OPTIMIZATION OF GROWTH CONDITIONS OF *Bacillus thuringiensis* ISOLATES FROM VARIOUS SOURCES IN KENYA AND TOXICITY ASSAYS OF THEIR DELTA-ENDOTOXIN AGAINST *Chilo partellus*

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Optimization of growth conditions of *Bacillus thuringiensis* isolates from various sources in Kenya and toxicity assays of their delta-endotoxin against *Chilo partellus*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my beloved parents, Mr Ernest Muhanji Kwallimwa and Mrs Alice Kwalimwa Khamoyi. I greatly acknowledge the affectionate help of my family, my grandparents, my sister Lucy and my brothers Kizito, Charles, David and John for their encouragement and patience. Thank you for providing me with uncountable favours through your benevolent cooperation.

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LIST OF ABBREVIATIONS AND ACCRONYMS

ATP	Adenosine triphosphate
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* subspecies *israelensis*
C: N	Carbon: nitrogen
CRY	Crystal toxins
CYT	Cytolysins
DNA	Deoxyribonucleaic acid
ICPs	Insecticidal crystal proteins
KDa	Kilo daltons
LC	Lethal concentrations
mRNA	Messenger ribonuclease acid
NYSM	Nutrient yeast synthetic medium
OD	Optical density
OTR	Oxygen transfer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloro acetic acid
WHO	World Health Organization

ABSTRACT

Chemical pesticides have disadvantages such as high production costs, short persistence, comparative low efficacy, development of resistance to toxin and causing ecological damage. In order to obtain cheaper biopesticides, many locally available and inexpensive agricultural/industrial byproducts have potential as culture media for *Bacillus thuringiensis* Crystal protein production. In this study, cost-effective media were developed, based on locally available raw materials namely legumes, potato, and whey. Optimization studies indicate that pH 6.4-7.5 was best for sporulation and OD 600 was highest at 37 °C for all isolates tested. The optical density, protein concentration yield, sporulation and *Chilo partellus* larvicidal action were studied by growing bacterial strains in these waste product and in comparison with the conventional medium (NYSM). Protein concentration yield of 27.60 µg/ ml, spore count of 5.60×10^8 and *Chilo partellus* larvicidal activity (LC_{50}) of 78 µg/ l against first-instar larvae were obtained with a 72 h culture of this bacterium. Based on media comparison between NYSM and other media, the legumes produced the highest spore counts, followed by potato and then whey; and, differences between media treatments were significantly different ($P \leq 0.05$). The SDS-PAGE profiles indicated that spore-crystal product from each treatment consisted of proteins with molecular weights of approximately 110-120 kDa and 60-70 kDa, suggesting the presence of bacterial insecticidal protoxins. Therefore the investigation suggests that legume, potato and whey-based culture media are more economical for the industrial production of *Bt* Insecticidal Crystal Proteins.

CHAPTER ONE

1.0 INTRODUCTION

Insects are the most abundant groups of organisms on earth. They often negatively affect humans in a variety of ways. They cause massive crop damage and act as vectors of both human and animal diseases, such as malaria and yellow fever. Therefore, humans have desired to control insects. As being parallel to development of chemistry, chemical substances have been used to control pests from the mid-1800s (Denolf *et al*., 1993; Yasutake *et al*., 2007). The use of inorganic chemicals and organic arsenic compounds were followed by organochlorine compounds, organophosphates, carbomates, pyrethroids and formamidines (Gill *et al*., 1992). These chemicals were very effective in killing and controlling many species of pests. However, they have many direct and indirect adverse effects on ecosystem including accumulation of toxic residues in nature, leading health problems in mammals and development of insect resistance (Fadel and Sabour, 2002; Sarfraz *et al*., 2005). The problems related with chemical pesticides oriented humans to find out safer and natural alternative ways of pest control.

In developing countries like Kenya, where agriculture is the main income generating activity, the excessive and widespread use of pesticides has attained immense momentum during the recent past. Pesticides are the largest group of possible hazardous chemicals that are introduced purposefully into the environment (Ghribi *et al*., 2007). These toxic pesticides produce residues which persist in the environment, causing pollution and

resistance in many target organisms against these chemicals (Brar *et al*., 2006). The residues persist in the soil and may contaminate ground water and following absorption, get accumulated in plants, fruits and vegetables. Pesticides affect wildlife, biological control agents and above all are dangerous to fish, man and mammals (Bhattacharya, 2000).

Presently, major concerns including insect resistance to chemicals, non-target effects of pesticides and the cost of production of new compounds have renewed interest in alternative forms of pest control, among which disease causing micro-organisms hold out particular promise (Ferre *et al*., 1991; Fadel and Sabour, 2002). Increased concerns about the potential effects of pesticides on health, the reduction in arable land per capita, and the evolution of pest complexes likely to be accelerated by climate changes also contribute to change in plant protection practices (WHO, 1985). Other drawbacks of synthetic insecticides include resurgence and outbreaks of secondary pests and harmful effects on non-target organisms (El-Bendary, 2006).

The widespread use of a single chemical compound confers a selective evolutionary advantage on the progeny of pests, since they acquire resistance to such chemicals. Another problem is that some pesticides affect non-target species with disastrous results (Lambert and Peferoen, 1992). Unexpected elimination of desirable predator insects has caused explosive multiplication of secondary pests. Other concerns include environmental persistence, toxicity of many pesticides and increased cost of developing new and safer

ones (Sayyed *et al*., 2000; Devi *et al.,* 2005). This calls for a demand for alternative control methods, including physical controls (Sayyed *et al*., 2004; Sarfraz *et al*., 2005). The drawbacks of using synthetic pesticides provide strong desire to find alternative approaches, formulations and cost-effective biopesticides production for pest control.

1.1 Pests in Croplands

Human population is estimated to increase to 8.5 billion by the year 2020 (United Nations, 2000). This increased population will cause an increase in the demand for agricultural production. However, the land suitable for agricultural production is limited due to restricted water availability, depletion of land sources and already cultivated highly productive soils. Under these limitations, it is important to develop the yield of agricultural production (Luna *et al*., 2004). It has been estimated that up to 15% of crops worldwide are lost annually due to insect damage only (Devi *et al*., 2005). Therefore, the need to exterminate insects that are destroying crops becomes urgent. Wheat, rice, maize and barley are the primary source for human nutrition worldwide and cover more than 40% of global croplands (Tyagi *et al*., 2002). Most of the pests giving damage to these grains belong to Coleoptera and Lepidoptera orders. In addition, some species of Arachnida, Orthoptera, Hymenoptera, Diptera and Psocoptera can also cause damage in stored grain products.

1.2 Pesticides

Early pesticides were largely composed of chemical constituents. Certain properties made them useful, such as long residual action and effective toxicity to a wide variety of insects (WHO, 1985). However, their use led to many negative outcomes. The chemical insecticides used today are considered as presumably safer to those used in the past, but there are still some concerns. Long-term exposure to these chemicals can cause cancer, liver damage, immunotoxicity, birth defects and reproductive problems in humans and animals (Bhattacharya, 2000; Zouari *et al*., 2002). Also, they can cause accumulation and persistence of toxic residues in soil, water and food; toxicity against beneficial insects and development of pest resistance (Luna *et al*., 2004). Nevertheless, chemical insecticides have a large market volume, and global sales of them are about $5 billion a year (Lambert and Peferoen, 1992).

1.3 Biopesticides

By contrast, microbial pesticides are safe for ecosystems. They are non-toxic and non-pathogenic to wildlife and humans. Their toxic action is often specific to a single group or species of insects, so they do not affect the other insect population in treated areas (Adams *et al*., 1999). Because they have no hazardous residues to humans or animals, they can also be applied when crop is almost ready for harvest (Ouoba *et al*., 2008). In spite of these attractive features, microbial pesticides represent about 2% of global insecticide sales.

Bacillus thuringiensis based pesticides account major share of the bioinsecticide market with 80-90% (Lambert and Peferoen, 1992).

For several reasons, the use of biopesticides as insecticide has grown slowly when compared to chemical ones. Microbial pesticides are generally more expensive to produce than many chemicals. Large quantities of toxins have to be applied to the field to ensure that each larva will ingest a lethal dose. However, the cost can be decreased by increasing demands. Many chemical pesticides have broad spectrum of toxicity, so pesticide users may consider microbial pesticides with a narrower range to be less convenient (Chang *et al.*, 2008). The use of biological pest control agents has been considered to be much safer than chemical ones for the ecosystem. Moreover, the future prospects of them seem to be positive. It is estimated that, the growth rate of usage of biopesticides use over the next 10 years up to 2015 will be 10-15% compared with 2% for chemical pesticides (Crickmore, 2005). Also, the cost of development of *Bacillus thuringiensis* insecticides is predicted to be $3-5 million, compared with $50-80 million for chemical insecticides (Krattiger, 1997). In addition, the use of chemical insecticides seems likely to decline in the future; restrictions for their registration will increase resulting in a smaller chemical pesticide market (Zouari *et al.*, 2002).

In nature, some microorganisms have the potential to produce some biological agents capable of infecting other living organisms including insects. Many of these infectious

agents have a narrow host range and, are not toxic to beneficial insects or vertebrates (Schnepf *et al*., 1998). Therefore, the use of these non-pathogenic microorganisms has been developed as the biological way of pest control. Insect viruses (baculoviruses), some fungi, protozoa and bacteria have been used as biological pest control agents. Among all, *Bacillus thuringiensis* is the most important microorganism with entamopathogenic activity against certain insect orders. It is ubiquitous, gram-positive and spore-forming bacterium which produces insecticidal crystal proteins during sporulation. The toxic activity due to proteins produced by plasmid encoded *Cry* genes varies with insect type. The native strains of this bacterium have been used nearly for 50 years safely, as an alternative to chemical pesticides (Aronson *et al*, 1986). *Bacillus thuringiensis* formulations account for 80-90% of world biopesticides market (Krattiger, 1997). By contrast, it represents only 2% of the total global pesticide market with $90 million worldwide sales (Lambert and Peferoen 1992).

Like all living things, insects are infected by bacteria, fungi, protozoa and viruses. The discovery of the larvacidal action of some bacterial strains from the genus *Bacillus* caused the introduction of biopesticides (Afkhami, 1993). *Bacillus thuringiensis* has been used for pest control since 1920s and still accounts for over 90% of the biological insecticide market (Adams *et al*., 1999; Crickmore, 2005). *Bacillus sphaericus* was next identified as a new biological insecticidal agent and its products appeared in the market (WHO, 1985). The name *Bacillus sphaericus* was first applied by Neide in 1904 to an aerobic bacterium that formed terminally located, spherical spores. Kellen during routine surveillance of rock

holes in California collected several moribund fourth instar larvae of *Culiseat incidens*. He isolated several bacteria, among which were strains of *Bacillus sphaericus*. Kellen's strain was later designated as strain K. It was the first reported active *Bacillus sphaericus* isolate. The isolation of strain SSII-1 renewed interest in *Bacillus sphaericus*. In early 1975, strain 1593 was identified. It was the first fermentation and population stable strain (WHO, 1985).

Insect viruses (baculoviruses) have achieved some modest commercial success as pest control agents. Still, further genetic manipulations of already defined agents or continuous isolation of new strains may well increase the utility of biological pest management strategies (Krattiger, 1997; Schnepf *et al.,* 1998). The use of *Bacillus thuringiensis* as a biopesticide was discovered in the first decade of this century when larvae of flour moths died suddenly. Research into their deaths led to the discovery that the presence of *Bacillus thuringiensis* was responsible for the death. However, it took 50 years before *Bacillus thuringiensis* became a widely used biopesticides with its registration in the USA in 1961 (Aronson *et al*., 1991). The percentage worldwide is estimated to be less than 1% with over half of the *Bacillus thuringiensis* biopesticides being used in the USA, with a total worldwide market of biopesticides of US$24 million in 1980 (Krattiger, 1997; Lambert and Peferoen, 1992).

Over 90% of the biopesticide sales are one single product type, such as the *Bacillus thuringiensis* based products. For biopesticide applications, the *Bacillus thuringiensis* protein is usually used in a formulation containing the spores and crystalline inclusions that are released upon lysis of *Bacillus thuringiensis* during its growth. The molecular potency of the toxin is 300 times higher than synthetic pyrethroids and the toxin breaks down quickly when exposed to ultraviolet light e.g. from sunlight.

1.4 History of *Bacillus thuringiensis*

The entamopathogenic bacterium *Bacillus thuringiensis* was first isolated by the Japanese scientist S. Ishiwata, in 1901, from silkworm larvae (*Bombyx mori*) exhibiting the sotto disease and named as Bacillus sotto. In 1911, Berliner formally described the species from a diseased Mediterranean flour moth larvae (*Anagasta kuehniella*) collected in a German town, Thuringia which gave the name to the species (Lambert and Peferoen, 1992). *Bacillus thuringiensis* first became available as a commercial insecticide, against flour moth, in France in 1938 (Schnepf *et al.*, 1998). In 1956, the main insecticidal activity of *Bacillus thuringiensis* against Lepidopteran insects was found to be due to parasporal crystals by the researchers Hanay and Fitz-James Angus. This discovery increased the interest of other researchers in crystal structure, biochemistry and action mechanism of toxins. In 1950s, *Bacillus thuringiensis* was developed for commercial use in the US. By 1961, *Bacillus thuringiensis* was registered as biopesticide by the United States Enviromental Protection Agency (EPA). Up to 1976, *Bacillus thuringiensis* was available

only for control of Lepidoptera (butterflies and moths), with a highly potent strain *Bacillus thuringiensis* subsp. *kurstaki* (Lambert and peferoen, 1992). This strain still forms the basis of many *Bacillus thuringiensis* formulations.

In 1976, with the discovery of *Bacillus thuringiensis* subsp. *israelensis* by Margalit and Tahori in Israel, disease causing dipteran insect pests such as mosquitoes and blackflies have been controlled (WHO, 1985). In 1980s, developments in biotechnology stimulated researchers to screen large number of natural *Bacillus thuringiensis* isolates in order to find different strains toxic against other insect orders. In 1983, *Bacillus thuringiensis* subsp. *tenebrionis* was described effective against the larvae of coleopteran insects (Lambert and peferoen, 1992). *Bacillus thuringiensis* subsp. *aizawai* active against both Lepidoptera and Diptera orders and *Bacillus thuringiensis* subsp. *sandiego* active against beetles were later introduced into markets (Schnepf *et al*., 1998).

At the end of the 1980s, the first report came on the insertion of genes encoding *Bacillus thuringiensis* toxic proteins into plants. The first transgenic plants expressing *Bacillus thuringiensis* toxins were tobacco and tomato (Wakisaka, *et al*., 1982). *Bacillus thuringiensis* field cotton was the first plant pesticide registered by United States EPA (USEPA, 1999). Rapidly developing recombinant DNA technology after 1990 became an important tool to develop genetically manipulated Bt pesticides. Today, major Bt transgenic crops include corn, cotton, potatoes and rice. They have been commercialized

and are in use widely in Canada, Japan, Mexico, Argentina, Australia and United States. Both *Bacillus thuringiensis* in the form of transgenic crop and spray formulations are still being widely used (USEPA, 1999). Furthermore, many research centres focus on the collection of native strains from different environments to find novel strains with high toxic potential to wider insect spectra.

1.5 *Bacillus thuringiensis* Formulations

Commercially available *Bacillus thuringiensis* formulations contain both spore and toxic crystal protein (Delta-endotoxin). In the production, spores and crystals obtained from fermentation are mixed with the additives including wetting agents, stickers, sunscreens and synergists (Brar *et al*., 2007). It is expected that UV inactivation of the crystal toxin is the major cause for the rapid loss of *Bacillus thuringiensis* activity. Several approaches, such as the use of some chromophores to shield *Bacillus thuringiensis* formulations against sunlight and enhancing the melanin-producing mutants of the organism, increase UV resistance and insecticidal activity. Besides, encapsulation of *Bacillus thuringiensis* in biopolymers reduces washing of the product from the plant by rain (Depieri and Ludow, 1992).

In the development of new formulations and optimization of the utilization of biopesticides, knowledge of insect feeding behaviour is a fundamental requirement (Aronson *et al*., 1986). Some formulations are used to stimulate feeding, such as the use of a

phagostimulant mixture or a yeast extract in a dustable granular form have been proposed to increase residual toxic activity and to attract to the feed selectively on the *Bacillus thuringiensis* product than the feed on the plant (Bravo *et al*., 2007). These approaches can help to increase the effectiveness of the new *Bacillus thuringiensis* formulations.

In agricultural use, *Bacillus thuringiensis* formulations are mostly applied with ground sprayers. Since high volumes of aqueous spray per unit area are needed for adequate coverage of the plant, ground spraying may not be practical in some cases. In recent years, air spraying with the use of a helicopter have reduced spray volume and made more effective and better controlling of the droplets (Tyagi *et al*., 2002). Also the use of air has increased spray penetration, plant coverage and reduces drifting to unintended areas. Low persistence of the spore-crystal product on the plant is an important problem in *Bacillus thuringiensis* applications (Aronson *et al*., 1986). When the products of *Bacillus thuringiensis* were applied to cotton and potato, persistence was observed as 48 hours (Luna *et al*., 2004). Therefore, timing is the major factor for determining the effectiveness of *Bacillus thuringiensis* applications. Application early in the season, according to monitoring egg hatching and after sunset instead of in the morning can increase the persistence of Bt formulations (Bravo *et al*., 2007). Laboratory and field assays have showed that younger larvae are more susceptible to *Bacillus thuringiensis* formulations than older ones (Broderick *et al*., 2006). Therefore, larval age is an important aspect in *Bacillus thuringiensis* applications.

The primary advantage of *Bacillus thuringiensis* products is their safety resulting from their selectivity which is affected by several factors. The delta-endotoxins are activated by alkaline solutions and different varieties may require different pH values (Dregval *et al.*, 2002). Also, crystals need to be broken down to toxic elements by certain enzymes that should be present in the insect's gut. In addition, certain cell characteristics in the insect gut encourage binding of the endotoxin and leading to pore formation (Gill *et.al.*, 1992). Therefore, each strain is capable of producing toxic proteins effective on one or few specific groups of insect. Non-target species such as beneficial insects and wildlife are not affected by these toxins. According to oral mammalian toxicology and *in vitro* digestibility studies which are demanded by the Environmental Protection Agency (EPA), *Cry* proteins (Cry1Ab, Cry1Ac, Cry3A) have not shown toxicity to mammals and they are rapidly degraded in simulated gastric fluid (USEPA, 1999). Additionally, *Bacillus thuringiensis* toxins are biodegradable and do not persist in the environment.

1.6 Concepts of local production

The term local production of microbial insecticides refers to the development of production (fermentation and formulation) facilities in developing countries, in contrast to the existing production facilities in more industrialized nations. In the conventional *Bacillus thuringiensis* production process, the cost of raw materials varied between 30-40% of the total cost depending on the plant production capacity. Therefore, local production of this insecticide in developing countries should depend on the use of production media made of

cheap, locally available sources including agro-industrial by-products. For large scale production of *Bacillus thuringiensis*, different approaches have been investigated to construct media that could support good production of spores and toxins at reasonable costs.

There are number of advantages in promoting development of local production facilities for larvacides. The most important advantage of local production concerns stability. One of the disadvantages of using microbial agents to control pests has been instability and the variation of the toxicity of the formulations. This lack of stability was most likely the result of the lengthy shipping periods and long and variable storage temperatures before the product reached the consumer. To avoid these instability and variability problems with microbial larvacidal agents, local production should be encouraged. The second advantage of local production concerns appropriate formulations. The development of such formulations will depend largely on the result of actual field-trial data due to very different environmental conditions between tropical or dry countries and temperate industrial countries. Thus, there will be no single formulation that will be effective for all field conditions and local production of microbial agents would be beneficial in providing material for conducting appropriate field studies and for developing formulations suitable for local environmental conditions. The last advantage of local production concept is the dependency on the locally available, cheap raw materials.

In summary, local production of biological larvacides would not only allow the development of general fermentation capability in developing countries, but would also provide more stable and more suitable product formulation i.e. with cheaper raw materials, shorter shipment period, shorter shelf-life requirement, and better quality of low-cost flowable formulations. Nonetheless, it appears that to achieve the aim of local production capability, the final price of the product has to be competitive with the commercially available products.

Various agricultural and industrial by-products used as raw material in *Bacillus thuringiensis* production are citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor (Obeta and Okafor, 1984; Ouoba *et al*., 2008). Recently, other wastes such as sludge and broiler poultry litter were utilized for biopesticides production (Chang *et al*., 2008). In general, two methods of fermentation are used for production of microbial products, submerged fermentation and solid state fermentation. In this study, we attempted to optimize lab-scale production of entomopathogenic crystal toxins of sixteen different biolarvacidal strains prior to stage of local industrial production.

1.7 Problem Statement

Chemical pesticides have certain disadvantages such as high production costs, short persistence, comparative low efficacy and development of resistance to toxin. The ecological damage occasionally caused by the lack of specificity in the toxic effects of insecticides has provided the impetus to seek alternative methods of insect control. This observation led to the development of bioinsecticides based on the insecticidal action of *Bacillus thuringiensis* (*Bt*). The discovery of biolarvicidal actions of *Bacillus thuringiensis* and *Bacillus sphaericus* opened a new perspective for insect control. In order to obtain these cheap biopesticides, many locally available substrates which are inexpensive agricultural and industrial byproducts would conduct to discover a culture medium for *Bacillus thuringiensis* cultivation that has a low price, locally valuable and most importantly, has satisfactory potential for Crystal protein production.

1.8 Justification

The use of isolated *Bacillus thuringiensis* strains in Kenya for pest protection will be a milestone in the reduction of chemical pesticides and hence reduce negative impacts of chemical pesticides in the environment. Discovery of cheap raw materials for production of biopesticides will reduce the costs making it affordable to the locals who depend on agriculture as their principle mode of income. The goal then becomes how to find alternative approaches for pest control by developing a cost-effective medium from locally available raw materials in Kenya e.g., soybean, groundnut seed meal extracts, gruel and

fish meal media, for large scale toxin production by *Bacillus thuringiensis* isolates exhibiting various insecticidal activities. There is also need to search for other toxic *Bacillus* species that are less likely to induce the development of resistance when used as bioinsecticides.

1.9 Objectives

1.9.1 General Objective

To characterize local isolates of *Bacillus thuringiensis* from various sources and test their efficacy of their toxins against *Chilo partellus*.

1.9.2 Specific Objective

1. To optimize conditions for low cost production of *Bacillus thuringiensis* toxin from *Bacillus thuringiensis* isolates.
2. To extract and characterize the delta toxin from the *Bacillus thuringiensis* isolates.
3. To carry out *in vivo* studies on the sensitivity of first-instar *Chilo partellus* to the *Bacillus thuringiensis* toxin from the new isolates.

1.10 Hypotheses

1. Growth, sporulation and protein production by local *Bacillus thuringiensis* isolates is not influenced by the carbon-source.
2. Local isolates differ in their delta-endotoxin production and toxicity to *Chilo partellus larvae.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General characteristics of *Bacillus thuringiensis*

The genus *Bacillus* comprises the Gram-positive rods that grow aerobically and form heat resistant spores (El-Bendary, 2006). The vegetative bacilli are large, straight and often grow in filamentous chains. These organisms are among the most difficult to classify because of their physiological characters and morphology (Manzano *et al*., 2009). In spite of these factors, different species of *Bacillus* have been studied and special recognition is given to *Bacillus thuringiensis* and its various strains (Agaisse and Lereclus, 1995).

Bacillus thuringiensis is a widely studied bacterium among microbial biological agents known for their use in pest management (Ferre *et al*., 1991; Adams *et al*., 1999). Nowadays, it is the most attractive commercial biopesticide having worldwide application (Broderick, 2006). In comparison to chemical pesticides, *Bacillus thuringiensis* is advantageous because of being biologically degradable, selectively active on pests and less likely to cause resistance (Alvarez *et al*., 2009).

The name *Bacillus thuringiensis* was first coined by the German biologist E. Berliner to describe pathogenic bacteria located in the pupae of the Mediterranean flour moth (*Ephestia kuehniella)* and other unspecified insect larvae living in stored grains in the city of Thuringen (formerly East Germany). This bacterium had previously been recognized by

the Japanese biologist Ishiwata (1901) as the causal agent of the flasherie, sotto disease, of silkworm (*Bombyx mori*) (Lambert and Peferoen, 1992).

Bacillus thuringiensis strains have been isolated worldwide from many habitats, including soil insects, stored-product dust, deciduous and coniferous leaves (Ohba *et al*., 2002; Yasutake *et al*., 2007). Isolation usually involves heat treatment to select for spores, sometimes with an acetate enrichment step or antibiotics selection (Hofte and Whiteley, 1989). The diversity in the flagellar H-antigen agglutination reactions is one indication of the enormous diversity among *Bacillus thuringiensis* isolates (Yamamoto, 1983; Knowles *et al*., 1986).

2.2 Sporulation

Bacillus thuringiensis has a two-phase growth cycle. During the vegetative cell cycle, the bacterium normally multiplies by cell division but when the nutrients are depleted or when the environment becomes adverse, it forms spores within the sporangium (Lambert and Peferoen, 1992). These spores are highly resistant to adverse conditions such as heat and drought, enabling the bacterium to survive in the periods of stress. Spores germinate and may restart a vegetative cell cycle under favourable conditions (Crickmore *et al*., 2005). Persistence of *Bacillus thuringiensis* spores in the laboratory, greenhouse, and field or forest environment has been well studied (Crickmore *et al*., 1995). *Bacillus thuringiensis*

spores can survive for several years after spray applications, although rapid declines in toxicity and populations have occurred (Kim *et al*., 1998).

Bacillus thuringiensis synthesizes an insecticidal cytoplasmic protein (ICP) inclusion during the stationary phase of its growth cycle (Schnepf *et al*., 1998). These crystalline inclusions comprise of relatively high quantities of one or more glycoproteins known as delta-endotoxins. The insecticidal activity of *Bacillus thuringiensis* has been attributed to these parasporal crystals (Bravo *et al*., 2007). These proteins are toxic to larvae of different orders of insects, including disease vectors and many agricultural pest insects (Devi *et al*., 2005).

These pesticidal delta-endotoxins are produced during sporulation and are accumulated both as an inclusion and as part of the spore coat (El-Bendary, 2006). Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores (Denolf *et al*., 1993). Once ingested, crystals are solubilised in the alkaline and reducing environment of the mid gut lumen and are activated by host proteases (Brar *et al*., 2007). On the other hand, the involvement of *Bacillus thuringiensis* proteases in processing inactive protoxins has also been reported (Brar *et al*., 2007).

2.3 Insecticidal Crystal Proteins (ICPs)

In 1989, Hofte and Whiteley, reviewed the known *Cry* genes and proposed a systematic classification. They distinguished four major classes of δ-endotoxin (Cry 1, -2, -3 and 4) and Cytolysins (*Cyt*) found in the crystals of the mosquitocidal strains, on the basis of their insecticidal activity and molecular properties (Table 1).

Table 1: The cryotoxin groups and the orders they are pathogenic to.

Spectrum and mode of action	**Delta-endotoxin**
Lepidopteran larvicidal	Cry1, Cry9, Cry15
Coleopteran larvicidal	Cry3, Cry7, Cry8, Cry14, Cry34, Cry35, Cry36, Cry38
Dipteran larvicidal	Cry4, Cry10, Cry11, Cry16 (Cry17), Cry19, Cry20
Lepidopteran and Dipteran larvicidal	Cry2
Nematicidal	Cry5, Cry6, Cry12, Cry13, Cry21
Active on Hymenopteran	Cry22

Source: Schnepf *et al*. (1998).

The δ-endotoxins belonging to each of these classes were grouped in class and subclass (A, B, C... and a, b, c...) respectively. Generally these proteins are active against insect and invertebrate groups: Cry1 toxic to lepidopterans, Cry2 toxic to lepidopterans and dipterans, Cry3 toxic to coleopterans, Cry4 and Cry6 toxic to nematodes.

Many of *Cry* genes have been cloned, sequenced and classified as *Cry* and *Cyt* genes (Rajamohan *et al*., 1996; Tounsi *et al*., 2006). Phylogenetic analysis of *Bacillus thuringiensis* based on PCR amplified fragment polymorphisms of flagellin genes also provides a fast, convenient and accurate way to classify all subspecies that cannot be classified using the H-antigen method (Hadjinicolaou *et al*., 2009; Kwon *et al*., 2009).

2.4 Mode of action

The mechanism of action of the *Bacillus thuringiensis* crystal proteins involves a series of events including solubilisation of the crystal in the insect mid gut, proteolytic processing of the protoxin by mid gut proteases, binding of the Cryotoxin to mid gut receptors, insertion of the toxin into the apical membrane to create ion channels or pores and cell lysis, ultimately leading to insect death (Younsten and Davidson, 1982; Ihara *et al*., 1993; Keeton *et al*., 1998; Luo *et al*., 1999; Karim and Dean, 2000; Jurat-Fuentes and Adang, 2001).

A single crystal may contain a million protein subunits of protoxin held together by inter-chain disulphide bonds, and thus cleavage of those disulphide bonds is a critical step in

crystal solubilisation (Couche *et al*., 1987). Upon ingestion by the susceptible target, the protoxins are solubilised and proteolytically processed to release the toxic fragment (Rajalakshmi and Shethna, 1980; Luo and Adang, 1994; Yamagiwa *et al*., 1999).

For most lepidopterans, protoxin are solubilised under alkali conditions of the insect mid gut (Schnepf *et al*., 1998). During proteolytic activation, peptides are removed from both amino- and carboxyl-terminal ends of the protoxin. For Cry1(Ac) toxin (133.33 kDa), proteolytic activation removed half of the molecules from the carboxyl-terminal, resulting in an active toxin fragment of 68 kDa located on the N-terminal (Knowles *et al*., 1986; Hofte and Whiteley, 1989; Schnepf *et al*., 1998; Lightwood *et al*., 2000). Correct activation of delta-endotoxin is likely to be a prerequisite for toxicity and insufficient processing or over digestion of a protoxin may render it inactive (Aronson *et al*., 1986).

The variety of mid gut protease that an insect processes is therefore likely to be a major determinant of toxin potency (Aronson *et al*., 1991). Apart from the exogenous and larval gut protease, the protoxin is also proteolysed by endogenous protease synthesized by *Bacillus thuringiensis* (Bravo *et al*., 2007). After proteolytic activation, the activated-toxins bind to receptors located on the apical brush border of the mid gut microvillae of susceptible insects (Nishiitsutsuji-Uwo and Endo, 1981; Bravo *et al*., 2007). A receptor binding is a key factor of specificity. Specific binding involves two steps, one that is reversible and the other that is irreversible. It has been suggested that the toxicity is

correlated to insertion of the toxin into the membrane and could also reflect a tighter interaction of the toxin with the receptor terminal (Crickmore *et al*., 1995; Keeton *et al*., 1998; Hofte and Whiteley, 1989; Schnepf *et al*., 1998). After binding, the toxin conformation is changed, allowing toxin insertion into the membrane.

Oligomerization of the toxin follows and this oligomer then forms a pore that leads to osmotic cell lysis (Hofte and Whiteley, 1989; Schnepf *et al*., 1998). After the mid gut epithelium of the larva is damaged, the haemolymph and gut contents proceed to mix. This results in favourable conditions for *Bacillus thuringiensis* spores to germinate. The resulting vegetative cells of *Bacillus thuringiensis* and the pre-existing microorganisms in the gut proliferate in the haemocoel causing septicemia, and may thus contribute to the mortality of the insect larva (Aronson *et al*., 1991).

2.5 The factors effecting sporulation, crystal formation and insecticidal toxicity of *Bacillus thuringiensis*

2.5.1 Medium composition

To develop a *Bacillus thuringiensis* formulation, it is important to cultivate the bacterial strain to obtain the highest yields of spore-crystal complexes. In general, the nutritional and cultural requirements vary with the *Bacillus thuringiensis* strain. The parameters optimized for one strain may never work well for another strain. The culture conditions for *Bacillus thuringiensis* must be optimized to give high cell yield, high crystal protein concentration

and high toxicity. Previously, it was believed to be important to end up with high spore counts, but it was later shown that high spore counts do not always mean high toxicity (El-Bendary, 2006).

The Bergey's manual of systematic bacteriology states that the fermentation of the bacteria is promoted by the substrate of glycolysis, and inhibited by increase in the metal concentration of the medium. Sporulation can be stimulated either by the depletion of the nutrient in the medium, or by increase of Ca^{2+}, Mg^{2+}, K^{+}, Mn^{2+}, Fe^{3+} and Zn^{2+} concentrations.

2.5.2 Energy and carbon sources

For the fermentation of *Bacillus thuringiensis*, carbohydrates are very important. However, when used at high concentrations, they can cause adverse effects because *Bacillus thuringiensis* produces acids from carbohydrates. The utilization of carbohydrates to produce acidic forms can decrease the pH to below 5.5 – 5.7 ranges at which most *Bacillus thuringiensis* strains cannot grow, thus the fermentation stops. To prevent such conditions, it is important to maintain a balance between the carbon and nitrogen ratio. The pH is controlled by adding alkali when required during the process. As to the carbon sources, sucrose, lactose, whey, glycerol, dextrin, maltose or inulin were found to be stimulatory, while starch, glucose or molasses were suppressive (Icgen *et al*., 2002b).

2.5.3 Nitrogen source

The variation of entomotoxicity could be attributed to the quality of available protein in the medium (Sikdar *et al.*, 1991). The insecticidal protein which makes up 20-30% of total protein of *Bacillus thuringiensis* is synthesized from amino acids derived from the complex nutrients (Sasaki *et al.*, 1998). Thus the use of $(NH_4)_2SO_4$ as a sole N-source is completely inadequate for *Bacillus thuringiensis* growth (Zouari *et al.*, 2002). According to the observation (Sikdar *et al.*, 1991), *Bacillus thuringiensis* requires some amino acids or peptides which are essential for growth.

Rajalakshmi and Shethna (1980) observed that certain concentrations of L-cystine in culture media stimulated exponential growth of *Bacillus thuringiensis*. They stated that cysteine might be important in membrane protein and fatty acids synthesis and might be essential for both toxicity and proteolytic activity. However, the presence of excess cysteine (>0.1 g/ l) in a culture media inhibited sporulation and endotoxin formation. Although cysteine does support growth and crystal synthesis at certain concentrations, in some media, the endotoxin produced at those concentrations may have poor toxicity.

The combination of yeast extract and $(NH_4)_2SO_4$ seems to be the best protein source (Sikdar *et al.*, 1991). More recent investigations have been focussed on industrial by products, to produce high crystal levels in low cost media. For this reason other components than yeast extract are highly desirable such as casein, peptone or casitone.

However, casein (4 g/ l) used as amino acid source negatively affected growth and crystal protein synthesis in *Bacillus thuringiensis* strain BNS3 (Zouari *et al*., 2002). Moreover, they also found that the substitution of yeast extract by either peptone from casein or casamino acids provided a positive effect on crystal production.

2.5.4 C: N ratio

Farrera *et al*. (1998) have shown that C: N ratio balance itself was directly important for the crystal protein production. They have investigated the range of 3:1 to 11:1 C: N ratio for the fermentation of *Bacillus thuringiensis kurstaki* HD-73 and although the spore count was the highest with 4:1 ratio, the crystal protein concentration was highest when C: N ratio was 7:1.

A higher amount of glucose concentration in the medium increases the size and potency of the crystal proteins. However, glucose is also known to repress several operons and genes in Gram-positive bacteria through carbon catabolite repression. In addition, the Cry4A toxin of *Bacillus thuringiensis* subsp. *israelensis* was not synthesized when the medium contained 0.4% glucose. The mRNA levels were very low when glucose was at that concentration when compared to the glucose depleted medium at the same hour of fermentation (Foda *et al*., 1985).

Another important component for the production of crystal protein is potassium. Wakisaka *et al*. (1982) cultivated *Bacillus thuringiensis* in a media containing several potassium salts and detected an increased crystal protein production. However, when potassium salts are replaced with the same salts of sodium, the same effect was not seen. A similar response is also reported by Foda *et al*. (1985).

2.5.5 Minerals

Other important components for the production of crystal proteins are the trace minerals. Sikdar *et al*. (1991) established that Iron, Manganese and Copper are required for the production of crystal protein while Mo had an inhibitory effect.

Sachinandham *et al*. (1997) demonstrated that several amino acids result in better carbon utilization and also improve the stability and volumetric productivity of biomass for *Bacillus thuringiensis* subsp. *galleriae*. Moreover, when tryptophan was supplied to the medium, the formation of spores and crystal proteins improved (Padilla *et al*., 2006).

The regulation of crystal protein biosynthesis has been investigated by Icgen *et al*. (2002a; 2002b). The two former reports focus on crystal protein biosynthesis by an anti-lepidopteran strain of *Bacillus thuringiensis*, isolated locally and designated *Bacillus thuringiensis* 81. The most important metals for the synthesis of crystal proteins were found to be Magnesium and Copper. Magnesium was found to be essential for the synthesis of the

crystal proteins as the level of crystal protein synthesis was almost zero when Magnesium was omitted from the medium.

The stimulatory effect of Copper was seen between the range of 10^{-6} to 10^{-7} M. Calcium and Zinc had no effect on toxin production while Manganese favoured the crystal protein synthesis in a range of 3×10^{-4} to 10^{-5} M (Icgen *et al*., 2002a). Co-regulation of sporulation and crystal protein synthesis by the minerals was not evident. Also, the suppression of crystal protein biosynthesis by inorganic phosphate over a range of 3 to 100 mM, as a general suggestion for secondary metabolism was not seen. The highest toxin concentrations were obtained when sucrose, lactose or inulin was used as carbon sources. Glucose, glycerol, maltose, starch and dextrin on the other hand, yielded low crystal protein concentrations. Of various organic and inorganic nitrogen sources investigated, peptone was found to be the best (Icgen *et al*., 2002b; Ozkan *et al*., 2003).

Ozkan *et al*. (2003) investigated the optimal parameters for the production of anti-dipteran crystal proteins from *Bacillus thuringiensis israelensis* HD500. According to this study, Fe, Zn and Cu negatively influenced the synthesis of the crystal proteins. Mg and Ca favoured the toxin production while Mn was found to be the most critical trace element. For the production of crystal proteins, high concentrations of inorganic phosphate were found to be beneficial. Also media supplemented with antibiotics are highly effective for some *Bacillus*

strains for example MYPGP agar supplemented with vancomycin is useful for quantification of *Bacillus popilliae* spores (Stahly *et al*., 1992).

2.6 Cultural conditions

2.6.1 Aeration

Oxygen plays an important role in fermentation processes and usually overall yields are closely related to its supply. In relation to this, it has been demonstrated that, for several species of the genus *Bacillus*, sporulation is highly related to oxygen supply. According to the (P/O) ration found for *Bacillus thuringiensis*, the main source of ATP production should be respiratory chain with only a minor contribution of substrate level phosphorylation (Ozkan *et al*., 2003).

The respiration rates of *Bacillus thuringiensis* grown in a culture medium containing sugar cane molasses and corn steep liquor increased when oxygen concentration increased from 2.5 to 10 % of the saturated value and decreased thereafter. Aeration rate of 1 vol/vol/min was recommended for *Bacillus thuringiensis* cultivation (Foda *et al*., 1985).

Foda *et al*., (1985) reported the production of crystals and spores of *Bacillus thuringiensis* var. *israelensis* under different aeration conditions. The results with 4 l batch culture showed that for oxygen non-limited cultures, cell yield, endotoxin production and spore count were constant for all oxygen transfer (OTR) ranging from 14.9 to 48.4 mmol O_2 / l/h

and also higher than those obtained in oxygen-limited culture. In addition spore count was not affected by an interruption of oxygen supply after 12 h of cultivation whereas endotoxin concentration reached was not significantly different to those reached in either oxygen-limited culture. Thus oxygen must be continuously supplied if high endotoxin concentrations are to be reached. Moreover, increasing the respiration capacity of *Bacillus thuringiensis* mutant strain with depressed expression of the terminal oxidase aa_3 during sporulation led to a modest increase in Crystal protein production.

2.6.2 Culture medium pH

The pH curve during the cultivation of *Bacillus thuringiensis* on a medium consisting of glucose has a typical pattern with decline in pH caused by the production of acetic acid during log phase of fermentation, followed by increase to pH level close to an initial pH as acetic acid is consumed. The cultivation with initial pH of 5 to 6 did not produce any spore-crystal complex consumed due to cell death at low pH. The culture medium should be brought to neutral pH at harvest because high pH is optional for *Bacillus thuringiensis* proteases and potentially damaging to the crystals (Icgen *et al*., 2002a).

2.6.3 Temperature

The normal temperature for growth and toxin production of *Bt* is 30 °C. Ozkan *et al*., (2003) found that Cry4Ba synthesis by *Bti* HD500 was the best when the organism was grown at 25 °C, whereas Cry11Aa synthesis was optimal at 30 °C. In comparable to these

finding, Depieri and Ludlow (1992) found the maximum sporulation yield as a percentage of viable counts of *Bs* 9602 was <10% at 10 °C and 12 °C, while it was >95% at 15 °C, 20 °C and 30 °C, however, at 40 °C *Bs* grew only vegetatively.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Source of organisms

The Kenya Agricultural Research Institute (KARI) isolates used in this study were from the *Bacillus thuringiensis* isolate germplasm stored in glycerine at the KARI-National Agriculture Research Laboratories, Nairobi. Ten *Bacillus thuringiensis* isolates (number: 30, 47, 21, 54, 20, 46, 12, 14, 53, and 37) were randomly selected from a pool of 68 *Bacillus thuringiensis* isolates and a reference standard *Bacillus thuringiensis* subspecies *kurstaki* HD-73 isolate also included in the assessment. All isolates were cultured on agar plates prepared by methods described manufacturer's instructions (Oxoid, England). The petri dishes (diameter = 8.5cm) with inoculated agar were incubated for seven days at 30 °C for development of the *Bacillus thuringiensis* cultures.

Also used in this study were *Bacillus thuringiensis* strains 24LBN30°C, 1SKAG37°C, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C isolated previously by Ntabo (2008) from soils and termite mounds collected from Kalunya Glade and Lirhanda Hill in Kakamega Forest and also from soil samples from JKUAT in Juja (Table 2). The isolates were preserved in the JKUAT GK laboratory at -80°C. The isolates were revived on sterilized 8.0 g/ l nutrient broth (HiMedia Lab, India) and a gelling agent 15.0 g/ l agar (HiMedia Lab, India) media at pH 7.0 and 25° C. The bacterial isolates were then

maintained on nutrient agar (HiMedia Lab, India) at 20° C for 72 h and stored at 4° C until used.

Table 2: A summary of sample unit collection sites and isolate codes

Location		Isolate	Code
1.	Kalunya parent soil A	1SKAG37°C	SKA
2.	Kalunya gut A	63KAG37°C	KAG
3.	Lirhanda parent soil A	58SLA25°C	SLA
4.	Lirhanda parent soil A	14SLA30°C	SLA
5.	Lirhanda gut B	62LBG37°C	LBG
6.	Lirhanda nest B	24LBN30°C	LBN

3.2 Optimum growth conditions of the isolates

In order to show the effect of temperature on the growth of *Bacillus thuringiensis* isolates, two parameters; optical density of the culture and number of cells, were measured as indicators of bacterial growth. 2 % vol/vol of bacteria culture (a loop full of bacteria inoculated in 50 ml of nutrient broth and 0.03% yeast extract wt/vol) was inoculated in 20 ml basal medium at 150 rpm and temperature was recorded in autoclaved four sets of basal

medium each of three test tubes and incubation done at four different temperatures, that is; 20 °C, 30 °C, 37 °C and 40 °C. After 10 h, bacterial growth was assessed by measuring absorbance at 600_{nm} using a spectrophotometer and determining the cell count with a haemocytometer.

2 % vol/vol of bacteria culture (a loop full of bacteria inoculated in 50 ml of nutrient broth and 0.03% yeast extract wt/vol) was inoculated in 20 ml basal medium at 150 rpm and the temperature was recorded in autoclaved thirteen sets of basal medium, each of three test tubes containing different pH, that is; 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10. After 10 h, bacterial growth was assessed by measuring absorbance at 600_{nm} using a spectrophotometer and determining the cell count with a haemocytometer.

3.3 Morphological characterization

Morphological characterization was done to confirm the identity of the isolates. The isolates were streaked on solidified 28.0 g/ l nutrient agar (HiMedia Lab, India) plates, pH 7.0, and incubated at room temperature for 5 days. Bacterial smears were prepared and colony, cell shapes, endospores and motility was observed and recorded. In order to study the morphology of the isolates, Gram stain was first performed as described by (Dussault, 1955).

3.4 Culture media

The basal medium consisted of 10.0 g/ l cow blood; 0.02 g/ l $MnCl_2.4H_20$ (s. d. fine-chem ltd); 0.05 g/ l $MgSO_4.7H_20$ (Lab Tech Chemicals); and 1.0 g/ l $CaCO_3$ (Sigma-Aldrich, Germany). Freshly collected cow blood was steamed for 30 min, oven dried at 50°C for 2 h, and homogenized in a commercial blender. The homogenate was dried in a stainless-steel tray at 60°C for 20 h and then ground to a fine powder. Nutrient broth (HiMedia Lab, India) supplemented with 0.03% (wt/vol) yeast extract (HiMedia Lab, India) was used for seed culture. The nutrient brioth pH was adjusted to 7.5 with 1 N NaOH (Loba chemie, India).

10.0 g of poultry litter was prepared by sieving in a kitchen sieve and mixed at 30 °C in distilled water for 1 h. The liquid was then decanted and centrifuged (3300 × g for 15 min) and its pH adjusted to 7.5 with 1 N NaOH (Loba chemie, India). These extract solutions were sterilized at 120 °C and 15 psi for 20 min. For each dilution, three replications of 135 ml volume in 500 ml shake flasks were inoculated with 15 µl broth containing cells of *Bacillus thuringiensis* having an optical density at 600_{nm} of 1.0 that is approximately 3.0 $\times 10^8$ CFU/ ml (Adams *et al.*, 1999).

10.0 g of legume seeds was prepared by mixing groundnut cake (*Arachis hypogea*), cow pea (*Vigna unguiculata*, white variety), soya beans (*Glycine soja*), cow pea (*Vigna unguiculata*, black variety), and njugumawe/jugo beans (*Voandzeia subterranean*). The

legume seeds were added as finely ground powders, except the groundnut cake, for which hard-pressed cylinders (1.1 by 4.5 cm) of the cake were ground to fine powder. Fifty grams of the powder was mixed with 500 ml of non-chlorinated tap water, boiled for 40 min, and filtered after cooling. The resulting filtrate was added to the basal medium and made up to 1,000 ml with similar tap water. These extract solution was sterilized at 120 °C and 15 psi for 20 min (Obeta and Okafor, 1984).

10.0 g of Wheat bran extract/cotton seed meal and whey were respectively placed in 250 ml Erlenmeyer flasks. Its pH adjusted to 7.5 with 1 N NaOH (Loba chemie, India) and these flasks were autoclaved at 120 °C and 15 psi for 20 min, cooled, and inoculated with 2.5 ml of seed culture, i.e., 10% (v/v) of *Bacillus thuringiensis* strain, cultured in 50 ml nutrient broth (HiMedia Lab, India) and 0.03% yeast extract (HiMedia Lab, India) for 48 h at 30 °C (Devi *et al*., 2005).

10.0 g of Nutrient Yeast extract Synthetic Medium (NYSM) which was used as reference medium in the present study was prepared by mixing 5.0 g glucose (R. P. Normapur), 5.0 g peptone (HiMedia Lab, India), 3.0 g beef extract (HiMedia Lab, India), 0.5 g yeast extract (HiMedia Lab, India). These flasks were autoclaved at 120 °C and 15 psi for 20 min, cooled, and inoculated with 2.5 ml of seed culture, i.e., 10% (v/v) of *Bacillus thuringiensis* strain, cultured in 50 ml nutrient broth (HiMedia Lab, India) and 0.03% yeast extract (HiMedia Lab, India) for 48 h at 30 °C.

10.0 g of potato broth was obtained by peeling their skin off, cutting them into small pieces and boiling them in tap water for 15-20 min (till the potatoes became soft). After cooling, potatoes were mashed thoroughly by hand and filtered through a muslin cloth. The resulting potato extract was made up to 1 L with tap water. The extract was dispensed into 500 ml Erlenmeyer flasks, its pH adjusted to 7.5 with 1 N NaOH (Loba chemie, India), sterilized at 120 °C and 15 psi for 20 min, cooled, and inoculated with 2.5 µl of seed culture, i.e., 10% (v/v) of *Bacillus thuringiensis* strain, cultured in 50 ml nutrient broth (HiMedia Lab, India) and 0.03% yeast extract (HiMedia Lab, India) for 48 h at 30 °C.

3.5 Growth Curves

An aliquot from overnight culture (500 µl) was used to inoculate 50 ml each of: poultry litter, legumes, wheat bran/cotton, NYSM, and potato broth media in 250 ml Erlenmeyer flasks. The culture was incubated at 30°C by shaking for 72 h at 200 rpm. Inoculation time was considered as time zero. Samples were taken from the culture at 6 h intervals and used for quantitative determination of growth which was measured spectrophotometrically at 600_{nm} (Maniatis *et al.*, 1982).

3.6 Spore-delta endotoxin recovery

The spore-delta-endotoxin complexes in the final whole culture were recovered as bacterial powders at the end of fermentation by the acetone precipitation method. 1 ml cell lysate, 8 ml 100% ice-cold acetone, and 1 ml 100% TCA were mixed in a 1:8:1 ratio and

precipitated at -20 °C for 1 h. The solution was centrifuged at 11, 500 rpm (18000 × g) for 15 min at 4° C in a microfuge. The supernatant was discarded and the protein pellet dissolved in the appropriate volume of 2-D rehydration buffer 1 by repeatedly pipetting up and down to break up the pellet. The sample was allowed to sit at room temperature for 1 h, vortexing every 10 min and transferred to an eppendorf tube and centrifuged at 14, 000 rpm for 10 min at room temperature. The supernatant was immediately transferred into a new eppendorf and stored at -80 °C until used (Wessel and Flugge, 1984).

3.7 Crystal Protein Staining

A bacterial smear was prepared; air dried and heat fixed. It was placed inside a staining tray, covered with blotting paper and the paper saturated with basic fuchsin. The slide was heat fixed and examined under oil immersion objective (Dougall, 1977).

3.8 Total viable cell count and spore count

Total viable cell and spore counts were determined in the final whole culture by the pour plate method. Serial decimal dilutions of the final whole culture were made in sterile 1% peptone water (Oxoid, England) and 0.5 ml of each dilution in triplicate was added to a petri dish, followed by the addition of 10 ml plate count agar (Oxoid, England) at 45 °C. The culture and agar were mixed thoroughly and allowed to set. Plates were incubated at 32 °C for 24-48 h. Plates with 30-300 colonies were counted with a colony counter

(Gallenkamp Ltd.). For spore counts, cultures were pasteurized at 65 °C for 20 min before serial dilutions were made (Hoben and Somasegaran, 1982).

3.9 Protein Extraction

A sample (10 ml) of 48 h bacterial culture was centrifuged at 4 000 rpm for 10 min. The pellet was re-suspended in 500 µl of 1 M NaCl (Sigma-Aldrich Lab, Germany). This mixture was transferred into an Eppendorf tube and centrifuged at 7000 rpm for 7 min. The pellet was re-suspended in 250 µl of TE buffer (Appendix B) and centrifuged at 7000 rpm for 7 min. Then, the pellet was suspended in 250 µl of dH_2O and centrifuged at 7000 rpm for 7 min. After discarding the supernatant, 150 µl of 10 mg/ ml lysozyme solution in TE buffer was added and the suspension was incubated at 37°C for 30 min. 25 µl of 10% SDS (Sigma-Aldrich Lab, Germany) solution was added into the suspension which was then vortexed for 30 sec. It was centrifuged at 6000 rpm for 10 min and 100 µl of 0.2% SDS (Sigma-Aldrich Lab, Germany) solution was added to the pellet.

For denaturation, 60 µl of gel loading buffer (Appendix B) was added to 30 µl of this mixture in another Eppendorf tube. Finally, this sample was incubated at 90°C in a water bath each time lasting 7 mins before cooling on ice and repeating this procedure three times. The final native and denatured samples were stored at -20°C. Protein concentrations in the denatured samples were adjusted to the same level prior to gel electrophoresis.

The protein concentrations of the samples were determined by Bradford method (Bradford, 1976). 10 µl of native sample was made up to 0.5 ml with dH_2O and vortexed after the final volume was brought to 5 ml with Bradford reagent (Appendix B). After 10 min, the mixture was vortexed again and the absorbance was measured at 595 nm. Concentrations of the samples were calculated from the calibration curve which was also prepared at each batch of the experiments. 25 µL samples from the test media final whole bacterial culture was then applied to gel after equalizing protein concentration.

3.10 SDS-Polyacrylamide Gel Electrophoresis

A protein molecular weight marker was from the Protein mixture, (Amersham Biosciences UK). The proteins were separated by using a vertical polyacrylamide gel apparatus. Electrophoresis was run at 20 mA at the beginning but when the samples reached the separating gel, the current was increased to 40 mA and the electrophoresis continued until the samples reached to the end of the gel (Laemmli 1970).

3.11 Bioassay of *Bacillus thuringiensis* isolates against *Chilo partellus*

Powders of *Bacillus thuringiensis* produced from the five local media and control were assayed against laboratory-reared first instar larvae of *Chilo partellus.* A standard primary powder of *Bacillus thuringiensis* subsp. *kurstaki* was included in the assay for comparison. One hundred milligrams of potato: legumes: whey powder mixed in ration 1:1:1 was suspended in 1,000 ml of distilled water containing 1% (vol/vol) Tween 80. Serial dilutions

of this suspension were made in distilled water. Fifteen larvae were added to 150 ml of each dilution in 250 ml white plastic cups. Three cups were used per dilution. Controls consisted of three cups each containing 150 ml of distilled water and 15 larvae for each powder assayed. Larval food was provided by adding a small portion of finely ground oaf flakes (Quaker) mixed with dried yeast powder for *Chilo partellus* larvae. Each experiment was incubated at 20 ±5°C for 48 h, and each assay repeated three times. Observations were made at 6 h for paralysis and knockdown effects. Mortality counts were made at 24 h and 48 h. A larva was presumed dead if it did not move when touched with a blunt needle (Obeta and Okafor, 1984).

3.12 Data analysis

A one-way ANOVA test was used to compare mean maximum spore count among media and pairwise comparison of the media was done using the Duncan's multiple comparison test based on least significance differences. Probit analysis for calculation of LC_{50} values was carried out using the statistical software SPSS 18.0 for windows.

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of Bacilli

The isolates were processed through gram's staining. All the isolates were identified as bacilli (Fig. 1), after staining, the colonies showed purple gram positive (+) rods. In all isolates, there was a uniform colony, slightly raised with cream white colour. There were spores present and, formation of one or more parasporal crystalline bodies/endospore adjacent to the spore (Table 3).

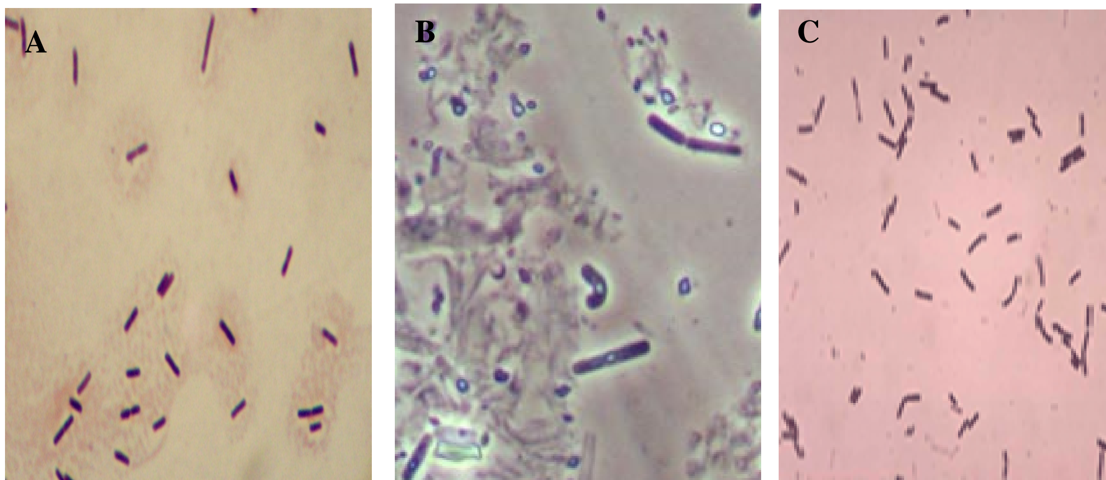

Fig. 1: Gram (+) rods (A and C) and spore/delta-endotoxins (B) produced from *Bacillus thuringiensis* isolates cultured in legume medium (×1000).

Table 3: Different isolates of *Bacillus thuringiensis* and their respective morphological characteristics

Isolates	**Gram**	**Cell shape**	**Endotoxin**	**Cell morphology**	**Colour**	**Pigment**	**Motility**
24LBN30°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
1SKAG37°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
62LBG37°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
63KAG37°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
58SLA25°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
14SLA30°C	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 30	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 47	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 21	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 54	+	Rod	+	Oval, slightly raised, entire	Cream white	-	+
KARI 20	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 46	+	Rod	+	Oval, slightly raised, entire	Cream white	-	+
KARI 12	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+
KARI 14	+	Rod	+	Oval, slightly raised, entire	Cream white	-	+
KARI 53	+	Rod	+	Oval, slightly raised, entire	Cream white	-	+
KARI 37	+	Rod	+	Circular, slightly raised, entire	Cream white	-	+

4.2 Effect of temperature and pH on growth of isolates

Regarding temperature effect, results pointed to the importance of incubation temperature which regulated the OD 600nm obtained from the experiments. At 20 °C (Table 4), KARI 37 recorded the highest growth of 0.60 and the lowest performing was observed in isolates: 62LBG37°C, KARI 47, 14SLA30°C, KARI 53 KARI 14 having an OD 600 of 0.24-0.34.

Table 4: The growth of *Bacillus thuringiensis* isolates after incubation at four different temperatures, as depicted by the optical density of the bacterial culture at 600 $_{nm}$, after 10 h

Isolate	20 °C	30 °C	37 °C	40 °C	CV%
KARI 46	0.55^{c}±0.12	0.60^{b}±0.15	0.72^{a}±0.22	0.55^{c}±0.17	5.6
KARI 37	0.60^{a}±0.12	0.63^{a}±0.15	0.64^{a}±0.22	0.46^{b}±0.17	3.13
62LBG37°C	0.26^{d}±0.12	0.44^{c}±0.15	0.81^{a}±0.22	0.62^{a}±0.17	5.17
KARI 30	0.40^{b}±0.12	0.49^{b}±0.15	0.55^{a}±0.22	0.42^{b}±0.17	5.7
KARI 20	0.43^{c}±0.12	0.51^{b}±0.15	0.60^{a}±0.22	0.45^{c}±0.17	7.2
KARI 21	0.10^{c}±0.12	0.36^{b}±0.15	0.77^{a}±0.22	0.32^{b}±0.17	7.77
KARI 47	0.26^{d}±0.12	0.32^{c}±0.15	0.93^{a}±0.22	0.45^{b}±0.17	5.34
63KAG37°C	0.37^{c}±0.12	0.41^{b}±0.15	1.25^{a}±0.22	0.39^{c}±0.17	5.43
14SLA30°C	0.24^{d}±0.12	0.39^{c}±0.15	1.03^{a}±0.22	0.51^{b}±0.17	4.61
KARI 53	0.29^{d}±0.12	0.36^{c}±0.15	0.69^{a}±0.22	0.42^{b}±0.17	5.73
24LBN30°C	0.33^{b}±0.12	0.41^{b}±0.15	1.00^{a}±0.22	0.35^{b}±0.17	13.5
1SKAG37°C	0.19^{c}±0.12	0.46^{b}±0.15	1.19^{a}±0.22	0.41^{b}±0.17	6.22
58SLA25°C	0.39^{c}±0.12	0.51^{b}±0.15	0.89^{a}±0.22	0.39^{c}±0.17	6.23
KARI 54	0.35^{c}±0.12	0.43^{b}±0.15	0.69^{a}±0.22	0.37^{c}±0.17	5.84
KARI 12	0.21^{c}±0.12	0.58^{b}±0.15	0.96^{a}±0.22	0.51^{b}±0.17	5.41
KARI 14	0.34^{d}±0.12	0.64^{b}±0.15	0.88^{a}±0.22	0.44^{c}±0.17	4.63

Value=Mean. Means of the same parameter followed by the same letter in the same row are not significantly different ($p \leq 0.05$) as calculated by Duncan multiple range test. S.D=Standard deviation.

Isolate KARI 37 was still the highest performing isolate at 30 °C with an OD 600 value of 0.63 while isolates 62LBG37°C, KARI 47,14SLA30°C, KARI 53 recorded high OD 600 of 0.32-0.44. Lastly, at 40 °C the highest OD 600 was obtained from 62LBG37°C with a value of 0.62 and isolates KARI 46, KARI 20, 63KAG37°C, 58SLA25°C, KARI 54 and KARI 14 recorded lowest OD 600nm values between 0.37-0.55. OD 600 was highest at 37 °C for all isolates tested, a temperature generally considered to be optimum to permit a good growth in Bacillus spp. Growth of isolate KARI 30 were optimal at both 30 °C and 37 °C. There was no significance growth at 37 °C ($P \leq 0.05$) for all the isolates because they were grouped together after performing the Duncan's multiple range tests.

Maximum growth was observed between pH 6.5-7.5 for all the *Bt* isolates (Table 5). At pH 6.5 the isolates; KARI 46, KARI 20, KARI 53, ISKAG37°C, 58SLA25°C, and KARI 14 recorded fairly high OD 600nm values while pH 7 was suitable for growth of isolates KARI 37, 62LBG37°C, KARI 30, KARI 21, KARI 47, 63KAG37°C, 14SLA30°C, 24LBN30°C, KARI 54 and KARI 14. For pH 7.5, it was also optimum for growth of isolates; KARI 46, KARI 37, 62LBG37°C, KARI 20, KARI 21, KARI 47KARI 53, 24LBN30°C, and 58SLA25°C. At pH 7, there was no significant growth ($P \leq 0.05$) for all the isolates because they were all grouped together after performing the Duncan's multiple range test.

Table 5: The growth of *Bacillus thuringiensis* isolates after optimization at different pH, as depicted by the optical density of the bacterial culture at 600nm, 10 h after inoculation at 37°C

Isolates				**pH**				
	4	**5**	**6**	**7**	**8**	**9**	**10**	**CV%**
KARI 46	$0.17^{e}\pm0.11$	$0.25^{d}\pm0.10$	$0.47^{c}\pm0.16$	$0.78^{a}\pm0.13$	$0.53^{b}\pm0.12$	$0.41^{c}\pm0.11$	$0.18^{e}\pm0.18$	13.8
KARI 37	$0.13^{d}\pm0.11$	$0.27^{c}\pm0.10$	$0.41^{b}\pm0.16$	$0.65^{a}\pm0.13$	$0.49^{b}\pm0.12$	$0.35^{c}\pm0.11$	$0.15^{d}\pm0.18$	15.4
62LBG37°C	$0.22^{e}\pm0.11$	$0.35^{c}\pm0.10$	$0.46^{b\pm}0.16$	$0.76^{a}\pm0.13$	$0.54^{b}\pm0.12$	$0.32^{d}\pm0.11$	$0.16^{e}\pm0.18$	10.1
KARI 30	$0.15^{d}\pm0.11$	$0.38^{c}\pm0.10$	$0.45^{b}\pm0.16$	$0.64^{a}\pm0.13$	$0.53^{b}\pm0.12$	$0.35^{c}\pm0.11$	$0.13^{d}\pm0.18$	13.8
KARI 20	$0.23^{d}\pm0.11$	$0.36^{c}\pm0.10$	$0.47^{b}\pm0.16$	$0.89^{a}\pm0.13$	$0.57^{b}\pm0.12$	$0.31^{d}\pm0.11$	$0.09^{e}\pm0.18$	14.5
KARI 21	$0.26^{d}\pm0.11$	$0.31^{c}\pm0.10$	$0.43^{b}\pm0.16$	$1.00^{a}\pm0.13$	$0.49^{bc}\pm0.12$	$0.37^{c}\pm0.11$	$0.15^{e}\pm0.18$	15.2
KARI 47	$0.16^{d}\pm0.11$	$0.33^{c}\pm0.10$	$0.41^{b}\pm0.16$	$0.83^{a}\pm0.13$	$0.55^{b}\pm0.12$	$0.33^{c}\pm0.11$	$0.16^{d}\pm0.18$	14.4
63KAG37°C	$0.22^{d}\pm0.11$	$0.29^{c}0.10$	$0.40^{b}\pm0.16$	$0.72^{a}\pm0.13$	$0.51^{b}\pm0.12$	$0.29^{cd}\pm0.11$	$0.15^{e}\pm0.18$	14.3
14SLA30°C	$0.14^{d}\pm0.11$	$0.25^{c}\pm0.10$	$0.42^{b}\pm0.16$	$0.67^{a}\pm0.13$	$0.49^{b}\pm0.12$	$0.30^{c}\pm0.11$	$0.22^{d}\pm0.18$	17.5
KARI 53	$0.19^{ef}\pm0.11$	$0.27^{cd}\pm0.10$	$0.46^{b}\pm0.16$	$0.73^{a}\pm0.13$	$0.41^{c}\pm0.12$	$0.35^{de}\pm0.11$	$0.19^{f}\pm0.18$	15.4
24LBN30°C	$0.11^{d}\pm0.11$	$0.26^{c}\pm0.10$	$0.42^{b}\pm0.16$	$0.68^{a}\pm0.13$	$0.4^{c}\pm0.12$	$0.31^{c}\pm0.11$	$0.16^{d}\pm0.18$	15.9
1SKAG37°C	$0.23^{de}\pm0.11$	$0.29^{c}\pm0.10$	$0.45^{b}\pm0.16$	$0.69^{a}\pm0.13$	$0.52^{b}\pm0.12$	$0.36^{cd}\pm0.11$	$0.12^{e}\pm0.18$	13.7
58SLA25°C	$0.19^{d}\pm0.11$	$0.34^{c}\pm0.10$	$0.46^{b}\pm0.16$	$0.75^{a}\pm0.13$	$0.58^{b}\pm0.12$	$0.35^{cd}\pm0.11$	$0.15^{d}\pm0.18$	14.9
KARI 54	$0.11^{e}\pm0.11$	$0.27^{cd}\pm0.10$	$0.41^{b}\pm0.16$	$0.64^{a}\pm0.13$	$0.45^{bc}\pm0.12$	$0.34^{d}\pm0.11$	$0.13^{e}\pm0.18$	17.9
KARI 12	$0.17^{d}\pm0.11$	$0.33^{c}\pm0.10$	$0.42^{b}\pm0.16$	$0.61^{a}\pm0.13$	$0.46^{bc}\pm0.12$	$0.29^{d}\pm0.11$	$0.14^{e}\pm0.18$	16.3
KARI 14	$0.18^{e}\pm0.11$	$0.31^{cd}\pm0.10$	$0.45^{b}\pm0.16$	$0.63^{a}\pm0.13$	$0.45^{bc}\pm0.12$	$0.38^{d}\pm0.11$	$0.15^{e}\pm0.18$	18

Value=Mean. Means of the same parameter followed by the same letter in the same row are not significantly different ($p\leq0.05$) as calculated by Duncan multiple range test. S.D=Standard deviation.

4.3 Effect of media on growth of *Bacillus thuringiensis* isolates

For NYSM medium, the isolates; KARI 46, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C recorded OD 600nm values of between 1.35-1.47 (Table 6). Legume medium recorded optical density values of 2.40-1.81 from isolates; 58SLA25°C, 62LBG37°C, 24LBN30°C, KARI 54, KARI 37, KARI 12 and KARI 21. Potato medium recorded OD 600nm values of between 1.003-1.331 from KARI 46, KARI 47, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C.

Wheat bran/cotton seed medium recorded maximum OD 600nm values of 0.54-0.474 from KARI 21, 58SLA25°C, 24LBN30°C, 1SKAG37°C and KARI 37. For whey medium; KARI 30, 58SLA25°C, 62LBG37°C 24LBN30°C, 14SLA30°C and KARI 37 recorded OD 600nm values of between 0.90-1.218 and Poultry litter medium recorded 0.41-0.37 OD 600nm values from isolates; 58SLA25°C, 14SLA25°C, 1SKAG37°C, 62LBG37°C, KARI 46, KARI 37 and KARI 47. Legume medium has the highest OD 600nm values, significantly different to that achieved for NYSM. Based on media comparison between NYSM and other media, the legumes, potato and whey produced the greatest OD 600nm; and, differences between media treatments were significantly different ($P \leq 0.05$). Based on OD 600nm, the *Bacillus thuringiensis* isolates growth in different media was significantly different between treatments.

Table 6: The optical densities of *Bacillus thuringiensis* isolates after 72 h fermentation at 37 °C in six different media

Isolates	NYSM	Legume	Potato	Whey	Wheat/cotton	Poultry
KARI 46	$1.47^{a}\pm0.22$	$1.809^{b}\pm0.22$	$1.003^{bc}\pm0.22$	$0.902^{cd}\pm0.22$	$0.538^{de}\pm0.22$	$0.368^{e}\pm0.22$
KARI 37	$1.48^{a}\pm0.23$	$1.927^{b}\pm0.23$	$1.079^{b}\pm0.23$	$0.919^{b}\pm0.23$	$0.448^{c}\pm0.23$	$0.394^{c}\pm0.23$
62LBG37°C	$1.45^{a}\pm0.22$	$1.829^{b}\pm0.22$	$1.218^{b}\pm0.22$	$0.936^{b}\pm0.22$	$0.436^{c}\pm0.22$	$0.412^{c}\pm0.22$
KARI 30	$1.4^{a}\pm0.2$	$1.926^{b}\pm0.2$	$1.331^{b}\pm0.2$	$0.92^{c}\pm0.2$	$0.474^{d}\pm0.2$	$0.391^{d}\pm0.2$
KARI 20	$1.44^{a}\pm0.19$	$1.847^{a}\pm0.19$	$0.712^{b}\pm0.19$	$0.991^{a}\pm0.19$	$0.408^{c}\pm0.19$	$0.349^{c}\pm0.19$
KARI 21	$1.35^{a}\pm0.21$	$1.918^{ab}\pm0.21$	$1.303^{bc}\pm0.21$	$1.011^{c}\pm0.21$	$0.389^{d}\pm0.21$	$0.329^{d}\pm0.21$
KARI 47	$1.33^{a}\pm0.2$	$1.955^{a}\pm0.2$	$0.515^{b}\pm0.2$	$0.996^{a}\pm0.2$	$0.423^{bc}\pm0.2$	$0.355^{c}\pm0.2$
63KAG37°C	$1.29^{a}\pm0.18$	$1.904^{a}\pm0.18$	$1.095^{b}\pm0.18$	$0.923^{b}\pm0.18$	$0.407^{c}\pm0.18$	$0.344^{c}\pm0.18$
14SLA30°C	$1.33^{a}\pm1$	$1.993^{a}\pm1$	$0.991^{a}\pm1$	$0.97^{a}\pm1$	$0.338^{a}\pm1$	$0.326^{a}\pm1$
KARI 53	$1.14^{ab}\pm0.21$	$1.64^{a}\pm0.21$	$1.218^{c}\pm0.21$	$0.829^{bc}\pm0.21$	$0.398^{d}\pm0.21$	$0.339^{d}\pm0.21$
24LBN30°C	$1.04^{b}\pm0.3$	$2.398^{a}\pm0.3$	$0.944^{c}\pm0.3$	$0.845^{c}\pm0.3$	$0.412^{d}\pm0.3$	$0.372^{d}\pm0.3$
1SKAG37°C	$1.05^{a}\pm0.2$	$1.669^{a}\pm0.2$	$0.728^{b}\pm0.2$	$0.836^{b}\pm0.2$	$0.383^{c}\pm0.2$	$0.351^{c}\pm0.2$
58SLA25°C	$1.38^{a}\pm0.22$	$1.932^{b}\pm0.22$	$1.279^{b}\pm0.22$	$0.837^{c}\pm0.22$	$0.408^{d}\pm0.22$	$0.318^{d}\pm0.22$
KARI 54	$1.35^{ab}\pm0.2$	$1.796^{a}\pm0.2$	$0.614^{c}\pm0.2$	$0.991^{b}\pm0.2$	$0.363^{c}\pm0.2$	$0.397^{c}\pm0.2$
KARI 12	$1.45^{a}\pm0.2$	$1.907^{ab}\pm0.2$	$1.132^{bc}\pm0.2$	$1.218^{c}\pm0.2$	$0.372^{d}\pm0.2$	$0.371^{d}\pm0.2$
KARI 14	$1.44^{a}\pm0.22$	$1.924^{a}\pm0.22$	$0.525^{b}\pm0.22$	$0.944^{a}\pm0.22$	$0.381^{c}\pm0.22$	$0.362^{c}\pm0.22$

Value=Mean. Means of the same parameter followed by the same letter in the same row are not significantly different ($p\leq0.05$) as calculated by Duncan multiple range test. S.D=Standard deviation.

The following; KARI 46, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C recorded spore counts of between 3.87-4.14 × 10^8 CFU/ ml in NYSM medium (Table 7). Legume medium recorded spore counts of 4.89-5.60 × 10^8 CFU/ ml from; 58SLA25°C, 62LBG37°C, 24LBN30°C, KARI 54, KARI 37, KARI 12 and KARI 21. Potato medium recorded spore counts of between 2.45-3.25 × 10^8 CFU/ ml from KARI 46, KARI 47, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C.

For wheat bran/cotton seed medium, maximum spore counts of 0.83-1.34 × 10^7 CFU/ ml were recorded from KARI 21, 58SLA25°C, 24LBN30°C, 1SKAG37°C and KARI 37. In whey medium; KARI 30, 58SLA25°C, 62LBG37°C 24LBN30°C, 14SLA30°C and KARI 37 recorded spore counts of between 1.72-2.44 × 10^7 CFU/ ml and poultry litter medium recorded 0.64-0.83 × 10^7 CFU/ ml spore counts from isolates; 58SLA25°C, 14SLA25°C, 1SKAG37°C, 62LBG37°C, KARI 46, KARI 37 and KARI 47. Therefore, after media comparison, the lowest performing medium was wheat bran/cotton seed followed by poultry litter medium. Based on media comparison between NYSM and other media, the legumes produced the highest spore counts, followed by potato and then whey; and, differences between media treatments were significantly different ($P \leq 0.05$). Based on spore counts, the *Bacillus thuringiensis* isolates growth in different media was significantly different between treatments.

Table 7: The degree of spore counts (No. of cells $\times 10^{8}$) in the final whole culture of *Bt* obtained from six different media, after 72 h

Media	**NYSM**	**LEGUME**	**POTATO**	**WHEY**	**WHEAT**	**POULTRY**	**CV%**
KARI46	$38^{b}\pm5.8$	$51^{a}\pm5.8$	$25^{c}\pm5.8$	$22^{c}\pm5.8$	$9^{d}\pm5.8$	$6^{d}\pm5.8$	23.2
KARI37	$38^{b}\pm3.4$	$50^{a}\pm3.4$	$29^{c}\pm3.4$	$25^{c}\pm3.4$	$6^{d}\pm3.4$	$5^{d}\pm3.4$	13.4
62LBG37°C	$26^{a}\pm9.7$	$41^{a}\pm9.7$	$24^{ab}\pm9.7$	$24^{ab}\pm9.7$	$6^{b}\pm9.7$	$6^{b}\pm9.7$	46.1
KARI30	$34^{b}\pm5.3$	$53^{a}\pm5.3$	$14^{d}\pm5.3$	$24^{c}\pm5.3$	$7^{d}\pm5.3$	$6^{d}\pm5.3$	22.8
KARI20	$34^{a}\pm6.2$	$41^{a}\pm6.2$	$20^{b}\pm6.2$	$21^{b}\pm6.2$	$6^{c}\pm6.2$	$5^{c}\pm6.2$	29.4
KARI21	$35^{a}\pm14.1$	$33^{ab}\pm14.1$	$13^{ab}\pm14.1$	$11^{ab}\pm14.1$	$6^{b}\pm14.1$	$6^{b}\pm14.1$	82.7
KARI47	$40^{a}\pm9$	$37^{a}\pm9$	$10^{b}\pm9$	$15^{b}\pm9$	$6^{b}\pm9$	$5^{b}\pm9$	47.4
63KAG37°C	$24^{abc}\pm10.1$	$37^{a}\pm10.1$	$26^{ab}\pm10.1$	$20^{abc}\pm10.1$	$11^{bc}\pm10.1$	$6^{c}\pm10.1$	49.3
14SLA30°C	$38^{a}\pm4.3$	$44^{a}\pm4.3$	$10^{c}\pm4.3$	$21^{b}\pm4.3$	$5^{c}\pm4.3$	$5^{c}\pm4.3$	21.0
KARI53	$35^{b}\pm3.9$	$49^{a}\pm3.9$	$30^{b}\pm3.9$	$21^{c}\pm3.9$	$11^{d}\pm3.9$	$7^{d}\pm3.9$	15.5
24LBN30°C	$27^{b}\pm10.5$	$47^{a}\pm10.5$	$22^{bc}\pm10.5$	$19^{bc}\pm10.5$	$9^{bc}\pm10.5$	$7^{c}\pm10.5$	48.2
1SKAG37°C	$30^{b}\pm5$	$47^{a}\pm5$	$20^{c}\pm5$	$20^{c}\pm5$	$9^{d}\pm5$	$5^{d}\pm5$	22.7
58SLA25°C	$30^{b}\pm7.8$	$48^{a}\pm7.8$	$25^{b}\pm7.8$	$25^{b}\pm7.8$	$9^{c}\pm7.8$	$4^{c}\pm7.8$	33.1
KARI54	$34^{b}\pm5.1$	$54^{a}\pm5.1$	$22^{c}\pm5.1$	$24^{c}\pm5.1$	$9^{d}\pm5.1$	$12^{d}\pm5.1$	19.9
KARI12	$33^{b}\pm7$	$53^{a}\pm7$	$19^{cd}\pm7$	$26^{bc}\pm7$	$9^{d}\pm7$	$10^{d}\pm7$	26.2
KARI14	$32^{b}\pm7.4$	$56^{a}\pm7.4$	$27^{b}\pm7.4$	$27^{b}\pm7.4$	$13^{c}\pm7.4$	$12^{c}\pm7.4$	26.6

Value=Mean. Means of the same parameter followed by the same letter in the same row are not significantly different ($p \leq 0.05$) as calculated by Duncan multiple range test. S.D=Standard deviation.

4.4 Protein concentrations

These were calculated using results of the BSA assay. A standard curve was constructed basing on the concentrations of the BSA protein standards and the corresponding OD 595nm values. The equation (Fig. 2) displays the relationship between protein concentration (in mg/ ml) and absorbance (OD 595nm).

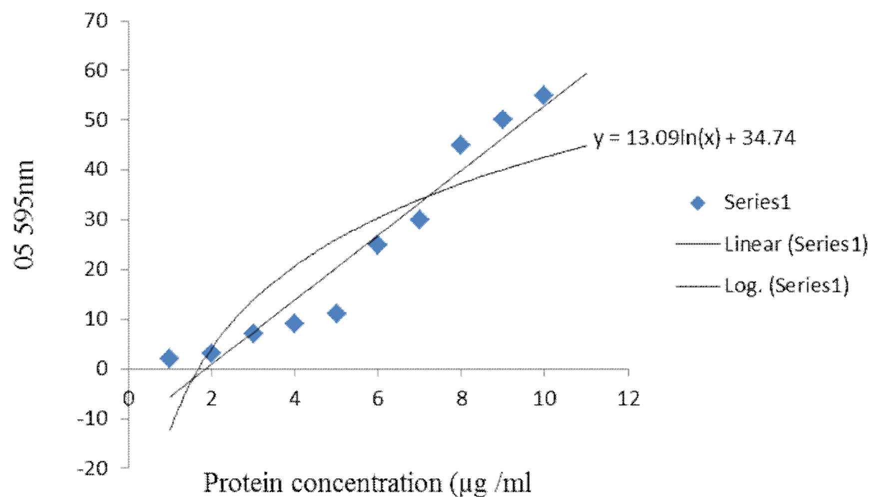

Fig. 2: The standard curve of *Bacillus thuringiensis* protein concentrations using the Bradford Standard Assay protein standards.

The following; KARI 46, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C recorded protein concentrations of between 22.96 - 28.75 µg/ ml in NYSM medium (Table 8). Legume medium recorded 18.64 - 25.79 µg/ ml from; 58SLA25°C, 62LBG37°C, 24LBN30°C, KARI 54, KARI 37, KARI 12 and KARI 21. Potato medium

recorded 13.30 - 20.61 µg/ ml from KARI 46, KARI 47, KARI 20, KARI 53, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C.

For wheat bran/cotton seed medium, 12.45 - 14.53 µg/ ml were recorded from KARI 21, 58SLA25°C, 24LBN30°C, 1SKAG37°C and KARI 37. In whey medium; KARI 30, 58SLA25°C, 62LBG37°C 24LBN30°C, 14SLA30°C and KARI 37 recorded 16.96 – 19.50 µg/ ml and poultry litter medium recorded 15.71 - 18. 47 µg/ ml from isolates; 58SLA25°C, 14SLA25°C, 1SKAG37°C, 62LBG37°C, KARI 46, KARI 37 and KARI 47. Thus, the overall protein production of *Bacillus thurigiensis* in NYSM was comparable to that of legume medium and the mean values were significantly different (P≤0.05). Protein production of *Bacillus thuringiensis* from potato and whey-based culture medium was significantly different (P≤0.05) between treatments.

Protein concentration in NYSM medium was 22.96 - 28.75 µg/ ml, 18.64 - 25.79 µg/ ml in legume, 13.30 - 20.61 µg/ ml in potato, and 16.96 – 19.50 µg/ ml in whey, respectively. These results are in tandem with toxicity results which show that legume medium had the highest protein concentration production with high toxicity compared to the conventional and other media. Addition of readily utilizable carbon and nitrogen supports high initial growth of *Bacillus thuringiensis* after inoculation, whereas that available in legume, potato and whey-based media is used for subsequent growth.

Table 8: The protein concentrations of *Bacillus thuringiensis* isolates after 72 h fermentation in six different media

Isolate	Protein concentrations (µg/ ml) from different media						
	NYSM	**Potato**	**Legume**	**Wheat/cotton**	**Whey**	**Poultry litter**	**CV%**
KARI 46	25.96^{a}±3.49	16.19^{b}±3.49	20.18^{a}±3.49	13.16^{b}±3.49	17.84^{b}±3.49	17.66^{b}±3.49	19.11
KARI 37	24.89^{a}±2.74	20.01^{b}±2.74	22.88^{a}±2.74	13.86^{b}±2.74	18.11^{b}±2.74	17.49^{b}±2.74	14.85
62LBG37°C	28.04^{a}±3.06	19.18^{b}±3.06	23.45^{a}±3.06	12.45^{b}±3.06	17.27^{b}±3.06	17.33^{b}±3.06	16.55
KARI 30	24.04^{a}±3.02	18.78^{b}±3.02	25.79^{a}±3.02	12.89^{b}±3.02	18.35^{b}±3.02	16.86^{b}±3.02	15.83
KARI 20	25.58^{a}±2.39	18.61^{bc}±2.39	21.45^{b}±2.39	13.60^{c}±2.39	17.62^{c}±2.39	16.50^{c}±2.39	13.19
KARI 21	22.96^{a}±3.2	19.11^{b}±3.2	22.89^{a}±3.2	14.53^{b}±3.2	17.59^{b}±3.2	17.19^{b}±3.2	16.81
KARI 47	28.55^{a}±2.59	16.83^{b}±2.59	23.12^{a} ±2.59	13.30^{b}±2.59	16.96^{b}±2.59	17.58^{b}±2.59	13.8
63KAG37°C	25.57^{a}±2.22	18.84^{b}±2.22	22.65^{a}±2.22	12.97^{b}±2.22	17.07^{b}±2.22	16.92^{b}±2.22	11.38
14SLA30°C	24.30^{a}±3.03	13.30^{c}±3.03	24.28^{ab}±3.03	13.29^{c}±3.03	19.50^{c}±3.03	17.64^{bc}±3.03	16.68
KARI 53	25.07^{a}±2.94	18.72^{b}±2.94	20.74^{a}±2.94	13.75^{b}±2.94	19.41^{b}±2.94	16.93^{b}±2.94	16.59
24LBN30°C	24.59^{a}±2.6	19.15^{b}±2.6	23.87^{a}±2.6	13.96^{b}±2.6	18.54^{b}±2.6	15.71^{b}±2.6	14.15
1SKAG37°C	27.47^{a}±2.81	20.61^{bc}±2.81	18.64^{ab}±2.81	14.00^{c}±2.81	17.15^{c}±2.81	17.43^{c}±2.81	15.28
58SLA25°C	28.75^{a}±2.95	19.32^{b}±2.95	27.60^{a}±2.95	14.08^{b}±2.95	19.23^{b}±2.95	18.47^{b}±2.95	15.12
KARI 54	28.05^{a}±2.99	18.94^{b}±2.99	23.16^{a}±2.99	13.89^{b}±2.99	18.06^{b}±2.99	16.47^{b}±2.99	16.44
KARI 12	25.91^{a}±2.45	19.15^{b}±2.45	23.04^{a}±2.45	12.84^{b}±2.45	16.47^{b}±2.45	16.86^{b}±2.45	13.75
KARI 14	25.31^{a}±2.65	19.37^{bc}±2.65	19.20^{b}±2.65	12.96^{c}±2.65	18.80^{c}±2.65	17.19 ^{c}±2.65	14.6

Value=Mean. Means of the same parameter followed by the same letter in the same row are not significantly different ($p \leq 0.05$) as calculated by Duncan multiple range test. S.D=Standard deviation.

4.5 SDS-PAGE results

When compared to high molecular weight standards in SDS-PAGE analysis, the solution and pellet of the dissolved spore-crystal product from each treatment had proteins with molecular weights of approximately 110-120 kDa and 60-70 kDa (Figure 3 and 4). The major polypeptides present in the spore-crystal complex of *Bacillus thuringiensis* produced from Legume medium (1SKAG37°C and 24LBN30°C), NYSM medium (58SLA25°C and KARI 20), potato medium (58SLA25°C and KARI 30) and whey medium (14SLA30°C and 24LBN30°C) were clear and conspicuous. The protein profiles as indicator of *Bacillus thuringiensis* Cryotoxins were correspondingly related to their larvicidal activity.

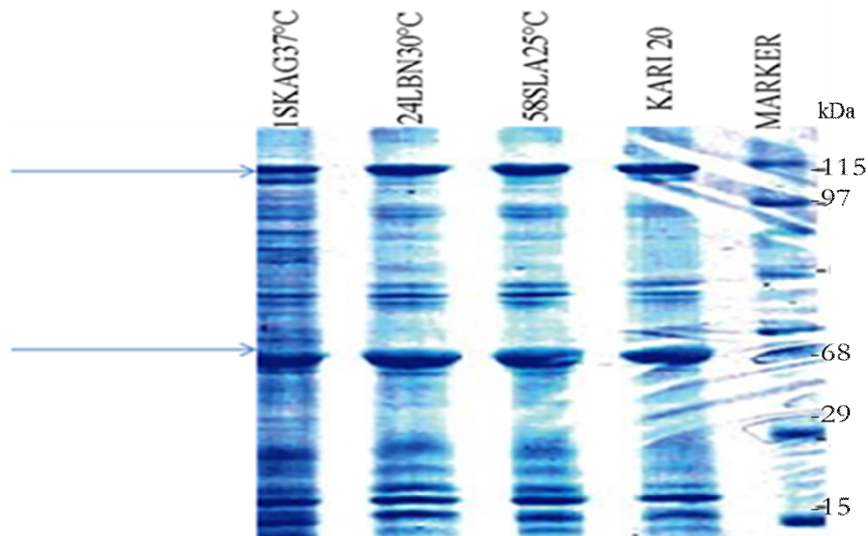

Fig. 3: The protein bands (arrows) of delta-endotoxin and spore mixture of *Bacillus thuringiensis* isolates during its fermentation from Legume medium (1SKAG37°C and 24LBN30°C) and NYSM medium (58SLA25°C and KARI 20) as determined by SDS-PAGE.

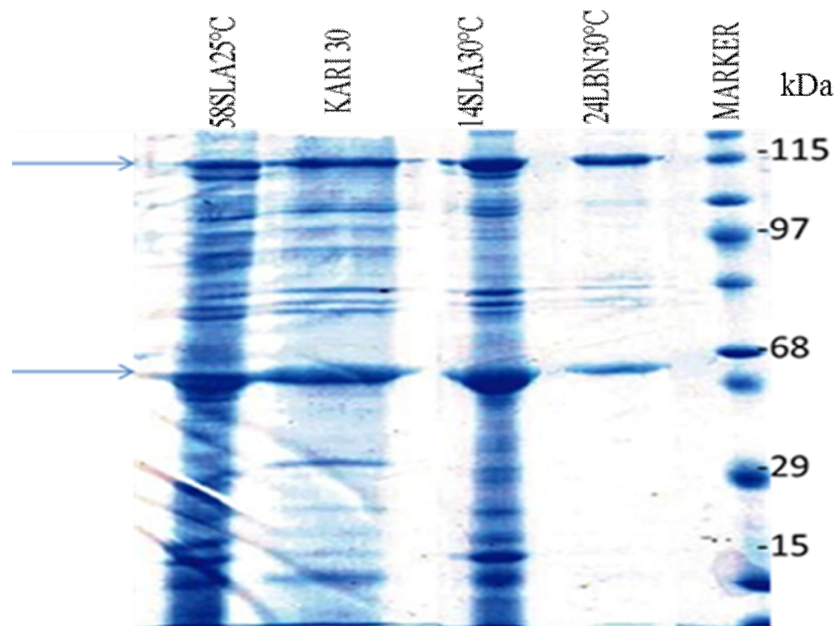

Fig. 4: The protein bands (arrows) of delta-endotoxin and spore mixture of *Bacillus thuringiensis* isolates during its fermentation in potato medium (58SLA25°C and KARI 30) and whey medium (14SLA30°C and 24LBN30°C) as determined by SDS-PAGE.

The wheat bran/cotton seed and poultry litter in the Figure 3 has offered a feeble visibility due possibility to a lesser degree of crystal proteins compared to that of legume/potato/whey induced banding patterns. There is no variation in the protein pattern between the toxins produced from the conventional and test media. The major polypeptides of *Bacillus thuringiensis* spore/crystal complex produced from *Bt* cultured in wheat/cotton and poultry litter media were (110-120 kDa and 60-70 kDa).

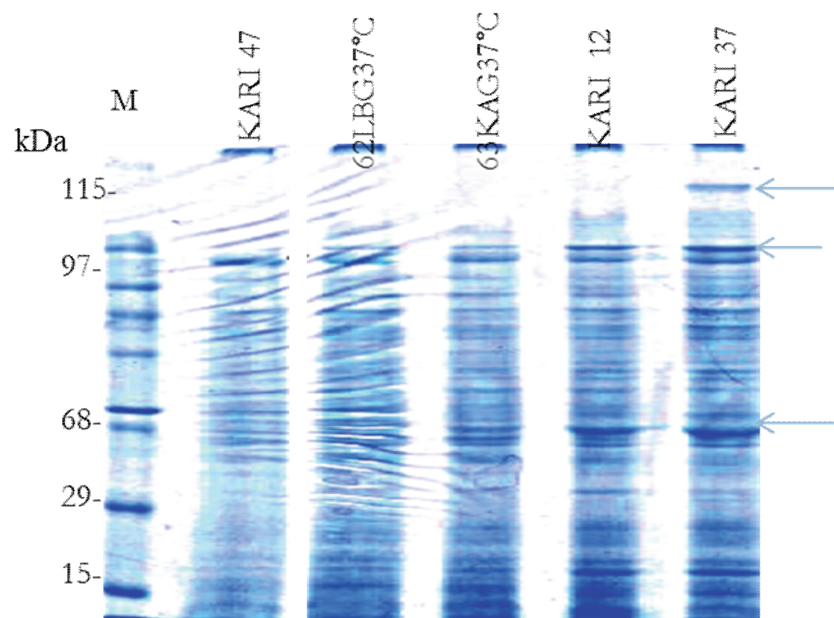

Fig. 5: Protein bands of delta-endotoxin of *Bt* isolates: Wheat/cotton (KARI 47 and 62LBG37°C and poultry (63KAG37°C, KARI 12 and KARI 37) as determined by SDS-PAGE.

4.6 Bioassay results of *Bacillus thuringiensis* isolates against *Chilo partellus*

Determination of the median lethal concentration of the standard, *Bacillus thuringiensis* subspecies *kurstaki*, against *Chilo partellus* was done to identify the right concentration treatment to use for screening the *Bacillus thuringiensis* isolates. From the five concentrations (15mg/ ml, 1.5 mg/ ml, 0.15 mg/ml, 0.015 mg/ml and 0 mg/ml) of *Bacillus thuringiensis* subspecies *kurstaki* prepared to infect the 1st instar (neonate) larvae of *Chilo partellus*, highest LC50 values were recorded at the 0.015 mg/ml concentration (Table 9).

Table 9: Percent mortality of neonate *Chilo partellus* larvae on treatment with five toxin concentrations of, the standard, *Bacillus thuringiensis* subspecies *kurstaki*

Isolate	**Concentration (mg/ ml)**	**Mean larval mortality (48 h)**	**Intercept**	**Slope (±SE)**	**LC50 (μg/ l)**	**χ^2 (df)**
Bt kurstaki	0	10%	1.2	0.8 ±0.33	15.074	5.462
Bt kurstaki	0.015	78%	2	1.1 ±0.25	83.787	
Bt kurstaki	0.15	72%	1.8	1.2 ±0.19	66.602	
Bt kurstaki	1.5	49%	1.7	1 ±0.21	45.462	
Bt kurstaki	15	20%	1.4	0.9 ±0.27	25.311	

Among the different *Bacillus thuringiensis* treatments; KARI 20, 63KAG37°C and 24LBN30°C recorded mortality (of 10%) at 48 h of observation (Table 9). KARI 37 recorded 20 % mortality towards the end of the observation, while isolates; KARI 54, 62LBG37°C, KARI 46, KARI 14, KARI 53 recorded 30 % mortality from 48 h. KARI 30 and KARI 47 recorded mortality of 60 % at 48 h. Maximum toxicity was recorded by isolate 58SLA25°C, that is, 73 % mortality at 48 h. No mortality was observed with isolates 14SLA30°C, 1SKAG37°C, KARI 21, KARI 12 and the control throughout the observation period (Table 10).

Table 10: Percent cumulative mortality of *Chilo partellus* first-instar larvae exposed to 0.015mg/ ml endotoxins from *Bacillus thuringiensis* isolates.

Isolates	**Mean larval mortality (48 h)**	**Intercept**	**Slope (±SE)**	**LC50 (µg/ l)**	**χ^2 (df)**
Bt kurstaki	83%	1.9	1.1 ±0.33	84	11.425 (2)
58SLA25°C	73%	1.8	1.1 ±0.33	74	
KARI 30	60%	1.8	1 ±0.25	63	
KARI 47	60%	1.8	1 ±0.25	63	
KARI 54	30%	1.5	0.8 ±0.19	34	
62LBG37°C	30%	1.5	0.8 ±0.19	34	
KARI 46	30%	1.5	0.8 ±0.19	34	
KARI 14	30%	1.5	0.8 ±0.19	34	
KARI 53	30%	1.5	0.8 ±0.19	34	
KARI 37	20%	1.3	0.6 ±0.21	24	
24LBN30°C	10%	1.1	0.5 ±0.27	14	
63KAG37°C	10%	1.1	0.5 ±0.27	14	
KARI 20	10%	1.1	0.5 ±0.27	13	
14SLA30°C	0				
1SKAG37°C	0				
KARI 21	0				
KARI 12	0				

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

The use of chemical insecticides has become detrimental because of a multiplicity of factors including physiological resistance in the vectors, environmental pollution resulting in bio-amplification of food chain contamination and harmful effects on beneficial insects. Hence, there has been an increased interest in recent years in the use of biological vector control agents. They have some advantages over conventional insecticides in pest control operations, that is, are safe for non-target organisms including humans and they are not hazardous to the environment.

This warrants developing cheaper media for the culturing of *Bacillus thuringiensis* and thus facilitating the production of biopesticides in a cost-effective manner. Media containing industrial byproduct, animal parts, fishmeal, soya bean and corn steep liquor for the production of *Bacillus sphaericus* and *Bacillus thuringiensis* serovar. *israelensis* have also been reported (Devi *et al*., 2005; Chang *et al*., 2008; Ouoba *et al*., 2008). Many studies have been done on local production of *Bacillus thuringiensis* delta-endotoxins but none have studied the influence of carbon-source on growth, sporulation and protein production by local *Bacillus thuringiensis* isolates and if the local isolates differ in their delta-endotoxin production and toxicity to *Chilo partellus* larvae.

In the present study, the production of *Bacillus thuringiensis* spore/crystal mixtures in five different media (potato, legume, wheat bran/cotton seed, whey and poultry litter) was measured and compared with the toxin production in a conventional laboratory medium (Nutrient Yeast Synthetic Medium or NYSM). The growth curve, based on OD 600, of Bt when grown on all these local media and NYSM had a similar pattern. Data obtained from this study indicate that with increasing culture time, culture density (measured by optical density at 600 nm) increased and reached a plateau in the range 2–2.5 in legume, potato and whey- based media except in *Bacillus thuringiensis* cultured in wheat bran/cotton seed and poultry litter media, where the sample density had reached a plateau in the range of 1–1.5.

All the above media formulated from locally available substrates were rich in carbon and nitrogen although supplemented with minerals to enhance the growth and sporulation of *Bacillus thuringiensis* isolates. İçgen *et al*., (2002) had obtained the highest cell densities and lowest sporulation frequencies in Yousten Synthetic medium containing glucose. Toxin yields on this sugar was markedly less than those obtained on sucrose which was a not good carbon source for growth, but supported the synthesis of large amount of crystal protein as well as high sporulation frequencies. In the present study, I obtained high cell density as well as a high sporulation frequency with high toxin yield by studying carbon source effect on different *Bacillus thuringiensis* isolates cultured in locally derived media. When carbon source were varied at 10 g/ l each of, legumes, potato, whey, wheat/cotton, and poultry litter, these conditions seemed to trigger a very high portion of vegetative cells

to sporulate. These, ratios could be preferred for further crystal protein production trials in view of the final cell and spore concentrations compared (e.g. 4.89-5.60 $\times 10^8$ CFU/ ml and 3.87-4.14 $\times 10^8$ CFU/ ml, respectively for carbon 10 g/ l legume and NYSM media). These values also indicated that protein concentration approached 22.96 - 28.75 µg/ ml. Thus, full-sporulation seemed to be achieved under this condition.

Ozkan *et al*., (2003) had studied the effects of increasing concentration of the nutrients in glucose-yeast extract medium on growth, sporulation and endotoxin formation in batch cultures of *Bacillus thuringiensis* subspecies *kurstaki* HD-1. They found that spore counts were increased from 1.08 $\times 10^{12}$ to 7.36 $\times 10^{12}$ spores/ mL and toxin level from 1.05 mg/ mL to 6.85 mg/ mL, when the concentration of glucose was increased from 8 to 56 g/L, with the corresponding increase in the rest of medium components. Based on their approach, I cultured *Bacillus thuringiensis* in carbon sources higher than 4:1 as well. However, when 10 g/ l wheat bran/cotton seed and 10 g/ l poultry litter were used, the growth was significantly inhibited and feint 110-120 kDa and 60-70 kDa *Bacillus thuringiensis* specific bands could be observed in SDS-Polyacrylamide gel electrophoresis.

When 10 g/ l each of legumes, potato and whey-based media were next tested at the same carbon source ratios as was made for 10 g/ l wheat/cotton and poultry litter, it showed different behaviors at the same conditions. While cell growth, and sporulation seemed to increase until 72nd hour in legume media, no more increase in numbers of cells and spores

was detected after 48th hour, in wheat/cotton and poultry litter media with carbon source at the same levels but decreased cell growth stopped at around 48th hour and death phase started. In spite of these, still 110-120 kDa and 60-70 kDa *Bacillus thuringiensis* toxin bands were visualized by SDS-polyacrylamide gel electrophoresis. Yet, the crystal protein level seemed to be comparable to those obtained from those of the 10 g/ l NYSM medium.

At the range of 10 g/ l NYSM carbon source decrease after 60th hour in the numbers of vegetative cells and spores were detected which was the case at 10 g/ l legume medium carbon with this low cost substrate. In addition, the expression levels of the 110-120 kDa and 60-70 kDa crystals are similar. When the entire quantitative toxin data for legumes, potato and whey-based media varying carbon source were compared, it was determined that the crystal protein concentrations had the highest value in 10 g/ l legume based medium.

The growth of *Bacillus thuringiensis* isolates were satisfactory in three media namely, legumes, potato and whey-based media and comparable to that of conventional medium, NYSM. These results suggests that local *Bacillus thuringiensis* isolates are capable of utilizing the carbon and nitrogen present in these media for growth even though these are not present in a readily usable form. Isolate 24LBN30°C cultured in legume media, it recorded an OD 600nm of 2.398. Addition of readily utilizable carbon and nitrogen supports high initial growth of *Bacillus thuringiensis* after inoculation, whereas that

available in legumes, potato and whey is used for subsequent growth. This indicates that legumes, potato and whey is not only a nutrient source but also provides the consistency suitable for providing sufficient air supply to support *Bacillus thuringiensis* growth.

37 °C was the most suitable for the maximum sporulation and toxicity of *Bacillus thuringiensis*. Regarding temperature effect, results pointed to the importance of incubation temperature which regulated the biosynthesis of Crystal proteins complements. KARI 37 had unique toxin production with OD 600nm not recording significant growth difference at 20 °C, 30 °C and 37 °C. 20 °C is a temperature generally considered to be low to permit a good growth in Bacillus spp. (El-Bendary, 2006) this is due to its isolation location where environmental factors in Nairobi area play a critical role in modulating the differentiation pattern and synthesis of toxins which form a distinct group of secondary metabolites.

Growths of the other isolates were optimal at 37 °C and 30 °C, respectively. This optimum temperature seems to concur with early findings of the studies carried out on *Bacillus thuringiensis* subspecies *kurstaki* spore production in batch culture using broiler litter extracts as complex media, Adams *et al*. (1999) recorded pH levels of 7.0-9.4 with an optimum temperature of 30 °C. In a later report, highest crystal protein yields were recorded when temperature was set at 30 °C while pH was adjusted to 7.2 after studying the production of *Bacillus thuringiensis* on standardized wheat bran based media in 250ml Erlenmeyer flasks (Devi *et al*., 2005). Similar results (pH 7 and temperature 30 °C) were

also reported by Ghribi *et al*. (2007) after researching on the use of sea water as salts source in starch- and soya bean-based media, for the production of *Bacillus thuringiensis* bioinsecticides.

All the isolates grew maximally at pH ranges of between 6.5-7.5. At pH 6.5 the isolates; KARI 46, KARI 20, KARI 53, ISKAG37°C, 58SLA25°C, and KARI 14 recorded fairly high OD 600nm values while pH 7 was suitable for growth of isolates KARI 37, 62LBG37°C, KARI 30, KARI 21, KARI 47, 63KAG37°C, 14SLA30°C, 24LBN30°C, KARI 54 and KARI 14. For pH 7.5, it was also optimum for growth of isolates; KARI 46, KARI 37, 62LBG37°C, KARI 20, KARI 21, KARI 47KARI 53, 24LBN30°C, and 58SLA25°C. Obeta and Okafor (1984) found out that crystal protein production was more efficient in buffered medium, especially when the initial pH was adjusted to 7.4 and temperature 32 °C. Results obtained from Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of Bacillus isolated from Soumbala and Bikalga, African alkaline fermented food condiments showed that 37 °C was the best for fermentation (Ouoba *et al*., 2008). Lastly, Zouari *et al.* (2002) and Yezza *et al.* (2007) found pH 7 and temperature of 30 °C to be adequate in different studies on the scale-up of biopesticide production processes using wastewater sludge as a raw material and production of delta-endotoxins by *Bacillus thuringiensis* strains exhibiting various insecticidal activities towards Lepidoptera and Diptera in gruel and fish meal media respectively. Therefore, optimum pH growth conditions determined for the growth of *Bacillus thuringiensis* isolates

will be helpful in getting best activity of the isolates, as conditions like pH play active role in determining their toxicity.

Growth of *Bacillus thuringiensis* on legume, potato and whey-based media enriched with synthetic minerals were the most efficacious in terms of toxin content (20.61 - 25.79 µg/ ml) and spore yield (4.89-5.60 $\times$ 10^8 CFU/ ml) when compared to the conventional laboratory medium (NYSM) toxin content 22.96 - 28.75 µg/ ml and spore yield 3.87-4.14 $\times$ 10^8 CFU/ ml. A maximum spore count of 3.87-4.14 $\times$ 10^8 CFU/ ml in NYSM medium, 4.89-5.60 $\times$ 10^8 CFU/ ml was observed in legume medium, followed by 2.45-3.25 $\times$ 10^8 CFU/ ml in potato medium, then 1.72-2.44 $\times$ 10^7 CFU/ ml in whey medium. Legume medium recorded the highest optical density values from isolates; 58SLA25°C, 62LBG37°C, 24LBN30°C, KARI 54, KARI 37, KARI 12 and KARI 21 spore counts were between 4.89-5.60 $\times$ 10^8 CFU/ ml with isolate 58SLA30°C having recorded 27.60 µg/ ml protein concentration. This compares with the findings of Obeta and Okafor (1984) on a suitable legume medium for crystal protein production who reported spore counts of 0.85-5.08 $\times$ 10^9 CFU/ ml. In order to produce, more economically bioinsecticides of *Bacillus thuringiensis*, NYSM was substituted by using 10 g/ l each of legumes, potato and whey. Results obtained with the different strains and isolates revealed that this medium is suitable for growth of *Bacillus thuringiensis* as reflected by the 4.89-5.60 $\times$ 10^8 CFU/ ml spore count and delta-endotoxin production.

Using local substrates as nitrogen source improved significantly the spore counts and delta-endotoxin production compared to that obtained in NYSM. As they are balanced media they do not need other nutrients for culturing the degrading bacteria. This fermentation technology facilitated the complete utilization of legumes, potato and whey, by avoiding any kind of resultant residual loss or wastage, resulting in an enhanced production of biopesticides and maintaining a cleaner environment. This study is, of much relevance, as it upholds the dual benefits of complete utilization of locally available agricultural and industrial produce from the environment and enabling the production of *Bacillus thuringiensis* delta-endotoxins.

The mean maximum spore counts differed significantly between media (F=14.54, df (12), $P \leq 0.05$). The mean spore count of legume medium is significantly higher than that of the standard, NYSM, (Post hoc Duncan's multiple range comparison test, $P \leq 0.05$), whereas the spore counts for wheat bran/cotton seed ($0.83\text{-}1.34 \times 10^7$ CFU/ ml) and poultry litter recorded ($0.64\text{-}0.83 \times 10^7$ CFU/ ml) were significantly lower than that of standard ($p \leq 0.05$). Legume medium showed maximum yield of spores (5.60×10^8 CFU/ ml) with reference to *Bacillus thuringiensis* this is due to its composition of high amounts of carbon and nitrogen as compared to other media and also the addition of yeast extract. Maximum spore production of *Bacillus thuringiensis* was achieved by isolate KARI 14 tested in legume medium. This is because the nutritional requirement of different strains of *Bacillus thuringiensis* is known to be variable. All isolates cultured in wheat/cotton and poultry litter

media produced low spore counts inefficient for biolarvicidal tests. Isolate 58SLA25°C only managed to produce 4.00×10^7 CFU/ m which which indicates that sporulation was hindered by lack of manganese, magnesium and calcium while previous studies have shown that high amounts of iron, zinc and copper negatively influence sporulation which could also be the case here (Ozkan *et al*., 2003).

The results produced from protein concentration content from fermentation of *Bacillus thuringiensis* isolates in legume and NYSM media recoded 22.96 - 28.75 μg/ ml values. It was observed that all the isolates were able to produce delta-endotoxins with isolates 58SLA25°C, KARI 30 and 14SLA30°C recording promising results. It was found that wheat/cotton and poultry litter media failed to produce appreciable levels of delta-endotoxins. It was noted that, the higher the isolate growth in media, the higher the amount of spore counts and delta-endotoxins in the same media for example, isolates cultured in legume and potato media recorded 18.64 - 25.79 μg/ ml protein concentration levels. Another observation noted was that protein concentration determined from wheat bran/cotton and poultry litter were appreciable although not significant, this is remarkable considering that protein concentrations from the same media were 12.45 - 14.53 μg/ ml. Growth of *Bacillus thuringiensis* on legumes, potato, and whey enriched with mineral supplements was the most efficacious in terms of toxin content and these results compares with *Bacillus thuringiensis* cultured on wheat bran medium without enrichment of carbon and nitrogen producing 26mg toxin. This indicates that legume, potato and whey-based

media is not only a nutrient source but also provides the consistency suitable for providing sufficient air supply to support optimal *Bacillus thuringiensis* delta-endotoxins production. An additional benefit is that the medium can be used for formulation of *Bacillus thuringiensis* as an inert carrier. With proper pre-treatment and mineral supplements, media from low-cost raw materials have the potential to be an excellent medium for the growth, sporulation and delta-endotoxin production for *Bacillus thuringiensis*.

The pH curve during the cultivation of *Bacillus thuringiensis* on a medium consisting of legumes, potato and whey has a typical pattern with decline in pH caused by the production of acetic acid during log phase of fermentation, followed by increase to pH level close to an initial pH as acetic acid is consumed. The cultivation with initial pH of 5 to 6 did not produce any spore-crystal complex consumed due to cell death at low pH. The culture medium brought to neutral pH at inoculation produced 18.64 - 25.79 µg/ ml from isolate 24LBN30°C cultured in legume medium.

The normal temperature for growth and toxin production of *Bt* is 30 °C. Ozkan *et al*., (2003) found that Cry4Ba synthesis by *Bti* HD500 was the best when the organism was grown at 25 °C, whereas Cry11Aa synthesis was optimal at 30 °C. In comparable to these finding, overall protein production of *Bacillus thuringiensis* in NYSM was comparable to that of legume medium and the mean values were significantly different ($P \leq 0.05$). This study indicates that optimum levels for delta-endotoxin production are 37 °C and pH 6.5-

7.5 for these conditions regulate the biosynthesis of locally isolated *Bacillus thuringiensis* delta-endotoxins complements.

Microscopic observation of *Bacillus thuringiensis* (Fig. 1) spores and crystals obtained from NYSM and test media after 72 h culture of growth indicates the sporulation in test media was comparable to NYSM. Isolates cultured in all media produced a characteristic two-stage process of vegetative growth and spore-crystal production. Every cell sporulating also appeared to produce a bipyrimidal parasporal crystal. This process of multiplication lasted for 72 h followed by lysis of the cells, which released the spore/crystal toxin complex (endotoxins) into the medium. Appreciable levels of sporulation were seen in other test media.

The protein profile of *Bacillus thuringiensis* produced from conventional and new culture media were analyzed by SDS–PAGE and the results were compared. Twenty five micrograms of *Bacillus thuringiensis* extracts were analyzed for protein profile. The major polypeptides present in the parasporal crystal proteins of *Bacillus thuringiensis* were clear and conspicuous. The bands produced from KARI 47, 62LBG37°C, 63KAG37°C, KARI 12 and KARI 37 in wheat bran/cotton seed and poultry litter media offered a feeble visibility due to possibility of a lesser degree of crystal proteins compared to that of legume/potato/whey induced banding patterns. There is no variation in the protein pattern between the toxins produced from the conventional and test media. The major polypeptides

of *Bacillus thuringiensis* spore/crystal complex produced from wheat bran/cotton seed and poultry litter media and NYSM were 110-120 kDa and 60-70 kDa.

The 110-120 kDa and 60-70 kDa polypeptides present in the spore-crystal complex of *Bacillus thuringiensis* were produced from Legume medium (1SKAG37°C and 24LBN30°C), NYSM medium (58SLA25°C and KARI 20), potato medium (58SLA25°C and KARI 30) and whey medium (14SLA30°C and 24LBN30°C). The protein profiles as indicator of *Bacillus thuringiensis* delta-endotoxins were correspondingly related to their larvicidal activity. When compared to high molecular weight standards in SDS-PAGE analysis, the solution and pellet of the dissolved spore-crystal product from each treatment had proteins with molecular weights of approximately 135 kDa produced by *Bacillus thuringiensis* subspecies *kurstaki* (Adams *et al*., 1999). Crystal proteins from different isolates share a number of common features. The most obvious of these has already been pointed out; namely, they usually share a common crystal shape and contain proteins in a common size range (110–120 kDa). The protein subunits are protoxin molecules that are converted to a toxic form after ingestion by a susceptible insect. Upon ingestion, the crystal becomes soluble in the insect midgut and is then activated, via proteolytic cleavage, to a toxin (mol wt = 68 kDa). The evidence suggests that the proteases responsible for toxin activation are insect-derived (Schnepf *et al*., 1998).

At 72 h, many spores and delta-endotoxins were outside the sporangium and separation of ICPs by SDS-PAGE was efficient, probably accounting for the clear and conspicuous protein bands in the ICP sample. The 60-70 kDa proteins were enriched in the course of fermentation but the 110-120 kDa protein did not evolve in the same way. The decrease in intensity of the 110-120 kDa protein in the course of fermentation may have resulted from its proteolytic cleavage to 60-70 kDa and to other fragments, which is known to occur during activation of this protoxin. At 72 h, all of the delta-entoxins were present in significant concentrations, possibly explaining why toxicity to *Chilo partellus* first-instar larvae became greater after this time. In view of the results, legumes, potato and whey turned out to be the best choice, 72 hour being the most appropriate time of incubation. These media have sufficient nutritional supplements for production of delta-endotoxins from local *Bacillus thuringiensis* strains namely, 110-120 kDa and 60-70 kDa as proved by this study.

Moreover, *Bacillus thuringiensis* delta-endotoxins from legumes, potato and whey-based media yielded significant cumulative larval mortality of 40 % at 28 h after treatment, and 73 % by 48 h. The comparative toxicities of *Bacillus thuringiensis* delta-endotoxins produced from test media and NYSM were shown in Table 10. The LC50 values against *Chilo partellus* for *Bacillus thuringiensis* were between 13-74 µg/ l and for, the standard, *Bacillus thuringiensis* subspecies *kurstaki* HD-73 was 84 µg/ l which were statistically similar (fiducial limits overlapping). The *Bacillus thuringiensis* delta-endotoxins produced

from legume, potato and whey-based media were effective against first-instar *Chilo partellus* larvae tested and found to be equally comparable to the toxins produced from *Bacillus thuringiensis* subspecies *kurstaki* HD-73. Paralysis was observed in within 2 h at a 0.015 mg/ ml concentration of each bacterial powder, including *Bacillus thuringiensis* subspecies *kurstaki* HD-73. The effects on the larvae were severe at 0.015 mg/ ml concentration; the larvae merely gathered at the top center of the assay cup and stopped feeding. At 24 h, approximately 40 % mortality was observed in the larvae at a concentration of 0.015 mg/ ml in all three locally produced *Bacillus thuringiensis* powders.

The average mean larval mortality in first-instar *Chilo partellus* larvae during the same period and at the same concentration was 74 % at 48 h. Maximum numbers of dead *Chilo partellus* first-instar larvae were recorded in all the *Bacillus thuringiensis* powders at 48 h. Increase in larval death after this period was very high. The powder from the control medium produced 83 % kill in assay cups containing 0.015 mg/ ml after 48 h in three separate experiments. The concentrations required to kill 50% of the larvae (LC50) indicated that three locally produced *Bacillus thuringiensis* powders compared favorably with *Bacillus thuringiensis* subspecies *kurstaki* in the first-instar *Chilo partellus* assays.

Isolates 58SLA25°C, KARI 30, KARI 47, KARI 54 and 62LBG37°C recorded satisfactory toxicity to *Chilo partellus* while other isolates showed considerable toxicity which could be enhanced with specific alterations in the fermentation designs. The toxicity is due to the

binding of the active delta-endotoxins to specific receptors present in the mid-gut brush border membrane. The delta-endotoxins were ingested along with the food material, by the *Chilo partellus* larvae and after solubilisation and proteolytic cleavage; the activated toxin interacted with the midgut epithelium, leading to the death of the larvae. Earlier reports indicated that the formation of toxic parasporal bodies in *Bacillus thuringiensis* subspecies *israelensis* generally occurs two to three hours after the end of exponential growth and during sporulation (Devi *et al*., 2005). The process of growth, sporulation, and toxin synthesis, we observed conform to these reports. Other authors have shown variability in aspects of *Bacillus thuringiensis* subspecies *israelensis* production using media derived from various nutrient sources.

Desai and Shethna (1991) used three fermentation media for bulk growth of *Bacillus thuringiensis* subspecies *israelensis* formulated using defatted groundnut cake (*Arachis hypogea*) as the first nitrogen source and gram flour (*Cicer arietinum*), soybean (*Glycine max*), and defatted milk powder as the second nitrogen source. The latter medium containing gram flour showed highest toxicity (LC50 14.45 µg/ l). Luna *et al*., (2004) re-used the supernatant arising after biomass separation of *Bacillus thuringiensis* subspecies *israelensis* by flocculation/sedimentation and supplemented it with 25, 50, and 75% (w/v) of the original culture medium, based on corn steep liquor, glucose, and mineral salts. This supplementation at 75% gave a spore concentration (1×10^{10} CFU/ ml) five times greater than that obtained with the other supplements (Luna *et al*., 2004).

A medium containing glucose solution, corn extract, sodium humate, and mineral salts resulted in an increase in biomass titer by 45%, endotoxin by 220% as compared to the initial medium (Dregval *et al*., 2002). These authors also found that a combined carbohydrate source consisting of soluble starch and cane sugar molasses, irrespective of the source of protein in the media, drastically reduced delta-endotoxin production, thereby reducing the potency of the primary products. Fermentation studies in shake flasks containing standard nutrient broth and soybean waste took 37 h, rice bran 50 h, soybean, grated coconut waste, and fish meal 50 h, standard nutrient broth 55 h, and grated coconut waste 28 h, respectively for the *Bacillus thuringiensis* subspecies *israelensis* to develop spores and crystals (Lee and Seleena, 1991). In Brazil, soya has been reported as a reference medium for the large scale production of *Bacillus thuringiensis* - based larvicides in studies where sewage sludge was used as a source for production of *Bacillus thuringiensis* (Tyagi *et al*., 2002). Results obtained with the different isolates in this study revealed that these media are suitable for production of *Bacillus thuringiensis* delta-endotoxins effective against first-instar *Chilo partellus* larvae.

In conclusion, legumes, potato and whey-based media is not only a nutrient source but also provides the consistency suitable for providing sufficient air supply to support Bt growth and sporulation. Results obtained with the different strains and isolates revealed that these media are suitable for growth of Bt as reflected by 1.81-2.40.

This study developed and studied a new Bt spore product culture medium formulized from legumes, potato and whey with appropriate efficacy (4.89-5.60 $\times$ 10^8 CFU/ ml). Its advantage is that it can be produced through the use of an industrial by-product at a relatively low price.

This study indicates that optimum levels for delta-endotoxin production are 37 °C and pH 6.5-7.5 for these conditions regulate the biosynthesis of Crystal proteins complements. Therefore, optimum pH growth conditions determined for the growth of Bt isolates will be helpful in getting best activity of the isolates, as conditions like temperature and pH play active role in determining their toxicity.

This study is, of much relevance, as it upholds the dual benefits of complete utilization of locally available agricultural and industrial produce from the environment and enabling the production of Bt delta-endotoxins. In view of the results, legumes, potato and whey turned out to be the best choice, 72 hour being the most appropriate time of incubation. These media have sufficient nutritional supplements for production of delta-endotoxins from local Bt strains namely, 110-120 kDa and 60-70 kDa as proved by this study.

Results obtained with the different isolates in this study revealed that these media are suitable for production of *Bacillus thuringiensis* delta-endotoxins effective at the 0.015

mg/ml concentration where isolate 58SLA25°C, recorded 73 % mean larval mortality at 48 h against first-instar *Chilo partellus* larvae.

Developing countries should search for new avenues to reduce costs for development of pesticides to counter the ever increasing insect pests. More formulations and trials should be done with other low cost media supplements.

These results form a basis for further investigation of the local *Bacillus thuringiensis* isolates showing significant efficacy against *Chilo patellus* such as determination of the cryotoxins therein and how temperature and other abiotic factors would affect their toxicity. It is also recommended that the toxicity of these isolates be investigated against other local Lepidopteran pests in order to determine their target range.

The null hypothesis that growth, sporulation and protein production by local *Bacillus thuringiensis* isolates is not influenced by the carbon-source is therefore rejected.

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APPENDICE

Appendix 1: Low cost media constituents

Basal medium: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; and $CaCO_3$, 1.0 g/ l.

Medium A: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and 10.0 g of poultry litter.

Medium B: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and 10.0 g of legume seeds consisting of a mixture of groundnut cake (*Arachis hypogea*), cow pea (*Vigna unguiculata*, white variety), soya beans (*Glycine soja*), cow pea (*Vigna unguiculata*, black variety), and njugumawe/jugo beans (*Voandzeia suberranean*).

Medium C: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and10.0 g of Wheat bran extract mixed with cotton seed meal.

Medium D: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and 10.0 g of Nutrient Yeast extract (HiMedia Lab, India) Synthetic Medium (NYSM)

Medium E: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and 10.0 g of potato broth.

Medium F: Cow blood, 10.0 g/ l; $MnCl_2.4H_20$, 0.02 g/ l; $MgSO_4.7H_20$, 0.05 g/ l; $CaCO_3$, 1.0 g/ l and 10.0 g of Whey.

Appendix 2: Synthetic chemicals

Nutrient agar (HiMedia Lab, India) (HiMedia Lab, India) Medium

Component	**Concentration (g/ l)**
Nutrient broth (HiMedia Lab, India)	8
Agar (HiMedia Lab, India)	15

The pH was adjusted to 7.3 and the medium sterilized at 121°C for 15 min.

TE Buffer

10 mM Tris HCl

1 mM EDTA

pH 8

Bradford reagent (Bradford, 1976)

100 mg Coomassie brilliant blue G will be dissolved in 50 ml absolute ethyl alcohol and mixed for 6 h (or overnight) with light protection. 100 ml of 85 % orthophosphoric acid will be added to this mixture and stirred for another 6 h (or overnight). The required volume from this stock will be diluted to the desired volume with distilled water.

Basic Fuchsin Stain

Basic Fuchsin stain was prepared by mixing solution A and solution B. Solution A was prepared by dissolving 0.3 g of basic Fuchsin stain (90% dye content) in 10 ml 95% ethyl alcohol. Solution B was prepared by dissolving 5 g of phenol in 95 ml of water.

Stacking Buffer

5 g of Trizma base will be dissolved in 80 ml distilled water, pH will be adjusted to 6.8 with concentrated HCl then the volume will be completed to 100 ml.

Buffer

36.6 g Trizma base

48 ml of 1 N HCl

Diluted to 100 ml with distilled water

Running Buffer

30 g Trizma base

144 g Glycine

5 g SDS

Volume will be made up to 5 L with distilled water.

Loading Buffer

Autoclaved glycerol	5 ml
10 % SDS	0.5 ml
β- mercaptoethanol	0.5 ml
Stacking buffer	2.5 ml
Sterile dH_2O	11.5 ml
Bromophenol blue	10 mg

The chemical constituents for preparation of the SDS-PAGE gels

	Stacking gel	**Separating gels**
	12 %	**3.98 %**
45 %	2 ml	9 ml
Stacking buffer	5 ml	12 ml
dH_20	12 ml	24 ml
Ammonium persulfate	5 mg	20 mg
10% SDS	200 µl	450 µl
TEMED	25 µl	50 µl

Staining of SDS-Polyacrylamide Gel (Baum *et al.*, 1990):

Step	**Solution**	**Time of treatment**
Fixation	50 % Methanol	o/n
	12 Acetic acid	
	0.05 % Formaldehyde	
Washing	50 % Ethanol	3 × 20 min
Pre-treatment	0.29 g/ l $Na_2S_2O_3$	1 min
Rinse	dH_2O	2 × 20 sec
Impregnate	2 g/ l $AgNO_3$	20 min
	0.75 ml/L Formaldehyde	
Rinse	dH_2O	2 × 20 sec
Development	60 g/ l Na_2CO_3	1-5 min
	0.5 ml/ l Formaldehyde	
	4 mg/ l $Na_2S_2O_3$	
Rinse	dH_2O	2 × 20 sec.
Stop	50 % Methanol	
	12 Acetic acid	
Store	50 % Methanol	