DUAL PRODUCTION OF ENDOTOXIN AND AMYLASE FROM *BACILLUS THURINGIENSIS* subsp. *KURSTAKI* BY FERMENTATION AND EFFICACY STUDIES OF ENDOTOXIN AGAINST ERIOPHYID MITE

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

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

Submitted by

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FEBRUARY, 2010

CERTIFICATE

This is to certify that the thesis entitled **" Dual production of endotoxin and amylase from *Bacillus thuringiensis* subsp. *kurstaki* by fermentation and efficacy studies of endotoxin against eriophyid mite"** is an authentic record of the research work accomplished by **Ms. R.B. Smitha,** at the Enzyme Technology Laboratory in the Department of Botany, University of Calicut under my supervision and that no part there of has been presented earlier for the award of any other degree or diploma.

Dr. Sailas Benjamin
(Research Supervisor)

Calicut University
27th February, 2010

DECLARATION

I, R. B. Smitha do hereby declare that this thesis entitled **"Dual production of endotoxin and amylase from *Bacillus thuringiensis* subsp. *kurstaki* by fermentation and efficacy studies of endotoxin against eriophyid mite"** is the summary of the research work carried out by me under the supervision of **Dr. Sailas Benjamin**, Reader, Department of Botany, University of Calicut in partial fulfillment of the requirement for the award of Ph.D. degree in Biotechnology 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
27th February, 2010

R.B. SMITHA

ABBREVIATIONS

α	:	alpha
∏	:	pi
Å	:	Angstrom
ATPs	:	Aqueous Two Phase Systems
B	:	Bacillus
BLA	:	Bacillus licheniformis amylase
Bt	:	Bacillus thuringiensis
Bti	:	Bacillus thuringiensis var.israelensis
Btk	:	Bacillus thuringiensis var.kurstaki
EDTA	:	Ethylene Diamine Tetraacetic Acid
ddH_2O	:	double distilled water
DNS	:	Dinitro Salicylic Acid
g	:	gram
g/L	:	gram per litre
gds	:	dry weight of the substrate
L	:	Litre
mA	:	milli Ampere
mg	:	milligram
mg/L	:	milligram per litre
mL	:	milli Litre
ß	:	beta
RT	:	Room temperature
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
U/mL	:	Units per milliliter

U/gds	:	Units per gram dry weight
w/v	:	Weight per Volume
γ	:	gamma
δ	:	delta
µg	:	microgram
µL	:	microLitre

EQUIPMENTS USED

Item	Brand	Country
Compound microscope	Magnus	India
Compound microscope with oculometer	Magnus	India
Chromatography column	BioRad Biologic LP	Italy
Deep freezer	Operon	Korea
Digital pH meter	Systronics	India
Distillation Unit	Borosil	India
Electrophoresis Unit	Biotech	India
Environmental shaker	Orbitek	India
Gel-documentation system	BioRad	Italy
Flourescent Microscope	Olympus	Japan
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
Refrigerated centrifuge	Plastocrafts/Remi	India
Refrigerator	Godrej	India
scanning electron microscope	JEOL, JWS 3000	Japan
UV-Visible spectrophtometer	Shimadzu	Japan
Vortex mixture	Kemi	India
Water bath	Scigenics Biotech	India
Web cam Companion 2.0	MEM 1300	Japan
Weighing balance (AX 200)	Shimadzu	Japan

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Dedicated

to…… my parents who dare me a dream
to…… my friends who made my dream into a reality
to……..my teachers who guided through the way
and
to…… God, the essence of all……..

CONTENTS

INTRODUCTION

The leading bio-rational pesticide - *Bacillus thuringiensis* (*Bt*) and its strains - is well known to produce toxins to combat various pests (mainly insects), which adversely affect the agricultural crops, but not known for its capacity to produce industrially useful α-amylase.

Bt is a ubiquitous soil-dwelling, Gram-positive, aerobic and sporulating bacterium that forms a parasporal crystal proteins during the stationary phase of its growth cycle. *Bt* was initially characterised as an insect pathogen, and its insecticidal activity was attributed largely or completely to this parasporal crystals. 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 of the class, Insecta. Later, *Bt* isolates were also found active against other insect orders and even effective against nematodes, mites and protozoa [1]. *Bt* is a proven alternative or supplement to synthetic chemical pesticide applications in commercial agriculture, forest management and mosquito control programmes. It is also a key source of genes for transgenic expression to integrate pest resistance in plants.

Over 30 strains of *Bt* with different specificities have been identified and characterised. Some common strains and their specificities towards their specific target pets are: *Bt aizawai* (*Bta*) is specific to wax moth larvae [2]; *Bt israelensis* (*Bti*) is specific to mosquitoes, black flies and some mites [3]; *Bt kurstaki* (*Btk*) is specific to gypsy moth and cabbage looper [4]; *Bt japonensis* (*Btj*) is specific to scarabid beetles [5]. *Bt sandiego* (*Bts*) and *Bt tenebrionis* (*Btt*) are specific to beetle species and the boll weevil [6]; *Bt tolworthi (Btto)* is specific to cutworms [7]; *Bt sotto* (*Bts*) is specific to cabbage butterfly [8]; *Bt berliner* (*Btb*) is specific to *Lygus spp.* [9]; and *Bt kenya (Btke)* is specific to species of *Helicoverpa and Scrobipalpula* [10].

Apart from *Bt*, some other species of *Bacillus* are also known to produce crystals and effectively used against various insects. For instance, crystals of *B.*

laterosporus are effective to combat *Musca domestica* and *Aedes aegypti* [11,12], and crystals of *B. sphaericus* are effective against *Culex quinquefasciatus* and *C. tarsalis* [13].

Commercial *Bt*-toxin is a mixture of endospores (which can give rise to new cells if the conditions are favourable to grow) and parasporal crystals. Current focus of *Bt*-research is based on the production of biopesticide, which accounts for about 1% of all pesticides [1]. *Bt* strains are known to produce two types of toxins. The principal type is the crystal (*Cry*) toxins, encoded by different cry genes, and this is how different types of *Bt* are classified [14]. The second types are the cytolytic (*Cyt*) toxins - which would augment the *Cry* toxins - enhance the effectiveness of insect control. Over 50 genes that encode the *Cry* toxins have now been sequenced, which enabled the toxins to be assigned to more than 15 subgroups on the basis of sequence similarities. The *Cry* protein is a highly insoluble protoxin (inactive) in normal conditions, so it is entirely safe to humans, higher animals and most insects. However, it gets solubilised at alkaline pH and thus became functional. The environment in the mid-gut of the pest larvae is suitable for the activation of this toxin. Once the protoxin is activated in the gut, the active component in the toxin damages the gut cells by signal transduction mechanism. It eventually casues the influx of ion into this damaged gut and thus pH is reduced. This acidc pH favours the germination of the endospores in the *Bt*-toxin leading, which leads to the total destruction of the insect.

Btk is a powerful biopesticide to combat lepidopteran insects, which include gypsy moth, spruce budworm, and hemlock looper, especially at the forest habitats [15]. *Btk* is commercially available as a biological insecticide under different trade names and is used in pest control programmes in forestry, agricultural and urban settings around the world [16]. The insecticidal activities of the CryIA(a-c) toxins from *Btk* Hd-1 were found much active against the larvae of *Choristoneura fumigferana*, *C. occidentalis*, *C.pinus*, *Lymantria dispar*, *Orgyia leucostigma*, *Malacosoma disstria* and *Actebia fennica* [17].

Apart from toxins, *Bt* is a good candidate for the production of extracellular enzymes, which could be useful to the industry. Production of amylase and protease from culture supernatant of *Bt* strains is less documented globally [18, 19]. A few unfocused studies show that *Bt* secretes traces of amylase during its growth. Kuppusamy and Balaraman [20] have studied the production of extracellular proteolytic and amylolytic enzyme to assess the ability of *Bt* H14 strain in utilising complex carbon and nitrogen sources. Amylase activity was also detected in the culture supernatants [20]. Vu *et al*. [21] found that *Btk* produced amylase (0.1 U/mL), while studying the effects of various pH control agents such as ammonium acetate, ammonium sulphate, sodium acetate and sodium sulphate upon biopesticidal activity of *Btk* HD-1. They showed that amylase production in the fermentation medium is an important factor, because it can support the growth and synthesis of *δ*-endotoxin and other components of *Btk* through hydrolysis of residual starch.

Being an industrial species, *Bt* has been a focused candidate for *Bt*-toxin (δ-endotoxin/*Cry* protein) production by submerged fermentation (SmF) or liquid culture. Efficacy of solid-state fermentation (SSF) to produce *Bt*-toxin has not been explored yet. Moreover, no attempt has been made to isolate and characterise amylase from any strain of *Bt*. If a strategy is developed for the isolation of industrially valuable amylase as a by-product from the culture supernatant during crystal production, such a strategy would further boost the *Bt* industry. Thus, this pioneering study is aimed at the dual production and purification of α-amylase and *Bt*-toxin during growth of *Bt* subspecies *kurstaki* (*Btk*) in various culture media compositions. Coupled to this, the efficacy trial of the produced *Btk*-toxin on the microscopic coconut pest, *Aceria guerreronis* (mandari) causing much havoc to the coconut yield in India and the world over is also envisaged in this study.

In the light of this, the prime objectives of this study are:

- To standardise the growth characteristics of *Btk* under the production conditions
- To check the efficiency of raw natural starches as production medium

- To maximise α-amylase production employing SmF and SSF systems
- To purify and characterise α-amylase
- To maximise the endospore production by SSF
- To maximise the *Btk*-toxin (δ-endotoxin/Cry protein) production by SSF, and
- To evaluate the efficacy of *Btk*-toxin to combat *A. guerreronis*

2. REVIEW OF LITERATURE

Present study on *Bacillus thuringiensis* subspecies *kurstaki* has various facets, *viz*., α-amylase production, the correlation between α-amylase production during δ-endotoxin (*Bt*-toxin/ crystal protein) synthesis and efficacy studies of this *Bt*-toxin to combat the microscopic mite, *Aceria guerreronis*. Hence, these aspects are reviewed focusing on δ-endotoxin and its toxicity, various aspects of α-amylase produced by bacillial species, and lastly *A. guerreronis* (mandari), a dangerous coconut mite.

2.1. *Bacillus thuringiensis*

Bt is a biopesticide with unusual properties that make it useful for pest control in certain situations. *Bt* is a naturally occurring bacterium common in soils throughout the world. Several strains can infect and kill insects. Because of this property, *Bt* has been developed for insect control [22]. However, it was not commercially available until the 1950s. In recent years, there has been tremendous renewed interest in *Bt*. New products have been developed, largely because of the safety associated with *Bt*-based insecticides [23]. *Bt* insecticides are most commonly used against some leaf- and needle-feeding caterpillars. Recently, strains have been produced that affect certain fly larvae, such as mosquitoes, and larvae of leaf beetles. *Bt* is considered safe to people and nontarget species, such as humans and wildlife [24]. Some formulations can be used essentially on all food crops.

Bacillus thuringiensis subspecies *kurstaki*, commonly referred to as *Btk*, is a bacterium found naturally in soils [25]. For about 30 years, it has successfully been used world-wide as a biological pest control agent to combat a variety of forestry and agricultural insect pests [26]. *Btk* produces a protein crystal during the spore-forming stage of its life cycle which is toxic only to the larvae of specific insect species [27]. When they feed on foliage treated with *Btk*, these microscopic crystals are ingested by the insects. In the alkaline environment of the susceptible insect's digestive system, the crystals are converted into toxic protein molecules that destroy

the walls of the insect's stomach, which eventually causes death. The insect usually stops feeding within hours and dies within two to five days. *Btk* works against a group of insects called lepidopterans**,** which includes destructive tree pests such as gypsy moths, spruce budworms and forest tent caterpillars [28]. *Bt* strains with their genes encoding various toxic proteins, crystal shape and MW of the crystal proteins, target pest, *etc*., are summarised in the Table 1 [3, 7 - 12, 28 - 64].

Table 1: *Bt* strains with their genes encoding various crystal proteins, MW with shape of the crystal proteins and target pest.

***Bacillus* spp.**	**Gene**	**Protein size (kDa)**	**Crystal shape**	**Target pest**	**Ref**
Bt BRL 43	Cry 2Aa	-	-	First instar larvae of cotton leaf worm, cotton boll worm and black cut worm	[29]
Bt IPS 78/11	CryIAc1 & Ab5	65 & 130	-	Manduca sexta	[30]
Bt	CryIAb	250	-	Manduca sexta	[31]
Bt	cryIA(c)	-	-	Cabbage butterfly (*Pieris brassicae*) larvae	[32]
Bt tolworthi	Cry9Ca1 & 1Ab5	-	-	Cutworms, Ostrinia nubilalis and Plutella xylostella, Spodoptera exigua and P. xylostella	[7]
Bt	CryIIIC	72	Bipyramidal	Colorado potato beetle larvae	[33]
Bt	cry19Aa	-	-	*Aedes aegypti*	[34].
Bt israelensis IPS78/11	Cyt1Aa & 2Aa	-	-	Sheep blowfly species (*Lucilia cuprina*, *L. sericata*, and *Calliphora stygia*).	[35]
Bt finitimus B-1166 VKPM	cry26Aa1 & 28Aa1	-	-	-	[36]

***Bacillus* spp.**	**Gene**	**Protein size (kDa)**	**Crystal shape**	**Target pest**	**Ref**
Bt	-	-	-	Lepidoptera and Diptera	[19]
Bt israelensis	Cyt1Aa, 2Ba & 1Ca	-	-	-	[37]
Bt	CryIAb	-	-	gypsy moth (*Lymantria dispar*).	[38]
B. laterosporus	-	-	Canoe-shaped	-	[11]
Btk HD1 & *Btk* HD73	cry1Ac	190 to 200	-	*Manduca sexta* larvae *Heliothis virescens*	[39]
Bt	Cry1Aa, 1Ba, 1Ca, & 9Ca	-	-	Bean shoot borer (*Epinotia aporema*) larvae	[40]
Bt H14	-	43	-	*Leishmania. major*	[41].
Bt sotto	CryIA(a)	-	-	Cabbage butterfly	[8]
B. sphaericus	-	-	-	*Culex pipiens*	[42].
Bt	-	-	-	Gypsy moth, *Lymantria dispar*	[43]
Bt berliner	-	-	-	*Lygus hesperus* (Hemiptera: Miridae)	[9]
Bt	Cry1Aa1	130 & 68	-	second instar larvae of *Spodoptera litura*	[44]
Bt israelensis	Cyt1, 2, Cry 4A & B, Cry10 & 11	-	-	*Aedes aegypti*	[3]
B. sphaericus & *Bt israelensis*	Cry4A, 4B, 11A, & Cyt1A	-	-	*Culex quinquefasciatus* *Cx. tarsalis*	[13]
B. laterosporus & *Brevibacillu*	-	-	-	*Musca domestica* and *Aedes aegypti*	[12]

***Bacillus* spp.**	**Gene**	**Protein size (kDa)**	**Crystal shape**	**Target pest**	**Ref**
s laterosporus					
Bt; Bt kurstaki; Bt kenyae	-	-	-	*Helicoverpa zea; Scrobipalpula absoluta*	[10]
Bt	Cry1A & 1Ja	-	-	*Cacyreus marshalli* (Lycaenidae), *Lobesia botrana* (Tortricidae), *Manduca sexta* (Sphingidae), *Pectinophora gossypiella* (Gelechiidae), *P. xylostella* (Plutellidae), and *Spodoptera exigua* (Noctuidae))	[45]
Bt	Cry2Aa & 2Ab	65	-	*Autographa californica* & *Spodoptera frugiperda*	[46]
Bt	Cry1Aa, 1Ab, 1Ac, 1C, & 1E	-	Bipyramidal & round shaped	*Spodoptera exigua*	[47]
Bt BT8	Cry1A	134	-	*Ostrinia furnacalis, Helicoverpa armigera, Chilo suppressalis*, and *Plutella xylostella*	[48]
Btk HD-1	CryBi & B2	71	Cuboidal	Dipteran (*Aedes aegypti*)& lepidopteran (*Manduca sexta)* larvae	[28]
Bt	Cry1Ca	65	Cuboidal	*Anticarsia gemmatalis* & *Spodoptera frugiperda*	[49]
Bt	Cry1Ca	-	-	-	[50]

***Bacillus* spp.**	**Gene**	**Protein size (kDa)**	**Crystal shape**	**Target pest**	**Ref**
Btk BNS3	Cry2Aa	71 & 130	-	-	[51]
Bt 4.0718	Cry1Aa, 1Ac, 2Aa, & 2Ab	130	Bipyramidal & cuboidal	*Plutella xylostella*	[52]
Bt	Cry1C	-	-	adult *Heliothis virescens & Spodoptera exigua*	[53]
Bti	Cry2A & Cry3A	135 & 70		*Spodoptera exigua*	[54]
Btk HD73	CryIAc	-	-	*Manduca sexta*	[55]
Btk HD1 & *Bti* 4Q2-72	Cry1Aa, 1Ab, 1Ac, 2Aa, 4Aa, 4Ba, 10Aa, 11Aa, Cyt1Aa & 2Ba	130 & 60	-	-	[56]
Bt & *Bt thuringiensis*	-	-	-	Bee moth (*Galleria mellonella* L.)	[57]
Bt	Cry1-hybrid SN19 & Cry3Aa	150 & 75	Flat square or rectangular & bipyramidal.	Colarado potato beetle	[58]
Bt.	Cry1Aa, 1Ab & 1Ac	65	-	*Helicoverpa armigera*	[59]
Bt	Cry1Ba & 1Ia	-	-	Colorado potato beetle	[60]
Btk (serotype H3a, 3b, 3c) strain BNS3	cry1Aa, 1Ac, 2Aa & 1Ia	62	-	*Prays oleae*	[61]

***Bacillus* spp.**	**Gene**	**Protein size (kDa)**	**Crystal shape**	**Target pest**	**Ref**
Bt	Cry5B & 6A	3 to 140	-	*Meloidogyne hapla, Pratylenchus scribneri, Tylenchorhynchus sp., Ditylenchus destructor and Aphelenchoides sp*	[62]
Btk HD 1	Cry1C, 1Aa, 1Ac, 2A & 2B	130 & 65	Bipyramidal	*Achaea janata*	[63]
Bt HD73	CryIC & IAc	137, 120, 115, 68, 65, 63 & 45	-	*Spodoptera frugiperda & S. exigua*	[64]

2.1.1. *Btk-toxin (*δ-endotoxin/ crystal protein)

Biotechnology offers sustainable solutions to the problem of pests, pathogens, and plant parasitic nematodes in the form of insecticidal protein genes [5]. *Btk* produces δ-endotoxins, which is effective against various pests [1]. Along with δ-endotoxins, *Btk* produces extracellular enzymes like protease and amylases [20].

Commercial *Bt*-toxin or *Bt*-pesticide is a mixure of endospores and parasporal cyrtals. *Bt-toxin* is available with different application modes (emulsion, spray, *etc*.) under various trade names. Upon injection (mainly larvae), the parasporal crystal is activated in the digestive tract of the host which ultimately damages the intestinal cells. This cell damage results in low pH, there the spores in the *Bt*-toxin germinate and cause further attack on the host eventually causing death.

In the present era of transgenic technology, insecticidal proteins of *Bt* assume considerable significance in the production of insect-resistant crops such as maize, cotton, potato, rice, vegetables, *etc*. [65]. *Bt* is a Gram-positive sporulating aerobic

bacterium isolated from a wide variety of environments like soil, insect cadavers, stored grain products and phylloplanes [66]. The importance of *Bt* is that it accumulates certain proteins in crystalline form during sporulation phase, these proteins accumulated as parasporal crystals in the spornagium [67]. These proteins are toxic towards larvae of different orders of insect pests (*e.g.*, Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera) with different efficacies [1]. The toxicity of *Bt* toxin is highly specific and it is non-toxic to mammals and beneficial insects. *Bt* was initially characterised as an insect pathogen, and its insecticidal activity was attributed largely or completely (depending on the insect) to this crystal (Cry) proteins. These δ-endotoxins, synthesised as protoxins, are produced in large quantities during sporulation and are packaged into intracellular inclusions.

More than 150 different *Cry* toxins have been cloned and tested for their toxicity on various insect species till date [68]. In an attempt to accommodate the growing list of new toxin genes/proteins, a new nomenclature has been formulated, wherein each toxin gene/protein will be having four-letter code, according to their amino acid sequence identity among them [68]. *Bt* and its toxins have been extensively studied for their molecular mechanism of action and toxin structure-function relationships.

2.1.2. Classification of *Bt*-toxins

Bt has been used commercially in the form of dried spores and crystal toxins in agriculture since 1930s. *Bt* strains produce two types of toxins. The main types are the *Cry* (crystal) toxins, encoded by different *Cry* genes, and this is how different types of *Bt* are classified [14, 69]**.** The second types are the *Cyt* (cytolytic) toxins, which can augment the *Cry* toxins, enhancing the effectiveness of insect control mechanism. Over 50 of the genes that encode the *Cry* toxins have now been sequenced and these toxins were assigned to more than 15 groups on the basis of sequence similarities [70]. Over 100 varieties of *Cry* proteins are known to exist in *Bt* [71].

2.1.3. Structure of *Bt*-toxin

Bt δ-endotoxins are globular protein molecules, which accumulate as protoxins in crystalline form during late stage of the sporulation [72]. Protoxins will be solubilised in the alkaline environment of midgut of the insect larvae, and C-terminal part cleaved off to release ~66 kDa active N-terminal toxic molecule. The protoxin contains well-conserved cysteine residues (as many as 16 in Cry1Ac), which helps in bridging the protoxin molecules through intermolecular disulphide bonds and thereby crystal formation [68]. Currently, 3-dimensional protein structures have been determined for different *Bt* toxins through X-ray crystallography. Among them, two are crystals forming (Cry) proteins or δ-endotoxins *viz*. Cry1Aa (Lepidoptera-specific) [73] and Cry3A Coleoptera-specific [74]. Since primary amino acid composition determines the final structure of a protein, closely related proteins - Cry1Aa and Cry3A - with 36% amino acid sequence identity showed superimposable structures with similar mode of action, whereas Cyt2A protein, which shares less than 20% amino acid sequence identity has a single domain with different functional properties [1]. The tertiary structure of δ-endotoxins comprised of three distinct functional domains connected by a short conserved sequence. Each domain of δ-endotoxin has independent and inter-related functions in the larval midgut, which brings out colloid osmotic lysis [71].

2.1.4. Mode of action

Bt crystals are aggregates of a large protein (about 130-140 kDa) that is actually a protoxin – it must be activated before it has to claim any effect. The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects [24]**.** However, it is solubilised in reducing conditions of high pH (above about 9.5) -the conditions commonly found in the mid-gut of insect larvae. For this reason, *Bt* is a highly specific insecticidal agent. Once it has been solubilised in the insect gut, the protoxin is cleaved by a gut protease to produce an active toxin of about 60 kDa [14, 58]. It binds to the midgut epithelial cells, creating pores in the cell membranes and leading to equilibration of ions. As a result, the gut is rapidly immobilised, the epithelial cells lyse, the larva stops

feeding, and the gut pH is lowered by equilibration with the blood pH [24, 58]. This lower pH enables the bacterial spores to germinate and the bacterium can then invade the host, causing a lethal septicaemia [75].

Recent studies on the δ-endotoxin structure show that it has three domains. Domain I is a bundle of 7 alpha helices, some or all of which can insert into the gut cell membrane, creating a pore through which ions can pass freely [14]. Domain II consists of three antiparallel beta–sheets, similar to the antigen-binding regions of immunoglobulins, suggesting that this domain binds to receptors in the gut. Domain III is a tightly packed beta–sandwich which is thought to protect the exposed end (C-terminus) of the active toxin, preventing further cleavage by gut proteases. Interestingly, the diphtherial toxin (of another bacterium) has an essentially similar structure to that of *Bt* [58].

2.1.5. Toxicity assay for δ-endotoxins

Ingestion of the inclusions by insect larvae leads to protoxin solubilisation and conversion to toxins, each specific for one of several orders of insects or other pests. The toxins form cation-selective channels in the membrane of cells lining the larval midgut with subsequent lethality [76]. Upon ingestion, it punctures the insect's midgut thus causing it to leakout the cellular contents and ions uncontrollably and consequently bringing about death. The conversion of δ-endotoxins of *Bt* into active toxins is mediated by trypsin, insect gut (exogenous) and bacterial (endogenous) proteases. These proteases also play a role in influencing the host range of toxin and in the development of resistance to toxin [77]. These observations led to the development of bioinsecticides based on *Bt* for the control of certain insect species among the orders; Lepidoptera, Diptera and Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga and also against nematodes, mites and protozoa [1].

There are different strains of *Bt*, each with specific toxicity to particular types of insects: *Bt aizawai* (*Bta*) is used against wax moth larvae in honeycombs; *Bt israelensis* (*Bti*) is effective against mosquitoes, black flies and some midges; *Bt kurstaki* (*Btk*) controls various types of lepidopterous insects, including the gypsy

moth and cabbage looper; strain *Bt sandiego* (*Bts*) is effective against certain beetle species and the boll weevil; and *Bt japonensis* (*Btj*) controls many species of scarabid beetles [14]. Monitoring the target insect population before application ensures that insects are in the vulnerable larval stage. More than 150 insects, mostly lepidoterous larvae, are known to be susceptible in some way to *Bt* [78].

Btk is effective against lepidopteran pests including the gypsy moth, spruce budworm, and hemlock looper. For instance, large-scale use of *Btk* against the gypsy moth and spruce budworm has been commercially employed over forested habitats [15]. *Btk* is commercially available as a biological insecticide under different trade names and is used in pest control programmes in forestry, agricultural and urban settings around the world [16]. The insecticidal activities of the CryIA(a - c) toxins from *Btk* Hd–1 were determined in force–feeding experiments with larvae of *Choristoneura fumigferana*, *C. occidentalis*, *C.pinus*, *Lymantria dispar*, *Orgyia leucostigma*, *Malacosoma disstria*, and *Actebia fennica* [17].

The δ-endotoxin found as a crystalline inclusion in *Btk* has potent insecticidal activities toward lepidopteran larvae. The major component of the crystals is a protoxin protein of molecular mass approximately 130-140 kDa. Upon ingestion by insect larvae, the crystals are subjected to the alkaline pH of the insect gut and as a result are cleaved with the release of a toxic peptide [79]. Fermentation of recombinant pseudomonads has been used to produce concentrated aqueous biopesticide formulations consisting of *Cry* inclusions encapsulated in dead cells. These encapsulated forms of the *Cry* proteins have been reported to show improved persistence in the environment [80]. The anti-lepidopteran toxin from sporulated *Btk* cells, generated by the proteolytic action of endogenous proteases on the protoxin, was purified and studied to identify the effect of such proteolysis on the biochemical nature of the toxin. The active toxin was purified employing anion-exchange chromatography to absolute homogeneity, as indicated by SDS-PAGE and Western blot techniques [81].

Otvos and Vanderveen [82] conducted an aerial insecticide spray programme to eradicate gypsy moths, employing *Btk* formulation under the trade – named Foray

48B. The active ingredients of Foray 48B were the spores and proteinaceous δ-endotoxin crystal of *Btk* strain HD-1, suspended in water at a concentration of 2.1% [83]. Screening of different adjuvants, *viz*, suspending agents, phagostimulants, stickers, antimicrobial agents and UV screens to develop aqueous biopesticidal suspensions of *Btk* HD-1 fermented broths, specifically, nonhydrolysed sludge, hydrolysed sludge, starch industry wastewater and soya (commercial medium), were investigated by several workers [84].

Briefly, it seems that *Btk* toxin production by liquid culrure (SmF) system is well established and their molecular biology and mode of action are well characterised, but solid-state fermention is not taken up in *Bt*-toxin production so far as a production strategy. These aspects would be discussed in appropriate chapters to follow.

2.2. α-Amylase

In this section, α-amylases have been reviewed focusing bacillial α-amylases, and it encompases a short general introduction on amylases, production of α-amylases by submerged and solid-state fermentations; nutrients and other factors required for maximising production; immobilisation strategies for whole cells or purified enzyme; an overview on the molecular weight of the enzyme; followed by distinct sections for purification, characterisation, stability and crystal structure; and finally applications of the α-amylases.

2.2.1. Introduction

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilise enzymes at some stage or the other**.** Industrial enzyme market is an oligopoly with few strong players, and amylases are one among them. Amylases are glycosidases which catalyse the hydrolysis of glycosidic linkage in starch to generate smaller sugars useful to bioindustry. Although amylase can be derived from several sources - such as plants, animals and microorganisms - the enzymes from microbial sources generally meet

industrial demands. The amylase family of enzymes has been well characterised through the study of various microorganisms, especially bacteria and fungi.

2.2.2. Classification of amylase

Amylases are classified into three viz., α–amylase, ß–amylase and γ–amylase. Unlike ß–amylase, α- and γ –amylases are produced in animal system abundantly, and all these three enzymes are produced by plants, yeast, fungi and bacteria. Four groups of starch converting enzymes have been identified *viz*., endoamylases, exoamylases, debranching enzymes and transferases [85]. Endoamylases cleave α-1,4 glycosidic bond in a random fashion present in the amylose or amylopectin chain (structural components of starch made of sugars) and α-amylase is a well known endoamylase. Exoamylases such as β-amylase and glucoamylase cleave both α-1,4, and/or α-1,6 glycosidic bonds [85].

2.2.2.1. ***Alpha amylase (EC 3.2.1.1)***: is alternatively known as 1,4-α-D-glucan glucanohydrolase or glycogenase [86 - 90]. The α-amylases are calcium metalloenzymes, *i.e*., the divalent calcium ion is invincible for its function [91]. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltose and maltotriose from amylose or maltose, glucose and 'limit dextrin' from amylopectin [92 - 93]. The hydrolytic products have α–configuration. As it can act anywhere on the substrate, α–amylase tends to be faster-acting than ß-amylase.

2.2.2.2. Beta amylase (EC 3.2.1.2): synonymous as 1,4-α-D-glucan maltohydrolase, glycogenase or saccharogen amylase synthesised by bacteria, fungi, and plants [94]. Working from the non-reducing end, ß-amylase catalyses the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time [95]. During the ripening of fruit, ß-amylase breaks starch into sugar, resulting in the sweet flavor of ripe fruit. ß-amylase is present prior to germination, whereas α-amylase and proteases appear once germination has begun. Amylase from the cereal grains is the key to the production of malt. Many microbes also produce amylase to degrade extracellular starches [96 - 98]. Animal tissues do not possess ß-amylase, although it may be present in microorganisms contained within the digestive tract.

2.2.2.3. Gamma amylase (EC 3.2.1.3): is also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase and 1,4-α-D-glucan glucohydrolase [99]. In addition to cleaving the last α-(1-4)-glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose, γ-amylase will also cleave α-(1-6)-glycosidic linkages [99]. Unlike the other forms of amylase, γ-amylase is best active efficient in acidic environments [100].

Amylases are traditionally produced by submerged or liquid fermentation (SmF). Of late, solid-state fermentation (SSF) advanced a lot to supersede the conventional SmF owing to its proven advantages.

2.2.3. α-Amylase production by submerged fermentation

Table 2 [85, 92 - 93, 101 - 112] illustrates an overview of amylase production by various species of *Bacillus*. *Bacilli* secrete a variety of enzymes including polysaccharases, proteases and nucleic acid hydrolysing enzymes. Amylase from *B. subtilis* was considered as an industrial enzyme as early as 1917, but commercial production of this enzyme began in the late 1940s with the introduction of submerged culture or fermentation (SmF) borrowed from antibiotic industry [113]. Currently, a limited number of strains of selected species are used to prepare major seven enzymes including α-amylases on a large scale. Bacillial species like *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* are the major producers of α-amylases [114]

SmF has been traditionally used for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH [115 - 117]. Utilisation of agroindustrial residues as substrate for the fermentation received growing interests as they are inexpensive energy-rich sources and also eliminates large-scale accumulation of the biomass and thus most of the commercial processes are based on SmF [116, 118].

Saui *et al.* [119] produced α-amylase from *Bacillus* sp. AS-1 and found that 2% starch was a good supplement for enzyme synthesis. They also found that

simple sugars like lactose, glucose and soluble starch could stimulate production. Effect of different nitrogen sources revealed that 1% peptone or 2-4% yeast extract could increased the enzyme production [119]. Employing *B. licheniformis*, the composition of fermentation media and conditions for SmF were optimised by Tsurikova *et al.*, [108]. They obtained a maximum of 260 U/mL α-amylase acitivity. Gangadharan *et al.* [118] optimised the cultural and production parameters for the synthesis of α-amylase by *B. amyloliquefaciens* in SmF using a combination of wheat bran and ground nut oil cake (1:1) as the substrate. Lower agitation rate would be better for maximal α-amylase synthesis [120].

Ajai and Fagade [101] found that *Bacillus* spp. grow at different rate with specificity to different substrates in culture medium. Results indicate that the amylase production values ranged from 0.22 x 10^2 U/cfu (Units of enzyme activity per colony forming units) by *B. circulans* to 0.912 x 10^2 U/cfu by *B. licheniformis* for corn starch and 0.01 x 10^2 U/cfu by both *B. megaterium* and *B. licheniformis* to 0.693 x 10^2 U/cfu by *B. subtilis* for soluble starch. All these species are the major producers of amylases [101]. Oguntoyinbo *et al.* [102] found that a wild strain of *B. subtilis* isolated from Nigeria showed 13 U/mL as the maximum amylase activity. According to Srivastava and Baruah [121], *B. sterothermophilus* grew better on complex and semisynthetic medium than on synthetic medium. They noticed that α-amylase production was higher on the complex medium containing beef extract or corn steep liquor compared to a semisyntheic medium containing peptone. Saui *et al*. [119] found that 2 % w/v starch supplementation was effective for amylase synthesis by *Bacillus* sp. AS – 1. Effect of peptone and yeast extract in the presence of 1.5 % starch increased the α-amylase production by *B. subtilis* strain [122].

In shake flask culture of *B. thermooleovorans*, a combination of starch and tryptone was ideal nutrients being carbon and nitrogen sources, respectively [123]. In this study, 2.2 fold increase in amylase production was observed at 12 h then it declined, which continued upto 45 h. Agrawal *et al.* [85] isolated *Bacillus* sp. KCA 102 strain from soil which produced theromostable amylase (94 U/mL) in a short fermentation time 18-20 h. Mishra and Behera 2008 [103] isolated a *Bacillus* strain from soil sample receiving kitchen waste and showed optimum growth at temperature 37 ^oC in a pH range 6.8- 7.2. The maximum enzyme activity (75.7 U/mL) was observed at 2% soluble starch concentration in the starch-agar medium

[103]. Heng *et al.* [107] reported that the extracellular α-amylase activities in *B. licheniformis* and *B. subtilis* were 1001 and 2012 U/mL.

Ikram-ul-Haq *et al.* [124] conducted selection study employing commercially available agricultural starchy substrates like hordium, pearl millet, rice, corn, gram and wheat starch for the production of α-amylase by *B. licheniformis*. 1.5 % pearl millet starch significantly enhanced amylase production. Rao and Satyanarayana [125] found that α-amylase activity on several starch substrates and their derivatives. The enzyme hydrolysed raw starch of pearl millet efficiently [125].

Table 2 reveals that *B. licheniformis* and *B. subtilis* are the major producers of bacillial α-amylase. In general perspective, considering the thermostability, α-amylase from *B. licheniformis* is mostly preferred by the industry.

Table 2: α-Amylase production by bacillial spp. in various media with or without starch supplement by SmF.

***Bacillus* sp.**	**Starch supplement**	**Yield (U/mL)**	**reference**
B. circulans,	Corn starch and soluble	22	[101]
B. licheniformis,	starch	91.2	
B. megaterium		1	
B. subtilis		69.3	
B. subtilis	Soluble starch	13	[102]
Bacillus sp.	Soluble starch	75.7	[103]
Bacillu sp. KCA 102	Soluble starch	94	[85]
B. subtilis	LB agar medium	41.4	[104]
B. subtilis	Soluble starch	28	[105]
B. subtilis KCC 103	Sugarcane bagasse hydrolysate	144.5	[92]
B. halodurans	Soluble starch	1.8	[106]
B. licheniformis	LB medium	1001	[107]
B. subtilis		2012	
Bacillus sp. strain TSCVKK	Soluble starch	592	[93]
B. licheniformis	Soluble starch	260	[108]
B. licheniformis M27	Soluble starch	480	[109]
B. flavothermus	Lactose	28.6	[110]
B. subtilis JS 2004	Waste potato starch	72	[111]
Bacillis sp. Strain SMIA 2	Soluble starch	37	[112]

2.2.4. α-Amylase production by solid-state fermentation

Current developments in biotechnology are making use of enzymes for newer applications. Solid state (substrate) fermentation (SSF) holds tremendous potential for the production of enzymes. SSF has been defined as the fermentation process occurring in the absence or near-absence of free water. SSF has become a very attractive alternative to submerged fermentation (SmF) for specific applications due to the recent improvements in reactor designs. SSF processes generally employ a natural raw material as carbon and energy source [115]. SSF can also employ on inert material as solid matrix, which requires supplementing a nutrient solution containing necessary nutrients as well as a carbon source.

SSF has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products [116]. Its application in bioprocesses such as bioleaching, biobeneficiation, bioremediation, biopulping, *etc*. has offered several advantages. SSF has several biotechnological advantages; though at present on a laboratory scale only, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialised for water-insoluble substrates and lower demand on sterility due to the low water activity used in SSF.

A survey as in Table 3 [102, 126 - 137] shows that agroresidues are used mostly as the solid substrate for the production of α-amylase by various *Bacillus* spp.

Table 3: α-Amylase production by bacillial spp. on various solid media by SSF.

***Bacillus* sp.**	**Solid substrate**	**Yield (U/gds)**	**reference**
B. licheniformis M27	Wheat bran	21,000	[126]
B. licheniformis	Potato peel and wheat bran	270; 175	[127]
B. megaterium 16M	Wheat bran	30,000	[128]
B. subtilis	Wheat bran and rice husk	159,520; 21,760	[129]
B. subtilis	Banana peel	9	[130]
B. licheniformis *B. subtilis*	Potato peel and wheat bran	600; 265	[127]
Bacillus sp.	Wheat bran and lentil husk	172,800; 216,000	[131]
Bacillus sp. PS-7	Wheat bran	4, 64,000	[132]
B. cereus	Wheat bran and rice flake manufacturing waste	122	[133]
B. amyloliquefaciens	WB + Ground nut oil cake (1:1)	62,470	[134]
Bacillus sp.	Potato peel	270	[127]
B. subtilis SDA3,	Dehulled cooked soybean into soy-daddawa (a condiment)	4.0	[135]
B. circulans (GRS313)	Wheat bran	590	[136]
B. subtilis DSM 347,	Okpehe, a traditional fermented condiment in Nigeria	13	[102]

The growth pattern and microbial biomass formed during metabolic activities of the *Bacillus* species viz., *B. circulans, B. licheniformis, B. megaterium* and *B. subtilis* on starchy substrates was determined by Ajai and Fagade [101]. Apart from soluble starch, the amylolytic *Bacillus* spp. utilised white corn starch substrate as a sole carbon [101]. α-Amylase activity was higher in corn starch

medium than of soluble starch. Rao and Satyanarayana [125] purified α-amylase from *Geobacillus thermoleovorans* and the enzyme exhibited activity on several starch substrates and their derivatives, hydrolysed raw starch of pearl millet efficiently.

Kokab *et al.* [130] used banana peel as the solid substrate for the production of α-amylase from *B. subtilis* by SSF. Effect of varying incubation period, substrate level, pH of the medium, incubation temperature, nitrogen source and micronutrients on the production of α-amylase was also investigated [130]. Shukla and Kar [127] found that potato peel was superior over wheat bran to produce α-amylase from two thermophilic isolates of *B. licheniformis* and *B. subtilis*. Production of alkaline α-amylase by *Bacillus* sp. under SSF was optimised by Baysal *et al.*, [129]. They examined the effect of wheat bran and lentil husk for enzyme production and lentil husk supported more enzyme production (Table 3). The appropriate incubation time, inoculum size, moisture level, and buffer solution requirement were determined by Baysal *et al.*, [131].

Gargi *et al.* [138] employed response surface methodology to study the cumulative interactive effect of the macronutrients of the media and to optimise their concentration to enhance the production of maltooligosaccharide-forming amylase from *B. circulans* GRS 313. Soybean meal, yeast extract and wheat bran were the nutritional constituents used in their work and the amylase production increased over 1.25 fold. The combined effect of macronutrients of medium on α-amylase production by *Bacillus* sp. was studied using response surface methodology by Tanyildizi *et al.*, [139]. The medium constituents were starch, glycerol, yeast extract and peptone. The results showed that yeast extract had no effect on amylase production and maximum α-amylase production was determined in media with starch, glycerin and peptone.

Interestingly, *in situ* fermentation using microbes bearing GRAS label to enhance the palatability and flavour of vegetables, pulses and other for raw food stuffs is getting more attention these days [140]. Oguntoyinbo *et al.* [102] used *B. subtilis* to make Okpehe, a traditional fermented condiment in Nigeria. Among other

enzymes, amylase (13 U/mL) also contributed to the nutritional enrichment. Omafuvbe [135] converted sterile dehulled cooked soybean into soy-daddawa (a condiment) using *B. subtilis* as a starter culture. α-Amylase activity increased and attained a peak at the 48 h and then droped in 30 and 35 ^{o}C. However, organoleptically soybean fermented at 35 ^{o}C was good.

SmF has traditionally been used for the production of industrially important enzymes, owing to its ease of handling and greater control of environmental factors such as temperature and pH. In comparison SSF with optimised production parameters seems to be better for enhanced amylase production.

2.2.5. Other nutrients favouring α-amylase production

Carbon and nitrogen sources, coupled with their proper ratio are important parameters required for offering better growth to the microbes during fermentation. With *B. sphaericus*, Al-Qodah *et al.* [141] showed that optimum starch concentration was 3.2% in the medium. *B. subtilis* DM03 showed maximum production in the presence of soluble starch and NH_4Cl [142]. Saui *et al.* [119] found that a combination of 2.0% starch as carbon source and 10 g/l peptone as nitrogen source was good for *Bacillus* sp.AS-1 to produce α-amylase. Studies with strains of *B. amyloliquefaciens* and *B. subtilis***,** El-Tayeb *et al.*, [143] found that multi-protein mineral media in bioreactor supplemented with starch with initial 2.5 or 3.5 % concentration and subsequent addition of 2% at about 24 h was a good medium for α-amylase production. For *B. subtilis* KIBGE-HAR, the optimum feed ingredients were: 15 g/l starch, 5 g/l peptone, 1g/l yeast extract and 20 mg/l $CaCl_2$ [122]. *B. flavothermusin* produced 28.6 U/mL α-amylase in a feed containing 2% yeast extract and 4% lactose [110].

Starch and tryptone were found to be ideal C and N sources, respectively for the cultivation of *B. thermooleovorans* [123]. But in a chemically defined medium consisting of glucose, riboflavin, cysteine, $MgSO_4$, K_2HPO_4 and NaCl, production increased by 2-fold. In a laboratory fermenter with optimised medium at near abolition of gas phase, 2.2 fold increase in production was observed with reduction in optimal production time from 12 to 4-5 h [123]. Wheat bran and ground nut oil

cake in 1:1 ratio was found to be better for α-amylase production in SmF by *B. amyloliquefaciens* [118]. Process parameters such as optimum substrate concentration, incubation period (42 h) and $CaCl_2$ (0.0275M) were identified using Plackett-Burman design [118].

Sodhi *et al.* [132] found that *Bacillus* sp. PS-7 could produce a maximum of 4,64,000 U/gds α-amylase on wheat bran supplemented with 1% glycerol (w/w), 1% soybean meal (w/w), 0.1% proline, 0.01% vitamin B-complex, 1% Tween 40, 1 mM $MgSO_4.7H_2O$ at 48 h and 37 ºC. Of different agricultural starchy substrates - starch, hordium, pearl millet, rice corn, gram and wheat - pearl millet was found better at 1.5% level for amylase production by *B. licheniformis* [124].

2.2.6. Other factors controlling production

Enrichment of medium for fermentation is a crucial factor to maximise production. Srivastava and Baruah [121] cultivated *B. stearothermophilus* on complex medium containing beef extract or corn steep liquor. Carbohydrates such as starch, dextrin, glycogen, cellobiose, maltoheaose, maltopentaose, maltotetraose and maltotriose were used in the medium [121]. Monosaccharides such as inositol and D-sorbitol have repressed the enzyme production. Organic and inorganic salts such as KCl, sodium malate and potassium succinate increased the enzyme production than Na and K ions. Aminoacids such as isoleucine, cysteine, phenylalanine and aspartic acid were vital for enzyme production. Detergents such as Tween 80 and Triton X-100 repressed the enzyme production, but it increased biomass. Yoon *et al.* [144] found that an optimum phosphate level is required for maximum production of α-amylase in SmF by *B. amyloliquefaciens*.

Enhasy [145] found that the enzyme production from *B. amyloliquefaciens* was influenced by aeration. Production could be modified greatly by changing the shape of flask (baffled and non-baffled), working volume of fermentation media and shaking intensity [145]. The baffled flask with 200 rpm, 10% working volume increased the enzyme production by about 2200 µkat/l. By increasing the aeration rate of 1 vv/m in 3L stirred tank bioreactor, amylase activity was increased to 3600 µkat/l. The activity again increased to 5300 µkat/l after 34 h by the intermittent

addition of soluble starch [145]. Saui *et al*. [119] found that an optimum temperature of 35 ºC at pH 7.0 with 0.02% $CaCl_2$ increased amylase production and stabilitiy of *Bacillus* sp. AS-1. Khan and Husaini [137] found that optimum temperature, pH and rpm were crucial for optimum growth of *B. amyloliquefaciens*. They used sago pith residue (hampas) (4% w/v) in 0.2 M citrate buffer at pH 6.0, temperature 40 ºC and incubated for 6 h at 100 rpm. The addition of 1% soluble starch increased the enzyme production [137]. Al-Qodah *et al.* [141] cultivated *B. sphaericus* in a laboratory scale fermenter at pH 7.0 and temperature 50 ºC. The kinetic study of cellular growth indicates μ_{max}, K_s, t_d, $Y_{x/s}$ and K_d were 0.53 h-l, 1:1 g/l, 1.98 h, 0.44 g/cells/g starch and 0.4 g/l h, respectively. The optimum starch concentration for the production was 32 g/l and higher concentrations showed substrate inhibition constant Ki 190 mg/L.

2.2.7. Immobilisation

In order to increase better stability and durability, immobilisation, chemical modification strategies are being employed. Immobilisation of enzymes generally stabilises their structure and better adaptability to pH and temperature. Immobilised amylases offer several advantages: they can be reused; the process can be operated continuously with better controls, easy separation of the products, simpler handling of the materials, alteration of activity and thermostability and effective reduction in process cost.

B. subtilis was entrapped in a carrageenan gel [146]. The bead suspensions were submitted to two aeration modes, one consisting of bubbling air into a round flask, the other involving sparging of air into an airlift fermenter. The latter system, which produces microbubbles, gave 40-70% increase in enzyme production over the former and a doubling of bacterial density within the beads was measured. The use of $CaCl_2$, instead of KCl as polymerisation agent led to a better yield of α-amylase [146]. Groom *et al.* [147] immobilised cells of *B. amyloliquefaciens* to produce extracellular α-amylase on a large-pore, macroreticular anionic exchange resin.

The immobilised cell reactor was observed to achieve larger volumetric productivities than either mode of stirred tank fermentations, but achieved an

enzyme activity concentration lower than that of the batch stirred tank fermenter. Agar, agarose and alginate were also found good for immobilising cells of thermophilic *Bacillus* sp. [148]. In a novel technique, Amritkar *et al*., [149] incorporated polymeric substrates or substrate analogue during cross-linking of cellulose to prepare rigid, porous, cross-linked composite affinity matrices for the purification of α-amylase from *Bacillus* sp. B3. Alginic acid (AA) with cellulose produced the best affinity matrix for purification by batch, packed bed as well as expanded bed protocols. The optimised expanded bed protocol resulted 51-fold purification on AA-CELBEADS with 69% recovery. Takada and Hirai [150] showed that addition of polyvinyl alcohol (0.25 wt%) on the reaction system of α-amylase from *B. subtilis* enhanced the activity by 5 fold to that of the native state.

Whitney *et al.* [151] showed that cells of a mutant strain (H_1) of *B. subtilis* grown in standard growth medium (cells in log, stationary and early lag phases) were immobilised onto and into channeled porous alumina (oxide) beads with an average diameter of 3.8 mm and pore size range from 2.5 – 12 µm. The immobilised cells (~5 x 10^{12} cells/g bead) were placed in the column of the bioreactor system and were continuously fed the growth medium for α-amylase production. Maximal amylase productivity (about 427 u/mL effluent/h of reducing sugar equivalents) was achieved using stationary-phase cells and feed stock containing 3% starch kept at 45 ^{o}C and 100 mL/h flow rate. Immobilised *B. subtilis* cells in calcium alginate beads (2% sodium alginate and 3.5% $CaCl_2$, w/v) for the semi-continuous production of amylase was achieved by Konsoula and Liakopolou-Kyriakides [152]. Immobilised biocatalyst sustained 90% of their initial productivity over five sequential batches in a 10 day period, while amylase production by free cells declined sharply after second day of use.

B. licheniformis 44MB82-A cells (12 h culture) were immobilised in 4% alginate/agar [153]. The optimal initial cell quantity was found to be 0·6–3·0% in agar gel and 0·4% in Ca-alginate gel with bead sizes of 3·0 and 5·0 mm, respectively. Significant increases (2·2-fold) in the enzyme yields within the fourth

cycle of repeated-batch runs with cells entrapped in agar gel pellets with bead size 5·0 mm was observed.

Covalent binding has been extensively used as a tool to immobilise the enzymes. *B. licheniformis* α-amylase was immobilised on various carriers and the properties of the enzyme were compared before and after immobilisation [154]. Compared to the free enzyme, the optimum pH after immobilisation enzyme changed to acidic range and the optimum reaction temperature was shifted slightly to 70 - 80 ºC. The thermal stability of the immobilised enzyme was found to be higher than that of the free one.

Among the tested salts, $CaCl_2$ exerted a stimulating effect on the activity of α-amylase. Satish and Aniruddha [155] employed cross-linked cellulose matrix (CELBEADS) as a support for the immobilisation of *B. licheniformis* α-amylase (BLA). Optimum pH and temperature, and Michaelis-Menten constants were determined for both free and immobilised BLA. Immobilised BLA was observed to produce a different saccharide profile than free BLA at any value of dextrose equivalent. It was observed that pH, temperature, and initial starch concentration has a significant effect on the saccharide profile of starch hydrolysate produced using immobilised BLA in the batch mode, whereas the ratio of concentration of enzyme units to initial starch concentration has no influence on the same. Hence immobilised BLA can be used as an additional tool for the production of maltodextrins with different saccharide profiles. Immobilised BLA has better thermostability than free BLA. Immobilised BLA was found to retain full activity even after eight batches of hydrolysis, each of 8 h duration at 55 °C and 90 mg/ml initial starch concentration.

A semi-empirical model has been used for the prediction of saccharide composition of starch hydrolysate with respect to time [155]. Gargi *et al.* [138] entrapped the α-amylase produced by *B. circulans* in calcium alginate beads. At the optimum pH and temperature of 4.9 and 57 ºC, the apparent activity was 25.6 u/g of beads, with almost 2-fold increase in activity. The immobilised enzyme showed a

high operational stability by retaining almost 85% of the initial activity after seventh use.

2.2.8. Purification and characterisation of α-amylase

Table 4 shows the summary of amylase purification from various strains of *Bacillus*. Column chromatography employing sephadex [86 - 87, 142, 156 - 159] or sepharose [86, 142, 156 - 157, 160 - 162] has been the major matrix used to purify the protein. Few others have used vaious affinity matrices for the purification [149, 163 - 166].

Table 4: Activity and yield of bacillial α-amylases purified by various strategies.

Bacillus species	Purification Strategy	Activity(U/mg)	Yield (%)	Purification (fold)	Reference
B. licheniformis	CMC column	882.7	42	211.67	[163]
Bacillus sp.	QAE column	18.5	2.4	-	[164]
B. licheniformis	Sephadex G-100, mono Q Sepharose anion exchange columns	178.5	15.9	3.08	[156]
B. amyloliquefaciens in E. coli	Q – Sepharose & SP – Sepharose columns	31354	-	-	[86]
Bacillus sp. B3	AA – CELBEADS immobilisation	60.8	-	11.9	[149]
Bacillus sp. YX – 1	DEAE – Sepharose Fast Flow & Sephadex G-75 columns	607	6.60	34	[157]
B. subtilis DM – 03	DEAE Sephadex A – 50, Sephadex G – 50 & RP-	1421	2.9	80.5	[142]

Bacillus species	Purification Strategy	Activity(U/mg)	Yield (%)	Purification (fold)	Reference
Geobacillus thermodenitrificans HRO-10	HPLC columns, DEAE Sephadex A – 50 & Superdex – 200 columns.	684.3	11.5	13.6	[158]
B. licheniformis	Starch column	507	51	230	[165]
B. subtilis KCC-103	DEAE Sephadex A 50	482.4	45.2	19.9	[87]
B. *kaustophilus*	Metal – chelate column	39.7	49	11.4	[166]
B. subtilis	Sephadex G – 75 column	916.7	23.4	187.1	[159]
Lactobacillus amylovorus	Sepharose 6B column	-	69	75	[160]
B. subtilis X – 23	Q – Sepharose & Phenyl Sepharose columns	362	19.5	-	[162]

α-Amylase from *B. licheniformis* CUMC305 was characterised by Krishnan and Chandra [163]. Its *Vmax* for the hydrolysis (mg/mL) of soluble starch 0.738, amylase 1.08, amylopectin 0.8 and glycogen 0.5 (mg of maltose equivalents). Cations like Ca^{2+}, Na, Mg^{2+} showed stimulatory effect, while Ag^{+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} and Al^{3+} were found inhibitory. Anions like azide, SO_3^{2-}, SO_4^{3-}, $S_2O_3^{2-}$, $MoSO_4^{2-}$ showed excitant effect. *p*-Chloromercuribenzoate and sodium iodoacetate were also inhibitory, while cysteine, reduced glutathione, thiourea, *β*-mercaptoethanol, sodium glycerophosphate afforded protection to enzyme. It was fairly resistant to EDTA treatment at 30 ºC, but heating at 90 ºC in the presence of EDTA resulted complete loss of activity, which could be reduced by the addition of Cu^{2+} and Fe^{2+}, but not Ca^{2+} [163].

Das *et al.* [142] found that 4-bromophenacyl bromide (4 mM) and phenylmethylsulphonylfluoride (1.5 mM) completely abolished the α-amylase

activity of *B. subtilis* DM03, documenting the essential role of histidine and carboxylic residues in the catalytic process. Ca^{2+} was found as significant metal ion for maintaining the stability of amylase [132]. Maximum activity of amylase from *Bacillus* sp. PS-7 showed at 60 ºC and pH 6.5. Thermal stability profile revealed the half-life was about 6 h at 60 ºC, 5.5 h at 70 ºC in the presence of Ca^{2+} [132]. El-Tayeb *et al.* [143] observed that α-amylase from strains of *B. amyloliquefaciens* and *B. subtilis* liquefied starch to dextrose at 95 ºC in pH 4-8.

Hmidet *et al.* [156] isolated a thermostable α-amylase from *B. licheniformis* NH 1, which was purified to homogeneity by 40–60% ammonium sulphate precipitation, Sephadex G – 100 gel filtration and Sepharose mono Q anion exchange chromatography, and found that the chelating agent EDTA inactivated the enzyme, but activated by the metal ions Hg^{2+} and Zn^{2+}. The enzyme was highly active over a wide range of pH from 5-10 and optimum temperature of the purified enzyme was 90 ºC. A maltooligosaccharide forming α-amylase was produced by a new soil isolate (*B. subtilis* KCC 103) and found that EDTA abolished 50% of the enzyme activity [87]. It was highly active over a borad pH range from 5 to 7, though optimum temperature was 65-70 ºC, it was rapidly deactivated at 70 ºC with a half-life of 7 min and at 50 ºC, the half-life was 94 min, and the *Km* and *Vmax* for starch hydrolysis were found to be 2.6 mg/mL and 909 U/mg, respectively [87]. Bernhardsdotter *et al.* [164] isolated an alakaliphilic amylase producing bacterium *Bacillus* sp. strain L1711. Contrary to the above reports, this enzyme was strongly inhibited by Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+} and Cu^{2+}, whereas presence of Na^{+}, Co^{2+} and EDTA significantly enhanced the enzyme activity, and this feature is interesting to investigate at the molecular level. The pH optima for activity below and above 40 ºC were 9.5 – 10 and 7 – 7.5, respectively. It was stable in the pH range 6-11 and was completely inactivated at 55 ºC. Its *Km* and *Vmax* values were 1.9 mg/mL and 0.051 μmol/min, respectively.

Arikan [167] produced amylase from *Bacillus* sp. A3-15 and found that $ZnCl_2$, NaCl, $CaCl_2$, Na-sulphide, EDTA, urea and SDS inhibited the enzyme activity. The partially purified enzyme showed optimum activity at pH 11 and 70 ºC.

The enzyme was highly active (95%) in alkaline range of pH (10 – 11.5) and it was almost completely active up to 100 ºC with 96% of the original activity remaining after heat treatment at 100 ºC for 30 min [167]. Demirkan *et al.* [86] produced α-amylase from *B. amyloliquefaciens* and a mutant strain and found that Mg^{2+}, Ba^{2+} and Cu^{2+} stimulated the activity and Fe $^{2+}$, Hg^{2+}, Zn^{2+} and Ag^{2+} were potent inhibitors on the enzyme activity. Huang *et al.* [166] found that the α-amylase from *Bacillus* sp. strain TS 23 was purified through a series of steps and Ni^{2+} and Mn^{2+} stimulated the enzyme activity.

The α-amylase from *B. subtilis* WB600 was purified by ammonium sulphate fractionation, anion exchange and gel filtration by Liu *et al.*, [157] and found that the metal ions Cu^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cs $^{2+}$, Cd $^{2+}$, Fe $^{2+}$, $Hg^{2+}$and Ni^{2+} inhibited the enzyme activity. The enzyme exhibited maximum activity at pH 5, performed stability over a broad range of pH 4. 5 – 11, and was optimally active at 45 – 50 ºC [157]. Konsula and Liakopoulou-Kyriakides [105] isolated a thermophilic *B. subtilis* strain from fresh sheep's milk, produced extracellular thermostable α-amylase. The *Km* and *Vmax* values were calculated for potato starch was 7.79 mg/mL/h and 11.176 mg/mL/h, respectively. Ezeji and Bahl [158] purified α-amylase secreted by *Geobacillus thermodenitrificans* HRO 10 through a series of steps and found that the α-amylase hydrolysed soluble starch with a *Km* of 3.05 mg/mL and a *Vmax* of 7.35 U/mL.

2.2.9. Molecular weight

In general, a survey shows that the molecular weight (MW) of α –amylase from *Bacillus* spp. varies between 50 – 60 kDa with some exceptions. Thermostable *α*-amylase from *B. licheniformis*, a monomeric enzyme with molecular mass of 55,200 Da (483 amino acid residues), shows a remarkable heat stability [168]. Liu *et al.* [169] found that the MW of *B. licheniformis*, a thermostable α-amylase as 53,130 Da. The extracellular α-amylase produced by the *B. licheniformis* 44MB82-A strain was 58,000 Da as judged by SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) [170]. *B. acidocaldarius* strain *agnano* 101 produced an inducible thermoacidophilic α-amylase [171]. The purified amylase contained a

single polypeptide chain of molecular weight 68,000 Da [171]. The apparent MW of the purified enzyme from *B. amyloliquefaciens* was 58 kDa as revealed by SDS-PAGE [118].

The nucleotide sequence of the *B. stearothermophilus* α-amylase gene and its flanking regions were determined [172]. An open reading frame was found, comprising a total of 1,647 base pairs (549 amino acids) and starting from a GUG codon as methionine. It was shown by NH_2-terminal amino acid sequence analysis that the extracellular amylase consisted of 515 amino acid residues, which corresponded to a MW of 58,779 Da. Thus, the NH_2-terminal portion of the gene encodes 34 amino acid residues as a signal peptide.

N-terminal signal peptides and C-terminal truncation have been characterised in many *Bacillus* spp. A novel liquefying α-amylase was found in cultures of an alkaliphilic *Bacillus* isolate, KSM-1378 corresponding to 516 amino acids that included a signal peptide of 31 amino acids. The calculated MW of the extracellular mature enzyme was 55,391 Da [173]. Carboxyl-terminal truncation has been observed on α-amylases of *B. subtilis* [174]. A *B. subtilis* amylase gene was inserted into a plasmid which was transferred to *Escherichia coli*. The active protein was purified to apparent homogeneity. Its MW (48 kDa) as estimated by SDS-PAGE, was lower than the molecular mass values calculated from the derived amino acid sequences of the *B. subtilis* complete α-amylase (57.7 kDa) [174]. Complete (Ba-L) and truncated (Ba-S) forms of α-amylases from *B. subtilis* X-23 were purified, and the amino- and carboxyl-terminal amino acid sequences of Ba-L and Ba-S were determined [162]. The amino acid sequence deduced from the nucleotide sequence of the α-amylase gene indicated that Ba-S was produced from Ba-L by truncation of the 186 amino acid residues at the carboxyl-terminal region. The molecular masses of the two purified enzymes were 47 and 67 kDa, and these enzymes were designated Ba-S and Ba-L, respectively [162].

2.2.10. Stability

The pH and temperature optima increased the stability of α-amylase. Krishnan and Chandra [163] reported maximum amylase production from *B.*

licheniformis at 90 ºC and pH 9.0 and 91% of the activity remained at 100 ºC. In the absence of substrate, the enzyme retained 91, 79, 71% maximal activity after 3 h of treatment at 60 ºC, 70 ºC and at 80 ºC, respectively. Srivastava and Baruah [121] reported liquefied thick starch slurries at 80 ºC and pH 6.9 increased the stability of the α-amylase from *B. stearothermophilus.* Ca^{2+} was also essential for thermostability. Das *et al.* [142] found that the thermostability of the enzyme from *B. subtilis* by heating at 95 ºC for 10 min resulted in loss of 60% of original activity. They reported that the temperature and pH optima for enzyme activity were 52-55 ºC and 9.0.

A thermostable glucoamylase (GA) from thermophilic *Bacillus* sp. showed optimum activity at 70 degrees C and pH 5.0. The thermostability of the enzyme was enhanced twofold in the presence of 0.5% (w/v) starch at 5 psi [175]. Natalia *et al.* [176] studied the properties of thermostable glucoamylase-type enzyme produced by thermophilic *B. acidocaldarius* RP1 isolated from Cimanggu Hot Spring, West Java, Indonesia. The glucoamylase-type enzyme activities were detected both as cell-associated and in the culture supernatant. The 20-40% saturated ammonium sulfate fraction of glucoamylase-type enzyme exhibited an optimum temperature of 65 °C and an optimum pH of 4.5. The enzyme showed a V_{max} of 600 milliunits/mg and K_m of 11.7 mg/mL.

Suzuki *et al.* [177] found that the α-amylase of *B. licheniformis* (BLA) is stable and active at high temperature. More than 80% of its activity was retained after heat treatment at 90 ºC for 30 min. In contrast, the α-amylase of *B. amyloliquefaciens* (BAA), the amino acid sequence of which showed 80% homology with that of BLA, was rapidly inactivated at 90 ºC. Various chimeric genes were constructed from the structural genes for the two enzymes, and their products were analysed for stability as to irreversible thermoinactivation. Two regions in the amino acid sequence of BLA comprising Gln178 (region I) and the 255^{th} – 270^{th} residues (region II), respectively were shown to determine the thermostability of BLA. Region I played crucial role in determing the thermostability. Tomazic and Klibanov [114] reported half-lives of *Bacillus* α-

amylases at 90 °C and pH 6.5 greatly increase in the series from *B. amyloliquefaciens* to *B. stearothermophilus* to *B. licheniformis*. This stabilisation is achieved by lowering the rate constant of monomolecular conformational scrambling, which was the cause of irreversible thermoinactivation of *B. amyloliquefaciens* and *B. stearothermophilus* α-amylases, so that for *B. licheniformis* α-amylase, another process, deamidation of Asn/Gln residues, emerges as the cause of inactivation. The extra thermostability of the thermophilic enzyme was found to be mainly due to additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys-253).

2.2.11. Crystal structure

The α-amylase family, is the largest sequence-based family of glycoside hydrolyses (GH13 family) and groups together a number of different enzyme activities and substrate specificities acting on α-glycosidic bonds [178]. α-Amylases are classical calcium-containing enzymes which constitute a family of endo-amylases catalyzing the cleavage of *α*-D-(1→4) glycosidic bonds in starch and related carbohydrates with retention of the *α*-anomeric configuration in the products [179]. Furthermore, these enzymes are used as targets for drug design in attempts to treat diabetes, obesity and hyperlipemia [179]. It has long been known that *α*-amylases require calcium for their enzymatic activity [180]. All known structures of holo *α*-amylases show a common calcium-binding site stabilising the interface between the highly homologous central A domain and the more variable B domain [180].

Crystal structure of many *α*-amylase are known to date (Table 5 [168, 180 - 184]). It seems that *α*-amylases from *B. licheniformis* and *B. subtilis* have been the target of many investigators, owing to their predominant role in starch and drug industry. Typically, the *α*-amylases bear a $(\beta/\alpha)_8$-barrel structure [185]. *α*-Amylases with the $(\beta/\alpha)_8$-barrel fold are involved in the catalysis of a wide variety of biochemical reactions [186]. The active sites of these enzymes are located on the C-terminal face of the central β-barrel [168]. Thermostable *α*-amylase from *B. licheniformis*, a monomeric enzyme with molecular mass of 55,200 Da (483 amino

acid residues), shows a remarkable heat stability [168]. Like other *α*-amylases, the polypeptide chain of *B. licheniformis* folds into three distinct domains. The first domain (domain A), consisting of 291 residues (from residue 3 to 103 and 207 to 396), forms a (β/α) 8-barrel structure. The second domain (domain B), consisting of residues 104 to 206, is inserted between the third β-strand and the third α-helix of domain A. The third C-terminal domain (domain C), consisting of residues 397 to 482, folds into an eight-stranded antiparallel β-barrel [168]. As many crystal structures are known for *α*-amylase, interrelatedness can easily be deduced by protein modeling [187].

Table 5: Strategies employed for the resolution of crystals from various α-amylases of *Bacillus* spp.

Sl.No	Bacillus sp.	Strategy	Resolution	Ref.
1	*Bacillus* sp. Strain KSM-K38	Molecular replacement method and refined to a crystallographic *R*-factor of 19.9% (*R*-free of 23.2%)	2.13-Å resolution	[181]
2	*B. subtilis*	Crystallised maltopentaose and acarbose, determined by multiple isomorphous replacement. Restrained crystallographic refinement has resulted in an *R*-factor of 19.8% in the 7.0 to 2.5 Å resolution range	2.5 Å resolution	[182]
3	*B. licheniformis*	Multiple isomorphous replacement in a crystal of space group $P4_32_12$ ($a=b$=119.6 Å,c=85.4 Å).	2.2 Å resolution	[183]
4	*B. licheniformis* 55,200 Da (483 amino acid residues),	Multiple isomorphous replacement method of X-ray crystallography	1.7 A resolution	[168]
5	*B. stearothermophilus*	-	2.0 A resolution	[180]
6	*B. halmapalus*	Complex with the (pseudo) tetrasaccharide inhibitor acarbose	2.1 Å resolution	[184]

2.2.12. Applications

With the advent of new frontiers in biotechnology, the spectrum of amylase application has been widened up to many other fields such as clinical, medicinal and analytical chemistries, as well as their widespread use in the industries such as textile, laundry, porcelain, detergents, paper, food, brewing, baking and distilling.

Enzymes are commonly used in the baking industry, as they can improve dough quality and texture and lengthen the shelf life of the final product. There is little published information highlighting exposure to enzymes (other than fungal alpha-amylase) in the baking industry [188]. α-Amylases have been used extensively in bread making to break down complex sugars such as starch (found in flour) into simple sugars [189]. Directed evolution coupled with a high-throughput robotic screen was employed to broaden the industrial use of the maltogenic α-amylase Novamyl from *Bacillus* sp. TS-25 [190]. Wild-type Novamyl is currently used in the baking industry as an anti-staling agent in breads baked at neutral or near neutral pH. However, the enzyme is rapidly inactivated during the baking process of bread made with low pH recipes and Novamyl thus has very limited beneficial effect for this particular application [190]. Very recently, Rajagopalan and Krishnan [92] used glucose and maltose forming α-amylases from *B. subtilis* in alcohol fermentation and sugar syrup formulation; and also for the food processing by malto-oligosaccharide-forming α-amylases.

The alkaline enzymes used in modern detergents are protease, cellulase, α-amylase, lipase, and mannanase [191]. Like proteases, alkaline amylases play crucial role in detergents designed for washing machines [164], [88]. Huge amounts of alkaline enzymes are used in the detergent industry, and they have widely been incorporated into heavy-duty laundry and automatic dishwashing detergents. Traditionally prepared from *B. amyloliquefaciens*, the enzyme from *B. licheniformis* going market shares because of its greater thermostability [156].

For a number of years α-amylase enzymes have been used for a variety of different purposes; the most important of which are starch liquefaction, textile desizing, starch modification in the paper and pulp industry [192]. The major uses of

amylases extend to textile and paper industries also. Its major use is for the liquefaction of starch (reduction of high molecular mass to about 10 or 11 residues) in the starch processing and related industries and for the designing of textiles [159]. α-Amylase is used to produce modified starches for the paper industry; to remove starch in the manufacture of textiles (desizing) [88]. In addition amylases have potential industrial applications, such as antisaling agent, production of cyclodextrins, sizing of textile fibres, and clarification of haziness in beer and fruit juices [85]. Variants of *B. licheniformis* α-amylase exhibit applications in textile desizing [192]. Alkaline α-amylase producing *Bacillus* sp. A3-15 found suitable in textile industries [167].

With the advent of various novel strategies in the pharmaceutical and chemical industries, amylases have been emerged as a major player in the synthesis of optically pure drugs and agrochemicals [189]. A method of curing and preventing obesity comprises orally administrating α-amylase inhibitor thereby inhibiting an α-amylase activity in saliva and pancreatic juice, and reducing digestion and absorption of starch (reduction of the calorie taken from meals). Obesity can be cured or prevented effectively while taking usual diet without giving any physical or mental pain [193]. Glucose and maltose are also produced depending on the degree of hydrolyses, because of this starch degrading ability of bacterial α-amylase – mostly from *B. subtilis*, it is widely used in pharmaceutical industry in various digestive aid preparations. Due to the presence of α-amylase, starch in the consumed food is better digested, this increases overall digestibility of food. Such digestive aid preparations are used for treatment of patients whose digesting power is reduced due to illness. Many such a commercial formulation of digestive aids either as syrup or as tablet are seen in many drug stores.

Table 6 [91, 96, 111 - 112, 167, 188 - 192, 194 - 198] shows that the bacillial α-amylases are mainly employed in food, detergent, textiles, paper and pharmaceutical industries.

Table 6: Major applications of α-amylases obtained from bacillial spp.

SI. No.	Industry	*Bacillus sp.*	Reference
1	Food industry	*Bacillus* sp. TS-25	[190]
		B. subtilis & *B. amyloliquefaciens*	[188]
		B. subtilis	[111]
		B. megaterium	[96]
		B. licheniformis	[194]
		Bacillus. sp. A3-15	[167]
		Bacillus. sp. strain SMIA-2	[112]
2	Detergent industry	*B. cohni*	[195]
		Bacillus strains	[191]
		B. licheniformis	[194]
		B. horikoshii	[192]
		Bacllus sp. B₃	[196]
3	Textile industry	*Bacillus* sp. A3-15	[167]
		B. licheniformis	[194]
		Bacillus sp. B₃	[196]
4	Paper industry	*Bacillus* sp. NCIMB 40916	[192]
		Bacillus sp. B₃	[196]
		B. licheniformis	[197]
5	Pharmaceutical industry	*Bacillus sp.*	[198]
		B.licheniformis	[91]
		Bacillus sp.	[189]

2.2.13. Conclusions

Three amylases (α, β, γ) known to present in microbial world are much useful to the industry [117]. Bifunctional industrially significant α and γ amylase fusion proteins are already available to the industry [199]. However, a trifunctional amylase embodying the catalytic activities of all known amylases is yet to be made available to the industry**.** Using available crystals and of other sequence

informations on mammalian and microbial amylases, the structure of α-amylase from novel strains of *Bacillus* can easily be predicted by homology modeling as long as we obtain similar amino acid sequences.

The engineering work performed on α-amylase from *B. licheniformis* (BLA) over the last decade provides a good example of the extent to which an enzyme can be remodelled in order to improve its natural performance and fulfill industrial requirements. Contrary to expectations, the thermal resistance of this highly thermostable α-amylase is far from being maximised in the wild-type enzyme and there seems to exist many ways to increase the thermostability even further. A fair set of stabilising substitutions have already been found in BLA and many more may be identified in the future. Given the success achieved so far, it may even be possible to increase BLA thermostability beyond the most thermostable enzymes found in hyperthermophiles.

Moreover, occupational diseases associated with bacilliary amylase have to be addressed properly amidst the industrial values of microbial amylases. Occupational asthma is a major threat in workers engaged in industries especially clothing and detergents. All these highlight that though we use *Bacillus* with GRAS status (unlike *B. anthracis* and *B. cereus*) for amylase production, when they come to the industrial perspective untoward risks have been sprouted up. These problems can be better solved by engineering such proteins with a view to improved enzymatic performance, including increased themostability and reduced calcium dependence.

Industry demands an ideal α-amylase with increased stability, durability, reusability – especially when used at immobilised state, or a trifunctional amylase embodying the functions of all amylases with maximum activity. Such a versatile fusion protein should be useful in starch processing, starch liquefaction, fermentation, starch saccharification, cleaning, laundrying, textile desising, baking, and biofilm removal.

2.3. *Aceria guerreronis* Keifer *vs.* coconut palm

Coconut, commonly referred to as “Tree of Life” as well as “KalpaVriksha” provides livelihood to millions of people across the world. Globally, coconut occupies an area of 12 million hectares with a total production of about 56 billion nuts. India, Indonesia, Philippines and Sri Lanka are major coconut-growing countries, contributing 78 percent of the total world production. India ranks third in the production and fourth in the productivity of coconut in the world with a production of 12.8 billion nuts in 2000-01 from 1.9 million hectares, accounting for about 22.36 percent of the total world production. Among the coconut producing states in India, Tamil Nadu ranks second in production. In Tamil Nadu, coconut is grown in an area of 3.24 lakh hectares with a total production of 3,158 million nuts with an average yield of 9,763 nuts/hectare/annum. Coconut provides food, drink, medicine and altogether health to millions of consumers as well. This crop is attacked by various pests, of which, rhinoceros beetle, red palm weevil, leaf-eating caterpillar, *etc*. are important. Of late, incidence of nut infesting eriophyid mite, the microscopic *Aceria guerreronis* Keifer (Eriophyidae: Acari) has become a major problem in many of the coconut-growing countries.

The coconut mite, *Aceria guerreronis* Keifer (Prostigmata: Eriophyidae), is a major pest in several coconut production areas worldwide [200]. Information on region of origin and sources of recent introductions of this mite are important aspects to guide evaluation of biological control agents and adoption of quarantine measures. Studies on the geographic pattern of morphological variation among populations of the coconut mite from different countries and continents can provide some of the biogeographic information [201]. These authors also reported that *A. guerreronis* is of American origin and that it was introduced to Asia from Africa, or from the same source as that of the African populations [201].

A study by leaf analysis of the nutritional levels present in coconuts in St. Lucia showed that less than 2.5 % of trees had adequate levels of all three major nutrients, N, P and K. N levels were generally inadequate as were P levels, though less seriously so. K levels were inadequate in some areas and excessive in others.

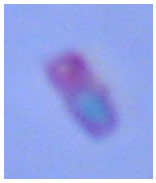
There were some associations between nutrient status and levels of damage by the coconut mite, *Aceria* (*Eriophyes*) *guerreronis*. Damage generally increased with increasing levels of N. At one site, K was associated with decreasing mite damage [202]. Being minute in size, eriophyoid mites can reach places that are small enough to be inaccessible to their predators. The coconut mite, *A. guerreronis*, is a typical example; it finds partial refuge under the perianth of the coconut fruit. However, some predators can move under the perianth of the coconut fruits and attack the coconut mite. In Sri Lanka, the phytoseiid mite *Neoseiulus baraki*, is the most common predatory mite found in association with the coconut mite [203]. The suppression of coconut mite *A. guerreronis* Keifer populations by augmenting the natural population of the predatory mite, *Neoseiulus baraki* (Athias-Henriot) (Acari: Phytoseiidae) by an inundative release with laboratory-bred *N. bar*aki [204]. Distribution pattern and numerical variability of the coconut mite *A. guerreronis* Keifer and its predator *Neoseiulus* aff. *paspalivorus* DeLeon (Phytoseiidae) on the nuts of 3- to 7-month-old bunches of coconut palms were studied at two sites in Sri Lanka by Fernando *et al.*, [205]. They also found that four Sri Lankan isolates of an entomopathogenic fungus, *Hirsutella thompsonii*, obtained from the coconut mite *A. guerreronis*, were tested as biopesticide treatments in two coconut mite-infested coconut estates [206].

It clearly indicates that no solution is available yet to combat *A. guerreronis* attacking coconut palms. The cap on the tender coconut buttons and young nuts offer a suitable environement for the best survival of these mites. Thus, in this study, new strategy has been tried to suucessfully combat the mite so as to rescue the dwindling coconut cultivation.

3. MATERIALS AND METHODS

3.1. Source of organism

3.1.1. Bacterium

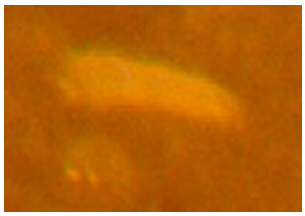


The standard strain of *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*) was procured from the Institute of Microbial Technology, Chandigarh (Strain designation: BA 83B and the MTCC number: 868), and maintained in Luria-Bertani (LB) medium. Figure 1 shows the single sporulated *Btk* cell.

3.1.1.1. Subculturing

Frozen stocks on agar slants were activated periodically (fortnightly) and maintained on LB-agar slants. For long-term storage, glycerol stocks were made and preserved at -72 ^{o}C (Operon, Korea).

3.1.2. Mite



The mite, *Aceria guerreronis* [Vernacular (Malayalam): mandari] was collected locally from the vicinity of Calicut University Campus [8^{o}29'N, 45-50 M, Mean Sea Level (MSL)] from the tender coconut buttons of the infested coconut palms, which were identified by Dr. B. Ramani, Professor, Department of Zoology, University of Calicut. Figure 2 shows a single mite.

3.2. Chemicals

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

3.3. Plastic/ Glasswares

Different glasswares made by Borosil, and Riviera were used for the whole study. Measuring jars (10, 100, 200, 500 and 1000 mL), pipettes (1.0,5.0, 10.0 mL), beakers (10 mL, 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL), conical flasks (100 mL, 250 mL, 500 mL), Petri- plates (100 x 15 mm) were used for the whole study. Micropipettes (0.5 – 10.0 µL, 20 – 200 µL, 200 – 1000 µL, 1 – 5 mL) from Accupipete, Biosystems and Microlit were used.

3.4. Cultivation of *Btk*

For the production of extracellular amylase, endospore and δ-endotoxin, *Btk* was cultivated in various media combinations as described in the succeeding sessions. LB was the basic medium used throughout the study. During the study, it was supplemented with soluble/raw starch, as written in the respective sessions. Prior to inoculation, all the media were autoclaved at 15 ψ, 121 ºC.

Table 7: Composition of LB medium.

LB medium	
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled Water	~1.0 L
pH	7.0

.*3.4.1. Submerged Fermentation (smF)*

3.4.1.1. SmF in LB medium without starch

LB medium without starch was used to monitor the extracellular amylase production by SmF. Samples were withdrawn for assays at 3 h intervals.

3.4.1.2. SmF with soluble starch

For SmF, LB medium was supplemented with 1% (w/v) (Himedia). No starch was added in the control.

3.4.1.3. SmF with natural raw supplements

The LB medium was prepared supplemented with 1% (w/v) various raw starch supplements *viz.* Banana powder (BP), Bengal gram powder (BgP), Jack seed powder (JP), Potato powder (PP), Tapioca powder (TP) and Taro powder (TaP). Of the raw starch powders, BP was purchased locally (Bana-tone), JP, PP, TP and TaP were home-made and BgP powdered locally in a mill. No starch was added in the control. Source of raw starch was maintained constant throughout the experiments.

3.4.2. Semisolid- or Solid-State Fermentation (SSF)

3.4.2.1. SSF with soluble starch

The LB medium prepared supplemented with different concentrations of 5%, 10%, 15%, 20% and 25% (all w/v) soluble starch (Himedia). No starch was added in the control.

3.4.2.2. SSF with natural raw supplements

The LB medium was prepared supplemented with different concentrations (5%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%, all w/v) of natural raw starch powders such as BP, BgP, JP, PP, TP, TaP. No starch was added in the control.

3.4.3. Seed culture

The frozen stock was streaked with a sterile toothpick on the LB-agar medium in the petri-plate. For the preparation of overnight seed culture (12 h), single colony from LB-agar plate was inoculated with a sterile toothpick in the liquid medium.

3.4.4. Inoculum

50 µL seed culture was used to inoculate 10 mL sterilised production medium, which was equivalent to 6.2 x 10^5 cfu (colony forming units) per 1mL medium. Cfu was calculated by serial dilution, and the colonies were counted by Magnus Compound Microscope (India).

3.4.5. Incubation

For SmF, the medium was incubated at 37 ºC with constant shaking (140 rpm) in an environmental shaker (Orbitek, India). For SSF, the samples were incubated in an oven (Technicho, India) at 37 ºC.

3.5. Crude extracellular α-amylase harvest

3.5.1. SmF

3.5.1.1. SmF in LB medium

The LB medium was centrifuged at 800g for 10 min at 4 ºC in a refrigerated centrifuge (Plastocrafts/Remi, India). 0.5 mL samples were withdrawn for assay at 3 h intervals. The supernatant was used for enzyme assay.

3.5.1.2. SmF in LB medium supplemented with 1 % (w/v) soluble/raw starch

0.5 mL samples were withdrawn for assay at 6 h intervals. The broth was centrifuged at 800g 10 min at 4 ºC in a refrigerated centrifuge. The supernatant was used for enzyme assay.

3.5.2. SSF

0.5 g samples were withdrawn for assay at 6 h intervals. The fermented matter was weighed and mixed with 5.0 mL double distilled water (ddH_2O) and centrifuged at 800g 10 min at 4 ºC in a refrigerated centrifuge. The supernatant was used for extracellular amylase assay.

3.6. α-Amylase assays

α-Amylase was assayed by employing the 3,5-dinitrosalicylic acid (DNS) method of Bernfeld [207], modified by Ezeji and Bahl [158].

3.6.1. DNS Reagents

DNS reagent contained DNS (1%)**,** potassium sodium tartarate (Rochelle salt, (1 M) and NaOH (0.4 M) and ddH_2O.

3.6.1.1. Procedure for 100 mL Reagent

Dissolve by stirring (Remi, India) at room temperature (RT) 1g DNS in 50 mL ddH_2O, then added 20 mL 2 M NaOH and 28.2 g Rochelle salt, finally made up to 100 mL by ddH_2O. The reagent was stored at RT.

3.6.2. Other solutions

- *Buffer:* 0.02 M sodium phosphate buffer (pH, 6.9) with 0.006 M sodium chloride.
- *Starch solution:* 1.0% starch solution was prepared fresh by dissolving 1.0 g soluble starch in 100 mL 0.02 M sodium phosphate buffer (pH, 6.9).
- *Maltose stock solution*: Dissolved 50 µg maltose in 50 mL ddH_2O in a standard flask and stored at 4 ^{o}C.

3.6.3. α-Amylase activity assay procedure for SmF (unless otherwise specified)

- Pipette out 0.5 mL enzyme solution (prepared as explained in **3.5.1**.) and incubate tubes at 25 ^{o}C for 3 min.
- Add 0.5 mL starch solution and incubate for 5 min (RT).
- Stop the reaction by adding 1 mL DNS reagent.
- Heat the solution in a boiling water bath for 5 min.
- Cool it in running tap water.

- Make up the volume to 10.0 mL by the addition of ddH_2O.
- Read the absorbance at 540 nm using UV-Vis Spectrophotometer (Schimadzu UV 1601, Japan).
- Blank is prepared without enzyme.
- Prepare a standard graph with 0 -100 µg maltose.

3.6.3.1. Calculation of enzyme activity Extinction coefficient (Σ)

For stock solution, 2g *D*-glucose was weighed into 1 L capacity volumetric flask and made upto 1 L with ddH_2O. 5, 15, 25, 50, 75 and 100 mL of stock solutions were pipetted into 100 mL volumetric flasks and made upto 100 mL with ddH_2O to give 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0 mg/mL glucose respectively. 2 mL 3,5, DNS reagent, 1mL of each of the different glucose concentrations and 1mL of 0.1 M acetate buffer, pH 5.5 were added into each of the reaction tubes. The mixtures were boiled in water bath for 5 min. and their extinction measured using Spectrophotometer at 540 nm. These extinctions were plotted against their concentrations and thus the glucose standard curve or regression equation was established.

$Y = 1.1576\ X - 0.0429\ (R^2 = 0.999)$……equation 1

X = 0.18 mg = 1 µM glucose

Substitute 1 µM glucose in equation 1,

Therefore Y = 0.165 cm^2/µM glucose = Extinction coefficient (Σ)

3.6.3.2. Calculation of α-amylase *activity in SmF*

One unit (U/mL) of *α*-amylase activity is defined as: the amount of protein (*α*-amylase) required to liberate 1 μmol (0.18 mg equivalence) of reducing sugar (*D*-glucose) from starch/min, under the assay conditions [158].

$$\text{Formula: } \alpha\text{-amylase activity (U/mL)} = \frac{\Delta E \cdot Vf}{\Delta t \cdot \Sigma \cdot Vs \cdot d}$$

ΔE = Absorbance at 540 nm

Vf = Final volume including DNS

Vs = Volume (mL) of α-amylase used

Δt = Time of hydrolysis

Σ = Extinction coefficient

d = Diameter of cuvette (1 cm for standard cuvette)

3.6.3.3. α-Amylase activity assay procedure for SSF (unless otherwise specified):

- Pipette out 0.5 mL enzyme solution (prepared as explained in 3.5.2**.)** and incubate tubes at 25 ºC for 3 min.
- Add 0.5 mL starch solution and incubate for 5 min (RT).
- Stop the reaction by adding 1 mL DNS reagent.
- Heat the solution in a boiling water bath for 5 min.
- Cool it in running tap water.
- Make up the volume to 10.0 mL by the addition of ddH_2O.
- Read the absorbance at 540 nm using UV-Vis Spectrophotometer.
- Blank is prepared without enzyme.
- Prepare a standard graph with 0 -100 μg maltose.

3.6.3.4. Calculation of α-amylase *activity in SSF*

One unit per gram dry substrate (U/gds) of *α*-amylase activity is defined as: the amount of protein (*α*-amylase) in one gram dry substrate (gds) required to liberate 1 μmol (0.18 mg equivalence) of reducing sugar (*D*-glucose) from starch/min, under the assay conditions [140]; [158].

Therefore, α-amylase activity is calculated as follows:

$$\text{Formula: } \alpha\text{-amylase activity (U/gds)} = \frac{\Delta E \times Vf \times Vs}{\Delta t \times \Sigma \times gds \times d}$$

ΔE = Absorbance at 540 nm

Vf = Final volume including DNS

Vs = Volume (mL) of α-amylase used

Δt = Time of hydrolysis

Σ = Extinction coefficient

gds = dry weight of the substrate in gram

d = Diameter of cuvette (1 cm for standard cuvette, the one used)

3.7. Protein estimation

Protein content was estimated using Lowry's method with bovine serum albumin (BSA) as the standard [208].

3.7.1. 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 (alkaline copper solution): 50 mL of Reagent A + 1 mL of Reagent B
- Folins-phenol reagent: Commercial Folins-phenol reagent was used after dilution in a 1:1 ratio with ddH_2O.

3.7.2. Procedure

- Pipette out 0.5 mL of the enzyme in test tube and make upto 1 mL with 0.1 N NaOH. Add 5.0 mL of alkaline copper reagent. 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 670 nm using Spectrophotometer.
- Calculations were done using the graph generated from the standard graph of BSA.

3.7.3. Standard BSA graph

- 1 mg/mL stock solution is prepared with BSA
- Pipette out different aliquots of stock solution (0.05, 0.10, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 µL) in test tubes and make upto 1 mL with 0.1 N NaOH. Add 5.0 mL of alkaline copper reagent. 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 670 nm using Spectrophotometer.
- The values were plotted against concentration vs optical denstity at 670 nm.

3.8. Enzyme purification and characterisation

Amylase was purified by the method of Ezeji and Bahl [158]. The strategy included fractionation by ammonium sulphate followed by dialysis and gel permeation chromatography. The purity was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [209].

3.8.1. Ammonium sulphate fractionation

Finely powdered ammonium sulphate was added slowly into the crude enzyme preparation until it reached 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 in an ice bath. The precipitated protein was removed by centrifugation at 2400g for 10 min at 4 ^{o}C. The pellet was resuspended in a minimum volume of 0.02 M sodium phosphate buffer (pH 6.9).

3.8.2. Dialysis

The precipitate obtained after ammonium sulphate fractionation was dialysed against sterile ddH_2O for 24 h at 4 ^{o}C with continuous stirring and occasional changes of the buffer. Cellulose membrane dialysis tubes were used for dialysis. The amylase activity and protein content of the dialysate were determined as described under **3.6.3**. and **3.7.2,** respectively.

3.8.3. Gel permeation chromatography

Gel permeation chromatography was done in a column (50 x 3 cm) packed with sephadex G 100 (Sigma Aldrich, USA) using a peristaltic pump (Riviera, India) in a cold room. The dialysate obtained by 40-60% ammonium sulphate fraction was used for gel permeation chromatography. It included the following steps.

- 2.5 g of sephadex G 100 was suspended in ddH_2O and kept for 72 h at 20 ^{o}C (as directed by the manufacturer).

- Swollen sephadex was poured into the chromatography column (50 x 3 cm) and allowed to settle under gravity while maintaining a slow flow rate through the column. Care was taken to avoid trapping of air bubbles in the column. The column was stabilised and equilibrated by passing about 1 L of buffer (0.02 M sodium phosphate buffer, pH 6.9). This step was repeated twice.

- The concentrated dialysate (5 mL) was loaded on the top of the column. The enzyme was eluted using phosphate buffer (0.02 M, pH 6.9) in a sequential

manner. Flow rate was adjusted to about 12 mL/h and fractions of 2.0 mL/10 min were collected.

- The absorbance of each fraction was read at 280 nm using a Spectrophotometer.
- The fractions with major peaks were subjected to amylase enzyme activity and protein content estimation.
- Those fractions with maximum amylase enzyme activity were subjected to SDS – PAGE [209].

The gel permeation chromatography (BioRad Biologic LP, Italy, 50 x 1.5 cm) was also done in National Institute for Interdisciplinary Science and Technology, Papanamcode, Thiruvananthapuram.

3.8.4. Electrophoresis

The purified enzyme was subjected to SDS–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 x 7 cm) slab with notched glass plate system (BioTech, India). Gels of 1.5 mm thickness were prepared for the whole study.

3.8.4.1. Stock solutions

- Acrylamide-bisacrylamide (30:0.8%) was prepared by dissolving 30 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100 mL of ddH_2O. The solution was filtered through Whatman No. 1 filter paper and stored at 4 ^oC in a dark bottle.
- 1.5 M Tris buffer, pH 8.8 – 23.6 g tris was dissolved in 40 mL of ddH_2O and titrated to a pH of 8.8 with 6.0 N NaOH and made upto a final volume of 100 mL. Filter through Whatman No.1 filter paper and stored in a refrigerator.

- 1.0 M Tris buffer, pH 6.8 – 15.7 g tris was dissolved in 48 mL of 1.0 N NaOH and made upto a final volume of 100 mL with ddH_2O. Filter through Whatman No.1 filter paper and stored in a refrigerator.
- 10% SDS
- 10% Ammonium persulphate (make fresh)
- TEMED (N,N,N',N'-tetra methyl ethylene diamine) was used as such which was stored in a dark bottle at 4 ^oC (make fresh).
- Electrophoresis buffer - 12 g tris, 57.6 g glycine and 2.0 g SDS was dissolved in 500 mL of ddH_2O and made upto a final volume of 1L and stored in a refrigerator.
- Sample buffer.

Table 8: Ingredients required for sample buffer

Ingredients	Quantity
0.6 M Tris buffer, pH- 6.8	1.0 mL
10% SDS	0.1 g
Sucrose	1.0 g
β-mercaptoethanol	0.05 mL
10 mM bromophenol blue	1.0 mL
20% Glycerol	0.4 mL

Make upto 10.0 mL with ddH_2O and store in a refrigerator.

3.8.4.2. Stacking gel composition

Table 9: Ingredients required for stacking gel

Ingredients	Quantity

Acrylamide-bisacrylamide	0.5 mL
Tris buffer	0.38 mL
10% SDS	0.03 mL
10% Ammonium per sulphate	0.03 mL
TEMED	0.003 mL
ddH_2O	2.1 mL

3.8.4.3. Composition of 12% gel

Table 10: Ingredients required for 12% gel

Ingredients	Quantity
Acrylamide-bisacrylamide	4.0 mL
Tris buffer	2.5 mL
10% SDS	0.1 mL
10% Ammonium per sulphate	0.1 mL
ddH_2O	3.3 mL
TEMED	0.004 mL

3.8.4.4. Sample preparation

Enzyme solution and sample buffer were mixed in the ratio of 3:1, respectively. The contents were mixed well in a clean eppendorf tube and heated in a boiling water bath for 3 min. For MW determination, wide range markers (Chromous, Bangalore) were used. The MW markers in the ladder were: lysozyme (14 kDa), lactoglobulin (18 kDa), BamHI (25 kDa), carbonic anhydrase (30 kDa), amylase (51 kDa), bovine serum albumin (66 kDa), Phosphotylase (97 kDa) and β - galactosidase (110 kDa). Electrophoresis was performed using a constant voltage (30 V) till the sample dye reached the bottom of resolving gel.

3.8.4.5. Protein staining

Gel was stained using 0.1% coomassie brilliant blue (CBB) G-250 in 50% methanol and 10% glacial acetic acid. It was stirred at room temperature for 60 min and filtered through Whatmann No. 1 filter paper. The solution was stable indefinitely at RT.

3.8.4.6. Destaining

The destaining solvent system contained 10% glacial acetic acid: 45% methanol: 45% ddH_2O.

3.8.4.7. Visualisation

The SDS-PAGE gels were visualised through Gel-documentation system (BioRad, Italy) and also by Canon digital camera (EOS 450D, Japan).

3.8.5. Characterisation of α-amylase

The 40–60% ammonium sulpahate fraction was used for the characterisation studies. The purified enzyme was characterised and its various properties were studied. Effects of pH, temperature, substrate concentration, chelating agents and different metal ions on enzyme activity were the factors studied.

3.8.5.1. Effect of pH on enzyme activity

Optimum pH on the enzyme activity was studied by performing the assay at different pH using acetate (pH 4.0 – 5.0) and phosphate (pH 6.0 – 8.2) buffers.

3.8.5.2. Effect of temperature on enzyme activity

Optimum temperature needed for enzyme activity was estimated by incubating the reaction mixture for 5 minutes at different temperatures (30^{o}C – 100^{o}C) at pH 6.0.

3.8.5.3. Effect of different metal salts on enzyme activity

Effect of different metal ions on amylase activity was determined by incubating the reaction mixture with different metal salts, *ie.*, Ag^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Mo^{2+}, Na^{2+}, SO_4^{2-}, SO_3^{-} and Zn^{2+} to a final concentration of 0.5 µM, 1.0 µM, 2.0 µM, 3.0 µM, 4.0 µM and 5 µM at pH 6.0 and temperature 60 ^{o}C.

3.8.5.4. Effect of complex compounds on α- amylase activity

The role of complex compounds like ethylene diamine tetra acetic acid (EDTA), β - mercaptoethanol, SDS and thiourea were tested. Cysteine was used for the effect of enzyme towards activity.

3.8.5.5. Effect of substrate concentration

The enzyme was treated with soluble starch with concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% and 4%. The reaction mixture was incubated with 5 min time intervals ranging from 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min and 60 min at pH 6.0, temperature 60 ^{o}C and the metal Ca^{2+}.

3.8.5.6. Calculation of Km and Vmax

The *Km* and *Vmax* values were calculated using the effect of soluble starch on enzyme activity using the software, Hyper 32.

3.9. Endospore production

3.9.1. Endospore production by SmF

Spore production also done with various raw starches as supplements. The medium prepared with JP, PP and TP at concentration of 1% w/v and 6 h time interval ranging from 6h to 72h.

3.9.2. Endospore production by SSF

Spore production also done with various raw starches as substrates. The medium prepared with JP, PP and TP at different concentrations of w/v (1%, 5%, 10%, 20% 30%, 40%, 50%, 60%, 80% and 100%) and 6 h time interval ranging from 6h to 72h. The supplements were mixed in a vortex mixture and incubated at 37 ^{o}C in an incubator.

3.9.3. Preparation of working spore solution

Culture medium was centrifuged at 8900g for 10 min. Pellets collected and resuspended in 4 ^{o}C sterile ddH_2O and made upto 10 mL. The resulting solution was centrifuged again at 8900g for 10 min. The pellet taken and the centrifugation procedure repeated 3 times more. Then the pellet collected and stored at 4 ^{o}C for overnight for bursting the vegetative cells. Spore suspension was centrifuged and the pellet taken and resuspended in 4 ^{o}C ddH_2O and centrifuged at 8900g for 10 min.

Final pellet diluted to 50% concentration with ddH_2O to make the working spore solution.

3.9.4. Slide preparation

10 µL working spore solution was applied on to a cleaned glass slide with ethanol. Using pipette tip, the sample was smeared into a circle about 1.5 cm in diameter. The slide was placed on a 42 ºC metal heating block to dry for ~ 3 min. Slide was then washed for 1 min in Coplin jar with 50 mL ice cold 100% absolute ethanol. The slide was removed from the ethanol and allowed to dry on a metal heating block (42 ºC) to dry for approximately 3 min.

3.9.5. Staining

3.9.5.1. Spore staining by malachite green

Malachite-green staining technique was used as demonstrated by Sherman and Cuppuccino [210]. Bacterial smears were prepared in the usual manner using 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. Counter stain with safranin for 1.0 min. Wash with running tap water and air-dried. The slides were observed under the binocular microscope (100 X). The photographs were taken by Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon digital camera (DXM 1200F, Japan).

3.9.5.2. Spore staining by Acridine orange

Acridine orange staining technique was used as demonstrated by Schichnes *et al.*, [211]. 10 µL of 0.1 µg/mL acridine orange staining solution was applied to the area of spore smear and applied a cover-slip. Slides were observed using an Olympus fluorescent microscope equipped with a BP 480/20 excitation filter fitted with Nikon digital camera (Japan).

3.9.5.3. Measurement of endospores

The average length and breadth of the endopores were measured using a micoscope attached with oculometer.

3.9.5.4. Visualisation of endospres

The spores stained by malachite-green were visualised by Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon digital camera (DXM 1200F, Japan). The spores stained by acridine orange were visualised by Olympus fluorescent microscope equipped with a BP 480/20 excitation filter fitted with Nikon digital camera (Japan). The fluorescent photographs were taken from St. Bazalios College Changanassery, Kottayam.

3.10. Production of *Btk*-toxin (δ-endotoxin/ Crystal protein)

3.10.1. Production of Crystal protein in SSF

Crystal production was done with various raw starches as supplements. The LB medium was prepared supplemented with JP, PP and TP at 10% (w/v) concentration and 6 h time interval ranging from 48h to 72h. The media were mixed in a vortex mixture and incubated at 37 ^{o}C.

3.10.1.1. Maximising Btk-toxin production

For maximisation of crystal production, the fermented matter at 12 h was centrifuged at 800g for 10 min in a refrigerated centrifuge. This step was to remove free water in the medium. The pellets were collected and incubated further at 37 ^{o}C.

3.10.2 Slide preparation

The technique employed for the preparation of slides for crystals was the same in **3.9.4.**

3.10.3. Purification of crystal protein

The sporulated culture were harvested by centrifugation and crystals purified on a step- wise sucrose gradient [19]. The band containing crystals was washed 3

times in 50 mM tris – HCl, pH 7.5, resuspended in 3 mL of ddH_2O containing 1.0 mM Phenyl Methane Sulfonyl Flouride (PMSF).

3.10.4. Crystal staining with safranin

Crystals were stained with malachite-green and safranin. The slides were observed and the photographs were taken by Image Analyser.

3.10.5. Scanning Electron Microscopy (SEM)

3.10.5.1. Procedure

Purified crystals were treated with sorensen phoshate buffer pH 7.2 and stored at 4 ^{o}C overnight. Fixed in gluteraldihyde phosphate buffer for 10 min. Washed with phosphate buffer 15 min.

Dehydration

- 30% ethanol: 15 min 2 changes
- 50% ethanol: 15 min 2 changes
- 70% ethanol: 15 min 2 changes
- 90% ethanol: 30 min 2 changes
- 100% ethanol: 30 min 2 changes

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). The SEM photomicrographs were taken from Sree Chitra Tirunal Institute for Medical Sceince and Technology, Poojappura, Thiruvananthapuram.

3.10.5.2. Protein Estimation

Protein estimation of the purified crystals was done by Lowry's method [208] as described under **3.7.**

3.10.5.3. SDS-PAGE

The purified crystals were analysed on 12% SDS – PAGE under standard conditions as described under **3.8.4.**

3.11. Efficacy studies of *Btk*-toxin against *A. guerreronis (*mandari)

3.11.1. Selection of palms for collecting coconut buttons

Coconut palms of about 10 M height growing near Calicut University Campus (Calicut University Botanical Garden, Villunnial and Kakkanchery) at 8º29'N and 45-50 M MSL were selected for the present study. Collections were made at about 10 AM in summer (April-May) days with an average day temperature ~33 ºC. It is during this time maximum infestation is seen in Kerala.

3.11.2. Collection of coconut buttons

The coconut buttons of about 30 days old were collected every day and used as fresh for culturing the mite *A. guerreronis* as described under **3.1.2.** Hundreds of coconut buttons were cultured repeatedly every week and standardised the culture conditions prior to toxicity assay.

3.11.3. Culturing of A. guerreronis

Commercially available borosil glass rods of 5 cm diameter were bored and cut as rings with internal diameter of 2.5, 3, 3.5 and 4 cm with a height of 1.5 – 2.5 cm and thickness of 0.5 – 1 cm. Cover glasses of 1 mm thickness were suitably cut and used as lids for these culture rings. Coconut buttons (developing small nuts) of about 1 month old were selected from non-infested healthy palms and the appropriate glass ring was fixed with the help of paraffin wax to each button in such a way that the ring touched the boundary between meristematic yellow (the exposed region after the removal of the perianth or cap) and non meristematic green regions. The area (meristematic region within glass ring) of the coconut buttons was calculated from the diameter using vernier calipers.

Diameter = MSR + VSR x LC

MSR = Mean Scale Reading

VSR = Vernier Scale Reading

$$\text{LC (Least count)} = \frac{\text{Value of the smallest division on the main scale}}{\text{Total number of division on the vernier scale}}$$

$$\text{Area} = \Pi r^2$$

$$\Pi = 3.14$$

Using a sterile brush, about 45 mites/cm^2 were carefully introduced in the meristematic region separated by the glass ring. After introducing the mites, the mouth (rim) was lined with a drop of water so that the cover glass remains intact at the mouth of the ring. This arrangement considerably helped in observing the behavior of individual stages of the mite through the cover glass and their manipulation according to the need. A plastic tray (8.5 cm diameter and 3. 5 cm height) was used for rearing *A. guerreronis*. The arrangement was placed in the centre of the plastic tray. The tray was then filled with ddH_2O up to the outer boundary of the glass ring only, so as to prevent the movement of the mites away from the meristematic zone and also to maintain proper humidity. Maximum aseptic conditions were maintained throughout the experiments. Cultures were maintained at 37 ^oC in an incubator.

3.11.4. Bioassay for A. guerreronis

For toxicity assay (bioassay), the standardised *A. guerreronis* cultures were used. 48 h raw pellet of PP (10% w/v) supplemented medium as described in **3.10.1.1.** was used as the crude *Btk*-toxin/*δ*-endotoxin for the bioassay. This crude *Btk*-toxin (including the remnants of PP starch supplement, endospores, bacterial debris) was dried in an oven (37 ^oC for 24 h) and then made into fine powder using mortar and pestle. 1.25, 1.88, 2.5, 3.13, or 3.73 µg/cm^2 crude powder was applied in the culture set-up containing standardised healthy mites. The control mites were fed with the powder prepared as above, which was not inoculated with *Btk.*

3.11.5. Monitoring the growth and mortality of mites

The life cycle of the mite and mortality rates were observed through a Magnus compound microscope. The photographs were taken using a digital camera attached to the microscope (Webcam companion 2.0 MEM 1300, Japan).

3.12. Statistics

All experiments described in this thesis were performed at least in triplicate. SigmaPlot 11.0 (Systat Software Inc) was used for making calculations and drawings. The *Km* and *Vmax* values of amylase were calculated using the software Hyper 32.

4. EXTRACELLULAR α-AMYLASE PRODUCTION BY SUBMERGED FERMENTATION (SmF)

The materials and methods for this piece of work have been described in **chapter 3, section 3.6.**

4.1. Results

4.1.1. Growth characteristics

Growth characteristics showed that *Btk* reached the stationary growth phase of about 18 h. The log phase was extended from 6 to 18 h (Fig. 3).

4.1.2. α-*Amylase activity profile at 3h intervals in LB medium*

Figure 4 gives the profile of α-amylase activity (3 h intervals) of *Btk* during SmF. It shows that maximum activity (~4.4 U/mL) was obtained at around 12 h cultivation (Fig. 4). This activity was persisted up to 18 h cultivation, and then it declined gradually.

4.1.3. α-*Amylase activity in LB medium supplemented by 1% (w/v) soluble starch*

Presence of 1% (w/v) soluble starch in the LB medium greatly enhanced the α-amylase activity (11.2 U/mL) compared to that of control (4.4 U/mL), which was maximum at 12 h (Fig. 5). α-Amylase activity using 1% (w/v) soluble starch as supplement was monitored at 3 h intervals, of which maximum activity was at 12 h (other data not shown). This was about 60% increase over the control. It suggests that presence of starch in the medium can induce elevated production of α-amylase.

4.1.4. α-*Amylase activity of Btk on LB medium supplemented by 1% (w/v) raw starch*

As described under **3.4.1.3**., the raw starch sources used were: BP, BgP, JP, PP, TP and TaP, at a concentration of 1% (w/v) supplement in LB medium. Except

TaP, all other supplements showed maximum activity at 12 h (Fig. 6). Beyond 1% supplementation, the texture of the medium became thicker and thicker, hence SmF studies were limited to 1% (w/v) of starch supplementation.

4.1.4.1. α*-Amylase activity of Btk on BP supplemented medium*

The amylase activity was maximum at 12 h (4.8 U/mL) (Fig. 6). It shows that there was no significant increase in α-amylase activity by BP.

4.1.4.3. α*-Amylase activity on BgP supplemented medium*

The maximum α-amylase activity was (1.9 U/mL) at 12 h (Fig. 6). This result showed that BgP retarded α-amylase activity by about 55% over the control.

4.1.4.4. α*-Amylase activity of Btk on JP supplemented medium*

The maximum α-amylase activity was (4.49 U/mL) was at 12 h (Fig. 6). It shows that there was similar activity in 1% (w/v) JP supplemented medium and in control.

4.1.4.5. α*-Amylase activity of Btk on PP supplemented medium*

The maximum α-amylase activity was (3.91 U/mL) at 12 h cultivation, which was about 10% less than that of control (Fig. 6).

4.1.4.6. α*-Amylase activity of Btk on TP supplemented medium*

The TP starch in the medium induced the α-amylase production by 18% higher (13.19 U/mL) than that of soluble starch (11.21 U/mL), while it was about 2 fold increase over the control (Figs. 5 & 6). This data clearly suggests that an increased level of enzyme production by TP supplementation.

4.1.4.7. α*-Amylase activity of Btk on TaP supplemented medium*

Unlike other supplements in the LB medium (maximum activity at 12 h), presence of TaP in the medium showed maximum activity at 6 h (9.83 U/mL), while at 12 h its activity was only, 2.28 U/mL (Fig. 6). As shown in Figure 6, this activity at 12 was comparable to that of control at 6 h (2.92 U/mL).

4.2. Discussion

The aim of this piece of study was to check whether *Btk* would produce extracellular α-amylase by SmF as a byproduct in the process of producing endospore and δ-endotoxin (parasporal crystal protein or *Bt*-toxin). To analyse α-amylase activity (at pH 6.9 and temperature ~32 ^{o}C for 1 % starch with 5 min incubation), crude supernatant obtained after 12 h fermentation was used. Results showed that the presence of starch in the LB medium could enhance α-amylase production by about 2 fold, compared to that of control (LB medium). Presence of starch in the growth medium has been found to increase extracellular α-amylase in the medium [85]. In the present study, in general, natural starch was not better than that of soluble starch (at 1% w/v) towards inducing extracellular amylase production. Nevertheless, TP showed significantly higher activity (18%) over soluble starch. Contrarily, presence of BgP in the medium even retarded amylase production by about 55%.

The novelty of this investigation is that, so far no focused study has been made to produce α-amylase by a strain of *Bt*, because main focus of all *Bt*-based studies hitherto has been its ability to produce *Bt*-toxin, the pesticide. However, a couple of preliminary studies reported that *Bt* strains could produce traces of extracellular amylase. Firstly, while screening the utilisation of complex carbon and nitrogen sources, Kuppusamy and Bamaraman [20] have observed that *Bacillus thuringiensis* H14 could secrete extracellular proteolytic and amylolytic enzymes. Secondly, *Btk* produced up to 0.11 U/mL amylase, while studying the effects of various pH control agents such as ammonium acetate, ammonium sulphate, sodium acetate and sodium sulphate upon biopesticidal activity of *Btk* HD-1 [21]. In this study, Vu *et al.* [21] used starch industry waste water as the medium for fermentation. But, the present study was intended solely to monitor the amylase production pattern of *Btk* under SmF.

Starch degrading bacteria are most important for food, fermentation, textile and paper industries [103]. Members of *Bacillus* are well known for their potential to secrete a number of degradative enzymes like amylase, cellulose and protease

[212]. Most researchers have reported that the biosyntheis of α-amylase is induced only in the presence of starch [105]. It was found in this study that the commercially available soluble as well as naturally available raw starches could induce α-amylase production during SmF. Vu *et al.* [21] showed that amylase production in the fermentation medium is an important factor, because it can support the growth and synthesis of *Bt*-toxin and other components of *Btk* through hydrolysis of residual starch. Moreover, starch is a best inducer of α-amylase production [85].

There are reports showing various species of *Bacillus* producing amylase upon supplementing different substrates and changing the growth conditions. *Bacillus* spp. grow at different rate with different specificities to different substrates in culture media [101]. Ajai and Fagade [101] showed that amylase production values range from 0.22 x 10^2 U/cfu by *B. circulans* to 0.912 x 10^2 U/cfu by *B. licheniformis* for corn starch and 0.01 x 10^2 U/cfu by both *B. megaterium* and *B. licheniformis* to 0.693 x 10^2 U/cfu by *B. subtilis* for soluble starch. All these species are the major commercial producers of amylases. Likewise, *B. subtilis* wild strains isolated from Nigeria, showed highest amylase activity of 13 AU/mL [213]. This activity is comparable to the report made herein.

Complex and semisynthetic media were better than synthetic medium for the growth and activity of *B. sterothermophilus* [121]. Srivastava and Baruah [121] also observed that α-amylase production was higher on the complex medium containing beef extract or corn steep liquor than on semisyntheic medium containing peptone. In the present study, the complex LB was the basic medium with or without starch supplements.

According to Saui *et al.*, [119], 20 g/L (2 % w/v) starch supplement was good for amylase synthesis by *Bacillus* sp. AS – 1. Effect of peptone and yeast extract in the presence of 1.5 % starch increased the α-amylase production by *B. subtilis* strain [122]. In shake flask culture of *B. thermooleovorans*, starch and tryptone were observed to be the ideal carbon and nitrogen sources, respectively [123]. In this study, 2.2 fold incerase in amylase production was observed at 12 h then it declined, which continued upto 45 h. Agrawal *et al.* [85] isolated *Bacillus* sp.

KCA 102 strain from soil which produced theromostable amylase (94 mU/mL) in a short fermentation time 18-20 h. *Bacillus* strain, an isolate from soil sample receiving kitchen waste and showed optimum growth at temperature 37 ^{o}C, pH 6.8 - 7.2, whose maximum α-amylase activity (75.7 U/mL) was observed at 2% soluble starch concentration in the starch agar medium [103].

α-Amylase production by supplementing naturally available raw supplements mentioned in the present study is rarely reported employing *Bacillus* sp. Ikram-ul-Haq *et al*. [124] conducted selection study employing commercially available agricultural starchy substrates like hordium, pearl millet, rice, corn, gram and wheat starch for the production of α-amylase by *B. licheniformis*. 1.5 % pearl millet starch significantly enhanced amylase production. *Geobacillus thermoleovorous* showed α-amylase activity on several starch substrates and their derivatives, but it hydrolysed raw starch of pearl millet efficiently [125].

Unlike *Btk*, most of the species of *Bacillus* as discussed above are known for their amylase production commercially. In the present study, 1% (w/v) starch was used as a supplement in the LB medium, and this concentration is comparable to the soluble or raw starch supplements used by other investigators (mostly 1-2 %) employing different *Bacillus* spp. Moreover, over 11 U/mL activities in the present study is a promising result. Like the study of α-amylase production by *Btk* in SmF, natural starch supplements used in this study were also new in terms of α-amylase production by any *Bacillus* sp. Thus, this piece of the whole study concludes that *Btk* is a good producer of amylase under SmF in the presence of suitable starch supplemented complex medium like LB, and that the role of starch as an inducer of α-amylase production is very much significant.

5. α-AMYLASE PRODUCTION BY SEMI-SOLID- AND SOLID-STATE FERMENTATIONS (SSF)

The materials and methods for this piece of work have been described in **chapter 3, section 3.7.**

5.1. Results

5.1.1. α-Amylase production by *Btk* on soluble starch as supplement

LB medium was supplemented with different concentrations of soluble starch (5%, 10%, 15%, 20% and 25%, all w/v). Upon starch supplementation (> 5%), the medium turned to be semi-solid or solid. At 5% (w/v), the medium was viscous, while at 10% (w/v) the medium became soft solid mass (Fig.7). During the progression of incubation and growth, the texture of the medium was softer than its initial state (Fig. 8). Since insignificant increase in α-amylase activity was noticed beyond 25% supplement, subsequent data are not presented here.

Maximum α-amylase activity (~ 28.59 U/gds) at 10% (w/v) starch concentration was obtained at 12 h cultivation, while the activity in the control LB (without starch) was about 4.36 U/mL (Fig. 9). This activity in the 10% (w/v) starch supplemented medium was about 6.6 fold increase over the control. For SSF, the unit of activity has been expressed in the conventional "U/gds", as described in **3.6.3.4.**

5.1.2. α-Amylase production by *Btk* on BP supplemented LB medium

α-Amylase activity was studied by replacing the soluble starch in basic LB medium with six locally available natural raw starch sources, *viz.*, BP, BgP, JP, PP, TP and TaP, as described under **3.4.1.3.** The LB medium was supplemented with one of these starch sources at 5%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%

(w/v) concentrations at a time. In all cases, α-amylase activity was maximum at 12 h at 10% (w/v) concentration of the respective starch, except BgP.

Maximum α-amylase activity in the BP supplemented medium was about 266 U/gds. This was 61 fold increases over the control (Fig. 5). The time interval assay data showed that α-amylase production increased from 6 h to 12 h and gradually decreased from 18 h to 24 h (Fig. 10).

5.1.3. α-Amylase production by *Btk* on BgP supplemented LB medium

In this, maximum activity (56.06 U/gds) observed at 18 h at 50% concentration of BgP (Fig. 11). This was the only starch supplement which induced maximum activity at 50% concentration, and was at 18 h. However, the activity was much lower, compared to other supplements.

5.1.4. α-Amylase production by *Btk* on JP supplemented LB medium

Maximum amylase activity was 460.27 U/gds, which was 105 fold increase over the control (Figs. 5 & 12).

5.1.5. α-Amylase production by *Btk* on PP supplemented LB medium

In this medium composition, maximum α-amylase activity was about 867 U/gds, which was 198 fold increase over the control (Fig. 5). This was the highest yield among 7 starch supplements including soluble starch used (Fig. 13). Hence, this combination was used for further studies.

5.1.6. α-Amylase production by *Btk* on TP supplemented medium

Maximum α-amylase activity on TP supplemented LB medium was 481.13 U/gds (Fig. 14), it was 110 fold increase over the control (Fig. 5).

5.1.7. α-Amylase production by *Btk* on TaP supplemented medium

The maximum α-amylase production was 268.99 U/gds (Fig. 15), which was about 61 fold increase over the control (Fig. 5).

5.2. Discussion

Main question of this piece of work was to check whether water-restricted environment (SSF) would enhance α-amylase production by *Btk*. For that, LB was supplemented with up to 100% (w/v) starch supplements. In 100% (w/v) starch-supplemented medium, starch to LB ratio was 1:1. As reported in the succeeding chapters, supporting goal of this SSF study was to check whether α-amylase production is correlated with *Bt*-toxin production. Hence, the SSF strategy adopted herein used a system with some water freely available in the initial stage. Unlike other solid substrates (mainly solid agro-residues) [117, 140], starch supplements used herein easily assumed a slurry state even at its low concentrations.

According to Benjamin and Pandey [140], SSF holds tremendous potential for the production of enzymes and it can be of special interest in those processes where the crude fermented product may be used directly as enzyme source. *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis*, coupled with some mycelial fungi are the major commercial strains of α-amylase production, especially by SSF [214]. Until this pioneering study, no attempt has been made to produce α-amylase by SSF employing any *Bt* strains. Moreover, the raw starch substrates used in this study are rarely used by other investigators for the cultivation of other bacterial or fungal species.

In the present study, 7 starch sources have been used; *viz*., the commercially available soluble and 6 natural raw starches. Being stored food, the natural raw starches offer a balanced diet to the embryo/buds in the seed or modified stems/roots to be developed later, thus they should contain many growth factors apart from the major feeder starch. In comparison, PP yielded maximum activity (867.55 U/gds), which was about 33 fold increase over the activity of commercially available soluble starch supplemented medium (28.59 U/gds), both at 10% (w/v) and 12 h of cultivation. Upon assay, all the supplements supported maximum α-amylase production at 12h (10% w/v), except BgP.

There are reports wherein potato residues (not potato powder as used in the present study) used as solid substrate in the fermentation medium and got maximum

enzyme production than other substrates [127]**.** Shukla and Kar [127] found potato peel as a superior substrate for SSF compared to wheat bran for the production of α-amylase by *B. licheniformis* and *B. subtilis*, their α-amylase activities were 270 U/mL and 600 U/mL, respectively. In a different study, potato waste was an efficient substrate for SSF by *Rhizopus oryzae* to produce α-amylase [215]. Present study shows that potato powder supplement was efficient than other starch supplements for enhanced production of α-amylase by *Btk* during SSF. It indicates that potato starch provided better growth conditions for *Btk* than other supplements.

SSF was carried out by Goes and Sheppard [216] using ground potato peel in perforated stainless steel trays with 80% moisture content. By using a mixed microbial culture (*B. subtilis* ATCC 21556 and ATCC 21332), α-amylase activity (290 U/gds) increased upto 10 times in comparison with that of *B. subtilis* ATCC 21556 (1 fold) and *B. subtilis* ATCC 21332 (25 fold), when used singly. Enhanced α-amylase production under SSF was observed employing *Aspergillus niger* under fermentation conditions involving 10% w/v soluble starch as supplement [217]. This finding clearly demonstrated that the yield in SSF was very high compared to SmF. Ramesh and Lonsane [109] found that α-amylase production by *B. licheniformis* M27 increased by 29 times with 42 fold increase in the presence of soluble and other starchy substrates in the SSF system.

α-Amylase production in the presence of naturally available raw supplements mentioned in the present study is rarely reported. Kokab *et al.* [218] used banana peel (not powder) as agricultural raw substrate for the production of α-amylase by fermentation employing *Bacillus* sp. α-Amylase production under SSF using various agricultural substrates was reported by several authors. Ramesh and Lonsane [126] conducted SSF process for amylase production using *B. licheniformis* M 27. A surge in enzyme production in the first 24 h of fermentation was observed in media with 75% and 85% moisture using wheat bran as substrate. *B. licheniformis M27* produced 21,000 units of α-amylase/g dry bacterial bran under SSF on wheat bran medium enriched with 3.3% di-ammonium hydrogen phosphate [126]. Ramesh and

Lonsane [128] also found that *B. megaterium*, a related species produced α-amylase (30,000 U/g dry bacterial bran) under SSF in wheat bran medium.

Production of extra-cellular α-amylase by thermo tolerant *B. subtilis* was studied during SSF [129]. Effects of wheat bran and rice husk were examined and in comparison to rice husk, enzyme yield was 7.3 fold higher with wheat bran medium (15%) in 0.1M phosphate buffer at pH 7.0 and at 24 h [131]. In the same study, alkaline α-amylase produced from *Bacillus* sp. under SSF using wheat bran medium showed maximum activity at 24 h. Laboratory isolates of *Bacillus* sp. AS-1 produced very high amounts of thermostable α-amylase during SSF using wheat bran [219]. The enzyme was stable at 15% starch and temperature 50 ^{o}C. Production of α-amylase under SSF by *B. cereus* MTCC 1305 has been investigated using wheat bran and rice flake manufacturing waste as substrates, and the highest activity was about 122 U/g at pH 5.0 and temperature 55 ^{o}C with substrate concentration ratio 1:1 [133].

Fourteen different agro-residues were screened for α-amylase production using *B. amyloliqefaciens* ATCC 2384 by Gangadharan *et al.*, [134]. Among these agro residues, wheat bran and ground nut oil cake in mass ratio of 1:1 was proved as the best substrate, and maximum enzyme titer was 62470 U/g [134]. Sodhi *et al.* [132] isolated *Bacillus* sp. PS-7 from the hot springs in India and found that the strain produced very high levels of thermostable α-amylase by SSF. They observed maximum enzyme production as 4, 64,000 U/g dry bacterial bran on wheat bran supplemented with glycerol (1.0% w/v).

The foregone discussion indicates that employing SSF, rice or what bran is the favourite agro-substrate for the production of α-amylase by bacillial spp. α-Amylase production even enhanced to 4,64,000 U/gds [132], and on an average it is about 30,000 U/gds [126 -127, 134]**.** It is evident that, apart from the solid media used, the ingredients supplemented to the medium played a crucial role in deciding the production [214]**.** However, in the present study, the activity of the crude supernatant was 867 U/gds in the potato starch supplemented medium. Compared to many reports as above, of course, this is moderately high activity. Furthermore, as

shown in the succeeding chapter, the partially purified enzyme from the same supernatant (as above) showed over 8000 U/gds activity, which in fact is over 80,000 fold more activity than the only earlier report for *Bt* strain [21]. Moreover, the prime objective of this study was not the production of α-amylase alone (*i.e.*, *Btk*-toxin production and efficacy studies as well, as shown in the succeeding chapters) [220]**,** nevertheless α-amylase produced as a by-product from a non-commercial *Bacillus* strain (*Btk*) offers much advantages to the *Bt*-toxin industry.

6. PURIFICATION AND CHARACTERISATION OF α-AMYLASE

The materials and methods for this piece of work have been described in **chapter 3, section 3.8.**

6.1. Results

6.1.1. Source of α-amylase produced by Btk for purification

As shown in Chapter 5, potato powder (PP) supported maximum α-amylase production at 10% (w/v) and 12 h fermentation (Fig. 13). Hence, supernatant obtained from 10% (w/v) PP supplemented LB medium after 12 h fermentation was used for the purification of α-amylase (Figs. 16-18 & Table 11).

6.1.2. Partial purification of α-amylase by ammonium sulphate fractionation

Of various ammonium sulphate fractions, 40-60% fraction showed maximum α-amylase activity (Fig. 16). This partially purified fraction was used for the characterisation studies of α-amylase. It was 3.45 fold purified fraction with 81% yield (Table 11). SDS–PAGE profile of crude as well as partially purified proteins showed that the apparent MW of the *Bt*-amylase is 51 kDa, the supposed α-amylase (Fig. 16).

6.1.3. Purification of α-amylase by Sephadex G-100 gel permeation chromatography

70 fractions (2 mL/10 min) were collected and the OD at 280 nm showed major peak, represented by 37–43 fractions (Fig. 17). These α-amylase active fractions were pooled and concentrated by lyophilisation. Purified enzyme was seen as single band on SDS-PAGE image, whose apparent MW was 51 kDa, as that of crude and partially purified ammonium sulphate preparations (Fig. 18). The

purification fold of sepahadex G-100 fraction α-amylase was 14.75 with 79% yield (Table 11).

Table 11. Summary of purification of extracellular *Btk* α-amylase from the supernatant on LB medium supplemented with 10% (w/v) raw potato powder.

Purification	Total protein (mg)	Total activity (U/gds)	Specific activity (U/ mg protein)	Yield (%)	Fold purification
Crude extract	3.36	2903.27	864.00	100%	1
40-60% $(NH_4)_2SO_4$ Fraction	1.8	5370.18	2983.43	81.36	3.45
Sephadex G-100 column Fraction	0.18	2295.27	127512	79.05	14.75

6.1.4. Enzyme characteristics

6.1.4.1. Effect of pH on enzyme activity

As large quantity of protein solution was required for repeated α-amylase assays, 40% - 60% ammonium sulphate fraction was used for the characterisation studies.

Maximum amylase activity (5643.64 U/gds) was noticed at pH 6.0 and the lowest activity (843.64 U/gds) was at pH 3.0 (Fig. 19). α-Amylase activity was considerably decreased at low acidic as well as at high basic pHs (Fig. 19).

6.1.4.2. Effect of temperature on enzyme activity

Results indicate that the temperature optimum for α-amylase from *Bt* was 60 ^{o}C and the activity was 5498.18 U/gds (Fig. 20) with comparable activities at 50 ^{o}C, 55 ^{o}C and 65 ^{o}C. However, about 17% activity was retained at 100 ^{o}C (930.91 U/gds).

6.1.4.3. Effect of complex compounds on amylase activity

Of the complex compounds, the chelating agent EDTA (552.73 U/gds) and detergent SDS (349.09 U/gds) have tremendously decreased the α-amylase, while β-mercaptoethanol completely abolished the activity. The amino acid cysteine partially retarded (15 %) the activity (4712.73 U/gds) (Table 12).

6.1.4.4. Effect of metal salts on amylase activity at concentration of 1µM

Table 12 summarises the effect of different metal salts on amylase activity. The optimum activity shown by Ca^{2+} and the activity was found to be 5934.55 U/gds while the Mn^{2+}showed lowest activity of 3810.91 U/gds. SO_4^{2-} and SO^{3-} were the anions showed activities of 3723.64 U/gds and 3374.55 U/gds.

Table 12. Effect of metal salts (µM) on partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* extracellular α-amylase from the LB medium supplemented with 10% (w/v) potato powder.

Metals	**Activity (U/gds)**	**Standard deviation**
Mn^{2+}	3810.91	±1.82
Fe^{2+}	5294.52	±4.84
Fe^{3+}	5410.91	±1.57
Thiourea	4218.18	±5.09
EDTA	552.727	±1.68
SO_4^{2-}	3723.64	±4.73
SO_3^{-}	3374.55	±4.84
Ag^{+}	5847.27	±5.77
Mg^{2+}	5880.00	±6.18
Na^{+}	3723.64	±1.2
Mo^{2+}	5498.18	±3.58
Ca^{2+}	5934.55	±1.68
Cu^{2+}	5556.36	±4.38
Zn^{2+}	5381.82	±9.87
Cysteine	4712.73	±5.36
SDS	349.09	±1.83
β-mercaptoethanol	0.000	0.000

6.1.4.5. ***Effect of different metal salts on amylase activity at different concentrations***

Table 12 shows that Ag^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mo^{2+}, were the metal ions mainly enhanced the α-amylase activity. The reaction mixture was incubated with these metals at 0.5 µM, 1.0 µM, 2 µM, 3 µM, 4 µM and 5 µM concentration. This result suggests that higher α-amylase activity at 3 µM concentration of Ca^{2+}, which was 6080 U/gds (Fig. 21). This was about 10% increase over other factors as described above.

6.1.4.6. Effect of substrate concentration

α-Amylase activities as described above were with 1% starch substrate and 5 min incubation. Here, effect of substrate concentration was tested 1-5% for 30 min incubation. From Figure 22, the optimum activity was about 8088.82 U/gds for 30 min at 2.5% starch concentration. Interestingly, α-amylase was almost stable for 1 h.

Briefly, optimised conditions for *Btk* α-amylase activity are: 2.5% starch at 6.0 pH and 60 ^{o}C incubation for 30 min in the presence of 3 µM Ca^{2+}.

6.1.4.7. Enzyme kinetics

The *Km* and *Vmax* values for *Btk* α-amylase were calculated using the data shown in 6.1.4.6. The *Km* and *Vmax* values were found to be 2.9 mg/mL and 0.05335 µmol/mL/min, respectively. This was calculated using the software, Hyper 32 (Fig. 23).

6.2. Discussion

No report is available in literature documenting the purification and characterisation of *Btk* α-amylase either by SmF or SSF. Present study, therefore, is the first ever attempt towards the purification and characterisation of *Btk* α-amylase [220]. Supernatant obtained from 10% (w/v) PP supplemented LB medium after 12 h fermentation of *Btk* was used for the purification and characterisation of α-amylase.

The first step was to fractionate the crude supernatant using ammonium sulphate. 40-60% fraction showed maximum amylase activity; hence that fraction was used for characterisation studies, and also for further purification by gel

filtration. *Btk* was capable of secreting significant amount of α-amylase and was purified (14.75 fold with 79.05% yield) through ammonium sulphate fractionation and sephadex G-100 gel permeation chromatography (Table 11). No reports are available demonstrating the purification of amylase from *Btk*, but there are reports supporting the present data from other *Bacillus* spp. Krishnan and Chandra [163] purified α-amylase produced by *B. licheniformis* CUM 305 to 212 fold with a 42% yield. Hmidet *et al.* [156] reported a thermostable α-amylase by a new isolate, *B. licheniformis* NH 1, which was purified to homogeneity through 40–60% ammonium sulphate precipitation, Sephadex G–100 gel filtration and sepharose mono-Q anion exchange chromatography. It showed 3.08 fold increase in specific activity and 15.9% recovery.

Amritkar *et al.* [149] purified (11.9 fold) α-amylase from *Bacillus* spp. B3 with 60.8% recovery. Purification and characterisation of a novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX – 1 resulted in 34 fold purification with 6.6% recovery [159]. Zhi *et al.* [221] found that the partial purification of α-amylase form *B. subtilis* attained a purification factor of 1.8 fold and 90% yield in a polyethylene glycol 1000–citrate aqueous two-phase systems (ATPS**)**. Das *et al.* [142] isolated *B. subtilis* strain DM–03 from starter culture being used for the production of alcohol by local Assam tribes, and the α-amylase purified by ion exchange, gel filtration and HPLC techniques to 80.5 fold purity. Ezeji and Bahl [158] purified α-amylase secreted by *Geobacillus thermodenitrificans* HRO 10 through a series of steps, which resulted in 13.6 fold purification with 11.5% recovery. Rao *et al.* [165] purified (230 fold) α-amylase from *B. licheniformis* to homogeneity by ammonium sulphate precipitation and affinity chromatography. α-Amylase from *Bacillus* sp. strain TS 23 was purified (11.4 fold) with 49% yield [166]. Liu *et al.* [157] purified α-amylase from *B. subtilis* WB600 to 187.1 fold purity by ammonium sulphate fractionation and anion exchange and gel filtration. All these findings show varying purification fold and yield. In the present study, about 15 fold purification and 79 % yield were obtained, which are within the limit of reported values for different *Bacillus* spp.

In the present study, the purified α-amylase from *Btk* showed a single protein band on SDS-PAGE with an apparent MW of 51 kDa. This finding corroborates well with previous reports as highlighted below on α-amylase from various bacillial spp. [214]. Demirkan *et al.* [86] obtained α-amylase produced by *B. amyloliquefaciens* and the purified enzyme was estimated to have a MW of 52 kDa as judged by SDS – PAGE. Faber *et al.* [222] reported that the MW of α-amylase from *B. licheniformis* was 55 kDa. Bernhardsdotter *et al.* [164] purified α-amylase from *Bacillus* sp. strain L1711 and MW was 51 kDa. Hmidet *et al.* [156] purified a thermostable α-amylase from a newly isolate *B. licheniformis* NH 1, whose MW was 58 kDa as shown by SDS – PAGE. The purified starch digesting α-amylase from a newly isolated *Bacillus* sp. YX – 1 showed a MW of 56 kDa, as judged by SDS-PAGE [159], while α-amylase from another strain of *B. lichenifromis* had MW of 55 kDa [88]. Lo *et al.* [223] produced α-amylase from *Bacillus* sp. strain TS -23 and its MW was 54 kDa. A maltooligosaccharide forming α-amylase produced by new soil isolate *B. subtilis* KCC 103 was purified whose MW 53 kDa [87]. The α-amylase from *B. subtilis* WB600 was purified by ammonium sulphate fractionation, anion exchange and gel filtration and the MW of the purified enzyme was 53 kDa [159]. It indicates that the MW of α-amylase secreted by the bacillial spp. varies from 51 - 58 kDa.

In the present study, the partially purified α-amylase from *Btk* showed its pH and temperature optima as 6.0 and 60 ^{o}C, respectively. At this conditions, the activity was persisted for over 1 h. These optimal pH and temperature are in the ranges reported for α-amylase from some *Bacillus* species by several authors. Demirkan *et al.* [86] purified α-amylases of *B. amyloliquefaciens* and a mutant strain, whose degradation ability with starch granules from various botanical sources was tested. They found that the purified enzyme was best active at pH 6.0 and temperature 55 ^{o}C [86]. Hmidet *et al.* [156] purified thermostable α-amylase from *B. licheniformis* NH 1, which was highly active at pH 6.5 and temperature 90 ^{o}C. Liu *et al.* [159] showed that α-amylase from *Bacillus sp.* YX – 1 had higher activity at pH 5.0 and temperature range was 40 –50 ^{o}C. Zhi *et al.* [221] studied partial purification of α-amylase from *B. subtilis* and found that its maximum activity was at pH 6.0.

The α-amylase from *Bacillus* sp. strain TS 23 was active at pH 8.0 and temperature 55 ^{o}C [166]. Kiran and Chandra [93] found that the pH and temperature optima of α-amylase from *Bacillus* sp. Strain TSCVKK were pH 7.5 and temperature 55 ^{o}C, repectively. Ohdan *et al.* [161] produced α-amylase by *B. subtilis* X 23, and its pH (5.5) and temperature (65 ^{o}C) optima were determined. A maltooligosaccharide forming α-amylase from *B. subtilis* KCC 103 was shown highly active over a broad range of pH from 5 to 7 and temperature 65–70 ^{o}C [87].

In the present study, complex compounds like the chelating agent EDTA and detergent SDS have tremendously decreased the α-amylase activity, while β-mercaptoethanol completely abolished it, and cysteine partially retarded the activity. The thermostable α-amylase from *B. licheniformis* NH 1 was almost inactivated by the chelating agent EDTA, retaining only 17% activity, while SDS and β-mercaptoethanol completely abolished the activity [156]. Krishnan and Chandra [163] observed that α-amylase from *B. licheniformis* CUM 305 was highly inactivated by EDTA, but EDTA retained 50% activity of α-amylase from *B. subtilis* KCC 103 [87]. Das *et al.* [142] isolated *B. subtilis* strain DM – 03, and found that the amylase activity was completely abolished in the presence of PMSF, bromophenacyl bromide and SDS. Arikan [167] produced amylase by *Bacillus* sp. A3-15 and found that $ZnCl_2$, NaCl, $CaCl_2$, Na-sulphide, EDTA, Urea and SDS inhibited the enzyme activity. These inhibitors should have irrecoverably complexed with the enzyme, so that its conformation would have changed leaving the enzyme with total loss or partial loss of activity.

Of different metal salts tested in this study to evaluate their effects on *Btk* α-amylase, 3 µM Ca^{2+} was the suitable metal ion for maintaining and enhancing its activity. Presence of Ag^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Mo^{2+} in the reaction mixture generally enhanced α-amylase activity, while other metal salts Mn^{2+}, Fe^{2+}, Fe^{3+}, Na^{+},Zn^{2+},SO_4^{2-},SO_3^{-} inhibited the enzyme activity up to 45%. This data gets support from several reports. Krishnan and Chandra [163] purified α-amylase from *B. licheniformis* CUM 305 and found that the cations Na^{+}, Ca^{2+} and Mg^{2+} showed stimulatory effect. Whereas Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, Fe^{2+}, Co^{2+}, Cd^{2+} Al^{3+} and

Mn^{2+} were inbihitory. The anions azide, So^{3-} and So^{4-} showed excitant effect. α-Amylase from *B.licheniformis* NH 1 showed enhanced activity by metal ions such as Hg^{2+} and Zn^{2+} [156]. Demirkan *et al.* [86] showed that α-Amylases from *B. amyloliquefaciens* was stimulated by the divalent metal ions like Mg^{2+}, Ba^{2+} and Cu^{2+}, while Hg^{2+}, Fe^{2+}, Zn^{2+} and Ag^{2+} strongly inhibited the activity. Huang *et al.* [166] found that Ni^{2+} and Mn^{2+} have stimulatory effects on the α-amylase from *Bacillus* sp. Strain TS 23. Srivastava and Baruah [121] grew *B. sterothermophilus* better on complex and semi-synthetic media than on synthetic medium. They found that medium containing $CaCl_2.2H_2O$ enhanced amylase production over that on Ca^{2+} deficient medium.

Briefly, divalent cations are potent enhancers of α-amylase as explained above. Ca^{2+} indicated as the most crucial ion in all these studies. Crystal studies show that Ca^{2+} is the most fovoured ion by α-amylases for their better stability and activity [214]. Binding of metal ions impart major conformational rearrangement on the enzyme molecule [182]. The metal induced disorder→order transition observed in *B. licheniformis* α-amylases leads to the formation of the extended substrate binding site and explains on a structural level the calcium dependency of *α*-amylases [179].

In the present study, the *Km* and *Vmax* values were found to be 2.9 mg/mL and 0.05335 µmol/mL/min, respectively. *Km* approximates the affinity of enzyme for its substrate. A small *Km* indicates high affinity, and a substrate with a smaller *Km* will approach V*max* more quickly. Literature shows that the *Km* of various bacillial *α*-amylases varies from 1.3 to 7.8 mg/mL. The *Km* and *Vmax* of extracellular thermostable α-amylase from *B. subtilis* were 7.79 mg/mL and 11.176 mg/mL/h, respectively towards potato starch substrate [105]. The *Km* and *Vmax* values were 0.738 mg/mL and 1.274 mg/mL, respectively for α-amylase from *B. licheniformis* CUM 305 [163]. Bernhardsdotter *et al.* [164] isolated an alakaliphilic amylase producing bacterium *Bacillus* sp. strain L1711, and the *Km* and *Vmax* values were calculated as 1.9±0.2 mg/mL and 0.051±0.006 µmol/min, respectively. Ezeji and Bahl [158] purified α-amylase secreted by *G. thermodenitrificans* HRO

10, whose *Km* and *Vmax* were 3.05 mg/mL and 7.35 U/mL, respectively. *Km* and *Vmax* of α-amylase from *B. subtilis* KCC 103 were 2.6 mg/mL and 909 U/mg, respectively [87]. From this, comparatively it is evident that *α*-amylase from *Btk* is a fast acting enzyme, which would find better position in industry. The substrate with the lowest *Km* upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes. In fact, in the present study, partially purified enzyme was used, and thus the kinetic behavior of pure enzyme from *Btk* would be far excellent.

7. ENDOSPORE PRODUCTION OF *Btk* BY SSF

The materials and methods of this piece of work section have been described under **chapter 3 section 3.9.**

7.1. Results

Six raw starch supplements and commercial starch were used to supplement the complex LB medium. In the preliminary studies, JP, PP and TP showed higher α-amylase activity. Compared to other starch supplements, endospore (and also crystals) production was higher in 10% (w/v) JP, PP or TP supplemented LB media. More emphasis was given to PP supplement, as it was emerged as the best support for both amylase and spore production. In all the Figures, the spores are seen greenish-blue, and crystals are in bluish-pink colours.

7.1.1. Production of endospore using potato powder (PP) supplement

Endospore production by *Btk* has been monitored up to 72 h with 6 h intervals. 6 h interval data are pooled and shown separately.

7.1.1.1. Endospore production at 6 h on PP supplemented LB medium:

The 6 h sample contained a few bacteria at 1%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100% (w/v) concentrations of the PP supplemented LB medium, but frequency of vegetative cells in LB control, 1% and 5% PP supplemented LB media was comparatively high (Fig. 24). There was no significant spore production in none of these samples (Fig. 24). As seen in the Figure, sporangial development started even at the lag phase of growth. The growth profile of *Btk* showed that its OD at 3h was 0.26 and at 6 h, it was 1.57 (Fig. 3). This increased OD further confirms the sporangial (green) development which resulted in the scattering of more light.

7.1.1.2. Endospore production at 12 h on PP supplemented LB medium:

The 12 h sample contained more number of vegetative cells in all the concentrations starch supplements and control. The endospore (green) formation was seen only in a few cells (Fig. 25). The smallest (0.75 – 1 µM length and 0.4 – 0.5 µM breadth) endospores noticed at 1% (w/v) and the largest (1.0 – 1.2 µM length and 0.6 – 0.75 µM breadth) endospores at 80%, while the spores in the control showed an average length of 0.5 – 0.6 µM with 0.4 – 0.5 µM breadth (Table 13). In this Figure, prominent endospores are seen.

7.1.1.3. Endospore production at 18 h on PP supplemented LB medium:

The 18 h sample contained about 50% of vegetative cells without endospores or with developing spornagia. Mature endospores in about 50% cells were noticed in all (10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%) starch supplemented media (Fig. 26). Also much swollen sporangia are seen in some starch supplemented media. The smallest (0.75 – 0.9 µM length and 0.5 – 0.7 breadth) endospores noticed at 20% (w/v) and the largest (0.9 – 1.0 µM length and 0.6 – 0.8 µM breadth) endospores at 100%, while the control having an average length of 0.5 – 0.6 µM and 0.4 – 0.5 µM with 0.4 – 0.5 µM breadth (Table 13).

7.1.1.4. Endospore production at 24 h on PP supplemented LB medium:

At 24 h, almost all cells contained endospores. The cells started rupturing to release the endospores. But in control, and samples with low starch concentration (1% and 5%), the most of the cells were intact with endospores. Size of the spore was much bigger in samples with higher concentration of starch (20%, 30%, 40%, 50%, 60%, 80% and 100%) (Fig.27). The smallest (0.75 – 1.0 µM length and 0.6 – 0.75 µM breadth) endospores noticed at 30% (w/v) and the largest (0.75 – 1.0 µM length and 0.5 – 0.75 µM breadth) endospores at 80%, while the control having an average length of 0.6 – 0.8 µM and 0.5 – 0.6 µM breadth (Table 13). In this Figure, the frequency of vegetative cells was less than that of sporangial cells. More sporangia lysed.

7.1.1.5. Endospore production at 30 h on PP supplemented LB medium:

The 30 h sample contained significantly higher levels of free endospores in all the starch supplemented media, except control (Fig. 28). 30% PP supplement onwards, the number of vegetative cells was comparatively very less. The smallest (0.75 – 1.0 µM length and 0.5 – 0.6 µM breadth) endospores noticed at 40 % (w/v) and the largest (0.8 – 1.0 µM length and .5 – 0.6 µM breadth) endospoares at 100%, while the control having an average length of 0.6 – 0.8 µM and 0.5 – 0.6 µM breadth (Table 13). Control still shows dividing cells, which give pseudo-filamentous morphology.

7.1.1.6. Endospore production at 36 h on PP supplemented LB medium:

The 36 h samples also contained significantly higher number of exposed endospores, which was less in the control. The endopore production was very high in starch supplemented media, and maximum spore production observed on 50% - 100% PP supplements (Fig. 29). The smallest (0.6 – 0.9 µM length and 0.5 – 0.6 µM breadth) endospores noticed at 10 % (w/v) and the largest (0.8 – 1.1 µM length and 0.5 – 0.6 µM breadth) endospores at 100%, while the control having an average length of 0.6 – 0.8 µM and 0.5 – 0.7 µM breadth (Table 13). Much bigger endospores are seen in starch supplemented media.

7.1.1.7. Endospore production at 42 h on PP supplemented LB medium:

Significant levels of endospore production were also noticed in all the starch concentrations. The endopore production was high in higher concentrations of starch (1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%) (Fig. 30). The smallest (0.75 – 1.0 µM length and 0.5 – 0.75 µM breadth) endospores noticed at 1% and 5% (w/v) and the largest (1.3 – 1.5 µM length and 0.75 – 1.1 µM breadth) endospores at 80%, while the control having an average length of 0.6 – 0.8 µM and 0.5 – 0.7 µM breadth (Table 13).

7.1.1.8. Endospore production at 48 h on PP supplemented LB medium:

The 48 h sample also contained significant levels of endospores production in all the concentrations except control (Fig. 31). At this 48 h stage of starch supplemented media, maximum sporangia were lysed and the endospores were exposed. The smallest (1.2 – 1.5 µM length and 0.75 – 1.0 µM breadth) endospores noticed at 1 % (w/v) and the largest (1.5 – 2.0 µM length and 0.75 – 1.0 µM breadth) endospores seen at 20%, while the control having an average length of 0.6 – 0.8 µM and 0.5 – 0.6 µM breadth (Table 13).

7.1.1.9. Endospore production at 60 h on PP supplemented LB medium:

Release of spores are similar to those in starch supplemented media at 48 h cultivation, but more sporangia lysed in the control (Fig. 32). The smallest (1.2 – 1.5 µM length and 0.75 – 1.0 µM breadth) endospores noticed at 1 % (w/v) and the largest (1.5 – 2.0 µM length and 0.75 – 1.0 µM breadth) endospores seen at 20%, while the control having an average length of 0.8 – 0.9 µM and 0.5 – 0.6 µM breadth (Table 13).

7.1.1.10. Endospore production at 72 h on PP supplemented LB medium:

Release of spores are similar to those in starch supplemented media at 48 h cultivation, but maximum sporangia lysed in the control (Fig. 33). The smallest (1.2 – 1.5 µM length and 0.75 – 1.0 µM breadth) endospores noticed at 1 % (w/v) and the largest (1.5 – 2.0 µM length and 0.75 – 1.0 µM breadth) endospores seen at 20%, while the control having an average length of 0.8 – 1.0 µM and 0.5 – 0.6 µM breadth (Table 13).

7.1.2. Production of endospore with jack seed powder (JP) supplement

Figure 34 shows the profile of endospores produced by 10% (w/v) JP supplemented medium at various cultivation times (Fig. 34). The results obtained from JP supplemented LB media showed comparable data as that of potato powder (PP) supplemented LB media.

7.1.3. Production of endospore with tapioca powder (TP) supplement

Figure 35 shows the profile of endospores produced by 10% (w/v) TP supplemented medium at various cultivation times (Fig. 35). The results obtained from JP supplemented LB media showed comparable data as that of potato powder (PP) supplemented LB media (Fig. 35).

7.1.4. Endospore production at water-restricted environment

For reducing the gestation time (incubation or cultivation period) for crystal production and to obtain high yield of crystals, the 10% (w/v) PP supplemented medium was incubated for 12 h, then the supernatant was removed by mild centrifugation for harvesting α-amylase. The pellet (with no free water) so obtained incubated further. After 12 h surprisingly higher amount of free and bigger spores and crystals were obtained (Fig. 36).

Table 13: The average spore length and breadth of endospores of *Btk*, when potato powder was used as supplement in LB medium.

Time	Sample	Length	Breadth
12 h	**Control (LB)**	**0.5 – 0.6**	**0.4 – 0.5**
	1%	**0.75 – 1.0**	**0.4 – 0.5**
	5%	0.8 – 1.0	0.4 – 0.6
	10%	0.8 – 0.9	0.5 – 0.6
	20%	0.9 – 1.0	0.4 – 0.6
	30%	0.9 – 1.0	0.5 – 0.6
	40%	0.9 – 1.2	0.5 – 0.6
	50%	0.8 – 1.1	0.6 – 0.75
	60%	0.9 – 1.1	0.6 – 0.75
	80%	**1.0 – 1.2**	**0.6 – 0.75**
	100%	1.0 – 1.2	0.5 – 0.6
18 h	**Control (LB)**	**0.5 – 0.6**	**0.4 – 0.5**
	1%	1.2 – 1.5	0.5 – 0.85
	5%	0.8 – 0.92	0.5 – 0.75
	10%	0.8 – 1.0	0.5 – 0.75
	20%	**0.75 – 0.9**	**0.5 – 0.7**
	30%	0.75 – 1.0	0.5 – 0.75
	40%	0.9 – 1.0	0.6 – 0.8
	50%	0.5 – 0.75	0.4 – 0.5
	60%	0.9 – 1.0	0.6 – 0.8
	80%	0.9 – 1.0	0.6 – 0.8
	100%	**0.9 – 1.0**	**0.6 – 0.8**
24 h	**Control (LB)**	**0.6 – 0.8**	**0.5 – 0.6**

Time	Sample	Length	Breadth
	1%	0.95 – 1.1	0.5 – 0.8
	5%	0.8 – 1.0	0.8 – 0.9
	10%	0.9 – 1.0	0.75 – 0.8
	20%	0.75 – 1.0	0.6 – 0.8
	30%	**0.75 – 1.0**	**0.6 – 0.75**
	40%	0.8 – 1.1	0.6 – 0.8
	50%	0.8 – 1.0	0.5 – 0.6
	60%	0.75 – 0.98	0.5 – 0.6
	80%	**0.75 – 1.0**	**0.5 – 0.75**
	100%	0.75 – 1.0	0.5 – 0.6
30 h	**Control (LB)**	**0.6 – 0.8**	**0.5 – 0.6**
	1%	0.8 – 1.0	0.7 – 0.8
	5%	0.8 – 1.0	0.5 – 0.6
	10%	0.75 – 1.0	0.8 – 1.0
	20%	0.8 – 1.0	0.8 – 0.9
	30%	0.7 – 1.0	0.5 – 0.7
	40%	**0.7 – 1.0**	**0.5 – 0.6**
	50%	0.8 – 1.0	0.5 – 0.6
	60%	0.8 – 1.0	0.5 – 0.6
	80%	0.8 – 1.0	0.5 – 0.6
	100%	**0.8 – 1.0**	**0.5 – 0.6**
36 h	**Control (LB)**	**0.6 – 0.8**	**0.5 – 0.7**
	1%	0.8 – 1.2	0.5 – 0.6
	5%	0.8 – 1.2	0.5 – 0.6
	10%	**0.6 – 0.9**	**0.5 – 0.6**
	20%	0.8 – 1.0	0.5 – 0.6
	30%	0.9 – 1.0	0.4 – 0.6
	40%	0.8 – 1.0	0.5 – 0.6
	50%	0.8 – 1.0	0.5 – 0.6
	60%	0.8 – 1.0	0.5 – 0.6
	80%	0.9 – 1.1	0.5 – 0.6
	100%	**0.8 – 1.1**	**0.5 – 0.6**
42 h	**Control (LB)**	**0.6 – 0.8**	**0.5 – 0.7**
	1%	**0.75 – 1.0**	**0.5 – 0.75**
	5%	0.75 – 1.0	0.5 – 0.75
	10%	1 – 1.2	0.75 – 1
	20%	1 – 1.5	0.75 – 1
	30%	1 – 1.5	0.8 – 1
	40%	1.1 – 1.5	0.75 – 1.0
	50%	1.2 – 1.5	0.75 – 1
	60%	1.2 – 1.5	0.75 – 1
	80%	**1.3 – 1.5**	**0.75 – 1.1**
	100%	1 – 1.5	0.75 – 1.0
48 h	**Control (LB)**	**0.6 – 0.8**	**0.5 – 0.7**
	1%	**1.2 – 1.5**	**0.75 – 1.0**
	5%	1.2 – 1.5	0.75 – 1.0
	10%	1.1 – 1.5	0.75 – 1.0
	20%	**1.5 – 2.0**	**0.75 – 1.0**
	30%	1.5 – 1.75	0.5 – 1.0
	40%	1.5 – 1.8	0.75 – 1.0

Time	Sample	Length	Breadth
	50%	1.5 – 1.75	0.5 – 0.75
	60%	0.75 – 1.25	0.5 – 0.75
	80%	0.75 – 1.25	0.5 – 0.75
	100%	0.8 – 1	0.75 – 0.8
60 h	**Control (LB)**	**0.8 – 0.9**	**0.5 – 0.6**
	1%	**1.2 – 1.5**	**0.75 – 1.0**
	5%	1.2 – 1.5	0.75 – 1.0
	10%	1.1 – 1.5	0.75 – 1.0
	20%	**1.5 – 2.0**	**0.75 – 1.0**
	30%	1.5 – 1.75	0.5 – 1.0
	40%	1.5 – 1.8	0.75 – 1.0
	50%	1.5 – 1.75	0.5 – 0.75
	60%	0.75 – 1.25	0.5 – 0.75
	80%	0.75 – 1.25	0.5 – 0.75
	100%	0.8 – 1	0.75 – 0.8
72 h	**Control (LB)**	**0.8 – 1.0**	**0.5 – 0.6**
	1%	**1.2 – 1.5**	**0.75 – 1.0**
	5%	1.2 – 1.5	0.75 – 1.0
	10%	1.1 – 1.5	0.75 – 1.0
	20%	1.5 – 2.0	0.75 – 1.0
	30%	1.5 – 1.75	0.5 – 1.0
	40%	1.5 – 1.8	0.75 – 1.0
	50%	1.5 – 1.75	0.5 – 0.75
	60%	0.75 – 1.25	0.5 – 0.75
	80%	0.75 – 1.25	0.5 – 0.75
	100%	**0.8 – 1**	**0.75 – 0.8**

7.1.5. Endospore staining with acridine orange

For comparing the results obtained with malachite green staining, endospores were also stained with acridine orange, a fluorescent stain. The endospore production with different supplements such as JP, PP, TP at 10% (w/v) also showed similar results with those obtained by malachite green staining (Fig. 37). But by fluorescent staining, the spores were much brighter and clear with less background (Fig. 37).

7.2. Discussion

Bacteria produce endospores normally at the senescence of their growth phase, which is controlled by many factors such as unfavorable environmental conditions like desiccation, heat, and ultraviolet radiations [224] and unavailability of carbon and nitrogen sources [225].

Btk is a gram positive, sporulating and soil-dwelling bacterium [53]. Staining is the routine procedure adopted to detect the bacillial endospores. Malachite green stain is commonly used for this purpose, which stained endospores greenish blue [226 - 227]. In the present study, malachite green was used as the primary stain for staining endospores and the vegetative cells were pinkish upon counter staining with safranin (Figures 24 – 35). Hamouda *et al.* [228] stained *Bt* spores at different stages of germinaton with malachite green and safranin, and found that spores stained greenish blue and vegetative by malachite green cells stained pink by safranin. Chilcott [229] 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.

In the present study, endospores from selected samples were also stained with acridine orange, a flouresecnt dye for comparing the results obtained from malachite green staining. The data showed increased frequency of exposed endospore at 30 h cultivation, which attained maximum in 10% starch supplemented samples at 48 h, as seen in malachite green staining. Several other workers also used acridine orange staining technique for obtaining fluorescent photomicrographs of bacterial endospores. Schichnes *et al.* [211] developed a quick and simple technique for the fluorescent staining of bacterial endospores. This technique was developed using *B. subtilis* endospores. Sharma and Prasad [230] used the *Bt* strains and proposed the use of aqueous acridine orange to differentiate non-viable (lemon green) and viable (orange red) spores. Briefly, though both staining techniques (malachite green and acridine orange) are equally good, flouresecnt technique is more advantageous as it eliminates more debrises on the backgraound and thus the spores would be much brighter. Of course, the simplicity of malachite green and low cost makes it a good candidate for fast screening.

Furthermore, sporulation time of *Btk* and their release by rupturing the sporangial wall are important factors to be considered. These two aspects need special mention because sporulation in closely associated with parasporal toxic crystal production. As already shown, clear developing sporangia were seen even at

6 h growth. Frequent sporangial rupturing and release of spores was much evident in samples from 24 h cultivation onwards. In the starch supplemented media, most of the spores were released at 48 h cultivation, but in LB control, similar tendency was noticed after 72 h cultivation. Interestingly, when the *Btk* was forced to grow at water-restricted environment, maximum sporulation and subsequent release of these spores were seen at 24 h.

Fadel and Sabour [22] cultivated four strains of *Bt* on locally available sugar cane molasses for monitoring the production profile of bioinsecticide. Highest yields were obtained after 90 h fermentation and found that total viable counts, total spores and percent of spores were different between four strains [22]. General trend is that *Bt* strains take 3-5 days for the maximum production of toxins under the normal culture conditions by SmF [19], [26], [231 - 232]. Contrary to this, Chestukhina *et al.*, [233] showed that a *Bt* strain produced copious amounts of spores and crystals at 48 h.

Main reason for the decrease in production time as described herein should be anoxic and water stresses developed by the solid medium used. One can expect that the sticky fermenting matters considerably reduced oxygen diffusion making it inaccessible for the strain-free growth of cells, and culture in the water-restricted environment (24 h culture) lacked free water, which in turn imparted water stress. Induction of stress would favourablely tune the *Bt* cells towards sporulation and coupled toxin production – a sign of stationary phase of growth and senescence [234]. Das and Danker [235] showed that anoxic and water stresses favour production of spores and crystals at an early stage of growth. Moreover, presence of higher sugar concentration in the medium would impart additional stress. In the present study, it is expected that α-amylase would have produced free sugars by hydrolysing the starch in the medium, even after harvesting α-amylase at the water-restricted state. Scherrer *et al.* [236] observed that increased glucose concentrations led to the production of bigger crystalline inclusions with a higher content of protein and insecticidal activity, and maximum yields of protein and endotoxin were

obtained in a semisynthetic medium that contained higher (0.8%) glucose concentrations in the medium.

A close observation of the data presented here shows that increasing starch concentration in the medium favoured the formation of bigger cells and spores, and also crystals. The reason for this must be the stoppage of motility of the cells. Importantly, the strategy here was to harvest α-amylase as by-product during *Bt*-toxin production. Thus, water-restricted fermentation strategy was adopted. To achieve this goal, free water was removed for harvesting α-amylase first by mild centrifugation and the pellet was left for further biological activities like sporulation. At the latter state, minimum water activity was expected in the fermenting medium with maximum stress by many factors as stated above. Surprisingly, maximum sporulation was noticed within 12 h of such induced stress. From these results, the monetarily attractive point to the industry is that the cultivation time for *Bt*-toxin could be reduced considerably with no yield loss in 24 h with considerable yield of α-amylase as by-product.

8. *Btk*-TOXIN (δ-ENDOTOXIN) PRODUCTION BY SSF

The materials and methods of this piece of work have been described in **chapter 3, section 3.10.**

8.1. Results

Though *δ*-endotoxin (crystal protein) production is focused here, production of *Bt* spores and crystals are very much inter-related. Since JP, PP and TP showed higher α-amylase activities, and also endospore production, only these 3 substrates were used for evaluating the *δ*-endotoxin (*Btk*-toxin/cry protein*)* production by *Btk*. Special emphasis was given to PP supplemented medium, because it supported maximum α-amylase activity as described in the chapter 5 and endospore production as described in chapter 7. In all the Figures (except those taken by SEM), the spores are seen greenish-blue, and crystals are in bluish-pink colours.

8.1.1. δ-Endotoxin production profile as revealed by malachite green staining

To evaluate the production of *δ*-endotoxin, *Btk* was grown in a medium containing naturally available raw substrates such as 10% (w/v) jack seed powder (JP), potato powder (PP) or tapioca powder (TP). Endospore production patterns was more or less similar in all these three starch supplemented media (Chapter 7). The δ-endotoxin production profile also showed similar pattern (Fig. 38). *δ*-Endotoxin production was very high (maximum at 48 h cultivation) in these starch supplemented media than the LB control (Fig. 3). Though crystal production was comparable among these three supplements, PP supplemented LB medium showed maximum crystal production (Fig. 38 C). The size and shape of crystals also showed variations. Size was much bigger in starch supplemented media than in control. Shape of the crystals showed much variation from cuboidal, rhomboidal to bipyramidal (Fig. 38). A comparative profile of the coupled production of endospores and crystals at different conditions is given in Figure 39, which reveals

that more or less same profile was obtained with 72 h LB control and 48 h 10% (w/v) PP supplemented medium (SSF) and 24 h 10% (w/v) PP supplemented medium (SSF at water restricted state).

8.1.2. δ-Endotoxin production profile as SEM

10% (w/v) jack seed powder (JP), potato powder (PP) or tapioca powder (TP) supplemented media after 48 h fermentation was used for the SEM analysis, because samples at this stage showed maximum crystal production, but crystals in the control were comparably less (Fig. 40). Vegetative cells, spores and crystals were seen on the control and shape of the crystal was rhomboidal (Fig. 40 A). The pellet of JP, PP or TP supplemented media showed maximum crystal production and variation in shape of the crystals was prominent (Fig. 40 B-D). Among the three supplements JP and PP showed best results in terms of crystal production. The shape of the crystals was bipyramidal and cuboidal, which were almost freed from the bacterial cells at 48 h (Fig. 40 B & C).

8.1.2.1. δ-Endotoxin production at water-restricted environment

For reducing the gestation time (incubation or cultivation period) for crystal production and to obtain high yield of crystals, the 10% (w/v) PP supplemented medium was incubated for 12 h, then the supernatant was removed by mild centrifugation for harvesting α-amylase. The pellet (with no free water) so obtained (Fig. 41 A) incubated further. After 12 h, these pellets showed surprising quantity of spores and crystals (Fig. 41 B&C), which was comparable to that obtained at 72 h LB control (Fig. 39 A) and 48 h cultivation in 10% (w/v) PP supplemented media (Fig. 39 B). By this technique, total fermentation time was reduced to 24 h. The size and shape of the crystals also showed much variation from cuboidal to bypiramidal (Fig. 41).

8.1.3. Purification of Btk-toxin by centrifugation

Sucrose-gradient centrifugation was found efficient for the separation of *Btk* toxin (Fig. 42). The figure shows that SSF is equally good as conventional SmF for *Btk*-toxin production. Moreover, the debris of SSF matter could easily be separated

by centrifugation. Upon quantification, it was found that about 10% (w/v) starch supplemented medium showed about 11% crystal protein, while that of control was about 5%. It shows that SSF could double the crystal protein production.

8.1.4. Protein profile of δ-endotoxin

The protein profile shows MWs of varying ranges (18-66 kDa). This result clearly supports the scanning electron micrographic images that the shape, size and number of crystals were higher in medium with raw supplements than the control (LB medium) (Fig. 43).

8.2. Discussion

Coupled production of endospores and parasporal toxic crystals (together known as *Bt* toxin) is a characteristic feature of *Bt*, which mainly occurs in the late growth phase of their life cycle, *i.e.*, after 18 h under normal conditions in SmF (Fig. 3). Insecticidal (mostly pyramidal shaped) *δ*-endotoxins formed juxtaposed to the endospores are the structural component in the *Bt* cells, which is released mainly when bacteria are lysed. During sporulation, *Bt* produces crystals of *δ*-endotoxin, and this mixture is the so-called *Bt*-pesticide [68]. Upon ingestion of these protoxins, bind to special receptors like α-amylase in their digestive tract of the insects [237], and activated by proteolytic cleavage. Its toxicity leads to the lethality of the pests.

Conventionally, *Bt*-crystals are being produced by using submerged or liquid fermentation (SmF) techniques. Complex media like Luria-Bertani [238] is used for the purpose, but recently many workers use nutrient-rich waste water or sludges from various treatment plants as the medium for the production of *Bt*-toxin [239]. However, in the present study solid-state fermentation (SSF) strategy was used to produce maximum *Bt*-toxin in limited time, for which naturally available cheap raw starches like 10% (w/v) potato powder (PP), jack seed powder (JP) and tapioca powder (TP) were used. Employing SmF techniques, unconventional media have been tried by various authors for the production of *Bt*-toxin [239 - 242]. From the data it is clear that SSF is better for doubling the crystal production at the experimental conditions explained here. Efficacy of soybean meal-based or waste

water sludge (2.5%) medium was compared by Vidyarthi *et al.*, [240] for the *Bt*-toxin production, and the entomotoxicity against *Choristoneura fumiferana* was higher with the *Bt*-toxin obtained with sludge media. In another study, gruel and fish meal based media were used for the *Bt*-toxin production by *Btk*, which showed strong larvicidal toxicity towards Lepidopteran larvae [19].

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 [26]. According to Farrera *et al.,* [241], 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.

Another crucial factor which needs discussion is the gestation time for the production of *Btk* crystals. In the normal LB control, it took about 72 h for the maximum release of the crystals from the cells, while in 10% starch supplemented media, as in the present study, it took only 48 h for the same result and at the water-restricted SSF, the production time has been further reduced to mere 24 h. Sporulation and crystal production are associated with the nutritional status in the medium [242]. It is expected that the raw starch supplement used in the present study offers a balanced diet for the optimum growth of *Btk.* Aeration plays a crucial role in sporulation [243]. Sarrafzadeh *et al.* showed that 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, and 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. The SSF strategy adopted in the present study restricted availability of oxygen to the cells, 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. Moreover, the water-restricted environment also imparted a much critical water stress on the cells, which resulted to terminate the growth phase still earlier, and thus maximum spores and crystals were obtained at 24

h of cultivation at the water-restricted environment (Fig. 41). Optimum time for the release of *Bt* spores and crystals is 72 h [24], but during SSF, the stress imparted by the water-restricted environments would considerably reduce the prolongation of microbial growth [140]. Moreover, the *Btk*-toxin yield was not affected by the SSF technique.

In the present investigation, δ-endotoxin from *Btk* showed various shapes ranging from rectangular, cuboidal, bipyramidal, rhomboidal to spherical, but the crystals obtained in the LB control were mostly rhomboidal in shape as seen in the conventional reports [244]. The protein profile as revealed by SDS-PAGE also showed varying MWs (18 – 66 kDa) for crystal proteins produced in various media (Fig.43). In the solid substrates, the shape was mostly rectangular. Variations in shape of the crystals have direct link to their varying MWs. *Bt*-toxin showed bipyramidal, flat, square and rectangular crystals with MWs ranging from 75 to 150 kDa [60]. Gitahy *et al*. [24] also reported bipyramidal, cuboidal and small spherical crystalline inclusions in *Bt* strain S76 with MWs ranging from 130, 70 and 38 kDa. From the reports, the MW (kDa) of *Btk* crystal proteins many be 63 [245], 71 [28], [51], between 68 – 43 [246] or between 130 and 71 [51]. Same strain would produce different crystal proteins [68], and such different proteins of same or different MWs would show different host-range specificities [28].

Taken together, the stress and strain induced by water- and air-restricted environments by SSF played significant role in inducing coupled production and release of spores and crystals marking the termination of the active life of the cells at an early stage, and the expected balanced nutrient in the raw starchy supplement would have further induced the production of morphologically different crystal proteins as reflected by variations in the MWs.

9. EFFICACY STUDIES OF *Btk*-TOXIN TO COMBAT *ACERIA GUERRERONIS*

The materials and methods for this piece of work have been described in **chapter 3, section 3.11.**

9.1. Results

9.1.1. Collection of coconut buttons

Figure 44 is the habit of the *A. guerreronis* (mandari) affected coconut palm with tender nuts showing different stages of infestation (with characteristic triangular creamy white V-marks) by *A. guerreronis*. 30 days aged coconut buttons were collected and cultured in lab conditions providing suitable temperature and humidity as described in materials and methods (Fig. 45), **Section 3.11.3**. The average area of the buttons (cap region) used in this study was about 8.6 cm^2 and diameter 3.23 cm, because *A. guerreronis* harbours inside this meristematic cap region sucking the juice.

9.1.2. Life cycle of mites

Life cycle of the mite, *A. guerreronis* was studied for checking its survival rate in lab conditions (Fig. 46). *A. guerreronis* completed its life cycle in 10 days. It reached adulthood after passing though egg, first nymph (6^{th} day) and second nymph (8^{th} day) stages (Fig. 46). Before subjecting to the toxicity assay, 5 consecutive generations of the mites were made in the laboratory. Theses were the standardised cultures.

9.1.3. Toxicity assayss

The standardised cultures were used for the efficacy trials. About 45 mites were transferred per cm^2 on the buttons. The *Btk*-toxin from potato powder (PP) (10% w/v) supplemented LB medium was used for the application study. The crude *Btk*-toxin (**as described in Section 3.11.4)** at 1.25µg/ cm^2, 1.88 µg/ cm^2,

2.5 µg/ cm^2, 3.13 µg/ cm^2 and 3.73 µg/ cm^2 concentrations was used for the efficacy study. Controls mites were fed with uninoculated matter as described in **3.11.4**. Each culture set for bioassay was monitored up to 10 days, until the end of their normal life. It was seen that only the control mites passed through all succeeding stages for next generation. The buttons applied with 1.25 µg/ cm^2 crude *Btk*-toxin showed a little survivability (~ 20%) of the mites after 24 h treatment (Figs. 47 & 48). In all other treatments, all mites were dead after 24 h treatment. Figure 47 illustrates the total number of mites transferred and the mortality rates after application of the *Btk*-toxin. From Figure 48, it is clear that application of 1.88 µg raw powdered fermented matter per cm^2 is enough to combat *A. guerreronis*. It is advantageous that no further purification of the crystals is required.

9.2. Discussion

The focus of this piece of work was to check whether the *Btk*-toxin is suitable to combat the *A. guerreronis.* Toxins of *Bt* strain HD1 subsp *kurstaki* 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 [23]. Direct feeding of crude pellet containing *Bt*-toxin [22], sprays [247], pollen diet containing the *Bt*-toxin [248], application of fermented broths [84] are the normal mode of applications being practiced in toxicity assays. But, none of these techniques is suitable to combat *A. guerreronis,* because these mites are microscopic air-bornes, and highly sensitive to moisture. Moreover, they reside inside the cap (perianth) of the coconut buttons. Thus, the efficacy of starchy fermented matter was tried, which would naturally stick to the surface of the coconut buttons proving suitable environment for feeding (ingestion upon sucking the juice from the tender part of the button) to the mite, avoiding the requirement of an adhesive in normal applications. This technique is the first in this kind and was found feasible for combating *A. guerreronis.*

Literature describes no consistent mode of application of *Bt*-toxins against insects, yet *Bt* products have been in commercial use for over 50 years. However, its use has largely been restricted due to the specific characteristics that limit the wider

use of *Bt* include limited host range specificity, inability to target cryptic feeding pests, slow action compared to chemical insecticides and lack of residual activity [19]. Some approaches were used to improve these characteristics. More attention is being given to the discovery of new *Bt* strains with activity on a spectrum of insects, and of late, certain beetles too. Mostly complex media are used for the *Btk*-toxin production by SmF. *Btk* δ-endotoxin produced using gruel and fish meal based media exhibited larvicidal toxicity towards Lepidopteran larvae [19]. The influence of the composition of media used for growth and δ-endotoxin production by *Btk* was studied using a cheap medium containing glucose and yeast extract [249]. It yielded 2.1 x 10^9 spores/mL, and was effective against *Galleria mellonella* larvae.

Upon application of *Btk*-toxin, the mortality rates of various insects have been demonstrated by various authors [78],[250]. Treatment of 0.02 ha pasture plots with *Bt* H14 resulted in effective control of *Aedes spp*. and *Culex tarsalis* at 1 kg/ha [247]. An aerial application on 12 ha duck club pond with *Bt* H 14 at 1 kg/ha resulted a 99% reduction of *C. tarsalis*, apparently without adverse affect on predator populations [247]. Crystals purified from *Bt* HD1 and HD73 were highly toxic to tobacco hornworm larvae (50% lethal dose, 0.01 µg per third instars larvae) and crystals purified from *Bt* HD1 were toxic to meal worm larvae (50% lethal dose 4 µg per second instar larvae) [232]. *Bt*-toxins act only on the actively feeding larvae of susceptible species by a mechanism which involved consumption and proteolytic processing of the toxic protein followed by binding to, and lysis of midgut epithelial cells [53]. Unlike in the above and many other studies [43]**,** where larvae were the primary target, in the present study, 100% mortality of the adult mites was noticed in 24 h of application at a concentration of 1.88 µg/cm^2 of crude matter, *i.e*., for about 45 mites/cm^2. Furthermore, data clearly indicates that the adult mites were sensitive to the *Btk*-toxin. The quantity of pure crystals in this crude matter was about 11% (*i.e*., 200 ng in 1.88 µg crude toxin), which reflects that the *Btk*-toxin is highly toxic to *A. guerreronis*. Since this is the first report showing efficacy of *Bt*-toxin to kill *A. guerreronis,* the biological mechanism behind this toxicity remain to be elusive.

Shape of crystals was another factor studied in various laboratories. Gitahy *et al.* [24] found that *Bt* strain S 76 produced cuboidal and bipyramidal crystals and were effective against *Diatraea saccharalis*, a major sugarcane pest. Bioassays were carried out using 500 µg/L of spore-crystal complex which promoted 69% mortality [24]. *Bti*-crystals (1ng/mL) of varying molecular weights effectively used against *A. aegypti* larvae [231]. The carboxy terminal extensions in the crystal proteins may be responsible for the union of small crystals into bigger ones, which in turn would alter the biological activities of the toxic proteins, and that the bi-pyramidal crystals formed in this way were more effective against Colorado Potato Beetle [58]. Large insecticidal CryIC1 (1mg/mL) toxin was found highly toxic to *Heliothis virescens* and *Spodoptera exigua* [53]. As seen in the present report, *Btk* produced mostly large rectangular (up to 5µ long) crystals under SSF, but smaller ones in the control SmF. The protein profile on SDS-PAGE also showed this variation even from and among the starch supplements used. Primarily, irrespective of the size, presence of toxic crystals in the feed (fermented matter used) should be accounted for the mortality of the mites. This is likely, because the control mites continued their normal life cycle.

Btk is known to produce various crystal proteins like CryIAa, CryIAb, CryIAc and CryIF, which were highly toxic to various insects and some beetles [251]. The crystals (0.064 µg/mL) named CryIA(c) from *Bt* was highly toxic to *Heliothis virescens* [252]. In another study, CryIAc (10 µg/mL) from a *Bt* strain was found highly toxic to pink boll worm [251]. The spore-crystal mixture recovered in the presence of whey ultrafiltrate showed high toxic effect against the larvae of *Spodoptera littoralis, S. exigua, Phthorimaeae operculella and Earias insulana*, whereas the recovered spore-crystal mixture in the presence of lactose solution was more toxic to *P. opercuella* [22]. Crystals (as pollen diet) from various strains of *Bt* (*Bta, Btk* and *Btt)* were found effective to kill different adult beetles like *Aethina tumida, Apis mellifera*, when applied on their combs [248]. The crystals produced by *Bt dendrolimus* strain T84A1 was effective against *Bombyx mori* [246]. Until this report, no report is available regarding the efficacy of *Bt* against the mite, *A. guerreronis* [220]. As demonstrated in the present study, *Btk*-toxin effectively used

against the hazardous *A. guerreronis* (mandari) [220]. The dried and powdered form of raw fermented matter with a concentration of 1.88 μg/cm^2 was 100% efficient to control about 45 mites under the assay conditions. It is important that the exact toxin content contained in the raw matter used to feed the mites was very little, which demands more studies including field trials.

10. GENERAL DISCUSSION

Solid-state fermentation (SSF) offers many advantages over the conventional submerged fermentation (SmF) system. Yet, no report is there in literature employing SSF process to produce the high-demanding δ-endotoxin *(Bt*-toxin) by any strain of *Bacillus thuringiensis (Bt)*. A production strategy for the industrially significant α-amylase from *Bt* is not initiated, the efficiency of the *Bt*-toxin to combat the notorious coconut mite *Aceria guerreronis* is also not attempted so far. Taken these facts together, this study addresses three questions founded upon SSF: firstly, whether *Bt* subspecies *kurstaki* (*Btk*) can be tamed to produce α-amylase as a by-product during the course of *Bt*-toxin production; secondly, whether endospore production is correlated to δ-endotoxin production; and lastly, whether δ-endotoxin produced by *Btk* be efficiently used to combat *A. guerreronis.*

It is important to evaluate why *Bt* and its subspecies and strains are getting much attention in eco-friendly pest management initiatives. The entomopathogenic Gram-positive rod-shaped sporulating bacterium and its toxins have extensively been used for pest control purposes in agriculture, forestry and public health programmes since 1930 [253]. *Bt* produces a crystalline inclusion known as parasporal crystal in sporulating cells. The parasporal crystals (about 200 variants) contain crystal proteins or the *δ*-endotoxins, which are toxic to certain pests. Commercial formulations differ but they essentially contain a mixture of *Bt* spores and crystals which are mostly sprayed on plants targeting the pest. There are more than 30 sub-species of *Bt* distinguished by differences in immunological properties of parasporal crystals [68]. In the recent three or four decades, with the advent of techniques of molecular rearrangement, the specificity of the bacterium for target insect pests has been refined. The *Bt* products now represent some 1% of the worldwide use of fungicides, herbicides and insecticides [244], [254]**.** In addition to spray formulations, transgenic crops carring *Bt* genes for the expression of the toxins (*Bt* plants) are commercially available since the mid 1990s and are grown on an

increasing percentage of the global agricultural area [253 - 255]. A main reason for the importance of *Bt* as a pesticide lies in the assumed environmental safety concluded from the high specificity of its endotoxins (Cry proteins) towards a limited number of target organisms, mostly distinct groups of pest insects [244].

Though *Bt*-pesticides are target-centered with no untoward harm on non-target organisms, their availability in the market is much scarce. SmF followed by centrifugation for the separation of spore-crystal mixture is the conventional strategy being employed for the industrial production of this pesticide. Luria-Bertani (LB) medium with or without modifications is used conventionally for the production of this pesticide by SmF. Many investigators now trying to use various agro-residues such as cheese whey, soya bean milk and molasses liquid [256]; and less expensive alternative culture media such as potato, common sugar, and bengal gram [257]. Of late, sludge-based media have been used by different investigators. For instance, wastewaters from sludge (non-hydrolysed and hydrolysed) [258]; starch industry [259] slaughterhouse and secondary sludges from wastewater treatment plants [259] or pretreated sludge [260] have been used as raw media for the production of *Bt*-based biopesticides. In addition to this, immobilisation techniques such as batch and fed-batch cultures using higher glucose concentration [261] or starch industry wastewater in the culture media [21] is an emerging strategy to significantly enhance *Bt* sporulation and coupled crystal production.

Nevertheless, no SSF strategy has been employed for the production of *Bt*-biopesticides [220]. SSF has emerged as a potential process technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products [214]. Its application in bioprocesses such as bioleaching, biobeneficiation, bioremediation, biopulping, *etc*. has offered several advantages [214]. Utilisation of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues [140]. Solid substrate (matrix), however, must contain enough moisture. Depending upon the nature of the substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads relatively high water

activity (a_w) on the solid/gas interface in order to allow higher rate of biochemical processes [262]. Low diffusion of nutrients and metabolites takes place in lower a_w conditions, whereas compaction of substrate occurs at higher water activity. Both states impart stress; water stress in the former, while aeration stress in the latter. Hence, maintenance of adequate moisture level in the solid matrix along with suitable a_w are essential elements for SSF processes. Smaller substrate particles provide larger surface area for microbial attack but pose difficulty in aeration/respiration due to limitation in inter-particle space availability [263]. Larger particles provide better aeration/respiration opportunities but provide lesser surface area. In bioprocess optimisation, sometimes it may be necessary to use a compromised size of particles (usually a mixed range) for the reason of cost effectiveness [262].

Thus, this study seeks answers for three questions founded on SSF strategy: (a). is *Btk* a good producer of α-amylase and if yes, is it feasible to produce α-amylase industrially without harming the sporulation and crystal production (*Bt*-toxin) process?, (b) is it possible to link *Bt*-toxin production with α-amylase harvest using the same production system without altering commercial levels of toxin prodction of *Btk*, and finally (c) is the *Bt*-toxin produced by this method is having potential to combat the notorious coconut pest, *A. guerrerinis*, and how?

10.1. **α-Amylase production by *Btk***

Bt and its strains are shown to have potentials to produce protease and amylases [20], but *Bt*'s potential for producing these industrial enzymes has not been exploited commercially. Nevertheless, some enzymes, especially proteases [75] have been studied to a certain extend owing to their interactions with crystal toxins at different levels (sporulation and insecticidal action) [75]. There exist a few isolated reports which describe *Bt* producing various enzymes like poly-3-hydroxybutyrate (PHB) depolymerase [264], chitinase [265], *etc*. These enzymes are expected to play important roles in their metabolic cascade, for instance, PHB is produced and utilised during spore formation and may have provided energy for the sporulation process [266].

Amylases are among one of the most demanding industrial enzymes and are of great significance in the present day biotechnology. Although they can be derived from several sources, such as plants, animals and microorganisms, enzymes from microbial sources generally meet industrial demands. The spectrum of amylase application widened in many other fields, such as clinical, medical and analytical chemistries as well as their widespread application in starch sacharification and in the textile, food, brewing and distilling industries [117]. Many bacillial spp., especially *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* are the major players in commercial production of α-amylase [214].

Interest in *Bt* and its strains is only due to their proven efficiency as a biopestcide. SmF system is the production strategy for the *Bt*-toxin [261], [267]. Routinely, after harvesting the spores and crystals (pellet, *i.e.*, *Bt*-toxin) from the fermented broth by centrifugation, the supernatant is discarded unused. If this supernatant is targeted for the isolation of industrially significant α-amylase (of course, other proteins too), the *Bt* industry would have much more attractive.

Thus, the present study made a pioneering attempt to purify the α-amylase from the fermented matter at an early state (after 12 h growth), followed by harvesting spores and crystals at maturity. In the present study, the crude supernatant (form control LB) showed only about 4.36 U/mL α-amylase activity, while the crude supernatant of 10% (w/v) PP supplemented LB medium showed about 200 fold (867 U/gds) increase in activity, which was further risen to 1840 fold (8022 U/gds) by partially purified α-amylase at standardised conditions. There exists only one previous report on amylase by *Bt*, which was only 0.1 U/mL [21]. Commercial strains of *Bacillus* produce yet more active α-amylases [214]. Moreover, the *Km* and *Vmax* values of the amylase reported here were 2.9 mg/mL and 0.05335 μmol/mL/min, respectively. *Km* indicates the affinity of enzyme for the substrate; a small *Km* shows high affinity, and a substrate with a smaller *Km* will approach V*max* more quickly. Literature shows that the *Km* of various bacillial *α*-amylases varies from 1.3 to 7.8 mg/mL [105].

Many authors categorically proved that SSF is much more advantageous over SmF [126]; but in this study, SSF system was employed with a different perspective. The question was whether α-amylase could be produced as a by-product during the process of toxin production by *Btk*. From the data presented, it is evident that amylase production was highest at the mid-log phase (12 h) of its growth. Being primary metabolite, secretion of more α-amylase was expected at this initial stage as the medium contained more substrate (starch). Many studies show that presence of starch in the medium can induce α-amylase by many commercial bacteria, and the production time spans between 18 – 48 h [85]. Other factors which controlled early production of enzymes would also be due the stress imparted by the non availability of oxygen, water, *etc*. for free life [235]. Strains of *Bt* were shown to grow well in sludge samples [268], one of the natural habitats of many *Bt* strains, including *Btk* [269], [270]. It indicates that stains of *Bt* can switch to a facultative anaerobic life, if anoxic situation is arisen, which would hasten the cells to sporulate, and thus hibernate during the unfavourable environment.

In fact, the prime objective of this study was not the production of α-amylase alone, but *Btk*-toxin production and efficacy studies as well [220], as shown in the preceding chapters. Nevertheless, α-amylase produced as a by-product from a non-commercial *Bacillus* strain (*Btk*) offers much monetary advantages to the *Bt*-toxin industry.

10.2. **Sporulation and crystal production by *Btk***

Sporulation and toxic crystal protein production is a synchronous biological process in *Bt.* Many *Bt* isolates produce crystalline inclusions (δ-endotoxin) upon sporulation [14]. The basis of the bacterium's insecticidal power is a protein δ-endotoxin (a toxin that remains inside the bacterium). The *Bt*-toxins are synthesised as protoxins - *i.e.,* the molecule must be processed to some other form before the toxic activity is present - during sporulation of *Bt* strains and are deposited in the parasporal crystal. Inside the bacterium the protoxin molecules collect together to form a crystal. These parasporal crystal proteins (about 200 identified so far) may

contain potent insecticidal δ-endotoxins (pyramidal or Δ-shaped) classed as either crystal toxins (Cry) or cytolytic toxins (Cyt) [68].

The potency and specificity of the *Bt* Cry toxins for their insect targets (with no harmful effects on non-target organisms, including man) makes them ideal for control of insect pests of medical and agricultural importance. Very recently, it was reported that the α-amylase in the lining of the digestive tract of mosquito larvae is the receptor for this toxic protein [237]**.** Similar receptors are essential for the binding of these toxic proteins in the respective pest-targets to elicit a cascade of reactions culminating at the death of the pest, but this may vary from pest to pests. Upon binding to the receptors, the toxic protein would be ready for endogenous protease attack [271]. The N-terminus is cleaved in all of the proteins and a C-terminal extension is cleaved in some members [272]. 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-toxin 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 beta-sandwich domain that interacts with the N-terminal domain to form a channel [272].

Sporulation is a characteristic feature of some bacterial genera, like *Clostridia* and *Bacilli* [273]. Sporulation and parasporal crystal production are a synergistic process in *Bt*. Apart from *Bt* and its strains, *B. laterosporus* [12] *B.sphaericus* [274], *B. subtilis* [228] are also known to produce crystal proteins along with sporulation. These biological phenomena (sporulation and crystal production) might have been inculcated in bacteria as a means of their pathogenic mode of life [275]. Of course, modern eco-friendly approach makes use of these biological phenomena as a tool to combat the notorious pests.

Bt strains were shown to tolerate various stresses due to less water, less aeration, high salt and sugar, *etc.* [234 - 236]. Free sugars released by amylase activity on starchy medium in the early phase of growth would have imparted a postitive impact on *Btk*-toxin production. For instance, increased glucose

concentrations led to bigger crystalline inclusions with a higher content of protein and insecticidal activity of *Bt* var. *thuringiensis* [236]. Maximum yields of protein and endotoxin were obtained in a semisynthetic medium containing 6 to 8 g/L [236]. Unlike in LB control, data from starch supplemented media presented else where clearly show that much bigger spores and crystals are seen embedded in the starchy fermented matter. It indicates that such variations in morphology and shape are the outcome of stress induced by SSF strategy. Acquisition of this increased size was true for the vegetative and sporulating cells too. As already explained, the stress due to SSF forced the cells to such adverse phase of life.

When production strategy is switched from conventional SmF over to SSF, one will doubt whether the *Btk*-toxin yield in the latter system would be affected. From the data shown, it is evident that SSF is equally or even more good to be adopted [220]. It shows that *Bt* is tolerant to swing towards both aerobic and anaerobic mode life, which would directly affect their metabolism too. For example, Ohara and Yahata [276] clearly demonstrated that *Bt* can produce higher quantity of pure lactic acid at anaerobic condition than aerobic cultivation, which was comparable to that produced by commercial *Bacillus* spp. The advantage of the SSF demonstrated in the present study is that *Btk*-toxin can be produced within a short time with high output.

10.3. **Efficacy of *Btk*-toxin against *A.guerreronis***

Bt and its strains are pathogenic to certain pests belonging to Lepidoptera (moths and butterflies) [24], Diptera (flies and mosquitoes) [51], Coleoptera (beetles) [232], hymenoptera (wasps, bees, ants, sawflies) [277], nematodes [278], and certain mites [279].

Btk-toxins are known toxic towards larvae of different orders of insect pests (Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera) with different efficacies [61]. *Btk* is being used against Lepidopteran pests including the gypsy moth, spruce budworm, and hemlock looper. For instance, large-scale use of *Btk* against the gypsy moth and spruce budworm has been commercially employed over forested habitats [15]. *Btk* is harmful to moths and butterflies at their caterpillar

stage. Caterpillars that eat *Btk* on trees and shrubs die when the *Btk* spores are activated in the insect's stomachs. *Btk* is commercially available as a biological insecticide under different trade names and is used in pest control programmes in forestry, agricultural and urban settings around the world [16]. The insecticidal activities of the CryIA(a-c) toxins from *Btk* Hd-1 were determined in force-feeding experiments with larvae of *Choristoneura fumigferana*, *C. occidentalis*, *C.pinus*, *Lymantria dispar*, *Orgyia leucostigma*, *Malacosoma disstria*, and *Actebia fennica*. The toxins were obtained from cloned protoxin genes expressed in *Escherichia coli* [17].

So far no report is available on *Btk* or any other *Bt* strain explain the efficacy to combat *A. guerreronis*. The present study demonstrates 100% pathogenicity of *Btk*-toxin against the mite, *A.guerreronis* causing severe damage to the coconut yield all over the world. *A.guerreronis* Keifer (Acarina: Eriophydae) was first discovered as a major pest of coconut (*Cocos nucifera*) from plantations along the coast of Guerrero, Mexico, in 1965 [280]. However, some predators and fungal applications have been used for controlling the hazardous effects of this mite [281].

Bt and its strains have been utilised worldwide as an environmentally benign biological pesticide. The quality control of formulations from *Bt* are usually performed based on bioassays with insects, although the test methods and the species of test insects differ among countries [282]. Still in normal toxicity assays, the *Bt*-toxin is applied as wet aerial spray [283], force feeding by sticky diet with 10-15% sucrose [284] or by an artificial diet in saline solution [22]. Unfortunately, none of these methods is applicable in the case of microscopic and air-borne *A.guerreronis,* because they are highly sensitive to moisture and resides inside the cap of the buttons. Thus, as described earlier, dried and powdered raw fermented matter containing residual medium components and bacterial spores and crystals was used as the feed. 1.88 µg of this preparation per cm^2 of culture area of the buttons (Chapter 9) was sufficient to kill about mature 45 mites. It has to be noted that the preparation used contained only a small proportion of actual toxin (~ 11%), *i.e*., about 200 ng in 1.88 µg crude toxin applied. In normal applications, much more

concentrations have been used. For instance, Sundaram and Sundaram [283] applied about 500 ng *Btk* crystal protein per cm^2 of foliage to control gypsy moth. The present study clearly indicates that *Btk*-toxin is much efficient at very low concentration to contol *A.guerreronis*.

Furthermore, as far as field application is concerned, it is proposed that the dried raw fermented matter (partially digested starchy substrate with bacterial spores and crystals) could be deposited in the leaf pockets of the coconut palm during rainy season [220]. It will undergo natural SSF (as the endospores grow receiving enough water) there using rain water and additional nutrients from the deposits in leaf the sheath. In the succeeding summer, the spores and crystals would be naturally brought by the wind to the developing coconut buttons, *i.e*., only one application is necessary in a palm.

Commercial *Bt* insecticides are classified as Generally Regarded as Safe (bearing GRAS status) by the Environment Protection Agency of the USA [285], and are approved for most organic certification programs. The *Bt* endotoxin is considered safe for humans, other mammals, fish, birds, and the environment because of its selectivity [285]. *Bt* has been available as a commercial microbial insecticide since the 1960s and is sold under many trade names. These products have an excellent safety record and can be used on many crops until the day of harvest [285].

10.4. Conclusions

Each organism makes conscious effort and establishes its own ecological niche to escape severe competition from others and thus to get the favour during the process of nature’s selection. Sporulation and coupled parasporal crystal production are the predominant features acquired by *Bt* during the process of their establishing such a niche. By inducing artificial stress, *Bt* can be compelled to enter into the sporulation stage to cross-over the adverse effects of stress, and thus crystal production will accompany this sporulation. In the present study, this strategy has successfully been accomplished by applying SSF system, which mainly imparts oxygen stress along with water stress. These two factors together would have

compelled the *Btk* to enter into sporulation at an early stage its life. Thus, maximum sporulation and coupled production of toxic crystals resulted at 24 h of cultivation. This advancement in their life cycle - by cutting short two-third of normal life span - is much advantageous to the entrepreneurs engaged in *Bt* industry.

On the other hand, as described in the preceding chapters, establishment of SSF strategy to produce industrially significant α-amylase as a by-product during *Btk*-toxin production is the pioneering attempt in *Bt* industry. Moreover, demonstration of *Btk*-toxin to combat the notorious coconut pest, *A. guerreronis* is another significant milestone which would further broaden the *Bt* industry. *In toto*, using raw natural starch as SSF system , this pioneering study puts forth three specific claims [220], which are: (a). *Btk* was successfully tamed for producing industrially significant α-amylase as a by-product during *Btk*-toxin production; (b). *Btk*-toxin production was maximised at 24 h cultivation by providing oxygen and water-restricted stress environements, thus the gestation period has been reduced considerably; and (c). *Btk*-toxin was proven efficient to combat *A. guerreronis,* which opens up a new facet of *Bt* application. On the reverse side of this study, limitations such as characterisation of completely purified α-amylase, amino acid sequence determination of the α-amylase and field study of *Btk*-toxin against *A. guerreronis* remain inconclusive, which demands immediate attention.

11. SUMMARY AND CONCLUSIONS

11.1. Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive, sporulating and mostly soil-dwelling bacterium. Owing to the production of insecticidal crystal proteins (the *Cry* proteins), *Bt* is popularly known as a biopesticide. This δ-endotoxin is the active ingredient of most (90%) of the microbial insecticides produced in the world. It also resides naturally in the caterpillars of some moths and butterflies, as well as on the surface of plants. Some commercially available subspecies/varieties of *Bt* include: *Bt aizawai*, *Bt israelensis*, *Bt. kurstaki (Btk)*, *Bt sandiego* and *Bt tenebrionis*. *Bt*-toxin is a mixture of endospores and *Cry* proteins. During sporulation, *Bt* cells produce parasporal protein inclusions juxtaposed to the endospore. These inclusions consist of one or more insecticidal protoxic proteins (insect crystal proteins or δ-endotoxin) in the form of a crystal or crystal-complex. Upon consumption by the insect, these protoxins are cleaved by protease in the insect gut to form active toxin, which kills the insect. The acidic environment created by the destruction of gut cells (by influx of ions) is utilised by the endospores for germination and subsequent attack.

Main focus of all *Bt*-based studies has been to produce the biopesticide. The δ-endotoxin found as a crystalline inclusion in the insect pathogen *Btk* has potent insecticidal activity toward lepidopteran pests including the gypsy moth, spruce budworm, and hemlock looper. Of late, there are inconclusive reports on the efficacy of *Btk* against the eriophyid coconut mite, *Aceria guerreronis* syn *Eriphyes guerreronis*. This pest (known as *mandari* in Malayalam) is a menace to coconut cultivation in the world, especially in Kerala and Tamilnadu.

Industrially significant extracellular amylases are neither produced nor characterised from *Bt* yet. In the process of harvesting the δ-endotoxin, supernatant containing amylases and other extracellular enzymes is left out as effluent by the *Bt*

industries. If such enzymes also purified from the effluents as a by-product and made available on a commercial level, the *Bt* market would become more attractive as it offers additional income. Thus, founded on solid-state fermentation, the specific objectives of this study are: (a) to produce, purify and characterise α-amylase from the culture broth/supernatant of *Btk*, (b) establishment of a fermentation strategy to maximising the production of *Btk* toxins (*δ*-endotoxin/*Cry* protein), and (c) to study the efficacy of *Btk* toxins (*δ*-endotoxin) to combat *A. guerreronis* (mandari), which severely damaging coconut palms and yield.

The thesis contains eleven chapters, *viz*., (1) introduction, (2) literature review, (3) materials and methods, (4) α-amylase production by Submerged Fermentation (SmF), (5) amylase production by Solid-State Fermentation (SSF), (6) purification and characterisation of *α*-amylase, (7) production of endospore, (8) production and purification of *Btk* toxins, (9) efficacy studies of *Btk* toxins to combat *A. guerreronis*, (10) general discussion, (11) summary and conclusions, followed by bibliography, and publications as appendix.

11.2. Summary of major findings is given below.

11.2.1. α-Amylase production by submerged/ liquid fermentation (SmF)

The standard strain *Bacillus thuringiensis* subsp. *kurstaki* was procured from Institute of Microbial Technology (IMTECH), Chandigarh (Strain designation: BA 83B and MTCC No. 868). Growth characteristics (λ = 600 nm) showed that the log phase was extended between 6 – 22 h. Under SmF, α-amylase production was accomplished by supplementing 1% (w/v) soluble starch (Merck) as supplement in the Luria-Bertani (LB) medium. 12 h seed culture (inoculum) containing about 6.2 x 10^5 cfu (colony forming units) was added per 1mL medium, and 0.5 mL samples were withdrawn for assay at 6 h intervals. α-Amylase activity in the supernatant was assayed by Dinitrosalicylic Acid (DNS) method. Maximum amylase activity in the starch supplemented medium was about 11.21 U/mL after 12 h growth, while the control showed only about 4.36 U/mL activity. When the soluble starch was replaced by various raw starch supplements [1% (w/v)], *viz*., banana powder, jack seed powder, potato powder, tapioca powder, taro powder and bengal gram powder;

maximum activity (13.19 U/mL) was obtained with tapioca powder, while the least activity (1.9 U/mL) was obtained with bengal gram powder supplement. Thus, the SmF study clearly suggests that some naturally available crude starches were suitable raw supplements for amylase production by *Btk*.

11.2.2. Amylase production by Solid (or semi-solid) State Fermentation (SSF)

Soluble starch (5, 10, 15, 20, 25 - all in % w/v) was supplemented to the LB medium for enrichment and incubated (37 ^{o}C) in an incubator. Data showed that maximum α-amylase activity (in units per gram dry substrate, U/gds) was obtained with 10% (w/v) starch (27.51 U/gds for 5%, 28.59 U/gds for 10%, 28.08 U/gds for 15%, 26.98 U/gds for 20% and 25.79 U/gds for 25%). For maximising the α-amylase production, the LB medium was supplied with various locally available raw starch substrates such as banana powder, jack seed powder, potato powder, tapioca powder, taro powder and bengal gram powder with different concentrations (w/v), *i.e*., 5%, 10%, 20%, 30%, 40%, 50%, 60%, 80%, 100%. Data showed increased α-amylase activity (12 h cultivation) at 10% and gradually decreased from 20% to 100%. Time dependence data showed an increased α-amylase activity at 12 h and gradually deceasing from 18 h to 24 h. At 12 h, all the supplements induced maximum α-amylase activity. Of these six raw starch supplements, 10% (w/v) potato powder induced maximum α-amylase activity (867.55 U/gds at 12 h harvest), while bengal gram yielded the least activity (4.31 U/gds).

11.2.3. Purification and characterisation of amylase

From the α-amylase production profiles by SmF and SSF, amylase produced by supplementing the LB medium with 10% (w/v) potato powder (which supported maximum activity) was chosen for purification. Ammonium sulphate fractionation, dialysis, Sephadex G100 gel permeation chromatography were the methods used for purifying the enzyme. 40-60% ammonium sulphate fraction was used for the characterisation of the enzyme. Upon characterisation, effects of pH, temperature and metal ion concentration were studied, and their optima were: 6.0 pH, 60 ^{o}C and 3 μM Ca^{2+}, respectively. Complex chemicals like EDTA and SDS adversely affected the enzyme activity and *β*–mercaptoethanol completely abolished the activity. Under

standardised assay conditions (pH 6.0, temperature 60 ºC, 3 µM Ca^{2+} , 2.5% starch, incubation 5 min), the crude extract showed a specific activity of 864 U/mg crude protein; 2983 U/mg by ammonium sulphate fraction and 12,751 U/mg activity by Sephadex G-100 column fraction. SDS–PAGE profile of the Sephadex G-100 column active fraction showed that the apparent molecular weight (MW) of the purified α-amylase was about 51kDa (14.75 fold purity). The *Km* and *Vmax* values were calculated as 2.9 mg/mL and 0.05335 µmol/mL/min by using the software Hyper 32.

11.2.4. Production of endospore

Endospore production was studied in the cultures supplemented by % (w/v) of jack seed powder, potato powder and tapioca powder. The spore production was studied for 3 days and the samples were withdrawn in 6 h intervals. Malachite-green was used for staining endospores and photographed by Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon (DXM 1200F, Japan) digital camera. Endospore production was noticed in the samples at 12 h, which attained optimum at 48 h and continued upto 72 h. Release of endospores from the cell was also optimum at 48 h in the starch supplemented media. But in the control, maximum spore release was noticed at 72 h culture. The spores showed about 1.5 – 2.0 µM length and 0.75 - 1µM breadth. Spore production was also studied by staining with acridine orange, a fluorescent dye, and photographed by Olympus fluorescent microscope fitted with Nikon digital camera (Japan). This data was in corroboration to that obtained by malachite-green staining.

11.2.5. Production of Bt-toxin (δ-endotoxin/ crystal protein)

Bt-toxin production was also studied with media supplemented by 10% (w/v) jack seed powder, potato powder and tapioca powder and samples were withdrawn at 6 h intervals. Malachite-green and safranin were used to stain crystals and photographed using Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon (DXM 1200F, Japan) digital camera. The presence of crystals was observed at 48 h to 72 h and the maximum crystals were seen at 48 h in

the starch supplemented medium and progressed upto 72 h. Photomicrographs taken by Scanning Electron Microscope (SEM) (JEOL, JWS 3000) also substantiated the above results. The shape of the crystals was cuboidal, bipyramidal, rhomboidal or spherical.

For reducing the harvesting time of crystal, the supernatant was discarded from the fermentation medium at 12 h by centrifugation (800 g for 10 min) and the pellets were incubated further to monitor the crystal production. 12 h pellet contained only vegetative bacteria and at 24 h, large amount of crystal production was observed, which was comparable to those obtained at 48 h of normal SSF. This observation was confirmed by SEM images. The crystals were purified in a discontinuous sucrose gradient. The purified crystals were analysed using 12% SDS-PAGE gel. SDS-PAGE profiled the crystal protein fractions of various MWs.

11.2.6. Efficacy studies of δ-endotoxin against mandari

Coconut buttons of about one month old were collected from the uninfected coconut palms of about 10 M height during March-May (~32 ^{o}C). For standardisation of the growth of the mites (*Aceria guerreronis)* in lab conditions, hundreds of coconut buttons were collected every week and cultured providing suitable temperature (30 ^{o}C) and water (for keeping the buttons alive). The control cultures showed all the three growth stages of the mites, *i.e*., adult nymph, eggs, first nymph and second nymph. After standardisation, adult mites (~ 2µ length) were transferred from the infected coconut palms and brushed on to the uninfected buttons under culture. About 45 mites per cm^2 were transferred on the top of the buttons (in the region where the cap was removed). The finely powdered *Bt*-toxin containing raw fermented matter was applied on the top of the buttons at different concentrations (µg/ cm^2), *i.e*., 1.25, 1.88, 2.5, 3.13 or 3.73. The cultures were kept in an incubator at 30 ^{o}C for 10 days. In the control buttons, some mites (91%) were dead and the remaining mites grew normally and laid eggs, which underwent further developmental stages like first nymph and second nymph. But on the experimental buttons, wherein 1µg/cm^2 crystals was applied, about 75% of mites were dead in the second day, while in all other treatments, all mites were dead in the second day.

11.3. *Conclusions*

The present study suggests that *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) is a potential organism for α-amylase production as a by-product in the process of *Btk*-toxin production by SSF, and thus for judicious industrial exploitation. Moreover, the *Btk*-toxin produced was found 100% effective to combat *A. guerreronis*, a dangerous coconut mite cuasing great economic loss.

- α-Amylase production was maximum, when potato powder (10% w/v) supplemented LB was used as the supplement. This medium composition was better for *Btk*-toxin production also.
- Optimum conditions for maximum α-amylase activity were: 6.0 pH, 60 ^{o}C temperature, 3μM Ca^{2+} and 2.5% starch.
- The Sephadex-G100 purification fold was 14.75.
- The *Km* and *Vmax* values were 2.9 mg/mL and 0.05335 μmol/mL/min, respectively.
- The *Bkt*-toxin production was optimum at 48 h in the 10% (w/v) potato starch supplemented medium, and size of the crystals varied from cuboidal, bypyramidal, rhomboidal to spherical; while it was maximum at 72 h in the control,
- The *Btk*-toxin production time was reduced to 24 h by the water-resticted cultivation strategy.
- Crude *Btk*-toxin is proved as an effective biopesticide to combat *A. guerreronis.*

11.4. Major outcomes

- Demonstration of the production, purification and characterisation of extracellular α-amylase from *Btk* for the first time by SSF.

- Demonstration of production and purification of *Btk*-toxin from the solid-fermented matter for the first time, and
- Demonstration of the efficacy of *Btk*-toxin to combat the coconut mite, *A. guerreronis* – a notorious coconut pest for the first time.

11.5. Limitations of the study

- In the present study, partially purified α-amylase was used for characterisation studies, compared to commercial strains of *Bacillus* moderate levels of enzyme activity were obtained. Purified enzyme has to be used to fix the exact characteristics of this enzyme, which, no doubt, would be very high.
- Purified α-amylase has to be subjected to amino acid sequencing for further confirmation.
- Quantification of the crystals (*Btk*-toxins) in large production vessels like fermenters has to be accomplished.
- Field trial of *Btk*-toxin on the *A. guerreronis* affected coconut palms has to be conducted.

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APPENDIX I

List of publications

a. Patent

A process for the dual production of α-amylase and *Bt*-toxin by *Bacillus thuringiensis* subsp. *kurstaki* and efficacy of *Bt*-toxin to combat coconut mite. Sailas Benjamin, **Smitha RB** and Jisha VN. **(**Patent Filed through the Department of Biotechnology, Ministry of Science and Technology, Government of India).

b. Research papers published

1. **Smitha RB**, Bennans T, Mohankumar C and Sailas Benjamin (2009). Oxidative stress enzymes in *Ficus religiosa* L.: Biochemical, histochemical and anatomical evidences. *Journal of Photochemistry and Photobiology B: Biology*, **95**: 17 – 25.

2. Abhilash P, Pradeep S, **Smitha RB**, Jisha VN, Balachandran S and Sailas Benjamin (2009). Effect of certain anti-diabetic ayurvedic drugs against microbes causing diabetes-dependent infections. *Journal of Pure and Applied Microbiology*, **3**: 503-516.

3. **Smitha RB,** Sonu KR and Sailas Benjamin (2009). Isolation and characterisation *Bacillus* spp. Proceedings of the national Seminar on "Genetics, Breeding and Biotechnology", Gregor Mendel Foundation, University of Calicut.

4. **Smitha RB,** Jisha VN, Pradeep S, Sreedevi S and Sailas Benjamin (2010). An insight in to the bacillial α-amylases. *Folia Microbiologica* (Under Review).

c. Papers under processing

5. **Smitha RB** and Sailas Benjamin (2010). Amylase production by submerged fermentation from *Bacillus thuringensis* subsp. *Kurstaki*. *Biodegradation.*

6. **Smitha RB** and Sailas Benjamin (2010). Amylase production by solid-state fermentation from *Bacillus thuringiensis* subsp. *kurstaki. Jounral of Biotechnology*.

7. **Smitha RB,** Madhavan Nampoothiri K, Chembakam B, Ichiro Yamato and Sailas Benjamin (2010). Purification and characterisation of amylase from *Bacillus thuringiensis* subsp. *kurstaki. Enzyme Microbial Technology* .

8. **Smitha RB,** Jisha VN, Joseph Job and Sailas Benjamin (2010). Production of endospore and *δ*- endotoxin from *Bacillus thuringiensis* subsp. *kurstaki. Applied and Environmental Microbiology.*

9. **Smitha RB,** Haq MA, Ramani N and Sailas Benjamin (2010). Efficacy studies of *δ*- endotoxins from *Bacillus thuringiensis* subsp. *kurstaki* against mandari. *Journal of Economic Entomology*.

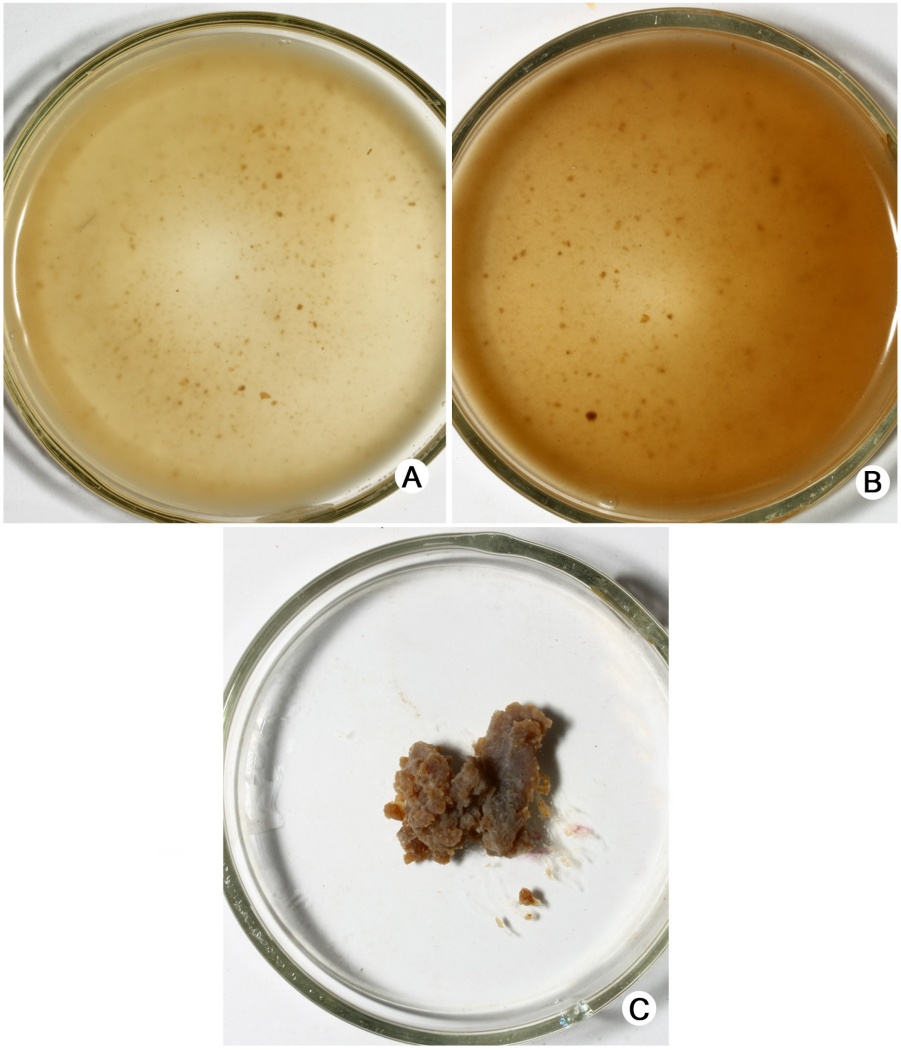

Figure 7: Texture of the fermented matter of potato powder (PP) supplemented LB medium (w/v) by *Btk* after 12 h incubation at 37 ^{0}C. A: 1%; B: 5% and C: 10%.

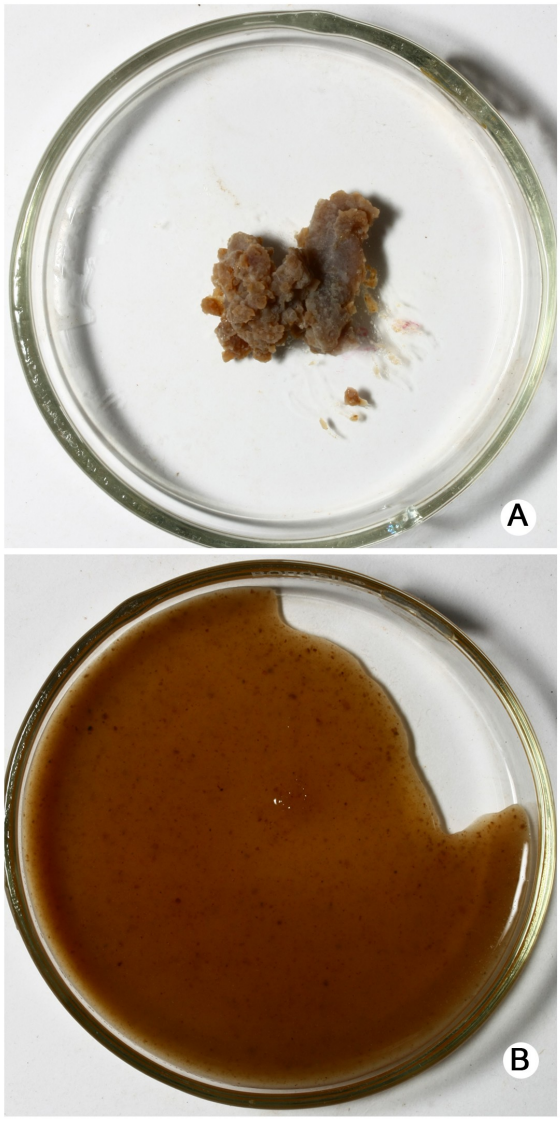

Figure 8: Texture of the fermented matter of potato powder (10% w/v) supplemented LB medium by *Btk* incubated at 37 ^{0}C. A: 12 h showing thick fermented matter and B: at 24 h the thick medium became a slurry.

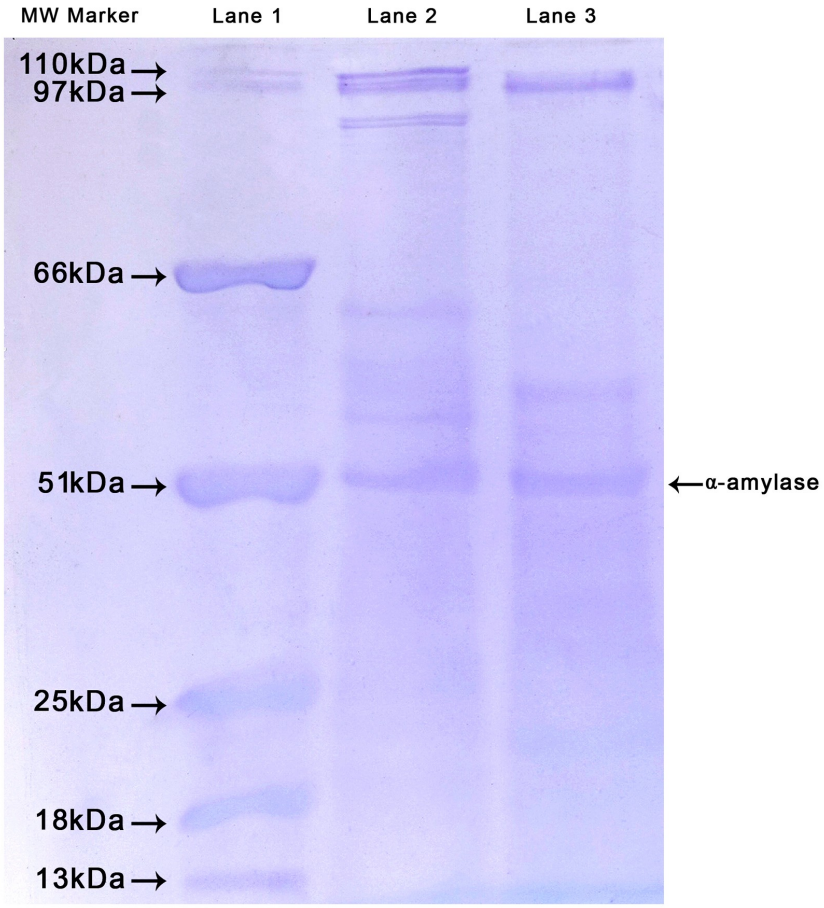

Figure 16: SDS-PAGE protein profile of the crude supernatant and 40 - 60% ammonium sulphate fraction showing α-amylase. Lane 1: molecular weight marker; Lane 2: crude extract and Lane 3: ammonium sulphate fraction.

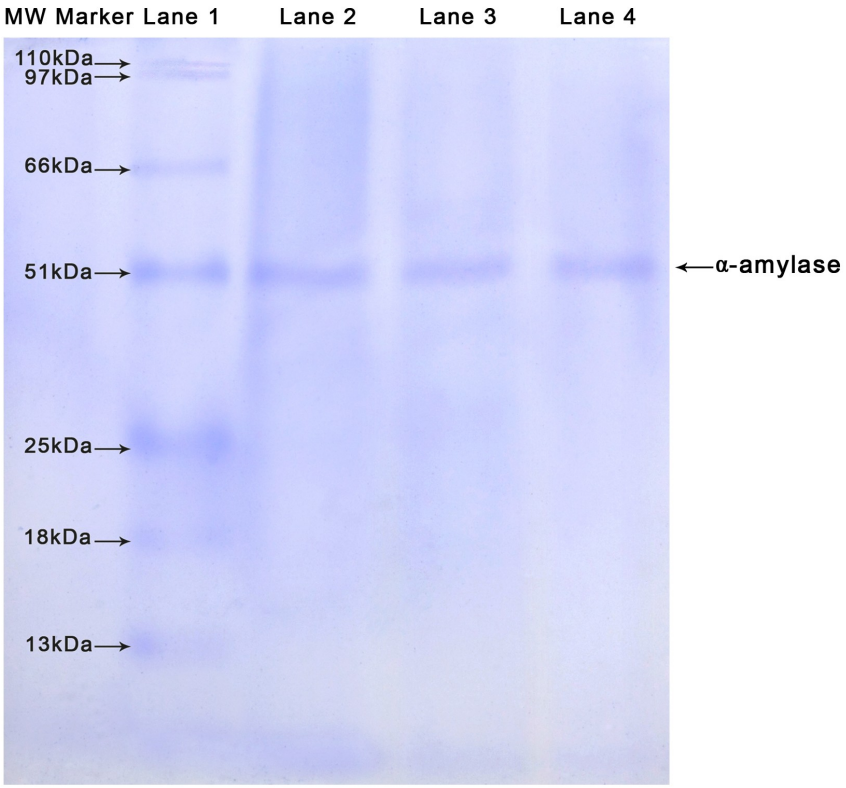

Figure 18: SDS-PAGE protein profile of the sephadex G-100 column fractions showing α-amylase. Lane 1: molecular weight marker; Lane 2: 39th fraction; Lane 3: 38th fraction and Lane 4: 40th fraction.

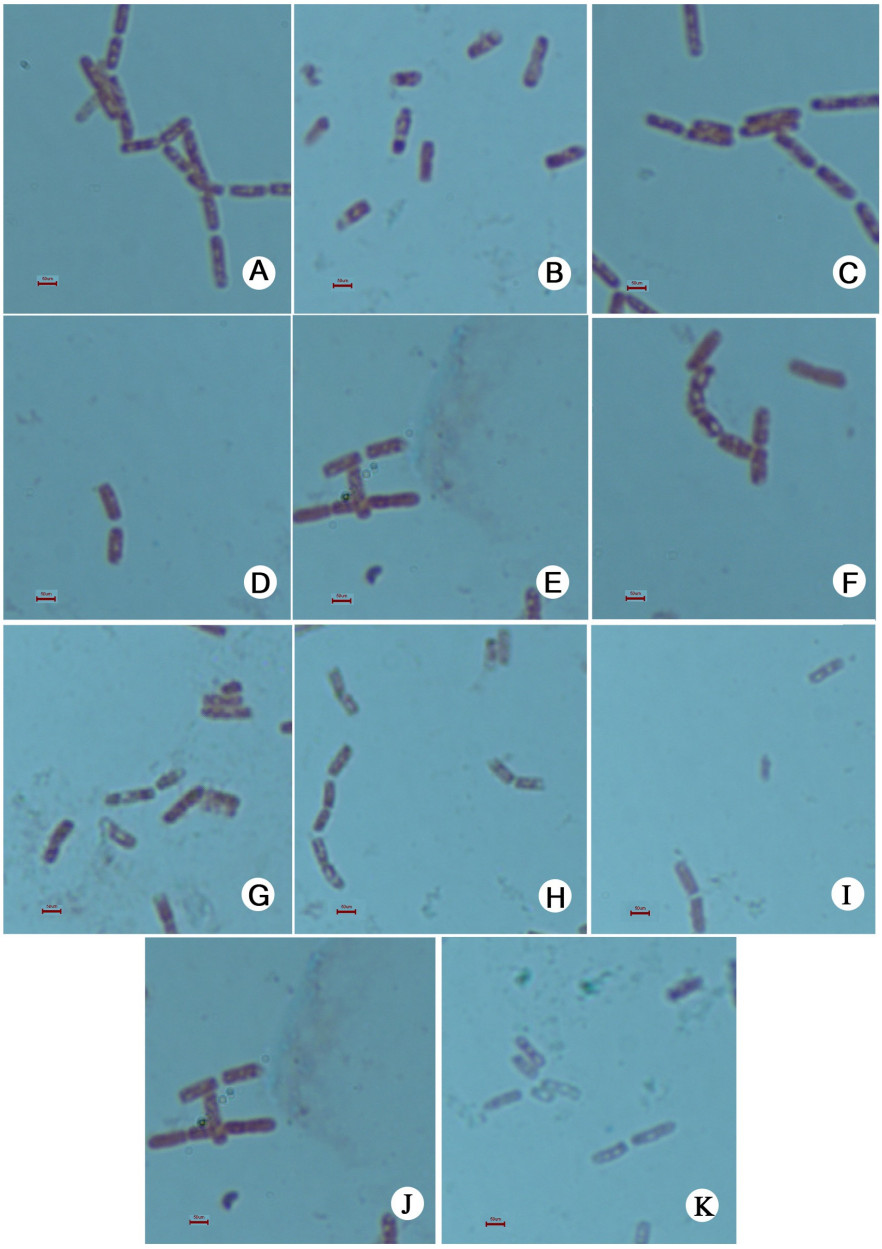

Figure 24:

Figure 24: Vegetative cells of *Btk* and cells with developing sporangia on various concentrations (w/v) of potato powder (PP) supplemented LB media and control (LB) at 6 h as revealed by Image Analyser (scale bar = 50 μM): **A:** control (LB) showing vegetative cells with sporangia; **B:** 1% showing vegetative cells with swollen sporangia; **C**: 5% showing vegetative cells with sporangia; **D:** 10% showing few vegetative cells with sporangia; **E:** 20% showing vegetative cells with sporangia; **F**: 30% showing vegetative cells with swollen sporangia; **G**: 40% showing vegetative cells with sporangia ; **H:** 50% showing vegetative cells with sporangia; **I:** 60% showing few vegetative cells with sporangia; **J:** 80% showing vegetative cells with sporangia and **K**: 100% showing vegetative cells with swollen sporangia.

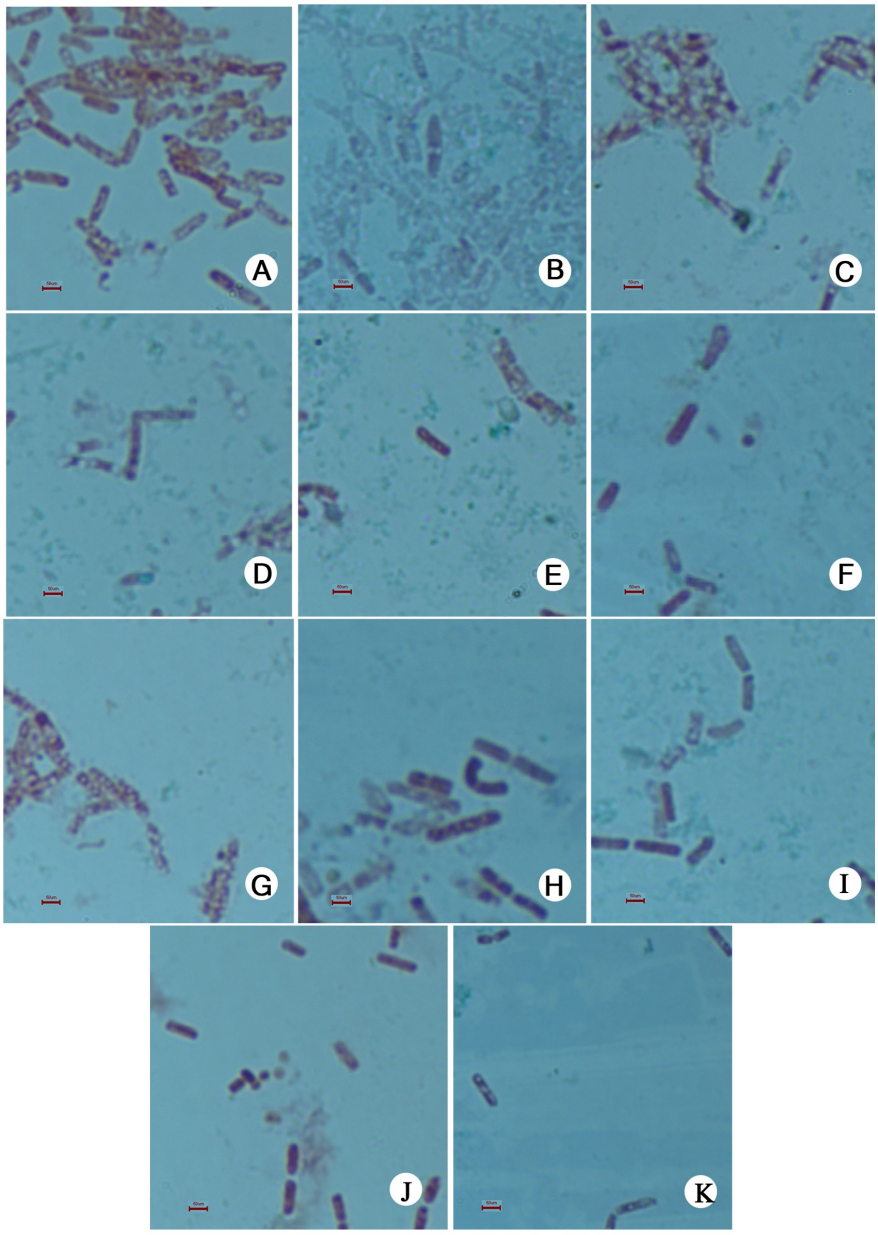

Figure 25

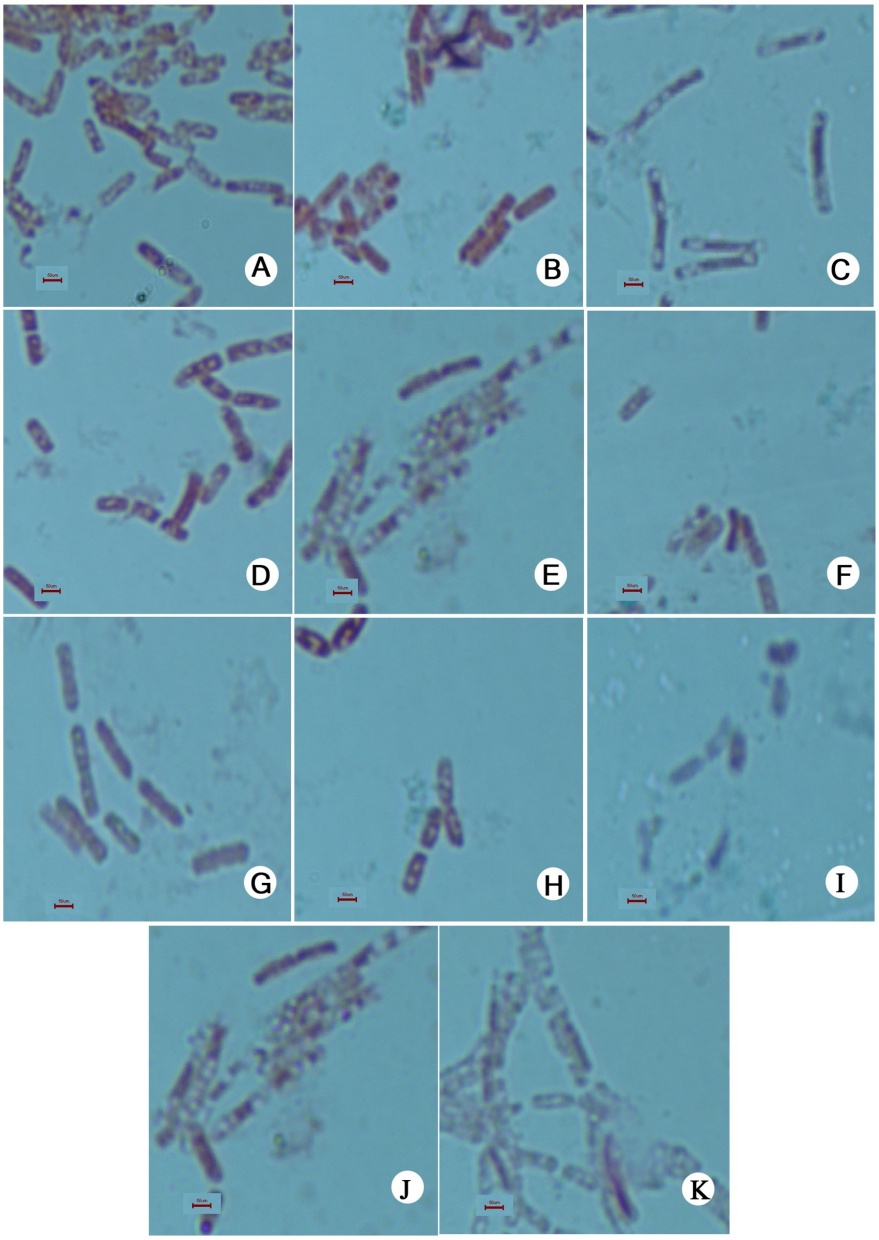

Figure 26

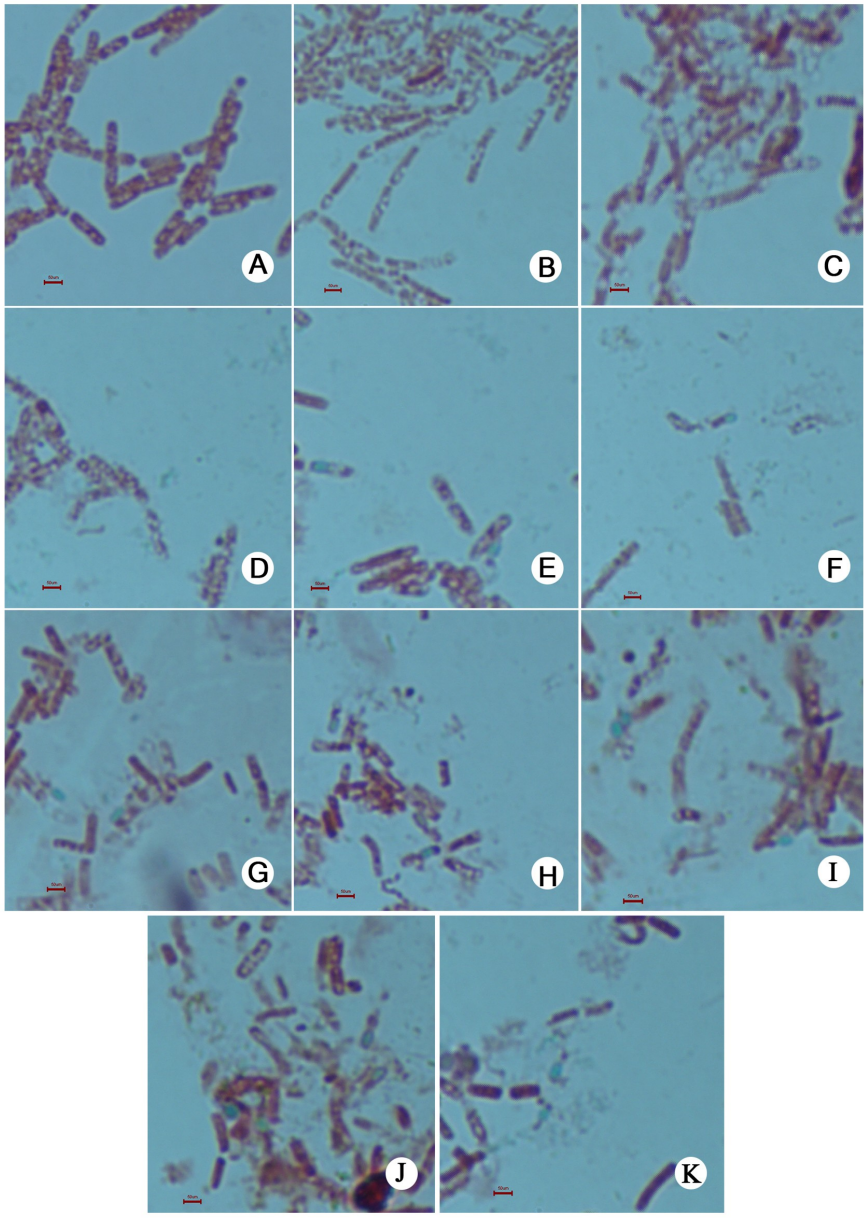

Figure 27

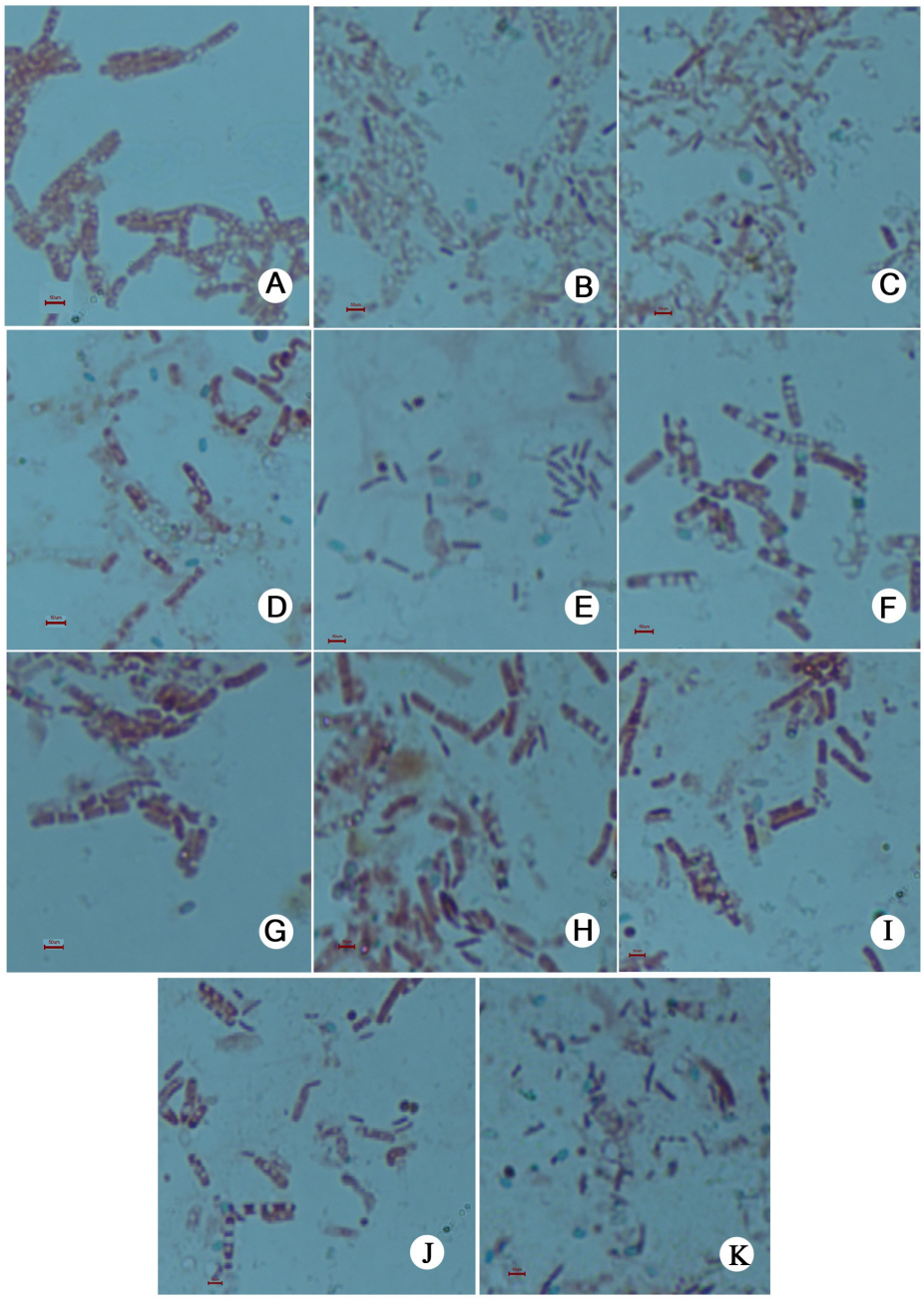

Figure 28

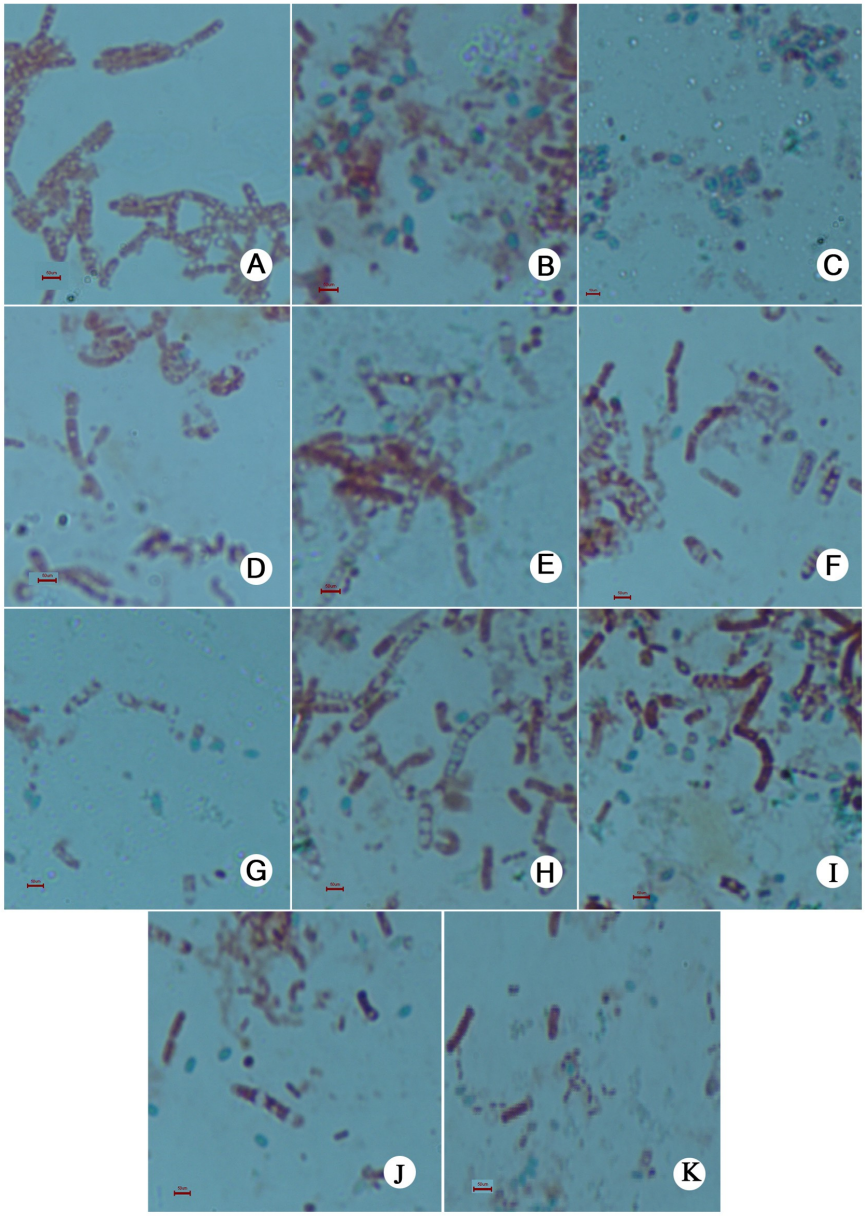

Figure 29

Figure 29: Endospore production pattern of *Btk* on various concentrations (w/v) of potato powder (PP) supplemented LB medium at 36 h as revealed by Image Analyser (scale bar = 50 µM): **A:** control (LB) showing numerous vegetative cells with swollen sporangia; **B:** 1% showing vegetative cells with numerous endospores; **C**: 5% showing few vegetative cells and numerous endospores; **D:** 10% showing few vegetative cells and endospores; **E:** 20% showing vegetative cells, swollen sporangia and endospores; **F**: 30% showing vegetative cells, swollen sporangia and endospores; **G**: 40% showing vegetative cells, swollen sporangia and endospores; **H:** 50% showing vegetative cells, swollen sporangia and endospores; **I:** 60% showing vegetative cells, swollen sporangia and endospores; **J:** 80% showing vegetative cells, swollen sporangia and endospores and **K**: 100% showing vegetative cells, swollen sporangia and endospores.

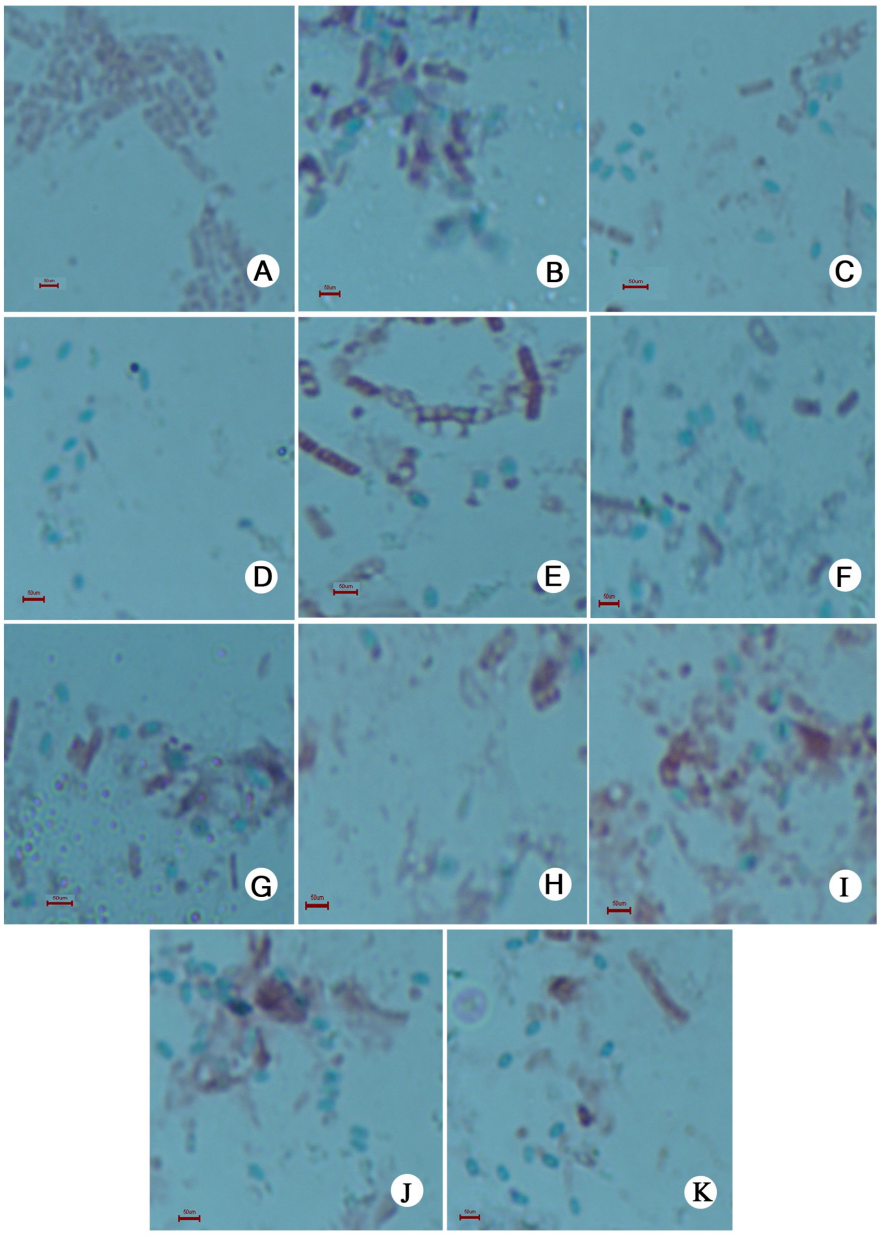

Figure 30

Figure 30: Endospore production pattern of *Btk* on various concentrations (w/v) of potato powder (PP) supplemented LB medium at 42 h as revealed by Image Analyser (scale bar = 50 μM): **A:** control (LB) showing numerous vegetative cells with swollen sporangia; **B:** 1% showing vegetative cells with numerous endospores; **C**: 5% showing vegetative cells and numerous endospores; **D:** 10% showing vegetative cells and numerous endospores; **E:** 20% showing vegetative cells, swollen sporangia and endospores; **F**: 30% showing vegetative cells and numerous endospores; **G**: 40% showing vegetative cells and numerous endospores ; **H:** 50% showing few vegetative cells and endospores; **I:** 60% showing vegetative cells and numerous endospores; **J:** 80% showing few vegetative cells and numerous endospores and **K**: 100% showing few vegetative cells and numerous endospores.

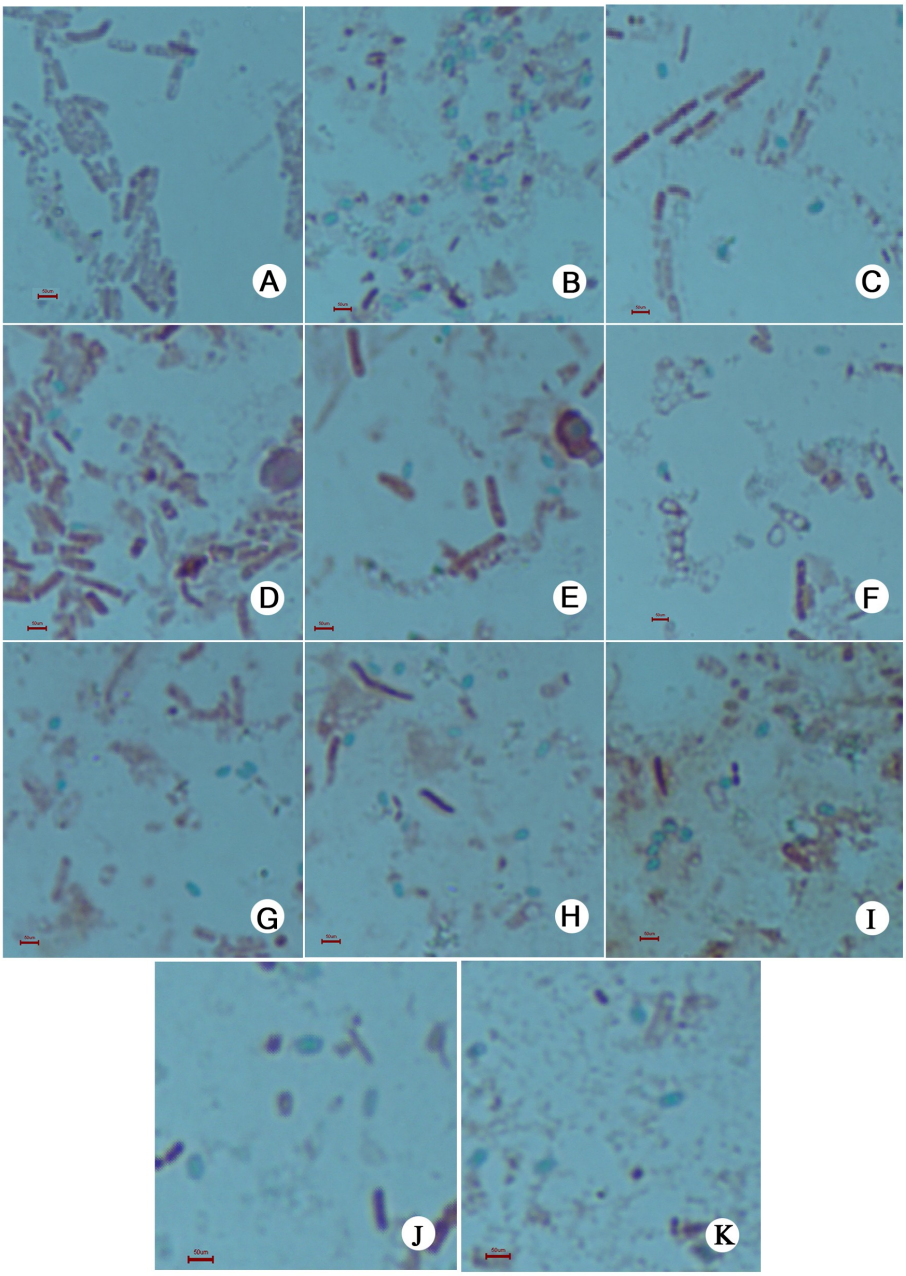

Figure 31

Figure 31: Endospore production pattern of *Btk* on various concentrations (w/v) of potato powder (PP) supplemented LB medium at 48 h as revealed by Image Analyser (scale bar = 50 μM): **A:** control (LB) showing vegetative cells with sporangia; **B:** 1% showing vegetative cells with numerous endospores; **C**: 5% showing vegetative cells and numerous endospores; **D:** 10% showing numerous vegetative cells and endospores; **E:** 20% showing vegetative cells and numerous endospores; **F**: 30% showing vegetative cells, swollen sporangia and numerous endospores; **G**: 40% showing vegetative cells and numerous endospores ; **H:** 50% showing vegetative cells and numerous endospores; **I:** 60% showing few vegetative cells and numerous endospores; **J:** 80% showing few vegetative cells and numerous endospores and **K**: 100% showing few vegetative cells and numerous endospores.

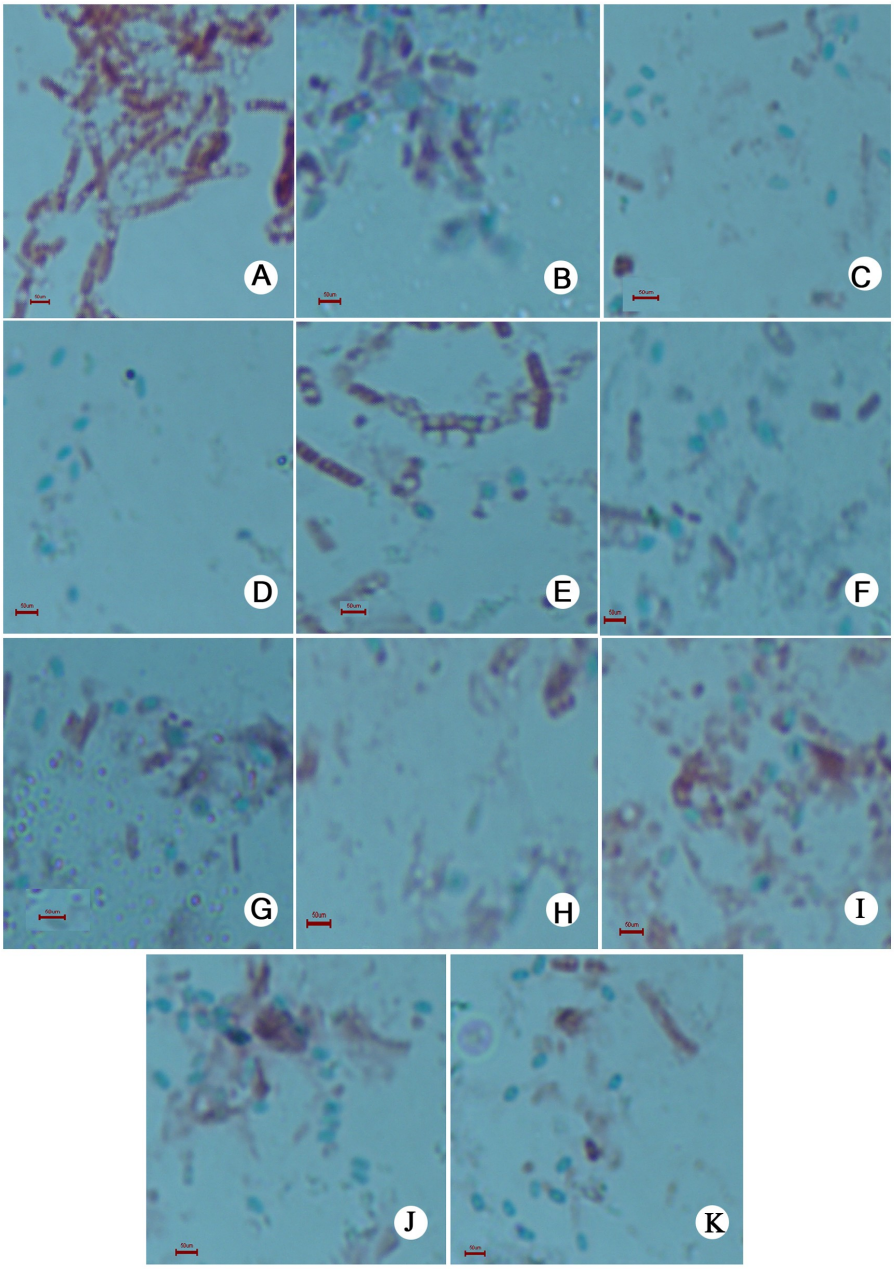

Figure 32

Figure 32: Endospore production pattern of *Btk* on various concentrations (w/v) of potato powder (PP) supplemented LB medium at 60 h as revealed by Image Analyser (scale bar = 50 µM): **A:** control (LB) showing numerous vegetative cells with swollen sporangia; **B:** 1% showing mumerous vegetative cells with endospores; **C**: 5% showing few vegetative cells with endospores; **D:** 10% showing very few vegetative cells with endospores; **E:** 20% showing very few vegetative cells with swollen sporangia endospores; **F**: 30% showing few vegetative cells with endospores; **G**: 40% showing few vegetative cells with endospores ; **H:** 50% showing vegetative cells with swollen sporangia and endospores; **I:** 60% showing vegetative cells with swollen sporangia and numerous endospores; **J:** 80% showing vegetative cells and numerous endospores and **K**: 100% showing few vegetative cells and numerous endospores.

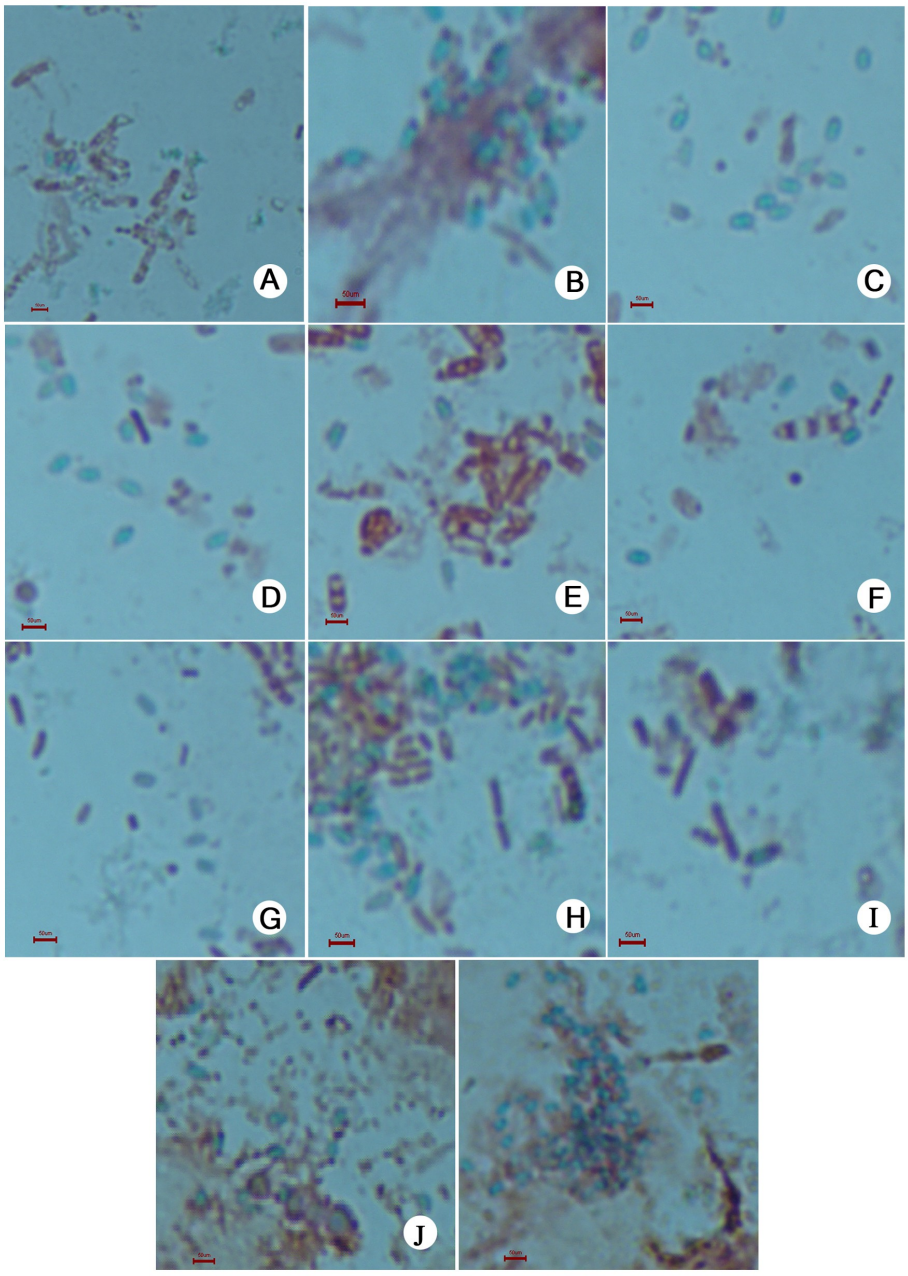

Figure 33

Figure 33: Endospore production pattern of *Btk* on various concentrations (w/v) of potato powder (PP) supplemented LB medium at 72 h as revealed by Image Analyser (scale bar = 50 μM): **A:** control (LB) showing numerous vegetative cells with sporangia and endospores; **B:** 1% showing few vegetative cells with numerous endospores; **C**: 5% showing very few vegetative cells and numerous endospores; **D:** 10% showing few vegetative cells and numerous endospores; **E:** 20% showing vegetative cells with swollen sporangia and numerous endospores; **F**: 30% showing few vegetative cells with swollen sporangia and numerous endospores; **G**: 40% showing very few vegetative cells and numerous endospores; **H:** 50% showing few vegetative cells and mumerous endospores; **I:** 60% showing very few vegetative cells and endospores; **J:** 80% showing very few vegetative cells and numerous endospores and **K**: 100% showing very few vegetative cells and numerous endospores.

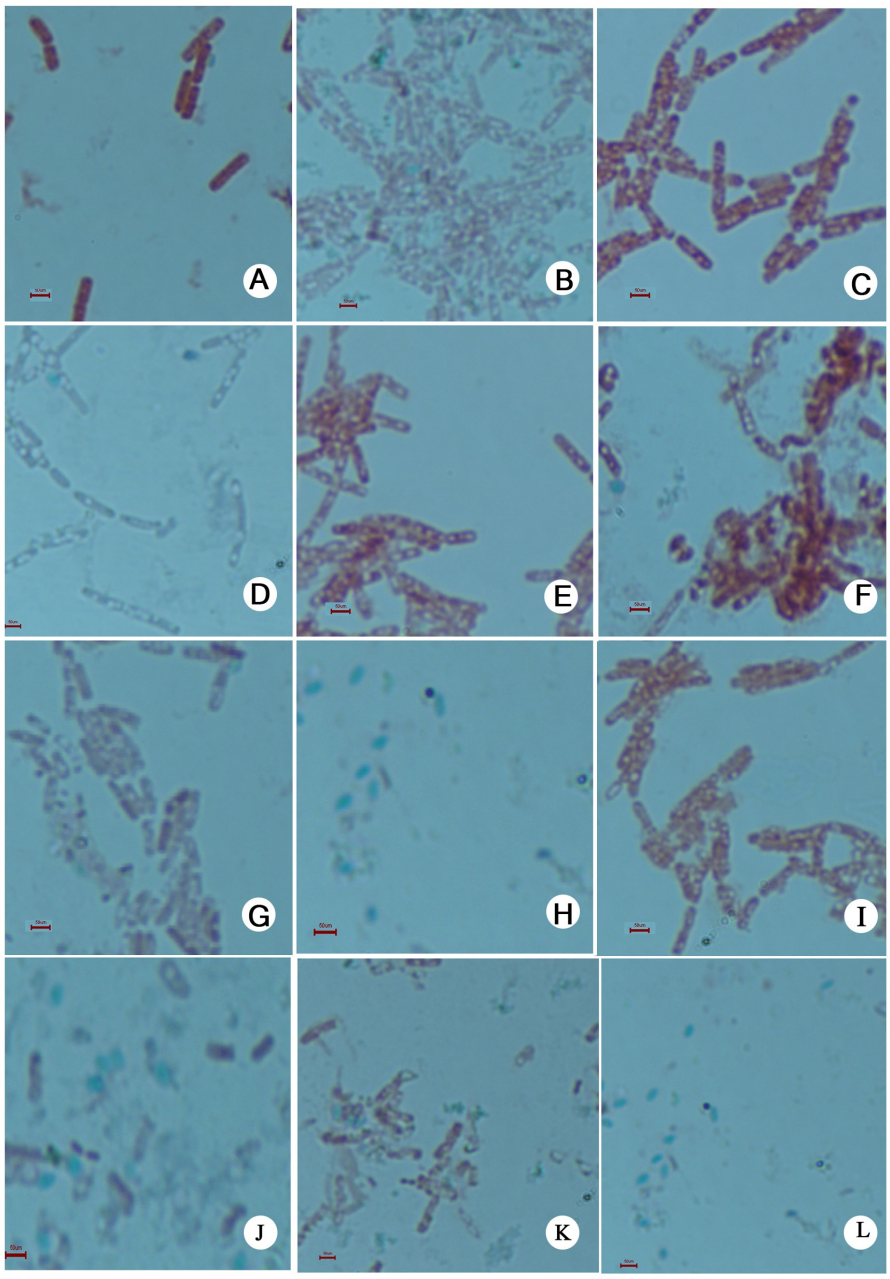

Figure 34

Figure 34: Endospore production of *Btk* with JP supplemented LB medium (10% w/v) and control (LB) as revealed by image analyser (scale bar = 50 μM):. A: 12 h control (LB) showing numerous vegetative cells with sporangia; B: 10% of JP at 12 h showing few vegetatve cells; C: 24 h control (LB) showing numerous vegetative cells with sporangia; D: 10% of JP at 24 h showing vegetative cells with swollen sporangia; E. 36 h control (LB showing numerous vegetative cells with swollen sporangia; F: 10% of JP at 36 h showing few vegetative cells with sporangia; G: 48 h control (LB) showing numerous vegetative cells with sporangia; H: 10% of JP at 48 h showing vegetative cells with endospores; I: 60 h control (LB) showing numerous vegetative cells with swollen sporangia; J: 10% of JP at 60 h showing vegetative cells, sporangia and endospores; J: 72 h control (LB) showing vegetative cells and endospores and K:10% of JP at 72 h showing numerous endospores.

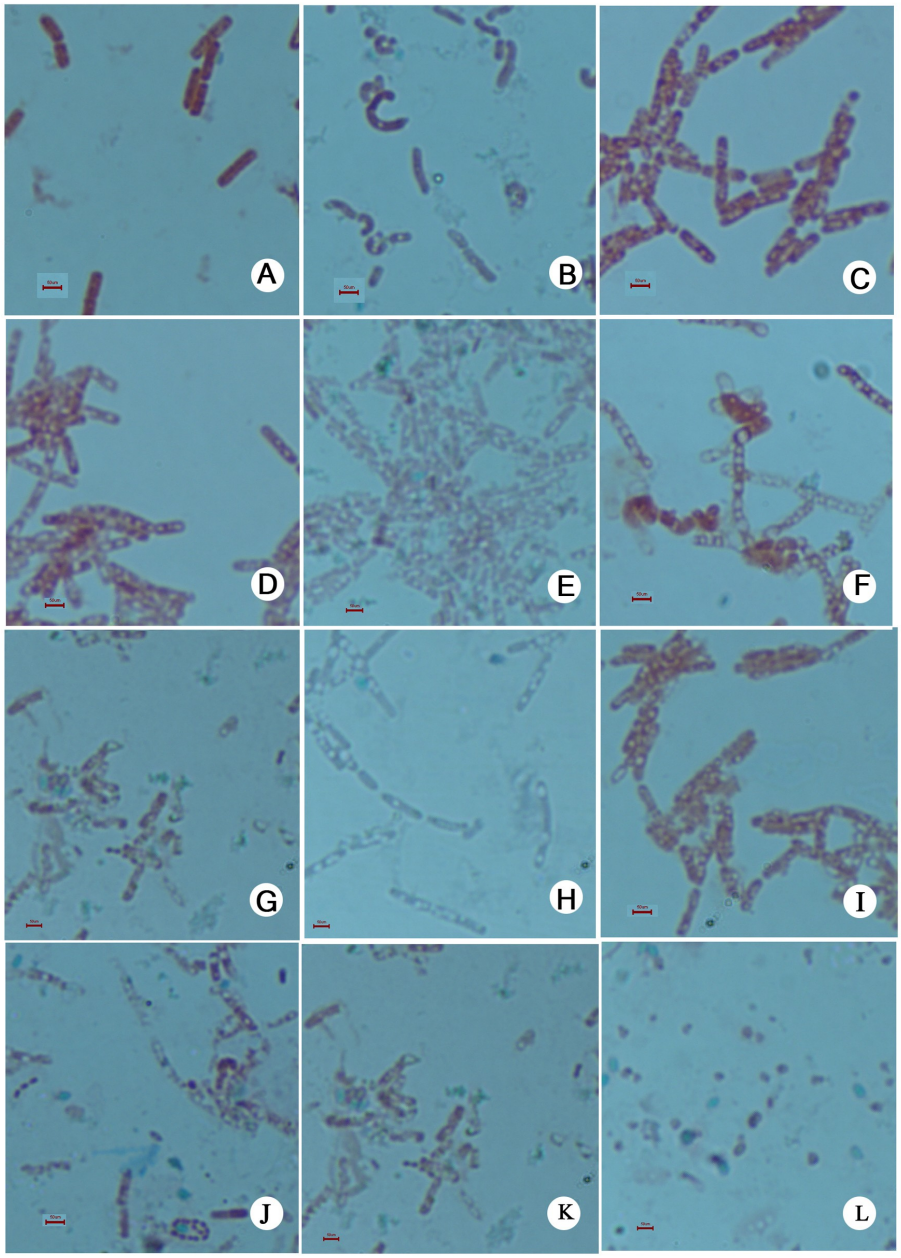

Figure 35

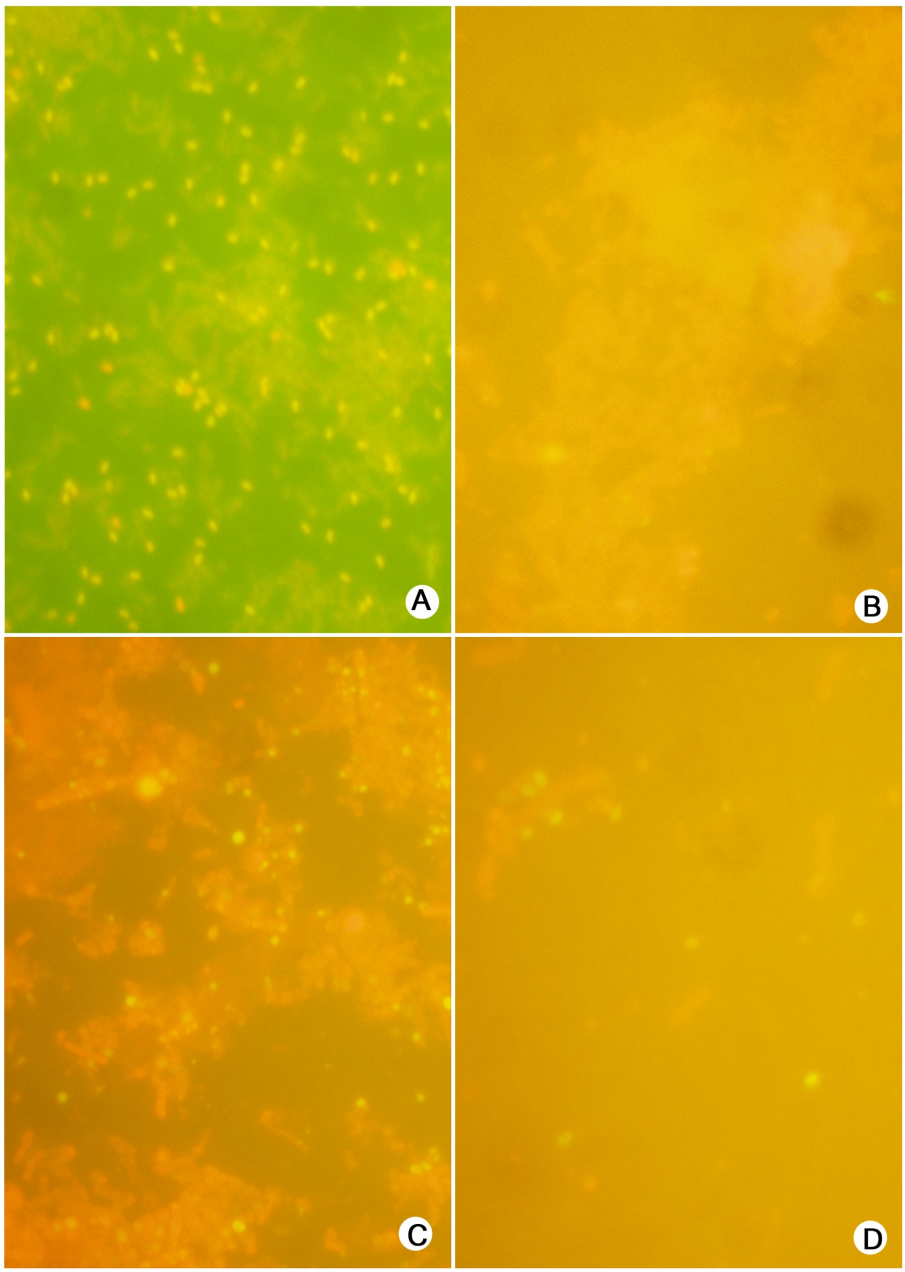

Figure 36: Endospore production pattern of *Btk* with jack seed powder (JP), potato powder (PP) and tapioca powder (TP) at 10% (W/V) supplemented LB medium and control as revealed by flourescent microscope (100x) at 48 h. A. control (LB) showing numerous endospores; B. 10% of jack seed powder showing very few endospores; C. 10% of potato powder showing big endospores; D. 10% of tapioca powder showing numerous big endospores.

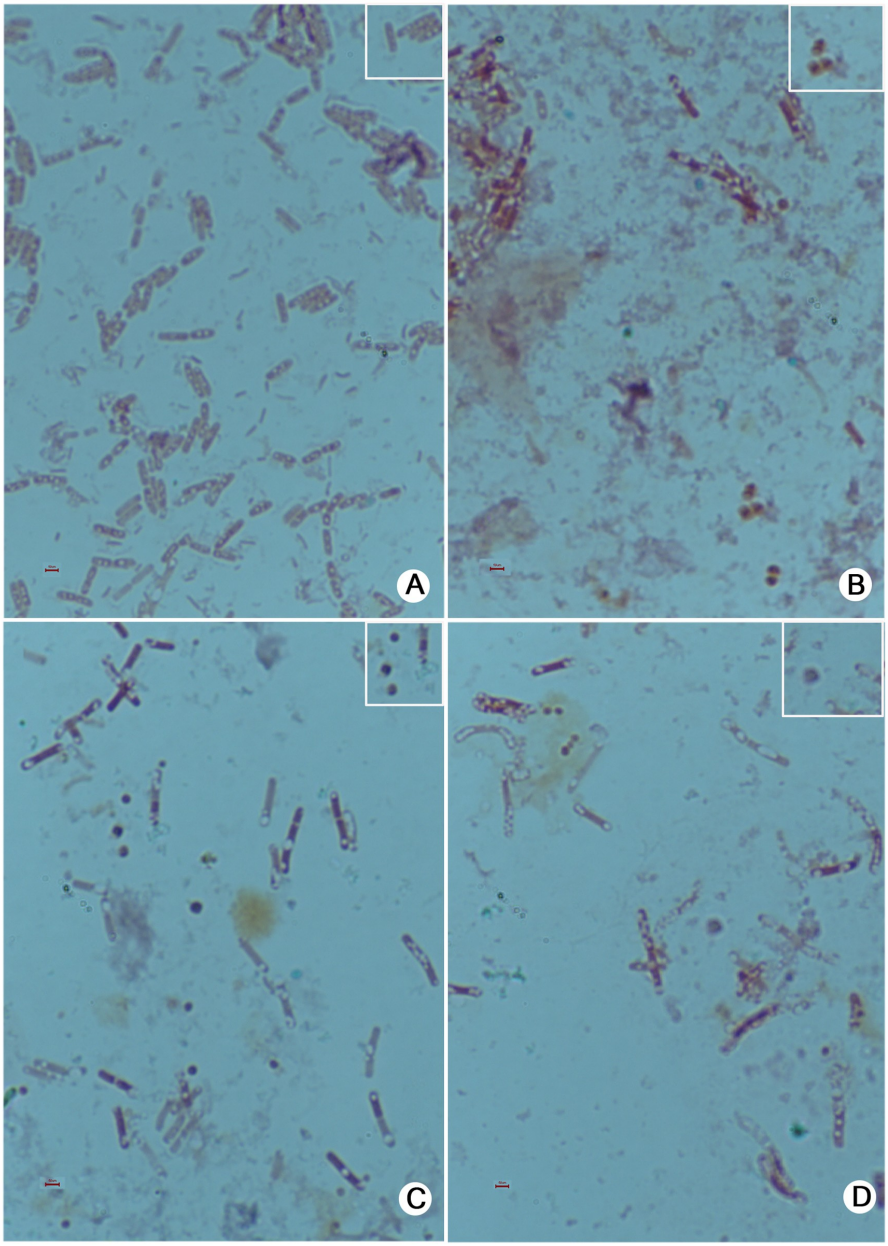

Figure 37: Delta endotoxin production pattern of *Btk* on jack seed powder, potato powder and tapioca powder at 10% (W/V) supplemented LB media as revealed by image analyser at 48 h. A: control (LB) showing vegetative cells with endospores and few crystals; B: 10% of jack seed powder showing vegetative cells with spores and crystals; C: 10% of potato powder showing few vegetative cells with endopores and many crystals and D: 10% of tapioca powder showing vegetative cells with endospores and few crystals.

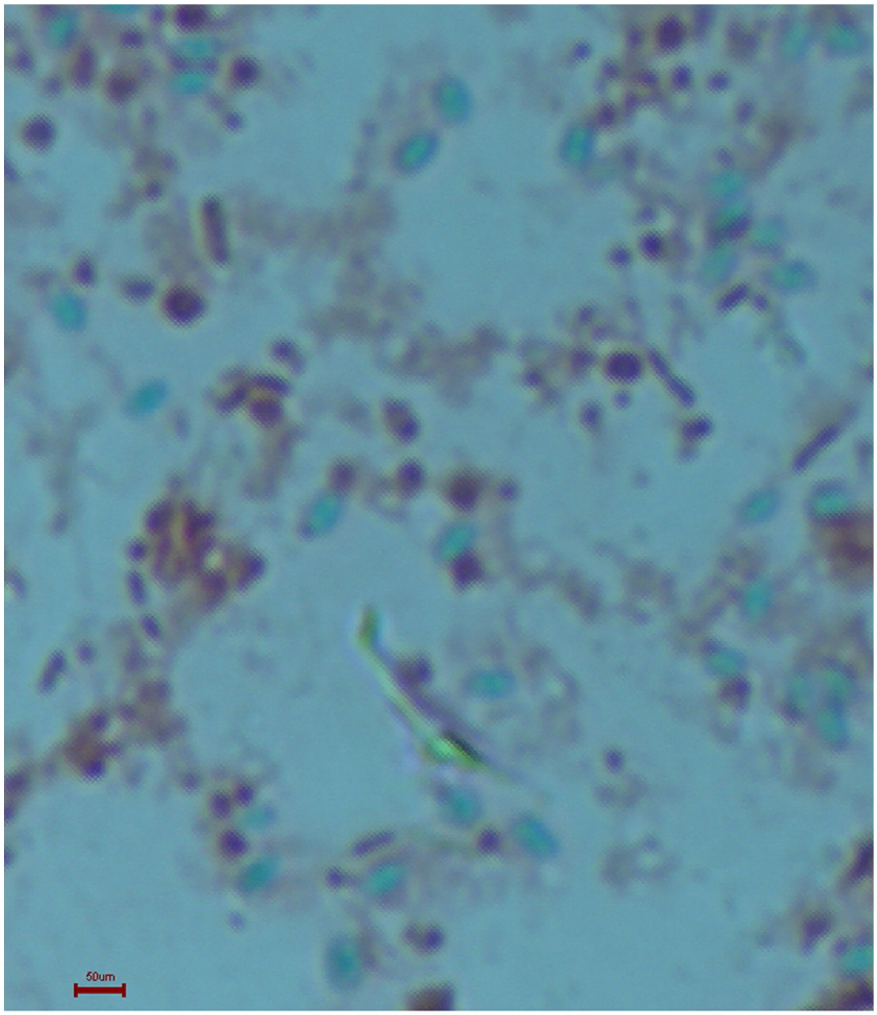

Figure 38: Endospores and crystals produced by *Btk* on PP (10 %) supplemented LB medium on water restricted environment at 24 h. Huge endospores (greenish blue) and crystals (bluish pink) are seen with very few vegetative cells or sporulating cells.

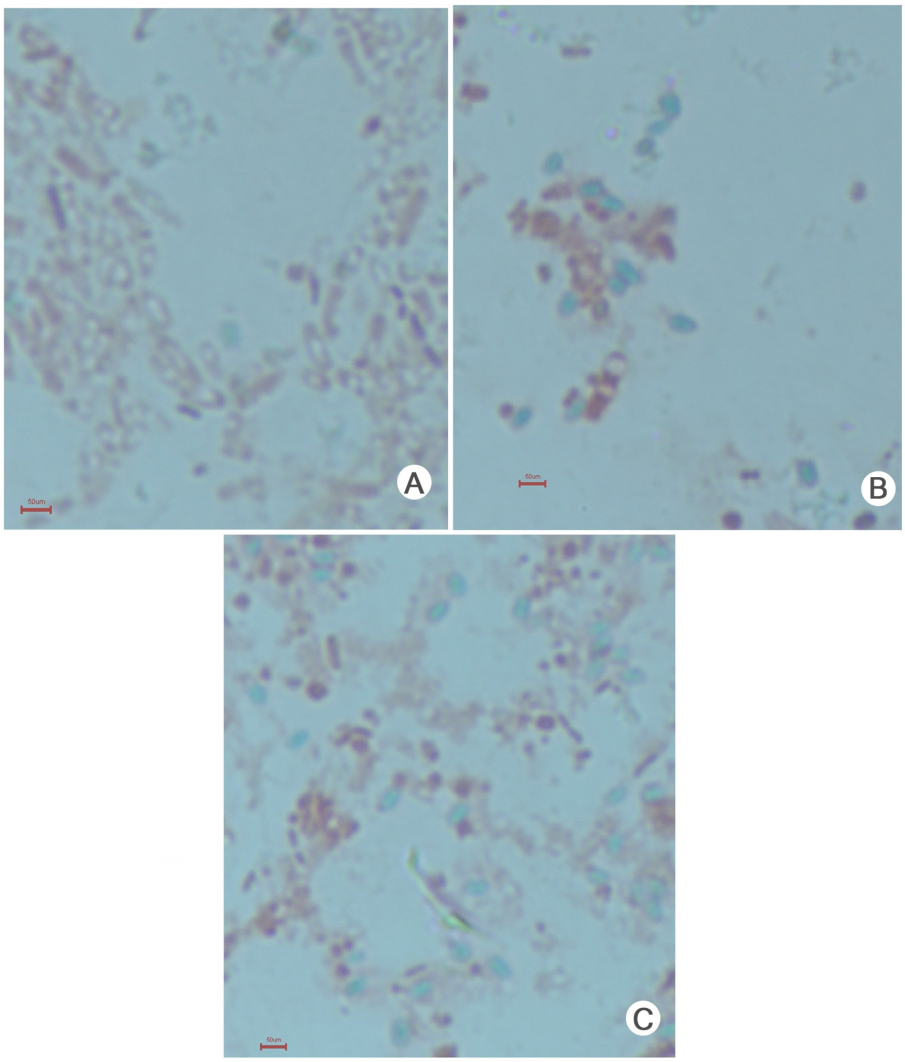

Figure 39: Comparative profile of coupled production of endospores and crystals by *Btk* at differnet conditions. A. 72 h control (LB) showing few vegetative cells, free endospores (greenish blue) and crystals (bluish pink); B: 48 h 10% of PP supplemented medium showing few vegetative cells, sporulating cells, many free endospores and crystals and C: 24 h 10% PP supplemented medium showing huge endospores (greenish blue) and crystals (bluish pink) are seen with very few vegetative cells or sporulating cells.

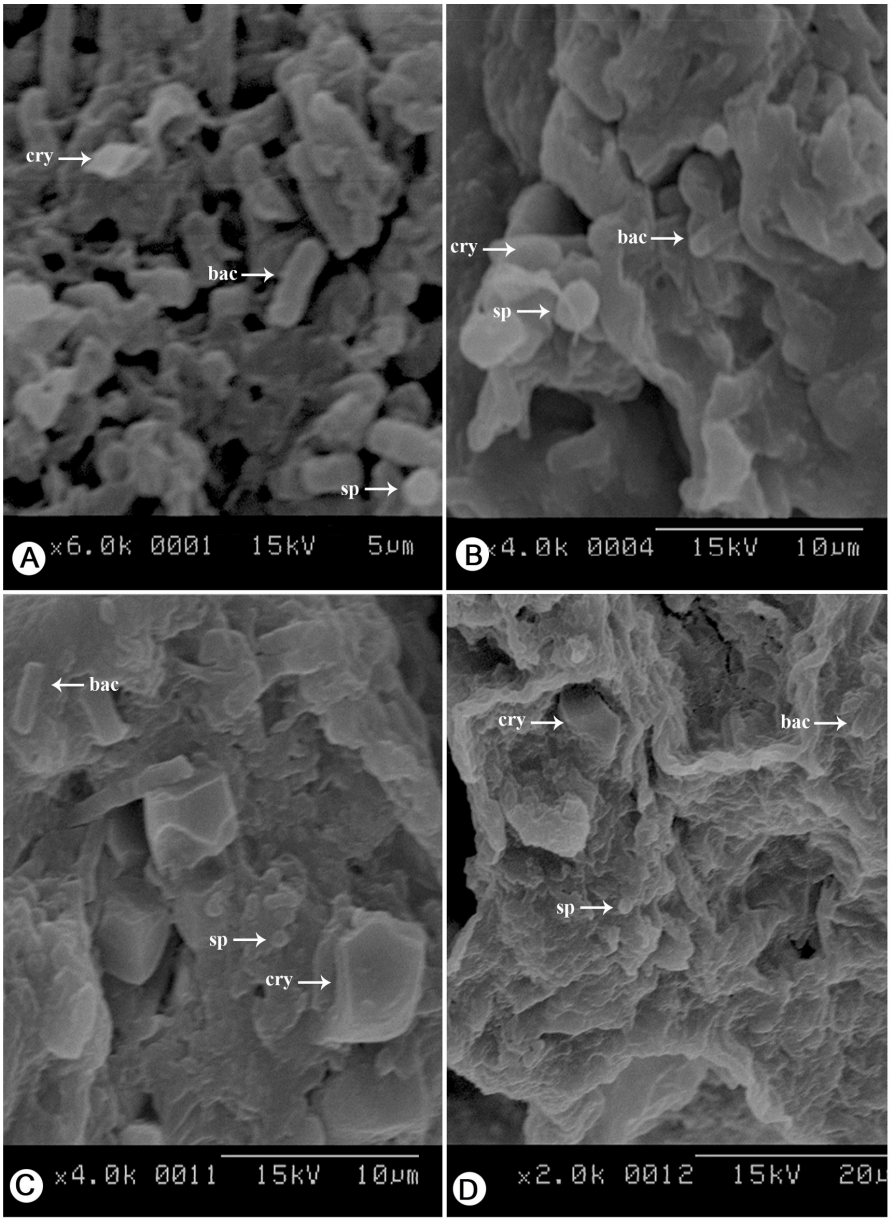

Figure 40: Scanning electron micrographic images of delta endotoxin production of *Btk* with jack seed powder, potato powder and tapioca powder (10% w/v) supplemented LB media at 48 h. A: control showing rhomboidal crystal with vegetative cells and spores; B: 10% of jack seed powder showing cuboidal crystals and spores; C: 10% of potato powder showing mostly rectangular large crystals with few spores and vegetative cells and D: 10% of tapioca powder showing few crystals, vegetative cells and spores.

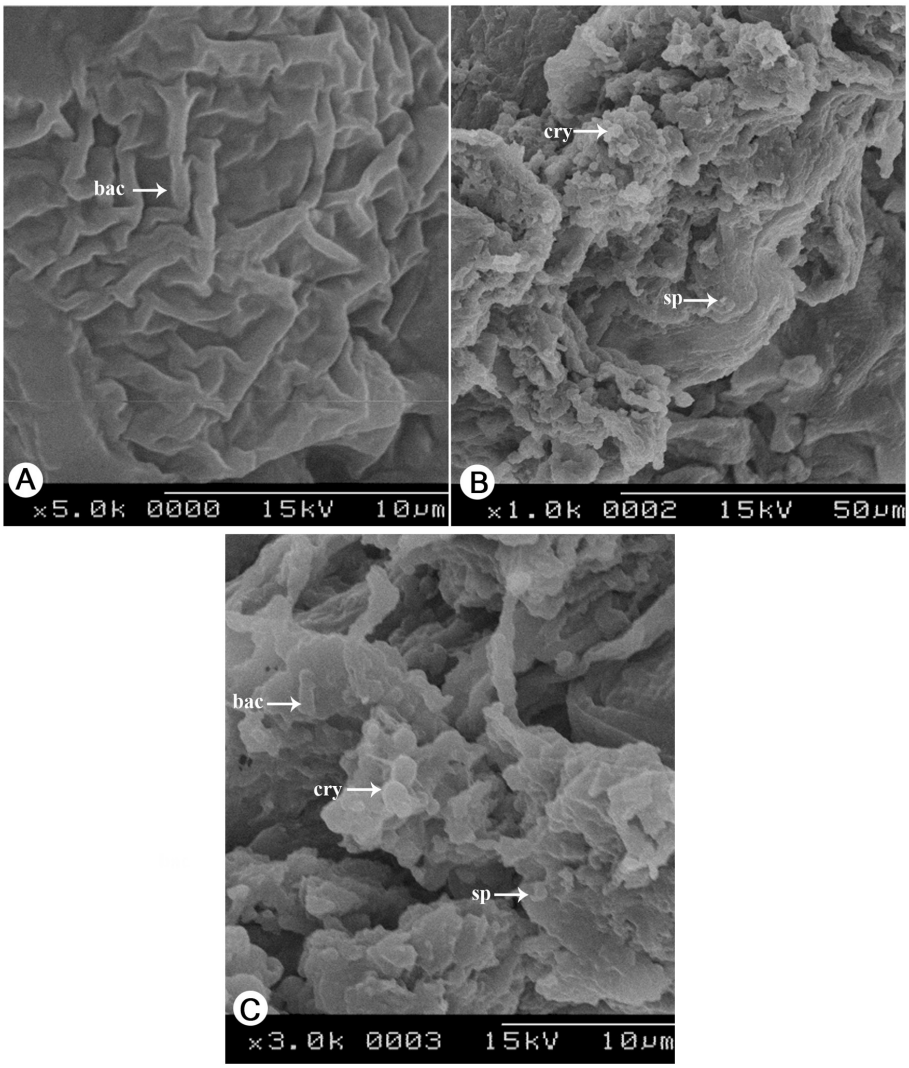

Figure 41: SEM images of *Btk*-toxin with endospores produced by *Btk* on PP supplemented LB medium (10% w/v) in water restricted environment. A: vegetative cells at 12 h; B: crystal at 24 h (1000 X) and C: 3 times magnified view of B with clusters of crystals.

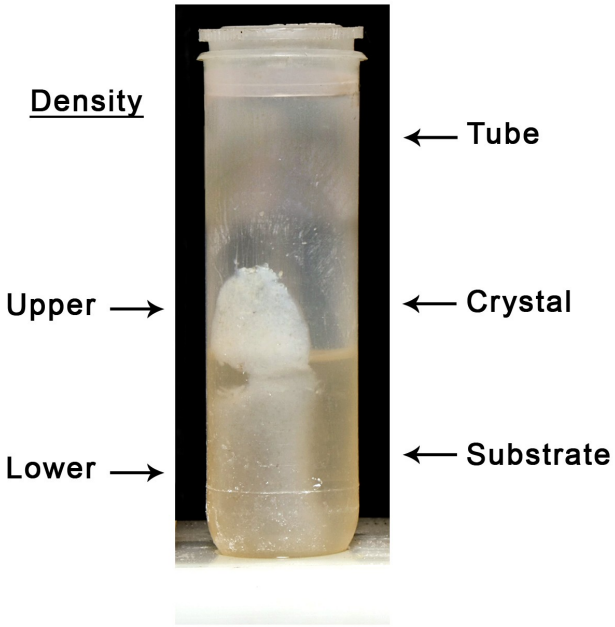

Figure 42 A

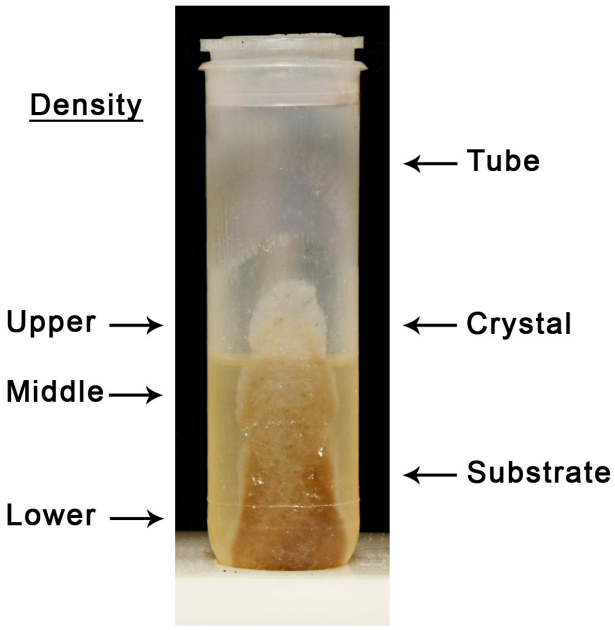

Figure 42 B

Figure 42 A: Sucrose gradient (45%, 67% and 87%) pattern of *Btk*-toxin prepared from LB medium (control). The white pellet of crystals was separated at the interphase of 67% and 87% gradients.

Figure 42 B: Sucrose gradient (45%, 67% and 87%) pattern of *Btk*-toxin proteins prepared from 10% (w/v) PP supplemented LB medium. The white pellet of crystals separated in the 87% gradients. Yet more *Btk*-toxin proteins embedded in the substrate.

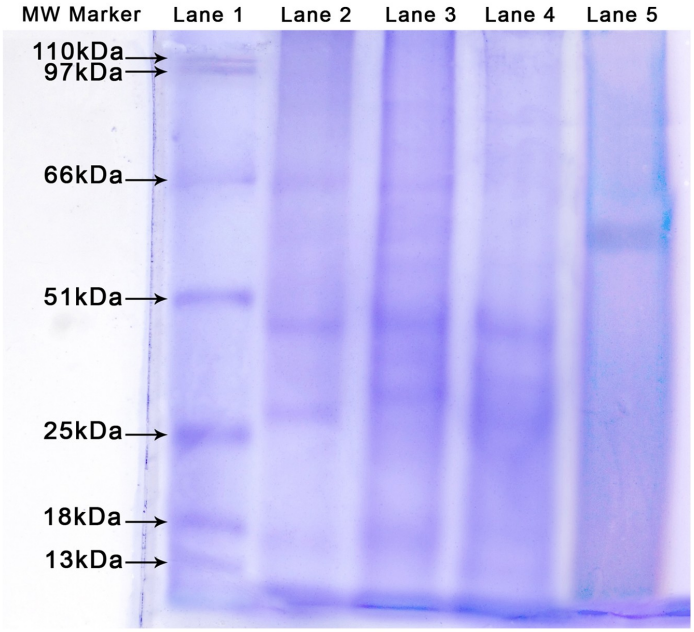

Figure 43 A

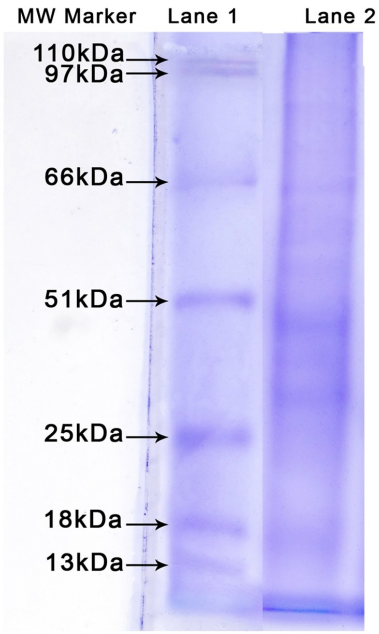

Figure 43 B

Figure 43 A: SDS-PAGE profile of *Btk*-toxin proteins purified from 48 h PP LB medium with or without starch. Lane 1: molecular weight marker; Lane 2: control showed 4 bands with approximate molecular weights of 18 kDa, 30 kDa, 50 kDa and 66 kDa; Lane 3: jack seed powder supplement showed 4 bands with approximate molecular weights of 18 kDa, 30 kDa, 50 kDa and 66 kDa; Lane 4: potato powder supplement showed 3 bands with approximate molecular weights of 18 kDa, 30 kDa and 50 kDa and Lane 5: tapioca powder showed a single band with approximate molecular weight of 60 kDa. This figure reveals that various shape of the crystals are dependent on the growth medium, which in turn are related to molecular weights as revealed by SDS-PAGE profile.

Figure 43 B: SDS-PAGE profile of the *Btk-toxin* proteins purified from 48 h fermented PP supplemented LB medium. Lane 1: molecular weight marker, Lane 2: sample from PP supplemented medium.

Figure 44: Habit of *Aceria guerreronis* (mandari) infested coconut palm with tender nuts showing different stages of infestation by mandari. A: *A. guerreronis* infested coconut palm; B: young buttons of 8 weeks old infested nuts develop triangular creamy white patches; C: buttons of 9 weeks old nut develop 1 to 2 patches and D: buttons of 11 week old nut develop longitudinal cracks.

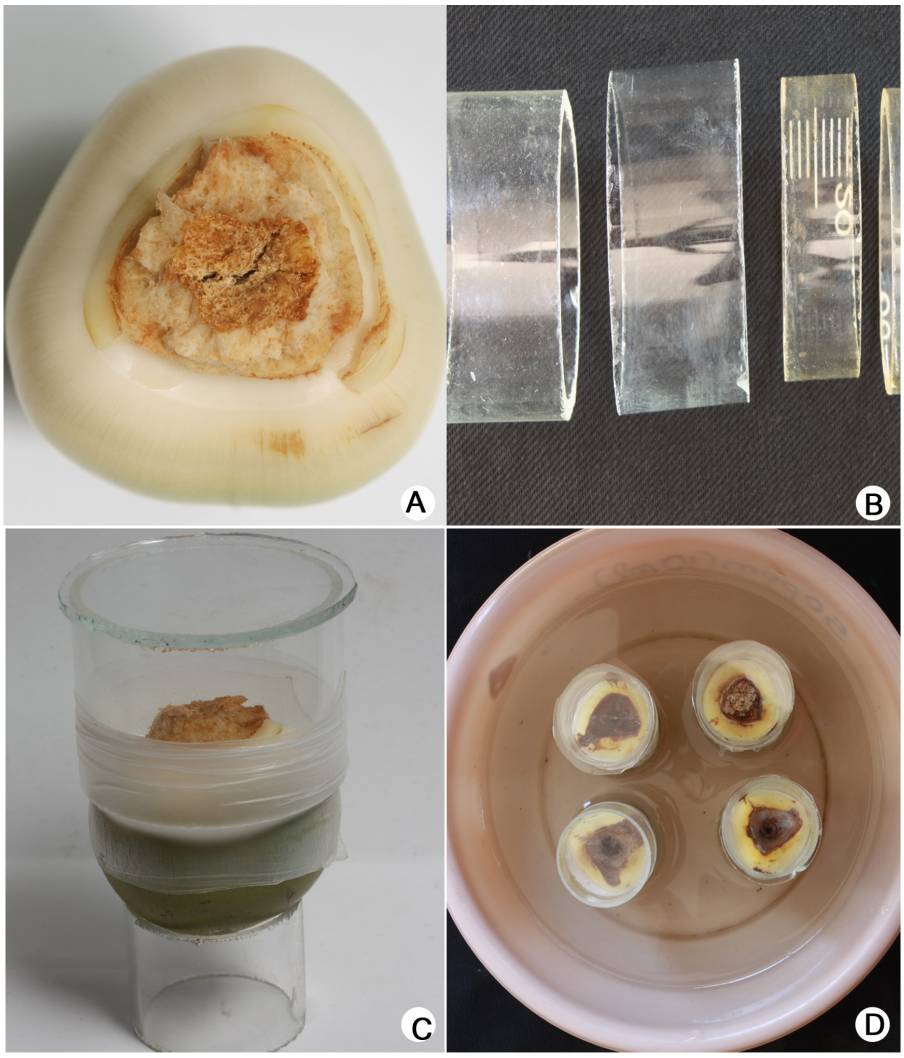

Figure 45: Coconut button and glass rings used for culturing of *A. guerreronis*. A: single 30 days aged button show cap region exposed; B; glass rings used for the culture of mites; C; glass rings attached to the button and sealed with parafilm and D: culture set-up (with 4 buttons) in a tray.

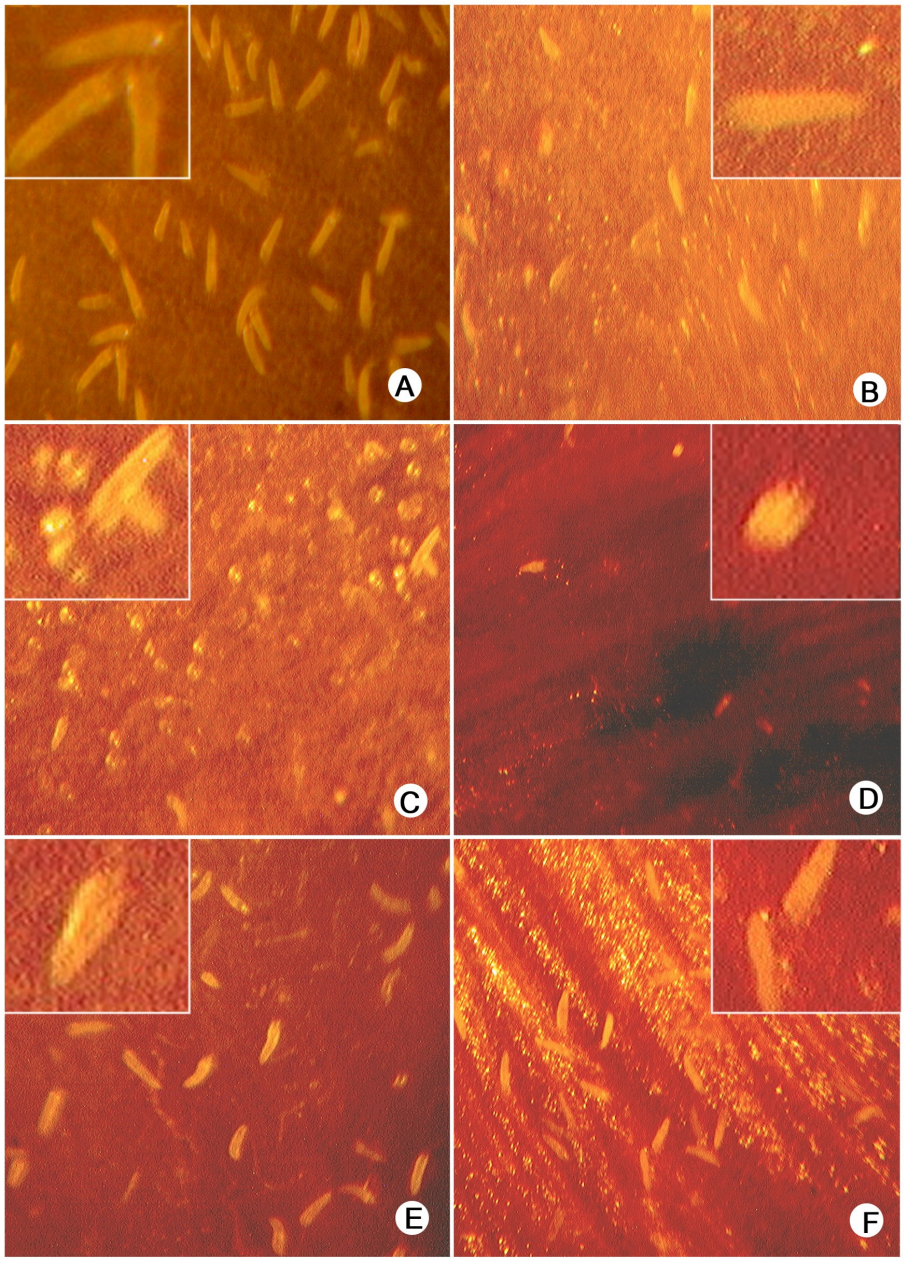

Figure 46

Figure 46: Detailed life cycle of *A. guerreronis* under culture conditions. A: first day showing adult mites; B: second day showing adult mites with eggs (1 mite with one egg at inset); C: fourth day showing adult mites with numerous eggs (1 mite with 7 eggs at inset); D: sixth day showing first nymph (one first nymph at inset); E: eighth day showing first and second nymphs (one second nymph at inset); F: tenth day showing new adult mites and second nymphs (one adult mite and one second nymph at inset).

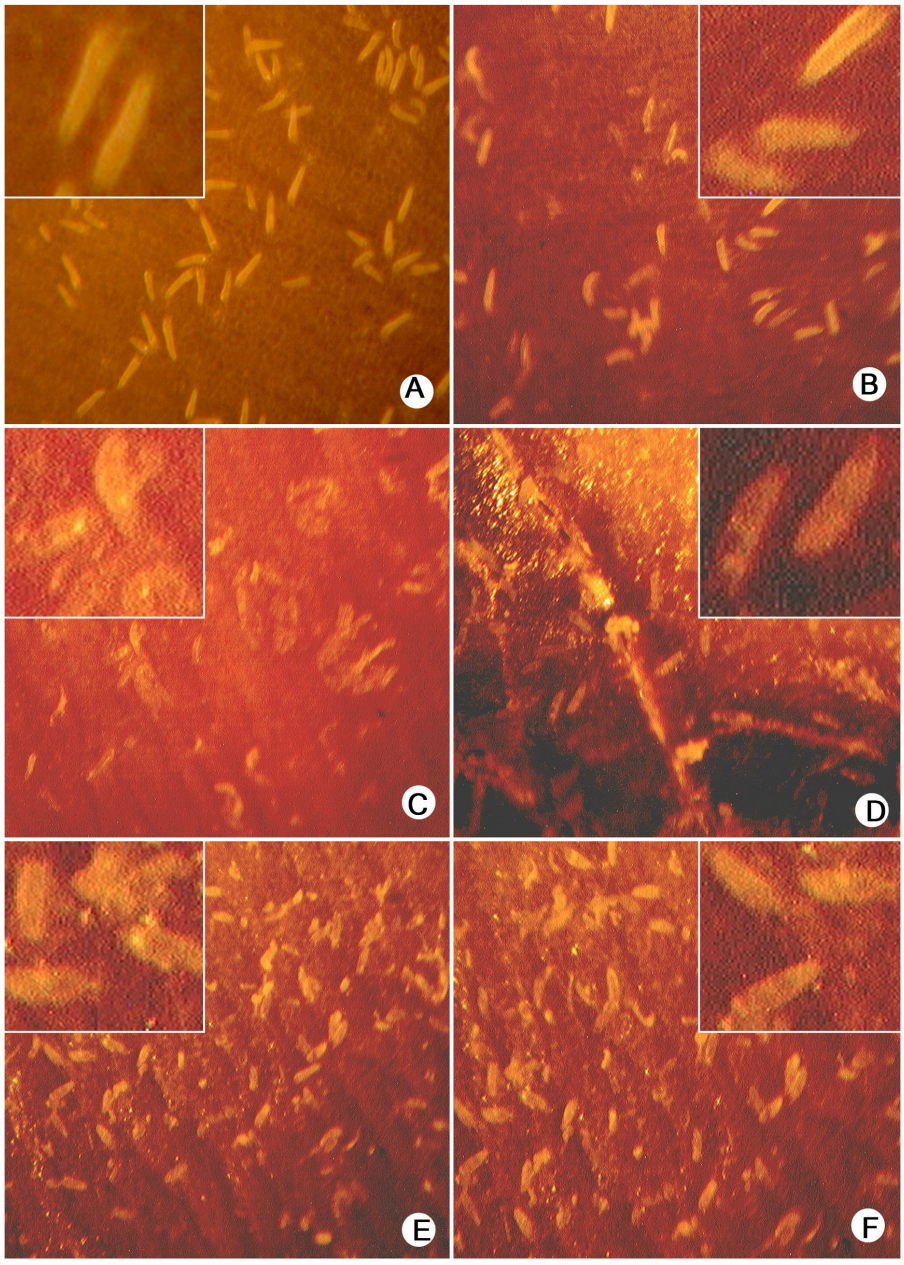

Figure 48

Figure 48: Toxicity assay using dry and powdered raw fermented 10% (w/v) PP supplemented LB medium (48 h) containing mixture of endospores, *Btk* crystals and substrate (µg/cm2). Treatment was made on the mites after 24 h observation of transferred mites in the culture set-up. Photographs were taken after 24 h treatment.

A: control mites treated with sticky uninoculated 10% (w/v) PP supplemented LB medium - here most of the mites were alive and active (above 90%) (two adult mites at inset); B: mites treated with 1.25 fermented matter – here some mites are alive but less active (about 20%) (one active mite with 2 dead mites at inset); C: mites treated with 1.88 fermented matter - here all mites were dead (3 dead mites at inset), D. mites treated with 2.5 fermented matter – here all mites were dead (2 dead mites at inset); E. mites treated with 3.13 fermented matter – here all mites were dead (5 dead mites at inset); F. mites treated with 3.73 fermented matter – here all mites were dead (3 dead mites at inset). From this, it is clear that application of 1.88 µg raw powdered fermented matter per cm2 is enough to combat *A. guerreronis*. In fact, original concentration of the *Btk*-toxin would be very little in the crude feed.

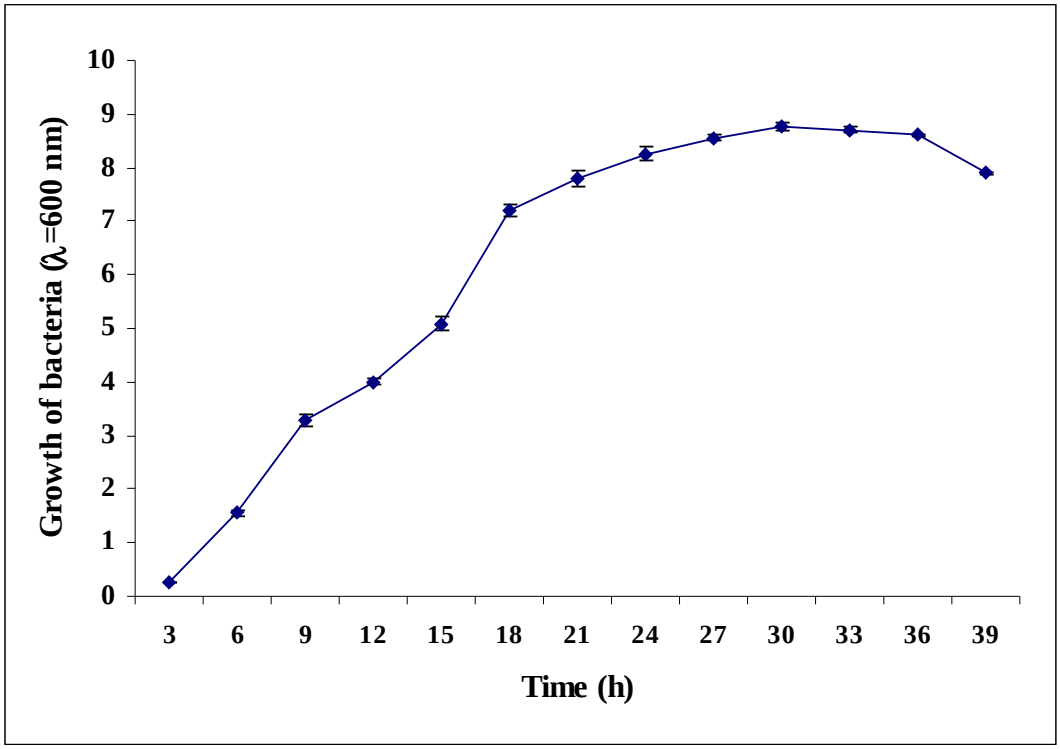

Figure 3: Growth curve of *Btk* on LB medium at 135 rpm and 37 °C.

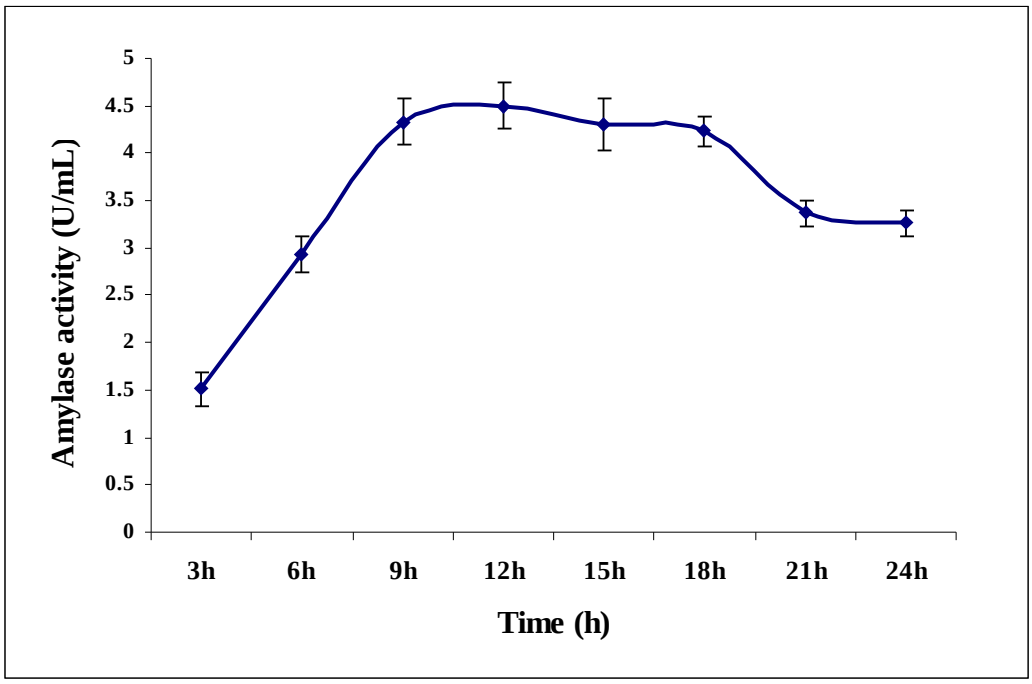

Figure 4: Amylase activity of *Btk* at 3 h intervals on LB medium at 135 rpm and 37 ºC.

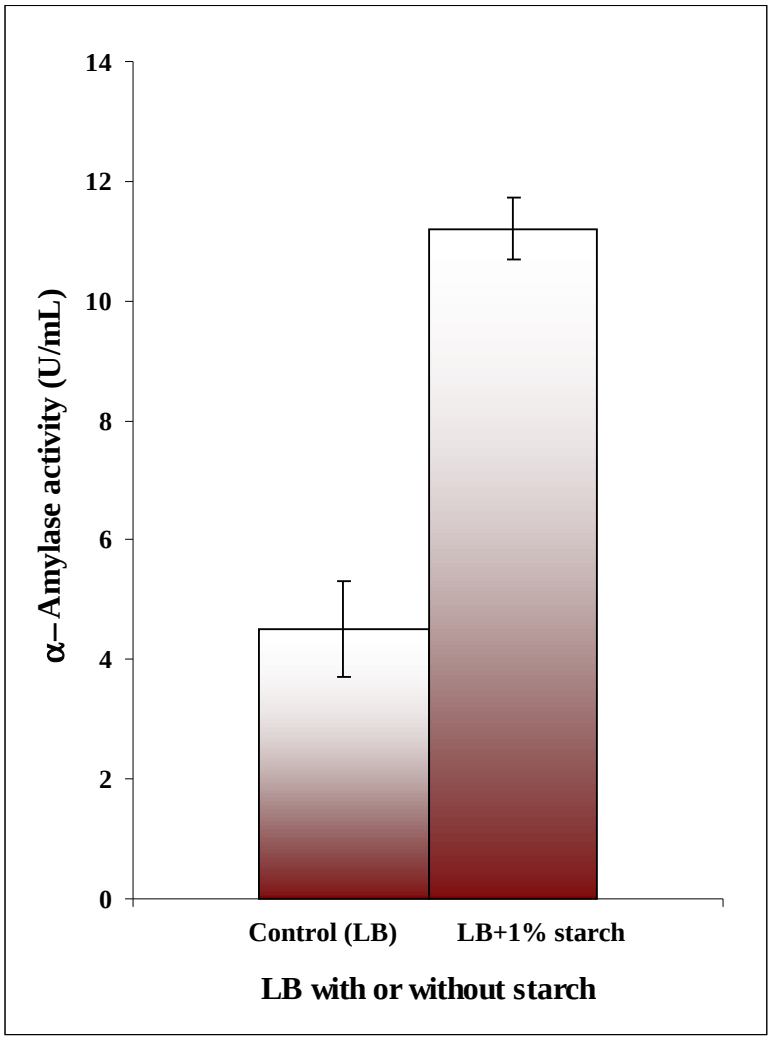

Figure 5: Amylase activity of *Btk* on 1% (w/v) soluble starch supplemented LB medium at 12 h at 135 rpm and 37 ^{o}C. Time interval activities not shown as maximum activity was 12 h.

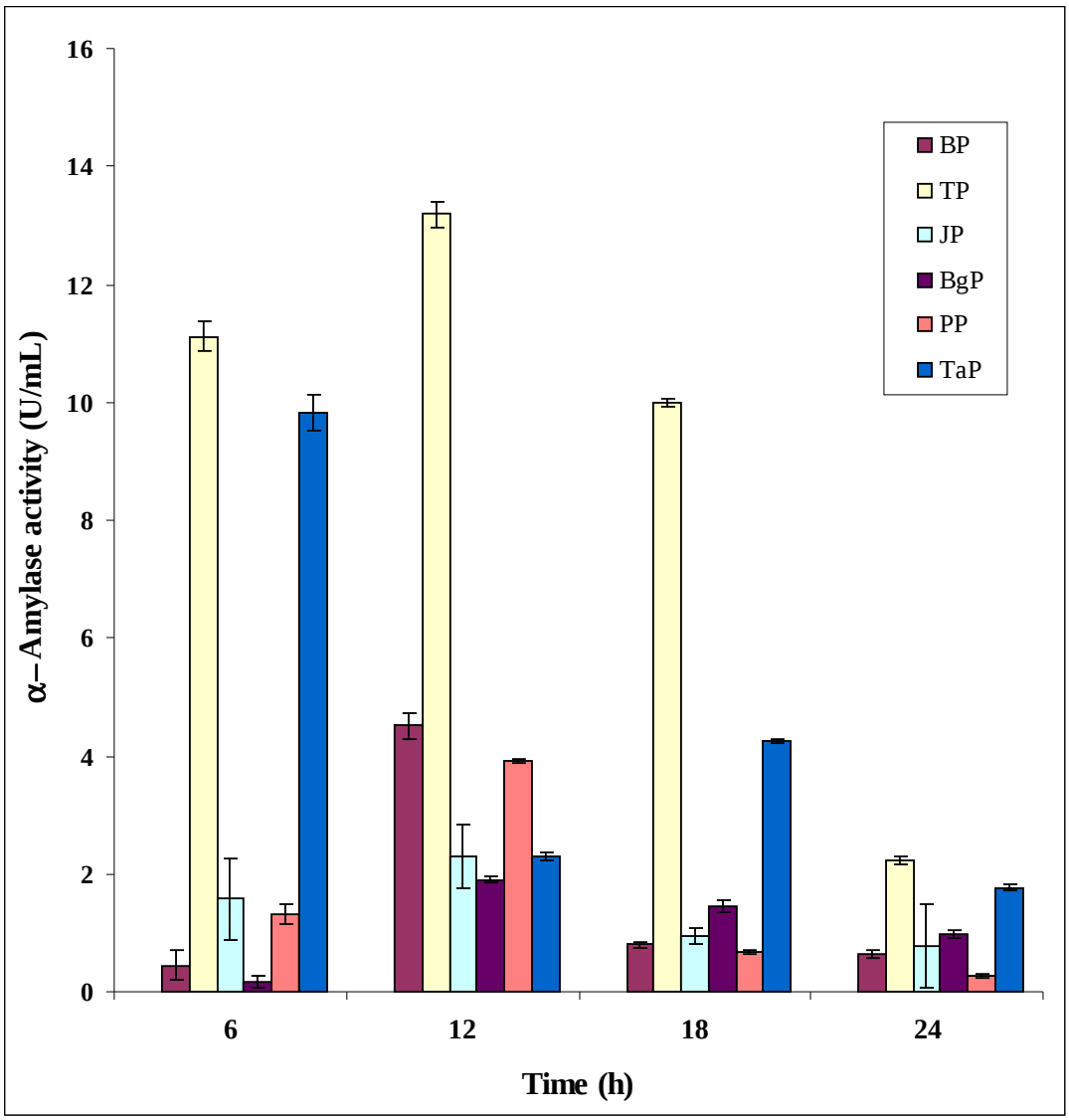

Figure 6: α-Amylase activity of *Btk* at 6h intervals on various naturally available raw starch substrates [banana powder (BP), tapioca powder (TP), jack seed powder (JP), bengal gram powder (BgP), potato powder (PP) and taro powder (TaP)] supplemented [1% (w/v)] in LB medium. SmF was at 135 rpm and 37 ºC.

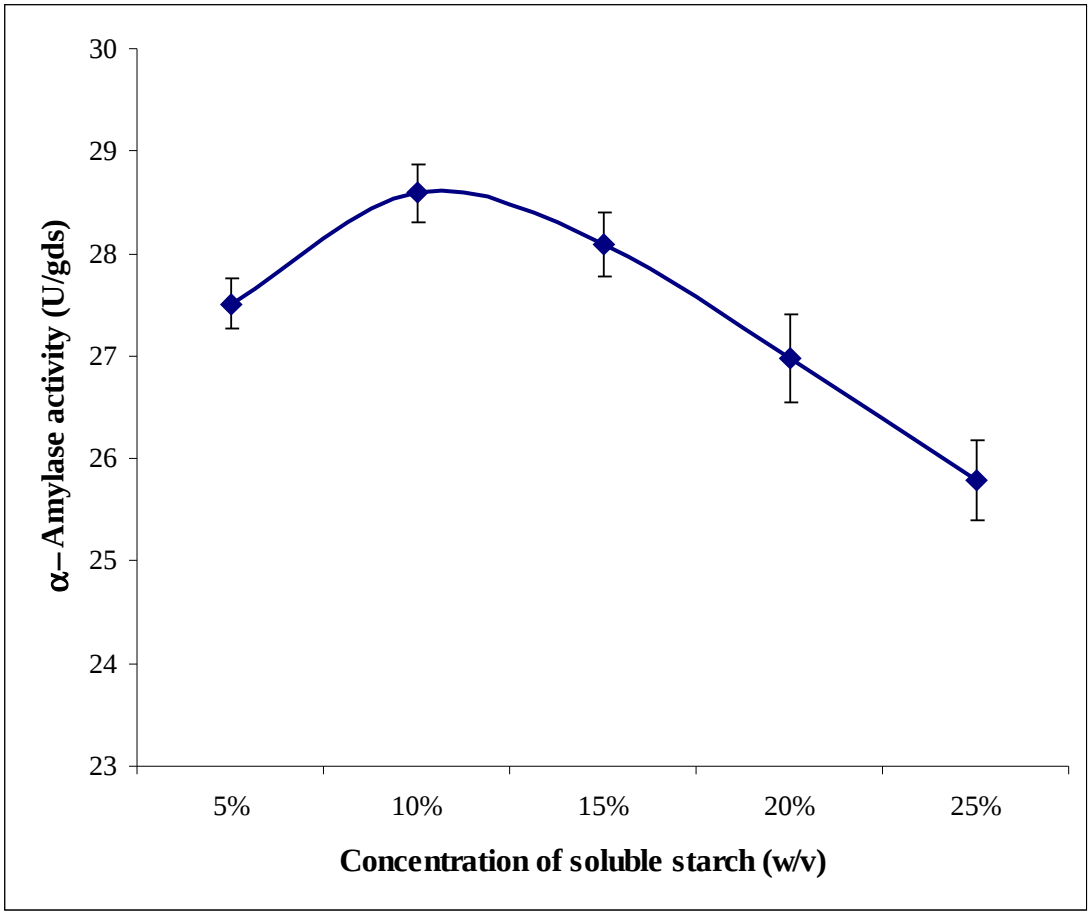

Figure 9: α–Amylase activity of *Btk* on soluble starch supplemented in LB medium at 12 h at 135 rpm and 37 ºC.

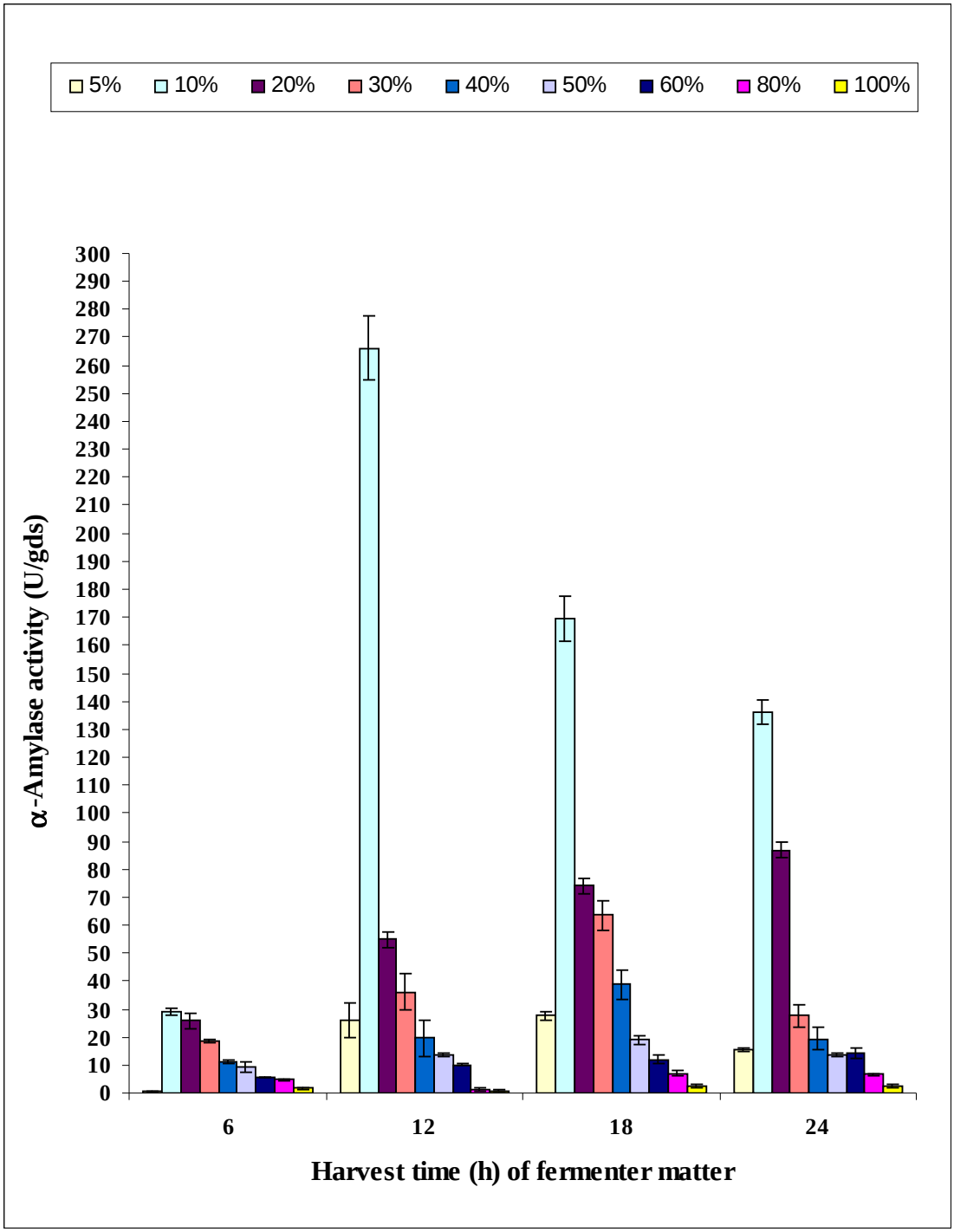

Figure 10: α-Amylase activity of *Btk* on banana powder [(BP), w/v] supplemented LB medium at 37 ºC incubation.

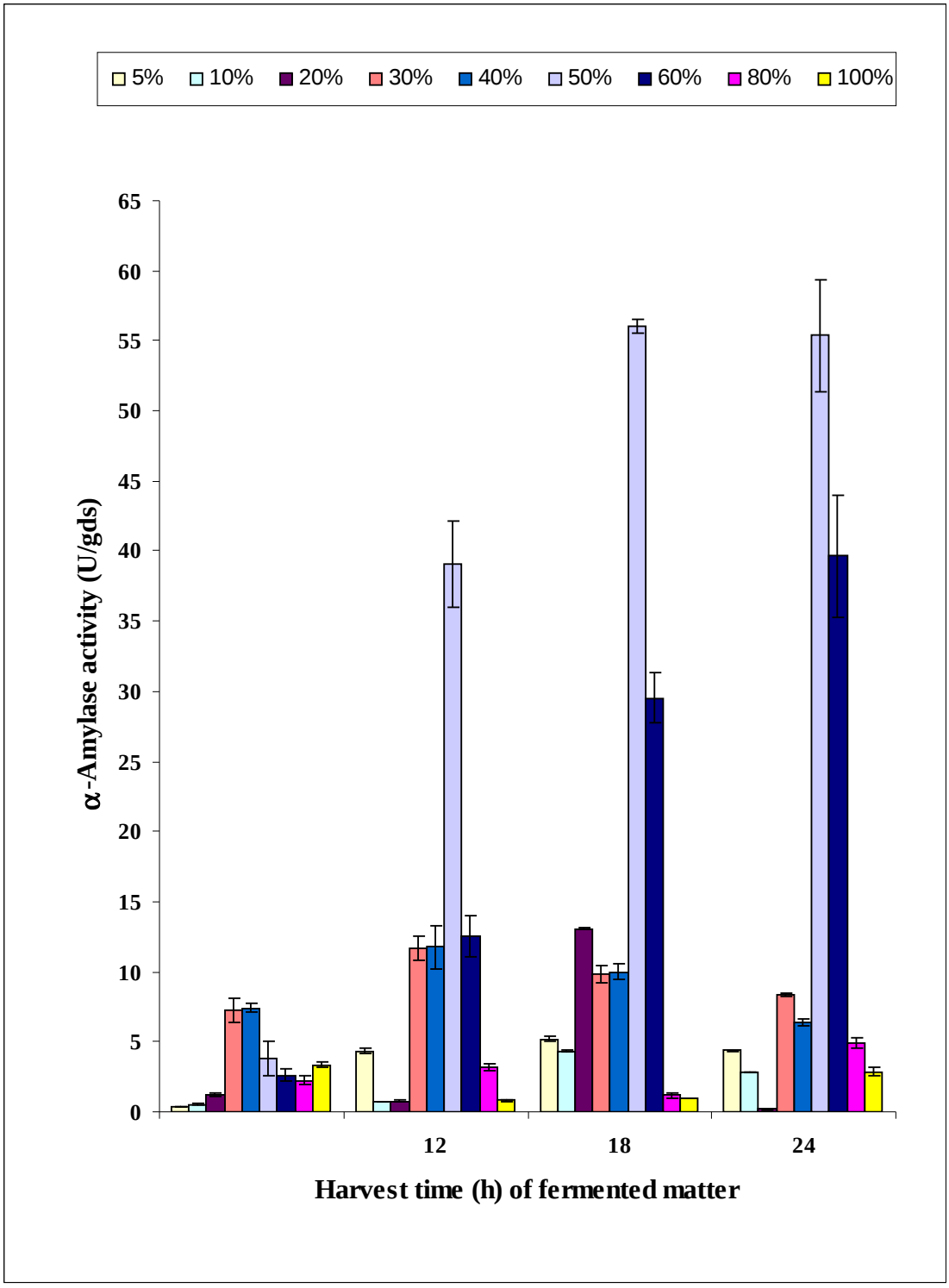

Figure 11: α-Amylase activity of *Btk* on Bengal gram powder [(BgP), w/v] supplemented LB medium at 37 ºC incubation.

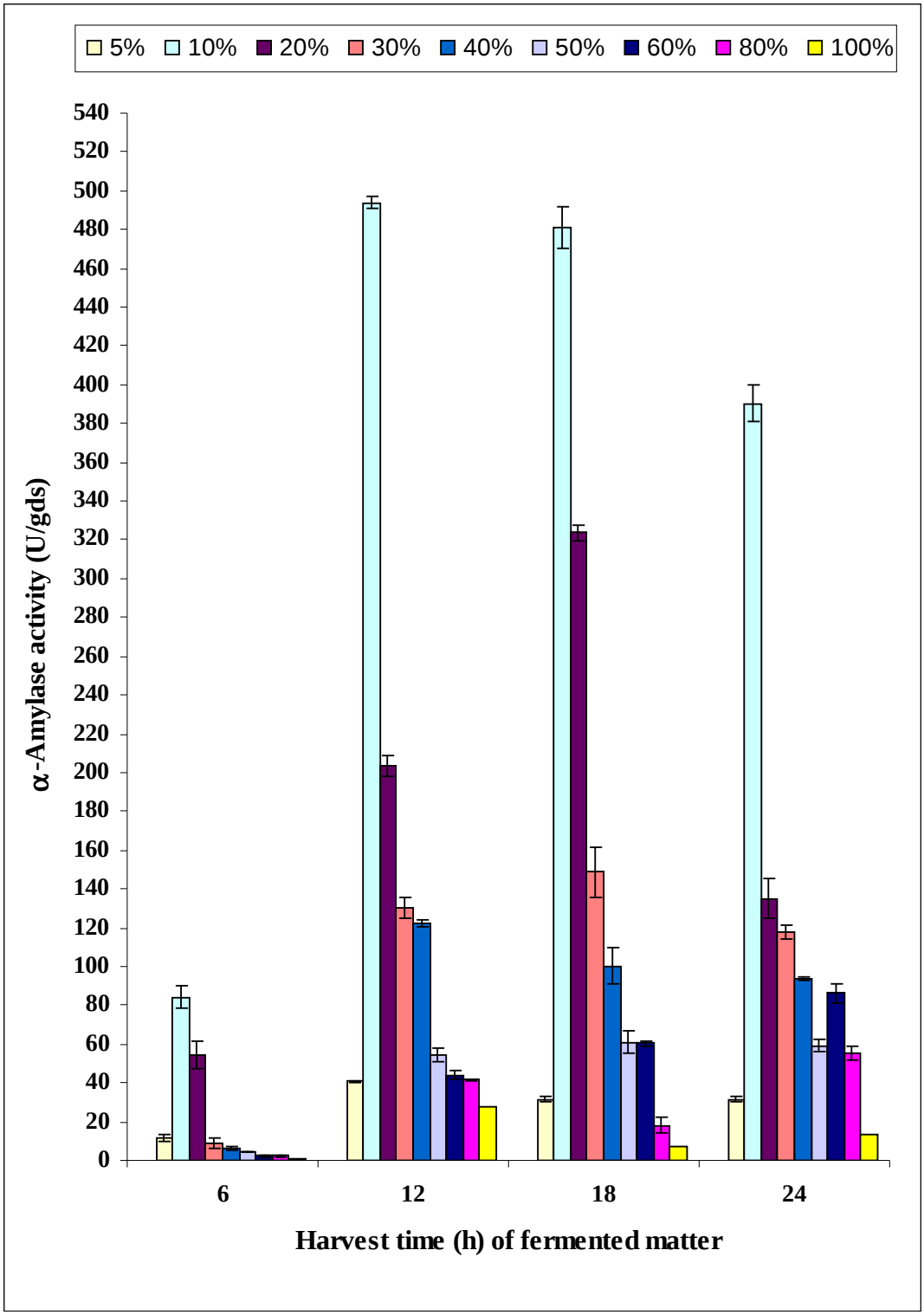

Figure 12: α-Amylase activity of *Btk* on jack seed powder [(JP), w/v] supplemented LB medium at 37 ºC incubation.

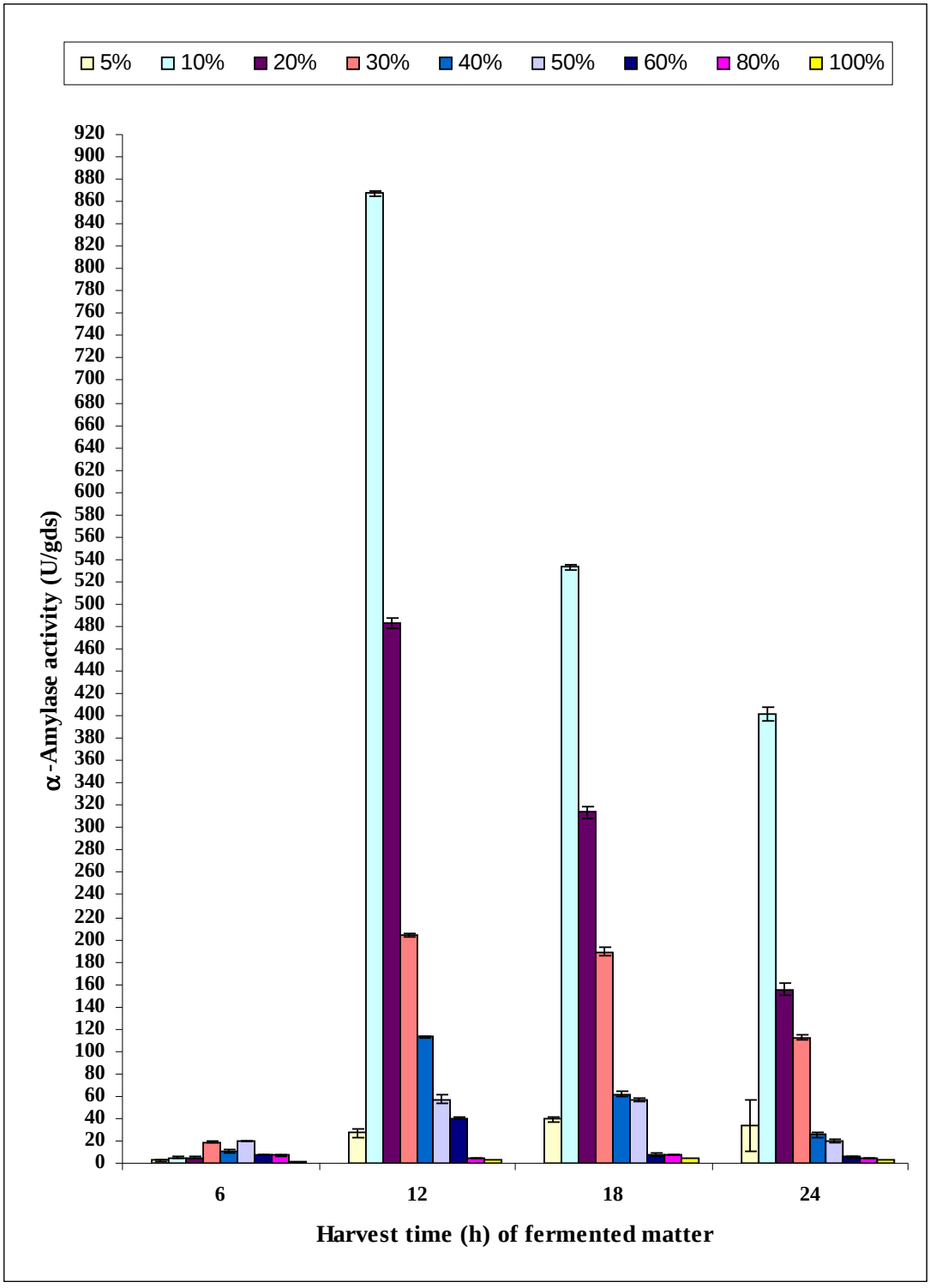

Figure 13: α-Amylase activity of *Btk* on potato powder [(PP), w/v] supplemented LB medium at 37 ºC incubation.

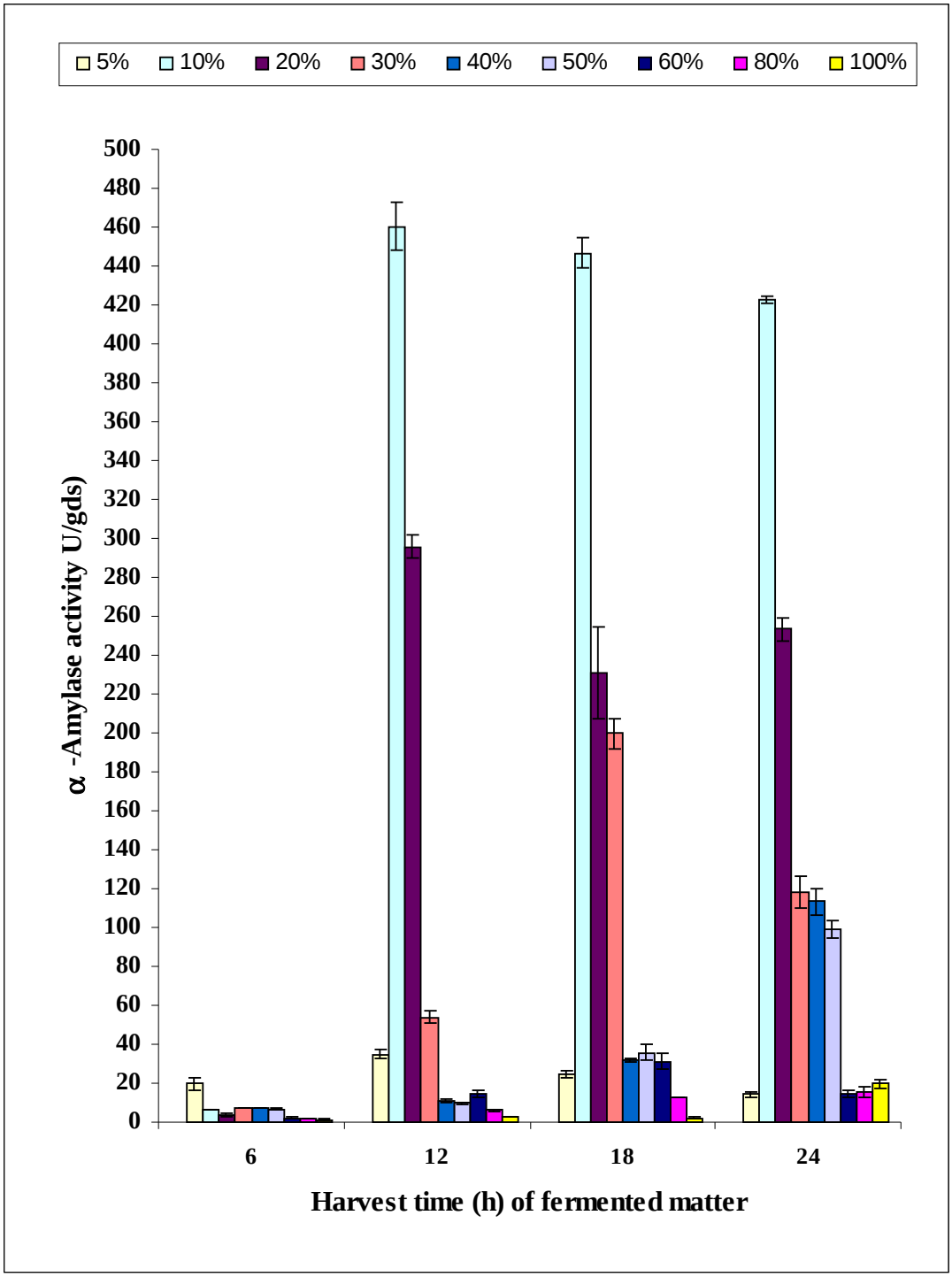

Figure 14: α-Amylase activity of *Btk* on tapioca powder [(TP), w/v] supplemented LB medium at 37 ºC incubation.

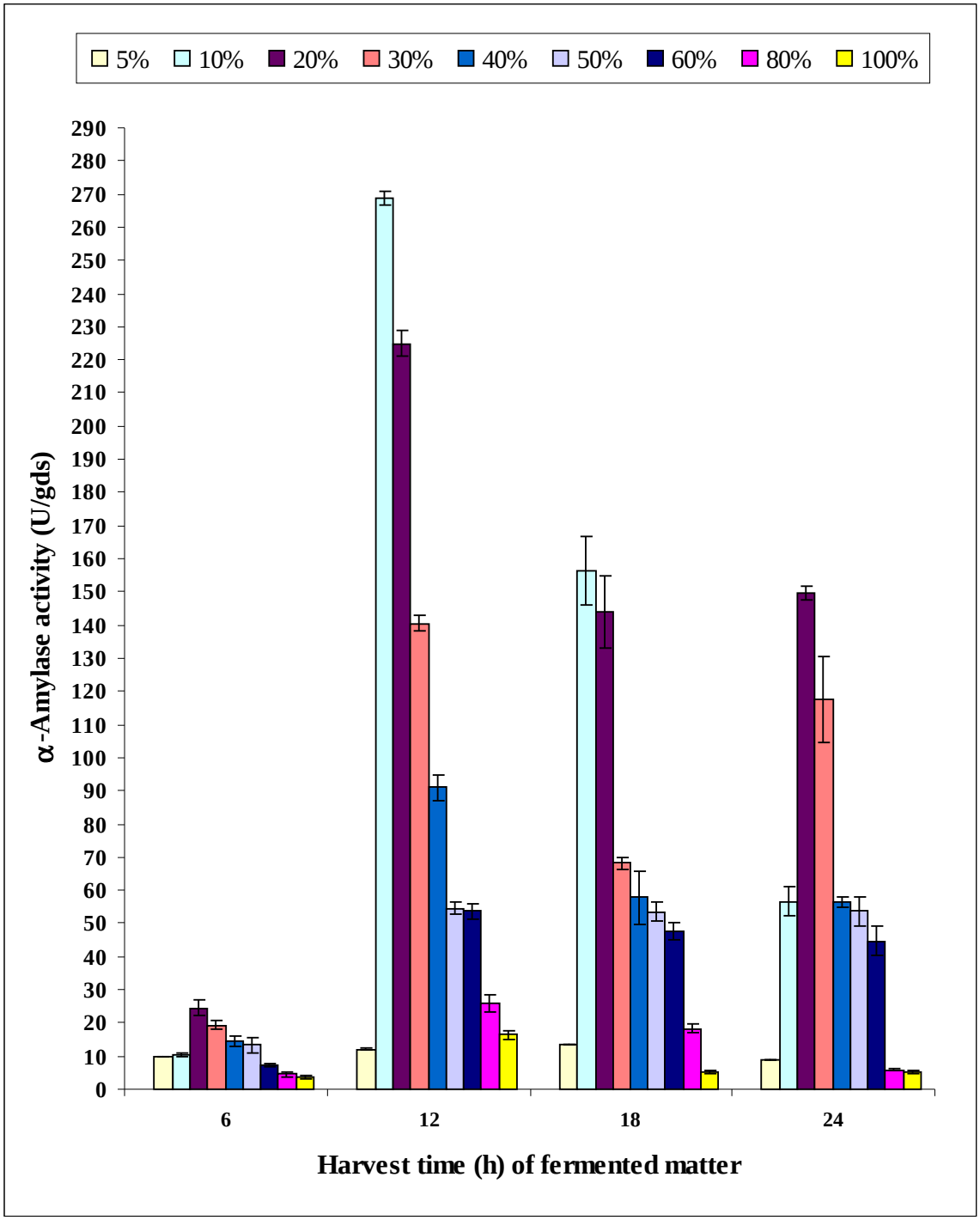

Figure 15: α-Amylase activity of *Btk* on taro powder [(TaP), w/v] supplemented LB medium at 37 ºC incubation.

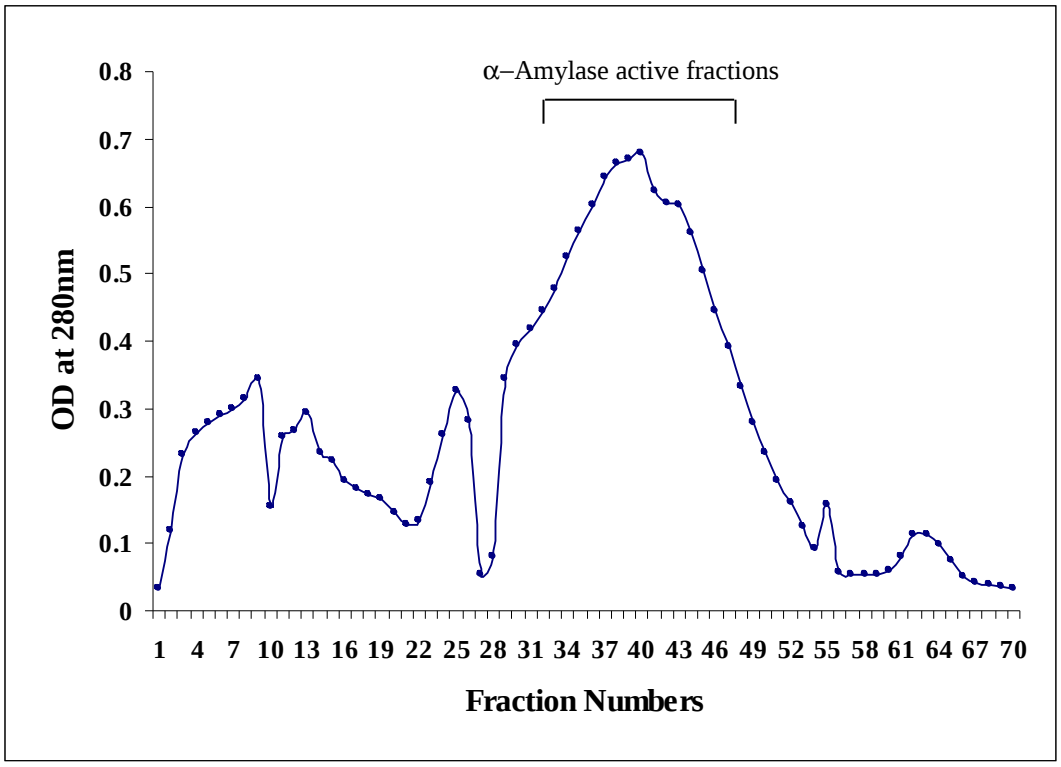

Figure 17: Sephadex G-100 elusion profile of the partially purified (40-60% ammonium sulphate protein fraction) α-amylase obtained by the cultivation of *Btk* on potato powder (10% w/v) supplemented LB medium after 12 h cultivation.

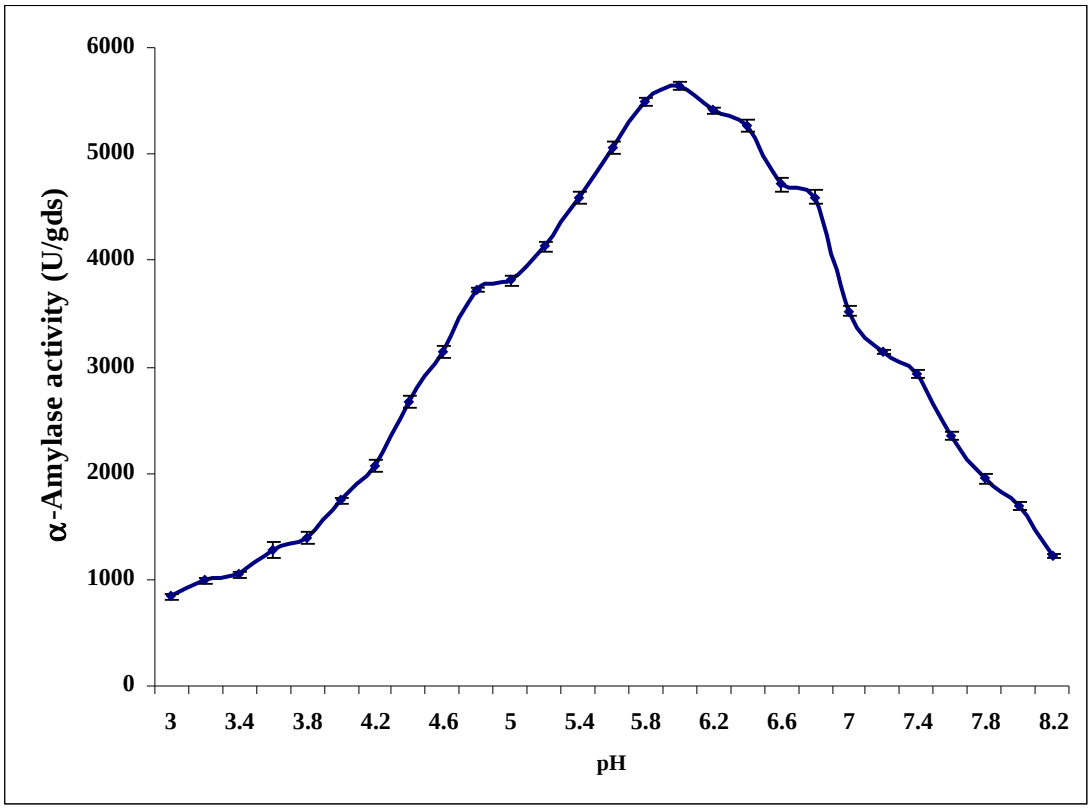

Figure 19: Effect of pH on partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* α-amylase obtained at 12 h fermentation of PP (10% w/v) supplemented LB medium. This activity was at ~32 ^{o}C (room temperature) and varying pH with 5 min incubation using 1% starch.

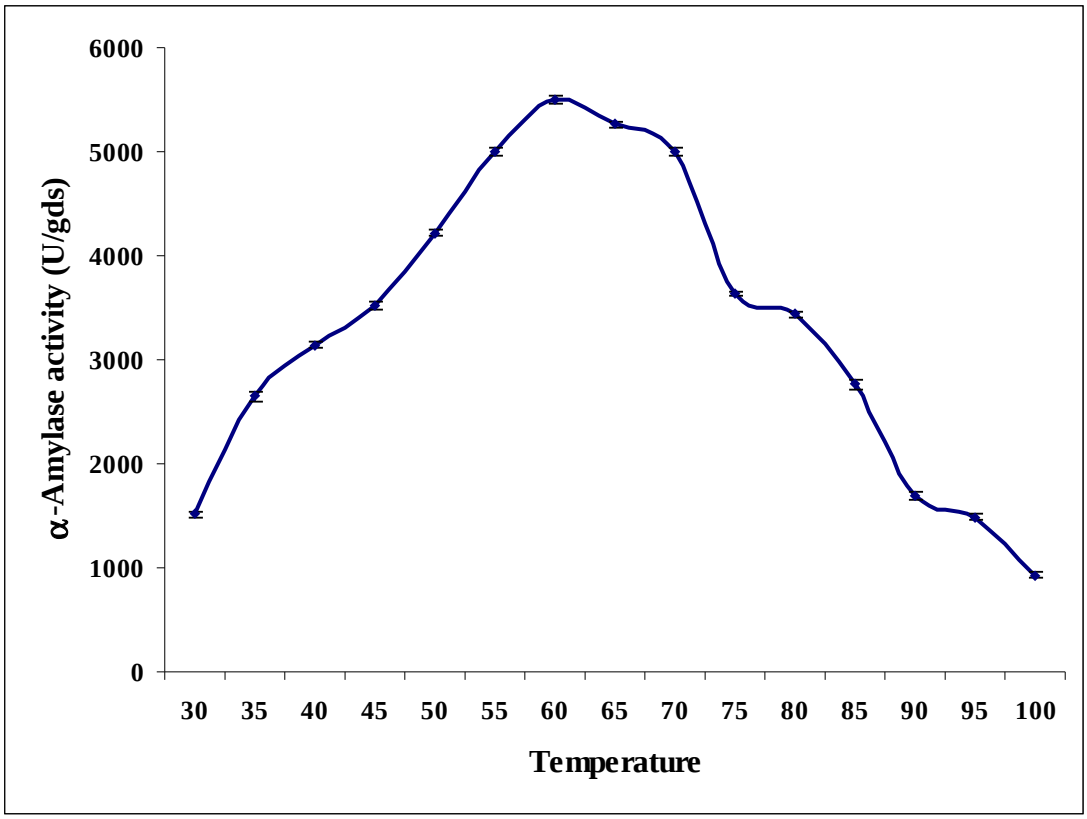

Figure 20: Effect of temperature on partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* α-amylase obtained at 12 h fermentation of PP (10% w/v) supplemented LB medium. This activity was at varying temperature and pH 6.0.

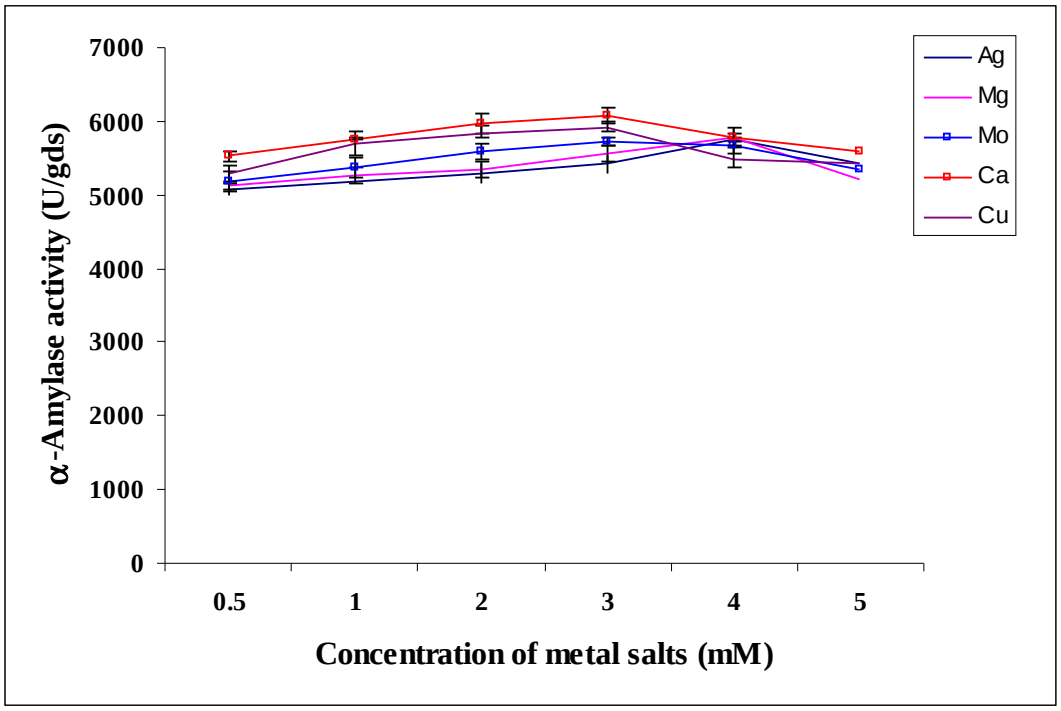

Figure 21: Effect of metal salts (µM) on partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* α-amylase obtained at 12 h fermentation of PP (10% w/v) supplemented LB medium. This activity was at 60 °C and 6.0 pH with 1% starch and 5 min incubation.

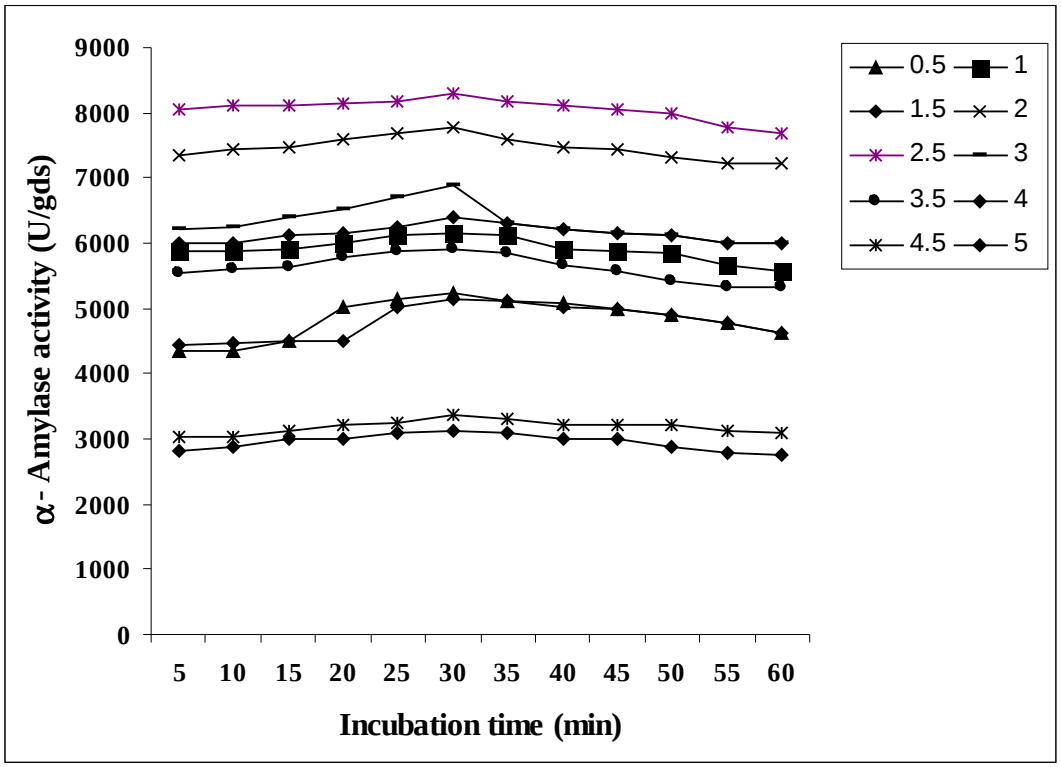

Figure 22: Effect of soluble starch concentration (%) on partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* α-amylase obtained at 12 h fermentation of PP (10% w/v) supplemented LB medium. This activity was at 60 ºC and 6.0 pH and 3 µM Ca^{2+}.

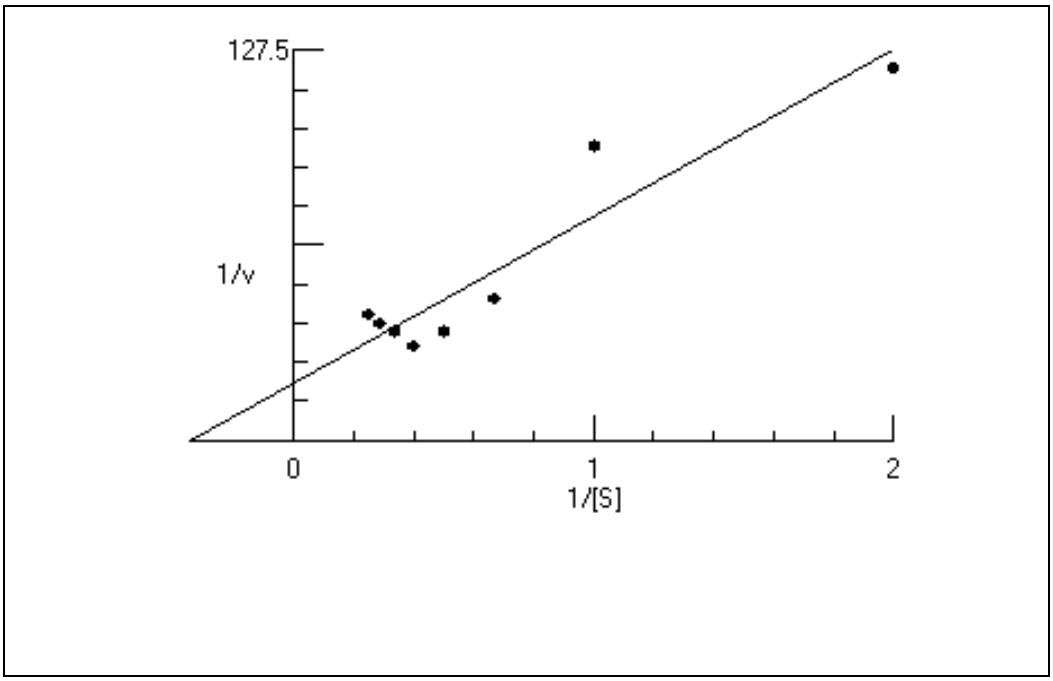

Figure 23: *Km* and *Vmax* of partially purified (40-60% ammonium sulphate fraction of the supernatant) *Btk* α-amylase obtained at 12 h fermentation of PP(10% w/v) supplemented LB medium.

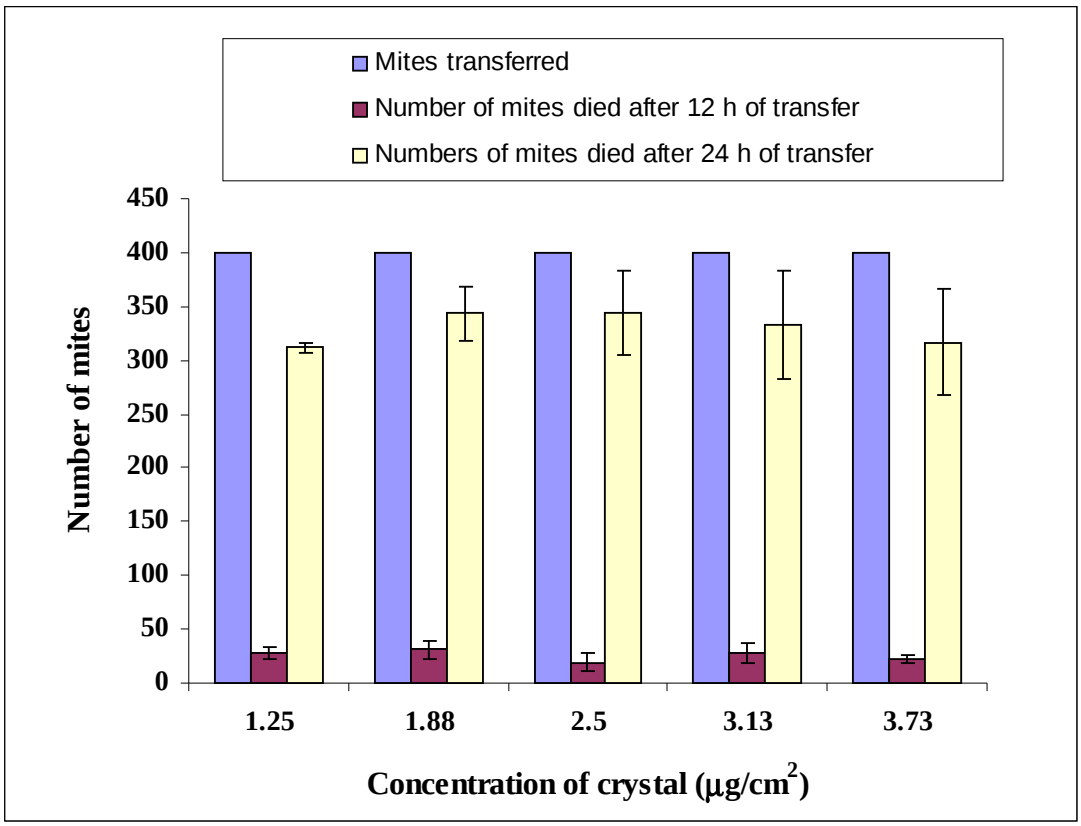

Figure 45: Histogram showing mortality rate of mites by the toxicity assay using dry and powdered raw fermented 10% (w/v) PP supplemented LB medium containing mixture of endospores, Btk crystals and substrate. Treatment was made on mites after 24 h observation of transferred mites in the culture set-up. Moratality rates of the before (0 h brown bars) and after (24 h) treatment are shown here (yellow bars). Some mites were dead (about 6%) after 24 h transfer in the culture set-up (brown bars), ie., before treatment. In the control, over 90% of the mites were alive and active at 48 h.