MOLECULAR CHARACTERIZATION OF *Bacilllus thuringiensis* STRAINS WITH DIFFERENTIAL TOXICITY TO *Chilo partellus*

BY

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LIST OF ABBREVIATIONS

α	alpha
β	beta
δ	delta
Bt	*Bacillus thuringiensis*
bp	Base pairs
BSA	Bovine serum albumin
Cry	Crystal
EDTA	Ethylene diamine tetra acetic acid
EMBL	European molecular biology laboratory.
GDP	Gross domestic product
GM	Genetically modified
ICIPE	International Centre for Insect Physiological and Ecology
ICP	Insecticidal crystal protein
IEBC	International Entomopathogenic Bacillus Centre
LB	Luria bertani
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
Tris- HCl	Tris-hydrochloric acid
TAE	Tris Acetate EDTA
UV	Ultra violet

UNIT ABBREVIATIONS

%	Percentage
µg	microgram
µl	micro liter
µm	micro meter
g	gram
hr	hour
k Da	Kilo Dalton
L	Liter
M	molar
mg/ml	milli gram/ milli liter
min	minute
mA	milli Amperes
ml	milli litre
mM	milli Molar
MT	metric tonnes.
ng	nanogram
nm	nano metre
°C	degrees centrigrade
r.p.m.	revolutions per minute
sec	Second
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/ weight

ABSTRACT

Bacillus thuringiensis (*Bt*) is one of more than 20 species of soil growing Bacilli, it is an ubiquitous gram positive, aerobic, spore- forming organism that forms parasporal crystals during the stationary phase of its growth cycle. *Bt* was initially characterized as an insect pathogen, and its insecticidal activity was attributed largely or completely depending on the insect and the parasporal crystals. This study addressed the need to characterize three *Bt* isolates with varying levels of toxicity to *C. partellus* using molecular characterization techniques. The intention was to establish the basis for differential toxicity to *C. partellus* and to determine if the unique bands and other properties can be used to screen other isolates. Growth rate of *Bt* isolates were determined by inoculating the isolates in separate flasks containing (Luria Bertani) LB broth then incubated at 37 ºC while shaking at 200 rpm. The absorbance at 600nm was then read at intervals of every 6 hrs from 0 hrs to 72 hrs. 1M isolates had a slightly elevated growth rate at the lag phase and exponential phase of growth than both K10-2 and V24-M isolates, although at the plateau phase the growth seems to be equal for all the three isolates. Smirnoff staining protocol was used to detect the level of crystal and spore formation over a period of 72 hrs. All crystals were bi-pyrimidal in shape and stained with an almost black luster with lilac blue tint. The spores stained pink, whereas bacterial cells and their fragments assumed a light lilac tint. In the process of *Bt* isolate growth; secreted protein concentrations were determined using the Lowry assay and absorbance taken at 750nm. The three *Bt* isolates showed varying levels of protein concentration with 1M isolate showing a significantly higher concentration than both V24-M and K10-2. Analysis of protein profiles using SDS- PAGE for the *Bt* isolates at time intervals of 54 hrs, 60 hrs and 72 hrs were performed respectively. The major protein bands in this study were of molecular weights, ~28kDA, ~65kDA and ~130kDA in all the isolates except isolates 1M that showed two protein bands of molecular weight ~28kDA and ~65kDA. Restrictions of plasmid DNA of the *Bt* isolates reveal unique bands in all the different isolates when digested with EcoRI, BamHI and HindIII. One particular unique band that was characteristic to all the digests were one of molecular weight ranging between 7,000 to 7,200bp. This was a unique band that was characteristic of all the toxic *Bt* strains and hence a crucial indicator of effective toxicity among *Bt* isolates in this study.

In conclusion growth rates, protein determination and restriction digestions on their own did not reveal much information on which *Bt* isolates that are most toxic. However when the techniques are used collectively they have proven to be effective tools in the characterization of different *Bt* isolates hence should be incorporated instead of lengthy and expensive bioassays

1.0 INTRODUCTION

1.1 Background information

Agriculture is the mainstay of Kenya's economy as it contributes over 25% of the gross domestic product (GDP). The agricultural sector provides food directly and employment indirectly to about 70% of the total work force in Kenya. Kenya has a wide range of climatic conditions that enables the growing of a wide variety of crops ranging from tropical to sub-tropical varieties. The country also has unique soils that are very fertile (Smaling and Nandwa, 2003).

Staple foods produced in Kenya comprise among others maize, pulses (beans, peas, and lentils), rice, wheat, millet and sorghum. Maize is the main staple food of Kenya, averaging over 80 percent of total cereals in the Kenyan diet, with a per capita consumption of 98 kilograms (Muchena *et al,* 2005).

Currently Kenya is facing a major food deficit attributed mainly to pre and post harvest crop losses, especially for graminaceous crops such as maize, rice, wheat and sorghum. In addition to drought and land degradation, a major threat to increased food production is damage to crops by insect pest both in the field and during storage. These are the major constraints to efficient cereal production in developing countries (Brownbridge, 1991).

Among the lepidopterous pests, the spotted stem borer (*Chilo partellus*) and the maize stem borer (*Busseola fusca*) rank as the most important stem borer pests of sorghum and maize in Africa. They are predominant borer species of economic importance in Eastern Africa and Southeast Asia and are a major constraint to increased productivity as they are of economic importance in most maize- growing countries throughout the world. They feed exclusively on graminaceous plants whereby heavy infestations may result in severe crop losses and hunger, given that maize and sorghum are staple foods in the East Africa region and mostly grown by subsistence farming (Brownbridge, 1991). The global losses due to all insect pests are 9%, equivalent to 52 million metric tonnes (MT) of maize, valued at $ 550 million. Losses associated with lepidopteran pests that can be controlled by crystal protein 1 Ab (cry1 Ab), are estimated to cause losses of 4.5% equivalent to half the total losses from insect pests of maize (Clive, 2005). However control measures have largely been based on chemical pesticides. Important problems

have however confronted the use of chemical pesticides. Non- degradable chemical residues accumulate to high levels in the environment. These chemicals find their way into the rivers therefore endangering the fauna and flora in these environments. Chemical pesticides have also disrupted resident natural enemies and allowed the development of secondary pests. Increased use of chemical pesticides also results in resistance of the target insects to chemical pesticides. They have also created an ecological imbalance through the destruction of beneficial non- target insects, and all are highly toxic to man. Furthermore, chemical pesticides have to be imported, hence expensive and are often unavailable to the small- scale farmers who form the bulk of the food producers in Africa. Alternatives have been sought, and the search is on for sociologically acceptable, sustainable and environmentally safe integrated pest management (IPM) strategies for these key crops. These includes cultural, chemical and biological control in order to come up with a more economic, sustainable and ecologically sound approach to pest management. Recent promising progress in agricultural entomology and microbiology demonstrates that there is a considerable unexploited potential. The microbial pesticides based on *Bt* can in an integrated control programme be used rapidly and efficiently to destroy insect pests (Dulmage, 1993). *Bt* has been exploited for more than 40 years to control agriculturally and medically important pest and disease- vector insects. In fact, *Bt* and Cry toxin constitute one of the most important environmentally compatible biological pesticides. Formulations of *Bt* pesticides are not toxic to vertebrates and non- target organisms and are widely used by organic farmers to ward off insect pests. Insecticidal Cry toxin genes have been incorporated into several major crops, where they provide a model for genetic engineering in agriculture.

The most distinctive property of *Bt* is its entomopathogenicity (insect pathogenicity) and production of insecticidal Cry toxin proteins that accumulate in the mother cell as crystalline inclusions during sporulation of the bacterium. The production of Cry toxins in the form of parasporal inclusions is a unique genetically regulated biological phenomenon that, probably, is related to offsetting dehydration during spore formation and affords an additional survival advantage by exerting pathogenic action that kills host insects. In turn, killing its host provides sufficient nutrients to allow germination of the dormant bacterial spore and its return to vegetative growth.

The entomopathogenicity of *Bt* spans a number of insect species including moths, mosquitoes, black flies, beetles, hoppers, aphids, wasps and bees as well as nematodes. *Bt*

entomopathogenicity involves various virulence factors common to many bacteria; However, Cry toxins can exert lethality without the presence of viable bacteria. The lethal action of Cry toxins is mediated by a specific cadherin receptor, BT-R1, which has been identified in the mid-gut of various lepidopteran larvae (Vadlamudi *et al.,* 2005). In host insect larva, toxin action destroys the mid-gut epithelium and brings sepsis, and ultimately death of the organism.

The action of Cry toxin in the entomopathogenicity of *Bt* appears to share a common mechanism with other bacterial pathogens that target host cell adhesion molecules to overcome or evade epithelial barriers in their hosts. Until the 1950s, relatively few people considered resistance to pesticides to be a serious threat to pest management. Although some insects had evolved resistance to DDT, the prevailing feeling was that resistance could be overcome by using ever-newer pesticides. However, in the 1970s, it became clear that at least some pests were evolving resistance faster than new and environmentally acceptable pesticides could be developed and brought to market. Indeed, two of the first three genetically modified *Bt* crops registered in the USA, cotton and potatoes, were targeted at markets essentially created by the recurrent evolution of resistance to insecticides in certain pests (Roush, 1997).

The concept of resistance management began to evolve in the late 1950s. The aim of resistance management programmes is to "slow the evolution of resistance and thereby extend the useful life of valuable toxicants" (Roush, 1997). There is no doubt that the potential development of resistance poses a significant challenge when attempting to develop effective insecticides or other means of control. Experience with conventional breeding to enhance insect resistance in crops and particularly experience with developing insecticides to control insect pests of cotton supports the cases that an insect management strategy is essential in order to preserve the durability of product effectiveness, irrespective of the source or mode of control. In the specific case of cotton pests and *Bt* there is ample evidence that cotton bollworm, *Helicoverpa armigera* as well as other lepidopteran pests have developed resistance to multitude of insecticides (Clive, 2005). Resistant to topical *Bt* spray applications has also developed in field populations of diamond black moth (Tabashink, 1994). Resistance management strategies try to prevent or diminish the selection of the rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently low for insect control. Strategy development generally relies heavily on theoretical assumptions and on computer models simulating insect population growth under various conditions. Proposed strategies include the use

of multiple toxins (stacking or pyramiding), crop rotation, high or ultra-high dosages, and spatial or temporal refugia. It is only recently that some of the proposed tactics been experimentally evaluated on a small scale. Retrospective analysis of resistance development does support the use of refugia. It is clear that the real value of the different proposed tactics can only be tested in larger-scale field trials (Tabashnik, 1994).

1.2 Microorganisms pathogenic to insects

The groups of microorganisms pathogenic for insects are varied and diverse. Among these are a broad range of viruses, bacteria and fungi. Each of these sub groups is composed of a spectrum of organisms that vary in their mode of infection, site of replication and mechanisms of pathogenicity. The major species of bacteria with mechanisms to infect and kill uncompromised, healthy insects are the spore-forming bacilli (*Bacilli thuringiensis* and *Bacillus sphaericus*) producing protoxin during sporulation. For these species, the hemolymph of insect larvae is an excellent nutritional environment for bacterial proliferation and sometimes for sporulation. However, some of these entomopathogens lack some of the attributes that would make them desirable as microbial control agents. An entomopathogen should have high pathogenicity, be able to persist in the environment of the host, have high transmission efficiency, be easy to produce and be safe to non- target fauna and flora. In the case of entomopathogenic fungi, several have being tested and commercial products have appeared in the market (Mc Coy, 1990).

1.3 Advantages and disadvantages associated with *Bt* Usage

Bt usage has several advantages and disadvantages such as production of stable spores that are readily formulated for use in conventional pest control and are remarkably safe for humans, other mammals and non- target fauna. Furthermore *Bt* has no phytotoxicity (Dulmage, 1993) and *Bt* resistance has been selected only in laboratory populations. Only *Plutella xylostella* (Black diamond moth) is the insect species in which very considerable resistance has been found to develop in the field. The low incidence of evolved resistance in the field is associated with the presence of multiple toxin genes in the individual *Bt* strains (Tabashanik, 1994). Additionally due to their demonstrated safety they are exempted from restrictions hence their use is permitted to the date of harvest, which is important for plant protection in vegetable crops. However

preparations applied to foliage is inactivated by sunlight therefore offering relatively short- term protection from pest populations following single applications. No single isolate is active against all pest species. For control of mosquito and blackfly larva using *B. thuringiensis subsp.israelensis* there is a requirement for formulations that keep the spores or inclusions in the feeding zone of the target pest in aquatic habitats (Tabashanik, 1994).

1.4 Isolation and screening of *Bt*

Several crystal forming *Bt* strains have been isolated from soils collected from various geographical regions in Kenya (Brownbridge, 1991). More than 150 *Bt* strains were isolated at the International Insect Centre for Insect Physiological and Ecology (ICIPE). Studies to screen isolates of *Bt* to elucidate virulent strains, which could be of use in the field were also undertaken with emphasis on the isolation of native *Bt* strains, due to possible objections to wider scale introduction and usage of exotic bacterial strains. Many of the identified exotic strains have been isolated from temperate zones, and may not be well suited for use in a tropical environment (Brownbridge, 1991).

Local isolates could potentially possess better characteristics such as greater field persistence due to adaptation to natural environment and toxicity at a higher temperature range (Brownbridge, 1991). From another study carried out by Brownbridge (1991), whereby they screened exotic and locally isolated *Bt* strains for toxicity to the *Chilo partellus* at ICIPE laboratories, they showed that some local isolates were apparently more toxic than any of the exotic strains obtained and tested. The results further highlighted the differences in the activity of different *Bt* subspecies, and showed that there was variation in the potency of strains within the same subspecies. Thus the origin of the strain appears to affect its toxicity to a particular target species (Brownbridge, 1991).

1.5 Cases of *Bt* biopesticide use in Kenya

In Kenya preliminary experiments using *Bt* based biopesticide have shown that the cereal stalk borer *Busseola fusca* and *Chilo partellus* can be controlled effectively (Kariuki, 1987). *B. thuringiensis* has also been used on coffee in Kenya to control *Ascotis selenaria* (Waikwa and Mathenge, 1977). However *Bt* has not been widely used in insect control programmes in Kenya, partly because of the high cost of commercial formulations from industrialized nations. There are

very few facilities for large-scale production of bio-pesticides operational in Kenya. In 1997 ICIPE established a bio-pesticide demonstration facility to produce *Bt* in Kenya with a view to lowering the costs of *Bt* products by producing them in Africa. However, *Bt* usage *in* Kenya is at its initial stages thus, there is a likelihood of influx of *Bt* based products into our market. *Bt* products represented 3% of the total pesticides imported into the country, which are based on *Bt. kurstaki*. The main target crops for which *Bt* is utilized include; vegetables, cereals, ornamentals and coffee (Ogoyi and Ochanda, 2002).

1.6 Problem statement

The use of *Bacillus thuringiensis* as a biopesticide has increased over the years due to environmental concerns arising from the use of conventional pesticides. However, *Bt* usage *in* Kenya is at its initial stages. The major constraint experienced by the farmers is the comparatively high costs of *Bt* products from industrialized nations compared to conventional insecticides. There are very few facilities for large-scale production of bio-pesticides operational in Kenya. Alongside mass production of bio- pesticides there is a requirement of data from research institutions based on studies conducted locally involving *Bt* isolates indigenous to Kenyan soils. Indeed such information may be crucial in formulation of more effective *Bt* formulations that are more adaptable to the local environment. As well as reducing costs of crop production. Usually imports are not as effective as the locally manufactured isolates due to specificity, greater field persistence resulting to adaptation to natural environment and toxicity at a higher temperature range. According to Brownbridge (1991), whereby they screened exotic and locally isolated *Bt* strains for toxicity to the *Chilo partellus* at International Centre for Insect Physiology and Ecology laboratories. Some local isolates were apparently more toxic than any of the exotic strains obtained and tested. Studies carried out by Wang' gondu and Ochanda (2001) revealed that there are differences in the activity of different *Bt* subspecies, and showed that there was variation in the potency of strains within the same subspecies. This is further illustrated in Table 1

1.7 Justification of the study

This study addressed the reasons for differential toxicities of these *Bt* isolates using molecular techniques. Indeed such information may be crucial in formulation of more effective *Bt* formulations and transgenic *Bt* crops that are more adaptable to the local environment, while taking into account the reality of being in a resource limited setting. The trend is to develop local isolates that may be used for pest control. However, there is need to find an alternative to bioassays which would allow for screening for a large number of isolates as well as developing molecular markers for such screening purposes. As any factor that will directly or indirectly lead to enhancing toxicity may be exploited in developing more potent *Bt* or *Bt* transgenics.

Table 1: Toxicity levels of *Bt* isolates to different corn borers (Wang' ondu, 2001)

***Bt* Isolate**	***C. partellus* %mean larval mortality**	***S. calamistis* % mean larval mortality**	***B. fusca* %mean larval mortality**
1M	100	62	45
VM-10	100	64	40
12F-K	93	47	20
K 10-2	91	31	27
44M	80	73	51
78	78	49	36
V24-M	77	58	44

Table 1 shows the % toxicity levels of different *Bt* isolates to different corn borers based on studies conducted by Wang' ondu and Ochanda (2001).

1.8 Objectives

The aim of the study to establish the molecular basis for selective toxicity of previously isolated *Bt* strains shown to vary in protection levels against *Chilo partellus.*

The specific objectives are:

(a) To determine the growth patterns of the different *Bt* isolates

(b) To determine the profiles of secreted proteins by the three *Bt* isolates.

(c) To isolate and compare the plasmid profiles from the three *Bt* isolates.

2.0 LITERATURE REVIEW

2.1 Historical background.

Bacillus thuringiensis was first discovered in Japan in 1901 by Ishawata and then in 1911 by Ernst Berliner as a pathogen of flour moths from the province of Thuringia, Germany, when a consignment of flour moths sent in from Thuringia was found to be infected by some contagious pathogen. It was first used as a commercial insecticide in France in 1938, and then in the USA in the 1950s. However, these early products were replaced by more effective ones in the 1960s, when various highly pathogenic strains were discovered with particular activity against different types of insect (Baum *et al.,* 1999).

For many years, *Bt* was available only for control of lepidopteran**,** using a highly potent strain namely *B. thuringiensis* var *kurstaki*. This strain still forms the basis of many *Bt* formulations. Further screening of a large number of other *Bt* strains revealed some that are active against larvae of coleoptera (beetles) or diptera (small flies, mosquitoes). The two most widely used *B. thuringiensis* subspecies in commercial insecticides are subspecies *kurstaki* and *Israelensis. B. thuringiensis subsp. kurstaki* is used for the control of larval lepidopterans while *B. thuringiensis subsp. Israelensis* is used for dipteran larvae control. Most of these strains have the same basic toxin structure, but differ in insect host range, perhaps because of different degrees of binding affinity to the toxin receptors in the insect gut. For example, the toxins produced by *B. thuringiensis* var *aizawai* have somewhat different toxins from those of *Bt* var *kurstaki* and they are highly specific to lepidoptera, with no effect on other insects. *Bt* is already a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control. It is also a key source of genes for transgenic expression to provide pest resistance in plants (Feitelson *et al.,* 1993).

In 2002, the global area of genetically modified (GM) crops was 58.7 million hectares grown in 16 countries by six million farmers, of whom five million were small resource- poor farmers in developing countries. The prinicipal GM crops continued to be soyabeans, maize, cotton and canola. Global deployment of the cry 1Ab gene in *Bt* maize that has the potential to increase maize production by up-to 35 million MT valued at $ 3.7 billion per year. Yield gains due to *Bt* maize are estimated at 5% in the temperate maize growing areas and 10% in the tropical areas,

where there are more and overlapping generations of pests leading to higher infestation and losses. The approved *Bt* maize events contain genes from the isolate *B. thuringiensis* var *kurstaki* that produces Cry 1 Ab protein and genes from *B. thuringiensis var aizawi* that produces Cry 1 Fa2 protein. All the *Bt* maize currently deployed contains one synthetic gene, a promoter and other sequences (Clive, 2005).

2.2 *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is one of more than 20 species of soil growing Bacilli. This bacterium is an ubiquitous gram positive, aerobic, spore- forming organism that forms parasporal crystals during the stationary phase of its growth cycle. *B. thuringiensis* was initially characterized as an insect pathogen, and its insecticidal activity was attributed largely or completely depending on the insect and the parasporal crystals. The most discussed of the other species are *Bacillus subtillis* a source of industrial enzymes. *Bacillus thuringiensis* in a lot of cases is confused with *Bacillus cereus* and *Bacillus anthracis*, the causative agent of anthrax. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis* are members of the *Bacillus cereus* group of bacteria, demonstrating widely different phenotypes and pathological effects. *B. anthracis* causes the acute fatal disease anthrax and is a potential biological weapon due to its high toxicity. Whereas *B. cereus* is a probably ubiquitous soil bacterium it is also an opportunistic pathogen that is a common cause of food poisoning. Indeed it is this ability to produce parasporal crystals that distinguishes *B. thuringiensis* from the common soil bacterium *Bacillus cereus* (Bernhard *et al.,* 1997).

All of these different Bacilli are mostly found in the soil, whereas *B. thuringiensis* has also been found in living and dead insects, insect faeces, and granaries on the surface of plants. Studies by Meadows suggests that *B. thuringiensis* has been more exclusively found in dead insect carcasses and stored product dust, than it was in the soil, although *B. thuringiensis* seems to be indigenous to many environments (Bernhard *et al.,* 1997).

2.3 Classification of *Bacillus thuringiensis*

Based primarily on flagellar antigen serotyping over 30 subspecies or varieties of *B. thuringiensis* have been recognized. Hence the diversity in flagellar H-antigen agglutination

reactions is one indication of the enormous genetic diversity among *B. thuringiensis* isolates. However in January 1999 the International Entomopathogenic Bacillus Centre (IEBC) suggested that there are currently 3,493 *B. thuringiensis* strains grouped into 69 serotypes based on their flagellar H antigens. More recent studies suggest that these strains are actually 82 serotypes. The two most widely used *B. thuringiensis* subspecies in commercial insecticides are subspecies *kurstaki* and *Israelensis. B. thuringiensis subsp. kurstaki* is used for the control of larval lepidopterans while *B. thuringiensis subsp. Israelensis* is used for dipteran larvae control (Bernhard *et al.,* 1997).

2.4 Virulence of *Bacillus thuringiensis*

Early work recognized the presence of a number of extracellular compounds that might contribute to virulence, including phospholipases, other heat-labile toxin activities, and β-exotoxins. More recent characterization has shown that proteases, chitinases and the secreted vegetative insecticidal proteins (VIPs) may contribute to virulence. *B. cereus* and *B. thuringiensis* also produce antibiotic compounds that have antifungal activity, one of these products synergizes crystal protein-induced intoxication of certain lepidopterans. The Cry toxins are therefore, the most prominent of a number of virulence factors allowing the development of the bacteria in dead or weakened insect larvae. A number of pesticidal proteins unrelated to the Cry proteins are produced by some strains of *B. thuringiensis* during vegetative growth. These VIPs do not form parasporal crystal proteins and are apparently secreted from the cell (Warren and Jonhansen, 1994).

2.5 Classification of Insecticidal Crystal Protein (ICP).

Bacillus thuringiensis strains produce two types of toxin. The main types are the crystal (Cry) toxins, encoded by different **cry genes**, and this is how different types of *Bt* are classified. The second types are the cytolytic (cyt) toxins, which can augment the Cry toxins, enhancing the effectiveness of insect control. Over 50 of the genes that encode the Cry toxins have now been sequenced and enable the toxins to be assigned to more than 15 groups on the basis of sequence similarities. The table below shows the state of such a classification in 1995, but an alternative classification has recently been proposed.

Table 2: Classification of Crystal genes (Carlton and Gonzalez, 2004)

Crystal shape	**Protein size (kDa)**	**Insect activity**	**Gene**
Bipyramidal	130-138	Lepidoptera larvae	Cry I [several subgroups: A(a), A(b), A(c), B, C, D, E, F, G]
Cuboidal	69-71	Lepidoptera and diptera	Cry II [subgroups A, B, C]
Flat/irregular	73-74	Coleoptera	Cry III [subgroups A, B, C]
Bipyramidal	73-134	Diptera	Cry IV [subgroups A, B, C, D]
Various	35-129	Various	Cry V-IX

The table elucidates the characteristics of crystals such as the gene, shape, size and insect species in which they are most active.

Further studies of the genes by Hofte and Whiteley (1989) revealed that there needed to be a systematic method of naming these genes. So in 1989 they defined four classes of crystal genes (Cry) classified as Cry I Cry II, Cry III, Cry IV proteins based on their insecticidal activities and two classes of cytolytic genes (Cyt) that actually produced the toxins that killed these insects. Cry II and Cry I are active against lepidopterans, Cry III and Cry IV are active against dipterans and Cry III is active against coleopterans. This classification system is being revised to rely solely on amino acid sequence similarities rather than on insecticidal properties as the criterion for assigning Cry designations. The original classification scheme by Hofte and Whitely was used for several years until the gene number went form 14 to more than 100. A survey from the European Molecular Biology Laboratory (EMBL) and Gene Bank databases reveals that over 90 Insecticidal crystal protein (ICP) genes have now been cloned and sequenced. (Feitelson *et al.,* 1993). A revision of the nomenclature scheme was suggested and accepted because of the large number of crystals and cytolytic genes discovered. Roman numerals were replaced with Arabic numbers and each toxin was given four ranks such as Cry50Aa1. The quaternary ranking is an optional ranking that is used only to distinguish between toxins that are more than 95% identical the quartenary ranking will be different such as Cry50Aa. Most subspecies of *B. thuringiensis* have insecticidal activity due to crystal production during sporulation. There are currently 37 families of crystal proteins and 2 families of cytolytic proteins and through cloning and sequencing technology over 100 genes have been revealed to form the crystal and cytolytic protein families (Crickmore *et al.,* 1995).

2.6 General structure of Crystal Proteins

Crystallography studies on the Cry IIIA protein toxin (Caroll and Ellar, 1991) delta-endotoxin structure indicate three structurally distinct domains. **Domain I** consists of a bundle of 7 alpha helices believed to be involved with membrane interactions and the insertion of the toxin into the insect's midgut epithelium and pore formation through which ions can pass freely. **Domain II** appears as a triangular column of three anti-parallel beta-sheets, similar to the antigen-binding regions of immunoglobulins, suggesting that this domain binds to receptors in the gut. **Domain III** consists of anti-parallel beta-strands in a "jellyroll" configuration forming a tightly packed beta-sandwich which is thought to protect the exposed end (C-terminus) of the active toxin, preventing further cleavage by gut proteases.

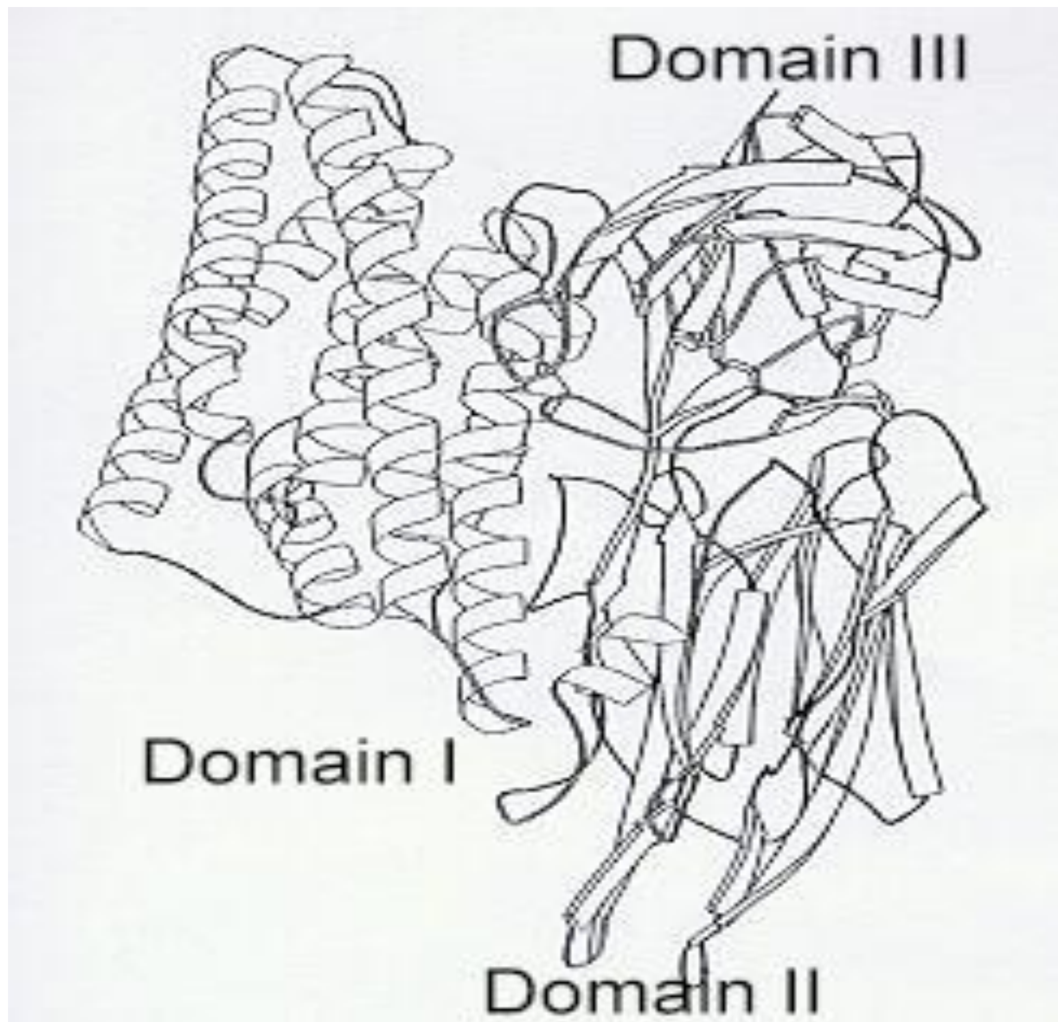


Figure 1. A three dimensional structure of δ- endotoxin. (Diagram based on Carroll and Ellar, 1991) The three dimensional schematic diagram illustrates the three domains of the δ- endotoxin

2.7 Mode of action of Insecticidal Crystal Proteins.

Certain structures common to *Bt* toxin genes suggest a kind of "built-in" variability that gives *Bt* great flexibility in its action in different target insects. Just as antibodies contain conserved as well as variable domains, so are toxin genes and proteins made up of alternating conserved and variable regions. The N-terminal part of the toxin protein is responsible for its toxicity and specificity and contains five conserved regions. The C-terminal part is usually highly conserved and probably responsible for crystal formation (Carlson *et al.,* 1996).

The crystals are aggregates of a large protein about 130-140 kDa that is actually a protoxin that must be activated before it has any effect. The Cry I type proteins are typically processed from a 130 kDa protoxin to the active 55- 65 kDa form. The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects. The highly acidic nature of most mammalian guts is not a favorable environment for the Cry toxin. The low pH of most mammal guts would solubilize and denature the Cry proteins, making them susceptible to hydrolysis by native gut proteases into inactive small peptides and free amino acids hence explaining why Cry toxin is not harmful to mammals and as a result a safe and reliable biopesticide. However, it is solubilized in reducing conditions of high pH about 9.5 conditions commonly found in the mid-gut of lepidopteran larvae. For this reason, *Bt* is a highly specific insecticidal agent (Carlson *et al.,* 1996).

The Cry proteins typically require both solubilization and activation steps before they become biologically active toxins. The ICPs contained within the parasporal crystals are released into the insect mid-gut after ingestion and solubilization of the crystals in the alkaline environment of the insect gut to proteins of 130 - 140 kDa size. The efficiency of crystal protein solubilization depends on the environment prevailing in the insect gut and the composition of the parasporal inclusion bodies. These events mark the first step of the infection cycle (Carlson *et al.,* 1996).

Once it has been solubilized in the insect gut, the protoxin is cleaved by a mid-gut protease to produce an active toxin of about 60kDa. These events mark the second step of infection cycle. This toxin is termed delta-endotoxin. The activated toxins bind to the brush- border membranes of the insect mid-gut epithelium, a step that frequently requires the presence of fortuitous (receptor) proteins. *In vitro* binding studies with radiolabeled toxins demonstrated a strong correlation between the specificity of *B. thuringiensis* toxins and the interaction with specific

binding sites on the insect mid-gut epithelium. This binding is followed by an apparent intercalation event in which the active toxin moiety, or a portion of it, contributes to the formation of ion channels by diffusion through the peritrophic membrane as well as aggregates to form larger pores within the brush- border membrane, which are impermeable to the solubilized, still unprocessed 130 - 140 kDa protoxins, and so acts as a kind of molecular sieve, this in turn leads to equilibration of ions, that causes osmotic imbalance, leading to consequent destruction of ion gradients. These pores also permit the vegetative *Bt* cells germinating from the spores to migrate into the haemolymph and promote the intoxication process through the ensuing bacterium. As a result, the gut is rapidly immobilized, the epithelial cells swell and lyse, and the gut pH is lowered by equilibration with the blood pH. This lower pH enables the bacterial spores to germinate, and the bacterium can then invade the host, causing a lethal septicemia. Intoxicated larvae stop feeding within minutes and eventually die due to massive water uptake (Marrone and MacIntosh, 1993).

2.8 ICP gene organization

Most of the crystal genes discovered have been linked to movable plasmids, which are extra- chromosomal DNA. It has been known since the 1980s that genes on plasmids of *B. thuringiensis* encode the Cry toxins. There are 5 or 6 different plasmids in a single *Bt* strain and these plasmids can encode different toxin genes. The plasmids can be exchanged between *Bt* strains by a conjugation-like process, so potentially there are a wide variety of strains with different combinations of Cry toxins. In addition to this, *Bt* contains transposons that are transposable genetic elements that flank genes and that can be excised from one part of the genome and inserted elsewhere. All these properties increase the variety of toxins produced naturally by *Bt* strains, and provide the basis for commercial companies to create genetically engineered strains with novel toxin combinations. Many of the *B. thuringiensis* subspecies have multiple crystal and cytolytic gene systems that are simultaneously expressed. This multiple expression has one major function, which is to increase the bacteria host killing range. *B. thuringiensis* strains have a genome size of 2.4 to 5.7 million bp (Carlson *et al.,* 1996).

2.9 Plasmid profiles of *Bacillus thuringiensis*

B. thuringiensis is well known for its numerous plasmids and complex plasmid arrays. Roughly 10- 20% of the potential genetic coding capacity of this organism maybe found on plasmids ranging in size from < 1.5 MDa to > 130 MDa. The ICP genes are typically located on large (> 30 MDa) plasmids although some ICP genes have been reported on the chromosome.

Native strains of *B. thuringiensis* frequently contain multiple ICP- encoding plasmids, some of which may harbour more than one ICP gene, particularly in the case of the Cry I and Cry IV genes. These multiple ICP genes may direct the synthesis of related proteins that form a heterogeneous crystalline inclusion or direct the synthesis of unique proteins that form separate crystals of distinct morphology. As a classic example, strain HD1 subspecies kurstaki, harbouring 12 resident plasmids, contains CryIAa, CryIAc, CryIIA, and a silent CryIIB gene on a ~110 MDa plasmid and a CryIAb gene one self- transmissible 44 MDa plasmid. The CryIAa, CryIAb, and CryIAc proteins contribute to the formation of the bipyramidal- shaped crystal of HD1, while the CryIIA protein forms the distinct cuboidal- shaped crystal. In the case of *B. thuringiensis subsp. israelensis,* the CryIVA, Cry IVB, CryIVD and CytA genes are all contained on a 72MDa plasmid and contribute to the formation of a complex parasporal body (Gill *et al.,* 2004).

3.0 METHODOLOGY

3.1 *Bt* Isolates

Bacillus thuringiensis isolates 1M, V24-M were isolated from soils in the Machakos area whereas K10-2 sample isolated from wsoils in the Kakamega area stored in the ICIPE LAB, University of Nairobi in refrigerated glycerol stocks maintained at –20 ºC. The source of the *Bt* isolates were from previous studies conducted by Wang'ondu 2001. The basis of *Bt* isolate selection was because of their differential toxicities with 1M which exhibits a very highly effective toxicity causing 100% larval mortality, K10-2 which is moderately toxic exhibiting 91% larval mortality and V24-M isolates which has low toxicity causing 77% larval mortality against *Chilo partellus.*

3.2 Media Preparation

3.2.1 Luria bertani (LB) agar plates

Five grams of Bacto tryptone, 2.5g of Bacto yeast extract, 5g of NaCl and 7.5g of agar were added into 500ml of distilled water in a one liter conical flask, the mixture was shaken vigorously to dissolve completely and the pH adjusted to 7.5 using sodium hydroxide solution. The mouth of the conical flask was firmly sealed with cotton wool rolled into a ball and covered with aluminium foil. The media was autoclaved for 35 minutes at 121 ºC at a pressure of 15lb/sq.in. The media was allowed to cool down to 45 ºC and about 20ml poured aseptically into each sterile petridishes. The plates were stored in the refrigerator at 5 ºC.

3.2.2 LB Broth

LB was prepared by mixing 5g of Bacto- Tryptone, 2.5g of Bacto- Yeast extract and 5g of NaCl into a two litre conical flask and 500ml of distilled water added. The mixture was mixed thoroughly to dissolve completely then the pH adjusted to 7.5 using 10M aqueous sodium hydroxide. The mouth of the conical flask was firmly sealed with cotton wool rolled into a ball and covered with aluminium foil. The media was autoclaved for 35minutes at 121 ºC at a pressure of 15lb/sq.in.

3.3 Analysis of Growth patterns

3.3.1 Determination of growth rate

Bt isolates were inoculated onto three separate plates and incubated at 37ºC overnight. A colony from the plates was inoculated into 150ml of the LB broth. 1.5ml of LB Broth containing the growing bacteria was placed in an eppendorf tube at an interval of every 6 hrs and absorbance read at 600nm for a period of 72hrs. A graph of absorbance versus time was plotted to determine the time for optimal growth.

3.3.2 Determination of optimal time for sporulation and crystal formation

A few drops of solution A (1.5gm-amido black dissolved in 50 parts of 90% methanol, 40 parts of distilled water and 10 parts acetic acid) were added to the heat fixed slides of *Bt* isolates taken at intervals of 6hrs for a period of 72hr, and washed after 70 seconds. 30% of solution B (1g of basic fuschin dissolved in10ml of 95% ethanol mixed with 5g of phenol dissolved in 90ml of distilled water) were added to the slide and left for 20 seconds. Washing was done in ice- cold tap water and later dried with filter paper. Examination was carried out by means of a standard light microscope with an oil immersion objective. The levels of crystal and spores formation was quantified and estimated

3.4 Analysis of Secreted proteins

One ml of the samples taken at an interval of every 6hrs for a period of 72 hrs were placed in separate eppendorf tubes and subjected to centrifugation at 10,000g for 2 minutes in order to separate the bacterial cells from the protein in solution. The supernatant and the pellet were retained in separate tubes and 0.1ml of the supernatant was incorporated in the Lowry assay protocol and concentration determined using Bovine Serum Albumin (BSA) standard curve as the standard protein.

3.4.1 Protein Determination

Lowry assay (1951) was used to determine the protein content in the bacterial supernatant. Serial dilutions of 0.3mg/ml solution of BSA were pipetted into separate tubes to cover protein concentration ranges of 0 to 1mg/ml. The total volumes of the tubes were brought to 1.0ml with distilled water and 1ml of Lowry reagent was added to all the tubes and vortexed gently to mix

them thoroughly. The tubes were incubated for 15 minutes and absorbance was measured at 750 nm. A standard curve was obtained and used to determine the protein concentration of the secreted proteins taken at an interval of every 6 hrs for a period of 72 hrs.

3.4.2 Determination of secreted protein content.

One ml of the samples taken at an interval of every 6hrs were placed in separate eppendorf tubes and subjected to centrifugation at 15000g for 15 minutes in order to separate the Bacterial cells from the protein in solution. The supernatant and the pellet were retained in separate tubes and absorbance of 0.1ml of the supernatant were taken at 750nm using a spectrophotometer. The concentration of the protein was then determined using the obtained BSA standard curve.

3.4.3 Analysis of secreted proteins through Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoresis on 12% SDS- polyacrylamide gels was carried out under reducing conditions on a vertical electrophoresis unit (BIO-RAD), using 25mM Tris-glycine buffer pH 8.3. Samples were diluted 1:1 with Laemmli sample buffer (Laemmli, 1970) containing 2% SDS, 6% β-mecarptolethanol, 10% glycerol, 0.002% bromophenol blue dye in 0.125M Tris- HCl buffer pH 6.8. Samples were boiled for 5 minutes before loading 25µl per well alongside standard molecular weight markers. Electrophoresis was carried out at 30mA until the tracker dye (Bromophenol blue) reached the bottom of the gel.

Staining solution was made by dissolving 0.1% Coomassie brilliant blue in 40% methanol, 10% acetic acid, and filtered after the dye had dissolved. Gels were immersed in the staining solution for 30 minutes at room temperature. Destaining was done at room temperature. Destaining was done in excess 40% methanol, 10% acetic acid with several changes of the destaining solution until a clear background was obtained.

The molecular weight of proteins (spore/crystal complex) of the various *Bt* isolates was estimated by measuring the relative movement of the standard markers through construction of a standard curve.

The relative movement was calculated by the formula

$$RF = \frac{\text{Distance Moved by each protein}}{\text{Distance Moved by tracker dye}}$$

Where RF = relative movement of the standard markers.
The relative movement of the different protein bands was then similarly determined.

3.5 Analysis of plasmid profile from *Bt* isolates

3.5.1 Harvesting of cells

Bacillus thuringiensis cells were obtained from refrigerated eppendorf tubes that contain the concentrates of interest 1M*, V24-M and K10-2 and inoculated in 150ml LB medium respectively. These cells were allowed to grow in a 250 ml baffle flask for 18 hr with major agitation using the Gerhardt's Shaker at the speed of 2000 rpm at 37 ºC. Cells were harvested through aspiration of supernatants using the Sorvall RC-5B refrigerated super-speed centrifuge and then retaining the pellet. The pellets were washed twice by vortexing with 2mls of solution I [STE/TES buffer: 0.01M Tris (pH 8), 0.01M EDTA, 1M NaCl].

3.5.2 Lysis by modified alkali method

Modified alkali lysis method was done according to Roderick *et al.,* (2005). In this step
The pellet was resuspended in 2ml of lysis buffer containing TE (0.025M Tris (pH 8), 0.01M EDTA, 25% sucrose, 4 mg/ml lysozyme and incubated at 37˚ C for 1hr. To this 2µl of solution II [2ml of 10N NaOH, 10ml of SDS, and 88ml of deionized water] were added. The tubes were inverted gently about five times and placed on ice for 5 minutes to equilibrate for the next step, which involved the addition of 1ml of 5M NaCl followed by a brief but gentle vortex. This was stored in ice for 3 to 5 minutes to equilibrate for the next step. This step involved centrifugation at 15,000 g for 5 minutes at 4 ºC in Sorvall RC-5B refrigerated super-speed microcentrifuge. The supernatant was then transferred into a fresh tube, 10 µl of RNase (500µg/ml) added and incubated at 37 ºC for a period of 10 minutes. An equal volume of phenol: chloroform was added and mixed gently then centrifuged at 15,000g for 15 minutes at 4 ºC in a microcentrifuge. The supernatant was transferred into a fresh tube.

3.5.3 Concentration of Plasmid DNA

Approximately 2.5- 3 volumes of an 95% ethanol and 5% 0.12M Sodium acetate solution was added to the plasmid DNA samples and placed in an ice-water bath for at least 10 minutes. DNA was then precipitated through centrifugation at 12,000 rpm for 15 minutes. The pellet was then washed using 70% ethanol and air dried before reconstitution in 50µl of TE (Tris- EDTA) pH 8.0.

3.5.4 Quantification of DNA

Five µl of plasmid DNA was diluted with 45µl of TE the absorbance read at both 260nm and 280nm. Their ratio was established to determine the purity of the DNA, usually pure preparations would have OD260/OD280 of 1.8 and 2.0. For quantification of DNA the readings were taken at a wavelength of 260nm for calculation of the nucleic acid in the sample, usually the OD of 1 unit corresponds to approximately 50µg/ml for double stranded DNA. By multiplication of the OD reading obtained with the dilution factor 10 the concentration of DNA can be determined through the following formula.

Quantity of DNA = 10(x/1 x 50 µg/ml), where x = OD of DNA at 260nm

3.5.5 Agarose Gel electrophoresis

Agarose gels of 0.7% were prepared by adding 0.35g of agarose to 50ml of 0.5M Tris Acetate EDTA (TAE) buffer and then dissolved by heating in a hotplate. As the solution was cooling the ends of the gel-casting tray were sealed with cello tape. Once the gel cooled to 60 ºC, 5.0µl of 10mg/ml of Ethidium Bromide was added to the agarose solution. The gel was poured into a casting tray with a plastic comb. This was left for 30 minutes until it sets then the comb and tape were removed and the gel mounted onto a horizontal electrophoresis tank. TAE buffer of strength 1/10 was used to cover the gel to a depth of about 1mm. The DNA samples were mixed with gel tracking dye and loaded into the sample wells. Electrophoresis was done at a voltage of 70V for 45mins at room temperature. Size markers were co-electrophoresed with DNA samples, appropriate for fragment size determination. After electrophoresis was done, the gel was placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern taken with a Polaroid camera.

3.5.6 Restriction digestion

Five types of restriction enzymes namely EcoRI, BamHI, SacI, KpnI and HindIII were initially used with the three *Bt* isolates. Using approximately 12µl of distilled deionized water, 1µg of DNA, 2 µl (10 units/µl) of restriction enzyme, in its respective 2µl buffer, were added and mixed uniformly then incubated at 37 ºC overnight for complete digestion. Size markers were co-electrophoresed with restricted DNA samples, appropriate for fragment size determination. It was then possible to estimate the size of fragments using standards to determine their molecular weights.

3.5.7 Statistical analysis

Statistical analysis was done using the SPSS program (version 7.0). This was for significance tests (t-test). Where P is the probabilities that the growth values for the different isolates at absorbance 600 nm are significantly different and where P is also the probabilities that the secreted protein concentration at absorbance 750nm are significantly different. The test of significance is based on two-tailed t- test. Where ∞=0.05 and $P<0.05$ meaning the results are statistically significant and $P>0.05$ results are not statistically significant.

4.0 RESULTS

4.1 Growth phases of *Bt* isolates

The *Bt* isolates grew very fast at 37 ºC they produced smooth creamish white colonies, which were slightly raised from the nutrient agar after overnight incubation. Of the three *Bt* isolates there was no difference observed in the physical appearance of the colonies.

The three *Bt* isolates 1M, V24-M and K10-2 obtained from refrigerated glycerol stocks and inoculated into 150ml of baffle flask containing LB broth, exhibited different growths rates demonstrated by their physical appearance after an interval of every 6 hrs (Fig 2). The general appearance of the LB broth at inoculation was clear yellow. After 6 hrs of inoculation the appearance for all the three isolates remained clear. Using levels of significance to compare the growth rates at absorbance 600 nm, 1M gave readings of 0.586±0.0025, 0.1±0.0023 for V24-M and K10-2 giving 0.029±0.0023. The results here indicated that the three values had no statistical significance as in all cases $P > 0.05$ when compared. Hence the 1M, V24-M and K10-2 growth levels at 6 hrs were significantly different from each other.

The general appearance of the media at 12 hrs remained the same as the appearance at 6 hrs, although all the absorbance's increased with1M registering readings of 1.344±0.034 compared to 1.269±0.037 for V24-M and K10-2 1.28±0.035. Further t-tests revealed that the growth rates were statistically significant as in all cases $P<0.05$.With 1M values being significantly greater than both K10-2 and V24-M. However, the K10-2 reading was not significantly greater than V24-M.

At 30 hrs the turbidity increased as the transparency and clarity of the media diminished. The absorbance readings at 600nm for 1M was 1.6±0.04, this was not significantly greater than both values for V24-M and K10-2 that had values of 1.56±0.0035 and 1.553±0.03 respectively as $P>0.05$ in all the cases. At 36hrs the optimum turbidity was achieved and the general appearance for all the isolates was milky yellow in appearance a trend that was continuous up-to 72hrs. The absorbance reading at 36hrs for 1M was 1.616±0.04 this was not significantly greater than both values for V24-M and K10-2 which were 1.565±0.0035 and 1.563±0.035 respectively as $P>0.05$. The absorbance reading at 72 hrs for 1M is 1.95±0.04, V24-M 1.94±0.005and K10-2 1.87±0.035 this indicated that 1M was not significantly greater than V24-M and K10-2 *Bt* isolates.

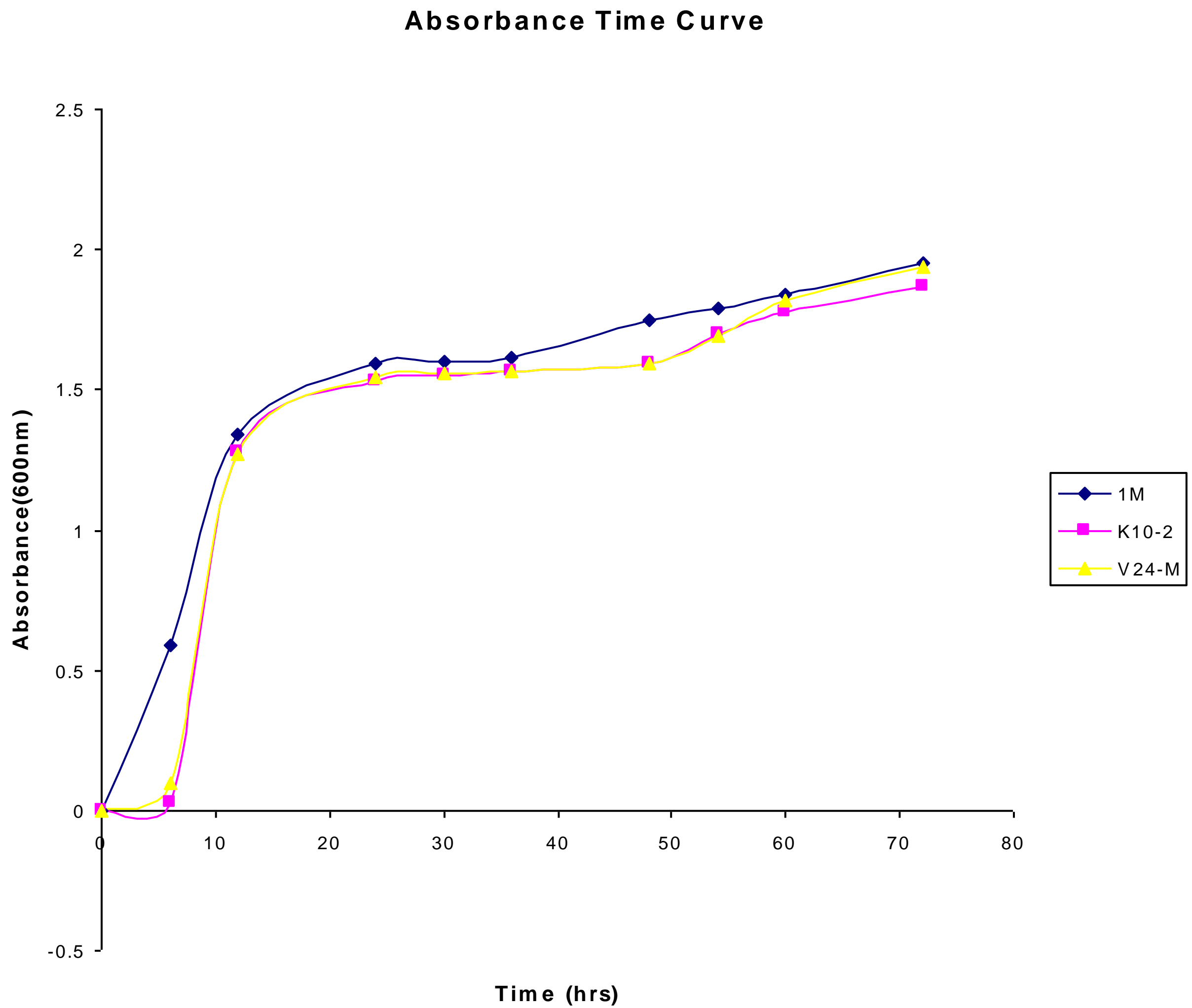
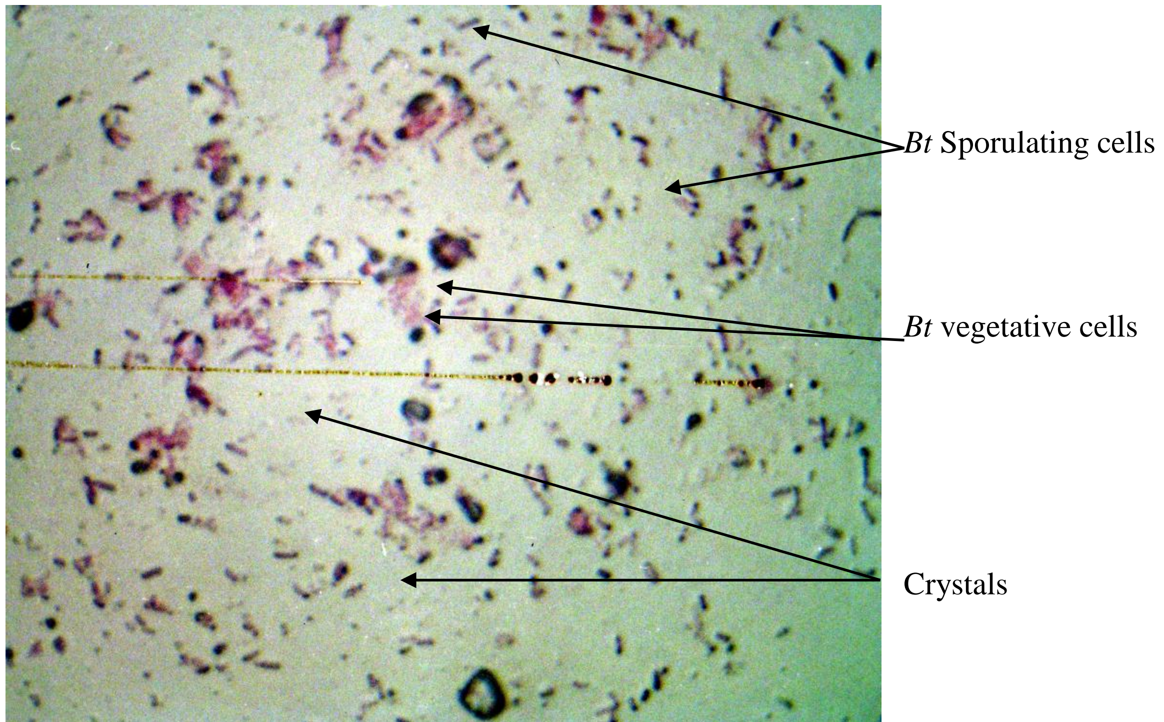


Figure 2: Growth patterns of the *Bt* isolates using a spectrophotometer, absorbance at 600nm

The figure 2 illustrates the growth patterns of *Bt* isolates 1M, V24-M and K10-2 measured after an interval of 6 hrs from 0 to 72hrs using the absorbance's at 600nm.

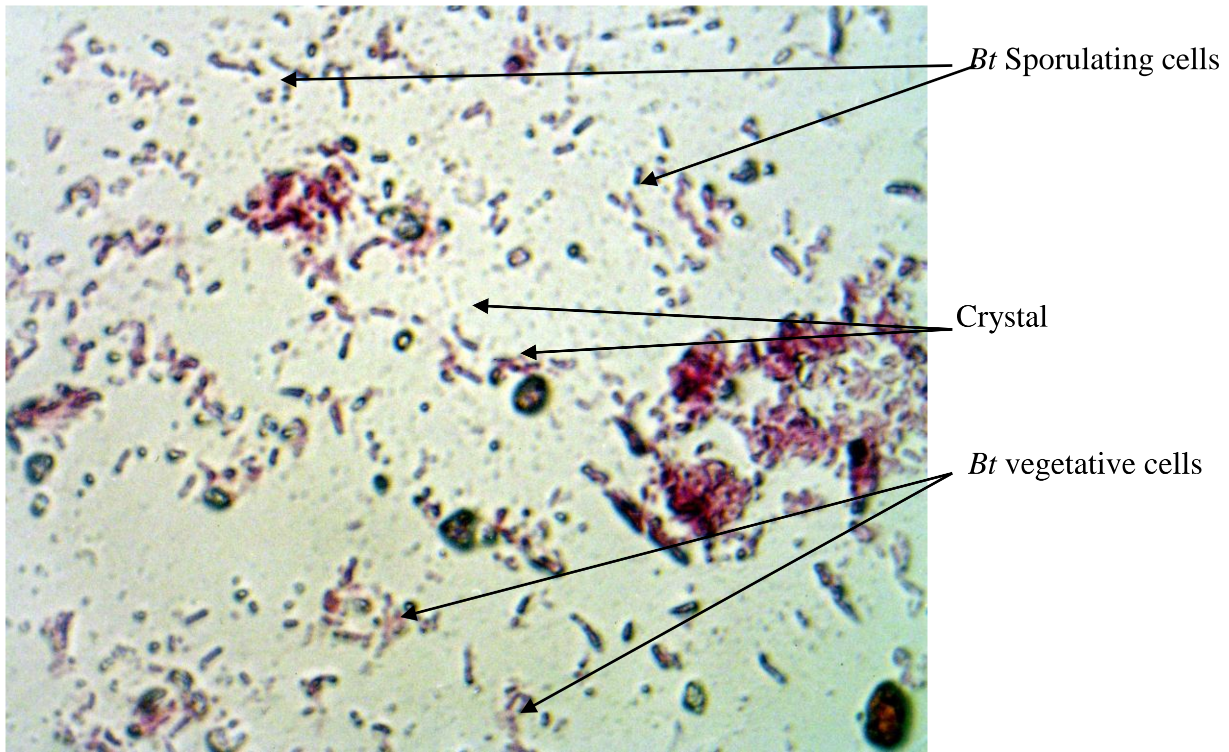
B. thuringiensis isolates were stained using the Smirnoff staining protocol (1962) at an interval of every six hours from 0 to 72hours to test for the prescence of parasporal bodies and crystals. During the time interval ranging from 0 to 36hrs there was no spore formation and crystals for all the three isolates 1M, V24-M and K10-2. 1M and V24-M isolates exhibited the first crystals at 48 hrs that were of minute proportions all of which were of bipyrimidal in shape (Wang'ondu and Ochanda, 2001). The first crystals of K10-2 were witnessed at 54hrs. There was then a gradual increase in the quantity of crystals produced with the optimum density experienced at 72hrs for all the isolates. All crystals stained with an almost black luster with lilac blue tint. The spores stained pink, whereas bacterial cells and their fragments assume a light lilac tints.



Magnification X 600

Figure 3. 1M *Bt* isolate cells and spores after 72 hrs of incubation.

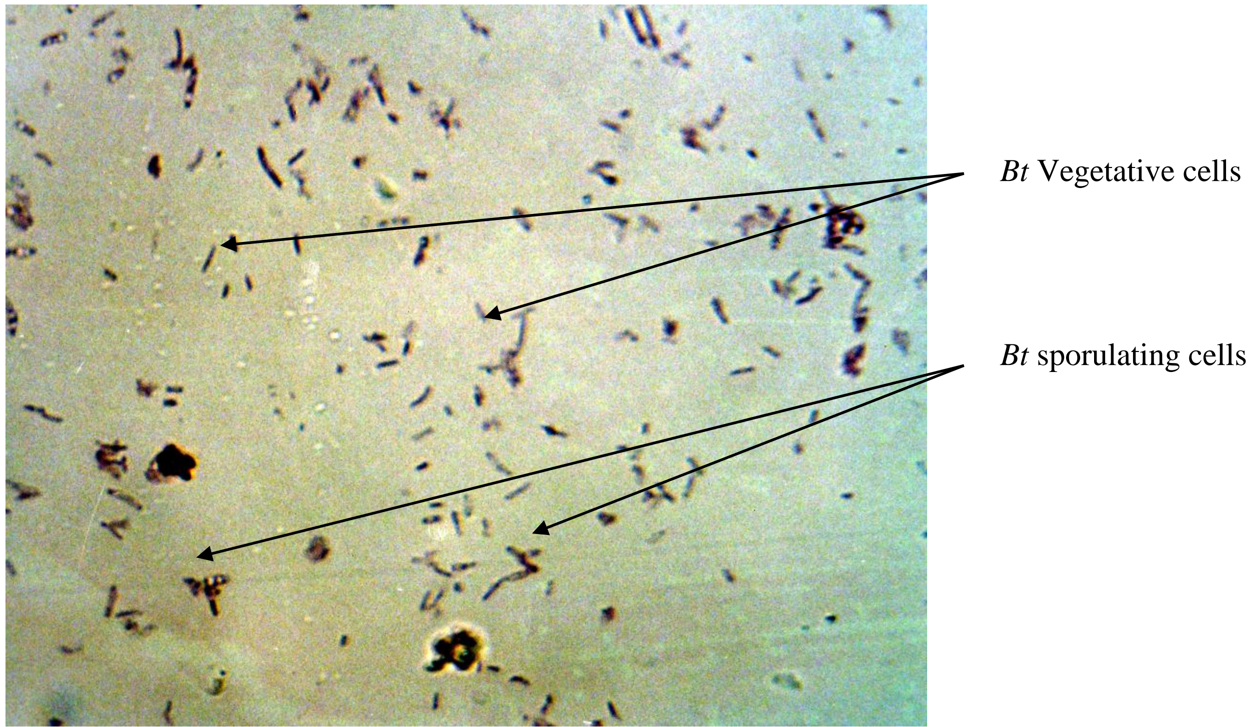
Bt isolate cells showing sporulating cells that have released crystals in the background.



Magnification X 600

Figure 4. V24-M, *Bt* isolate cells and spores after 72 hrs of incubation.

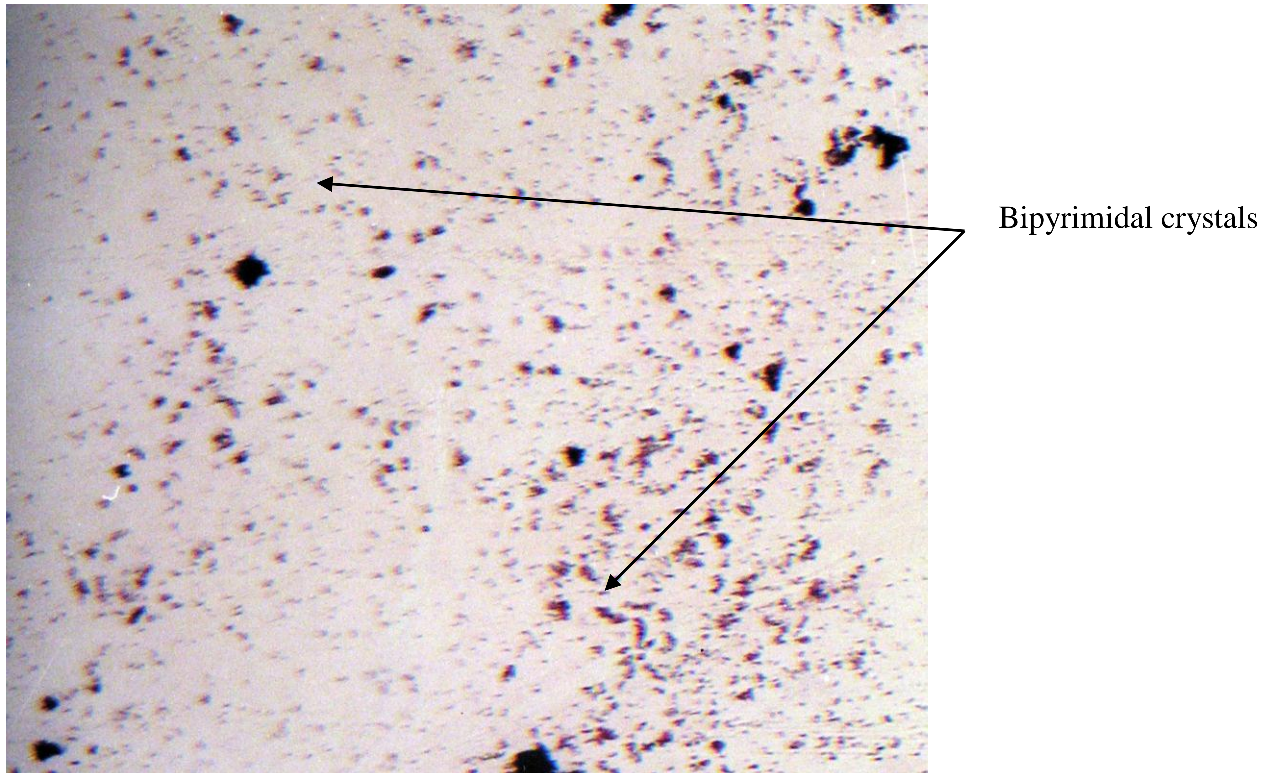
Bt isolate cells showing sporulating cells that have released crystals in the background.



Magnification X 600

Figure 5. K10.2, *Bt* isolate cells and spores after 72 hrs of incubation.

Bt isolate cells showing sporulating cells that have released crystals in the background.



Magnification X 1000

Figure 6: Diagram of Crystal proteins after 72 hrs of incubation.

A microscopic slide at magnification X 1000 showing bipyrimidal crystal proteins of 1M isolates.

4.2 Secreted proteins of *Bt* isolates 1M, V24-M and K10-2

Secreted proteins of the *Bt* isolates exhibited a varied concentration over a period of 72 hrs with 1M isolate recording a markedly rapid increase as compared to isolates V24-M and K10-2 (Fig 7). The first 12 hours was marked by an exponential increase in the protein concentration for all the *Bt* isolates with 1M having significantly greater protein concentration than both V24-M and K10-2 respectively as1M exhibited a protein concentration of 0.26±0.03 mg/ml whereas K10-2 0.24±0.02 mg/ml and V24-M 0.2±0.02 mg/ml hence giving values of $P<0.05$. However, K10-2 and V24-M values in comparison were not statistically significant with $P>0.05$.

After the first 12 hrs the trend changed as the protein concentration for all the *Bt* isolates exhibited a plateau phase where there was no remarkable change in protein concentration between the 12th hour to the 30th hour. Although the plateau phase for 1M isolate was greater than K10-2 that was also greater than V24-M isolate. From the 30th hour to the 72nd hour there was a rapid increase in protein concentration in all the *Bt* isolates with all of them maintaining the same pattern.

At 30 hrs the protein concentration for 1M was 0.34±0.038 mg/ml whereas for K10-2 was 0.30±0.02 mg/ml and V24-M was 0.264±0.03 mg/ml. Comparison of the levels of significance indicated that 1M protein concentration was significantly greater than K10-2, whereas K10-2 was significantly greater than V24-M with $P<0.05$. However, 1M and V24-M did not exhibit any statistical significance as $P>0.05$. At 60 hrs 1M isolates had a protein concentration of 0.56±0.0625 mg/ml which was not statistically significant when compared with V24-M that had a protein concentration of 0.435 ±0.05 mg/ml and K10-2 that had a protein concentration of 0.445±0.05 mg/ml as it gave values of $P>0.05$. However, V24-M and K10-2 protein concentrations were found to be significantly different with K10-2 being significantly greater than V24-M hence giving $P<0.05$. The same trend that occurred at 60 hrs was again repeated at 72 hrs as1M isolates had a protein concentration of 0.63±0.07 mg/ml which was not statistically significant when compared with V24-M that had a protein concentration of 0.49 ±0.05 mg/ml and K10-2 that had a protein concentration of 0.51±0.06 mg/ml as it gave values of $P>0.05$. However, V24-M and K10-2 protein concentrations were found to be significantly different with K10-2 being significantly greater than V24-M hence giving $P<0.05$.

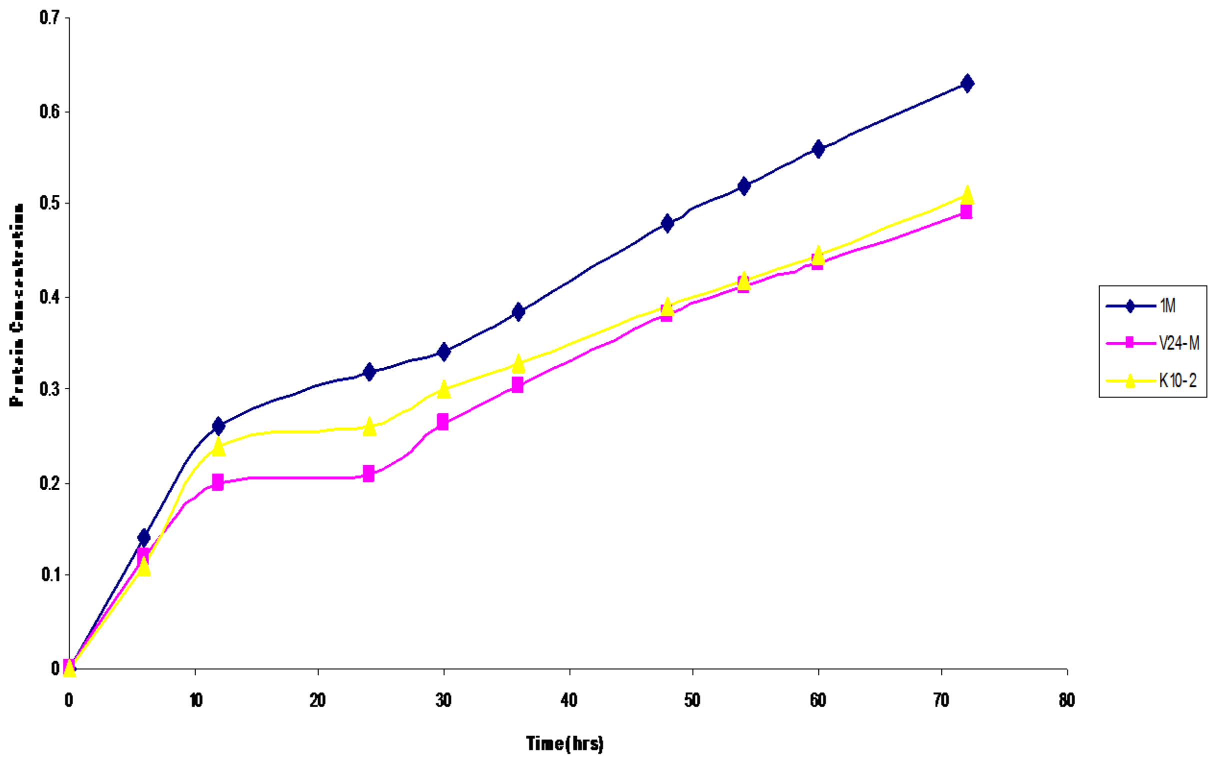


Figure 7: Protein concentration of *Bt* isolates 1M, V24-M and K10-2

Graph showing the protein concentrations of the various *Bt* isolates over a period of 72 hrs.

4.2.1 SDS PAGE.

4.2.1.1 Estimation of molecular weights by polyacrylamide gel electrophoresis.

Bt secreted protein complex of the different isolates were analysed for molecular weight by electrophoresis on a 12% SDS polyacrylamide gel. Electrophoretic analysis of proteins (crystal/spore complex) of the different *Bt* isolates revealed three major protein subunits of molecular weight ~28kDA, ~ 65kDA protein bands and ~ 130kDa for all the isolates (Fig 8). It was notable that 1M at 72 hrs has a remarkable approximately 28kDa protein band that was absent in both K10-2 and V24-M isolates. Additionally it was quite notable that 1M isolate is missing a protein band corresponding to ~130kDa that is present in both V24-M and K10-2 (Fig 8).

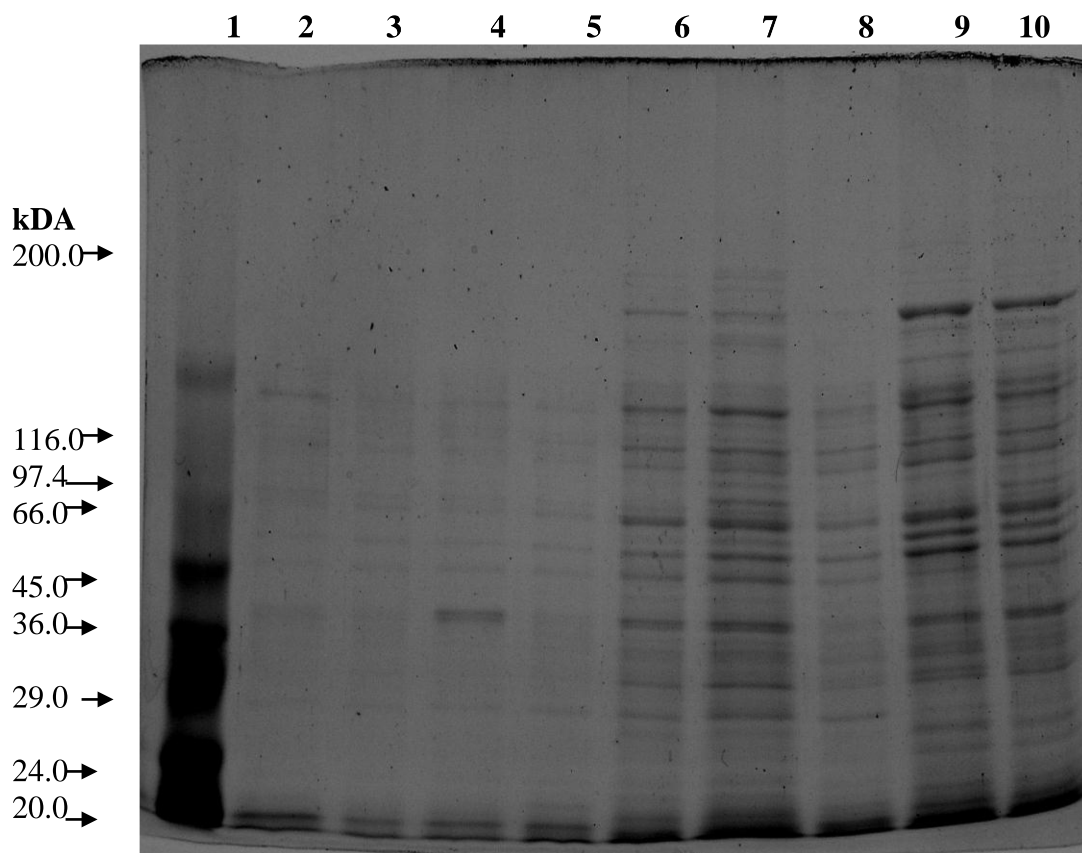


Figure 8:12% SDS-PAGE of proteins secreted by the *Bt* isolates. Lane 1, Molecular weight markers. The numbers alongside the gel indicate molecular weights (kDA) of standard marker proteins; 2, Isolate 1M at 54hrs, 3, Isolate V24-M at 54 hrs; 4, Isolate K10-2 at 54hrs; 5, Isolate 1M at 60 hrs; 6, Isolate V24-M at 60 hrs; 7, Isolate K10-2 at 60 hrs; 8, Isolate 1M at 72 hrs; 9, V24-M at 72 hrs; 10, Isolate K10-2 at 72 hrs. 100 mg of protein was loaded into each of the wells using the protein concentration curve (Appendix V) to estimate the various concentrations.

4.3 *Bt* plasmid profile

4.3.1 Concentration and purity of DNA

1M isolates had a slightly elevated DNA purity than both V24-M and K10-2 although the values were not different as 1M exhibited a 260/280 ratio of 1.99, V24-M 1.946 and K10-2 1.902 respectively (Table 4). This indicated the success of the isolation protocol as there was very high efficiency depicted by high 260/280 ratios. The three *Bt* isolates showed varying levels of DNA concentration, with 1M having a concentration of 47.987µg/ml, V24-M having a concentration of 32.5µg/ml and K10-2 isolate having a concentration of 31.562µg/ml. 1M isolate showed a significantly elevated concentration than both V24-M and K10-2. This was attributed to the elevated growth rate of 1M and to a greater extent a greater copy number of the plasmids for the 1M *Bt* isolates.

Table 4: Concentration and purity of plasmid DNA.

***Bt* Isolate**	**DNA Concentration**	**DNA (260/280)**
1M	47.987µg/ml	1.990
V24-M	32.5µg/ml	1.946
K10-2	31.563µg/ml	1.902

The table shows the purity of DNA using the 260/280 ratio and the concentration of the plasmid DNA for the *Bt* isolates.

4.3.2 Analysis of plasmid DNA.

Analysis of unrestricted plasmid DNA revealed that the *Bt* isolates had similar plasmids owing to their uniform banding patterns. Further analysis revealed that all of the isolates were having molecular weights greater than 23,130 bp in the ladder (Fig 9).

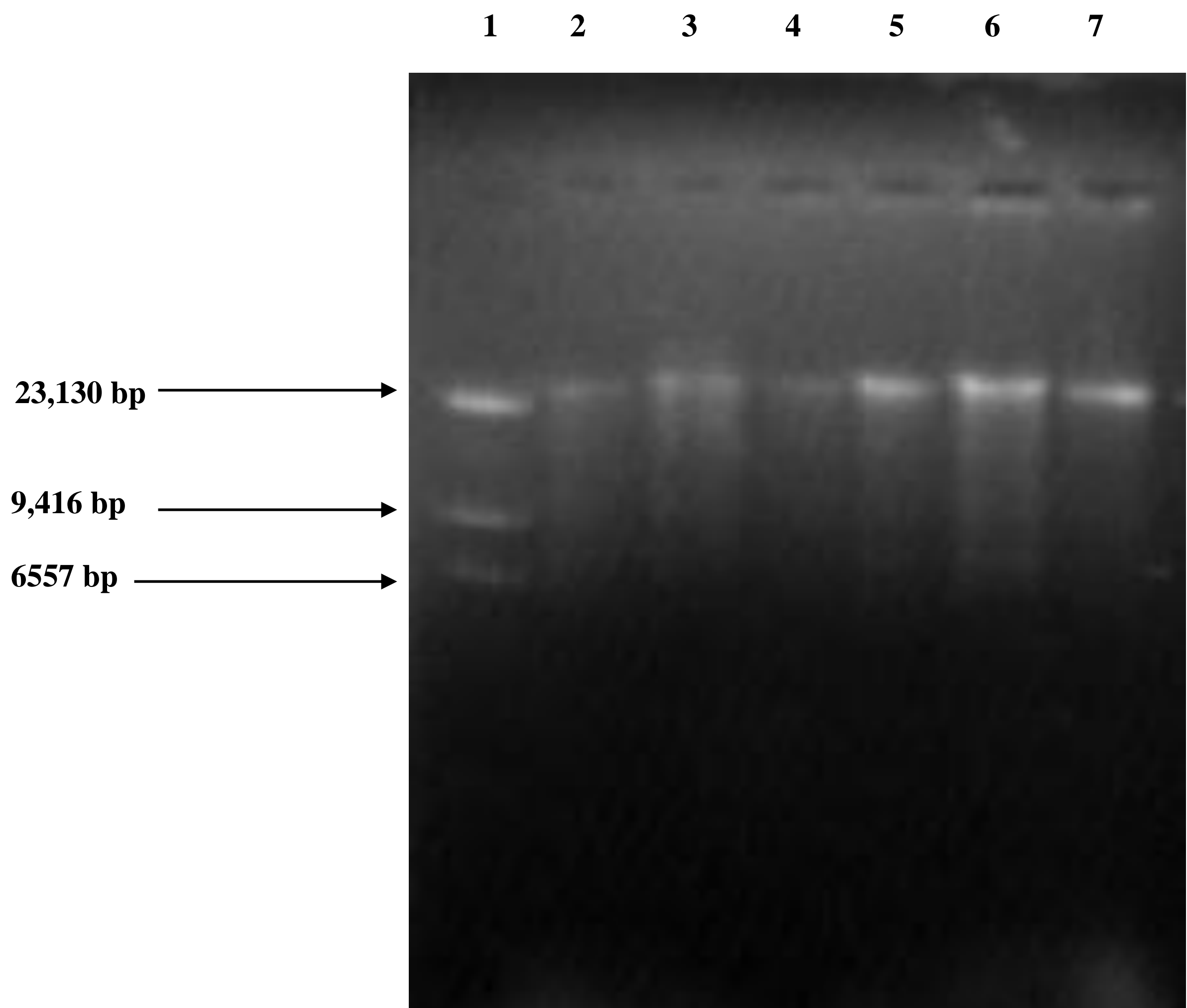


Figure 9: 0.7 % Agarose gel electrophoresis of isolated plasmid DNA. Lane1, DNA Standards; Lane2, 1M(isolate a); Lane 3, 1M (isolate b); Lane 4, V24-M (isolate a); Lane 5, V24-M (isolate b); Lane 6, K10-2 (isolate a); Lane 7, K10-2 (isolate b).

4.3.3 Restriction digestion profiles

DNA profiles preliminary restricted with an array of restriction enzymes such as EcoRI, BamHI, HindIII, KpnI and SacI. *Bt* isolate 1M was digested with all the 5 enzymes revealing an array of bands, whereas V24-M and K10-2 were digested only with EcoRI and HindIII. (Fig 10). Molecular weights were determined using HindIII digested λDNA molecular weight markers.

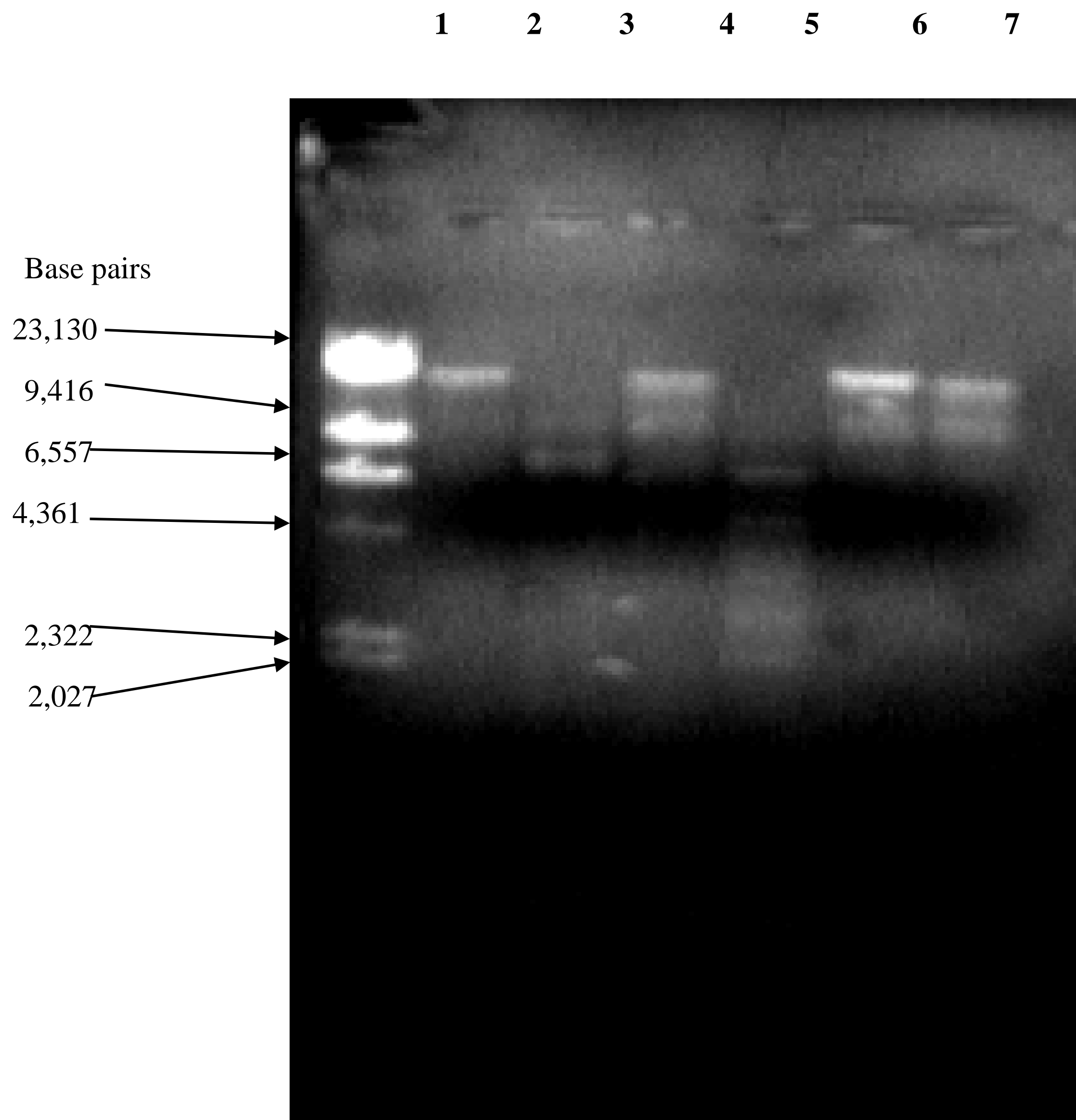


Figure 10: Restricted plasmid DNA (1M isolates). Lane 1, DNA Standard; Lane 2, 1M unrestricted Plasmid; Lane 3,1M restricted (EcoRI); Lane 4,1M restricted (BamHI); Lane 5, 1M restricted (HindIII); Lane 6, 1M restricted (KpnI); Lane 7, 1M restricted (SacI).

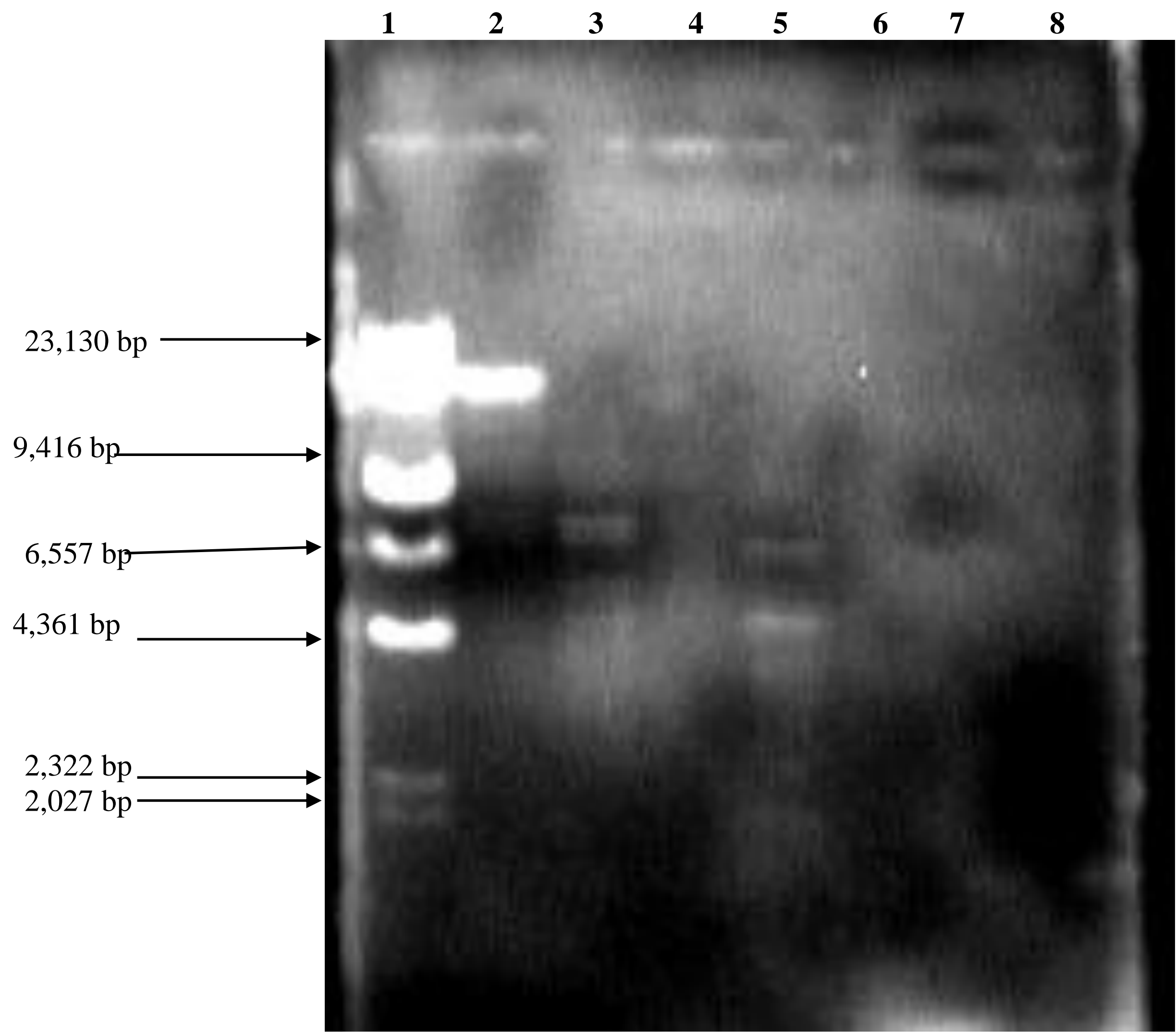


Figure 11: Restricted DNA, V24-M isolates. Lane 1, DNA Standards; Lane 2,Unrestricted; Lane 3, EcoRI; Lane 4, BamHI; Lane 5, HindIII; Lane 6, KpnI; Lane 7, SacI.

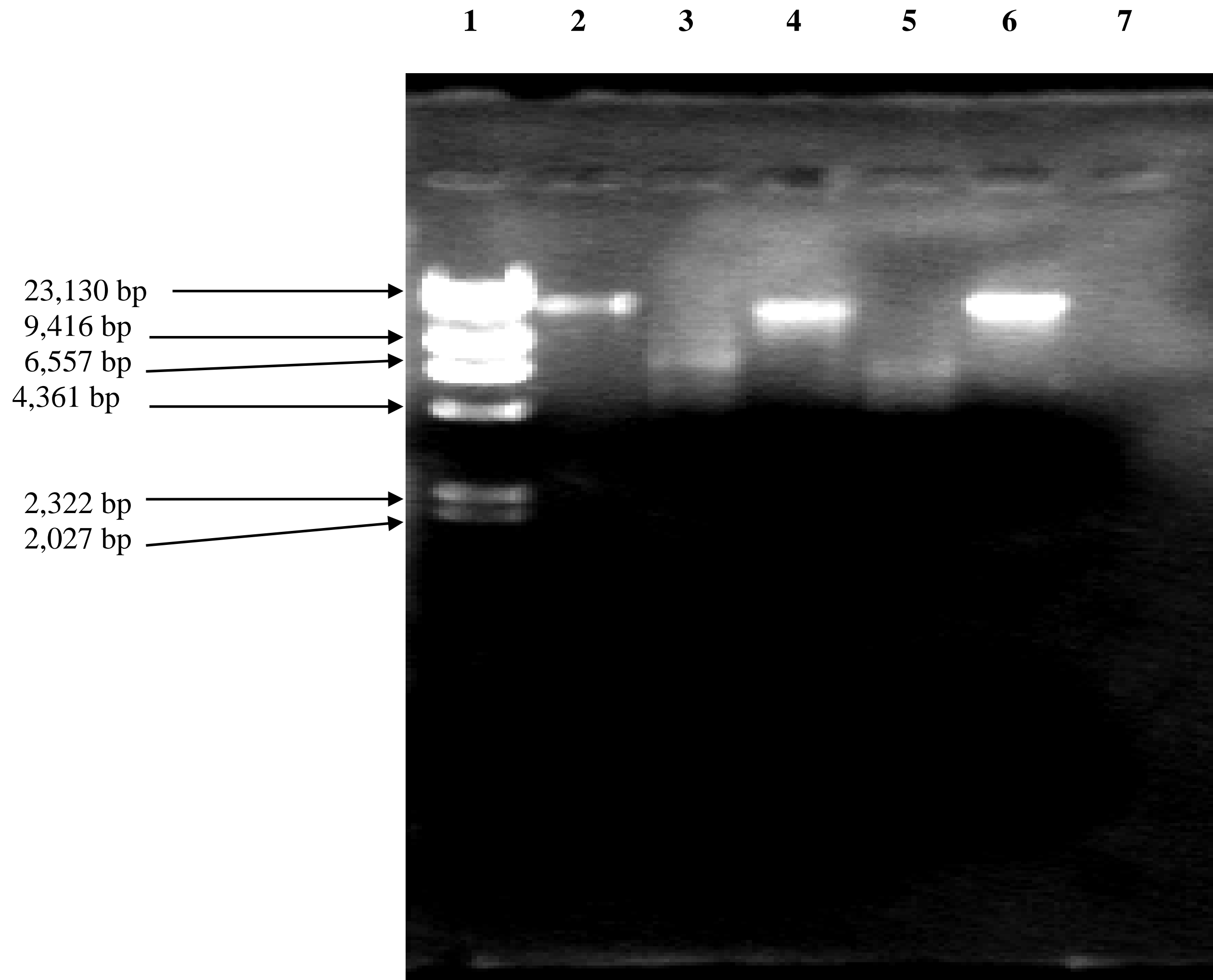


Figure 12: Restricted Plasmid DNA (K10-2) isolate. Lane 1, DNA Standards; Lane 2; unrestricted K10-2; Lane 3, EcoRI; Lane 4, BamHI; Lane 5, HindIII; Lane 6, KpnI; Lane 7, SacI.

From the preliminary restriction digestion setup by an array of restriction enzymes it was evident that there was a unique band that is approximately 7,000bp to 7,200bp. For a more conclusive analysis of the restriction profiles for the three *Bt* isolates. EcoRI and HindIII were done comparatively on a single gel (fig 13).

4.3.4 Comparative analysis of restriction patterns of Eco RI and Hind III digest of *Bt* isolates.

Restriction patterns for EcoRI and HindIII digests for the *Bt* isolates were performed to compare the patterns for any unique bands that may in turn be used in characterization and identification of effective toxicity (fig 13).

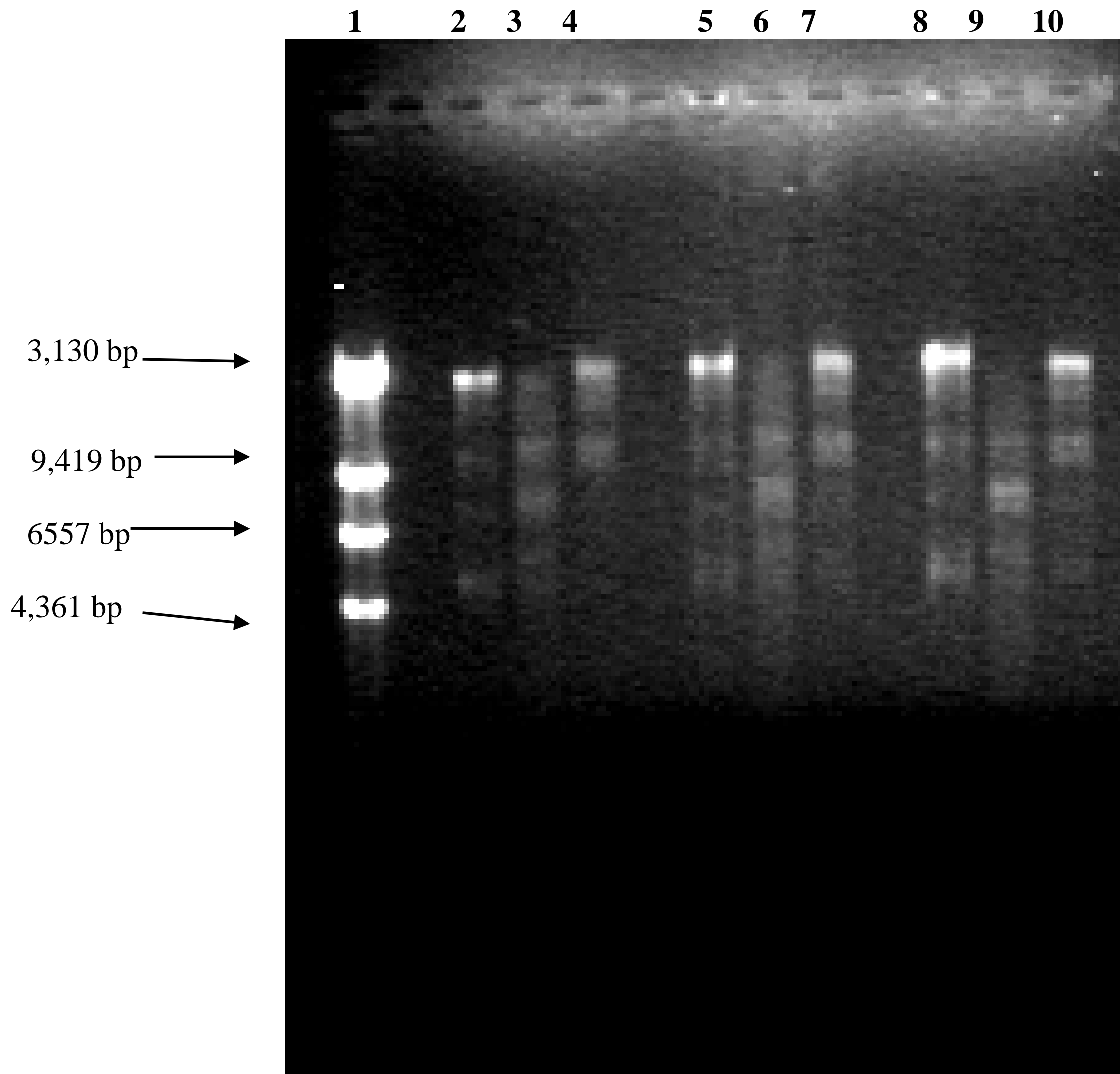


Figure 13: Restricted plasmid isolates of 1M, V24.M and K10.2. Lane 1, DNA Standards, Lane2, 1M unrestricted; Lane 3, 1M(EcoRI); Lane 4, 1M (HindIII); Lane 5, V24-M (unrestricted); Lane 6, V24-M (EcoRI); Lane 7, V24-M (HindIII); Lane 8, K10-2 (unrestricted); Lane 9, K10-2 (EcoRI); Lane 10; K10-2, (HindIII).

5.0 GENERAL DISCUSSION AND CONCLUSIONS

The morphological and colony characteristics of the *Bt* isolates 1M, K10-2 and V24-M were rod shaped forming whitish creamish colonies on nutrient agar with rough edges. Therefore this technique on its own cannot be used in effective characterization of the *Bt* isolates as the morphological and colony characteristics of the *Bt* isolates were similar to those obtained from previous studies conducted by Wangondu (2001).

1M isolates had a slightly elevated growth rate at the lag phase and exponential phase than both K10-2 and V24-M isolates, although at the plateau phase the growth appeared to be equal for all the three isolates (Fig 2). Hence 100% mortality exhibited by the 1M isolates were attributed to factors such as elevated growth rate.

Smirnoff staining protocol revealed that for the time interval ranging from 0 to 36 hrs there was no spore formation and crystals for all the three isolates 1M, V24-M and K10-2. 1M and V24-M isolates exhibited the first spores and crystals at 48 hrs that were of minute proportions. The first crystals and spores of K10-2 were witnessed at 54hrs. There was then a gradual increase in the quantity of crystals produced with the optimum density experienced at 72 hrs for all the isolates (Appendix III). All crystals stained with an almost black luster with lilac blue tint. The crystals obtained were bipyrimidal in shape hence indicating that the *Bt* isolates are lepidopteran specific (Wang' ondu 2001). The spores stain pink, whereas bacterial cells and their fragments assume a light lilac tint. However 1M isolates appear has a greater density of crystals and also spores than either V24-M or K10-2 (Fig 3). This was similar to other previous findings characteristic of *Bt* isolates crystals and spores hence Smirnoff staining on its own is not an effective characterization tool in the elucidation of effective toxicity of *Bt* isolates.

The protein concentration of the three isolates indicated a general characteristic trend over a period of 72 hrs for all the *Bt* isolates showing a rapid increase in protein concentration during the first 12 hrs, then a phase where there wasn't any significant change from 12 hours to 30 hours (lag phase). There was then a gradual increase in growth for all the isolates between 30hours and 72hours (Fig 7). Up to date protein concentration graphs have not been exploited to compare and to characterize *Bt* isolates with differential toxicity as there is no credible evidence to suggest this, hence this technique incorporated with other simple tests may prove to be vital in the characterization of effective *Bt* isolates as elevated protein secretion as well as patterns and trends can yield vital information as it remains a relatively simple yet effective tool.

Different *Bt* varieties produce δ-endotoxins that differ in their biochemical properties and host specificity. This has been reported by Dulmage, (1993), reported that analysis of *Bt* crystals using SDS-PAGE, revealed that most Lepidopteran-active crystals contain 130- and/or 65 k DA proteins. The major protein bands in this study were of molecular weights, ~28kDA, ~65kDA and ~130kDA for all the isolates except isolate 1M, which showed two protein bands of molecular weight ~28kDA and ~65 kDA (Fig 8). The results are related to the findings of Wang'ondu (2001) hence the protein pattern of the crystals described in this report were thus similar to those of other varieties active against lepidopteran larvae. It was notable that 1M at 72 hrs had a remarkable approximately 28kDa protein band that was absent in both K10-2 and V24-M isolates. Additionally it was quite notable that 1M isolate was missing a protein band corresponding to ~130kDa that was present in both V24-M and K10-2. This can again explain why the different *Bt* isolates exhibited varying toxicities to lepidopteran larvae, however further studies should be conducted to ascertain the composition of the protein bands and investigate their insecticidal properties.

All *Bt* strains were shown to harbor at least one plasmid based on earlier work on *Bacillus thuringiensis* (Carlson *et al;* 1996) besides the genomic DNA. These plasmids have been linked to δ-endotoxin genes. Previous workers have shown that most of the δ- endoxin genes are linked to movable plasmids. More importantly it had been suggested that crystal genes were not just located on plasmids but mega sized plasmids. (Martin and Travers, 1989). Many of the *B. thuringiensis* subspecies have multiple crystal and cytolytic gene systems that are simultaneously expressed. This multiple expression had one major function, which was to increase the bacteria host killing range. This corresponded to the conclusive findings of this study that reveal that the sizes of the plasmids for all the *Bt* isolates were far greater than 22,387 bp suggesting that the crystal genes must reside within these mega plasmids.

The three *Bt* isolates showed varying levels of plasmid DNA concentration with 1M isolate having a remarkable elevated concentration than both V24-M and K10-2. This was attributed to the fact that 1M isolates had a greater growth rate than both V24-M and K10-2 due to a faster exponential phase which could yield greater number of cells hence it would be expected to yield DNA of greater concentration and purity (Table 4).

DNA profiles preliminary restricted with an array of restriction enzymes such as EcoRI, BamHI, HindIII, KpnI and SacI. *Bt* isolate 1M was digested with all the five enzymes revealing

an array of bands, whereas V24-M and K10-2 were digested only with EcoRI and HindIII. Further analysis of the restricted bands revealed that a unique band existed that had approximately 7,200 bp for all the *Bt* isolates cut with both restriction enzymes EcoRI and HindIII (Fig 13).

Conclusions.

Increase in the number of *Bt* collection has led to an increase in the discovery of new *Bt* isolates with insecticidal activity against a diverse range of insects or with increased insecticidial activity (Martin and Travers, 1989). This study elucidated the need for isolation of more local *Bt* isolates and a method of rapidly detecting effective *Bt* isolates rather than expensive and lengthy bioassays. Analysis of restriction digests has indeed made it possible to identify unique bands that may be used as markers of effective toxicity. This information will go hand in hand in providing a large genetic resource base for the utilization of *Bt* as a microbial insecticide or the incorporation of the gene coding for the toxic proteins into plants and or other micro-organisms. As even in the same order, different *Bt* strains show different host specificity.

Presence of bi-pyrimidal crystals and a remarkable selective toxicity against lepidopteran pests was a crucial indicator of their sub –species that corresponds to the *B. thuringiensis subsp. kurstaki*.

Secreted proteins of the *Bt* isolates exhibited a varied concentration over a period of 72hrs with 1M isolate recording a markedly rapid increase as compared to isolates V24-M and K10-2. It was notable that 1M at 72 hrs had a remarkable 28kDa protein band that is absent in both K10-2 and V24-M isolates. Additionally it was quite notable that 1M isolate was missing a protein band corresponding to ~130kDa that is present in both V24-M and K10-2.

The three *Bt* isolates show varying levels of plasmid DNA concentration with 1M isolate showing a remarkable elevated concentration than both V24-M and K10-2. This was as a result of 1M having a greater copy number than both V24-M and K10-2. Analysis of unrestricted plasmid DNA revealed that the *Bt* isolates had plasmids exhibiting one thick hazy band of molecular weight greatly exceeding 23,130 bps.

Mortality of 100% exhibited by the 1M isolates was attributed to many factors such as elevated growth rate, greater plasmid copy number that was indicated by the relative greater amount of plasmid DNA than either V24-M and K10-2. This in turn translated to greater number of plasmids that harbored greater number of crystal genes that may be expressed simultaneously leading to more potent crystal proteins that ensures propagation of a greater population than both the V24-M and K10-2.

A unique band exists that has approximately 7,200 bp for all the *Bt* isolates cut with both restriction enzymes EcoRI and HindIII. Hence this distinct band will be used as an effective tool

in characterization of potent *Bt* isolates instead of lengthy bioassay to screen for potent *Bt* isolates. However this information cannot be used in isolation and must be used with other simple and short protocols previously conducted in this study. Thus the methodology employed in this study has proven to be effective in the successful characterization of *Bt* isolates as the findings of this study has effectively accounted for the selective toxicity of the *Bt* isolates.

Further work to investigate the unique bands of interest using sophisticated assays such as a sequencing, sequence analysis and PCR, would act to verify with certainty of the sub-species of these isolates and also determine with greater accuracy the reasons for differential toxicity. Additionally it would be crucial to investigate and compare why 1M is more toxic than V24-M and K10-2 *Bt* isolate using these advanced techniques. From these investigations it should be possible to incorporate the factors that contribute to 1M toxicity into transgenic crops. Further studies that would provide an insight as to why *Busseola fusca* remains resistant to most of these *Bt* isolates should also be conducted.

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APPENDIX

Appendix I

Molecular weight markers employed for estimation of molecular weight by SDS-PAGE.

Marker	Molecular weight (kDA)
Myosin	200
β- galactosidase	116
Phosphorylase b	97.4
Bovine serum albumin	66
Egg albumin	45
Glyceraldehyde –3-phosphate	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20

Appendix II

List of solutions

Solution I			
[Stock]	**Reagent**	**Amount**	**Final**
1M	Glucose	5ml	50mM
1M	Tris-HCl (pH =8)	2.5ml	25mM
0.5M	EDTA (pH = 8)	1ml	10mM
--	H_2O	90.5ml	--

Solution II			
[Stock]	**Reagent**	**Amount**	**[Final]**
10N	NaOH	20µl	0.2N
10%	SDS	100µl	1%
--	H_2O	880µl	--
Freshly prepared			

Solution III	
Reagent	**Amount**
Potassium Acetate	44.1675 g
Glacial Acetate Acid	11.5ml
H_2O	To 100ml
Dissolve the Potassium acetate in ~ 60 ml water, add the acetic acid then add water to reach 100ml.	

Tris- EDTA (TE)			
[Stock]	**Reagent**	**Amount**	**[Final]**
1M	Tris	1ml	10mM
0.5M	EDTA	0.2ml	1mM
--	H_2O	98.8ml	--

LB BROTH	
Constituent (Per 500ml)	**Amount**
Bacto tryptone	5 g
Bacto yeast extract	2.5 g
NaCl	5 g
H_2O	To 500ml
Adjust pH to 7.5 using Sodium hydroxide and autoclave.	

TAE (Electrophoresis Buffer) 10x		
[Stock]	**Reagent**	**Amount**
--	Tris- HCl (pH = 8)	121g Tris Base
0.5M	EDTA (pH = 8)	50ml
--	Glacial Acetate	28.55ml
--	H_2O	421.55ml

STE (CELL RESUSPENSION SOLUTION)			
[Stock]	**Reagent**	**Amount**	**[Final]**
1M	Tris	500μl	0.01M
0.5M	EDTA (pH = 8)	100 μl	0.001 M
5M	NaCl	10ml	1M
--	H_2O	39.4ml	--

LYSIS BUFFER			
[Stock]	**Reagent**	**Amount**	**[Final]**
1M	Tris	1.25 ml	0.025 M
0.5M	EDTA (pH = 8)	1ml	0.01 M
50mg/ml	Lysozyme	160 µl	8mg/ml
--	H_2O	37.75ml	--
Autoclaved. Sucrose was added to a final concentration of 25% under sterile conditions			

Appendix III

Quantification of spores, crystals and cells of *Bt* isolates after Smirnoff staining.

Time (hrs)	1M			V24-M			K10-2		
	CD	**S**	**C**	**CD**	**S**	**C**	**C D**	**S**	**C**
0	-	-	-	-	-	-	-	-	-
6	+	-	-	+	-	-	+	-	-
12	+	-	-	+	-	-	+	-	-
24	++	-	-	+	-	-	+	-	-
30	++	-	-	+	-	-	++	-	-
36	+++	-	-	++	-	-	++	-	-
48	+++	+	+	+++	+	+	+++	+	+
54	+++	++	++	+++	+	+	+++	+	+
60	+++	++	++	+++	+	+	+++	++	++
72	+++	+++	+++	+++	+++	+++	+++	+++	+++

KEY

- Absent

+ Sparsely present

++ Moderate

+++ Densely present

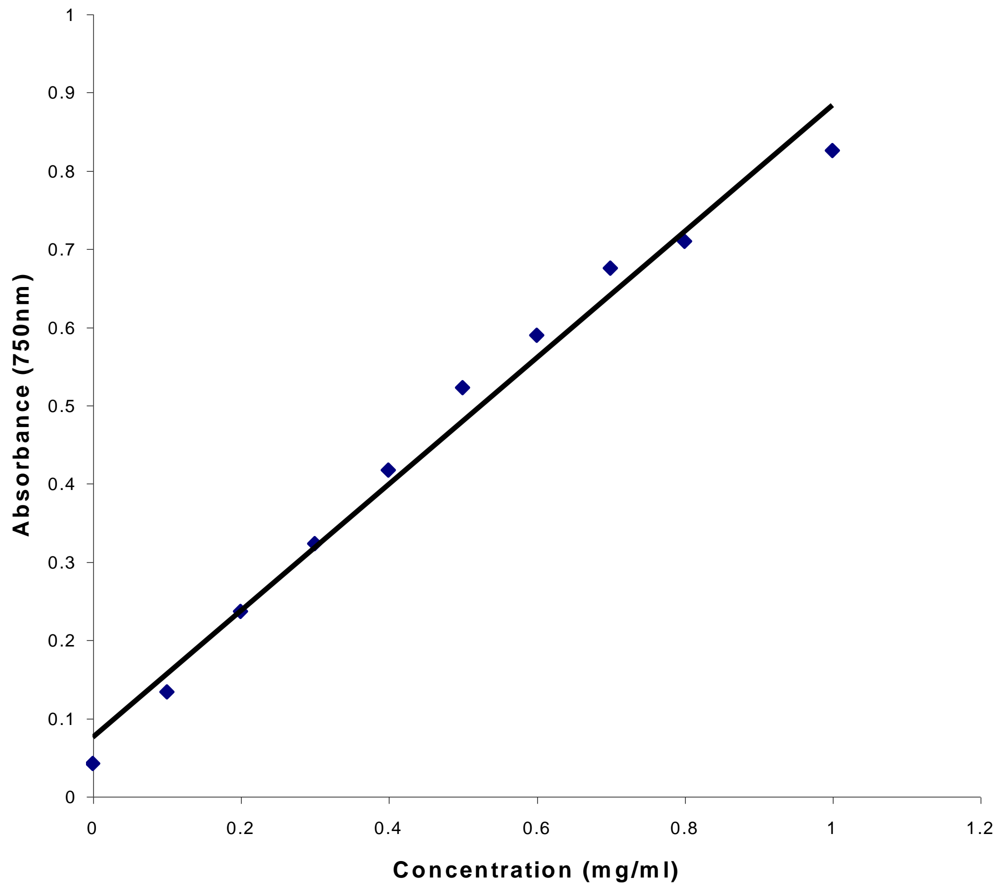
CD Cell Density

S Spores

C Crystal

The table compares the amount of spores, crystals and cells produced after every 6 hr interval for all the *Bt* isolates.

Appendix IV



y = mx + c

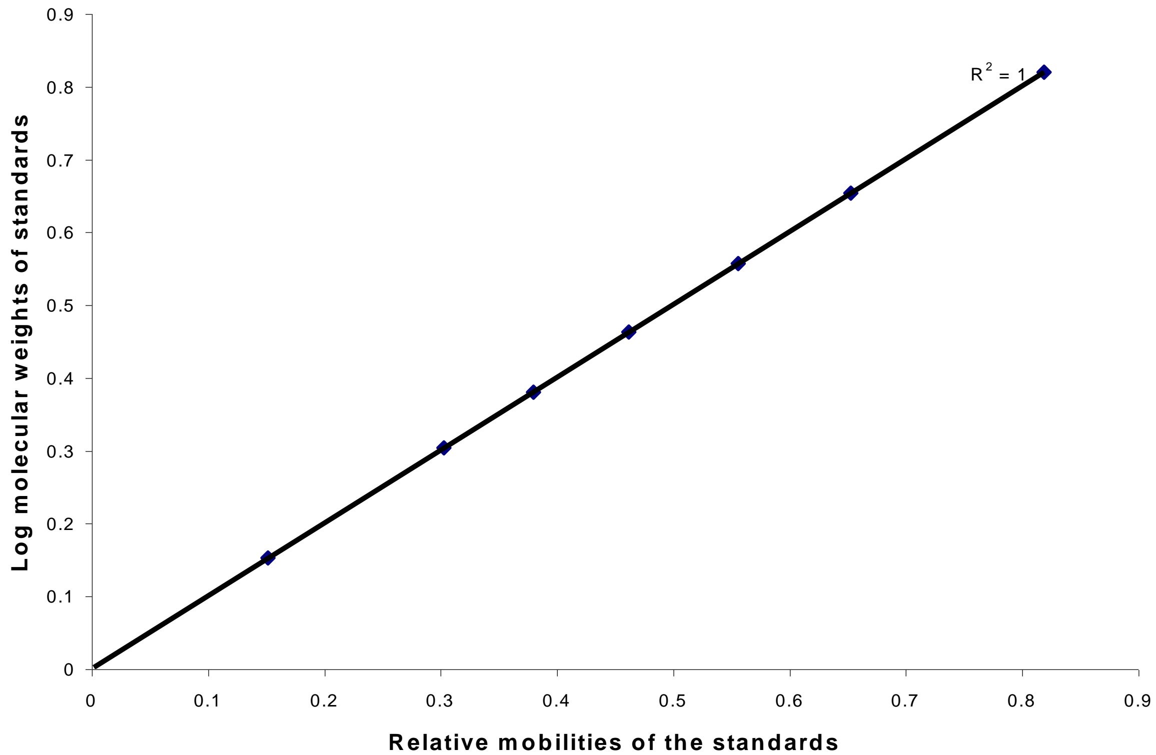
OD750nm = 0.04195 + 0.64754* Protein conc. (mg/ml)

Correlation: r = 0.99426

Standard curve for protein concentration determination.

The standard curve was constructed using BSA of varying concentrations and absorbance's taken at 750nm.

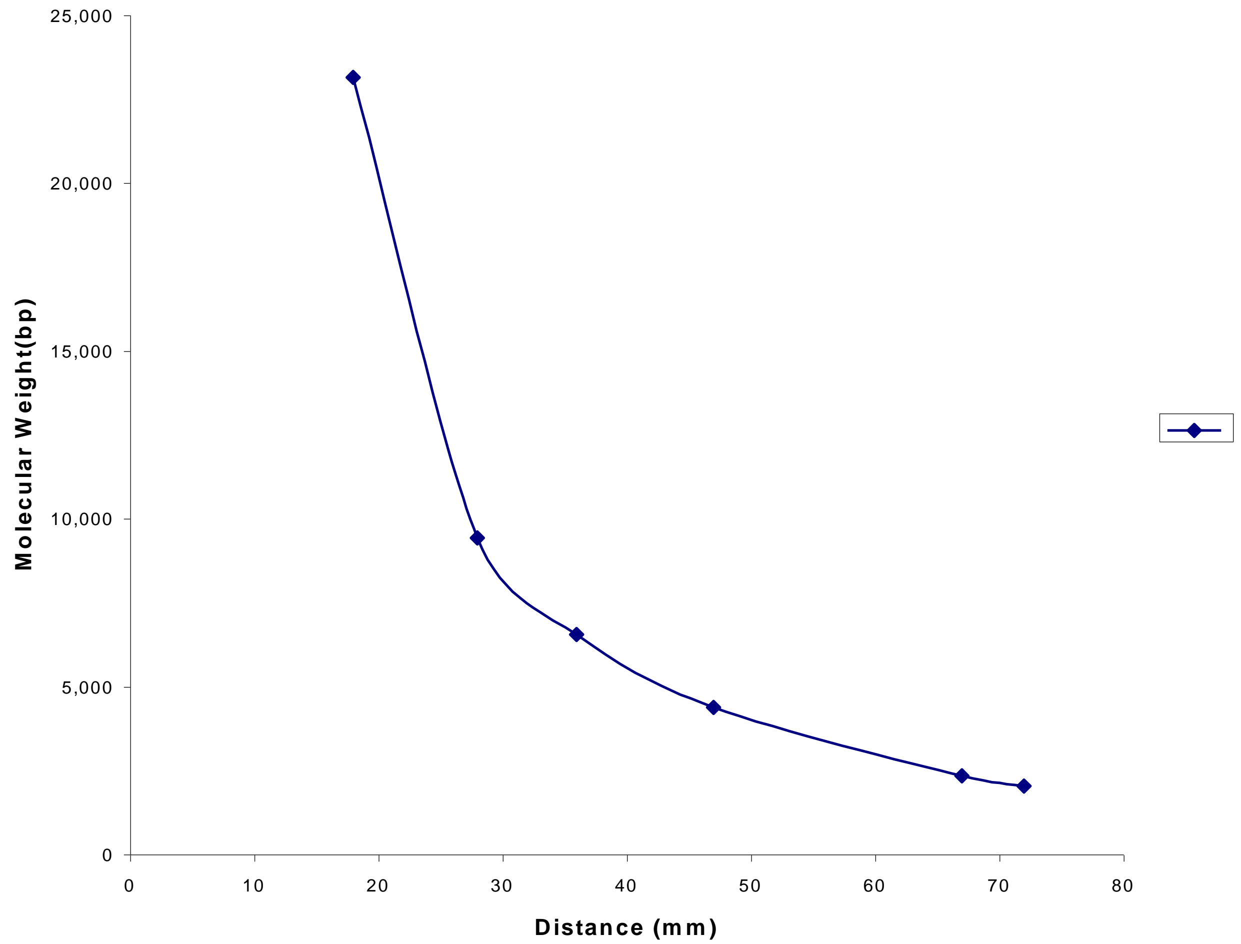
Appendix V



Standard curve for estimation of molecular weights SDS-PAGE.

Measuring the relative movement of molecular weight markers on a 12% SDS polyacrylamide gel electrophoresis and plotting against log molecular weights of BSA the standard curve was then constructed.

Appendix VI



Semi log graph of HindIII digested lambda DNA.

The standard curve was constructed by measuring the relative movement of molecular markers on a 0.7% agarose gel then plotting against log molecular weight.