99)00650-X

Wright DJ, Iqbal M, Granero F and Ferre J (1997). A change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*. ***Applied and Environmental Microbiology,*** **63**: 1814–1819.

Xia LQ, Zhao XM, Ding XZ, Wang FX and Sun YJ (2008). The theoretical 3D structure of *Bacillus thuringiensis* Cry5Ba. ***Journal of Molecular Modeling***, **14**: 843-848. doi:10.1007/s00894-008-0318-8

Xiubao Q, Hong D, Ying Y and Ying Y (1990). Studies on alkaline proteinase from alkalophilic *Bacillus pumilus.* I. Some properties and applications. ***Acta Microbiologica Sinica***, **30**: 445-449.

Xu Z, Yao B, Sun M and Yu Z (2004). Protection of mice infected with *Plasmodium berghei* by *Bacillus thuringiensis* crystal proteins. ***Parasitology Research***, **92**: 53-57. doi:10.1007/s00436-003-0990-7

Yadav JS, Chowdhury S and Chaudhuri SR (2010). Purification and characterization of an extracellular protease from *Pseudomonas aeruginosa* isolated from East Calcutta wetland. ***Journal of Biological Sciences***, **10**: 424-431.

Yang S and Huang C (1994). Protease production by amylolytic fungi in solid state fermentation. ***Journal of Chinese Agricultural Chemical Society,*** **32**: 589-589.

Yang SS and Wang JY (1999). Protease and amylase production of *Steptomyces rimosus* in submerged and solid state cultivations. ***Botanical Bulletin of Academia Sinica***, **40**: 259-265.

Yashodha P and Kuppusamy C (2008). A Bioefficacy of *Bacillus thuringiensis* var *kurstaki* against egg plant borer *Leucinodes orbonalis* (Pyraustidae: Lepidoptera). ***International Journal of Integrative Biology***, **3**: 209-213.

Yeo IO, Choi SH, Lee JS and Kim CJ (1995). Characteristics of an alkaline protease from *Alteromonas* sp. ***Agricultural Chemistry and Biotechnology***, **38**: 106-110.

Yezza A, Tyagi RD, Valéro JR and Surampalli RY (2005). Production of *Bacillus thuringiensis* based biopesticides by batch and fed-batch culture using wastewater sludge as a raw material. ***Journal of Chemical Technology and Biotechnology***, **80**: 502-510.

Yezza A, Tyagi RD, Valéro JR and Surampalli RY (2006). Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis*

based biopesticides in pilot fermentor. ***Bioresource Technology***, **97**: 1850-1857. doi:10.1016/j.biortech.2005.08.023

Yossan S, Reungsang A and Yasuda M (2006). Purification and characterisation of alkaline protease from *Bacillus megaterium* isolated from Thai fish sauce fermentation process. ***Science Asia***, **32**: 377-383.

Yum DY, Chung HC, Bai DH, Oh DH and Yu JH (1994). Purification and characterisation of alkaline serine protease from an alkalophilic *Streptomyces* sp. ***Bioscience Biotechnology and Biochemistry***, **58**: 470-474. doi:10.1271/bbb.58.470

Yun SW, Lee K P, Yu JH, Shin, CS and Oh DH (1989). Purification and properties of alkaline protease from *Streptomyces* sp. YSA-130. ***Korean Journal of Applied Microbiology and Bioengineering***, **17**: 358-364.

Zakirov MZ, Shchelova SS and Karavaeva NN (1975). *Torulathermophila*, strain UzPT-1-A thermo-philic producing agent of proteolytic enzymes. ***Prikl Bio- khim Mikrobiol***, **11**: 686-690.

Zhang L (2008). Biology and pest management of spider mites. department of regional development, primary industry, fisheries and resources. Factsheet ent4.

Zhang L, Li J and Zhou K (2010). Chelating and radical scavenging activities of soy protein hydrolysates prepared from microbial proteases and their effect on meat lipid peroxidation. ***Bioresource Technology***, **101**: 2084-2089.

Zhuang L, Zhou S, Wang Y, Liu Z and Xu R (2011). Cost-effective production of *Bacillus thuringiensis* biopesticides by solid-state fermentation using wastewater sludge: Effects of heavy metals. ***Bioresource Technology***, **102**: 4820-4826. doi:10.1016/j.biortech.2010.12.098

Zouari N and Jaoua S (1999). Production and characterisation of metalloproteases synthesized concomitantly with δ-endotoxin by

Bacillus thuringiensis subsp. *kurstaki* strain grown on gruel-based media. ***Enzyme and Microbial Technology***, **25**: 364-371. doi:10.1016/S0141-0229(99)00054-X

Zouari N, Achour O and Jaoua S (2002). Production of delta-endotoxin by *Bacillus thuringiensis* subsp *kurstaki* and overcoming of catabolite repression by using highly concentrated gruel and fish meal media in 2- and 20 dm3 fermenters. ***Journal of chemical Technology and Biotechnology***, **77**: 877-882. doi:10.1002/jctb.650.

Zouari N, Dhouib A, Ellouz R and Jaoua S (1998). Nutritional requirements of a strain of *Bacillus thuringiensis* subsp. *kurstaki* and use of gruel hydrolysate for the formulation of a new medium for δ-endotoxin production. ***Applied Biochemistry and Biotechnology***, 69: 41-52. doi:10.1007/BF02786020