CLONING OF GENES ENCODING INSECTICIDAL PROTEINS (*cry* / *vip* genes) OF *Bacillus thuringiensis* FROM WESTERN GHATS OF KERALA

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Cloning of genes encoding insecticidal proteins (*cry/vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Neema.P.M

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ABBREVIATIONS

bp	Base pair
BLAST	Basic local alignment search tool
cm	Centimeter
cfu	Colony forming unit
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DMFO	Dimethyl formamide
DNA	Deoxy Nucleotide Triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic acid
E- PCR	Exclusive PCR
g	Gram
ICP	Insecticidal crystal protein
IPTG	Isopropyl thio galactoside
IPM	Integrated pet management
KAU	Kerala Agricultural University
kb	kilo base
kDa	kilo dalton
LB	Luria Bertani
LC	Lethal concentration
M	Mole
MDa	Mega dalton
ml	Millilitre
mM	Millimole
µg	Microgram
µl	Microlitre
µM	Micromole
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer

OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenyl methyl sulfonyl flouride
pH	Hydrogen ion concentration
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodum dodecyl sulphate
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
vip	Vegetative insecticidal protein
v/v	Volume by volume
w/w	Weight by volume
X-gal	5- bromo 4 chloro 3- indolyl β-D galactosidase

Dedicated to my family,
Loving memory of my
mother and
to my beloved teacher
Dr.D.Girija

1. INTRODUCTION

Over half a century, the continuous use of synthetic pesticides has led to serious problems like environmental degradation and development of resistance in insect pests. This has created an urgent need for the development of environment friendly pesticides to reduce contamination and the likelihood of insect resistance (Sheltan *et al.*, 2002). Recently, there has been a renewed interest in the development of biological alternatives to chemical pesticides.

Bacillus thuringiensis Berliner is considered as one of the most versatile microbial insecticides. It is a gram positive, spore forming soil bacterium. The insecticidal activity is based on the ability of the bacterium to produce large quantities of larvicidal proteins known as delta-endotoxins. In addition to this, some strains are capable of producing insecticidal crystal proteins during the vegetative growth period. These are known as vegetative insecticidal proteins or exotoxins.

Exploiting the insecticidal activity of the bacterium, several bioinsecticides have been developed for the control of insect species among the orders Lepidoptera, Coleoptera and Diptera. There are reports on *B. thuringiensis* isolates active against other insect orders like Hymenoptera, Homoptera, Orthoptera, and Mallophaga and also against nematodes, mites and protozoa (Fietelson, 1993).

As a result of the increased selection pressure and continuous exposure to a single kind of toxin, insect pests have evolved varying levels of resistance. In order to face the certainity of wide spread *Bacillus thuringiensis* resistance, wise management strategies have to be adopted. Intensive screening programmes are going on worldwide to isolate large number of *B. thuringiensis*, in order to identify new strains with increased levels of insecticidal activity against a broader spectrum of insect pests. *B. thuringiensis* seems to be indigenous to many

environments. Different bacterial strains have been isolated from many habitats including soil, insects, stored products, deciduous and coniferous leaves etc. Hence, novel genes of *B. thuringiensis* from new isolates need to be explored.

Another option for resistance management is the expression of multiple crystal proteins in crops. Expression of two or more toxins in transgenic plants that act independently against the same insect will reduce the probability of resistance development in insect populations. Recently, cloning of insecticidal crystal protein genes and their expression in plant associated microorganisms or transgenic plants have provided alternative strategies for the protection of crops against insect damage.

In this study, an attempt has been made to isolate *B. thuringiensis* strains from the Western Ghats of Kerala. The Western Ghats of India is one among the eighteen hotspots of biodiversity of the world and is expected to harbour novel isolates of *B. thuringiensis*. In this context, the present study was taken up with the following objectives:

1. Isolation of ten strains of *Bacillus thuringiensis* from soil samples collected from the Western Ghats of Kerala.
2. Morphological and biochemical characterization of *Bacillus thuringiensis* isolates.
3. Screening for biocontrol efficiency against pumpkin caterpillar (*Diaphania indica* Saunders).
4. Cloning of *cry/vip* genes from *B. thuringiensis* isolates.

Review of Literature

2. REVIEW OF LITERATURE

The damage caused by pests and diseases in various crops in the field and in stored grains is estimated to be 30-90 per cent, which accounts for an annual loss of around Rs.10,000 crores in India. While chemical pesticides have played an important role in increasing food production in India, their indiscriminate use has led to several environmental problems such as pesticide residues in the food products. Application of chemical control agents against insect pests has severe drawbacks related to the appearance of insect resistance, emergence of secondary pests, the impact on non-target organisms, environmental pollution and residues on agricultural products.

Recent trends in pest management clearly demonstrate the scope for biological control in integrated pest management systems in agriculture. With the recognition of the concept of integrated pest management, biological control has assumed a special meaning over the past decade or two and newer concepts have emerged involving tritrophic interactions in place of two level trophic systems laying adequate stress on chemical ecology and behavioural aspects. There is a tremendous resurgence in biocontrol practices and efforts are on the way to define and redefine biocontrol, through genetic engineering approaches (Ananthakrishnan, 1992).

During the past few years, the development of plant genetic engineering as a tool for crop improvement has led to the development of crop plants resistant to insect attack by using *Bacillus thuringiensis* toxins. The use of biocontrol agents is a promising alternative to ecologically disruptive pest control measures (Jayaraj and Rabindra, 1992).

Several crystal toxin genes of *Bacillus thuringiensis* have been coned and expressed in bacteria like *Pseudomonas*, baculoviruses like Nucleopolyhedrosisviruses and plants like tobacco. Recent advances in molecular

biology and genetic engineering have enabled to modify the genetic constitution of biocontrol organisms with the view of achieving better or desirable traits.

In this study, attempts have been made to characterize and clone *cry* genes from *B. thuringiensis* isolated from the Western Ghats of Kerala. A comprehensive review of the previous research studies related to the topic had been done in accordance with the objectives of the present study. The contents of this chapter are presented below under the following headings:

Origin and ecology of *B. thuringiensis;* insecticidal crystal proteins produced by the bacterium; use in integrated pest management; insect resistance and its management; isolation and characterization of *B. thuringiensis*; bioactivity on different insects and cloning of insecticidal genes of *B. thuringiensis.*

2.1 ORIGIN OF *Bacillus thuringiensis*

The first published reference to a microorganism with the characteristics of *Bacillus thuringiensis* appeared from Dr. Ishiwata Shigetane in 1901, when he identified the bacterium in silkworm farms as the causal agent of 'sotto' bacillus disease (Aizawa, 1983).

Later in 1911, Berliner discovered the insecticidal activity and coined the name *Bacillus thuringiensis*, after isolating a strain toxic to the larvae of the Mediterranean flour moth (*Anagasta kuhniella* Zeller), from a granary in Germany. He recognized the possibility of controlling the moth by adding *B. thuringiensis* to infested flour and proved Koch's postulates of Bacteriology.

Bacillus thuringiensis is a gram positive, facultatively anaerobic, rod shaped spore forming soil bacterium. The primary insecticidal activity of *B. thuringiensis* is due to insecticidal crystal proteins (Aronson, 1994). Existence of parasporal inclusions in the bacterium was noted in 1915 by Berliner. During sporulation, it produces parasporal inclusions adjacent to the endospore. Endospores are resistant to inactivation by heat and desiccation and persist in the

environment under adverse conditions. They provide a mechanism for long term survival of *B. thuringiensis*.

2.2 INSECTICIDAL CRYSTAL PROTEINS

The parasporal inclusions consist of one or more insecticidal proteins in the form of a crystal or crystal complex. These are known as insecticidal crystal proteins (ICPs) or delta-endotoxins. Hannay and Fitz-James in 1950 rediscovered the parasporal inclusions and showed that the crystal was composed of protein. Angus (1956) proved that the insecticidal activity was caused by parasporal inclusions, by separating the toxin from spores.

The insecticidal crystal proteins are protoxins and must be activated before these produce any effect. They are commonly designated as cry proteins encoded by *cry* genes. The genes that encode ICPs are mostly on plasmids. A subspecies can synthesize more than one type of insecticidal crystal protein (Kronstad *et al.*, 1983). They have various forms such as bipyramidal, cuboidal, flat rhomboidal or composite with two or more crystal types (Chilcott and Wigley, 1994).

More than 150 insects belonging to the orders Lepidoptera, Diptera and Coleoptera are known to be susceptible to *B. thuringiensis* (Beegle and Yamamoto, 1992). Fietelson *et al.* (1992) reported *Bacillus thuringiensis* isolates active against other insect orders like Hymenoptera, Homoptera, Orthoptera, Mallophaga and also against nematodes, mites and protozoa. Insecticidal crystal proteins are extremely potent and toxic to target insect larvae at pico mole concentrations. Different domains of insecticidal crystal proteins are responsible for host susceptibility and toxicity.

2.2.1 Vegetative insecticidal proteins

In addition to the crystal associated toxic polypeptides, some insecticidal proteins produced during vegetative growth of the bacteria had also been

identified. These proteins, called vegetative insecticidal proteins (vip), were reported from about 15 per cent of the *B. thuringiensis* strains analyzed (Estruch *et al.*, 1996). The vegetative insecticidal proteins represent a structurally different group of insecticidal toxins produced by different strains of *Bacillus thuringiensis*. With several laboratories reporting development of resistance in insects against insecticidal crystal proteins, the vip toxins offer a promise of extending the usefulness of toxins to delay the onset of resistance in insects (de Maagd *et al.*, 1999; Tabashnik *et al.*, 2000), especially since the structural divergence of vip is a suggestive of a different mode of action.

During vegetative growth, some subspecies of *Bacillus thuringiensis* viz. *galleriae* and *darmstadiensis* produce beta-exotoxin, an ATP analogue, water-soluble and heat soluble secondary metabolite. Beta-exotoxin is an inhibitor of RNA polymerase and acts competitively with ATP in various biological processes. It has broad-spectrum insecticidal activity and is toxic to all forms of life including humans (Bajwa and Kogan, 2001).

2.2.2 Cytocidal proteins

The other class of toxic polypeptides is cytocidal proteins (cyt), which acts together with cry to effect larvicidal activity of the parasporal crystal (Park *et al.*,2002). This class includes cyt1, cyt2, cytC, and cytD. Variations of these have been found in *Bacillus thuringiensis* subspecies *israelensis* (Guerchicoff *et al.*, 1997).

Classification of crystal proteins

Cry proteins were classified according to their insect specificity (Bravo *et al.*, 1998).

Table 1. Insecticidal spectrum of crystal proteins

Cry group	Insect specificity
Cry 1	Lepidoptera
Cry 2	Lepidoptera & Diptera
Cry 3	Coleoptera
Cry 4	Diptera
Cry 5	Coleoptera & Lepidoptera

According to the latest classification of *B. thuringiensis* toxins by Crickmore *et al.* (1998), crystal protein genes have been classified under 53 groups, with each group having different subgroups within them. The other class of toxic peptides known as cytocidal proteins was grouped mainly under two classes, cyt 1 and cyt2, with different subclasses among them.

2.3 MODE OF ACTION OF *Bacillus thuringiensis*

Insecticidal crystal proteins, endospores or beta-exotoxins dominate bioactivity of *Bacillus thuringiensis.* To be effective as an insecticide, the insecticidal crystals must first be ingested by *B. thuringiensis* sensitive insects. These crystals are then exposed to alkaline conditions in midgut. The crystals are solubilized and processed concurrently by the alkaline gut juice into an active toxin by the action of midgut proteinases (Luthy and Ebersold, 1981). The alkaline solubilized crystal proteins are about 130kDa and require activation by insect gut proteins (Huber *et al.*, 1981). The crystal proteins therefore are called protoxins, signifying that it requires activation (Aronson *et al.*, 1991). The *cry*1A

type protoxins of 130kDa are soubilized and activated by gut enzymes to 55 to 65kDa toxins (Knowles and Dow, 1993). When artificially solubilized in an alkaline solution without proteinase, most crystal proteins, particularly those of 130kDa classes are not toxic to insect cells (Yamamoto and Powell, 1993).

It is generally accepted that the toxin recognizes specific receptors at the brush border membrane surface of gut epithelial cells (Hoffman *et al.*,1988; Van Rie *et al.*, 1989; Honee *et al.*,1991) and that the toxin receptor complex forms a pore for potassium ions from lumen to haemolymph through the cell membrane. The insect cells affected by the toxin lose their ability to regulate osmotic pressure and eventually lyse due to their massive water intake (Knowles and Ellar, 1987). Feeding paralysis is caused by the increase of potassium ions in the haemolymph. The mode of action of delta-endotoxin involves proteolytic activation, binding of toxin to receptors, pore formation leading to ionic imbalance, cessation of feeding, septicemia and eventually death (Thomas and Ellar, 1983; Knowles and Ellar, 1987; Hofte and Whitely, 1989; Slatin *et al.*, 1990; Gill *et al.*, 1992, Schwartz *et al.*, 1991). The rate of crystal solubilization is an important factor that will influence the bioactivity of *B. thuringiensis*.

The infected caterpillars become inactive and stop feeding. The caterpillar becomes flaccid and dies, usually within days. The body contents turn brown to black as they decompose (Aronson *et al.*, 1986)

2.4 ECOLOGY OF *Bacillus thuringiensis*

Bacillus thuringiensis has been found extensively in the phylloplane. Numerous subspecies have been isolated from coniferous trees, deciduous trees and vegetables. These have also been recovered from soil samples and stored products. Spores persist in soil and vegetative growth occurs when nutrients are available.

2.5 USE OF *Bacillus thuringiensis* IN INTEGRATED PEST MANAGEMENT (IPM)

B. thuringiensis is the most versatile biopesticide for use in pest management. It is used in agricultural crops, harvested produce in storage, ornamentals, bodies of water and around the home to control various groups of insects, depending on the type of toxin produced by the specific isolate of *B. thuringiensis*. There are different strains or varieties of the bacterium available that have been selected for the control of specific insects.

The first large scale *B. thuringiensis* based product was released in 1957, which was a *Bacillus thuringiensis* var. *kurstaki* strain (Feitelson, 1993). Goldberg and Maegalit (1977) discovered *B. thuringiensis* var *israelensis* active against mosquito (*Aedes aegypti*) and black fly larvae (*Simulium* sp). This was the first report of bacterial strain killing non-lepidopteran target and this doubled the number of known susceptible insect orders. The range of *B. thuringiensis* toxicity was once again expanded by the discovery of bacterial strains active on Colorado potato beetle (*Leptinotarsa decemlineata*) and elm leaf beetle (*Xanthogaleruca luteola*).

Trumble (1985) demonstrated the benefit of use of *B. thuringiensis* var. *kurstaki* in a pesticide rotation to decrease the development of resistance by target insects. Sears *et al.* (1983) reported the successful use of *B. thuringiensis* in an IPM programme against cabbageworm (*Pieris rapae*). Broza *et al.* (1984) demonstrated by conducting tests, the use of *Bacillus thuringiensis*, as a potential component in IPM programmes for Egyptian cotton leaf worm (*Spodoptera litura*).

Bacillus thuringiensis tenebrionis active on coleopteran pests have been shown to control Colorado potato beetle under field conditions and is now being used commercially (Ferro and Gelerntes, 1985). *B. thuringiensis* had been used

operationally for controlling forest caterpillar pests for many years (Cunningham, 1988). Bowen (1991) reported the potential of the bacterium in IPM for arboriculture.

Bacillus thuringiensis based microbial pesticides are used extensively in integrated pest management programmes in vegetables, concerned about residue on harvested products or to decrease the selection pressure from the use of conventional pesticides for control of diamond back moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*) on cole crops, lettuce and tomato (Zalom *et al.*,1992). He also reported the use of *B. thuringiensis* in IPM against leaf roller management in tree fruit crops. It also has the potential to be used as a bioinsecticide against peach twig borer. Edwards and Fords (1992) cited the use of the bacterium as a crop protectant in corn for European corn borer (*Ostrinia nubilalis)* and in soyabeans for green clover worm (*Hypena scabra*) and soyabean looper (*Pseudoplusia includens*).

New genetically engineered *B. thuringiensis* products may provide more opportunities and choices for growers using IPM programmes. The most successful products will be ones that provide efficacy and consistency competitive with traditional chemicals.

2.6 INSECT RESISTANCE TO *Bacillus thuringiensis*

The development of resistance to pesticides has long been a major concern within the agricultural community. More than 645 insect and mite species have already become resistant to various synthetic insecticides in the absence of integrated pest management (Georghiou and Tajeda, 1991; Rajamohan, 1998).

Resistance to *Bacillus thuringiensis* insecticides was for a long time believed to be a remote possibility, primarily because no field resistance had been observed in some twenty years of use. Resistance to the bacterium was first reported in Indian mealmoth (*Ploidia interpunctella)* larvae collected from grain storage facilities that had been treated with *B. thuringiensis* (McGaughey and Beeman, 1988; Gelernter, 1997). Development of resistance to the bacterium in

the field was reported from diamond back moth. (*Plutella xylostella)* (L.) (Tabashnik *et al.,* 1990; Ferre *et al.,* 1991; Shelton *et al.,* 1993; Tabashnik *et al.,* 1994). Substantial resistance to crystal proteins was found in insect populations inhabiting water stress fields treated with *B. thuringiensis* insecticides (Tabashnik, 1998).

Laboratory selection resulted in *B. thuringiensis* resistance in the tobacco budworm (*Heliothis virescens)* and Colorado potato beetle (Whalon *et al.,* 1993). Kranthi *et al.* (2000) reported seventy six fold resistance in an Indian population of Cotton bollworm (*Helicoverpa armigera*) against *cry*1Ac after ten generations of selection. Indian populations of *Helicoverpa armigera* were significantly less susceptible to *cry*IIAa than *cry*I (Chakrabarthi *et al.,* 1996; Babu *et al.,* 2002).

2.7 RESISTANCE MANAGEMENT

Tabashnik (1990), Croft (1990) and Denholm and Rowlan (1992) emphasized the need to develop resistance management programmes that integrate into IPM systems to take advantage of reduced selection pressure. Several strategies were proposed for resistance management (Tabashnik *et al.,* 1991; Bosch *et al.,* 1994; Hokkanen and Deacon, 1994; Kennedy and Whalon, 1995; Gelernter, 1997; Schnepf *et al.,* 1998). These include the use of non-treated refugia, high dosage, seed mixtures, toxin mixtures and the rotation or alternation of *B. thuringiensis* toxins.

Transgenic crops expressing insecticidal protein genes from *B. thuringiensis* are revolutionizing agriculture. *B. thuringiensis* toxins had been engineered into major crops. Since continuous exposure of insect pests to a single kind of bacterial toxin had led to rapid development of resistance in insects, it is essential to focus on identification of more potential genes, for the success of *B. thuringiensis* transgenic crops in India.

2.8 ISOLATION *OF B. thuringiensis* FROM DIFFERENT ENVIRONMENTS

The natural habitat of *B. thuringiensis* varies from phylloplane, soils and bodies of dead insects. *B. thuringiensis* has been isolated from diverse environmental conditions. In undisturbed ecological niches, lot of diversity is expected in the bacterial strains as a result of shuffling of toxic genes amongst the strains.

Martin and Travers (1989) developed sodium acetate selection to isolate *Bacillus thuringiensis*. Isolation of *B. thuringiensis* strains was reported from soil samples collected from various habitats (Yu *et al.,* 1990; Chilcott and Wigley, 1993; Johnson and Bishop, 1996; Widiastuti *et al.,* 1996; Benhard *et al.,* 1997; Chang *et al.*, 1999; Kim *et al.*, 1998; Lee *et al.*, 2001; Arango *et al.*, 2002; Asano *et al.*, 2003; Valicente and Barreto, 2003; Leithy *et al.*, 2004; Fuchu *et al.*, 2004; Obeidat *et al.*, 2004; Hernandez *et al.*, 2005; Chak *et al.*, 1994). Rampersad and Ammons (1995) reported the isolation of the bacterium from environmentally diverse sources including beach sand, forest soil, aquatic and intertidal sediments and soils from urban, rural and agricultural areas.

Isolation of *Bacillus thuringiensis* strains was done from phylloplane (Koo *et al.,* 1995) and agricultural fields (Petras and Casida, 1985) and analyzed for their insecticidal activity. Kawalek *et al.* (1995) isolated a new mosquitocidal strain, *B. thuringiensis* subspecies *jegathesan.* Johnson *et al.* (1996) reported the isolation of *B. thuringiensis* subspecies *tenebrionis* from rice grain dust. Hossain *et al.* (1997) obtained 650 *B. thuringiensis* isolates from soil samples collected randomly from agricultural lands of Bangladesh. Uribe *et al.* (2003) isolated 108 *B. thuringiensis* native strains from soil samples obtained from agricultural crops and wild ecosystems of Columbia.

Porcar *et al.* (1999) isolated two new *Bacillus thuringiensis* strains from dust in a corn silo in the province of Navarro and from horticultural soil in the

province of Lleida, Spain. Porcar and Caballero (2000) reported the isolation of a new *B. thuringiensis* belonging to the serovar *aizawai* from the dead larvae of the lepidopteran, Cosmoplitan (*Mythemna loreyi*) collected in a corn crop in Spain during a natural epizootic. Kim (2000) isolated *Bacillus thuringiensis* strains from sericultural farms, soils and granary samples of Korea. Kaelin and Gadani (2000) conducted a worldwide survey to evaluate the frequency and distribution of bacterial populations on cured tobacco leaves during post harvest storage. Soo *et al.* (2000) isolated the bacterium from soil samples collected at mushroom houses.

Prabagaran *et al.* (2002) reported the isolation of *B. thuringiensis* strains from different agro climatic regions of India. Jensen *et al.* (2002) isolated the bacterium from feacal samples of green house workers exposed to the use of *B. thuringiensis* based insecticides. Swiecicka and Mahillon (2005) recovered several *B. thuringiensis* isolates from the intestines of small wild rodents and insectivores.

2.9 MAINTENANCE OF *Bacillus thuringiensis*

After isolation of *B. thuringiensis*, it is essential to maintain the organism for further studies. To maintain bacterial cultures there are several methods such as overlaying cultures with mineral oil, lyophilisation (freeze drying), storage in sterile water and storage in glycerol.

2.10 MORPHOLOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *Bacillus thuringiensis*

Many phenotypic and genotypic methods are presently being employed for microbial identification and classification (Louws *et al.*, 1996). Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, and biovar to the strain specific level.

B. thuringiensis isolates were characterized based on the growth habits on culture medium. Colony morphological features like shape, size, colour, elevation etc were used as criteria for cultural characterization. *B. thuringiensis* isolates were usually characterized and grouped based on biochemical tests. Martin and Travers (1989) identified *B. thuringiensis* strains by using biochemical tests such as starch hydrolysis, urease production, mannose, sucrose and salicin fermentation, esculin utilization and lecithinase production.

Benhard *et al.* (1997) studied the crystal characteristics of the bacterial strains isolated from different countries. The parasporal inclusions were examined to study the crystal morphology, crystal size relative to the spore, the number of crystals in each spore and whether each cell produced a crystal. Crystal morphology was classified as bipyramidal, spherical, rectangular or cuboidal, irregularly spherical or irregularly pointed.

Porcar and Caballero (2000) compared the morphological characteristics of new *B. thuringiensis* strains isolated from the dead larva of lepidopteran, Cosmopiltan *(Mythemna loreyi)* with standard strains of *Bacillus thuringiensis* var. *kurstaki and aizawai*. The isolated strains showed similar morphological and biochemical characteristics to the standard strains. Kaelin and Gadani (2000) analyzed a total of 133 tobacco samples of different types and origins. Nine percent of samples showed the presence of *B. thuringiensis* and majority of the isolates produced bipyramidal crystals, and 75 per cent of them showed a second type of crystal protein (cuboidal or heterogeneous crystals). Only some isolates showed rhomboidal crystal morphology characteristic of anti-coleopteran *B. thuringiensis* subspecies *tenebrionis*.

Jung *et al.* (2003) studied seven *B. thuringiensis* strains upon their *cry*I type genes, crystal shapes, crystal protein patterns and insecticidal activities. Silva *et al.* (2004) analyzed the Brazilian strains of the bacterium using scanning electron microscopy for their crystal protein morphology. Strains S701 and S764 showed the presence of bipyramidal, cuboidal and round crystals, like in the strain

HD1 and bipyramidal and round crystals like in the strain S1265. Leithy *et al.* (2004) analyzed a total of 36 soil samples collected from different locations and specifications for total spore counts and for the occurrence and incidence of *B. thuringiensis*. These isolates were classified using biochemical tests. Obeidat *et al.* (2004) studied the parasporal crystal morphology of 26 *B. thuringiensis* strains isolated from different habitats of Jordhan, which revealed the presence of bipyramidal, spherical and cuboidal crystal inclusions.

2.11 BIOACTIVITY OF *Bacillus thuringiensis* AGAINST VARIOUS INSECT SPECIES

During sporulation, many strains of *B. thuringiensis* produce protein inclusions that are lethal to lepidopteran, dipteran and coleopteran insects. With the increased resistance of insects to single kind of toxins, there is a need for searching novel genes with broader spectrum of insecticidal activity.

The insecticidal activity of the delta-endotoxins of *Bacillus thuringiensis* belonging to 12 subspecies were determined by conducting bioassays against various lepidopteran pests (Jaquet *et al.*, 1987; Widner and Whiteley, 1989; Navon *et al.* 1990; Tang *et al.*, 1995; Rampersad and Ammons, 1995; Benhard *et al.*, 1997; Chang *et al.*, 1999; Carozzi *et al.*, 1991; Werneck *et al.*, 1999; White *et al.*, 1999; Kim, 2000; Porcar and Caballero, 2000; Arango *et al.*, 2002; Yong *et al.*, 2003; Polanczyk, 2003; Mohan and Gujar, 2002; Hernandez *et al.*, 2005; Silva *et al.*, 2004; Obeidat *et al.*, 2004; Leithy *et al.*, 2004). *Bacillus thuringiensis* strains causing 100 per cent mortality in the larvae were selected for potency determinations.

Chilcott and Wigley (1993) tested the activity of *Bacillus thuringiensis* against eight, first instar larvae of *Planotortrix octa* and *Tenebrio molitor*. Byung *et al.* (1995) found that *cry*VI protein was toxic to diamond back moth (*Plutella xylostella*) and Chinese silk worm (*Bombyx mori*) whereas *cry*V465 protein was toxic only to *Plutella xylostella*. Kawalek *et al.* (1995) conducted bioassays using parasporal inclusions isolated from the new mosquitocidal strain, *B. thuringiensis*

subspecies *jegasethan* against southern house mosquito (*Culex quinquefasciatus*), yellow fever mosquito (*Aedes aegypti*), Asian tiger mosquito (*Aedes albopictus*), and anopheles mosquito (*Anopheles maculatus*). The LC_{50} values of crystal inclusions for each species indicated that the parasporal inclusions from the new subspecies possessed mosquitocidal toxicity comparable to *B. thuringiensis* subspecies *israelensis*.

Johnson *et al.* (1996) conducted bioassays with the toxins in *B. thuringiensis* subspecies *tenebrionis* and recombinant *E. coli* against rice weevil. Widiastutti *et al.* (1996) studied the toxicity of indigenous *B. thuringiensis* isolates from various habitats in Indonesia, against inch worm moth (*Hyposidra talaca*). All the parasporal crystal forming bacteria obtained and three reference strains were not toxic to inch worm moth larvae. This study also indicated that toxicity of bacterial isolate did not always depend on the shape of its crystal protein. Peyronnet *et al.* (1997) performed bioassays with second instar larvae of the European gypsy moth (*Lymantria dispar*) and Chinese silk worm (*Bombyx mori*) to detect the ability of *B. thuringiensis* toxins to form pores in the midgut epithelial cell membrane of susceptible insects.

Kim *et al.* (1998) studied the toxicity effects of 58 strains of *B. thuringiensis* isolated from the soils of various regions in Korea republic. In toxicity tests, 35 per cent of all the isolates were toxic to lepidoptera, 20 per cent were toxic to diptera, and 9 per cent were non toxic isolates. A particularly large number of lepidopteran / dipteran active isolates were found. Fourty lepidopteran active isolates produced typical rhomboidal inclusions and the remainder belonging to the dipteran active and non toxic isolates was spherical in shape. Two isolates named S-333 and S-225 synthesized PCR products of *cry*IC gene, but the S-333 isolate which produced rhomboidal inclusion was toxic to both diamond back moth and mosquito whereas the isolate S-225 showed toxicity only to mosquito and produced spherical inclusion.

Jyoti and Brewer (1999) conducted bioassay experiments with banded sunflower moth larvae to *B. thuringiensis* to determine the median lethal concentration and its efficacy. Dobrzhanskaya *et al.* (2002) identified a new strain of *B. thuringiensis* 2-7 and found that it was toxic to Coleoptera. Balasubramanian *et al.* (2000) carried out the bioassay of a novel strain of *B. thuringiensis* subspecies *yunnanensis,* and it exhibited weak toxicity against larvae of diamond back moth. Bioassays showed no toxicity against several moths and mosquitoes. Asano *et al.* (2003) reported that a strain of *B. thuringiensis* subspecies *galleriae* isolated from Japan was highly toxic to the cupreous chafer (*Anomala cuprea*).

Lee *et al.* (2003) studied the mode of action of vegetative insecticidal protein (vip), the 88kDa *vip*3A full length toxin (vip 3A-F) was proteolytically activated to an approximately 62kDa toxin either by trypsin on lepidopteran gut juice extracts. Biotinylated *vip*3A-G demonstrated competitive binding to lepidopteran midgut brush border membrane receptors. In voltage clamping assays with dissected midgut from susceptible insect tobacco hornworm (*Manduca sexta)*, vip3A-G clearly formed pores whereas vip3A-F was incapable of pore formation.

Bhalla *et al.* (2005) reported toxicity of vip from 24 serovars of *B. thuringiensis* on larvae of *Spodoptera litura* and *Plutella xylostella.* Rajesh *et al.* (2006) studied the diversity of *B. thuringiensis* isolates of the Western Ghats of Kerala and Karnataka and their bioefficacy against *Spodoptera litura* and *Helicoverpa armigera.* Cabrare *et al.* (2007) performed bioassays with a laboratory strain of *Sesamia nonagrioides,* the Mediterranean corn borer to determine the mode of action of cryIAb protein and to search for other cry proteins that could be used to control the pest.

2.12 MOLECULAR CHARACTERIZATION OF *Bacillus thuringiensis*

DNA based methods have recently emerged as a more reliable, simple and inexpensive way to identify and classify microorganisms. The assignment of genera / species has traditionally been based on DNA-DNA hybridization

methods and modern phylogeny is increasingly based on 16S rRNA sequence analysis.

2.12.1 PCR based methods

Carozzi *et al.* (1991) reported the use of multiplex PCR with novel and specific primers to detect *cry* and *cyt* genes. A method referred to as Repetitive Extragenic Palindrome Polymerase Chain Reaction (Rep–PCR) genomic fingerprinting, a DNA amplification based technique has been found to be extremely reliable, reproducible, rapid and highly discriminatory (Versalovic *et al,,* 1994; Louws *et al., 1996*).

Ceron *et al.* (1995) used PCR strategy to rapidly identify *B. thuringiensis* strains that harbor any of the known *cryI or cryIII* genes. A general primer set, which amplifies DNA fragments from the known *cryI* or *cryIII* genes, was selected from conserved regions. Rampersad and Ammons (1995) conducted PCR amplification of toxin genes of *B. thuringiensis* isolated from different environmentally diverse areas, and probe hybridization with *cry* and *cyt* gene probes was carried out followed by sequencing.

Widiastuti *et al.* (1996) analyzed indigenous *B. thuringiensis* isolates by PCR, using lepidopteran specific primers. Four isolates contained *cryIA* gene and other *cry* genes and three isolates contained *cry* genes other than *cryI*. Lambert *et al.* (1996) reported the full characterization of a novel insecticidal crystal protein, cry9Ca1 from *B. thuringiensis* serovar *tolworthi*. Restriction Fragment Length Polymorphism of PCR amplified DNA was used to identify novel *cry* type genes (Kuo and Chak, 1996).

Extended multiplex PCR was used by Israeli collaborators to identify and classify *B. thuringiensis* strains containing *cry* genes against lepidopteran, dipteran and coleopteran pests (Ben-Dov *et al.*, 1997). Mexican isolates were characterized for the *cry* genes and results indicated the presence of strains harboring potentially novel *cry* genes as well as strains with combinations of less

frequently *cry* genes (Bravo *et al.*, 1998). Masson *et al.* (1998) used the approach of E-PCR with high-pressure liquid chromatography to analyze the gene content in *Bacillus thuringiensis* HD133.

A novel two step PCR technique named exclusive PCR that allows to clone genes for which no specific primers are available and in which a variable region exists between two conserved regions was used to detect novel *cry* genes (Juarez- Perez *et al.*, 1997). Kim *et al.* (1998) carried out PCR analysis using *cry*I, *cry*II, *cry*III, *cry*IV and *cry*V gene specific primers. The study revealed that *cry*IC gene predominated (57%), followed by *cry*IAb (45%) and *cry*IIA genes (34%). However, *cry*IE, *cry*IF, *cry*III, *cry*IVC and *cry*V genes were not reactive. Several isolates had universal PCR products and multiple insecticidal proteins. PCR result showed varied distribution of the *cry* type gene.

Kim (2000) characterized *B. thuringiensis* strains isolated from sericultural farms, soil and granary samples in Korea. PCR analysis was done using *cry*I, *cry*II, *cry*III, *cry*IV *and cry*V gene specific primers for determination of the *cry* gene contents of *Bacillus thuringiensis* isolates. It indicated that *cry*IA, *cry*IC, *cry*ID *and cry*II predominated among *cry* genes and the *cry*IB, *cry*IE, *cry*IF, *cry*IG and *cry*IV were not popular. In contrast no PCR products were detected for the *cry*III and *cry*V templates. Leithy *et al.* (2004) detected lepidopteran toxin gene *cry*I and dipteran toxic gene *cry*IV by PCR.

Porcar and Caballero (2000) characterized a new *Bacillus thuringiensis* strain isolated from dead larvae of lepidopteran, Cosmopiltan (*Mythemna loreyi)* using SDS PAGE, plasmid pattern and PCR analysis. DNA amplification was obtained with primers corresponding to six genes. The sequence information of amplified fragments revealed that these corresponded to *cry*IAa, *cry*IAb, *cry*ICa, *cry*IDa, *cry*IIAb and *cry*I1a.

Ben-Dov *et al.* (2001) designed a set of universal and specific primers for multiplex rapid screening of all known five genes for *cry*IX group. Loguerico *et al.* (2002) studied the compatibility of feeding bioassays using culture-supernatant proteins combined with PCR as a first tier screening strategy for *vip*3A-like genes efficient against beet army worm (*Spodoptera frugiperda*). The total protein concentrated from the culture supernatant of the strain HD125 yielded significantly high armyworm mortality and an intense band of predicted size for vip3A protein in SDS PAGE. However, PCR and sequencing data indicated *vip* like genes were ubiquitous in tropical *B. thuringiensis* isolates.

Jensen *et al.* (2002) analyzed *B. thuringiensis* strains isolated from feacal samples of greenhouse workers after exposure to *B. thuringiensis* based pesticides. The isolates were characterized by PCR with four general primer sets to detect various groups of insecticidal toxin genes and isolates gave signals with primer sets against *cry*II and *cyt*.

Uribe *et al.* (2003) characterized 100 *Bacillus thuringiensis* strains based on the presence of crystal proteins by SDS PAGE and multiplex PCR with general and specific primers for *cry*I, *cry*III, *cry*VII and *cry*VIII gene detection. About 75 per cent of *B. thuringiensis* strains reacted with *cry*I general primers. 27.8 per cent with *cry*III, *cry*VII and *cry*VIII general primers and 17.8 per cent did not react with any of these.

Yong *et al.* (2003) analyzed the *cry* type genes from a new strain of *Bacillus thuringiensis* isolated from soil in Hebei province, China by PCR- RFLP. The strain contained three types of insecticidal crystal protein genes *cry*I, *cry*IIAb, *cry*11A. SDS PAGE analysis showed that crystal proteins could be divided into 130, 79, 66, 60 and 58kDa fragments.

Fuchu *et al.* (2004) obtained amplification of *cry*1 and *cry*II genes in isolates from Taiwan. Soo *et al.* (2004) reported that the plasmid and protein

profiles of *Bacillus thuringiensis* 656-3, isolated from soil sample were similar to that of its reference strain *B. thuringiensis* subspecies *morrisoni* PG-14. However, PCR analysis using *cry* gene primers showed that *B. thuringiensis* 656-3, unlike its reference strain had *cry*IVA, *cry*IVB, *cry*XA, *cry*XI A, *cry*IAc genes suggesting it as a unique strain with respect to gene type. The bacterial strain showed a high level of toxicity against mushroom flies (*Lycoriella mali*) and mosquito (*Culex fuscipes).*

Swiecicka and Mahillon (2005) studied the genetic relationship among 103 natural *B. thuringiensis* isolates recovered from the intestines of small wild rodents and insectivores. Bacterial strains harboring genes coding for toxins active against Lepidoptera (*cry*I, *cry*II, *cry*IX*)* was 64 per cent and that of Diptera specific strains (*cry*IV) was 14 per cent. Bhulla *et al.* (2005) screened several *B. thuringiensis* strains by PCR to detect the presence of *vip*-like sequences by using a *vip*3Aa1 specific primer. Vip-like gene sequence was identified in eight serovars.

Hernandez *et al.* (2005) characterized a strain of *B. thuringiensis* against beet army worm and potato tuber worm (*Pthorimaea operculella*). It was characterized in terms of serotyping, crystal morphology, protein profile and *cry* gene content. PCR was performed with primers amplifying genes from the *cry*I, *cry*II, *cry*III, *cry*IV, *cry*VII, *cry*VIII and *cry*IXAa families. The toxic strains presented bipyramidal crystals, with a band of 130kDa in SDS PAGE and showed an amplification product with *cry*I family primers. RFLP confirmed the presence of a novel gene and sequence comparison showed that this gene had homology to *cry*IG.

Brousseau *et al.* (2005) applied arbitrary primer polymerase chain reaction technology for the identification of commercial strains of the bacterium by using total DNA extracted from single bacterial colonies as templates. When a

single primer was used this method was capable of discriminating DNA fingerprints for 33 unknown serovars. Rajesh *et al.* (2006) studied the molecular diversity of *B. thuringiensis* isolates obtained from the Western Ghats of Kerala and Karnataka. The isolates were subjected to molecular diversity analysis and characterization by specific PCR. Three *cry* specific primers were used for the amplification of full length genes viz. *cry*1Ac, *cry*3Aa and *cry*10Aa. Escudero *et al.* (2006) reported the molecular and insecticidal characterization of a novel *cry* gene encoding a protein of cryII group with toxicity towards insects of families Noctuidae, Tortricidae, Plutellidae and Chrysomelidae. PCR analysis detected a DNA sequence with an open reading frame of 2.2kb which encoded a protein with a molecular mass of 80.9kDa. The deduced sequence of the protein had homologies with *cry*IIa, *cry*IIb1 and *cry*IIc1. According to the cry protein classification, the protein was named as cryIIa7.

2.12.2 Nucleic acid hybridization methods

Byung *et al.* (1995) conducted DNA dot blot hybridization with *cry*V and *cry*I specific probes to screen 24 *B. thuringiensis* strains for their gene contents. The *cry*V specific probe hybridized to 12 of *B. thuringiensis* strains. Most of the *cry*V positive strains also hybridized to the *cry*I specific probe, indicating that *cry*V genes are closely related to *cry*I genes.

Kuo and Chak (1996) designed two pairs of universal oligonulcleotide primers to probe the most conserved regions of all known *cry*I type gene sequences so that the amplified PCR fragments of the DNA template from *Bacillus thuringiensis* strains may contain all possible *cry*I type gene sequences. The RFLP patterns of PCR amplified fragments revealed 14 distinct *cry* type genes from 20 *B. thuringiensis* strains. Those *cry* type genes included *cry*IAa, *cry*IAb, *cry*IAc, *cry*IB, *cry*IC, *cry*ICb, *cry*ID, *cry*IE, *cry*IF *and cry*III genes. Among these, the sequence of *cry*IAa, *cry*IAb, *cry*IB, *cry*IC, *cry*IF and *cry*III were found to be different from the corresponding published *cry* gene sequence.

Interestingly five *cry* genes *cry*IAa, *cry*IB, *cry*IC, *cry*ICb, *cry*IF and seven *cry* type genes *cry*IAa, *cry*IAb, *cry*IB, *cry*IC, *cry*ICb, *cry*IF *and cry*III were detected from *B. thuringiensis* subspecies *morrisoni* HD1 and *wuhanensis* respectively.

Oreshkova *et al.* (1999) analyzed the genomic DNA of *B. thuringiensis* by genomic fingerprinting technique. The biotin labeled single stranded DNA of the phage M13 was used as the marker of hyper variable sequences and a procedure for analyzing the differentiation among various strains was developed.

2.12.3 Protein based techniques

Chang *et al.* (1999) analyzed the plasmid and protein profile of a strain *B. thuringiensis* STB-1, isolated from soil sample in Korea, which was toxic against *Spodoptera exigua* with the reference strains *Bacillus thuringiensis* subspecies *kurstaki* and *kenyae* to verify the gene type. PCR analysis was performed with *Spodoptera* specific *cry* gene primers. *Bacillus thuringiensis* STB-1 had *cry*IAa, *cry*IAb, *cry*IAc and *cry* IE suggesting it as a unique strain with respect to gene type.

Werneck *et al.* (1999) analyzed a Brazilian strain of *Bacillus thuringiensis* subspecies *kurstaki* S93, regarding its *cry* gene and protein content. The spore crystal mixture analyzed by SDS PAGE showed two major polypeptides of 130 and 65kDa, corresponding to *cry*I and *cry*II toxins, respectively. Western blot analysis showed that these proteins were immunologically related to *cry*IA protein from *B. thuringiensis* subspecies *kurstaki* HD73. The PCR analysis using total DNA from S93 and specific primers showed the presence of *cry*IAa, *cry*IAb, *cry*IAc and *cry*IA type gene was localized in a plasmid of about 44MDa.

Arango *et al.* (2002) characterized *Bacillus thuringiensis* strains toxic to beet army worm which included crystal protein profile, plasmid profile, plasmid restriction patterns, *cry* gene composition, qualitative determination of beta

exotoxin production, RAPD and serotyping. All strains contained *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry1B, cry*1C *and cry*1D genes.

Silva *et al.* (2004) characterized Brazilian strains of *Bacillus thuringiensis* namely S701, S764, S1265 regarding their *cry* gene, protein content and crystal type. The spore crystal mixtures of the isolates were analyzed by SDS PAGE and showed similar protein pattern as *Bacillus thuringiensis* subspecies *kurstaki* strain HD1 (protein approximately 130 and 65kDa) for isolates S701 and S764, respectively and only one major protein of approximately 130kDa for isolate S1265.

2.13 CLONING OF GENES IN *Bacillus thuringiensis*

Widner and Whiteley (1989) cloned and sequenced two genes encoding insecticidal crystal proteins from *Bacillus thuringiensis* subspecies *kurstaki*. HD1. Both genes, designated *cry*B1 and *cry*B2 encoded polypeptides of 633 aminoacids having a molecular mass of 71kDa.

Santoso *et al.* (2004) reported the cloning of *cry* genes from genomic DNA of *Bacillus thuringiensis* isolates using direct cloning of PCR products in pGEMT. Rai *et al.* (2006) constructed a plasmid DNA library on pUC18 vector of a toxic native *Bacillus thuringiensis* strain S58. Two clones designated as pRKS1 and pRKSII encoding crystal protein were identified by screening the plasmid library using 732bp *Eco*R1 fragment which is a conserved sequence of *cry* gene across species. Pang *et al.* (1992) expressed full length 72K and truncated 61K *cry*IVD mosquitocidal protein of *B. thuringiensis* subspecies *morrisoni* in *S. frugiperda* cells and larvae of *Trichoplusia ni* using a baculovirus vector to investigate the role of *cry* IVD peptides in toxicity.

Chak and Jen (1993) determined the sequence of a *cry*1Ab gene from *B. thuringiensis* subspecies *aizawai* HD1. The *cry* gene coded for 130kDa proteins of 1155 amino acids. Gleave *et al.* (1993) screened a number of *B. thuringiensis* serotypes for the presence of *cry*V type genes and cloned and

sequenced a *cry*V type gene from *Bacillus thuringiensis* subspecies *kurstaki* DSIR73. They reported the expression of *cry*V type gene in *E. coli* and the insecticidal activity of 81kDa of *cry*V gene product.

Byung *et al.* (1995) cloned two *cry*V type genes, *cry*VI and *cry*V465 from *B. thuringiensis* subspecies *kurstaki* HD1 and *entomocidus* BP465 respectively. Johnson *et al.* (1996) cloned *cry*IIIA gene into pUC18 and transformed into *E. coli* strain DH5α. Expression of the cloned *cry*IIIA in *E. coli* was tested by utilizing pET-3, a bacterial expression vector. Juarez-Perez (1997) demonstrated a new PCR based approach known as E–PCR or exclusive PCR, for the systematic large scale screening of *B. thuringiensis* isolates to identify known *cry* genes, and most importantly to identify novel *cry* genes by the use of same initial set of primers. They also cloned PCR products related to *cry*IAc, *cry*IG and *cry*IB genes. Corona *et al.* (1998) cloned a new *cry* gene and sequenced it from a *B. thuringiensis* isolate native to Mexico LBIT-147.

Werneck *et al.* (1999) isolated a *cry*IAb gene from Brazilian strain of *B. thuringiensis* subspecies *kurstaki*. It showed 91.6 per cent and 85.9 per cent identity with *cry*IAa1 and *cry*IAc1 genes respectively. The deduced amino acid sequence showed a high degree of similarity with the amino acid sequences of cryIAb (100%) and cryIAa1 (93.8%) proteins. Dobrzhanskaya (2000) compared the *cry* gene of new strain of *B. thuringiensis* 2-7 and the *cry*IIIA gene of *B. thuringiensis* subspecies *tenebrionis* and showed that their nucleotide sequences were identical.

Balasubramanian *et al.* (2002) cloned a novel *cry*32Aa gene of *B. thuringiensis* subspecies *yunnanensis*. Misra *et al.* (2002) amplified a 1.9kb *cry* gene using PCR and the PCR amplified fragment was first cloned into pBluescript and then subcloned in pET3a and pET28a for expression in *E. coli*.

Tzeng *et al.* (2002) amplified a full length DNA 3.6 kb of *cry*IAc gene encoding insecticidal crystal proteins from a locally isolated *B. thuringiensis*

strain by PCR. This *cry*IAc gene was constructed into *E. coli* plasmid vector peRTMII and then sub cloned into the expression vector pSB909.5 to form pIAcSB. Full length of *cry*ICb2 gene and it was inserted into vector pET-21b and highly expressed in *E. coli* strain BL 21(DE 5). The expressed products were highly toxic to *Plutella xylostella* larvae.

Sheng *et al.* (2003) cloned a new *cry* gene *cry*ICa9 and sequenced it from a *Bacillus thuringiensis* isolate native of Taiwan. The *cry*IC type gene designated as *cry*ICa9 consisted of an open reading frame of 3567 bp encoding a protein of 1189 amino acid residues. Asano *et al.* (2003) cloned a gene encoding cryVII crystal protein in *E. coli* using antiserum directed to the protein. The gene expressed in *E. coli* produced a 130kDa protoxin to cupreous chafer (*Anomala cuprea).*

Stobdan *et al.* (2004) cloned a *cry*IAb gene from a new isolate of *B. thuringiensis* by PCR. Nucleotide sequencing and homology search revealed that the toxins showed 95 per cent homology with the known cryIAb toxins. Avisar *et al.* (2004) reported the cloning and expression of *cry*IC and *cry*IE genes using the pET expression system in the *E. coli* strain BL21 (DE3).

Bhalla *et al.* (2005) cloned the *vip* gene from *B. thuringiensis* and sequenced it. The deduced amino acid sequence of *vip*3Aa14 gene from *B. thuringiensis* subspecies *tolworthi* showed considerable differences as compared to those of vips reported so far. The *vip*3Aa14 gene from *B. thuringiensis* subspecies *tolworthi* was expressed in *E. coli* vector using expression vector pET 29a.

A novel gene encoding a leukemic cell killing parasporal protein, designated parasporin-4, was cloned from an isolate of *B. thuringiensis* serovar *shandongiensis.* The amino acid sequence of parasporin-4, deduced from the gene sequence had low level homologies of less than 30 per cent with the established *B. thuringiensis cry* proteins including three known parasporins. When the gene

was expressed as a recombinant of *E. coli* 3L 21 (DE3), the parasporin-4 formed intracellular inclusion bodies (Saitoh *et al.*, 2006).

Escudero *et al.* (2006) detected the presence of a novel *cry*II type gene in *Bacillus thuringiensis by* PCR. A general primer pair recognizing both *cry*IIa and *cry*IIb genes were used in combination with oligonucleotides specifically recognizing *cry*IIa primers. Amplified products were cloned into pGEMT vector. The cloned amplicons were sequenced and analyzed.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Cloning of genes encoding insecticidal proteins (*cry/vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala' was carried out in the Molecular Biology Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005-2007. The materials used and the methodologies adopted in this study are described below.

3.1 MATERIALS

3.1.1 Chemical, glassware and plastic ware

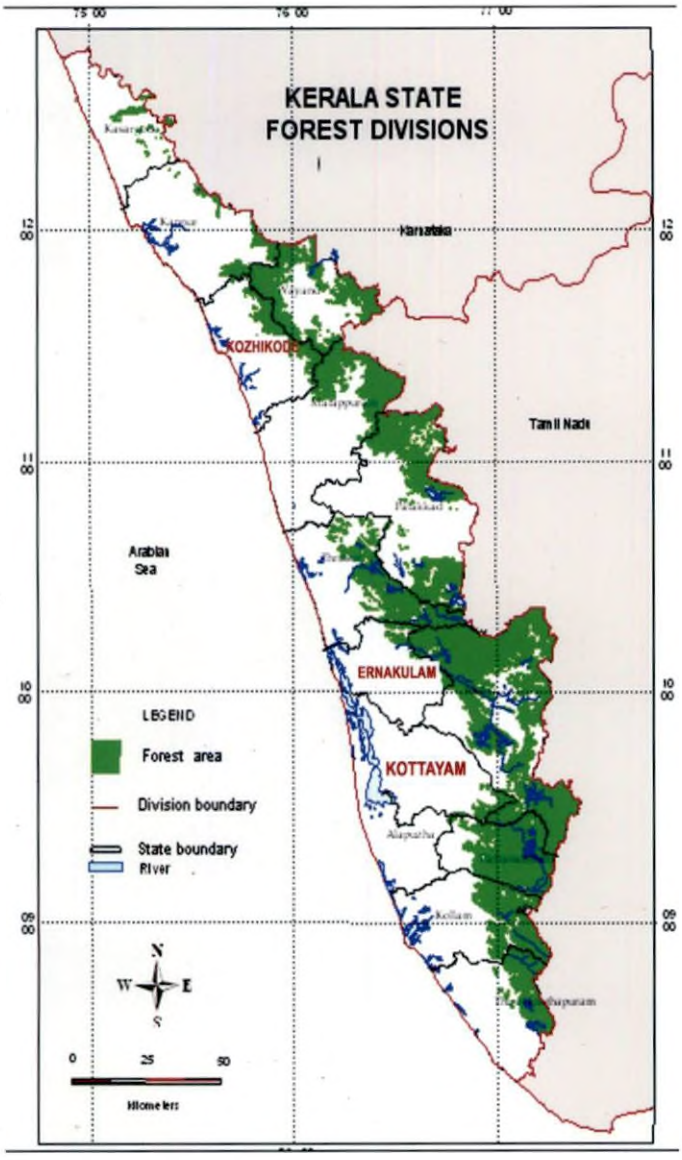
The chemicals used for the study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular Biology Grade enzymes and buffers were supplied by Banglore Genei Ltd. All the plastic ware used was obtained from Axygen and Tarson India Ltd. The primers were synthesized from Integrated DNA Technologies, USA.

3.1.2 Equipment and machinery

The equipment items available at Centre for Plant Biotechnology and Molecular Biology were used used for present study. Incubation of cultures was done in Incubator Shaker, Dai Ki- S1010 (Dai Ki Scientific Co.). Centrifugation was done in KUBOTA centrifuge. A compound binocular microscope (CETI) was used for viewing the slides of bacterial cultures. DNA amplification was carried out in Eppendorf Master Cycler, Gradient (Eppendorf, Germany). Alpha imager TM1200 was used for imaging the gel.

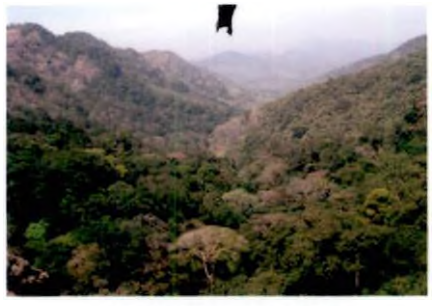
3.2 COLLECTION OF SOIL SAMPLES

Soil samples were collected from the Western Ghats of Kerala, coming under three districts, Kottayam, Kozhikode and Ernakulam (Plate 1). Samples



A. Edamalayar

B. Muthachipara



C. Kolahalamedu

Plate 1. Western Ghats of Kerala showing the districts surveyed for soil sample collection

were taken from the top 5 to 10cm soil in polythene covers. The location, altitude and latitude were recorded with the help of Geographical positioning System (GPS Model Etrex, Garmin, USA)

3.3 ISOLATION AND MAINTENANCE OF *B. thuringiensis*

Bacillus thuringiensis was isolated from soil samples collected from Western Ghats of Kerala according to the procedure described by Travers *et al.* (1987).

3.3.1 Procedure for isolating Bacillus thuringiensis

Initially 0.5 g of soil sample was added to test tubes containing 9ml of Luria-Bertani (LB) broth (composition is given in Annexure I) buffered with 0.25M sodium acetate. The broth was incubated for four hours at 30^0C with shaking on an incubator at 200 rpm. Aliquots of 1ml were transferred to sterilized test tubes and heated to 80^0C for 3 minutes. 1ml from this was transferred to a 9ml sterile water blank to get 10^{-1} dilution. This was mixed thoroughly and ten fold dilutions were made upto 10^{-3}. 100µl each taken from 10^{-2} and 10^{-3} dilutions were pour plated on LB agar medium. The plates were then incubated at 28 ± 2^0C for 24-48 hours. The colonies formed were picked up based on their morphological similarities with standard *Bacillus thuringiensis* colonies.

3.3.2 Purification and maintenance of cultures

The selected colonies were purified by repeated streaking on T3 agar medium (composition given in Annexure I) until pure single colonies were obtained. The cultures were maintained as stabs in cryostorage vials in LB agar medium and stored under refrigerated conditions.

3.4 IDENTIFICATION AND CHARACTERIZATION OF *Bacillus thuringiensis* ISOLATES

3.4.1 Morphological characterization

The bacterial isolates were characterized using staining reactions like Gram, endospore and crystal protein staining. The reference strain HD1 (Kronstad and Whitely, 1986) was used in all the studies. Chemical composition of the reagents used for staining reaction are given in Annexure II.

3.4.1.1 Gram staining

The isolates were subjected to Gram staining reaction following the procedure of Hucker and Conn (1923).

1. A smear of the culture was prepared on a clean slide, air dried and heat fixed.
2. One to two drops of crystal violet stain was added and the smear washed after one minute.
3. Gram's iodine solution was added for one minute and then washed.
4. Decolourized with ethyl alcohol for 30 seconds.
5. The smear was treated with the counter stain safranine for one minute.
6. Finally the slide was washed under running tap water, air dried and observed under100X objective of a compound binocular microscope.

3.4.1.2 Endospore staining

Endospore staining was done according to procedure developed by Smirnoff (1962).

1. Thin smears were prepared on a clean dry glass slides, from 48h old T3 cultures.

2. A few drops of amidoblack (1.5%) added and allowed to stay for 70 seconds.
3. The smears were washed and counterstained carbol fuchsin (1%) for 20 seconds.
4. The slides were observed under the 100X objective of a compound microscope after washing and air drying.

3.4.1.3 Crystal protein staining

The cultures were examined for the presence of crystal protein inclusions by staining with Coomassie brilliant blue as described by Shariff and Alaeddinoglu (1983).

1. Smears of the cultures grown on T3 agar medium for 48 hours were heat fixed.
2. The slides were stained with Coomassie brilliant blue (0.25%) for 3 minutes.
3. Slides were then washed under running tap water and air dried.
4. The smears were observed for the presence of crystal proteins under the 100X objective of a compound binocular microscope.

3.4.2 Cultural characterization

Cultural characteristics of the isolates were studied by streaking it on LB agar medium in petriplates. Colony characters like pigmentation, size, form, margin, elevation, optical characteristics and diameter of each isolate were observed after 24 hours of streaking, following Cappuccino and Sherman (1992).

3.4.3 Biochemical characterization

Bacillus thuringiensis isolates were characterized using seven different biochemical tests. All the biochemical tests were carried out following Cappuccino and Sherman (1992). Chemical composition of media used is given in Annexure I.

3.4.3.1 Solubility in three per cent KOH

At the centre of a sterile glass slide, two drops of three per cent KOH solution was placed and a loopful of bacterial growth was rapidly agitated on it in a circular motion. After five to eight seconds the loop was gently raised and lowered just above the slide surface. Presence or absence of viscous strands between the loop and slide surface was observed.

3.4.3.2 Urease test

Bacillus thuringiensis isolates were determined for their ability to degrade urea by means of an enzyme, urease. The presence of urease was detected by inoculating deep tubes containing urea agar with bacterial cultures. The deep tubes containing inoculated cultures were incubated for 24-48 hours at 30^0C and observed for development of deep pink colour, which was the positive reaction for urease test.

3.4.3.3 Gelatin hydrolysis

Bacillus thuringiensis isolates were analysed for their ability to produce a proteolytic extra cellular enzyme, gelatinase which hydrolyzes the protein gelatin to aminoacids. This was carried out by stab inoculating nutrient gelatin deep tubes with the bacterial isolates. The tubes were incubated for 24-48 hours at 30^0C. After incubation, tubes were transferred to a refrigerator at 4^0C for 30 minutes. Gelatinase positive cultures remained liquified while in others the medium remained solid.

3.4.3.4 Starch hydrolysis

Starch hydrolysis was carried out in order to determine the ability of isolates to excrete hydrolytic extra cellular enzymes capable of degrading the polysaccharide, starch. It was done by streaking the bacterial strains in Petriplates containing starch agar medium. The plates were incubated for 24-48 hours 30^0C. Plates were flooded with Lugol's Iodine solution which produced blue colour on

the plate. A clear zone of hydrolysis surrounding the growth of organism when flooded with Lugol's iodine indicated starch hydrolysis.

3.4.3.5 Voges-Proskauer Test

Voges-Proskauer test was done to determine whether the isolates were capable of producing non-acidic/neutral end products such as acetyl methyl carbinol from organic acids that result from glucose metabolism. In Voges-Proskauer test, the bacterial strains were inoculated into Voges-Proskauer medium and incubated for 48 hours at 30^0C. After incubation. 10 drops of Barritt's reagent A was added followed by the addition of Barritt's reagent B. The cultures were examined for the development of pink colour 15 minutes after addition of Barritt's reagent, which indicated a positive reaction.

3.4.3.6 Lecithinase test

Lecithinase test was done by streaking the bacterial cultures on Petriplates containing nutrient agar medium supplemented with egg yolk and incubated for 24-48 hours at 30^0C. Presence of a clear zone surrounding the growth indicated positive reaction.

3.4.3.7 Esculinase test

The isolates were tested for their ability to produce the enzyme esculinase by streaking them on Petriplates containing esculin agar. After incubation at 30^0C for 24 hours, the cultures were examined for blackening around the growth which indicated hydrolysis of esculin.

3.5 BIOASSAY AGAINST PUMPKIN CATERPILLAR (*Diaphania indica*)

The *B. thuringiensis* isolates obtained from soil samples were screened for their insecticidal activity against pumpkin caterpillar (*Diaphania indica*), a serious pest on cucurbitaceous vegetables. The standard strain HD1 was used as

positive control, in the study to compare the per cent mortality. The bioassay studies were carried out at Banana Research Station, Kannara.

3.5.1 Mass rearing of *Diaphania indica* larvae

Larvae were collected from cucurbitaceous vegetables such as snakegourd, coccinia and cucumber from the farm of Department of Olericulture, College of Horticulture, Kerala Agricultural University. Larvae collected from the field were reared on coccinia leaves in plastic containers covered with muslin cloth at a temperature of 28 ± 2^{0}C. Larvae were reared to the adult stage. The adult moths were released to net cages of dimension 38cm x 38cm x 38cm for mating and egg laying (Plate 2). Fifty per cent honey solution soaked on cotton swab was given as the food for adult moths.

Eggs were laid on the sides of the cage and on the leaves / vines provided inside the cage. Fresh leaves were kept inside the cage for collecting wandering larvae. The leaves and vines harbouring eggs were removed daily and kept in plastic containers of dimension 22cm x 15cm x 7cm for emergence of larvae. The neonates were transferred into plastic containers and fed with the leaves of coccinia. Four to five days old larvae were transferred to the artificial diet which was earlier standardized in the bioassay lab at Banana Research Station, Kannara. Composition of the artificial diet is given in Annexure I.

3.5.2 Harvesting of crystal protein from *Bacillus thuringiensis* isolates

A loopful of culture on T3 agar was inoculated in 5ml T3 broth contained in test tubes. After 24 hours incubation at 30^{0}C, 2ml of the culture was transferred into 50ml T3 broth and incubated for 48 hours at 30^{0}C with shaking at 160 rpm. Cells were observed under microscope for cell lysis. When lysed spores along with crystals were observed, crystal protein harvesting was carried out. Chemical composition of the reagents used for protein isolation is given in Annexure II.

First instar larvae

Mature larvae

Larvae feeding on artificial diet

Plastic containers for rearing larvae

Net cages for rearing moths

Penicillin vials for bioassay

Plate 2. Rearing of punpkin caterpillar for bioassay

1. 50ml of T3 grown culture was centrifuged at 10, 000 rpm at 4^0C for 10 minutes.
2. The supernatant was discarded and pellet was dissolved in 50ml 10X Tris-, EDTA (TE) buffer and 1ml Phenyl Methane Sulfonyl Flouride (PMSF) and centrifuged at 10, 000 rpm at 4^0C for 10 minutes.
3. Supernatant was discarded and pellet dissolved in 50ml 0.5M NaCl and centrifuged at 10,000 rpm at 4^0C for 10 minutes.
4. Pellet was again dissolved in 50ml 10X TE and 0.5ml PMSF and centrifuged at 10,000 rpm at 4^0C for 10 minutes.
5. The pellet was dissolved in 50ml 10X TE and 0.5ml PMSF, centrifuged at 10, 000 rpm at 4^0C for 10 minutes.
6. The final pellet obtained was centrifuged to remove water if any present.
7. The pellets were dissolved in 1ml of 50:1 PMSF and stored at -20^0C, in Eppendorf tubes.

3.4.4 Bioassay on Pumpkin caterpillar

The cry toxins were tested for their toxicity against four to five day old larvae of *Diaphania indica* by diet contamination method, as described by Schesser *et al.* (1977) incorporating the crude extract containing spore and crystal protein toxins in the artificial diet fed to the larvae. Three replications were maintained for each isolate and ten larvae were used for one replication. Bioassay was done in sterilized autoclaved small penicillin vials with cotton plugs.

Crude preparation of 1ml each, from each isolate was incorporated into 100ml freshly prepared artificial diet at 40 to 45^0C. The diet containing the crystal protein was dispensed in penicillin vials in equal quantities of 3ml each. After solidification of the diet, larvae were released at the rate of one per vial. The penicillin vials were closed with cotton plugs to prevent the escape of the larvae. 100ml of diet mixed with 1ml PMSF solution served as control.

The activity of crystal proteins was expressed in terms of mortality. Symptoms seen on the larvae fed with artificial diet containing protoxins were also observed. Mortality was observed daily till all the larvae died.

The per cent mortality was corrected using Abbots formula, as follows:

$$\text{Per cent mortality (corrected)} = \frac{X-Y}{X} \times 100$$

X= % live insects in control
Y= % live insects in treatment.

Statistical analysis was done using Kendall's coefficient of Concordance by SPSS software, a statistical package (Kendall and Smith, 1939). The data obtained on percent mortality of larvae was fed to SPSS software so as to find out Kendall's coefficient of concordance W. The analysis was carried out separately on fifth day and eighth day of mortality. The isolates were ranked on the basis of mortality observed on fifth and eighth days and also on the number of days taken to produce 50 per cent mortality.

3.6 AMPLIFICATION OF *cry* GENE FROM *Bacillus thuringiensis*

Total DNA was isolated *B. thuringiensis* strains obtained from the Western Ghats of Kerala, in order to amplify the *cry* genes. Specific primers for the amplification of *cry* genes were designed based on the conserved regions obtained after multiple sequence alignment. PCR was carried out using the specific primers at annealing temperatures specified for each primer.

3.6.1 Isolation of total DNA from *Bacillus thuringiensis*

Reagents

1. LB media
2. 10mM Tris Cl
3. 100mM NaCl

4. TE buffer
5. Lyzozyme
6. RNase
7. 2% SDS
8. Proteinase K
9. Phenol:Chloroform
10. Chloroform: isoamylalcohol
11. 3M Sodium acetate
12. Chilled isopropanol
13. 70% ethyl alcohol

(Chemical composition of reagents are given in Annexure III)

The total DNA was isolated from *Bacillus thuringiensis* isolates and reference strains following the protocol of Sambrook and Russel (2001).

1. 25ml of overnight grown culture of *Bacillus thuringiensis* in LB broth was centrifuged at 10,000 rpm for 10 minutes at 4^0C.
2. The pellet was resuspended in 10mM Tris Cl and 100mM NaCl.
3. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4^0C.
4. The pellet was resuspended in 2.5ml TE and 500µl of lyzozyme from a stock of 50mg/ml and incubated at 37^0C for 20 minutes.
5. 25µl of RNase was added from a stock of 10mg/ml.
6. Incubated at room temperature for 10 minutes.
7. 2.5ml of SDS was added and incubated at 50^0C for 45 minutes.
8. 50µl of proteinase K was added from a stock of 20mg/ml and incubated at 50 to 55^0C for 10 minutes.
9. An equal volume of phenol was added, mixed gently and centrifuged at 10,000 rpm for 10 minutes at 4^0C.
10. The aqueous phase was transferred to a fresh tube and an equal volume of phenol: chloroform (1:1) ratio was added. The mixture was mixed gently.

11. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4^0C and the aqueous phase was pipetted into a fresh tube.
12. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently.
13. Centrifugation was carried out at 10,000 rpm for 10 minutes at 4^0C and the aqueous phase was separated.
14. $1/10^{th}$ volume of 3M sodium acetate was added and kept in ice for 20 minutes.
15. An equal volume of isopropanol was added and incubated for 5 to 10 minutes.
16. Centrifugation was carried out at 10,000 rpm for 15 minutes at 4^0C and the supernatant was decanted.
17. The pellet was washed in 70 per cent alcohol, by giving a centrifugation at 10,000 rpm for 3 minutes. The supernatant was discarded.
18. The pellet was dried and dissolved in 25 to 30µl of TE buffer.

3.7 CHECKING THE QUALITY OF DNA

3.7.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA and also to separate the amplified products.

Materials

1. Agarose (Bangalore Genei, Low EEO)
2. 50X TAE buffer (pH 8.0)
3. Electrophoresis unit (Biorad, USA), power pack, casting tray, comb
4. 6X Loading/ Tracking dye (Bangalore Genei, Bangalore)
5. Ethidium bromide solution (stock 10mg/ml; working concentration, 0.5µg/ml)
7. Gel documentation and analysis system (Alpha imager TM 1200)

(Chemical composition of the buffer and dyes are given in Annexure IV)

3.7.2 Agarose Gel Elecrophoresis

1. 1X TAE buffer was prepared from 50 X TAE stock solution.
2. Agarose (1.0 per cent (w/v) for genomic DNA and 0.8 per cent (w/v) for PCR) was weighed and added to 1X TAE. It was boiled till the agarose dissolved completely and then cooled to lukewarm temperature.
3. Ethidium bromide was added to a final concentration of 0.5µg/ml as an intercalating dye of DNA and mixed well.
4. The open ends of the gel casting tray were sealed with a cellophane tape and placed on a perfectly horizontal leveled platform.
5. The comb was placed properly and molten agarose was poured into the tray, allowing it to solidify.
6. After the gel was completely set (15 to 20 minutes at room temperature), the comb and cellophane tape were carefully removed.
7. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1 X TAE to a depth of 1cm.
8. A piece of cellophane tape was pressed on a solid surface and 1µl 6X loading dye was dispensed in small quantity on the tape. A quantity of 3 to 5µl of DNA was added to each slot (in the case of PCR products, 10.0 to15.0µl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded in the wells, with the help of micropipette. λDNA/*Eco*R1+ *Hind*III double digest (Bangalore Genei) was used as the molecular weight marker.
9. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 60 volts.
10. The power was turned off when the tracking dye reached at about 3cm from the anode end.

3.7.3 Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using Alpha Imager TM 1200 documentation and analysis system.

3.8 PURITY OF DNA

The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm. 260/280 ratio was used to assess the purity of nucleic acids. A ratio of 1.8 to 2 indicated good quality DNA.

3.9 PRIMER DESIGNING

The sequence information available in the public domain NCBI was accessed for collecting the recent information about *cry* genes. The following steps were used:

1. Complete nucleotide sequences encoding *cry*1A gene reported for different species of *B. thuringiensis* were downloaded from NCBI Genbank (http://www.ncbi.nlm. nih.gov) and copied in FASTA format into a notepad
2. Multiple sequence alignment of nucleotide sequences downloaded was done using 'Clustal W1.83 '(www. ebi.ac.uk/clustal).
3. Based on the homology, conserved boxes of 18 to 24 bases were selected throughout the sequence.
4. The forward and reverse primers were selected from conserved boxes in such a way that
   a) The conserved boxes selected should have GC content not less than 50 per cent
   b) Melting temperature (Tm = 4GC + 2AT) ranged between 60^0C and 70^0C.
   c) The distance between the primers ranged from 500 to 1000 base pairs.

d) It is preferable to have GC content at 3' end.
e) There should not be any complementarily between forward and reverse primers.
f) Repeats of single base should not appear within the primer sequence.
g) The distance between forward and reverse primer should be greater than 500bp.
h) Each primer should be 18 to 24bp long

5. For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Based on these parameters, a pair of primer for amplification of *cry*1A gene was designed during this study. The forward and reverse primers were selected from highly conserved regions. The details of the gene specific primers used and details of annealing temperature and expected amplicon size for selected primers are given in Table 2 & 3.

A set of universal primers Un1(d), Un1(r), Un4(d) and Un4(r) (Ben-Dov *et al.*, 1997) were used for the amplification of *cry*1 and *cry*4 genes in *Bacillus thuringiensis*. A pair of primers I(+) and I(-) for the amplification of variable region of *cry*1 gene with a fragment size of 1500 to 1600 bp (Juarez-Perez *et al.*, 1997) was also used.

3.9.1 PCR Reaction

The total DNA extracted from *B thuringiensis* isolates was amplified by PCR. Initially, all the isolates were screened for the presence of *cry*1 and *cry*4 genes using universal primers designed by Ben-Dov *et al.* (1997). Those isolates which gave the *cry*1 amplicon of expected size, were subjected to amplification of *cry*1A gene using primers designed during the study and also the variable region of *cry*1 using the family of primers designed by Juarez-Perez *et al.* (1997). Polymerase chain reaction was carried out using *cry* gene specific primers in Eppendorf Master Cycler, Gradient (Eppendorf, Germany).

3.9.1.1 Composition of the reaction mixture for PCR (25.0µl)

a) Genomic DNA (25ng)	-	1.0µl
b) 10X Taq assay buffer	-	2.5µl
c) d NTP mix (10mM)	-	1.0µl
d) Forward primer (8pM)	-	1.0µl
e) Reverse primer (8pM)	-	1.0µl
f) Taq DNA polymerase (0.3 U)	-	2.0µl
g) Autoclaved distilled water	-	16.5µl
		25.0µl

The total volume for one PCR reaction was 25.0µl. The reaction was set in a 200µl microfuge tube chilled over ice flakes. A momentary spin was given for the reaction and set in thermal cycler for polymerase chain amplification under suitable programme. A positive control was set up using reference strain HD1 for amplification of *cry1* genes in *B. thuringiensis*. Reference strain 4Q1 was used as positive control for amplification of *cry*4 genes from the bacteria. A negative control was also kept using water as template.

3.9.1.2 Thermal cycler programme

The following programme was set in order to amplify *cry* gene from the template DNA.

Step 1: 94^0C for 2 min - initial denaturation
Step 2: 94^0C for 45 sec - denaturation
Step 3: 55^0C for 1 min - annealing
Step 4: 72^0C for 2 min - extension
} 30 cycles
Step 5: 72^0C for 10 min - final extension
Step 6: 4^0C for infinity to hold the sample

The PCR product was checked on 0.8 per cent agarose gel and documented.

Table 2. Details of gene specific primers used in the study

Sl. No.	Primer	Primer sequence	Length (bp)	Tm (^{0}C)	Source
1	*cry*1A forward	5' AGTGAATTTGTTCCCGGTGCTG 3'	22	66	Designed during the study
2	*cry*1A reverse	5'RMGGRAAHGYGAATTCTGGYCC 3'	20	66	
3	Un1(d)	5' CATGATTCATGCGGCAGATAAAC 3'	23	54. 7	Ben-Dov *et al.*, 1997
4	Un1(r)	5' TTGTGACACTTCTGCTTCCCATT 3'	23	57	
5	Un4(d)	5' GCATATGATGTAGCGAAACAAGCC 3'	24	56.6	Ben-Dov *et al.*, 1997
6	Un4(r)	5' GCGTGACATACCCATTCCAGGTCC 3'	25	61.6	
7	I(+)	5' TRACRHTDDBDGTATTAGAT 3'	20	45.5	Juarez-Perez *et al.*,1997
8	I(-)	5' MDATYTCTAKRTCTTGACTA 3'	20	44.6	

Un1(d) - Universal *cry*1 direct
Un1(r) - Universal *cry*1 reverse
Un4(d) - Universal *cry*4 direct
Un4(r) - Universal *cry*4 reverse
I(+) - *cry*1, forward
I(-) - *cry*1, reverse

Table 3. Details of annealing temperature and amplicon size expected for selected primers

Sl. No.	Primer	Annealing Temperature (^{0}C)	Expected amplicon size (bp)
1	*cry*1A	61	824
2	Universal *cry*1	55	277
3	Universal *cry*4	55	439
4	*cry*1 family	45	1500- 1600

3.10 GEL ELUTION OF PCR AMPLIFIED FRAGMENTS

Products obtained in different PCR reactions were loaded separately on 0.8 per cent (w/v) agarose gel and desired band in each case was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences) (Procedure followed as per the manufacturer's guide lines).

1. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel while avoiding much exposure to UV on a transilluminator.
2. Gel slice was weighed in a colourless 1.5ml micro centrifuge tube.
3. 3X gel volume of solubilization buffer (w/v) was added.
4. The gel was resuspended in gel solubilization buffer by vortexing. It was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 minutes to accelerate gel solubilization.
5. 0.5X gel solubilization volume of binding buffer was added and mixed properly.
6. Once the gel slice was completely dissolved, DNA fragments less than 400 bp was supplemented by adding 1X gel volume of isopropanol and mixed briefly by inversion.
7. A spin column was placed in a 2ml collection tube.
8. Solubilized gel slice was transferred into the spin column that was assembled in the 2ml collection tube and centrifuged at 12,000g for 1 minute.
9. The filtrate was discarded. 500µl of wash buffer was added to the spin column and centrifuged at 12, 000g for 30 seconds.
10. The filtrate was discarded. 700µl of desalting buffer was added.
11. Centrifugation was carried out at 12,000g for 30 seconds.
12. As a second wash, 700µl of desalting buffer was added

13. Centrifugation was carried out at 12,000g for 30 seconds ensure the complete removal of salt.
14. The filtrate was discarded. The spin column was again placed on collection tube.
15. Column was again centrifuged for 1 minute at 12,000g to remove any residual buffer.
16. Spin column was transferred to a fresh 1.5ml centrifuge tube. The eluent was prewarmed at 65^0C to improve the elution efficiency. To elute the DNA, 25µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 minute at room temperature. Then it was centrifuged at 12,000g for 1 minute.
17. Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel and stored at -20^0C.

3.11 TRANSFORMATION AND CLONING OF DNA

3.11.1 Preparation of competent cells

Competent cells for plasmid transformation were prepared using Genei Competent cell preparation Kit (B) from Bangalore Genei, following the manufacturer's guideline. Composition of media and reagents used are given in Annexure V.

Day 1

1. *Escherichia coli* JM 109 was streaked on LB agar plate from stab and incubated at 37^0C for 16 to 18 hours.

Day 2

1. 100ml SOC broth in 1 litre conical flask was inoculated with 10 to 12 moderately sized colonies from SOC plates.

2. Overnight incubation was given at 37^0C in a shaker at 200 rpm. When the OD_{600} reached 0.3 (3 to 3.5 h only) growth was arrested by chilling. The flask was chilled for 20 minutes.
3. The entire culture was transferred into a 50ml centrifuge tube and centrifuged at 3500 rpm for 15 minutes at 4^0C.
4. The supernatant was discarded. Keeping the tubes on ice, resuspended the bacterial pellet very gently in 33.3ml ice cold solution A.
5. The tubes were kept on ice for 20 minutes and then centrifuged at 3500 rpm for 15 minutes at 4^0C.
6. Supernatant was discarded and pellet chilled on ice. The pellet was resuspended in 5 to 6ml of ice cold solution A.
7. The suspension was kept on ice for 10 minutes and aliquots of 100µl were dispensed in chilled 1.5ml Eppendorf tubes.
8. The tubes were frozen on ice for few minutes before storing at -70^0C.

The competence of the cells prepared was confirmed by transformation using a plasmid containing an amplicillin resistance marker. The cells were plated on a plate containing LBA + 50mg/l ampicillin.

3.11.2 Ligation

The eluted product was ligated in pGEMT vector (Plate 3) using pGEMT Easy Vector System (Promega Corporation, USA) (Procedure followed as per the manufacturer's protocol).

1. Reaction mixture was prepared as described below:

2X rapid ligation buffer	-	5.0µl
pGEMT Easy Vector (50ng)	-	1.0µl
PCR product	-	1.0µl
T4 DNA ligase (3 units/µl)	-	1.0µl
Deionised water	-	2.0µl
		10µl

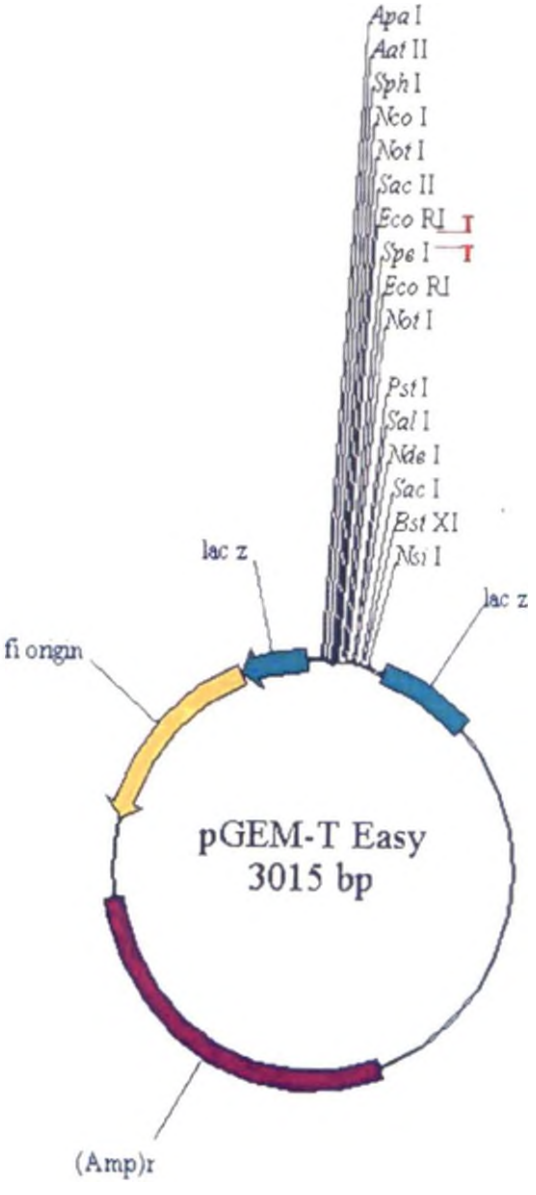


Plate 3. pGEMT – Easy vector (Promega) used for cloning PCR products. The lac Z region and multiple cloning sites are shown in the figure

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was taken for transformation in competent cells of *E. coli.*

3.11.3 Cloning of ligated DNA into competent cells

1. The vial containing competent cells was thawed on ice.
2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 minutes.
3. The tube was rapidly taken from ice; heat shock was given at 42^0C exactly for 90 seconds without shaking and placed back on ice for 5 minutes.
4. 250µl of LB broth was added and the tube was inverted twice
5. The tube was incubated at 37^0C for one hour with shaking.
6. 100 and 250 aliquots of the transformed cells were plated on LB/ ampicillin (50mg/l) plates layered with IPTG (6µl) and X-gal (60µl). (Stock: Ampicillin-5 mg/ml in water, IPTG-200mg/ml in water, X-gal-20 mg/ml in DMFO) and incubated overnight at 37^0C.
7. The recombinant clones were selected based on blue- white screening.

3.12 ISOLATION OF RECOMBINANT PLASMID DNA

Plasmid DNA was isolated from single white colonies using alkaline mini- prep procedure as given by Birnboim and Doly (1979). Composition of the reagents used are given in Annexure V.

3.12.1 Procedure for plasmid isolation

1. Cells were pelleted from overnight grown recombinant *E. coli* culture containing plasmid DNA by centrifugation at 12,000 rpm for 1 minute at 4^0C.
2. 100µl of ice cold solution I was added to the bacterial pellet and resuspended.

3. 200µl of freshly prepared lysis buffer was added, mixed gently by inverting the tube for five times.
4. 150µl of ice cold solution III was added to the tubes, mixed well and kept on ice for 5 minutes.
5. The contents were centrifuged at 12,000 rpm for 5 minutes at 4^0C and the pellet was discarded.
6. To the supernatant, 0.6 volume of ice cold isopropanol was added and kept at room temperature for 5 minutes.
7. The contents were centrifuged at 12,000 rpm for 5 minutes at 4^0C and the supernatant was discarded.
8. The pellet was rinsed with 1ml of 70 per cent (v/v) ethanol and mixed gently.
9. The tube was centrifuged at 12,000 rpm for 10 minutes at 4^0C.
10. The supernatant was discarded and the pellet air dried for 10 minutes.
11. Pellet was finally dissolved in 30µl TE buffer.
12. Plasmids isolated were checked by electrophoresis on 1 per cent agarose gel and documented.

3.13 CONFIRMATION OF PRESENCE OF INSERT

3.13.1 PCR amplification of recombinant plasmid DNA

Polymerase chain reaction was carried out as described in Section 3.9.1 except that the recombinant plasmid DNA isolated by alkali lysis method was used as template in place of bacterial DNA. The PCR products were analysed on 0.8 per cent (w/v) agarose gel as described in Section 3.7.1

3.14 SEQUENCING OF DNA CLONES

The recombinant clones from the isolates KY5, EM11 and EM10 were sequenced at Banglore Genei using T7 primer to obtain 5'-3'sequence information of the insert from the forward region, using automated sequencer (ABI-31100

Genetic Analyzer, USA). In the case of *cry*1A from KY3 and *cry*4 from KY1, the PCR products were directly sequenced using the corresponding forward primer.

3.15 THEORETICAL ANALYSIS OF SEQUENCE

3.15.1 Vector screening

The sequence obtained was subjected to vector screening using Vecscreen programme of NCBI to remove vector regions from the clones.

3.15.2 Nucleic acid sequence analysis

Homology search was carried out using Basic Local Alignment Search Tool (BLAST).

The following BLAST programes were used:

1. Nucleotide- nucleotide BLAST (Blastn)
2. Protein- protein BLAST (Blastp)

The BLAST programme (Blastn) was obtained from http://www.ncbi.nm.nih.gov/blast/ (Altschul *et al.*, 1997). Multiple sequence alignment was performed using Clustal W (www.ebi.ac.uk./clustalw/; Thompson *et al.*, 1994). To find the open reading frame of the insert nucleotide sequence, the programme ORF finder of NCBI was used (www.ncbi.nlm.gov/gorf/gorf). To predict exons and peptide in the sequences, gene prediction tools GENSCAN (www.gene.mit.edu/GENSCAN; Burge and Karlin, 1997) was used. Nucleotide composition of the given sequence was determined by nucleotide statistics (NASTATS) tool offered by Biology Workbench (http://seqtool.sdsc.edu/). Restriction sites available in the DNA fragment for the restriction enzymes were detected by restriction site analysis (TACG) offered by Biology Workbench.

3.15.3 *Amino acid analysis*

Physical and chemical properties of the given protein from the deduced aminoacid were determined by aminoacid statistics (AASTATS) tool offered by Biology Workbench. The secondary structure prediction of the aminoacid sequences were done using SOPMA programme. Hydrophobicity analysis of the sequences were done by Kyte and Doolittle hydropathy plots (Kyte and Doolittle, 1982). Important functional domains were detected using InterProScan' (www.ebi.ac.uk/InterProScan/; Zdobnov *et al.*, 2001).

Results

4. RESULTS

The results of the study on 'Cloning of genes encoding insecticidal proteins (*cry/vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala' undertaken during the period 2005-2007 at the Centre for Plant Biotechnology and Molecular Biology are presented below.

4.1 ISOLATION AND MAINTENANCE OF *Bacillus thuringiensis* ISOLATES

Eleven soil samples were collected from the Western Ghat regions of Kerala, coming under three districts namely Kottayam, Kozhikode and Ernakulam (Plate 1). The details of the soil samples collected and *Bacillus thuringiensis* isolated are given in Tables 4 and 5. A total of 30 spore forming bacteria were obtained, out of which 19 were *B. thuringiensis* isolates. The bacteria were isolated on LB agar medium. Creamy white colonies with typical morphology of *Bacillus thuringiensis* were selected and purified by repeated streaking on T3 medium. Out of these, 11 isolates which produced large number of crystal proteins were selected for the present study. The cultures were maintained as stabs in cryostorage vials in LB agar medium and stored under refrigerated conditions.

4.2 CHARACTERIZATION OF *Bacillus thuringiensis* ISOLATES

4.2.1 Cultural characterization

The cultural characteristics of the isolates were studied on LB agar medium. The isolates of *B. thuringiensis* from different locations showed slight variations in cultural characteristics. All the isolates produced creamy white, puffy colonies 24 hours after inoculation on LB agar medium (Plate 4). Except for the isolates KY3, KK8 and EM11, which produced medium sized colonies, all other isolates produced large sized colonies. Form of the colonies varied from circular to irregular, with flat elevation. Margin of the colonies varied from entire to

Table 4. Details of soil samples collected from the Western Ghats of Kerala

Sl. No.	Sample No.	District	Location	GPS reading
1	S1	Kottayam	Paruthumpara	N: 9^0 38.250 E: 76^0 56.510 Ele: 3210 ft
2	S2	"	"	N: 9^0 38.250 E: 76^0 56.510 Ele: 3210 ft
3	S3	"	"	N: 9^0 34.125 E: 76^0 32.210 Ele: 3278 ft
4	S4	"	Kolahalamedu	N: 9^0 38.520 E: 76^0 55.134 Ele: 3606 ft
5	S5	"	"	N: 9^0 38.210 E: 76^0 55.175 Ele: 3230 ft
6	S6	"	"	N: 9^0 35.531 E: 76^0 52.213 Ele: 3532 ft
7	S7	Kozhikode	Muthachipara	N: 11^0 31.489 E: 75^0 49.557 Ele: 340(109) ft
8	S8	"	Thumbithullumpara	N: 11^0 28.432 E: 75^0 3.678 Ele: 2255 (84) ft
9	S9	"	Thusharagiri	N: 10^0 28.402 E: 76^0 3.554 Ele: 1813(59) ft
10	S10	Ernakulam	Edamalayar	N: 10^0 12.556 E: 76^0 42.318 Ele: 989(102) ft
11	S11	"	"	N: 10^0 13.212 E: 76^0 43.554 Ele: 1012 ft

N- North
E- East
Ele- Elevation

Table 5. Details of *Bacillus thuringiensis* isolates obtained from soil samples from the Western Ghats

Sl. No.	District	Soil sample	No. of colony forming units (cfu)/ 0.5 g soil			*B.thuringiensis selected*
			Total no.	Spore formers	*B. thuringiensis*	
1	Kottayam	S1	Many	3	2	KY1
2	"	S2	"	3	2	KY2
3	"	S3	"	1	1	KY3
4	"	S4	"	5	3	KY4
5	"	S5	"	2	1	KY5
6	"	S6	"	2	1	KY6
7	Kozhikode	S7	"	3	1	KK7
8	"	S8	"	2	1	KK8
9	"	S9	"	1	1	KK9
10	Ernakulam	S10	"	3	1	EM10
11	"	S11	"	5	5	EM11
Total				**30**	**19**	

Table 6. Colony characteristics of native isolates of *Bacillus thuringiensis*

Sl. No.	Isolate	Pigmentation	Size	Form	Margin	Elevation	Optical characteristics	Diameter (mm)
1	KY1	Creamy white	Large	Circular	Entire	Flat	Opaque	0.6
2	KY2	Creamy white	Very large	Circular	Entire	Flat	Opaque	0.9
3	KY3	Creamy white	Medium	Circular	Entire	Flat	Opaque	0.4
4	KY4	Creamy white	Large	Irregular	Undulate	Flat	Opaque	0.5
5	KY5	Creamy white	Large	Circular	Undulate	Flat	Opaque	120
6	KY6	Creamy white	Large	Circular	Entire	Flat	Opaque	0.5
7	KK7	Creamy white	Large	Circular	Entire	Flat	Opaque	0.6
8	KK8	Creamy white	Medium	Circular	Entire	Flat	Opaque	0.4
9	KK9	Creamy white	Large	Circular	Undulate	Flat	Opaque	0.7
10	EM10	Creamy white	Large	Irregular	Undulate	Flat	Opaque	0.5
11	EM11	Creamy white	Large	Circular	Entire	Flat	Opaque	0.6
12	HD1	Creamy white	Large	Circular	Entire	Flat	Opaque	0.7

Table 7. Crystal protein morphology of native isolates of *Bacillus thuringiensis* and the reference strain HD1

Sl. No.	Isolate	Shape of crystal protein
1	KY1	Spherical & bipyramidal
2	KY2	Spherical
3	KY3	Spherical
4	KY4	Irregular
5	KY5	Spherical & bipyramidal
6	KY6	Spherical
7	KK7	Spherical & bipyramidal
8	KK8	Triangular & spherical
9	KK9	Irregular
10	EM10	Spherical
11	EM11	Spherical
12	HD1	Bipyramidal

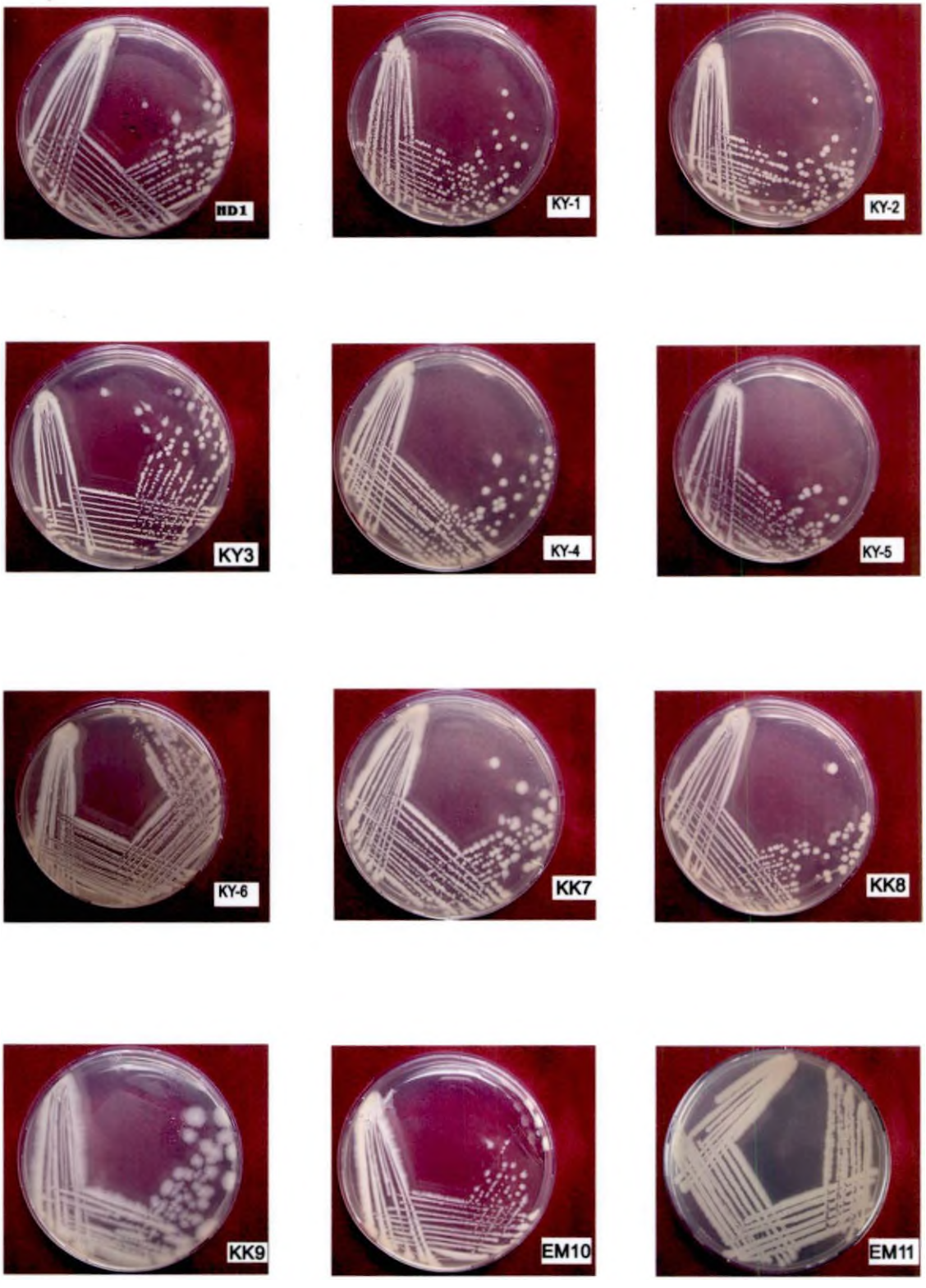


Plate 4. Colony morphology of native *B. thuringiensis* isolates

undulate. Observations made on the colony characteristics of *Bacillus thuringiensis* isolates are given in Table 6.

4.2.2 Staining Reactions

All isolates were found to have blue colour after Gram staining indicating Gram positive nature. The cells were rod-shaped and arranged in a chain. Endospore staining of the isolates showed the presence of spores as translucent and the crystals as bluish black in colour. Staining of the isolates with Coomassie brilliant blue revealed the presence of dark bluish crystal proteins (Plate 5). The shape of crystal proteins varied from irregular, spherical, triangular and bipyramidal for different isolates. Isolates KY1, KY5 and KK7 produced a composite of spherical and bipyramidal crystal proteins. Triangular and spherical crystal proteins were observed in KK8. Isolates KK9 and KY4 produced irregular crystal proteins. Of the total 11 isolates analyzed, eight produced spherical crystal proteins (Table 7).

4.2.3 Biochemical characterization

The isolates were characterized by seven biochemical tests (Table 8).

4.2.3.1 Solubility in three per cent KOH

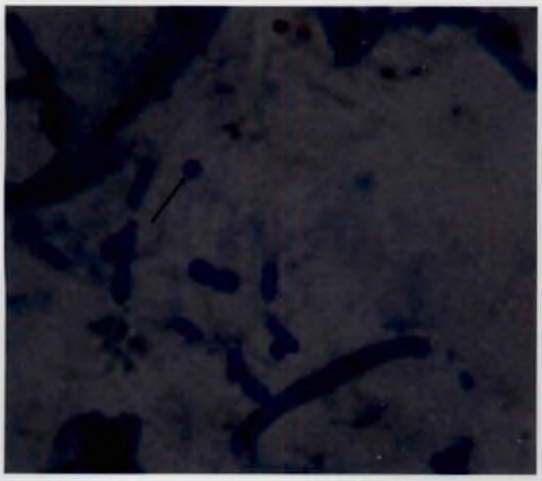
All the isolates formed non-viscous strands with three per cent KOH, confirming the Gram positive nature.

4.2.3.2 Gelatin hydrolysis

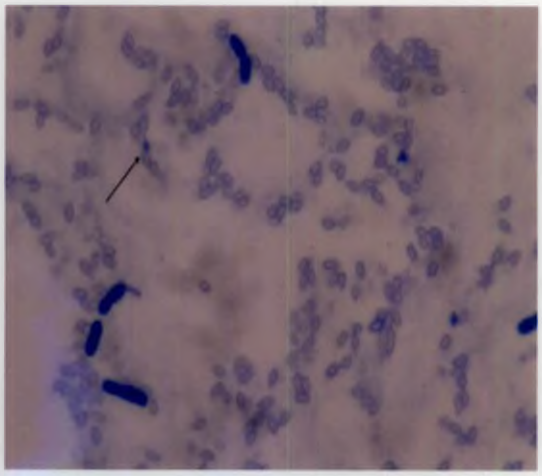
None of the isolates restored the gel characteristics of the gelatin agar medium, which indicated a negative reaction for gelatin hydrolysis.

4.2.3.3 Urease test

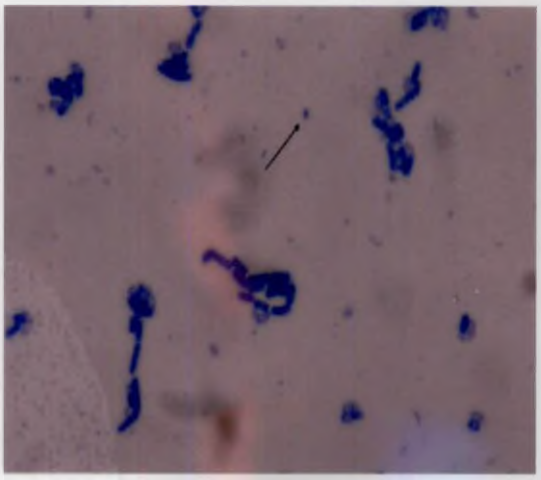
Urea broth containing phenol red as indicator was used for urease test. Six isolates namely KY1, KY5, KY6, KK7, KK8, EM11 and reference strain HD1



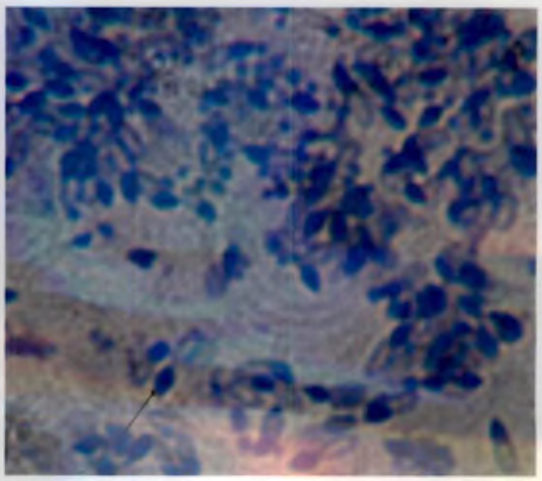
a) Spherical



b) Triangular

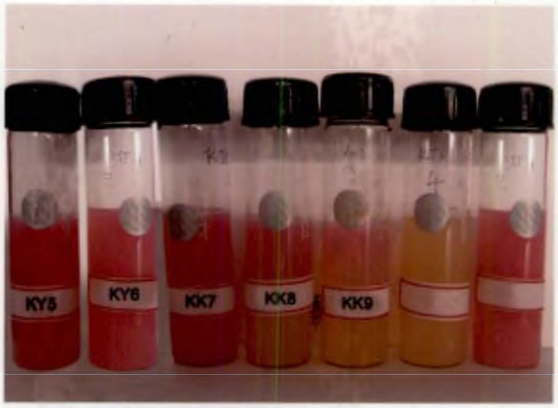


c) Irregular

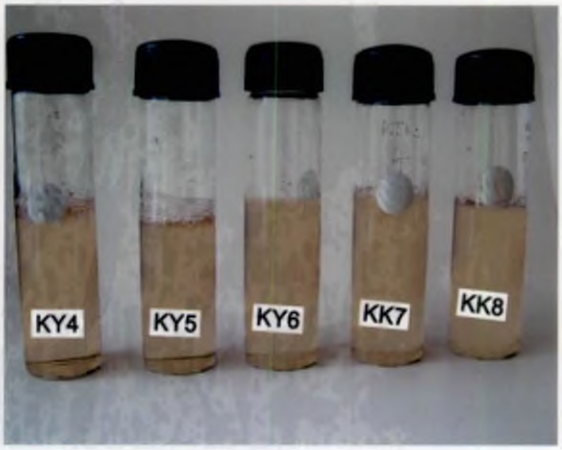


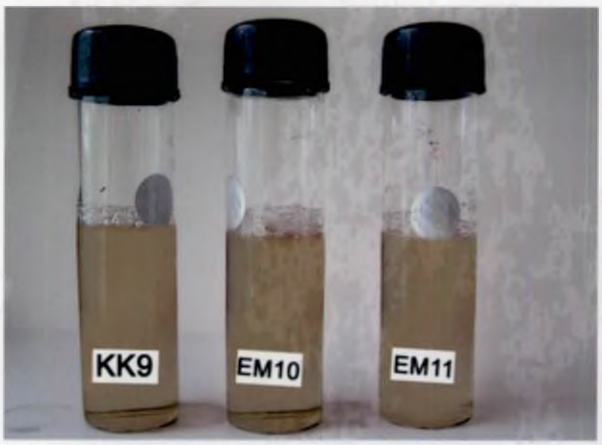
c) Bipyramidal

Plate 5. Crystal protein morphology of native isolates of *B. thuringiensis*



A) Urease test





B) VP test

Plate 6. Hydrolysis of urea and VP test

degraded urea, which was indicated by colour change of the medium to deep pink. Five isolates, namely KY2, KY3, KY4, KK9 and EM10 did not produce any colour change, indicating that these did not hydrolyze urea (Plate 6A).

4.2.3.4 Starch hydrolysis

A clear zone of hydrolysis surrounding the growth of bacteria when flooded with Lugol's iodine was observed in ten isolates and the reference strain indicating positive reaction. Absence of clear zone in KK9 showed that it was not capable of hydrolyzing starch (Plate 7).

4.2.3.5 Voges Proskauer test

All the isolates were negative for Voges Proskauer test indicating the absence of non-acidic/neutral end products such as acetyl methyl carbinol from glucose metabolism (Plate 6B).

4.2.3.6 Esculinase test

Six isolates namely KY1, KY2, KY4, KY5, KY6, EM11 and the reference strain HD1 produced blackening around the growth indicating the hydrolysis of esculin. The other five isolates namely KY3, KK7, KK8, KK9 and EM10 remained with no colour change, indicated a negative reaction for esculinase test (Plate 8).

4.2.3.7 Lecithinase test

All the isolates including reference strain HD1 produced a clear zone around the growth which indicated a positive reaction for lecithinase (Plate 9). This confirmed the ability of isolates to hydrolyze lecithin present in egg yolk medium.

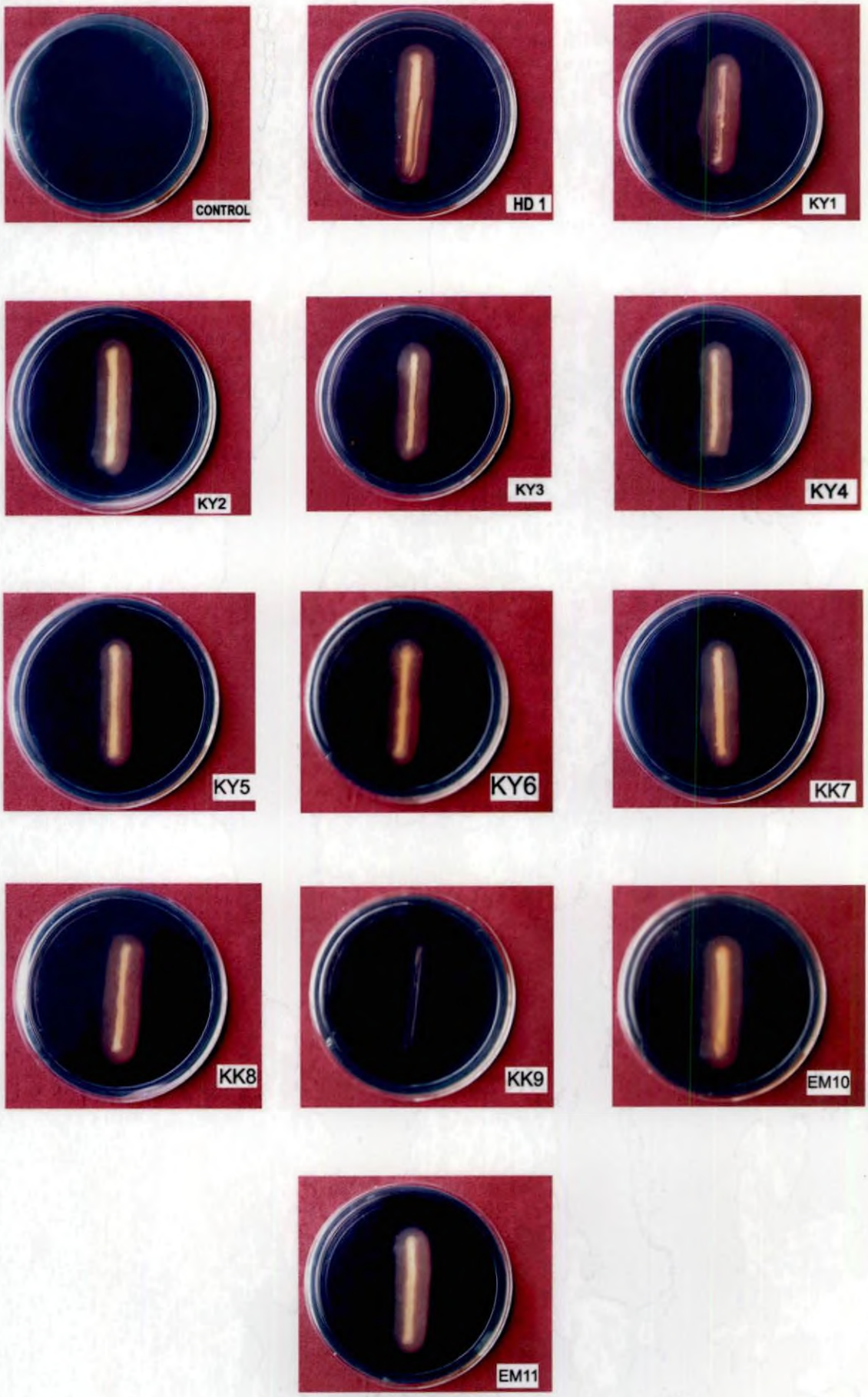


Plate 7. Hydrolysis of starch by *B. thuringiensis*

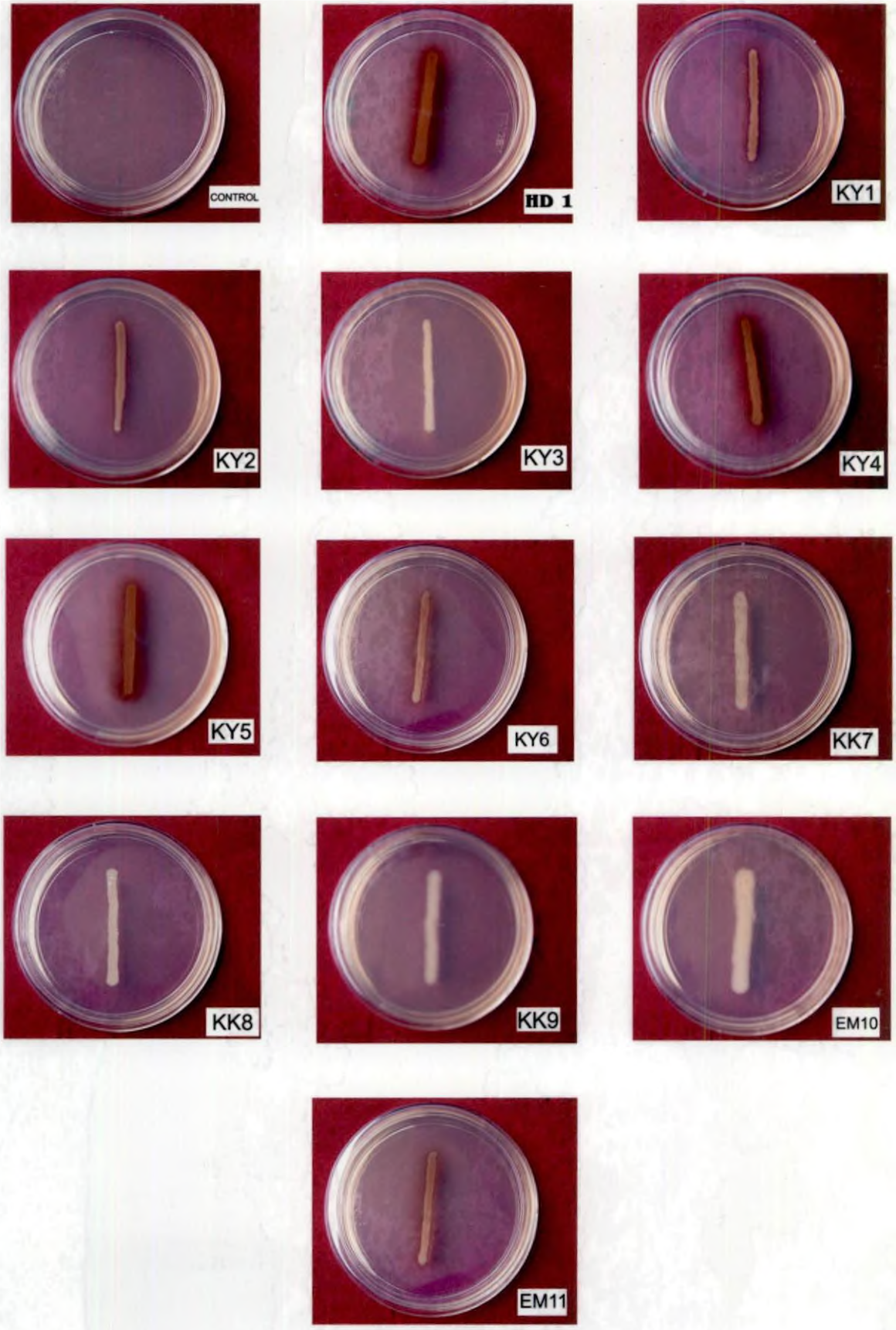


Plate8. Hydrolysis of esculin by *B. thuringiensis* isolates

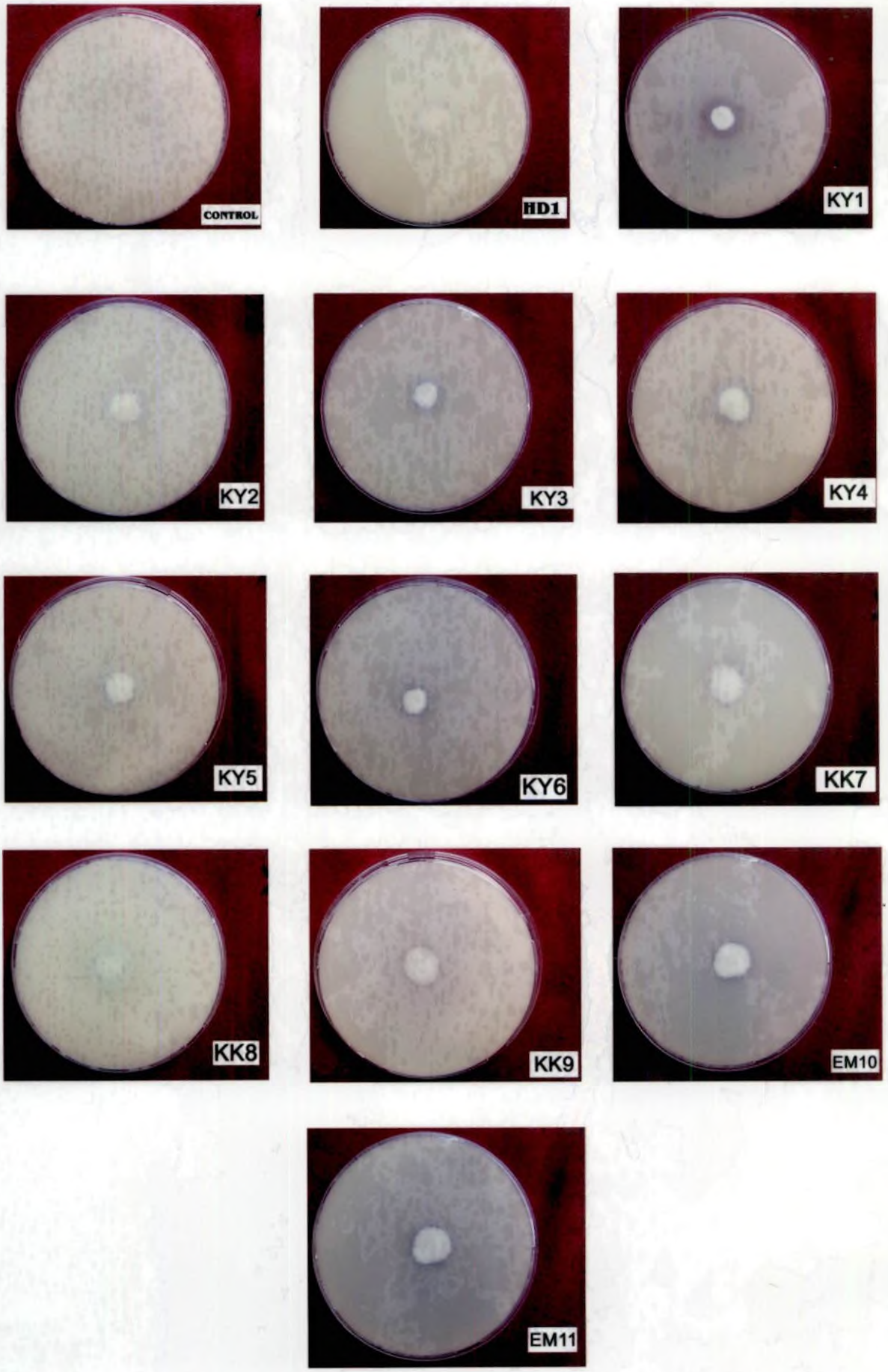


Plate 9. Hydrolysis of lecithin by *B. thuringiensis*

Table 8. Biochemical characteristics *of Bacillus thuringiensis* isolates obtained from soil samples from the Western Ghats

Sl. No.	Isolate	BIOCHEMICAL TESTS						
		Urease	Gelatin hydrol-ysis	Starch hydrol-ysis	Voges-Proskauer	Esculin-ase	Lecithin-ase	Solubility in 3 % KOH
1	KY1	+	-	+	-	+	+	+
2	KY2	-	-	+	-	+	+	+
3	KY3	-	-	+	-	-	+	+
4	KY4	-	-	+	-	+	+	+
5	KY5	+	-	+	-	+	+	+
6	KY6	+	-	+	-	+	+	+
7	KK7	+	-	+	-	-	+	+
8	KK8	+	-	+	-	-	+	+
9	KK9	+	-	-	-	-	+	+
10	EM10	-	-	+	-	-	+	+
11	EM11	+	-	+	-	+	+	+
12	HD1	+	-	+	-	+	+	+

+ Positive
- Negative

Table 9. Effect of crystal proteins from *B. thuringiensis* isolates on larvae of pumpkin caterpillar

Sl. No.	Isolate	Per cent mortality of larvae on different days after feeding									
		1	2	3	4	5	6	7	8	9	10
1	KY1	10	40.7	40.7	46.7	57.7	66.5	74	94.4	100	
2	KY2	10	40.7	49.9	60.1	70.1	79.6	83.8	84.4	84.4	100
3	KY3	3.3	25.9	25.9	45.4	65.1	94.7	94.7	100		
4	KY4	0	18.5	20.3	40.5	70.1	74.6	93.8	94.4	94.4	100
5	KY5	6.6	33.3	48.1	61.4	70.1	79.1	83.3	100		
6	KY6	3.3	44.4	44.4	48	62.6	79.1	89.6	94.4	94.4	100
7	KK7	20	40.7	44.4	65.1	85	89.7	95	95	100	
8	KK8	10	40.7	44.4	70.1	85	90	95	100		
9	KK9	3.3	48.1	48.1	66.5	70.2	79.8	88.5	89.2	89.5	100
10	EM10	6.6	40.7	40.7	50.4	50.4	60.1	79.1	88.8	88.8	100
11	EM11	3.3	10.7	29.2	29.2	38.3	47.9	56.7	76.1	76.1	100
	HD1	13.3	66.6	94.4	100						

4.2.3.8 Subspecies identification of *B. thuringiensis* isolates

Using various biochemical tests, two isolates of B. thuringiensis were identified. Isolate KY1 was identified as *B. thuringiensis* subspecies *brasilensis* and isolate KK7 was identified as *B. thuringienis* subspecies *seoulensis.*

4.3 BIOASSAY OF *Bacillus thuringiensis* ISOLATES AGAINST PUMPKIN CATERPILLAR (*Diaphania indica*)

The insecticidal activity of *Bacillus thuringiensis* isolates were assessed by diet contamination method. Crystal protein harvested from the isolates was mixed with the artificial diet. Reference strain HD1 was used to compare the rate of mortality.

4.3.1 Symptoms observed on the larval body

After feeding on the diet, the larval body became yellowish brown. Some larvae stopped feeding on the diet, became sluggish in movement and started drying. Later, a prominent black discolouration was noticed in the midgut region of the larval body. After death, the larval body decomposed and turned dark brown to black. In some of the larvae, the whole body changed to a black putrefied mass, with black coloured fluid oozing out from the body (Plate 10). Only one larva which was treated with the isolate EM11, survived till pupal stage. But pupal malformation was observed after three days with black fluid oozing out from the body.

4.3.2 Statistical analysis of data on bioassay

. Analysis of data on per cent mortality of larvae, treated with *Bacillus thuringiensis* crystal proteins, showed the reference strain HD1 as the most efficient isolate with hundred per cent mortality by fourth day. Among the native isolates, highest mortality was obtained for isolate KK8, with 70.1 per cent mortality on fourth day. This was followed by isolates KK9 and KK7 with 66.5 and 65.1 per cent mortality. Observations made on percent mortality of larvae are given in Table 9.

a) HD1

b) KY1

c) KY2

d) KY3

Plate 10. Bioassay of native *B. thuringiensis* isolates against pumpkin caterpillars

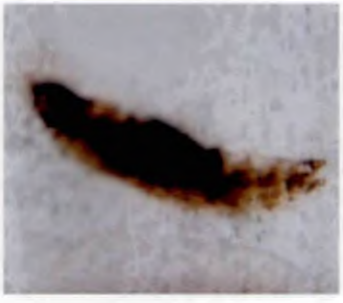
Plate 10. contd.....



e) KY4



f) KY5

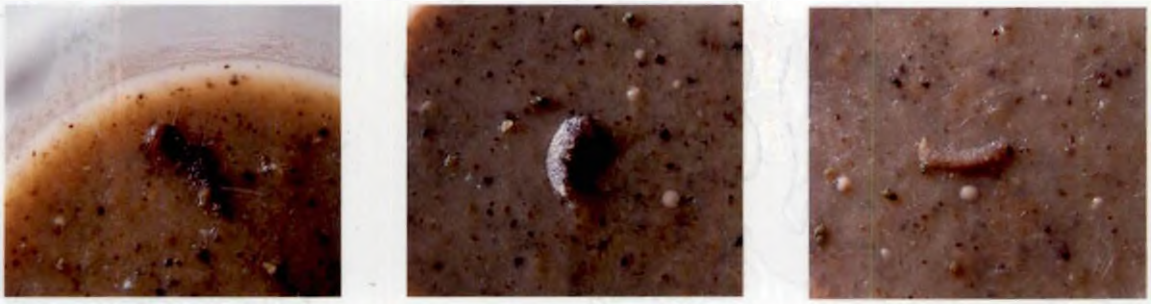




g) KY6



h) KK7

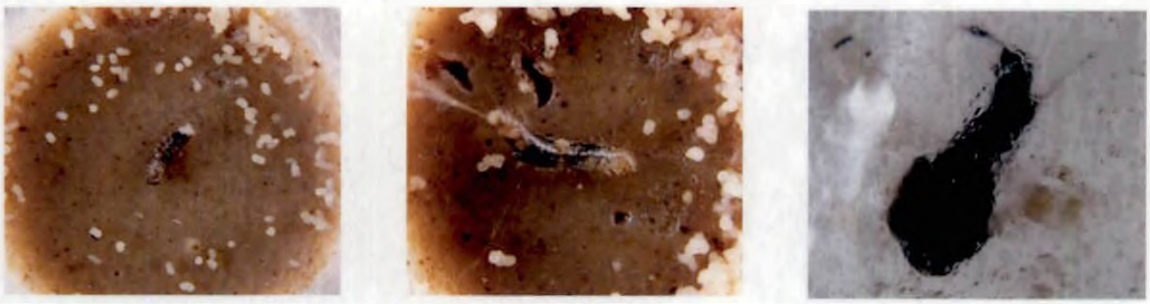
Plate 10. contd.....



i) KK8

j) KK9

k) EM10



l) EM11

Plate 10. Contd ....

Table 10. Statistical Analysis of larval mortality using Kendall's Coefficient of Concordance

Sl. No.	Isolate	Mean rank score of the isolates based on mortality	
		5^{th} day	8^{th} day
1	HD1	11. 75	12.50
2	KK7	8. 75	9. 14
3	KK8	8. 50	9.29
4	KK9	8. 50	8. 14
5	KY2	8. 50	7. 57
6	KY5	7. 00	6. 36
7	KY6	6. 38	6. 00
8	KY1	6. 13	4.64
9	EM10	6. 00	4. 34
10	KY3	2. 88	5.21
11	EM11	2. 13	1.64
12	KY4	1. 50	3.57

Table 11. Ranking of *B. thuringiensis* isolates based on no. of days taken for 50 per cent mortality

Sl. No.	Isolate	No. of days taken	Rank
1	HD1	2	I
2	KY2	3	II
3	KK7	4	III
4	KK8	4	
5	KK9	4	
6	KY5	4	
7	KY3	4	
8	KY4	4	
9	KY1	5	IV
10	KY6	5	
11	EM10	6	V
12	EM11	7	VI

Kendall's coefficient of concordance was used to differentiate the efficacy of twelve isolates as judged from the mortality. Kendall's coefficient of concordance W was found to be highly significant. The analysis was carried out separately on fifth and eighth days of mortality Statistical analysis of daily mortality was carried out using Kendall's Coefficient of Concordance based on mortality on 5^{th} and 8^{th} days (Table 10). The isolates were ranked based on number of days taken for 50 per cent mortality (Table 11).

From the table 10, it is evident that reference strain HD1 was the most efficient one with the highest rank scores (11.75 and 12.50) on 5^{th} and 8th days respectively. Isolates KK7, KK8, KK9 and KY2 were judged as having the same efficacy, but not as effective as HD1. The other isolates in their order of decreasing order of performance were KY5, KY6, KY1 and EM10. Isolates KY3, EM11 and KY4 proved to be the least effective ones.

The isolates were ranked based on number of days taken for fifty per cent mortality. HD1 ranked first and took only two days to reach fifty per cent mortality. Among the native isolates, KY2 ranked first which took three days. Isolates KK7, KK8, KK9, KY5, KY4 and KY3 took four days to achieve fifty per cent mortality.

4.4 AMPLIFICATION OF *cry* GENE FROM *Bacillus thuringiensis*

4.4.1 Total DNA isolation from *Bacillus thuringiensis* isolates and gel separation

Total DNA isolated from *Bacillus thuringiensis* was checked on 0.8 per cent agarose gel to detect the purity (Plate 12A). A single sharp band was obtained, which indicated that the DNA was intact. No RNA contamination was observed.

4.4.1.2 Quantification of DNA

The quantity of DNA present in the sample was determined using NanoDrop® ND-1000. The quantity of DNA as assessed by spectrophotometry using nanodrop varied from 1.337μg/ml to 20.132μg/ml of bacterial culture. The OD_{260}/OD_{280} ranged between 1.78 and 1.97 (Table 12) indicating that there was no protein contamination. The quantity of DNA was found to be highest in the isolate KY6 (20.132μg/ml) and the lowest was observed in isolate EM10 (1.337μg/ml).

4.4.2 Primer designing

Based on the parameters described in Section 3.9, a pair of primer for *cry*1A gene were designed. The complete nucleotide sequences of *cry*1A gene of different species of *Bacillus thuringiensis* were downloaded from NCBI databank (http://www. ncbi.nlm.nih.gov). The accession numbers of the sequence used are provided in Table 13.

Multiple sequence alignment of these nucleotide sequences was done using ClustalW 1.83 available from European Bioinformatics Institute (www.ebi.ac.uk/clustal). The conserved boxes selected through multiple sequence alignment at nucleotide level are provided in Plate 11. Based on these conserved regions, forward and reverse primers were designed. The conserved regions starting from bases 142 to 163 and from bases 988 to 1009 was used for designing forward and reverse primer respectively. The primers had an annealing temperature of 61^0C and the expected amplicon size was 824bp.

4.4.3 Profiling of *cry*1 and *cry*4 genes from *Bacillus thuringiensis* isolates

All the *B. thuringiensis* isolates were screened for the presence of *cry*1 gene using the universal *cry*1 primer designed by Ben-Dov *et al.* (1997). Amplification of a single band of less than 250bp was obtained with universal

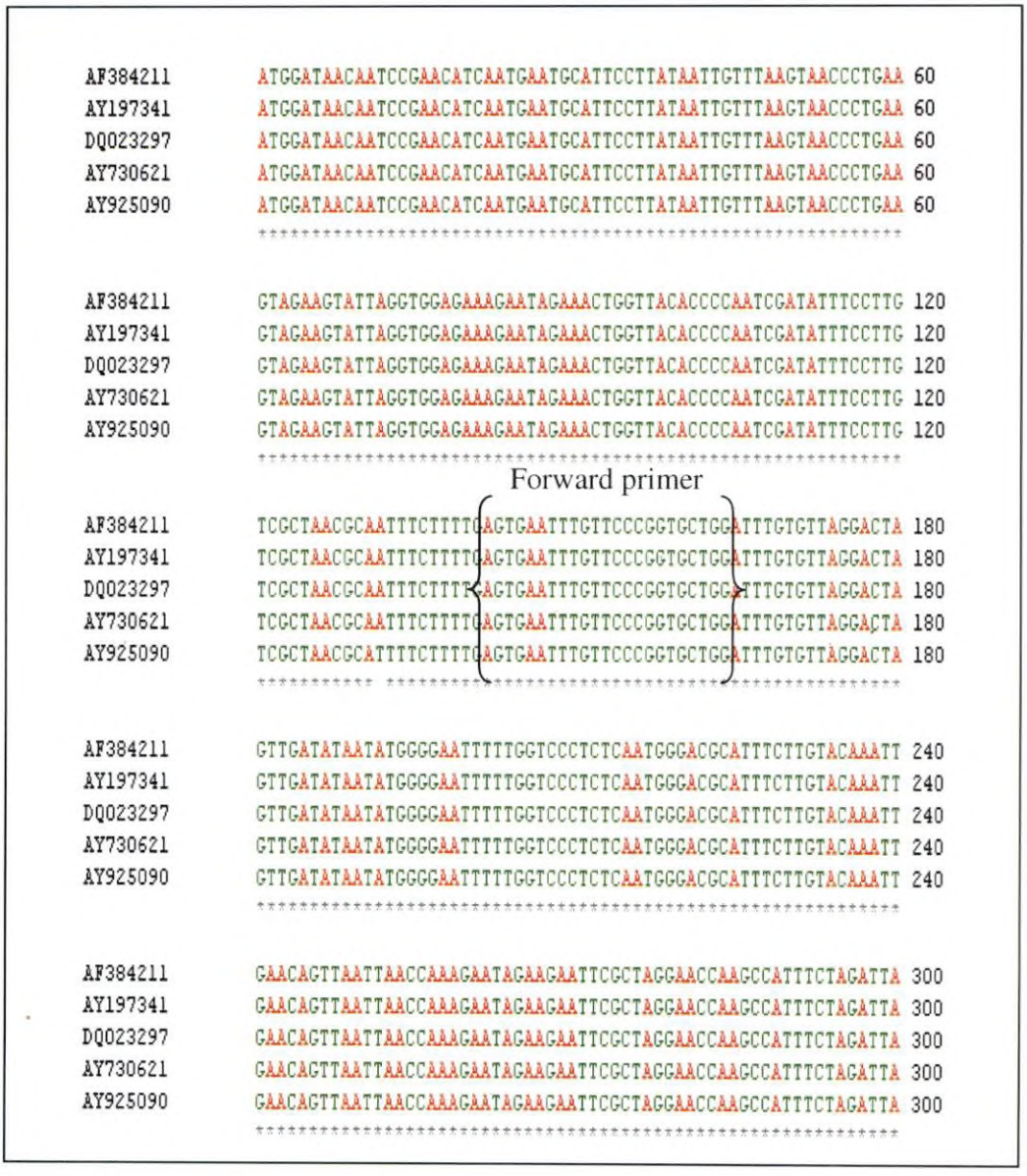


Plate 11. Multiple sequence alignment of *Bacillus thuringiensis cry*1A genes. The conserved sequences used for designing forward and reverse primers are shown in brackets.

Plate 11. Contd...

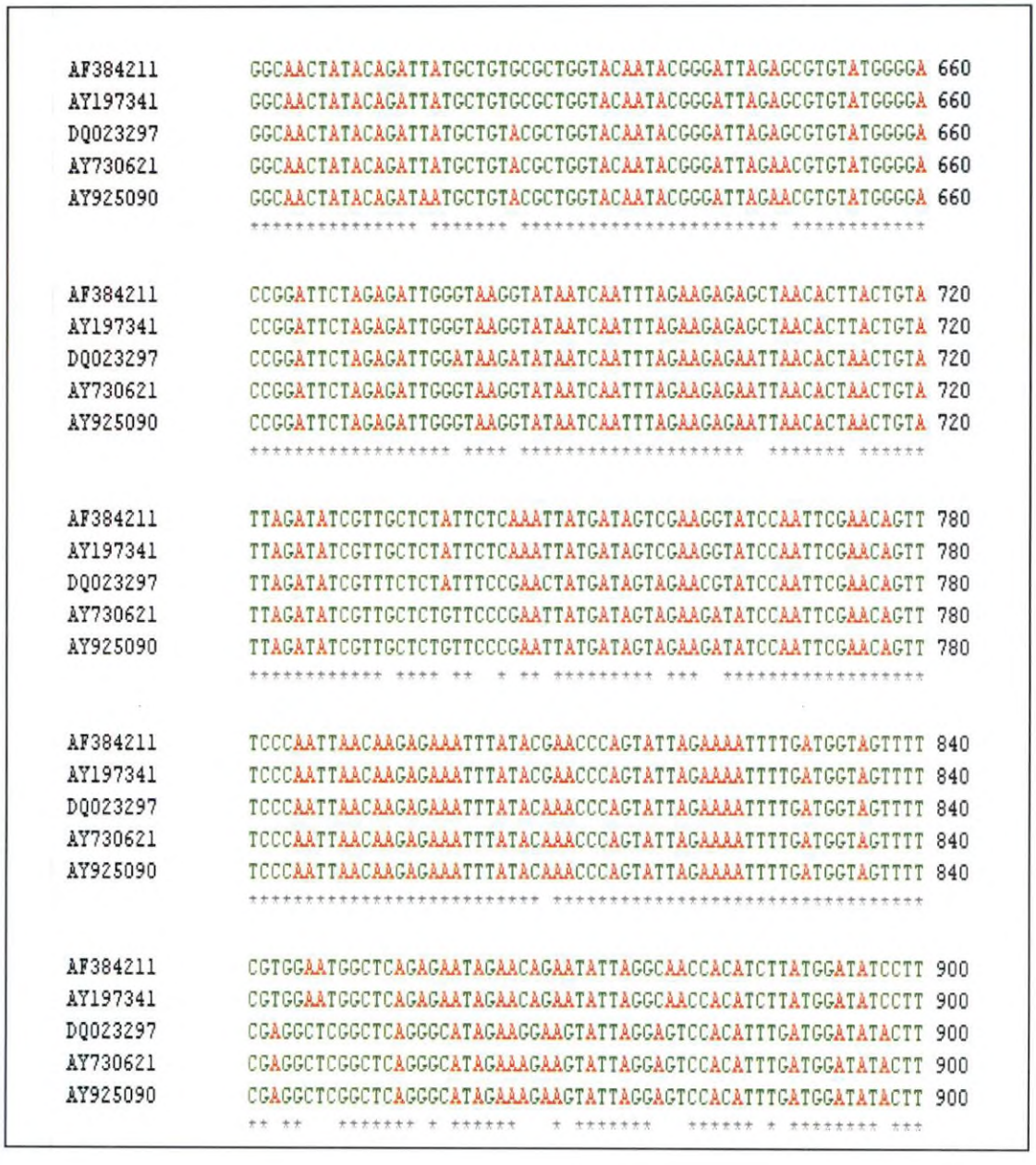


Plate 11. Contd...

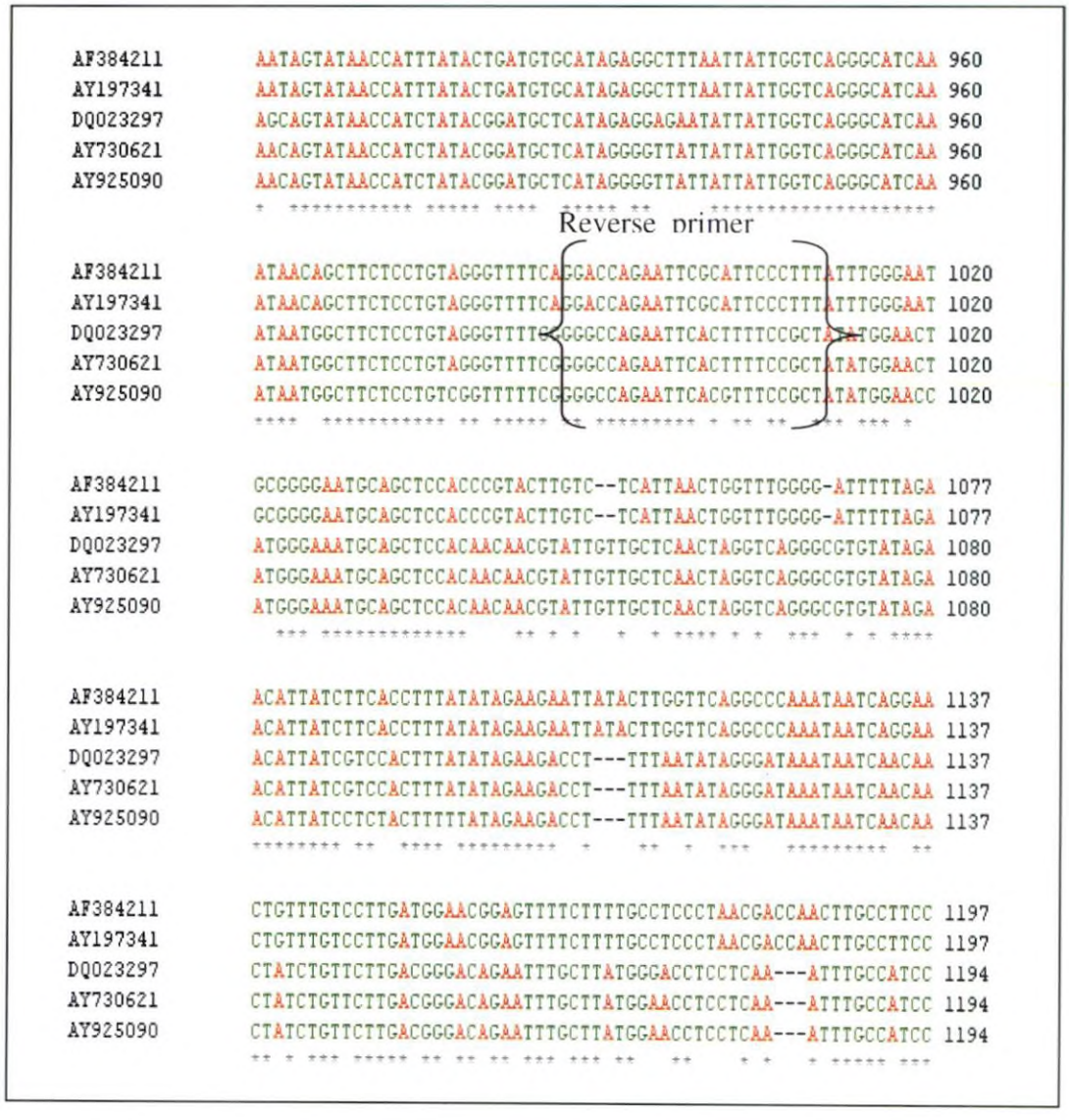
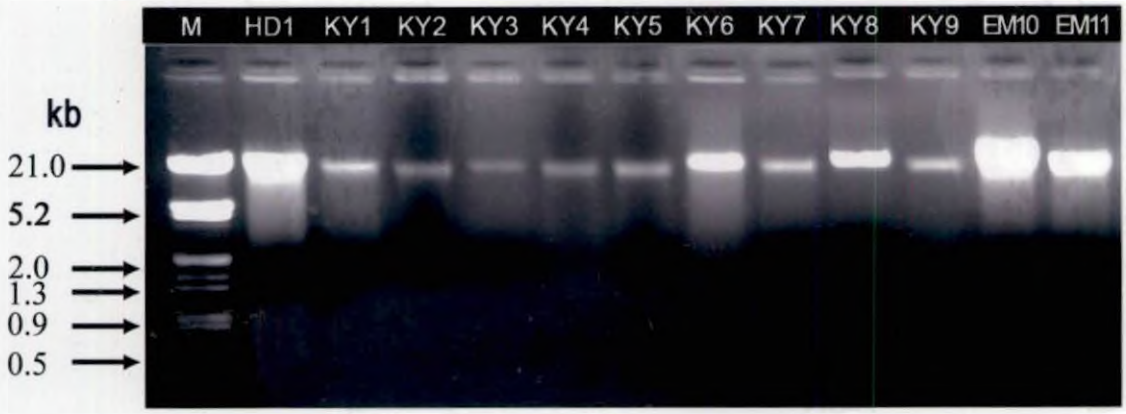
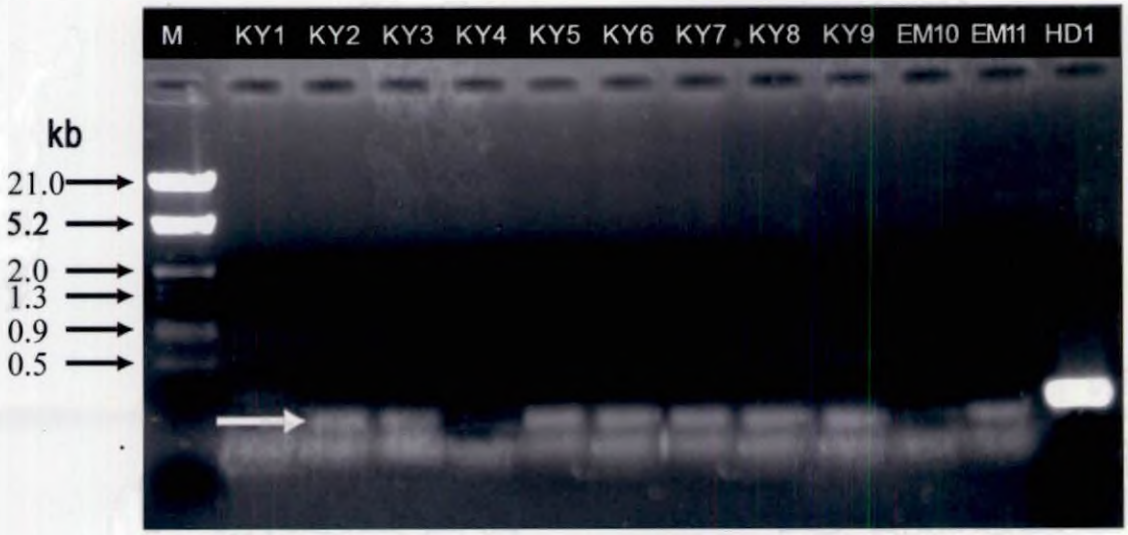


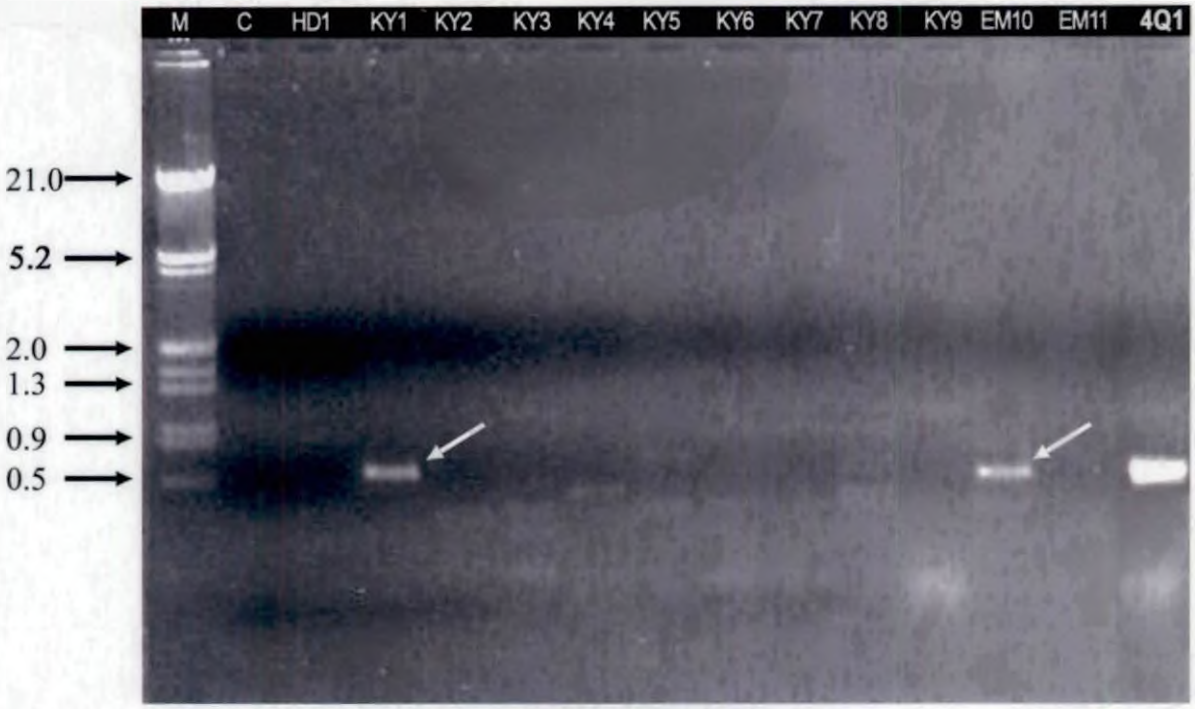
Plate 11. Contd...



A) Total DNA isolated from *Bacillus thuringiensis* isolates



B) *cry*1 profile



C) *cry*4 profile

Plate 12. *Cry* gene profile of native *B. thuringiensis* isolates

*cry*1 gene primer for eight isolates KY2, KY3, KY5, KY6, KK7 KK8, KK9 and EM11 along with the reference strain HD1 (Plate12B). No amplification was obtained for isolates KY1, KY4 and EM10 (Table14). Amplicon size of the fragment being 250 bp, which was slightly less than the expected, the amplicons obtained for isolates KY5 and EM11 were used for cloning so as to confirm the gene sequence. These amplicons were named as *cry*1ky5 and *cry*1em11.

Screening of *Bacillus thuringiensis* isolates was carried out for *cry*4 gene with universal *cry*4 primer (Ben-Dov *et al.*, 1997). PCR amplification of standard strain 4Q1 with universal *cry*4 primer showed a single band of 524bp (Plate 11C). Amplification for *cry* 4 gene was obtained for isolates KY1 and EM10. Single band of 575 bp and was obtained for isolate KY1. For isolate EM10, three bands of size 531, 942 and 1245 bp were obtained (Plate 12C and Table 14). Amplicons of desired molecular weight from these isolates were sequenced. These amplicons were named as *cry*4em10 and *cry*4ky1.

4.4.4 Gel elution of PCR products

The amplicons *cry*1ky5, *cry*1em11 and *cry*4em10 were eluted from the gel. The eluted products when observed on agarose gel showed good concentration suggesting that recovery from the gel slice was good (Plate 14A).

4.5 AMPLIFICATION OF *cry*1A GENE FROM *B. thuringiensis* ISOLATES

PCR of all the isolates found to be positive in *cry*1 gene profiling, was done with *cry*1A primer. This yielded the amplification of *cry*1A gene in isolates KY3 and KY7 (Plate 15A). A single band of size 600 bp was obtained for the isolate KY3. Isolate KK7 produced a single band of size 554 bp. The amplicon obtained for the isolate KY3 was sequenced after purification of PCR product. The amplicon was named as *cry*1Aky3.

Table 12. Quality and quantity of total DNA extracted from native *Bacillus thuringiensis* isolates

Sl. No.	Isolate	Optical Density Ratio OD_{260}/OD_{280}	Quantity of DNA (µg/ml)	Remarks on quality
1	KY1	1. 78	3.062	Good
2	KY2	1. 82	8.783	Good
3	KY3	1. 97	2.362	Good
4	KY4	1. 88	4.778	Good
5	KY5	1. 93	1.514	Good
6	KY6	1. 80	20.132	Good
7	KK7	1. 91	4.094	Good
8	KK8	1. 87	3.720	Good
9	KK9	1. 93	2.790	Good
10	EM10	1. 79	1.337	Good
11	EM11	1. 86	17.630	Good

Table 13. Details of *cry* gene sequences used for primer designing

Sl.No.	Bacterial strain	Accession number in NCBI
1	*B. thuringiensis* SK 22	DQ023297
2	*B. thuringiensis*	AY197341
3	*B. thuringiensis* strain ly 30	AF384211
4	*B. thuringiensis* serovar *kenyae* strain HD549	AY925090
5	*B. thuringiensis*	AY730621

Table 14. Details of amplicons obtained in *cry1* and *cry*4 gene profile

Sl. No.	Isolate	Details of amplicons					
		*cry*1			*cry*4		
		No. of bands observed	Mol. wt of amplicon (bp)	Expected size of amplicon (bp)	No. of bands observed	Mol.wt of amplicon (bp)	Expected size of amplicon (bp)
1	KY1	-	-	-	1	575	439
2	KY2	1	~250	277	-	-	-
3	KY3	1	~250	277	-	-	-
4	KY4	-	-	-	-	-	-
5	KY5	1	~250	277	-	-	-
6	KY6	1	~250	277	-	-	-
7	KK7	1	~250	277	-	-	-
8	KK8	1	~250	277	-	-	-
9	KK9	1	~250	277	-	-	-
10	EM10	-	-	-	3	531, 942, 1245	439
11	EM11	1	~250	277	-	-	-
12	HD1	1	~300	277	-	-	-
13	4Q1	-	-	-	1	524	439

4.6 TRANSFORMATION AND CLONING OF PCR PRODUCT

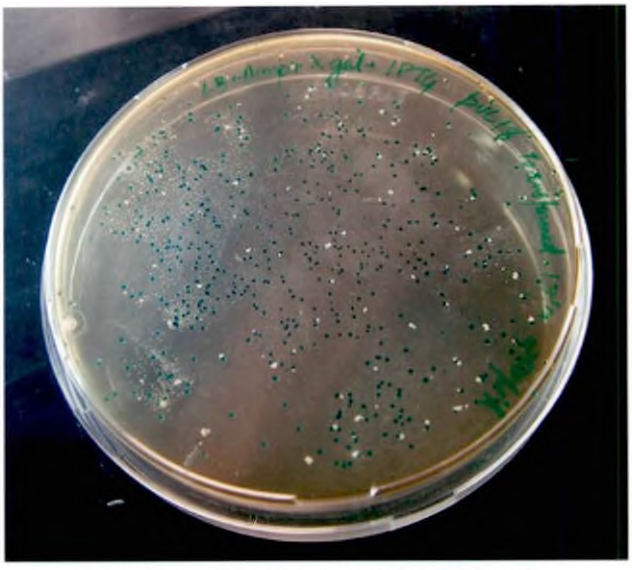
4.6.1 Preparation and screening of competent cells

Competent cells were prepared from *E.coli* JM 109 strain. Large numbers of colonies were obtained when the competent cells were checked for competence by transformation using the pUC18 plasmid containing ampicillin resistance marker. The transformation efficiency of competent cells was calculated as $5x10^8$cfu/µg DNA. The cells were competent and showed a high degree of transformation efficiency. The colonies showed luxuriant growth, with no other contamination in LBA ampicillin plates (Plate 13A). Thus the competent cells prepared were found to be efficient for transformation and further cloning purposes.

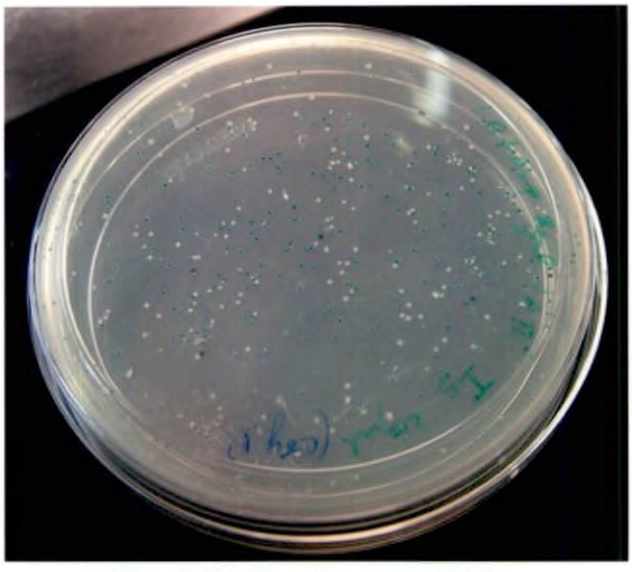
The eluted bands corresponding to each isolate was ligated into pGEMT vector. After confirmation of competence, the ligated product was transferred into competent *E. coli* JM109 cells using the heat shock method at 42^0C. When the transformed *E.coli* cells were grown in LBA ampicillin plates overlaid with X-gal and IPTG, a combination of blue and white colonies were obtained after overnight incubation confirming successful transformation (Plate13B and 13C). The recombination efficiency of transformation is presented in Table 15.

4.7 SCREENING OF THE TRANSFORMED COLONIES

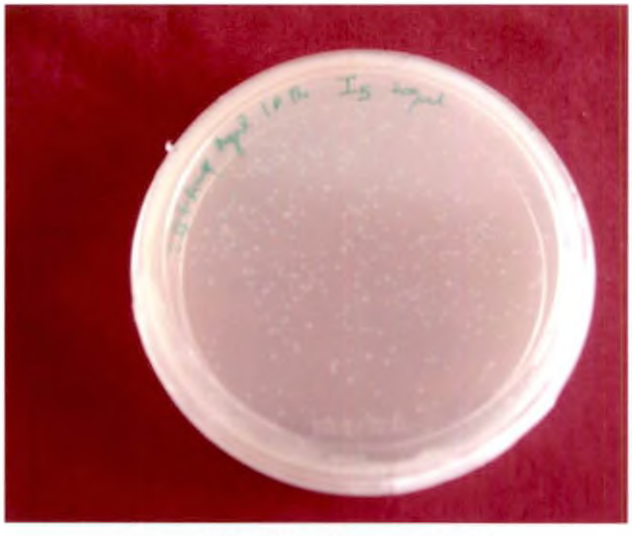
The LB ampicillin agar plates containing the transformed colonies were screened for recombinant plasmid. Three white colonies were picked from each plate and grown in LB ampicillin media separately. One blue colony was also inoculated in order to set the negative control. Plasmids were isolated and checked on 0.7 per cent agarose gel (Plate14B). The plasmids isolated from white colonies had high molecular weight when compared with plasmid isolated from blue colonies. This confirmed presence of the insert in white plasmid.



A). Transformants with pUC 18



B). pGEMT with *cry*4em10



C). pGEMT with *cry*1ky5

Plate 13. Blue- White screening of recombinant *E. coli*

Table 15. Recombination efficiency in *E. coli* cells transformed with different amplicons

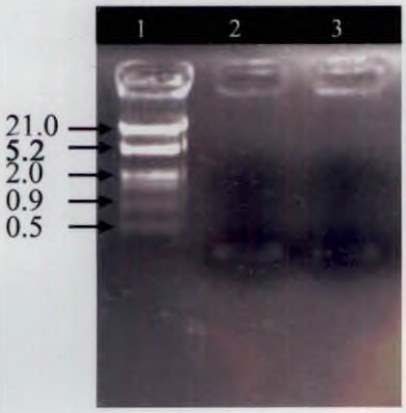
Sl. No.	Details of amplicon	No. of white colonies	No. of blue colonies	Total no. of colonies	Recombination efficiency (%)
1	*cry*1ky5	121	69	190	63.6
2	*cry*1em11	108	46	154	70.1
3	*cry*4em10	212	96	308	68.8

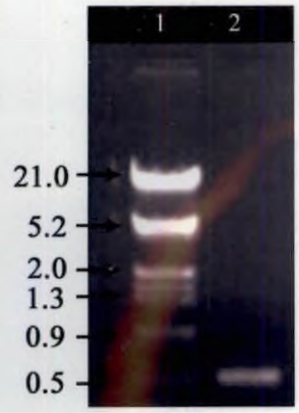
Table 16. Details of amplicons from native *B. thuringiensis* isolates used for sequencing

Sl. No.	Sequence name	Sequence length (bp)	
		Nucleotide	Aminoacid
1	*cry*1ky5	146	48
2	*cry*1em11	147	48
3	*cry*4em10	625	234
4	*cry*4ky1	452	142
5	*cry*1Aky3	559	185

Table 17. Nucleotide statistics of sequences from *B. thuringiensis* isolates

Sl. No.	Gene sequence	Nitrogen base percentage (%)					
		A	T	G	C	A and T	G and C
1	*cry*1ky5	28.4	20.9	27.7	21.6	49.3	49.3
2	*cry*1em10	27.5	20.1	28.2	22.8	47.7	51.0
3	*cry*4em10	30.8	24.1	23.3	21.8	54.9	45.1
4	*cry*4ky1	26.6	31.9	21.1	20.4	58.5	41.5
5	*cry*1Aky3	38.6	25.0	20.8	15.6	63.7	36.3

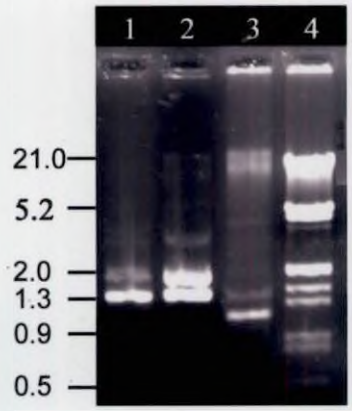


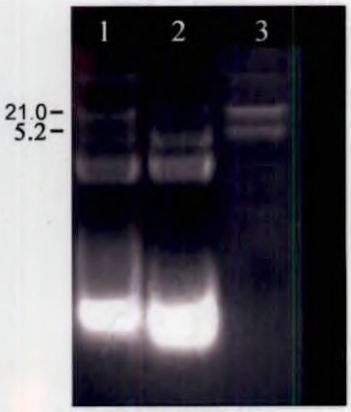


A) Eluted bands of *cry*1 and *cry*4

Lane 1 - λDNA/*Eco*RI/*Hind*III
Lane 2 - *cry*1ky5
Lane 3 - *cry*1 em11

Lane 1 - λDNA/*Eco*RI/*Hind*III
Lane 2 - *cry*4em10

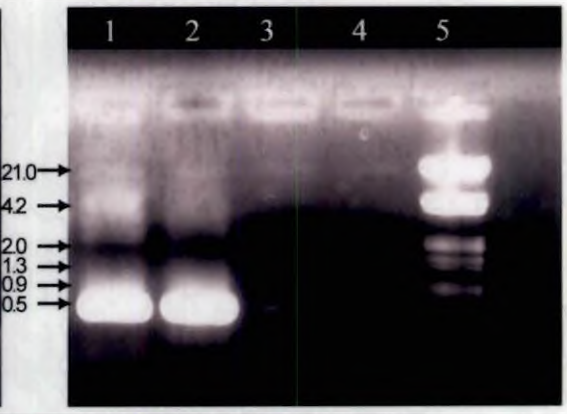




B) Plasmids isolated from recombinant and non-recombinant colonies

Lane 1, 2 - *cry*1ky5
Lane 3 - Non-recombinant
Lane 4 - λDNA/*Eco*RI/*Hind*III

Lane 1 - *cry*4em10
Lane 2 - Non-recombinant
Lane 3 - λDNA/*Eco*RI/*Hind*III



C) Reamplification of genes from recombinant plasmids

Lane 1 - λDNA/*Eco*RI/*Hind*III
Lane 2, 3, 4 - *cry*1ky5
Lane 5 - *Insert DNA*
Lane 6,7,8 - *cry*1em11
Lane 9 - Non-recombinant

Lane 1, 2, 3 - *cry*4em10
Lane 4 - Non-recombinant
Lane 5 - λDNA/*Eco*RI/*Hind*III

Plate 14. Cloning of *cry*1 and *cry*4 amplicons from native isolates of *Bacillus thuringiensis*

4.7.1 Confirmation of recombination by PCR

The plasmid was checked for the presence of insert by PCR confirmation. The plasmid DNA was used as template and insert was amplified using gene specific primers. Amplification was obtained in the plasmids isolated from white colonies. No amplification was detected in the plasmids isolated from blue colonies. The PCR products were checked on 0.7 per cent agarose gel (Plate 14C). The insert used for transformation was also loaded along with the amplified plasmid PCR products, to confirm the presence of insert in the plasmid.

4.8 SEQUENCING OF THE CLONE AND AMPLICONS

The screened colonies in which the presence of insert was confirmed were used for automated sequencing using T7 universal primer. The amplicons *cry*4ky1 and *cry*1Aky3 were directly sequenced from the purified PCR product. The isolate name and the sequence length are given in Table 16.

4.9 SEQUENCE ANALYSIS USING VARIOUS COMPUTER ALGORITHMS

The nucleotide sequence of the clones obtained from the isolates KY5, EM11, EM10 and the sequences of amplicons obtained from the isolates KY1 and KY3 are given in Plates 16A, 17A, 18A, 19A and 20A. The graphical outputs of each sequence are presented (Plates 16D, 17D, 18C, 19C and 20C).

4.9.1 Nucleotide sequence analysis

The nucleotide sequences were screened for the presence of vectors using VecScreen program of NCBI. The sequences cloned from isolates KY5 and EM11 showed significant similarity with vector regions. *cry*1ky5 showed similarity to vectors starting from the region 146 to 611 bases. *cry*1em11 showed similarity to vectors starting from 147 to 487 bases. No significant similarity with vectors was observed for *cry*4em10. Results of vector screen of cloned sequences are

displayed in Plates 16B, 17B and 18A. The sequences after deletion of vectors are given (Plates 16C and 17C).

Homology search of nucleotide sequences of clones from isolates KY5, EM11 and EM10 and those of the amplicons obtained from the isolates KY1and KY3 with other reported *cry* gene sequences were carried out. *cry*1ky5 and *cry*1em11 showed significant homology with *cry*1, *cry1*A, *cry*1Aa, *cry*1Ac, *cry*1B, *cry*1Ba5 gene sequences of various species of *Bacillus thuringiensis* present in NCBI databank. Query coverage for *cry*1ky5 was 66 per cent and for *cry*1em11, 65 per cent with two accessions namely, CP000485 and AEO17355. The percentage identity was 100 in *cry*1ky5 with the 123 Blast hits whereas in *cry*1em11 also it remained the same with 111 Blast hits reported (Plates 16E and 17E)

*cry*4em10 showed homology with *cry*4A, *cry*4BLB, and other crystal protein genes present in different species of *B. thuringiensis.* The query coverage was 16 per cent in *cry*4em10 among two accessions CP000485 and AEO17355. There was 100 per cent identity with 13 Blast hits reported (Plate 18D). *cry*4ky1 showed 23 and 32 per cent query coverage and 100 per cent identity with two accessions CP000485 and AEO17355 respectively.

*cry*1Aky3 showed homology with spore coat protein D present in different species of *B. thuringiensis.* The query coverage of *cry*1Aky3 was 86 per cent in two accessions. The per cent identity was 100 in three accessions CP000485, AEO17355 and DQ363750 (Plate 20C).

Homology search through protein-protein BLAST (Blastp) using deduced amino acid sequence showed that both *cry*1ky5 and *cry*1em11 had similarity with delta- endotoxin *cry*iiia (Bt13) gene of *B. thuringiensis* (Plates 16F and 17F). *cry*4em10 showed significant similarity to mosquitocidal/pesticidal crystal protein genes *cry*4Aa, *cry*4Ba of *B. thuringiensis.* Similarity was also observed with GTP

pyrophosphokinase, transcriptional regulator and riboflavin biosynthesis protein of *B. thuringiensis.* (Plate18G). The *cry*4ky1 showed similarity with delta-endotoxin genes (Plate 19D). *cry*1Aky3 showed homology with crystal protein cryE6L, cytosolic, ATP binding, membrane binding and glucokinase regulatory proteins present in *B. thuringiensis* (Plate 20D).

Multiple sequence alignment of *cry*4em10 with delta endotoxin genes of *B. thuringiensis* reported in the public domain was carried out (Plate 18E). Significant levels of conserved regions existed for *cry*4em10 with *cry*4 genes among different species of *B.thuringiensis.* Evolutionary relationship of the clone with other *B. thuringiensis* strains are depicted in (Plate 18F). *cry*4em10 showed close similarity with *cry*4A gene and *cry* gene in two accessions (EF 208904 and Y00423.1).

Nucleotide composition of the above sequences was determined using Biology Work Bench (http://seqtool.sds.edu/). The A+T and C+G base pair composition was same (49.3%) in *cry*1ky5, while *cry*1em11 was rich in C+G (51.0%). *cry*4em10 was rich in A+T (54.9%) as compared with C+G (45.1%). *cry*4ky1 was comparatively rich in A+T (58.5%). The A+T content of *cry*1Aky3 was high (63.7%) and C+G was comparatively low (36.3%). The details of nucleotide composition of sequences are given in Table 17.

The sequences were translated in all six opening reading frames (http://www.ncbi.nlm.nih.gov.ORF finder). There were two open reading frames in *cry*1ky5, with the longer one located on +2 strand starting from base 5 to145, having a length of 141 bases and the other on -2 strand starting from the base 2 to 112, with a length of 111 bases (Plate 16G) *cry*1em11 had two open reading frames, with the longer one on +3 strand with a length of 127 bases, starting from base 21 to 146 and the other on -2 strand with a length of 113 bases starting from base 1 to 113 (Plate 17G)

Table 18. Open reading frames of *cry* genes cloned from different isolates

Sl .No.	Gene	ORF location	ORF length (bp)	Reading frame
1	*cry*1ky5	5- 145	141	+2
		2-112	111	-2
2	*cry*1em11	21- 146	127	+3
		1-113	113	-2
3	*cry*4em11	94- 558	465	+1
		1-182	182	-2
4	*cry*4ky1	1- 132	132	-3
		270- 371	102	+3
5	*cry*1Aky3	3- 115	114	-1
		3- 115	114	+3
		2- 115	114	+2
		1- 114	114	-3
		1-114	114	+1

*cry*4em10 possessed two open reading frames (Plate 18H) with the longest one located on +1 strand with a length of 465 bp, starting from base 94 to558. The second one was encoded on the -2 strand, starting from base 1 to 182, with a length of 182 bp. There were two ORFs in *cry*4ky1, with the largest one located on -3 strand, having a length of 132 bp starting from base 1 to132. The other ORF was located on the +3 strand, starting from base 270 to 371, with a length of 102 bp (Plate 19E).

*cry*1Aky3 sequence had five open reading frames, three of them located on the plus (+1, +2, +3) strands and two in the minus (-1, -3) strands. The length of all the ORFs was 114 bp. Results of ORF analysis are displayed in Plate 20E. The location and length of ORFs are specified in Table 18.

All the sequences were analyzed for the presence of restriction sites for ten enzymes (http://seqtool.sdsc.edu/). The restriction enzymes *Hae*III and *Hinf* III had three sites in *cry*1ky5, whereas *Alu*I and *Bsa*HI had only one site (Plate 16K). In *cry*1em11, there were two restriction sites for *Hae*III and *Hinf* III, but restriction sites were absent for *Alu*I and *Bsa*HI (Plate 17K). Restriction enzymes *Bam*HI and *Dpn*I and *Mbo*I lacked restriction sites both on *cry*1ky5 and *cry*1em11.

The frequent cutter *Alu*I had six sites followed by *Hae*III with five restriction sites in *cry*4em10 (Plate 18N). There were no restriction sites for the enzymes *Bam*HI and *Dpn*I. It had single restriction site for *Btg*I, *Bsa*HI, *Mbo*I and *Sac*II. In case of *cry*4ky1, *Alu*I had five sites. There were no restriction sites for the enzymes *Bsa*HI, *Mbo*I, *Bam* HI and *Dpn*I. All the other enzymes had one restriction site each on *cry*4ky1 (Plate 19J).

The sequence cry1Aky3 was found to possess two restriction sites each for *Alu*I and *Mbo*I, whereas there were no sites for *Btg*I, *Bsa*HI, and *Sac*II (Plate 20J). Details of the restriction analysis of the sequences are given in Table 19.

Table 19 A. Theoretical restriction analysis of *cry*1ky5

Sl. No.	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG'CT	1	123	23, 121
2	*Bam* HI	G'GATC_C	0	-	-
3	*Btg* I	C'CryG_G	2	34, 40	34, 106
4	*Bsa* HI	Gr'CG_yC	1	14	14, 132
5	*Dpn* I	GA'TC	0	-	-
6	*Hae* III	GG'CC	3	10, 30, 39	9, 10, 20, 107
7	*Hinf* I	G'AnT_C	3	46, 53, 131	15, 46, 78
8	*Mbo* I	'GATC_	0	-	-
9	*Msp* A1I	CmG'CkG	1	42	42, 104
10	*Sac* II	CC_GC'GG	1	43	43, 103

Table 19 B. Theoretical restriction analysis of *cry*1em11

Sl. No	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG'CT	0	-	-
2	*Bam* HI	G'GATC_C	0	-	-
3	*Btg* I	C'CryG_G	1	41	41, 106
4	*Bsa* HI	Gr'CG_yC	1	15	15, 132
5	*Dpn* I	GA'TC	0	-	-
6	*Hae* III	GG'CC	2	11, 31	11, 20, 116
7	*Hinf* I	G'AnT_C	2	47, 54	7, 47, 93
8	*Mbo* I	'GATC_	0	-	-
9	*Msp* A1I	CmG'CkG	1	43	43, 104
10	*Sac* II	CC_GC'GG	1	44	44, 103

Table 19 C. Theoretical restriction analysis of *cry*4em10

Sl. No	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG'CT	6	215, 236, 326, 467, 488, 511	21, 23, 90, 141, 194, 215
2	*Bam* HI	G'GATC_C	0	-	-
3	*Btg* I	C'CryG_G	1	41`	41, 664
4	*Bsa* HI	Gr'CG_yC	1	15	15, 690
5	*Dpn* I	GA'TC	0	-	-
6	*Hae* III	GG'CC	5	11, 40, 637, 648, 702	3, 11, 11, 29, 54, 597
7	*Hinf* I	G'AnT_C	2	409, 589	116, 180, 409
8	*Mbo* I	'GATC_	1	446	123, 582
9	*Msp* A1I	CmG'CkG	2	43, 326	43, 283, 379
10	*Sac* II	CC_GC'GG	1	44	44, 661

Table 19 D. Theoretical restriction analysis of *cry*4ky1

Sl. No	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG'CT	5	20, 112, 136, 157, 298	20, 21, 24, 92, 141, 157
2	*Bam* HI	G'GATC_C	0	-	-
3	*Btg* I	C'CryG_G	1	391	64, 391
4	*Bsa* HI	Gr'CG_yC	0	-	-
5	*Dpn* I	GA'TC	0	-	-
6	*Hae* III	GG'CC	1	390	65, 390
7	*Hinf* I	G'AnT_C	1	212	212, 243
8	*Mbo* I	'GATC_	0	-	-
9	*Msp* A1I	CmG'CkG	1	298	157, 298
10	*Sac* II	CC_GC'GG	1	22	22, 443

Table 19 E. Theoretical restriction analysis of *cry*1Aky3

Sl. No	Restriction enzyme	Recognition sequence	No. of cut(s)	Position of restriction sites	Fragment sizes (bp)
1	*Alu* I	AG'CT	2	249, 360	11, 199, 249
2	*Bam* HI	G'GATC_C	1	242	242, 317
3	*Btg* I	C'CryG_G	0	-	-
4	*Bsa* HI	Gr'CG_yC	0	-	-
5	*Dpn* I	GA'TC	1	244	244, 315
6	*Hae* III	GG'CC	1	533	26, 533
7	*Hinf* I	G'AnT_C	1	74	74, 485
8	*Mbo* I	'GATC_	2	242	242, 317
9	*Msp* A1I	CmG'CkG	1	360	199, 360
10	*Sac* II	CC_GC'GG	0	-	-

Gene prediction analysis of the sequence using the tool Genscan (www.genes.mi.edu/genscan/) revealed that *cry*1ky1 and *cry*1em11 had no exons. *cry*4em10 had an initial exon with a length of 456 bp, starting from base 14 to 496 (Plate 18I). *cry*4ky1 possessed a terminal exon having a length of 224 bp (Plate 19F). The terminal exon of *cry*1Aky3 had a length of 440 bp, located from the region of base 87 to 552 (Plate 20F).

4.9.2 Amino acid sequence analysis

The proportion of each amino acid in cloned sequences were calculated using 'AASTAT' tool (http://seqtool.sdsc.edu/). Both *cry*1ky5 and *cry*1em11 had aminoacid serine with highest molar per cent of 18.18 and 12.50 respectively. This was followed by glycine (13.14%) cry1ky5 and phenylalanine (10.24%) in *cry*1em11. The amino acids glutamine, glutamic acid, valine, methionine, leucine and tyrosine residues were absent in *cry*1ky5. Glutamine was absent in both *cry*1ky5 and *cry*1em11.

*cry*4em10 had amino acid alanine with the highest content (8.55%), closely followed by glycine. Histidine content was least (0.43%) in *cry*4em10. The *cry*4ky1 fragment had leucine with the highest share of 16.90 per cent. Aspartic acid residue was absent. In *cry*1Aky3, the aminoacid with highest molar percentage was lysine (10.27%) followed leucine and isoleucine (9.19%). The details of aminoacid composition of the sequences are given in Table 20.

The 'Motif Scan' of aminoacid sequence of *cry*4em10 revealed similarity with tyrosine kinase region. The functional domains of *cry*4em10 sequence were located using 'InterProScan' (www.ebi.ac.uk/InterProScan). The sequence had anaphylatoxin/fibulin, thiolase, ferredoxin- iron sulphur binding sites and EGF like region. Results are displayed in Plate 18M

Table 20. Amino acid composition of different *cry* protein sequences

Amino acid			Molar percentage of amino acids (Mol%)				
			*cry*1ky5	*cry*1em11	*cry*4em10	*cry*4ky1	*cry*1Aky3
Non polar		Gly	13.64	4.17	8.12	3.52	5.95
		Ala	4.55	4.17	8.55	2.11	4.86
		Val	0.00	2.08	4.70	4.23	5.14
		Leu	0.00	4.17	6.84	16.9	9.19
		Ile	4.55	6.25	5.13	4.23	9.19
		Met	0.00	4.17	2.99	2.11	2.70
		Pro	4.55	4.17	5.98	2.82	5.95
		Phe	2.27	10.42	3.42	4.23	2.70
		Trp	4.55	2.08	1.71	2.11	0.54
Polar	Uncharged	Ser	18.18	12.50	6.84	9.15	5.14
		Thr	9.09	4.17	7.69	9.15	7.03
		Cys	6.82	2.08	2.99	7.04	0.54
		Tyr	0.00	2.08	3.88	7.04	4.32
		Asn	2.27	6.25	6.14	3.52	3.24
		Gln	0.00	0.00	2.14	2.82	2.16
	Basic	Lys	6.82	2.08	6.84	3.52	10.27
		Arg	9.09	8.33	5.98	5.63	3.24
		His	9.09	8.33	0.43	4.93	2.70
	Acidic	Asp	4.55	6.25	5.98	0.00	5.41
		Glu	0.00	6.25	3.42	4.93	8.11

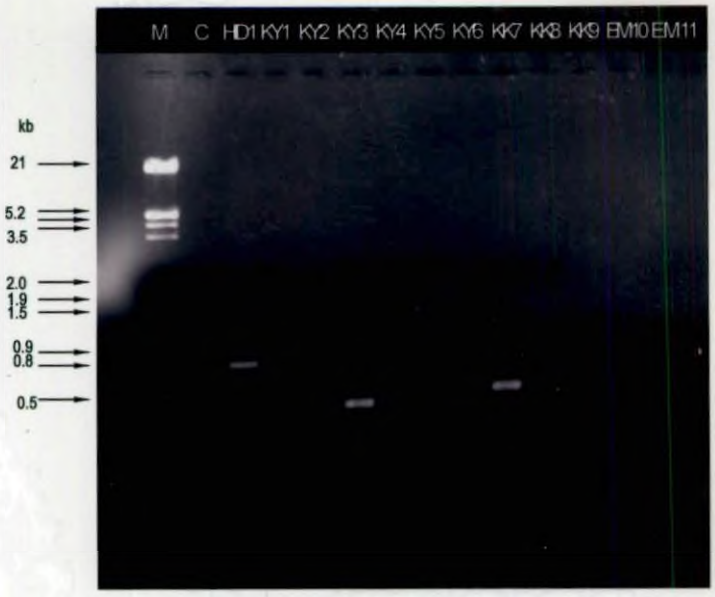
The secondary structure prediction of proteins was done using SOPMA programme provided by Expasy tools. The proportion of each structure type was found out. The sequences *cry*1ky5 and *cry*1em11were comparatively richer in random coils (43.75%, 64.58 %) and alpha helices (41.67%, 14.58%). Results are displayed in Plates 16H and 16I. The share of extended strands came to about 14.58 percent in *cry*1ky5 and 18.75 per cent in *cry*1em11. The beta turns were completely absent in *cry*1ky5, whereas it contributed to 2.08 per cent in *cry*1em11 (Plates 17H and 17I).

Analysis of secondary structure of *cry*4em10 revealed the share of random coils as high (41.03%), followed by extended strands (27.35%) and α-helix (24.79%). Alpha helices contributed maximum (33.80%), closely followed by random coils and extended strands (31.69% and 24.65 % respectively) in *cry*4ky1(Plates 19G and 19H). The β-turns contributed to 6.84 and 9.86 per cent in *cry*4em10 and *cry*4ky1. Secondary structure analysis of *cry*1Aky3 showed that α-helix contributed to 32.79 per cent, closely followed by random coils accounting to 32.43 per cent. The proportion of extended strands and β-turns were 23.24 and 11.35 per cent respectively. Results are displayed in Plates 20G and 20H.

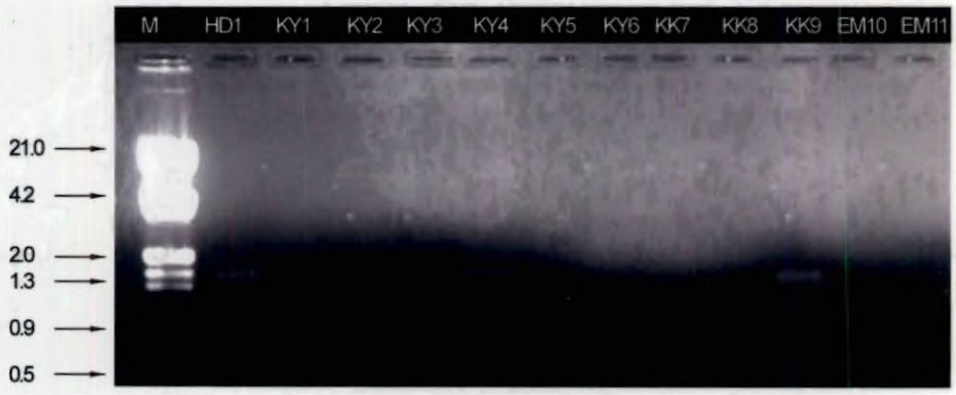
Hydropathy plot of the sequences was constructed by means of Kyte Doolittle Hydropathy Profile. All the sequences were, in general, rich in hydrophilic aminoacids, but *cry*4ky1 and *cry*1Aky3 contained both hydrophilic and hydrophobic aminoacids. The sequences were analyzed for the presence of transmembrane regions also. All the sequences were devoid of transmembrane regions. Results are displayed (Plates 16J, 17J, 18K, 19I and 20I).

4.10 AMPLIFICATION OF VARIABLE REGION OF *cry*1 GENE

In order to amplify *cry* gene having fragment size of 1500 to 1600 bp, PCR was carried out using *cry*1 primers designed by Jaurez-Perez *et al.* (1997). Amplification was obtained for isolates KY4 and KK9 (Plate 15B). Single band of 1545 bp was obtained for isolate KY4 and a band of 1532 bp was obtained for isolate KK9.



A) *cry*1A



B) *cry*1 variable region

Plate 15. Amplification of *cry*1A and *cry*1 variable region of *B.thuringiensis* isolates

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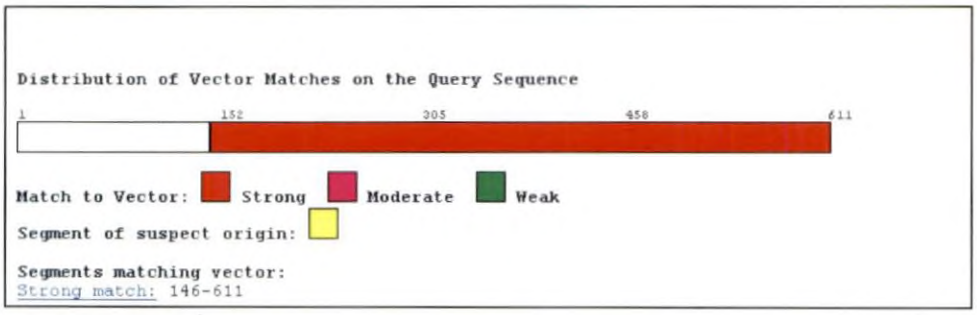
AATCGTTTTGCTGGAAAACATGTCATTCAAGTGCCGGTAGCTGATGGGATTCGAAAAACGGAAAAGCTTCTTAATCGAGCTGT
CAAACAAGATGCACCAATATTTAAAGGTGTAAAAGAAAATGGGAAGCAGAAGTGTCACAAAATCACTAGTGCGGCCGCCTG
CAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTA
ATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTA
AAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGA
CTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGG
ATAACGCAGGAAAGACATGTGAGCAAAAGG

A) Nucleotide sequence

Query= cry1ky5
Length=691



B) VecScreen output

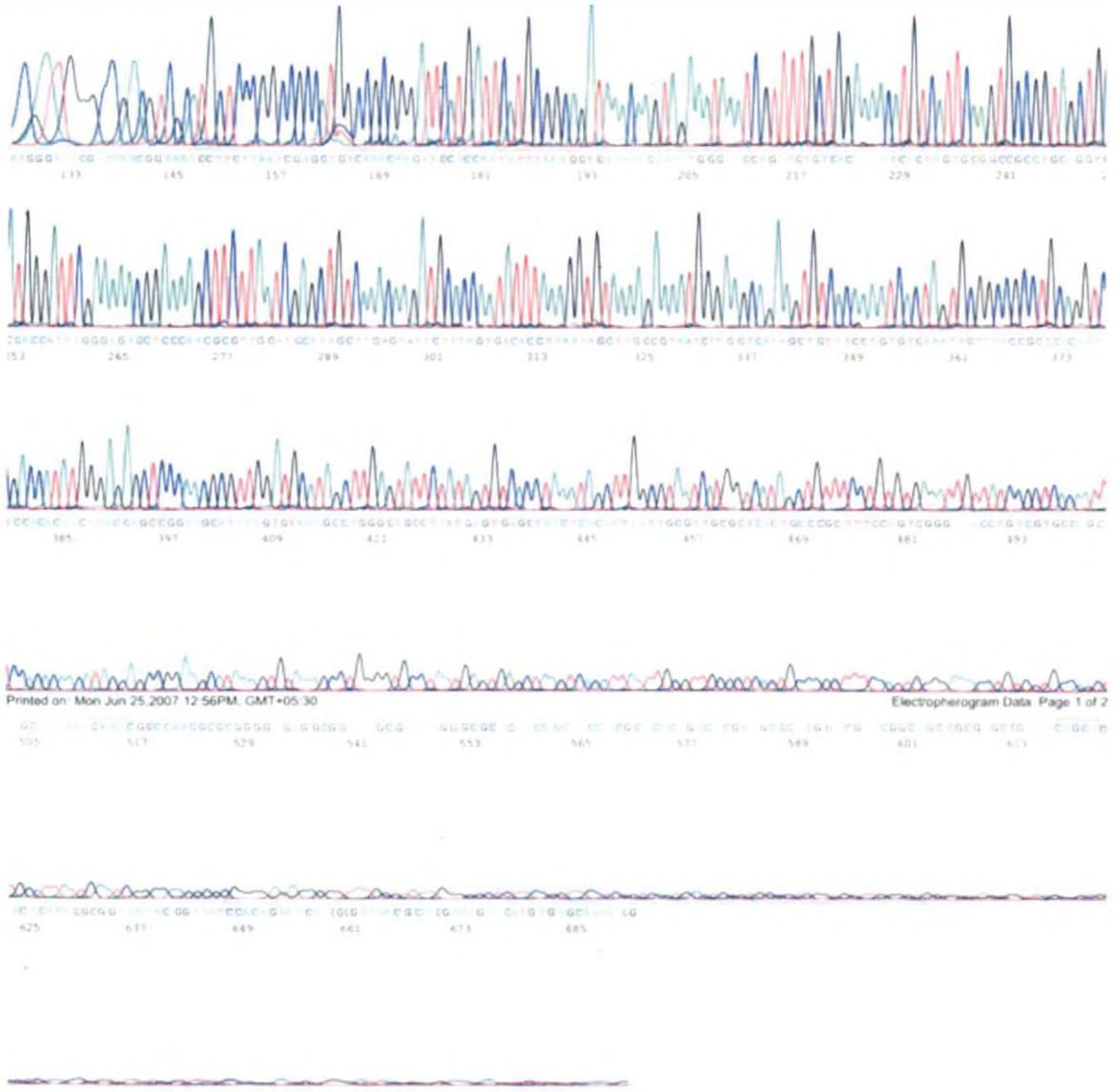
```
I  T  I  G  P  T  S  H  A  P  G  R  H  G  R  G  I  H  D  S  C
1ACGATTGGGCCGACGTCGCATGCTCCCGGCCGCATGGCCGCGGGATTCATGATTCATGC

21 G  R  -  T  S  R  N  K  S  F  C  W  K  T  C  H  S  S  A  G
61GGCAGATAAACAAGTAGAAATAAATCGTTTTGCTGGAAAACATGTCATTCAAGTGCCGGT

41 S  -  W  D  S  K  N  G
121 AGCTGATGGGATTCGAAAAACGAAA
```

C) Nucleotide and deduced aminoacid sequence after deleting vector sequence

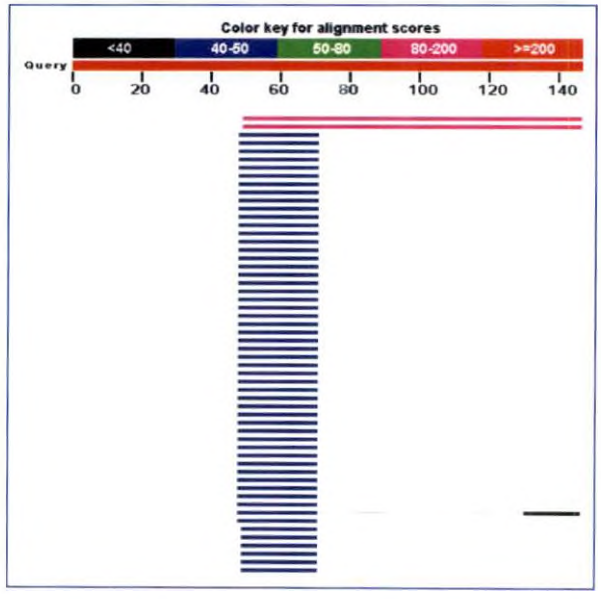
Plate 16. Sequence analysis of *cry*1ky5



D). Graphical output

Plate 16 . Contd.

Distribution of 123 Blast Hits on the Query Sequence

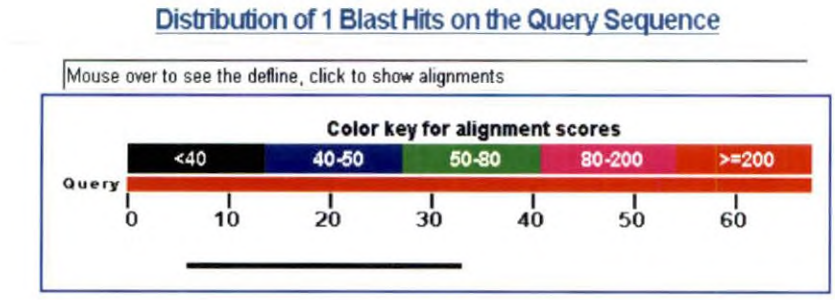


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
CP000485.1	Bacillus thuringiensis str. Al Hakam, complete genome	131	448	66%	6e-31	100%	
AE017355.1	Bacillus thuringiensis serovar konkukian str. 97-27, complete genome	131	423	66%	6e-31	100%	
AY925090.2	Bacillus thuringiensis serovar kenyae strain HD-549 Cry1Ac (cry1Ac)	42.8	42.8	15%	2e-04	100%	
DQ023297.1	Bacillus thuringiensis isolate SK-222 Cry (cry) gene, complete cds	42.8	42.8	15%	2e-04	100%	
DQ023296.1	Bacillus thuringiensis isolate SK-729 Cry (cry) gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF541112.1	Bacillus thuringiensis plasmid S907 Cry1B gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF432793.1	Bacillus thuringiensis strain sfw-12 insecticidal protein Cry1BaS (cry1E	42.8	42.8	15%	2e-04	100%	
EF102874.1	Bacillus thuringiensis strain S601 plasmid Cry1B gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF094884.1	Bacillus thuringiensis 146-158-01 crystal protein (cry1Ac) gene, comp	42.8	42.8	15%	2e-04	100%	
DQ285666.1	Bacillus thuringiensis delta-endocytoxin (cry1Ac) gene, complete cds	42.8	42.8	15%	2e-04	100%	
DQ438941.1	Bacillus thuringiensis strain INTA TA24-6 insecticidal crystal protein (C	42.8	42.8	15%	2e-04	100%	
AF492767.1	Bacillus thuringiensis Cry1Ac gene, complete cds	42.8	42.8	15%	2e-04	100%	
AF368257.1	Bacillus thuringiensis plasmid encoded Cry1Ba (cry1Ba) gene, comple	42.8	42.8	15%	2e-04	100%	
AY319967.1	Bacillus thuringiensis cry1A toxin gene, complete cds	42.8	42.8	15%	2e-04	100%	
AF384211.1	Bacillus thuringiensis strain ly30 delta-endotoxin (cry1Aa) gene, comp	42.8	42.8	15%	2e-04	100%	
AY122057.1	Bacillus thuringiensis insecticidal crystal protein Cry1Ac (cry1Ac) gene	42.8	42.8	15%	2e-04	100%	
DQ195217.1	Bacillus thuringiensis serovar kurstaki strain C-33 insecticidal crystal	42.8	42.8	15%	2e-04	100%	
DQ062690.1	Bacillus thuringiensis strain INTA Mo1-12 Cry1Aa gene, partial cds	42.8	42.8	15%	2e-04	100%	
DQ062689.1	Bacillus thuringiensis strain INTA Mo1-12 Cry1Ac gene, partial cds	42.8	42.8	15%	2e-04	100%	
Z22513.1	B.thuringiensis encoding crystal protein	42.8	42.8	15%	2e-04	100%	
Z22511.1	B.thuringiensis encoding crystal protein	42.8	42.8	15%	2e-04	100%	
Y09663.1	B.thuringiensis mRNA for delta-endotoxin	42.8	42.8	15%	2e-04	100%	
X95704.1	B.thuringiensis cry1Ba2 gene	42.8	42.8	15%	2e-04	100%	
X13535.1	Bacillus thuringiensis cryA gene for parasporal crystal toxin	42.8	42.8	15%	2e-04	100%	
X06711.1	B. thuringiensis cryA4 gene for delta-endotoxin	42.8	42.8	15%	2e-04	100%	
AJ130970.1	Bacillus thuringiensis gene encoding crystal toxin protein	42.8	42.8	15%	2e-04	100%	
AJ002514.1	Bacillus thuringiensis kurstaki cry218 gene	42.8	42.8	15%	2e-04	100%	
AF363025.1	Bacillus thuringiensis subsp. entomocidus delta-endotoxin Cry1Ba2 (c	42.8	42.8	15%	2e-04	100%	
AF358862.1	Bacillus thuringiensis cry1Db operon, partial sequence	42.8	83.7	15%	2e-04	100%	
AF327927.1	Bacillus thuringiensis subsp. kunthalanags3 insecticidal crystal protein	42.8	42.8	15%	2e-04	100%	
AY225453.1	Bacillus thuringiensis Cry1Ac (cry1Ac) gene, complete cds	42.8	42.8	15%	2e-04	100%	
D00348.1	Bacillus thuringiensis serovar aizawai gene, complete cds	42.8	42.8	15%	2e-04	100%	
AY730621.1	Bacillus thuringiensis plasmid Cry1Ac (cry1Ac) gene, complete cds	42.8	42.8	15%	2e-04	100%	
AF081790.1	Bacillus thuringiensis insecticidal crystal protein (cry1) gene, complete	42.8	42.8	15%	2e-04	100%	
AF081248.1	Bacillus thuringiensis plasmid-encoded lepidoteran-specific toxin (cry1	42.8	42.8	15%	2e-04	100%	
U87397.1	Bacillus thuringiensis kurstaki crystal protein (CryIA(c)) gene, comple	42.8	42.8	15%	2e-04	100%	
AF077326.1	Bacillus thuringiensis Cry1Be1 delta-endotoxin gene, complete cds	42.8	42.8	15%	2e-04	100%	
U89872.1	Bacillus thuringiensis Cry1Ac delta-endotoxin gene, complete cds	42.8	42.8	15%	2e-04	100%	
U87793.1	Bacillus thuringiensis kurstaki insecticidal delta-endotoxin CryIA(c) (c	42.8	42.8	15%	2e-04	100%	
AY176063.1	Bacillus thuringiensis insecticidal crystal protein gene, complete cds	42.8	42.8	15%	2e-04	100%	
U35780.1	Bacillus thuringiensis crystal toxin gene, complete cds	42.8	42.8	15%	2e-04	100%	

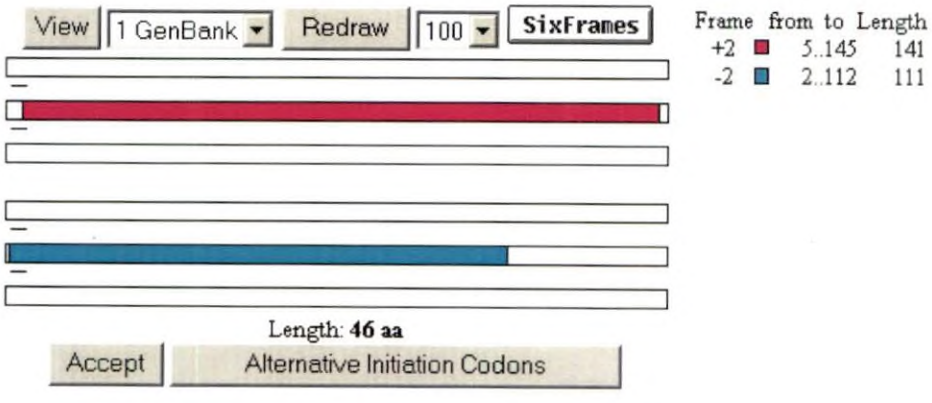
E) Blastn output

Plate 16.Contd...



ng significant alignments:	Score (Bits)	E Value
, Delta-Endotoxin Cryiiia (Bt13)	14.6	6.1

F) Blastp output



G) Open reading frame

Plate 16. Contd...

```
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          |          |          |          |
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ccccccchhhhhhhhhhhhccchhhhhhhhccceeeeccccccc

Sequence length :    48

GOR4 :
   Alpha helix      (Hh) :    20 is  41.67%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     7 is  14.58%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    21 is  43.75%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
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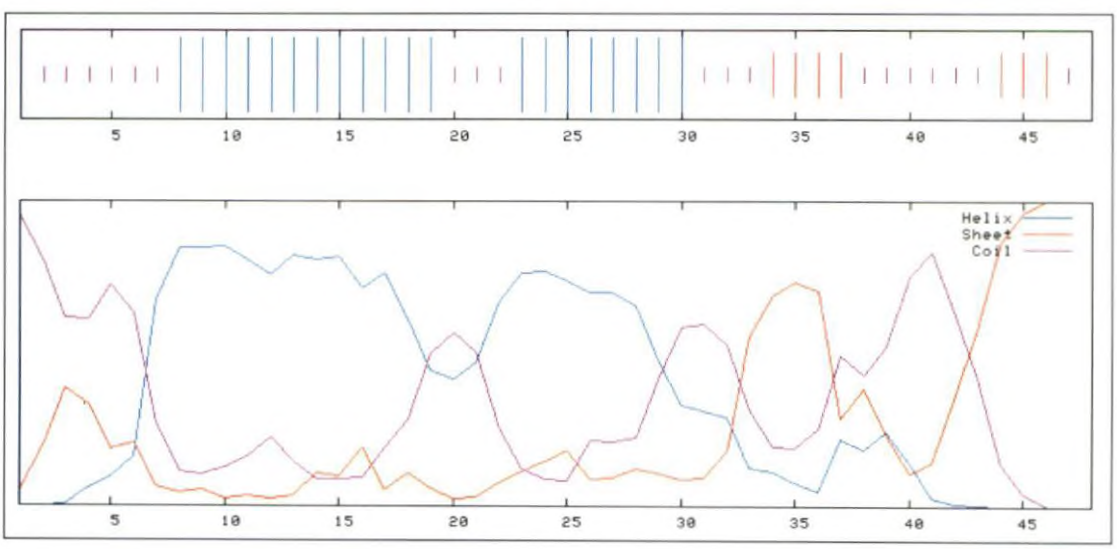
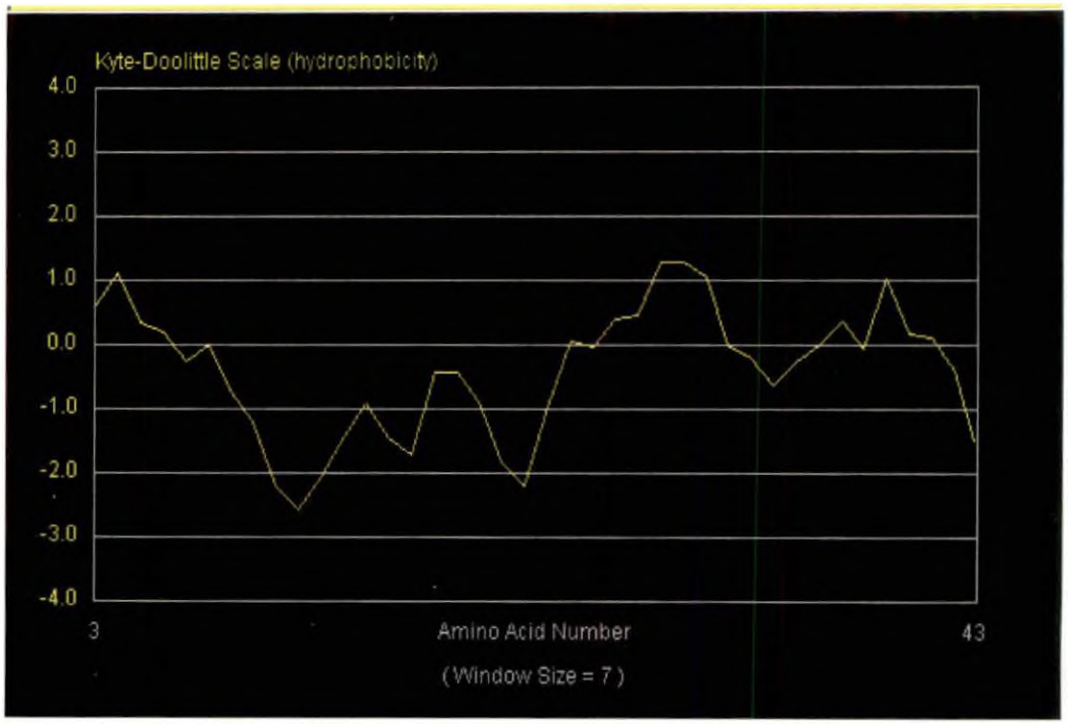
H) Predicted secondary structure

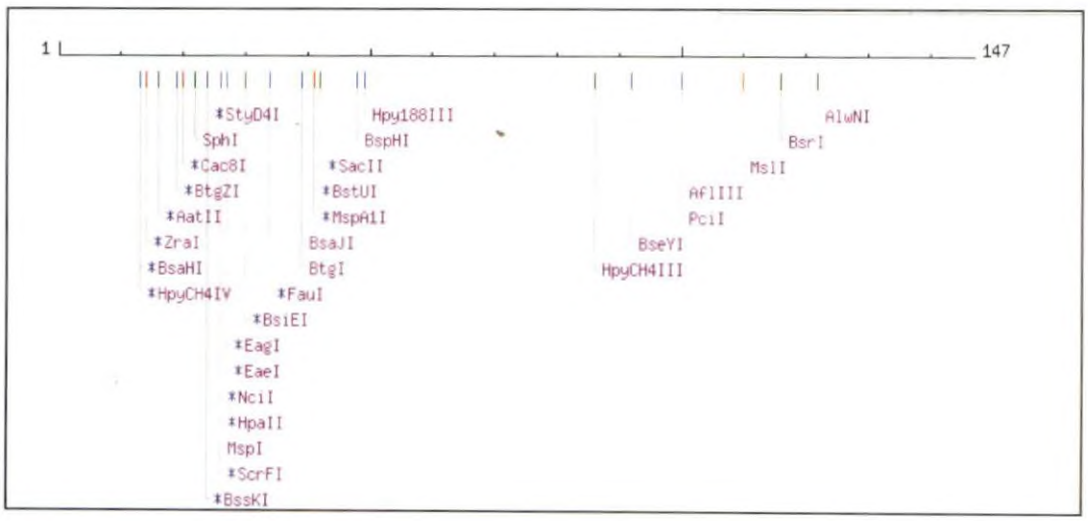


I) Graphical representation of secondary structure

Plate 16. Contd...



J) Kyte Doolittle Hydropathy Plot for deduced proteins



K) Restriction map

Plate 16. Contd...

ACGATTGAGGCCGACGTCGCATGCTCCCGGCCGCCATCGCCGCGGGATTCATGATTCATGCGGCAGATAAACAAGTAGAAAT
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GTAAAACAGGATGCGCCAATCTTTAAAGGAATAAAGGAAAATGGGAAGCAGAAGTGTCACAAAATCACTAGTGCGGCCGCC
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TAAAGCCTGGGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTTCCAGTCGGGAAAACC
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A) *cry*1em11

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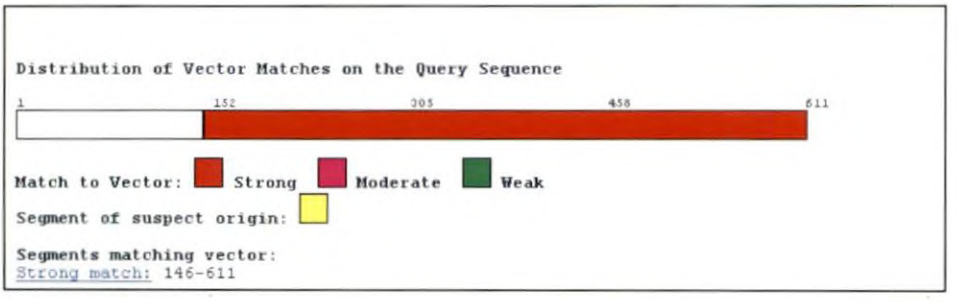

B) VecScreen output

1 X X D - G R R R M L P A A I A A G F M I
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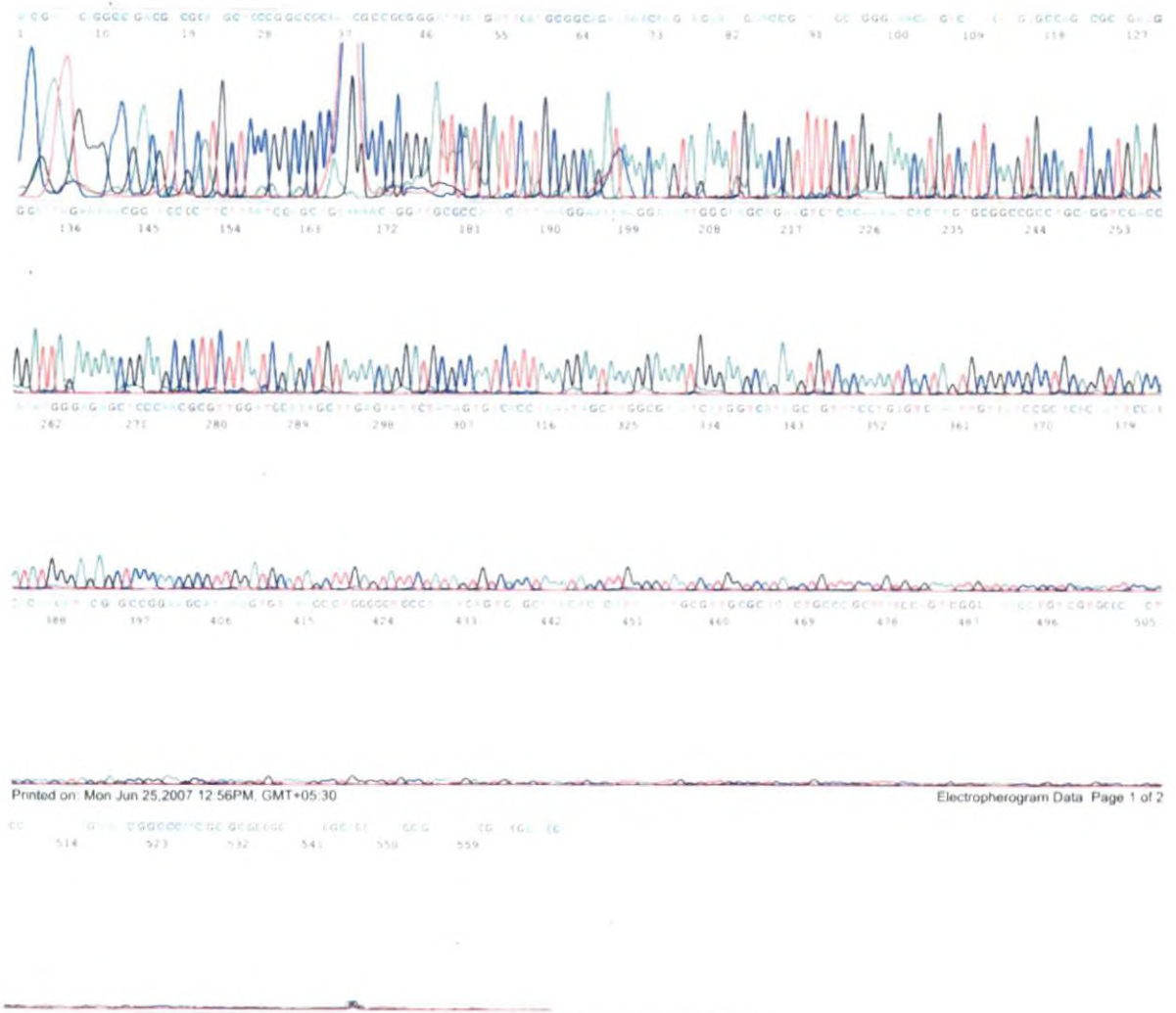
21 H A A D K Q V E M N R F A G K H V I Q V
61CATGCGGCAGATAAACAAGTAGAAATGAACCGTTTTGCTGGGAAACATGTCATTCAAGTG

41 P V A D G I R K T E
121 CCAGTCGCTGATGGGATTAGAAAAACGGAAG

C) Nucleotide and deduced aminoacid sequence after deleting vector sequence

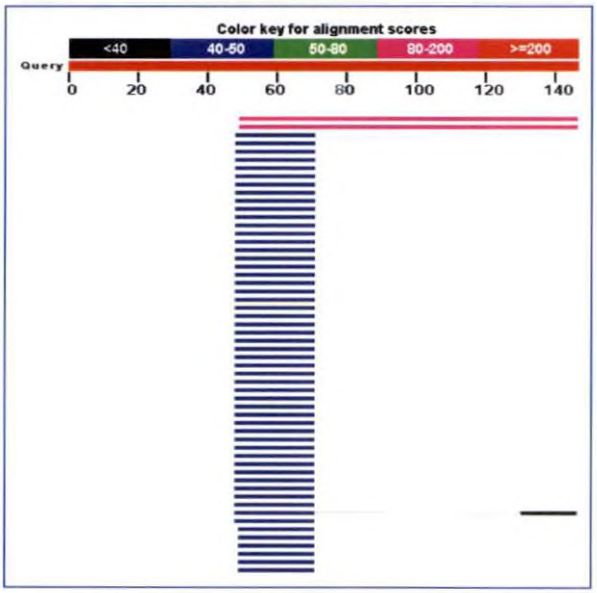
Plate 17. Sequence analysis of *cry*1em11



D). Graphical output

Plate 17 . Contd.

Distribution of 111 Blast Hits on the Query Sequence

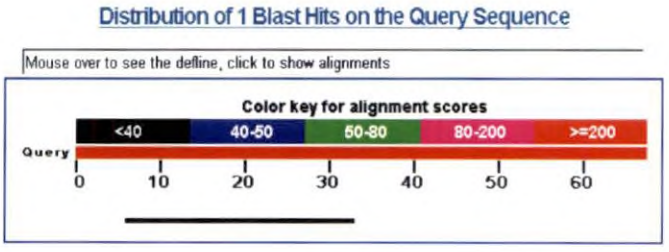


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
CP000485.1	Bacillus thuringiensis str. Al Hakam, complete genome	141	257	65%	3e-34	92%	
AE017355.1	Bacillus thuringiensis serovar konkukian str. 97-27, complete genome	141	313	65%	3e-34	100%	
AY925090.2	Bacillus thuringiensis serovar kenyae strain HD-549 Cry1Ac (cry1Ac)	42.8	42.8	15%	2e-04	100%	
DQ023297.1	Bacillus thuringiensis isolate SK-222 Cry (cry) gene, complete cds	42.8	42.8	15%	2e-04	100%	
DQ023296.1	Bacillus thuringiensis isolate SK-729 Cry (cry) gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF541112.1	Bacillus thuringiensis plasmid S907 Cry1B gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF432793.1	Bacillus thuringiensis strain sfw-12 insecticidal protein Cry1Ba5 (cry1E	42.8	42.8	15%	2e-04	100%	
EF102874.1	Bacillus thuringiensis strain S601 plasmid Cry1B gene, complete cds	42.8	42.8	15%	2e-04	100%	
EF094884.1	Bacillus thuringiensis 146-158-01 crystal protein (cry1Ac) gene, comp	42.8	42.8	15%	2e-04	100%	
DQ285666.1	Bacillus thuringiensis delta-endocytoxin (cry1Ac) gene, complete cds	42.8	42.8	15%	2e-04	100%	
DQ438941.1	Bacillus thuringiensis strain INTA TA24-6 insecticidal crystal protein (C	42.8	42.8	15%	2e-04	100%	
AF492767.1	Bacillus thuringiensis Cry1Ac gene, complete cds	42.8	42.8	15%	2e-04	100%	
AF368257.1	Bacillus thuringiensis plasmid encoded Cry1Ba (cry1Ba) gene, comple	42.8	42.8	15%	2e-04	100%	
AY319967.1	Bacillus thuringiensis cry1A toxin gene, complete cds	42.8	42.8	15%	2e-04	100%	
AF384211.1	Bacillus thuringiensis strain ly30 delta-endotoxin (cry1Aa) gene, comp	42.8	42.8	15%	2e-04	100%	
AY122057.1	Bacillus thuringiensis insecticidal crystal protein Cry1Ac (cry1Ac) gene	42.8	42.8	15%	2e-04	100%	
DQ195217.1	Bacillus thuringiensis serovar kurstaki strain C-33 insecticidal crystal	42.8	42.8	15%	2e-04	100%	
DQ062690.1	Bacillus thuringiensis strain INTA Mo1-12 Cry1Aa gene, partial cds	42.8	42.8	15%	2e-04	100%	
DQ062689.1	Bacillus thuringiensis strain INTA Mo1-12 Cry1Ac gene, partial cds	42.8	42.8	15%	2e-04	100%	
Z22513.1	B.thuringiensis encoding crystal protein	42.8	42.8	15%	2e-04	100%	
Z22511.1	B.thuringiensis encoding crystal protein	42.8	42.8	15%	2e-04	100%	
Y09663.1	B.thuringiensis mRNA for delta-endotoxin	42.8	42.8	15%	2e-04	100%	
X95704.1	B.thuringiensis cry1Ba2 gene	42.8	42.8	15%	2e-04	100%	
X13535.1	Bacillus thuringiensis cryA gene for parasporal crystal toxin	42.8	42.8	15%	2e-04	100%	
X06711.1	B. thuringiensis cryA4 gene for delta-endotoxin	42.8	42.8	15%	2e-04	100%	
AJ130970.1	Bacillus thuringiensis gene encoding crystal toxin protein	42.8	42.8	15%	2e-04	100%	
AJ002514.1	Bacillus thuringiensis kurstaki cry218 gene	42.8	42.8	15%	2e-04	100%	
AF363025.1	Bacillus thuringiensis subsp. entomocidus delta-endotoxin Cry1Ba2 (c	42.8	42.8	15%	2e-04	100%	
AF358862.1	Bacillus thuringiensis cry1Db operon, partial sequence	42.8	83.7	15%	2e-04	100%	
AF327927.1	Bacillus thuringiensis subsp. kunthalanags3 insecticidal crystal protein	42.8	42.8	15%	2e-04	100%	

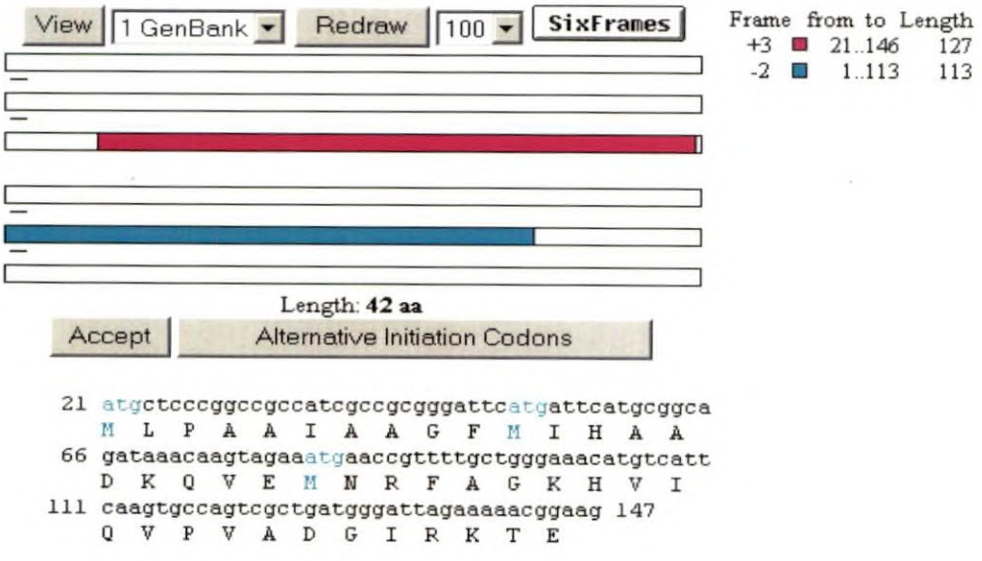
E) Blastn output

Plate 17. Contd...



ng significant alignments:	Score (Bits)	E Value	
, Delta-Endotoxin Cryiiia (Bt13)	14.6	6.1	S

F) Blastp output



G) Open reading frame

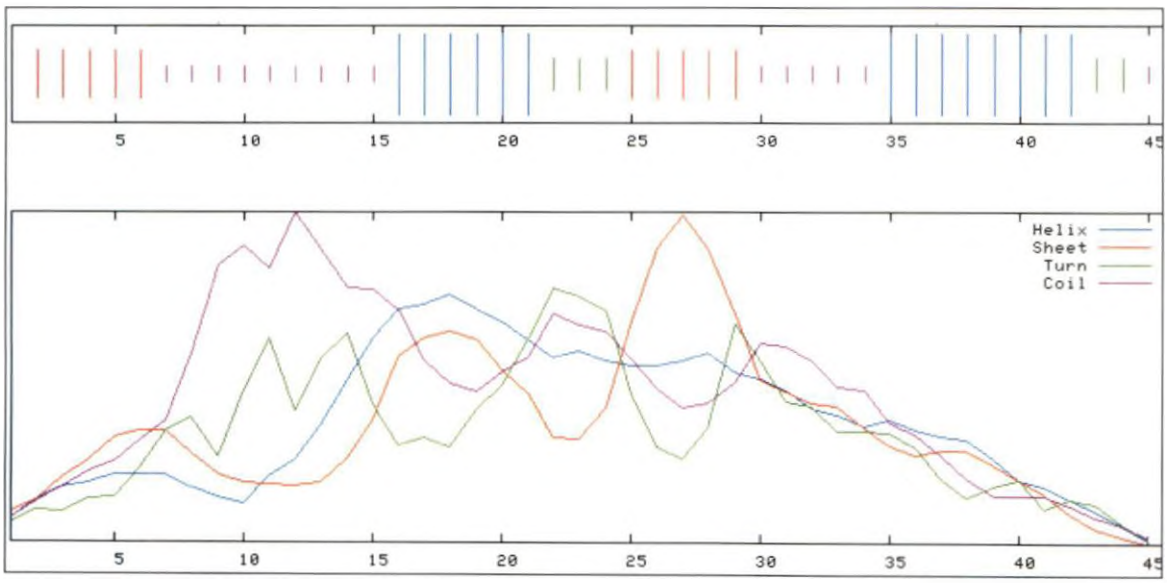
Plate 17. Contd...

```
        10         20         30         40
         |          |          |          |
TIEADVACSRPPSPRDSFMRQINKKTVLLGNMSFKCQSLMGLEKRK
eeeeeeeccccccccchhhhhhttteeeeeccccchhhhhhhttcc

Sequence length :    46

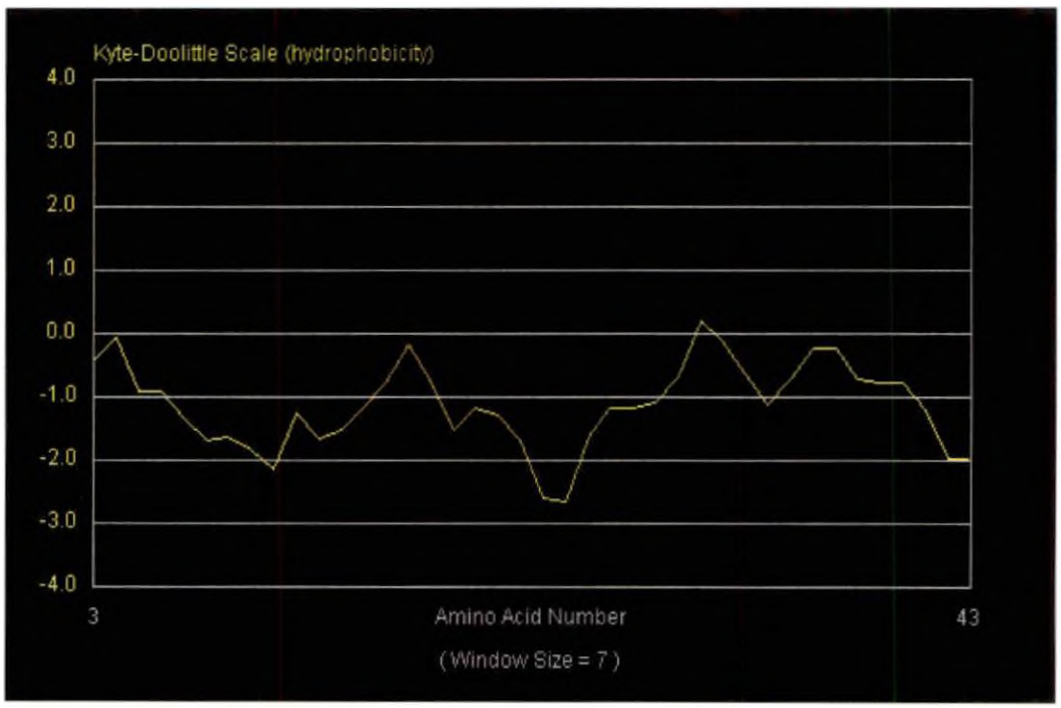
SOPMA :
   Alpha helix      (Hh) :    14 is  30.43%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    12 is  26.09%
   Beta turn        (Tt) :     5 is  10.87%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    15 is  32.61%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

H) Predicted secondary structure

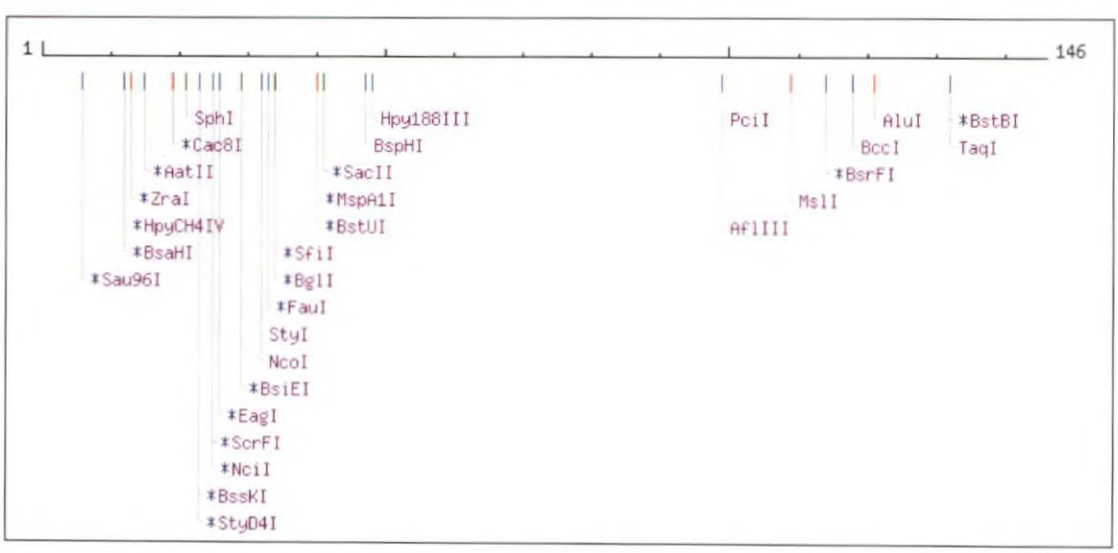


I) Graphical representation of secondary structure

Plate 17. Contd...



J) Kyte Doolittle Hydropathy Plot for deduced proteins



K) Restriction map

Plate 17. Contd...

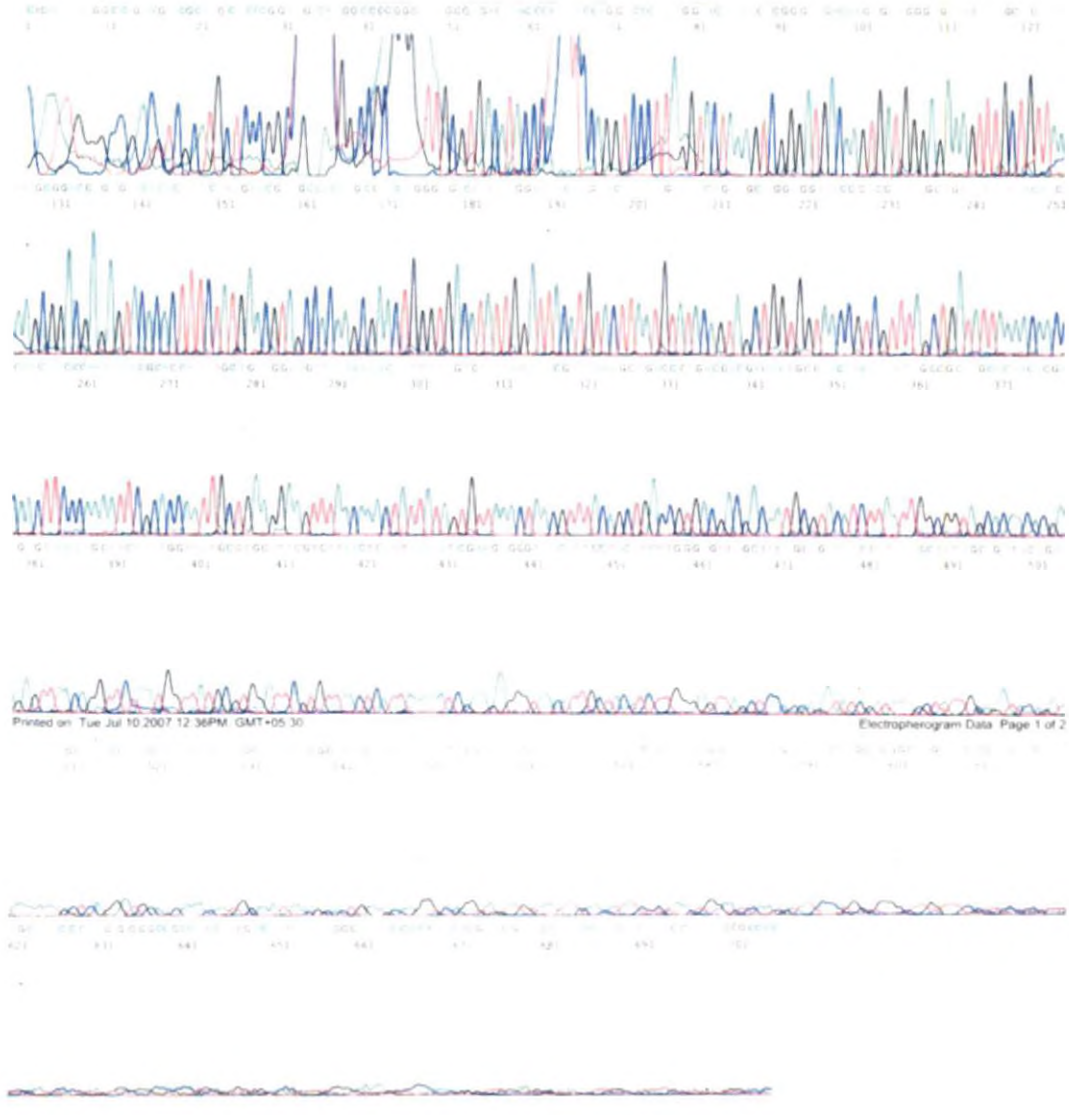
No significant similarity found.

A) VecScreen output

```
   R  N  K  A  D  V  A  C  S  R  Q  S  R  P  R  D  C  V  T  Y
1 CGGAATAAGGCCGACGTCGCATGCTCCCGGCAGTCAAGGCCGCGGGATTGCGTGACATAC
  21  P  F  P  G  P  L  G  T  K  V  G  M  T  S  D  G  R  K  F  A
61 CCATTTCCAGGTCCCTTAGGTACTAAAGTCGGGATGACAAGTGATGGGAGAAAGTTTGCT
  41  V  K  S  G  R  E  Y  T  L  S  M  N  V  A  T  S  E  L  G  D
121GTTAAAAGCGGACGAGAGTACACACTTTCTATGAACGTAGCCACAAGCGAACTGGGTGAC
  61  I  L  D  Y  M  Y  L  M  Y  T  V  A  G  G  N  R  R  L  A  D
121 ATATTGGATTACATGTACTTAATGTATACAGTAGCTGGTGGTAACCGTCGTTTAGCTGAT
  81  I  K  T  T  N  F  P  K  F  A  P  I  A  E  G  S  L  T  N  Y
241 ATTAAAACAACCAACTTCCCAAAATTCGCACCAATTGCTGAAGGAAGTTTAACAAACTAT
 101  Y  S  V  K  L  T  F  T  A  D  R  D  D  N  A  Y  I  L  I
301 TATAGTGTTAAACTAACGTTTACAGCTGACCGTGACGACAATGCGTACATCTTAATT
 121  G  G  S  T  T  R  E  L  T  G  S  N  G  Y  A  W  I  R  V  N
361GGCGGTAGCACAACACGAGAGTTAACAGGTTCTAATGGTTATGCGTGGATTCGTGTTAAC
 141  S  L  K  I  E  E  G  N  I  A  T  S  W  E  L  A  P  A  D  I
421  TCATTAAAAATCGAAGAGGGTAACATTGCTACATCTTGGGAGTTAGCTCCAGCAGATATC
 161  D  K  A  I  A  D  T  D  K  K  L  R  M  H  K  Q  C  N  N  A
481GATAAAGCTATTGCAGATACAGATAAAAAGCTCAGGATGCACAAACAATGCAATAACGCA
 181  L  N  K  V  R  -  C  N  S  T  F  S  N  W  I  K  I  P  C  W
541 CTGAACAAGGTCAGGTAGTGTAATTCAACATTCTCTAACTGGATCAAGATTCCCTGCTGG
 201  A  C  F  A  T  S  M  Q  S  L  V  G  P  P  A  G  Q  Q  M  G
601GCTTGTTTCGCTACATCAATGCAATCCCTAGTGGGGCCGCCTGCAGGCCAACAAATGGGA
 221  K  P  P  R  V  G  C  N  L  E  F  P  K  G  P
661 AAACCCCCACGCGTTGGATGCAACCTTGAATTTCCAAAGGGCCCC
```

B) Nucleotide and deduced aminoacid sequence

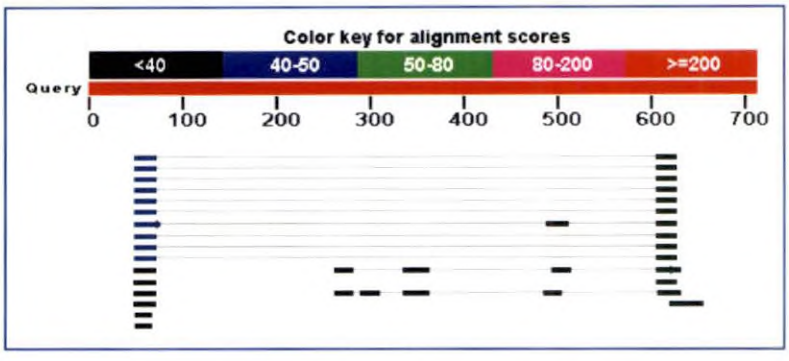
Plate 18. Sequence analysis of *cry*4em10



C). Graphical output

Plate 18 . Contd.

Distribution of 43 Blast Hits on the Query Sequence

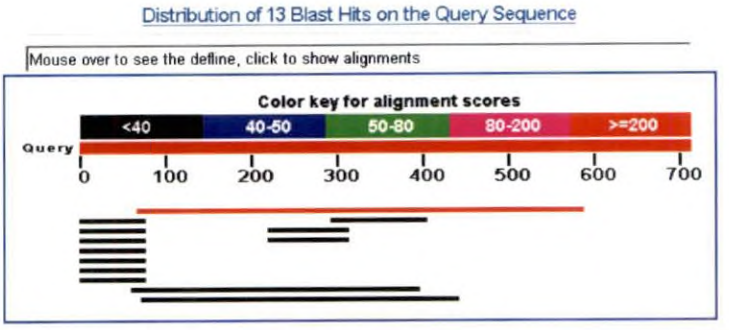


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
EF208904.1	Bacillus thuringiensis Cry4A (cry4A) gene, complete cds	46.4	81.9	6%	1e-04	100%	
AY729887.1	Bacillus thuringiensis serovar israelensis delta-endotoxin (cry4BLB) g	46.4	81.9	6%	1e-04	100%	
AY847707.1	Bacillus thuringiensis strain PBT602 130 kDa crystal protein (cry) gen	46.4	81.9	6%	1e-04	100%	
Y00423.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	81.9	6%	1e-04	100%	
X07423.1	Bacillus thuringiensis israelensis bt8 gene for 130 kDa crystal protein	46.4	81.9	6%	1e-04	100%	
X07082.1	Bacillus thuringiensis gene for 130 kDa delta-endotoxin	46.4	81.9	6%	1e-04	100%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	46.4	272	10%	1e-04	100%	
D00248.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticida	46.4	81.9	6%	1e-04	100%	
D00247.1	Bacillus thuringiensis israelensis plasmid gene for 130 kDa insecticida	46.4	81.9	6%	1e-04	100%	
M20242.1	B.thuringiensis mosquitocidal protein (CryD2) gene, complete cds	46.4	81.9	6%	1e-04	100%	
CP000485.1	Bacillus thuringiensis str. Al Hakam, complete genome	35.6	159	13%	0.18	100%	
DQ174290.1	Bacillus thuringiensis Cry4A (cry4a) gene, partial cds	35.6	35.6	3%	0.18	95%	
AE017355.1	Bacillus thuringiensis serovar konkukian str. 97-27, complete genome	35.6	163	16%	0.18	95%	
AB074413.2	Bacillus thuringiensis serovar aizawai cry39A and 39orf2 genes for m	35.6	35.6	3%	0.18	91%	
AB251642.1	Bacillus thuringiensis serovar aizawai cry30like, orf2-30like genes for	35.6	35.6	3%	0.18	91%	
AB112346.1	Bacillus thuringiensis serovar aizawai cry40-like and ORF2 genes for p	35.6	35.6	3%	0.18	91%	
AB074414.1	Bacillus thuringiensis serovar aizawai cry40A and 40orf2 genes for pu	35.6	35.6	3%	0.18	91%	
AF122897.1	Bacillus thuringiensis subsp. finitimus Cry26Aa1 protein (cry26Aa1) g	33.7	33.7	2%	0.64	100%	
DQ242519.1	Bacillus thuringiensis serovar finitimus crystal protein Cry26Aa (cry26	33.7	33.7	2%	0.64	100%	
EF197878.1	Bacillus thuringiensis chitinase (chi74) gene, complete cds	30.1	30.1	5%	7.8	77%	

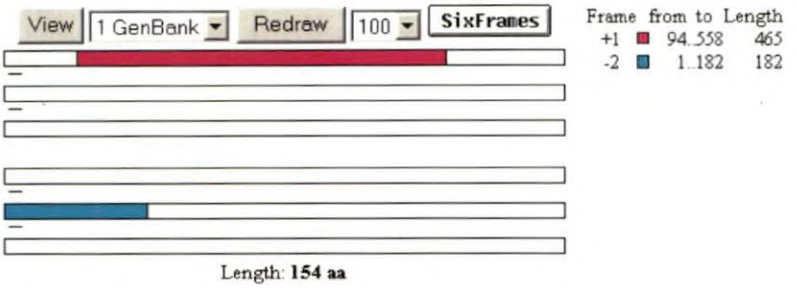
D)Blastn output

Plate 18. Contd...



gb|AAW55474.1|	delta-endotoxin [Bacillus thuringiensis serovar i	26.6	5.4
emb|CAD30099.1|	putative pesticidial crystal protein [Bacillu...	26.6	5.4
emb|CAA30114.1|	unnamed protein product [Bacillus thuringiensis]	26.6	5.4
gb|AAA22337.1|	mosquitocidal protein	26.6	5.4
sp|P16480|CR4AA_BACTI	Pesticidal crystal protein cry4Aa (Inse...	26.6	5.4
sp|P05519|CR4BA_BACTI	Pesticidal crystal protein cry4Ba (Inse...	26.6	5.4
dbj|BAA00178.1|	130 kDa insecticidal protein (ISRH3) [Bacillu...	26.6	5.4
ref|YP_894993.1|	GTP pyrophosphokinase [Bacillus thuringiensi...	26.2	7.1
ref|YP_036650.1|	transcriptional regulator, TetR family [Baci...	25.8	9.2
ref|YP_038170.1|	riboflavin biosynthesis protein [Bacillus th...	25.8	9.2
ref|ZP_00743425.1|	Transcriptional regulator, TetR family [Ba...	25.8	9.2

G) Blastp output



Length: **154 aa**

```
 94 atgacaagtgatgggagaaagtttgctgttaaaagcggacgagag
    M  T  S  D  G  R  K  F  A  V  K  S  G  R  E
139 tacacactttctatgaacgtagccacaagcgaactgggtgacata
    Y  T  L  S  M  N  V  A  T  S  E  L  G  D  I
184 ttggattacatgtacttaatgtatacagtagctggtggtaaccgt
    L  D  Y  M  Y  L  M  Y  T  V  A  G  G  N  R
229 cgtttagctgatattaaaacaaccaacttcccaaaattcgcacca
    R  L  A  D  I  K  T  T  N  F  P  K  F  A  P
274 attgctgaaggaagtttaacaaactattatagtgttaaactaacg
    I  A  E  G  S  L  T  N  Y  Y  S  V  K  L  T
319 tttacagctgaccgtgacgacgacaatgcgtacatcttaattggc
    F  T  A  D  R  D  D  D  N  A  Y  I  L  I  G
364 ggtagcacaacacgagagttaacaggttctaatggttatgcgtgg
    G  S  T  T  R  E  L  T  G  S  N  G  Y  A  W
409 attcgtgttaactcattaaaaatcgaagagggtaacattgctaca
    I  R  V  N  S  L  K  I  E  E  G  N  I  A  T
454 tcttgggagttagctccagcagatatcgataaagctattgcagat
    S  W  E  L  A  P  A  D  I  D  K  A  I  A  D
499 acagataaaaagctcaggatgcacaaacaatgcaataacgcactg
    T  D  K  K  L  R  M  H  K  Q  C  N  N  A  L
544 aacaaggtcaggtag 558
    N  K  V  R  *
```

H) Open reading frame

Plate 18. Contd...

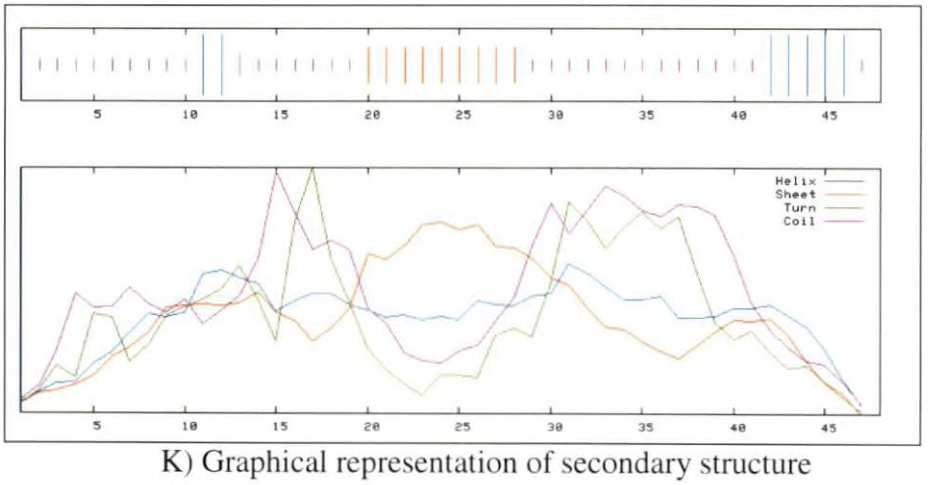
I) Exon sequence

```
         10         20         30         40
          |          |          |          |
FRFSNPISDWHLNDMFPSKTVHFYLFICRMNHESRGDGGREHATSASI
ccccccccccchhtccccccceeeeeeeeecccccccccccchhhhhc

Sequence length :    48

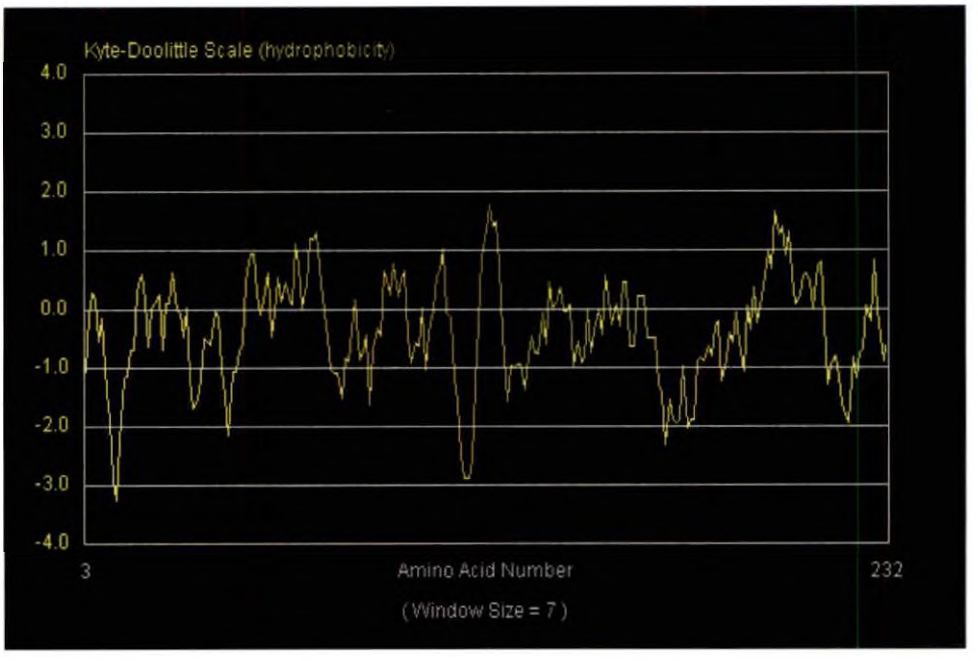
SOPMA :
   Alpha helix      (Hh) :     7 is  14.58%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     9 is  18.75%
   Beta turn        (Tt) :     1 is   2.08%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    31 is  64.58%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

J) Predicted secondary structure



K) Graphical representation of secondary structure

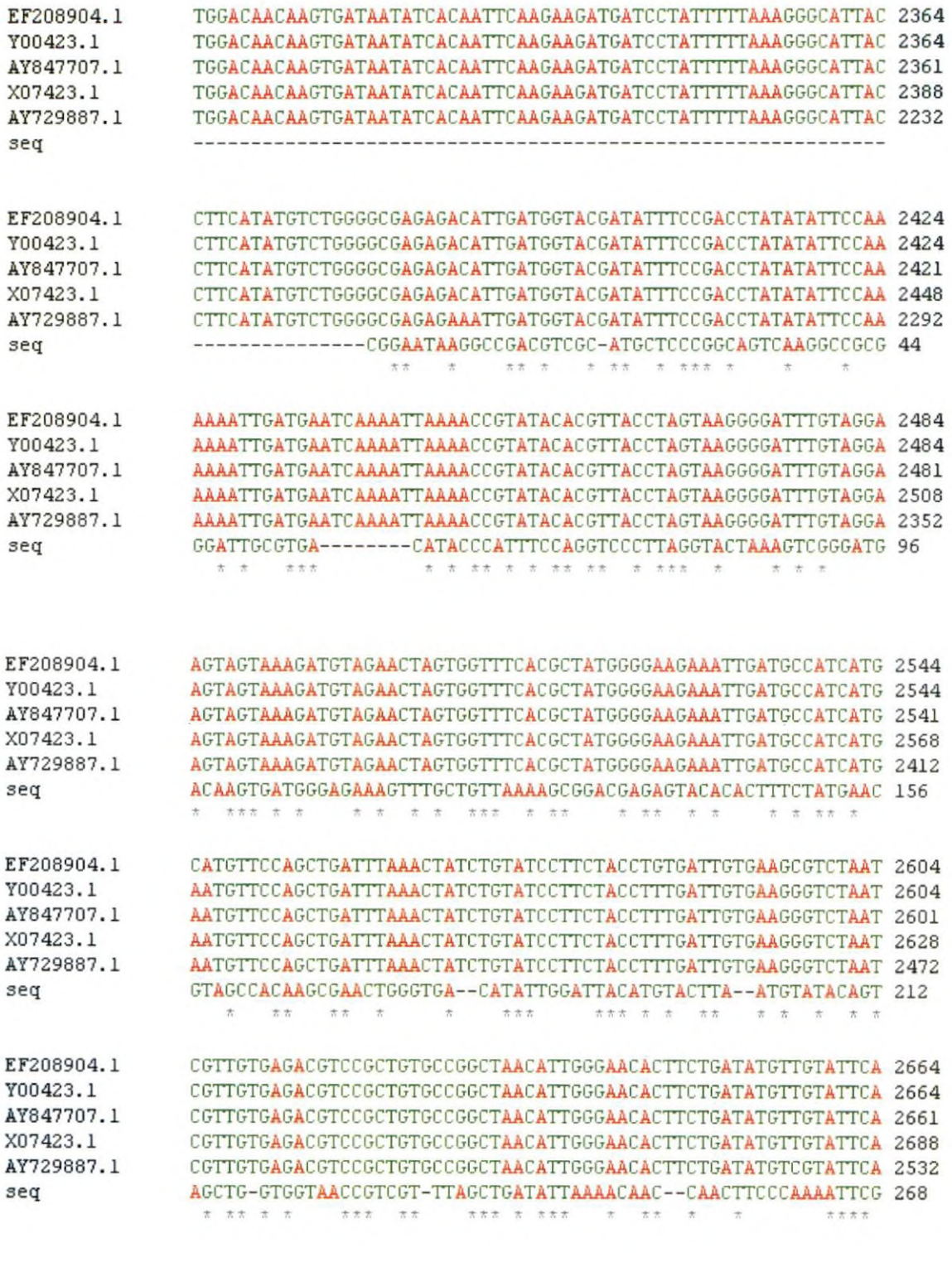
Plate 18. Contd....



L) Kyte Doolittle Hydropathy Plot for deduced proteins

M) Functional domains detected through InterProscan

Plate 18. Contd....



E) Multiple sequence alignment of *cry*4em10 with *cry* gene sequences on the databank. Locations with star sign indicate conserved regions.

Plate 18. Contd....

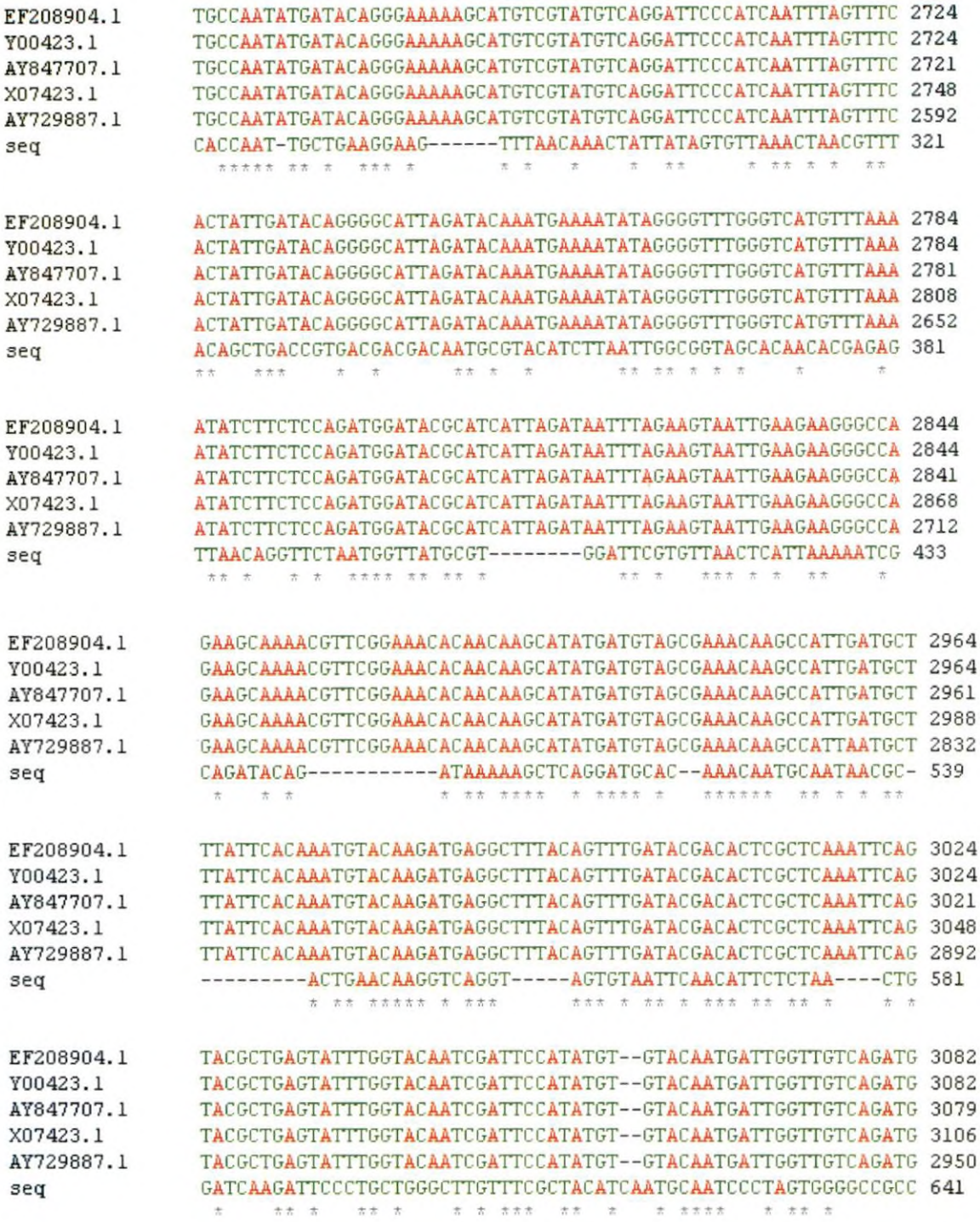
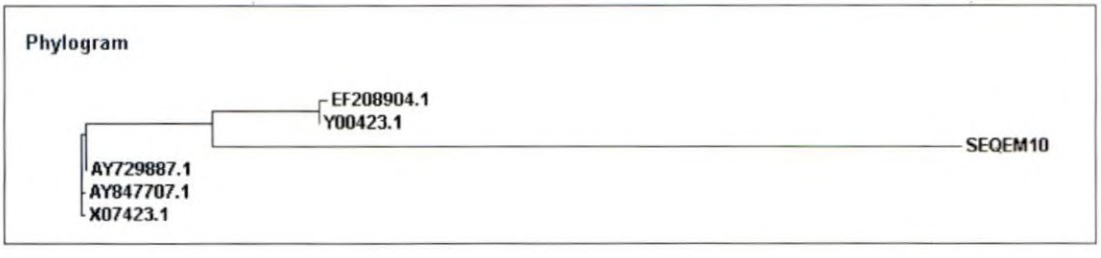


Plate 18. Contd....

```
EF208904.1      TTCCAGGTATGAATTATGATATCTATGTAGAGTTGGATGCA----CGAGTGGCACAAGCG 3138
Y00423.1        TTCCAGGTATGAATTATGATATCTATGTAGAGTTGGATGCA----CGAGTGGCACAAGCG 3138
AY847707.1      TTCCAGGTATGAATTATGATATCTATGTAGAGTTGGATGCA----CGAGTGGCACAAGCG 3135
X07423.1        TTCCAGGTATGAATTATGATATCTATGTAGAGTTGGATGCA----CGAGTGGCACAAGCG 3162
AY729887.1      TTCCAGGTATGAATTATGACATCTATGTAGAGTTGGATGCA----CGAGTGGCACAAGCG 3006
seq             TGCAGGCCAACAAATGGGAAAACCCCCACGCGTTGGATGCAACCTTGAATTTCCAAAGGG 701
                * *  *  *  ** *  ** * *      * **********     ** *  *  *** *

EF208904.1      CGTTATTTGTATGATATAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATG 3198
Y00423.1        CGTTATTTGTATGATATAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATG 3198
AY847707.1      CGTTATTTGTATGATACAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATG 3195
X07423.1        CGTTATTTGTATGATACAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATG 3222
AY729887.1      CGTTATTTGTATGATACAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATG 3066
seq             CCCC---------------------------------------------------------- 705
                *

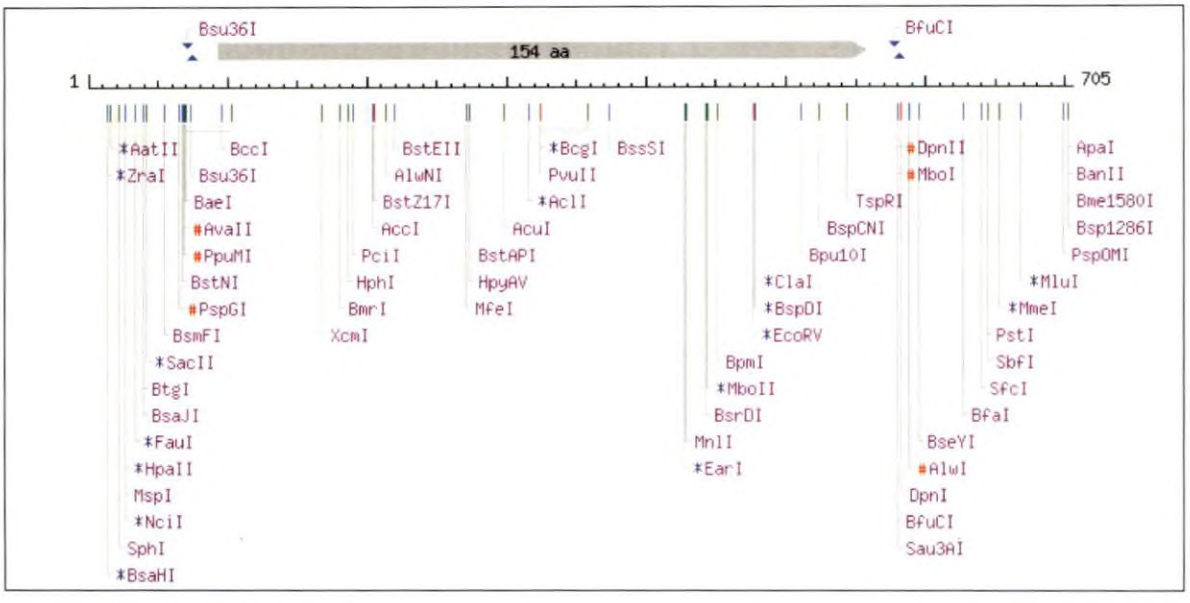
EF208904.1      GGGTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
Y00423.1        GGGTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3258
AY847707.1      GGGTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3255
X07423.1        GGGTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3282
AY729887.1      GGGTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTT 3126
seq             ------------------------------------------------------------
```



EF 208904.1 - *B. thuringiensis cry*4A gene
Y00423.1 - *B. thuringiensis cry*4em10
AY729887.1 - *B. thuringiensis* serovar *israelensis cry*4BLB
AY847707.1 - *B. thuringiensis* strain PBT602 *cry* gene
XO7423.1 - *B. thuringiensis israelensis* bt8 gene

F) Phlyogram of *cry*4em10 showing evolutionary relationship with *cry*4 genes in NCBI databank

Plate 18. Contd....

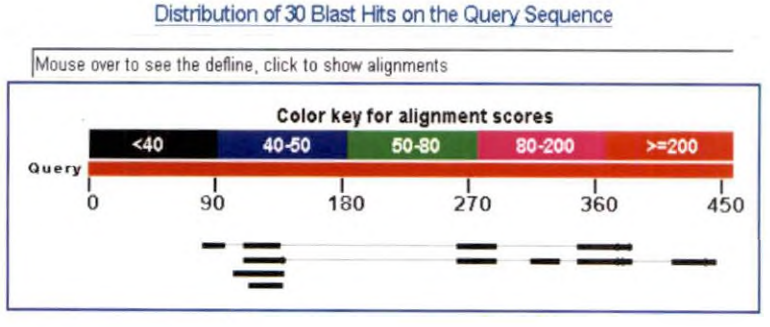


N) Restriction map

Plate 18. Contd....

```
1   X  X  Q  H  L  A  V  S  S  V  R  D  M  L  -  Q  Y  V  D  L
1 NCNNTTCAGCATCTCGCGGTGAGCTCTGTACGTGACATGCTCTAGCAGTATGTTGATTTA
21  H  Y  L  T  L  L  H  A  A  L  F  A  L  F  A  A  S  -  A  F
61 CACTACCTGACCTTGCTTCACGCTGCGTTATTGCATTGTTTGCTGCATCCTGAGCTTTT
41  L  S  V  S  A  I  A  L  S  I  S  A  G  A  N  S  Q  D  V  A
121 TTATCTGTATCTGCAATAGCTTTATCGATATCTGCTGGAGCTAACTCCCAAGATGTAGCA
61  M  L  P  S  S  I  F  N  E  L  T  R  I  H  A  -  P  L  E  P
181 ATGTTACCCTCTTCGATTTTTAATGAGTTAACACGAATCCACGCATAACCATTAGAACCT
81  V  N  S  R  V  V  L  P  P  I  K  M  Y  A  L  S  S  S  R  S
241 GTTAACTCTCGTGTTGTGCTACCGCCAATTAAGATGTACGCATTGTCGTCGTCACGGTCA
101  A  V  N  V  S  L  H  Y  N  S  L  L  N  F  L  Q  Q  L  V  R
301 GCTGTAAACGTTAGTTTACACTATAATAGTTTGTTAAACTTCCTTCAGCAGTTGGTGCGA
121  I  F  K  K  -  L  F  N  I  N  W  P  R  L  G  E  Q  L  D  R
361 ATTTTTAAAAAGTAATTGTTTAATATAAACTGGCCACGGTTGGGCGAGCAACTCGATAGA
141  K  S  K  G  M  G  K  K  R  N  G  W  G
421 AAAAGTAAAGGAATGGGCAAAAAACGAAACGGTGGGGG
```

A) Nucleotide and deduced aminoacid sequence

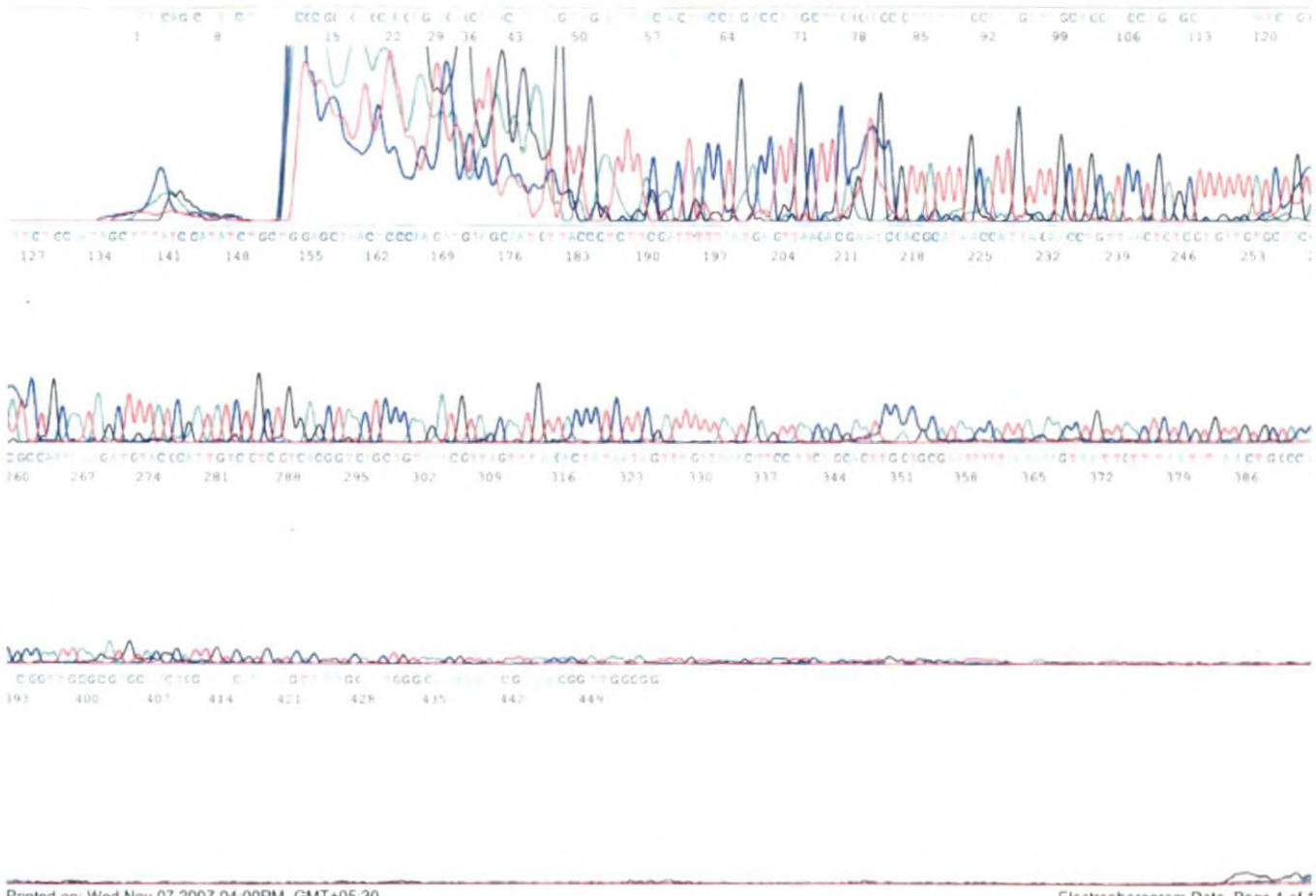


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
CP000485.1	Bacillus thuringiensis str. Al Hakam, complete genome	33.7	345	23%	0.41	100%	
AE017355.1	Bacillus thuringiensis serovar konkukian str. 97-27, complete genome	33.7	537	32%	0.41	100%	
DQ377253.1	Bacillus thuringiensis serovar tolworthi strain IEBC-T09 001 flagellin (I	31.9	31.9	7%	1.4	81%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	30.1	30.1	5%	5.0	87%	

B) Blastn output

Plate 19. Sequence analysis of *cry*4ky1

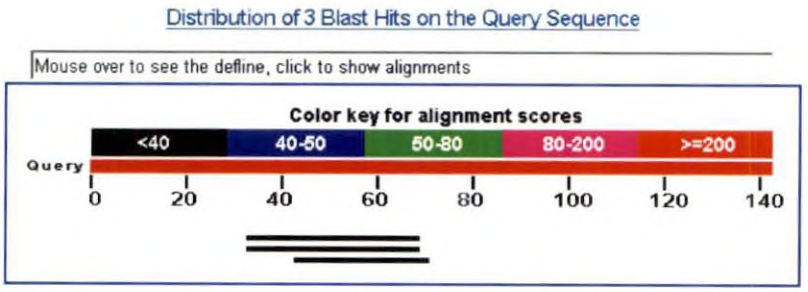


Printed on: Wed Nov 07,2007 04:00PM, GMT+05:30

Electropherogram Data Page 1 of 1

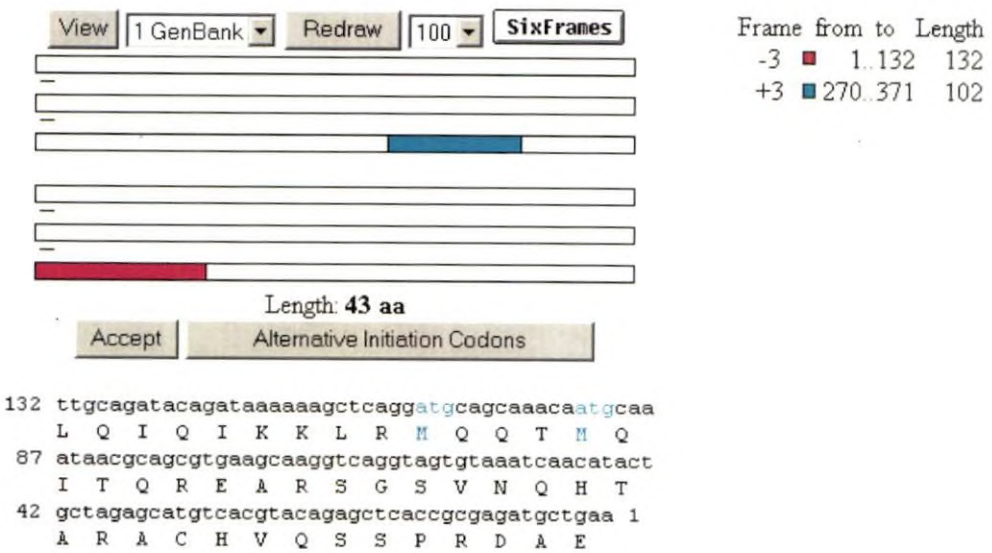
C). Graphical output

Plate 19 . Contd.



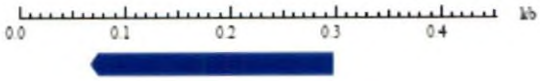
gb|AAY24695.1|	Cry [Bacillus thuringiensis]	25.0	6.7

D) Blastp output



E) Open reading frame

Plate 19. Contd...



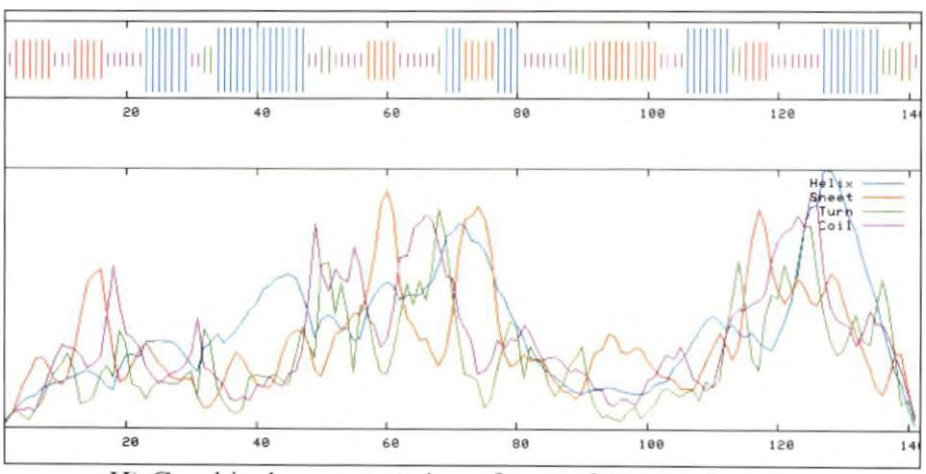
F) Exon sequence

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
SISRALYVTCSSSMLIYTTPCFTLRYLHCLLHPELFYLYLQLYRYLLELTPKMQCYPLRFLMSHESTHNH
hheeeeeeccceeeeeccccchhhhhhhcctthhhhhhhhhhhhhhhcctteeeeeeeeeccccccth
NLLTLVLCYRQLRCTHCRRHGQLTLVYTIIVCTSFSSWCEFLKSNCLITGHGWASNSIEKVKEWAKNETV
hheeeeehhhhccccccttteeeeeeeeeeeccchhhhhhhttteeeeccccccchhhhhhhhhttte
GG
ec

Sequence length :   142

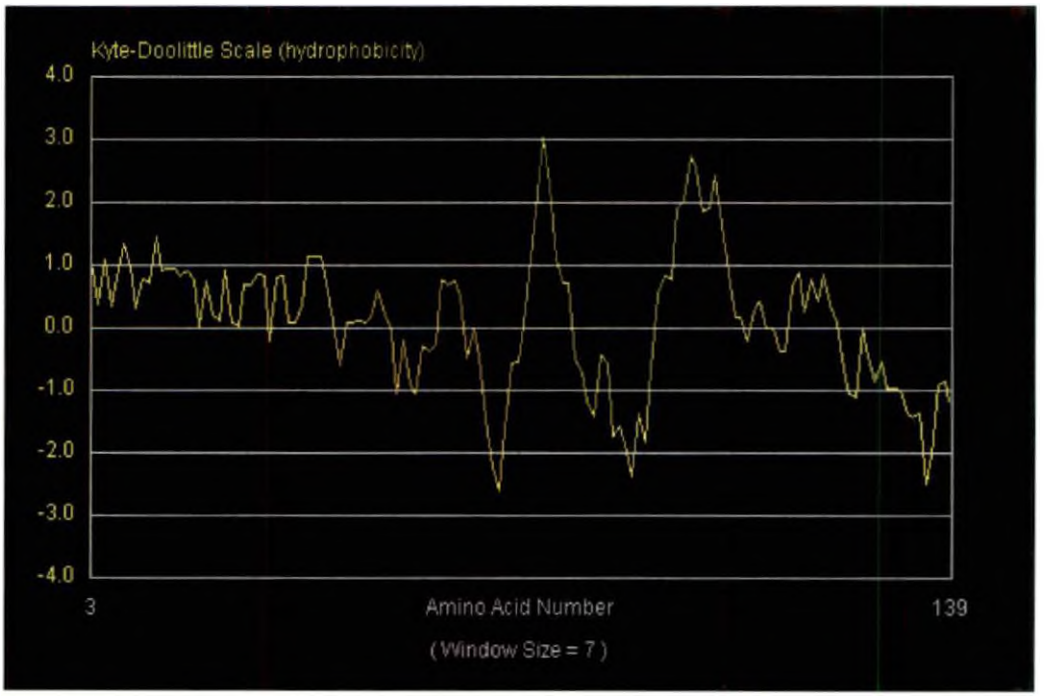
SOPMA :
   Alpha helix      (Hh) :    46 is  32.39%
   3_10  helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    38 is  26.76%
   Beta turn        (Tt) :    13 is   9.15%
   Bend region      (Cc) :     0 is   0.00%
   Random coil      (Cc) :    45 is  31.69%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

G) Predicted secondary structure

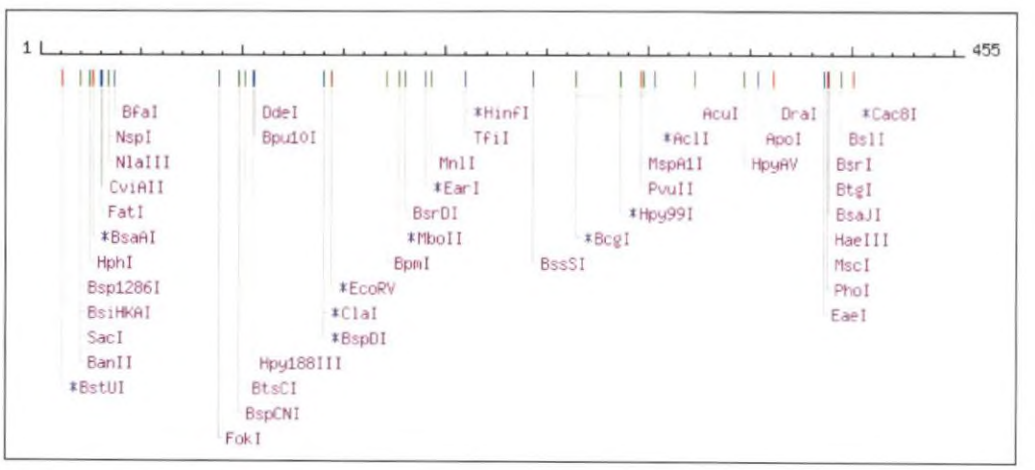


H) Graphical representation of secondary structure

Plate 19. Contd...



I) Kyte Doolittle Hydropathy Plot for deduced proteins

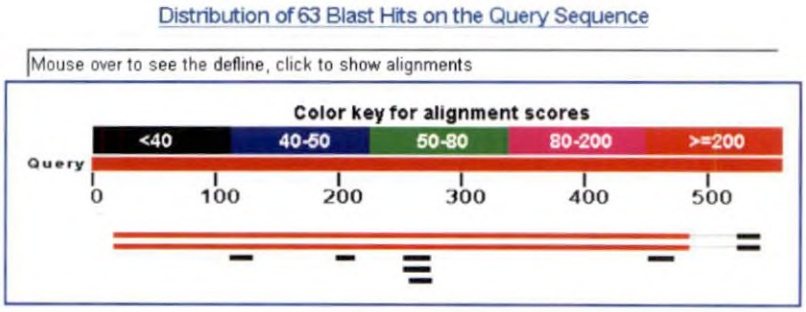


J) Restriction map

Plate 19. Contd...

```
1   T   A   -   S   V   T   -   W   H   -   V   L   Q   C   R   P   Y   S   S   S
1   ACAGCATAGTCTGTGACGTAGTGGCATTAGGTATTGCAGTGCAGACCTTATTCATCATCT
21  E   -   R   T   D   S   I   Y   R   K   R   K   G   T   R   N   D   I   K   R
61  GAATAACGGACCGATTCTATTATAGAAAAAGAAAAGGTACCCGAAATGACATTAAAAGA
41  N   K   T   I   K   S   A   R   N   E   R   W   N   T   N   Y   G   D   G   G
121 AATAAAACGATTAAATCCGCTCGGAACGAAAGATGGAACACAAATTATGGTGATGGGGGA
61  C   R   C   I   Y   P   -   A   I   K   R   V   -   S   K   A   N   K   R   N
181 TGTCGATGCATCTATCCTGAGCAATTAAAAGAGTATAAAGTAAAGCAAATAAAAGAAAAC
81  G   S   S   S   Y   I   C   K   R   C   G   -   R   I   C   R   Y   N   R   K   L
241 GGATCCAGCTACATTTGCAAAAGATGTGGATAAAGAATATGCCGATATAACAGGAAGTTA
101 P   K   -   C   Y   Y   R       F   V   -   R   R   R   T   F   I   Y   N   T   S
301 CCCAAATATGTTATTATCGGTTCGTTTGAAGAAGAAGGACGTTTATATACAACACCCAG
```

A) Nucleotide and deduced aminoacid sequence

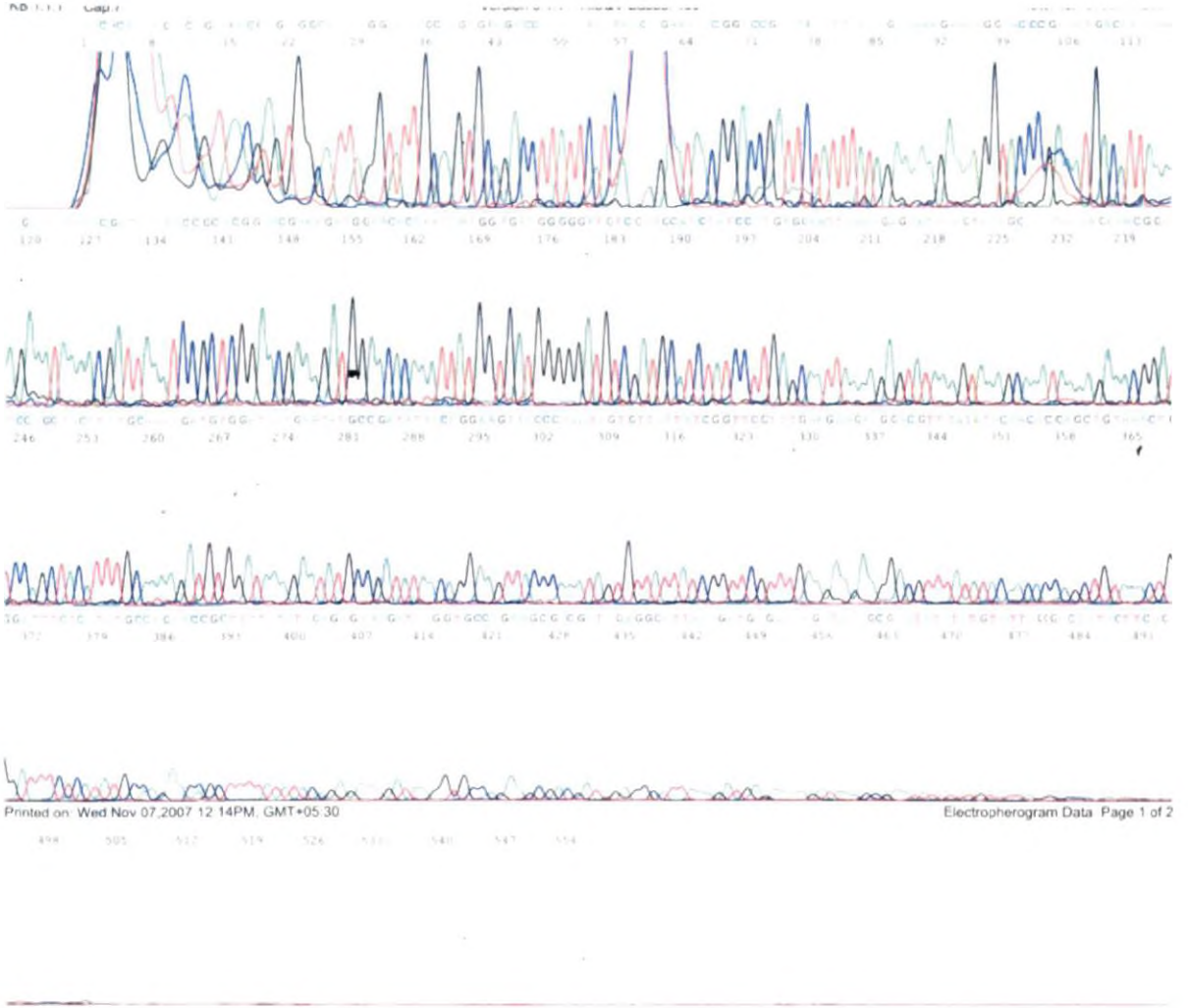


Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AE017355.1	Bacillus thuringiensis serovar konkukian str. 97-27, complete genome	807	1644	86%	0.0	100%	
CP000485.1	Bacillus thuringiensis str. Al Hakam, complete genome	720	1602	86%	0.0	100%	
AL731825.1	Bacillus thuringiensis subsp. israelensis plasmid pBtoxis	31.9	31.9	3%	1.8	90%	
AJ296640.1	Bacillus thuringiensis subsp. israelensis pBtoxis plasmid, subclone pT(	31.9	31.9	3%	1.8	90%	
CP000047.1	Bacillus thuringiensis serovar konkukian str. 97-27 plasmid pBT9727,	30.1	30.1	3%	6.1	90%	
AY550111.1	Bacillus thuringiensis serovar galleriae holin-like protein, pBt10-like pl	30.1	30.1	3%	6.1	94%	
DQ363750.1	Bacillus thuringiensis plasmid pBMB67, complete sequence	30.1	30.1	2%	6.1	100%	
Y11173.1	Bacillus thuringiensis plasmid DNA ORF1-ORF4, ORF6-ORF10, ORF14 a	30.1	30.1	3%	6.1	94%	G

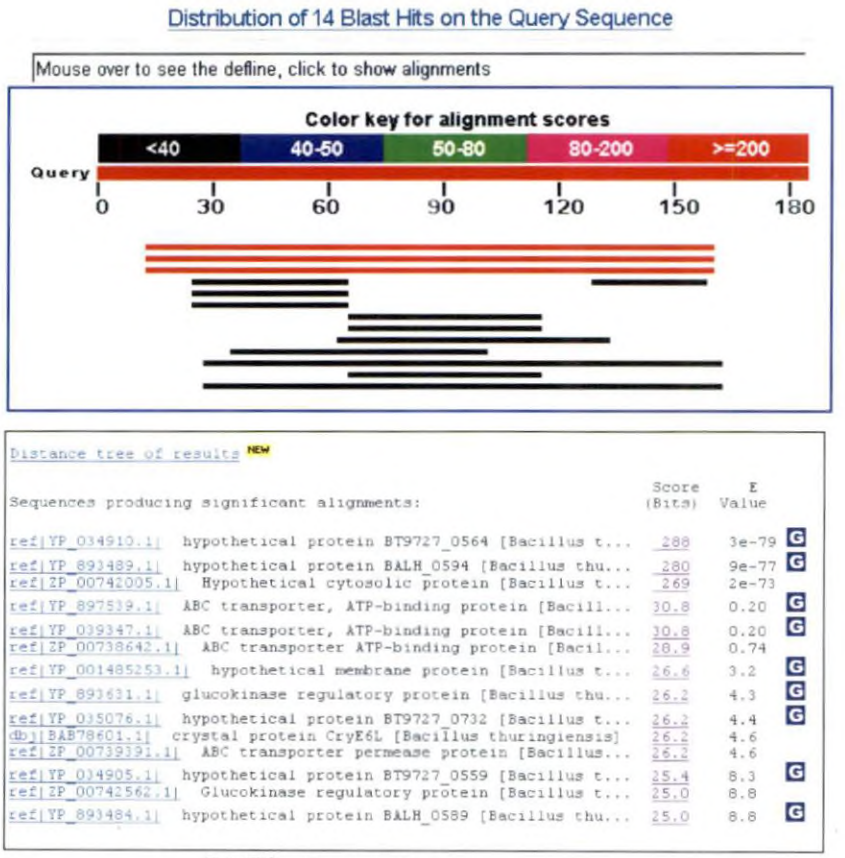
B) Blastn output

Plate 20. Sequence analysis of cry1Aky3

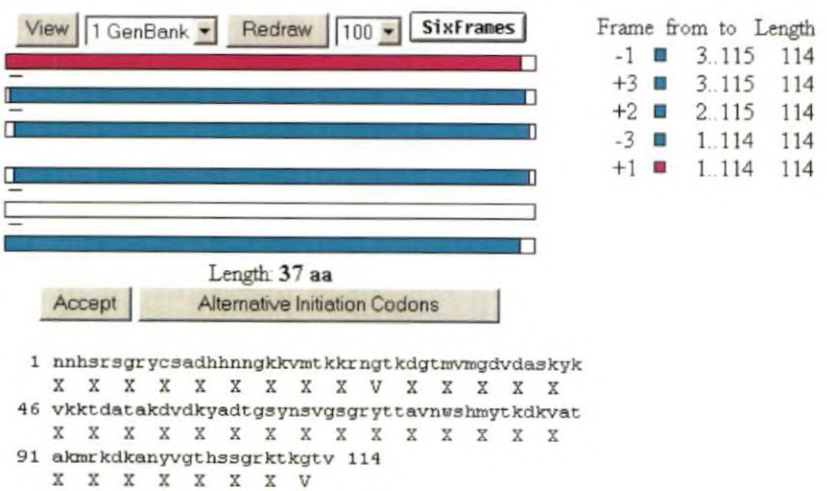


C). Graphical output

Plate 20 . Contd.

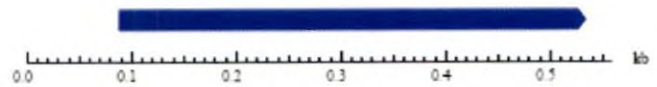


D) Blastp output



E) Open reading frame

Plate 20. Contd...



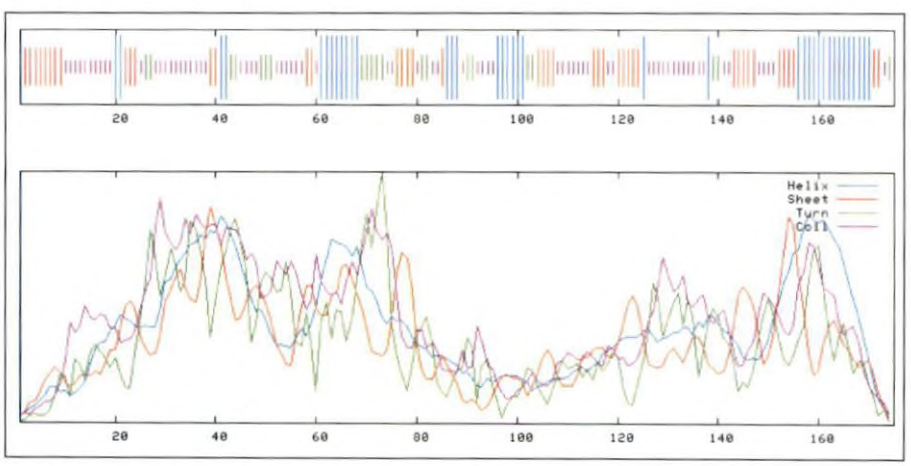
F) Exon sequence

```
        10        20        30        40        50        60        70
        |         |         |         |         |         |         |
TASVTWHVLQCRPYSSSERTDSIYRKRKGTRNDIKRNKTIKSARNERWNTNYGDGGCRCIYPAIKRVSKA
cceeeeeeeeccccccccchheeecttccccccccccceehhttccccttccccccceechhhhhhhht
NKRNGSSYICKRCGRICRYNRKLPKCYYRFVRRRTFIYNTSCKLDFSYARTAFIYRERGARSDDRGIKDE
ttttcceeeecttccechhhcttcccchhhhhhttcccccccccceeeccceeeeehccccccccccht
KRSEYICIRDHTSLFLTRAENHLLKRAINCVFFKN
tccceeeeeccccceeeehhhhhhhhhhhhhhheeet

Sequence length :   175

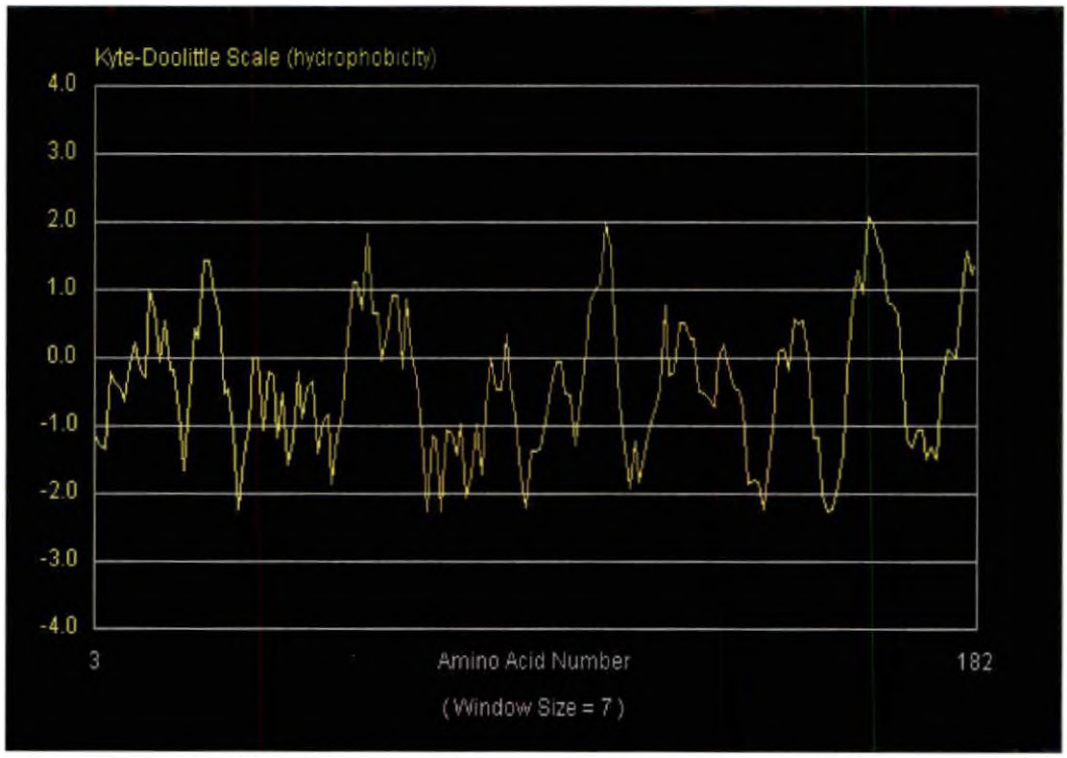
SOPMA :
   Alpha helix      (Hh) :    38 is  21.71%
   3_10 helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    43 is  24.57%
   Beta turn        (Tt) :    21 is  12.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    73 is  41.71%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

G) Predicted secondary structure

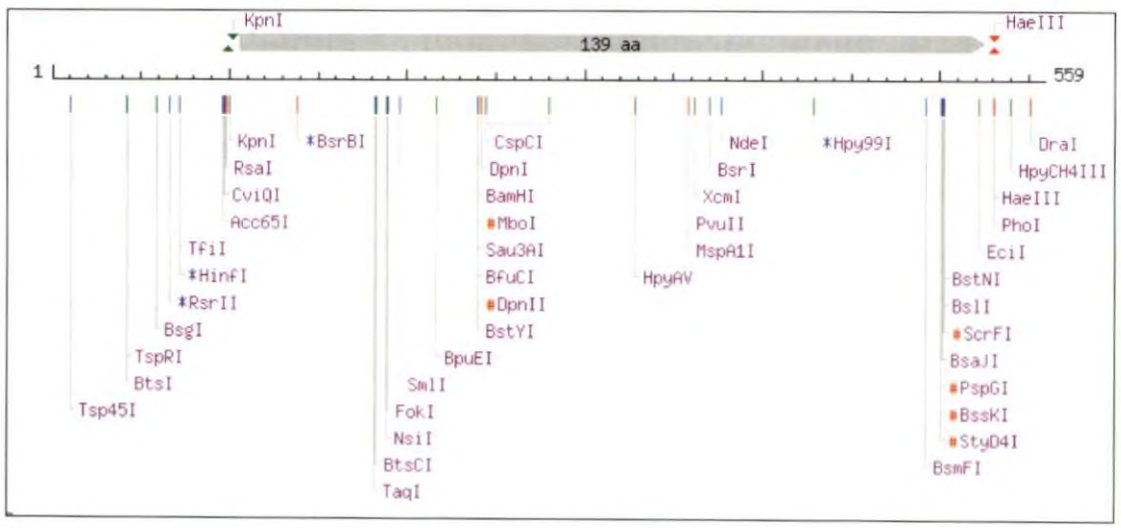


H) Graphical representation of secondary structure

Plate 20.Contd...



I) Kyte Doolittle Hydropathy Plot for deduced proteins



J) Restriction map

Plate 20.Contd...

Discussion

5. DISCUSSION

Overdependence on pesticides and their indiscriminate use over the last four to five decades have resulted in many negative effects like pest resurgence, development of pesticide resistance and accumulation of pesticide residues in the environment, leading to serious health hazards. Consequently, there has been a great interest in developing alternative strategies for insect control.

Microbial pest control agents are a primary means of biological control for insect pests. Among microbial biocontrol agents, *Bacillus thuringiensis* is reported to be the most successful one used commercially (Rajesh *et al.*, 2006). *Bacillus thuringiensis* is a gram positive, facultatively anaerobic endospore forming bacterium. It is recognized by its parasporal inclusion bodies, which is proteinaceous in nature and possesses insecticidal activity. It exhibits wide range of toxicity to different insect orders (Aranda *et al.*, 1996).

The insecticidal activity of *B. thuringiensis* is associated with the proteinaceous toxins, located in parasporal inclusion bodies, also known as parasporal crystals. These are produced at the time of sporulation and account for up to 30 per cent of the total protein content of the bacterium (Hofte and Whiteley, 1989; Aronson, 1993). The toxins found in parasporal crystals are collectively referred as δ-endotoxins. These compounds become toxic by ingestion for many insect larvae belonging to orders of Lepidoptera, Diptera and Coleoptera. Some bacterial strains also secrete insecticidal proteins during their vegetative growth phase; known as vegetative insecticidal proteins, which are toxic to insects of various orders.

The need for novel cry proteins with toxic potential against different organisms with specificity for a much broader range of pests has resulted in continuous search for unique bacterial strains from diverse ecosystems. *Bacillus thuringiensis* is a wide-spread bacterium, detected in different habitats

including soil, grain, dust, insect larvae and sericultural environments (Obeidat *et al.*, 2005).

The present study was aimed at the isolation of *B. thuringiensis* strains from soil samples collected from the Western Ghats of Kerala. The study emphasized on morphological, biochemical and molecular characterization of the strains isolated. The insecticidal activity of the strains against a major lepidopteran pest of cucurbitaceous vegetables, the pumpkin caterpillar (*Diaphania indica*) was also studied. *cry*1 *and cry*4 gene fragments were amplified using specific primers, cloned and sequenced from a few isolates.

5.1 ISOLATION OF *Bacillus thuringiensis* FROM WESTERN GHATS OF KERALA

The Western Ghats is an area of exceptional biodiversity and possesses an undisturbed ecological niche. Great biodiversity among *B. thuringiensis* isolates collected from this area is due to its co-evolution along with the insects or by conjugal transfer of plasmid to other strains.

Very few attempts have been made by scientists to explore the microbial diversity of Western Ghats. There is only a single report on the isolation of *B. thuringiensis* strains from the Western Ghats. Rajesh *et al.* (2006) isolated *Bacillus thuringiensis* from the Western Ghat region of Karnataka and Kerala. They studied the *cry* gene diversity of 15 strains of *B. thuringiensis* isolated from these areas.

In the present study, attempts have been made to isolate the bacterium from different environments, potentially undisturbed and without much human interference. Soil samples were collected from three districts namely Kottayam, Kozhikode and Ernakulam, coming under the Western Ghats region of Kerala, for the isolation of *B. thuringiensis*. Earlier workers have collected samples from different geographical locations such as forests, agricultural lands, industrial areas

and plant surfaces. Intensive screening programmes identified bacterial strains from these sources (De Lucca *et al.*, 1991; Chilcott and Wigley, 1993; Benhard *et al.*, 1997; Valicente and Barreto, 2003). Isolation of *Bacillus thuringiensis* strains active against lepidopteran/ dipteran/ coleopteran species were reported from soils and wild ecosystems by other workers (Yu *et al.* 1991; Prabagaran *et al.* 1999; Uribe *et al.*, 2003; Arango *et al.*, 2004; Kim, 2004).

Recent ecological surveys on the distribution of the bacterium have shown that soil is the natural habitat of *Bacillus thuringiensis* and its nature is heterogeneous in terms of phenotypic characters (Padua *et al.*, 1982; Obha and Aizawa, 1986; Martin and Travers, 1989; Hastowo *et al.*, 1992; Benhard *et al.*, 1997; Soo *et al.*, 2004).

Mainly three methods were reported for isolating *B. thuringiensis* from soil samples. They are as follows:

1. Samples in nutrient broth medium heated for 15 minutes and plated on sporulation plates with antibiotics like penicillin (Saleh *et al.*, 1969).
2. Samples in selective/ non selective media heat shocked for 15 minutes and plated on sporulation plates without antibiotics.
3. Pre-incubation of samples in LB buffered with sodium acetate for four hours, followed by heat shock and plating on sporulation plates (Travers *et al.*, 1987)

Of the three methods available, sodium acetate selection method is considered as the best method. Hence in the present study, the same method was followed for isolating *B. thuringiensis* from soil samples. Placing the soil samples in growth media and incubating them allowed the maximum germination of unwanted spores. Subsequent heat treatment eliminated the excess contaminating organisms. Sodium acetate inhibited the germination of *B. thuringiensis* spores, so that other spores germinated. The soil samples were analysed for total spore

counts and occurrence of *Bacillus thuringiensis*. Similar method was adopted by earlier scientists (Leithy *et al.*, 2004, Carrozi *et al.*, 1991). The isolates produced creamy white puffy large colonies, typical of *Bacillus thuringiensis* when grown on LB agar medium.

After pure cultures of the bacterium were obtained, single colonies were stab inoculated in cryostorage vials in LB agar medium and stored at 4^0C. Soil samples collected from wild ecosystems of Western Ghats of Kerala, yielded a total of 19 *B.thuringiensis* isolates, by sodium acetate selection method.

5.2 STAINING REACTIONS

Gram staining of *Bacillus thuringiensis* isolates revealed the presence of blue coloured, rod-shaped cells arranged like a chain. Upon endospore staining, spores appeared translucent, with crystals bluish black in colour.

Staining with Coomassie Brilliant Blue showed dark blue coloured crystal proteins. Three isolates KY1, KY5 and KK7 produced composite of crystal proteins, i.e., spherical and bipyramidal shaped crystal proteins. Isolate KK8 produced both triangular and spherical shaped crystal proteins. Isolates KK9 and KY4 produced irregular crystal proteins. Of the total 11 isolates analysed, four isolates produced composite of crystal proteins. Eight isolates produced spherical crystal proteins, revealing the fact that soil samples collected from the specific locations in the Western Ghats of Kerala, harboured a higher proportion of bacerial isolates producing spherical crystal proteins. Composite parasporal inclusion bodies have been earlier reported by Obeidat *et al.* (2004).

The parasporal inclusion bodies of *Bacillus thuringiensis* were observed as morphologically heterogeneous (Ohba *et al.*, 2001). Benhard *et al.* (1997) observed crystal protein morphology of *Bacillus thuringiensis* isolates as diverse ranging from spherical, bipyramidal, rectangular, irregularly spherical and

irregular pointed. No correlation between the type of insecticidal activity and crystal morphology was reported earlier (Obha and Aizawa, 1986; Martin and Travers, 1989).

5.3 CULTURAL CHARACTERIZATION

The cultural and morphological characters of the isolates were studied on LB agar medium. The isolate HD1 was used as a reference strain for comparison of colony morphology. All the isolates produced creamy white, puffy, large colonies within 24 hours after streaking on the medium. The colony size ranged from medium to large; with circular form, flat elevation and margin varied from undulate to entire. Similar variations in the morphological characteristics of *B. thuringiensis* isolates on nutrient agar medium were recorded by Chatterjee *et al.* (2006).

5.4 BIOCHEMICAL CHARACTERIZATION

The *B. thuringiensis* isolates were characterized using seven biochemical tests. Biochemical characterization helps in subspecies identification of bacterial strains.

Testing the solubility of bacterial cells in three per cent of KOH is an effective supplement to Gram staining, proven to be useful for rapid and accurate differentiation of bacteria (Suslow *et al.*, 1982). None of the isolates formed viscous thread-like structure with KOH, indicating gram positive reaction.

In the present study, the bacterial isolates were checked for their ability to degrade urea by means of enzyme urease. Nine isolates namely KY1, KY5, KY6, KK7, KY8, KK9, EM11 along with HD1 were found to degrade urea. This was indicative of presence of urease enzyme. The remaining isolates KY3, KY4 and EM 10 gave negative results for urease test. Differential response of bacterial isolates to hydrolysis of urea had been reported by earlier workers (Hernandez *et*

al., 1998; Demir *et al.*, 2002; Chatterjee *et al.*, 2006; Sharmin and Rahmin, 2007). Urease is a hydrolytic enzyme that attacks nitrogen and carbon bonds in amide compounds such as urea and forms the alkaline end product ammonia. Colour change of the medium to deep pink, is due to the reason that as the substrate urea splits into products, presence of ammonia creates an alkaline environment that causes phenol red to turn deep pink.

Starch hydrolysis test was carried out to determine the ability of the bacterium to excrete hydrolytic extracellular enzyme which is capable of degrading polysaccharide. A clear zone of hydrolysis was observed around bacterial growth when flooded with Lugol's iodine. Except for the isolate KK9, all other isolates including HD1 gave a positive reaction, indicating the presence of amylase, the starch splitting enzyme. Similar observations were made by Demir *et al.* (2002) who characterized *B. thuringiensis* isolates using starch hydrolysis reaction and all gave positive response.

In gelatin hydrolysis test, after 24 hours incubation, gelatin remained soldified indicating that the isolates were not capable of producing proteolytic extra-cellular enzyme gelatinase. Negative reaction of bacterial isolates to gelatin hydrolysis was reported by earlier workers (Hernandez *et al.*, 1998; Demir *et al.*, 2002).

Voges-Proskauer test was done to determine the ability of isolates to produce non-acidic or neutral end products such as acetyl methyl carbinol from organic acids that result from glucose metabolism. All the isolates gave a negative reaction for Voges- Proskauer test. Reports on negative reaction of bacterial isolates to Voges- Proskauer test were made by various scientists (Hernandez *et al.*, 1988; Demir *et al.*, 2002; Lopez and Alippi, 2005).

All the isolates gave positive reaction for lecithinase test, indicating the ability of the bacterium to produce the enzyme lecithinase which hydrolyses

lecithin present in egg yolk medium. A clear zone was observed around the growth of bacteria which indicated a positive reaction. Hydrolysis of lecithin by bacterial strains isolated from diverse locations was reported by various scientists (Hernandez *et al.*, 1989; Demir *et al.*, 2002).

Esculinase test was carried out to detect the ability of the bacterium to hydrolyze esculin. When the glycoside esculin is hydrolyzed to from esculetin and dextrin, the esculetin reacts with ferric citrate to produce dark brown or black phenolic iron complex. Isolates KY1, KY2, KY4, KY5, KY6, and EM11along with HD1 hydrolyzed esculin which was indicated by a blackening around the growth of bacterium (Plate 8). The remaining isolates gave a negative reaction. Reports on bacterial isolates that differ in their reaction to esculinase were made by various scientists (Gordon *et al.*, 1973; De Lucca *et al.*, 1981; Hernandez *et al.*, 1998).

Using the different biochemical tests subspecies identification of bacterial isolates was carried out. Isolate KY1 was identified as *B. thuringiensis* subsp. brasilensis and isolate KK7 as *B. thuringiensis* subsp. *seoulensis.*

Among the different biochemical tests used for the characterization of *B. thuringiensis* isolates, differential response was obtained for urea hydrolysis, starch hydrolysis and esculinase test. The response of the bacterial isolates to three per cent KOH, gelatin hydrolysis, Voges-Proskauer test and lecithinase test remained uniform. The differential response of bacterial isolates to biochemical tests is indicative of the diversity in locations from which *B. thuringiensis* were isolated.

5.5 BIOASSAY AGAINST PUMPKIN CATERPILLAR

The main focus in the studies of *Bacillus thuringiensis* strains is the production of insecticidal crystal inclusions by these organisms during sporulation. It has been reported that these crystal proteins exhibit toxic activity against larvae of very different insect orders (Fietelson *et al.*, 1992; Hofte and Whiteley, 1989). The genes encoding crystal proteins have been organized into six different groups based on their sequence similarities and range of specificities. The cryl proteins are toxic to lepidopteran larvae, whereas cry4 proteins are toxic to dipteran larvae. Within the cryl protein group there are ten different subclasses. Each subclass of cryl protein has a specific range of activity against different lepidopteran insects.

The insecticidal activity of *B. thuringiensis* crystal proteins have been traditionally investigated by using crude preparations of spore crystal mixtures. Spore crystal preparations generally contain other toxic agents such as beta-exotoxins and toxic spore components. Several attempts had been made to determine the insecticidal activity of *B.thuringiensis* strains isolated from different environments against the target insect species of orders Lepidoptera, Coleoptera and Diptera using spore crystal preparations (Hofte and Whiteley, 1989; Carrozi *et al.*, 1991; Johnson *et al.*, 1996, Bravo *et al.*, 1998).

Pumpkin caterpillar (*Diaphania indica*) is one of the major pests of cucurbitaceous vegetables in Kerala. It causes damages to crops such as melon, cucumber and gourds by feeding on leaves, flowers and young developing fruits. The caterpillar binds together leaves and feeds on them. In this study, the bioefficacy of *B. thuringiensis* against pumpkin caterpillar was determined.

Bioassay of *B. thuringiensis* isolates against pumpkin caterpillar (*Diaphania indica*) was carried out by diet contamination method. Crude crystal protein preparation from *B. thuringiensis* isolates were mixed with semi synthetic

diet and larvae of pumpkin caterpillar were allowed to feed on the diet. Similar method had been used by many workers in their bioassay experiments (Thomas *et al.*, 1972; Held *et al.*, 1982; Travers and Martin, 1989; Benhard *et al.*, 1997; Tang *et al.*, 1995; Chak *et al.*, 1994; Peyronnet *et al.*, 1997; Jyoti and Brewer, 1999; Herrero *et al.*, 2001; Prabagaran *et al.*, 2002; Valicente and Barreto, 2003; Obeidat *et al.*, 2004; Escudero *et al.*, 2006). The solubilization buffer for crystal protein PMSF along with diet served as the control.

Analysis of data on per cent mortality of larvae clearly demonstrated that HD1 was highly toxic with a mortality of hundred per cent by the fourth day. Isolates KY2, KK7 and KK8 achieved greater than 65 per cent mortality on fourth day. Mortality per cent of greater than 45 was obtained for the remaining isolates KY1, KY3, KY6 and EM 10. Observations made on the percentage mortality of larvae on the eighth day revealed that except for isolate EM11, all other isolates produced nearly 90 per cent mortality. Similar results were obtained by Valicente and Barreto (2003) who identified efficient strains as those, which killed more than 70 per cent of larvae on the eighth day of evaluation.

The isolates were ranked based on the number of days taken for fifty per cent mortality. The highest score was observed in HD1, followed by the isolate KY2. Six isolates namely KY3, KY4, KY5, KK7, KK8 and KK9 took only four days to produce fifty per cent mortality. Chak *et al.* (1994) reported that certain bacterial isolates were capable of producing more than 50 per cent mortality in bioassay against lepidopteran larvae. Isolates which recorded about fifty per cent mortality when compared to standard strains were ranked as highly efficient and toxic by various scientists (Benhard *et al.*, 1994; Hossain *et al.*, 1997).

Stastical analysis of data was carried out using Kendall's coefficient of concordance. It provides a measure of agreement or concordance between sample rankings or dependence of the samples (Siegel, 1975). Isolates were also ranked

based on the mortality on fifth and eighth days. The results confirmed that standard reference strain HD1 was the most efficient one in controlling larvae of *D. indica.* Among the native isolates, KK7 and KK8 were the most efficient with the highest rank score. Only two isolates (EM11 and KY4) were ranked low. This might be due to the fact that certain insects have a low susceptibility for bacterial crystal proteins owing to the inefficient solubilization of crystals in the midgut. The delta-endotoxin is released by the bacterial isolate as protoxin and it should be converted to the toxic form, by the digestive enzymes in the midgut of insect larvae. Solubilization of crystals significantly enhanced the toxic activity (Jaquet *et al.*, 1987).

Since *Bacillus thuringiensis* strains simultaneously produce more than one type of crystal protein, bioassays can be greatly influenced by the relative proportion of different proteins within the crystal. Hence it is difficult to accurately determine the spectrum of individual proteins causing toxicity.

5.6 DNA ISOLATION

For any molecular assay, the most important pre-requisite is good quality DNA. Hence the protocol for DNA isolation is very important. Total DNA was isolated from all the bacterial isolates and reference strain according to the procedure of Sambrook and Russel (2001). Same procedure had been used by several workers for isolating DNA from *Bacillus thuringiensis* (Ben- Dov *et al.*, 1997; Beron *et al.*, 2004; Rajesh *et al.*, 2006). The bacterial strains were cultured overnight in LB broth with vigorous shaking before isolating DNA. Similar method was adopted by several scientists (Held *et al.*, 1982; Kawalek *et al.*, 1995; Juarez Perez *et al.*, 1997; Ben-Dov *et al.*, 1997).

Tris EDTA is one of the reagents used in DNA isolation. EDTA present in it could effectively chelate magnesium ions and mediate aggregation of nucleic acid. Lyzozyme helps in lysis of bacterial cells. The detergent used was SDS which acts as a nuclease inhibitor and also dissolves membranes.

Efficient extraction of cell extracts or solutions containing nucleic acids are most often performed with a series of phenol and phenol: chloroform extractions. Both phenol and chloroform denature proteins, get solubilized in organic phase or interphase, while nucleic acids remain in the aqueous phase. Chloroform is mixed with phenol to increase the efficiency of nucleic acid extractions. The increased efficiency is due to the ability of chloroform to denature proteins. It helps in removal of lipids, thus improving separation of nucleic acids into the aqueous phase. Chloroform: isoamyl alcohol improves deproteinization.

Ribonuclease catalyses the hydrolysis of RNA into smaller components. Proteinase K degrades almost every protein and rapidly inactivates enzymatic activities. Isopropanol was used for initial precipitation of DNA at low temperature and two volumes of ethanol were used for final precipitation. The pellet was dissolved in TE buffer for long term storage.

In the present study, the DNA when isolated following the procedure of Sambrook and Russel (2000) yielded a single sharp band on 0.8 per cent agarose gel (Plate12A). No RNA contamination was observed. The optical density of DNA was found out using nanodrop. The OD_{260}/OD_{280} values of DNA ranged between 1.78 and 1.97 (Table 12) indicating that DNA was good without much protein contamination. Quantity of DNA was found to be highest in the isolate KY6 (20.132 µg/ml of bacterial culture) and the lowest in EM10 (1.337 µg/ml).

5.7 PRIMER DESIGNING

The strategy devised for cloning of *Bacillus thuringiensis cry* genes was based on the assumption that the genes encoding for crystal proteins, which in turn confer toxicity against target insects, may be conserved during evolution among species.

The insecticidal crystal protein gene sequences provide the basis for the construction of gene specific primers to screen the *B. thuringiensis* isolates by PCR analysis for the presence of known nucleotide sequences and characterizing insecticidal crystal proteins from new bacterial isolates (Perfontaine *et al.*, 1987). As a result of the development of resistance of insect against the insecticidal crystal proteins of *Bacillus thuringiensis*, emphasis should be laid on identifying and isolating more diverse and novel *cry* gene. Novel *cry* genes will help in combating resistance development in insects.

The hypothesis that *cry* genes may be conserved among species during evolution was supported by the results of Juarez-Perez *et al.* (1997), who aligned nucleotide sequences available from Genbank and two conserved regions from all the *cry*1 genes were selected for designing degenerate or family primers to match any of the known *cry* gene. Hence primers could be designed from the *cry* gene sequence of different species of *Bacillus thuringiensis*.

In the present study, a pair of primer for *cry*1A gene was designed based on the multiple sequence alignment of nucleotide sequences. Multiple sequence alignment of *cry*1A genes of *B. thuringiensis* using Clustal W showed more homology in conserved boxes. Specific primes were designed by various scientists for amplification of *cry* gene (Ceron *et al.*, 1994; Ben-Dov *et al.*, 1997; Beron *et al.*, 2004).

The primers for the amplification of *cry*1A gene were designed in such a way that a stretch of single nucleotide and complementarities between forward and reverse primers were avoided. The *cry*1A primers designed have an annealing temperature of 61^{0}C and a GC content of 50 per cent. The forward primer had no degeneracy whereas reverse primer contained degeneracy.

5.8 PROFILING OF *cry*1 AND *cry* 4 GENES IN *Bacillus thuringiensis*

PCR is a tool that has been widely used in characterizing genes encoding crystal proteins and for analysis of bacterial collections. Carrozi *et al.* (1991)

introduced this technique to identify *cry* genes in order to predict the insecticidal activity.

DNA amplification being very sensitive to PCR conditions, various parameters like the concentration of DNA and Taq DNA polymerase, annealing temperature and number of cycles for the thermal cycler programme were standardized for the specific amplification of *cry* gene.

Profiling of *cry*1 gene in *B. thuringiensis* was done using universal primer (Un1(d) and Un1(r), (Ben-Dov *et al.*, 1997). *cry*1 gene is reported to be toxic to lepidopteran and *cry*4 to dipteran larvae. Amplification was obtained for eight isolates KY2, KY3, KY5, KY6, KK7, KY8, KY9 and EM11 along with reference strain HD1. Screening of the bacterial isolates for *cry*4 gene, using universal *cry*4 primer Un4(d) and Un4(r), (Ben-Dov *et al.*, 1997) resulted in the amplification in two isolates namely KY1 and EM10, with an amplicon size of 531bp and 575bp.

Amplification was obtained for *cry*1 gene primer, at an annealing temperature of 55^0C. The amplicon size obtained was less than the expected size of 277bp. Salem *et al.* (2006) identified *cry*1 gene, by producing fragments of 277bp for *cry*1 and 439bp for *cry*4 genes respectively. Similar reports had been made by earlier workers (Carrozi *et al.*, 1991; Chak *et al.*, 1994). The amplicon size obtained with *cry*1 universal primer being less than 277bp, cloning and sequencing of the amplicon was carried out, in order to confirm that the amplicon was part of the *cry*1 gene. Hence, the amplicons obtained from two isolates KY5 and EM11 were eluted and used for cloning. The sequence information confirmed it as *cry*1 gene.

5.9 AMPLIFICATION OF *cry*1A GENE FROM *B. thuringiensis* ISOLATES

PCR was carried out with all isolates found positive in *cry*1 gene profiling, with *cry*1A primer designed during this study. Amplification was obtained for two

isolates namely KY3 and KY7 with *cry*1A primer. The amplicon obtained in the isolate KY3 was subjected to sequencing, after purifying the PCR product. The amplicon was named as *cry*1Aky3.

5.10 CLONING AND SEQUENCING OF *cry*1 and *cry*4 amplicons

Amplified DNA fragments of *cry*1ky5, *cry*1em11 and *cry*4em10 were ligated into pGEMT vector and transformed into E. coli JM 109 cells. Various scientists have cloned the PCR amplified DNA fragments with *cry* gene specific primers after ligating to the pGEMT vector, which was further used for transforming *E.coli* cells (Held *et al.*, 1982; Widner and Whitely, 1988; Aronson *et al.*, 1986; Johnson *et al.*, 1996; Ceron *et al.*, 1995; Escudero *et al.*, 2006). The amplicons obtained with *cry*4 primer in the isolate KY1 and with *cry*1A primer in the isolate KY3 were subjected to sequencing, after purifying the PCR product.

Competence of *E.coli* JM 109 cells was confirmed by transforming the cells with a pUC18 plasmid containing an ampicillin resistance marker. There was luxuriant growth of the competent cells in LBA-ampicillin plates overlaid with X-gal and IPTG, indicating high transformation efficiency, which was in the tune of $5x10^8$cfu/ μg DNA. *E. coli* cells alone could not grow in the media containing ampicillin, since they lacked the resistance encoding sequence. But all the competent cells harbouring the plasmid could grow in that media.

The cloning vector used was pGEMT easy vector having a size of 3kb. It is specially designed for direct cloning of PCR products. It has a 3' terminal thymidine at both ends and the presence of this at the insertion site greatly improves the efficiency of ligation of PCR product onto the plasmids, by preventing recircularization of the vector and provides a single stranded overhang for PCR products generated by certain thermo-stable DNA polymerases. It contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β- galactosidase.

Insertional inactivation of the α-peptide allows the recombinant clones to be directly identified by colour screening of the indicator plate. Ligated product containing *cry* gene sequence was used to transform the *E. coli* cells, which would later be picked up from the media containing 5-bromo 4-chloro 3-indolyl β-D galactosidase (X-gal) and isopropyl thiogalactoside (IPTG) based on blue white screening. The pGEMT vector contained polycloning sites inside a β-galactosidase gene. The bacterial cell and vector together provided the complete protein as a result of α complementation (Ullman *et al.*, 1967). The colonies which have not taken up the plasmid can further utilize the substrate and appear as blue colonies on X-gal chromogenic substrate (Horwitz *et al.*, 1964). All transformed colonies harbouring the recombinant plasmid appeared in white colour due to the disruption of α-complementation.

Plasmids isolated from blue and white colonies gave bands with different molecular weights. All the plasmids isolated from white colonies had higher molecular weight than the pGEMT vector alone. Plasmid from blue colony was self-ligated vector alone, since no recombination has occurred. A band corresponding to 3.1 kb corresponding to the actual size of plasmid was obtained.

5.11 THEORETICAL ANALYSIS OF THE SEQUENCE

The sequences obtained after cloning, were subjected to vector screening to delete the sequences of vector if any present. Vector screening of *cry*1ky5 showed significant similarity with vector, from region starting from 146-611 base pairs. Vector screen of *cry*1em11 showed strong match to vectors starting from region 147 to 487 base pairs. The vector screen of the *cry*4em10 showed no significant similarity to vectors. Hence these regions showing similarity to vector were delected and only the sequences of gene were retrieved.

When the cloned sequences were subjected to Blastn, they were identified as partial *cry*1 and *cry*4 genes. After homology search, *cry*1ky5 and *cry*1em11,

showed hundred per cent similarity with *cry*1, *cry1A*, *cry*1Aa *cry*1Ac, *cry*1B and *cry*1Ba5 gene sequences of various species of *Bacillus thuringiensis* present in NCBI databank. The percentage identity was 100 in *cry*1k5 with the 123 Blast hits whereas in *cry*1em11 also it remained the same with 111 Blast hits reported. Significant homologies was obtained with *cry* gene sequences of *Bacillus thuringiensis* strain Al Hakam (Challacombe *et al.*, 2007), *B.thuringiensis* serovar *konkukian* strain 97-27 (Brettin *et al.*, 2004), *cry*1Ac sequence of *B. thuringiensis* kenyae strain HD-459 (Hire *et al.*, 2005) and *B. thuringiensis* isolate SK-222 (Kaur *et al.*, 2005) and *B. thuringiensis* isolate SK-729 (Kaur and Allam, 2005). Hofte and Whitely (1989) identified *cry*1 gene sequences from bacterial strains.

The *cry*4em10 when subjected to Blastn showed homology with *cry*4A, *cry*4BLB, and other crystal protein genes present in different species of *B. thuringiensis*. Hundred per cent similarity was obtained with that of *cry*4A gene sequence from *B. thuringiensis*, *cry*4BLB gene sequences from *B. thuringiensis* serovar *israelensis* (Zqhal *et al.*, 2007) and delta endotoxin gene of *B. thuringiensis* (Misra *et al.*, 2002). The query coverage was 16 per cent in *cry*4em10 among two accessions CP000485 and AEO17355. cry4ky1 showed significant similarities with cry gene sequences present in different species of *B. thuringiensis*. The *cry*4ky1 showed 23 and 32 per cent query coverage and 100 per cent identity with two accessions CP000485 and AEO17355 respectively. Nucleotide blast analysis of cry1ky3 showed significant homologies with spore coat protein D of *B. thuringiensis* isolates present in NCBI databank.

*cry*1ky5 and *cry*1em11 when subjected to Blastp analysis, showed similarity to δ-endotoxin genes *cry*iiia (Bt13) gene of *Bacillus thuringiensis*. The *cry*4em10 had significant similarity to mosquitocidal protein genes cry4Aa, cry4Ba of *B. thuringiensis* serovar israelensis (Zqhal *et al.*, 2007). The multiple sequence alignment of *cry*4em10 shared conserved regions with *cry*4A and *cry* genes of different species of *B. thuringiensis* in NCBI databank. Phylogram analysis revealed that *cry*4em10 was more related to two strains of bacterium with

accession numbers EF 208904 and Y00423. *cry*4ky1 showed similarity with gene encoding crystal protein of the bacterium.

*cry*1Aky3 showed homologies with crystal protein cryE6L, cytosolic, ATP binding, membrane binding and glucokinase regulatory proteins present in *B. thuringiensis*. A direct comparison of the nucleotide sequences of several crystal protein genes indicated that there is extensive conservation of the amino acid sequences at the carboxy terminal of the gene sequences.

The analysis for discovering nitrogen base composition in *cry*1ky5 indicated that A+T and C+G base pair composition was same in *cry*1ky5. *cry*1em11 revealed that C+G composition (51.0%) was more than A+T (47.7%). *cry*4em10 was comparatively rich in A+T (54.9%) than C+G (45.1%). *cry*4ky1 was comparatively rich in A+T (58.5%). The A+T content of *cry*1Aky3 was high (63.7%) whereas C+G was comparatively low (36.3%). Johnson *et al.* (1996) made similar observations in which the cloned sequences of *cry* genes were rich in A+T compared to C+G bases.

When the ORFs of the cloned sequence were examined, *cry*1ky5 encoded the longest ORF on +2 reading frame, with a length of 141bp. The longest ORF was located on the +3 reading frame for *cry*1em11 sequence, with a length of 127bp. The ORF analysis of *cry*4em10, revealed that the longest ORF (465) was encoded on +1 reading frame. The longest ORF of *cry*4ky1 was located on -3 strand, having a length of 132bp. The *cry*1Aky3 sequence had five open reading frames, three of them located on the plus (+1, +2, +3) strands and two on the minus (-1, -3) strands. The length of the ORF was 114bp.

The theoretical restriction analysis for ten enzymes showed that *Hae*III and *Hinf* III had three sites in *cry*1ky5, whereas *cry*1em11 had two restriction sites for *Hae*III and *Hinf*III. Restriction enzymes *Bam* HI and *Dpn*I and *Mbo*I lacked restriction sites both in *cry*1ky5 and *cry*1em11. The frequent cutter *Alu*I

had six sites in *cry*4em10. In case of *cry*4ky1, *Alu*I had five sites. It lacked restriction sites for *Bsa*HI, *Mbo*I *Bam*HI and *Dpn*I. All the other enzymes had one restriction site in *cry*4ky1. Restriction enzymes *Alu*I and *Mbo*I had two restriction sites whereas *Btg*I, *Bsa*HI, *Sac*II lacked restriction sites in *cry*1Aky3.

'Genscan' tool was used to analyze the exons present in the cloned sequence. No exons were detected in *cry*1ky5 and *cry*1em11. An initial exon has been detected in *cry*4em10 sequence with a length of 465bp. *cry*4ky1 possessed a terminal exon having a length of 224bp. The terminal exon of *cry*1Aky3 had a length of 440 bases, located from the region of base 87 to 552.

Aminoacid analysis revealed composition of different aminoacids in *cry* gene sequences cloned. The molar percentage of serine was found to be highest (18.18%, 8 residues), followed by glycine (13.14%, 6 residues) in *cry*1ky5 sequence. The *cry*1em11 when subjected to aminoacid analysis, identified the aminoacid with highest molar percentage as serine (12.50%, 6 residues) followed by phenylalanine (10.42%, 5 residues). Aminoacid analysis of *cry*4em10 revealed that alanine was the one with highest molar percentage (8.55%, 7 residues), followed by glycine (8.12%, 5 residues). The *cry*4ky1 fragment had leucine with the highest share of 16.90 per cent and 24 residues. In *cry*1Aky3, the amino acid with highest molar percentage was lysine (10.27%, 19 residues) followed leucine and isoleucine (9.19%, 17 residues).

The secondary structure prediction of the cloned sequences showed the proportion of different structures namely alpha helix, beta sheet and random coils. Penetrating through most parts of *cry*1ky5, random coils (43.75%) and α-helix (41.67%) formed the most abundant structural elements, while beta bridges were completely absent. Prediction of secondary structure of *cry*1em11 showed that the sequence possessed mostly random coils accounting to 64.58 per cent. Analysis of *cry*4em10 revealed, that it contained more of random coils (41.07%) followed by extended strand (27.35%), α-helix (24.79%) and β-turns (6.84%). Secondary

structure analysis of *cry*1Aky3 showed that α-helix contributed to 32.79 per cent, closely followed by random coils accounting to 32.43 per cent.

Functional aspects of domains of *cry*4em10 sequence discovered through 'InterProscan' revealed anaphylatoxin/fibulin, thiolase, ferredoxin- iron sulphur binding sites and EGF like region. The 'Motif Scan' of *cry*4em10 revealed similarity to tyrosine kinase region.

The *cry*1ky5, *cry*1em11, *cry*4em10, *cry*4ky1 and *cry*1Aky3 were analyzed for the presence of transmembrane helices. All the sequences were devoid of transmembrane helices. Kyte and Doolittle hydropathy plot analysis was carried out. The sequences were in general rich in hydrophilic amino acids, whereas *cry*4ky1 and *cry*1Aky3 contained both hydrophilic and hydrophobic aminoacids.

5.12.1 AMPLIFICATION OF *cry*1 VARIABLE REGION

PCR of the isolates was carried out with *cry*1gene primer designed by Jaurez- Perez *et al.* (1997). This degenerate primer pair detects any gene belonging to the *cry*1 gene family, since it is designed to match a highly conserved region flanking a variable region. The family band of 1500 to 1600 bp will be detected when multiple *cry*1 genes are present in a single strain. Type primers cannot detect a *cry*1 gene if it is different from all those already known to be present, since these are designed based on a variable region specific to a particular cry1 gene.

In the present study, PCR with family primer gave amplicons of expected size in two isolates, KY4 and KK9. The remaining isolates gave no amplification. The primer used was highly degenerate and amplification was obtained at an annealing temperature of 42 ^{0}C. The results agree with findings of Misra *et al.* (2002) who amplified *cry* gene sequences of 1500bp from *B. thuringiensis* using specific primers.

Some bacterial strains did not react with *cry*1 family primer. This might be due to the reason that they possess other *cry* genes which are not identified by the specific primer used. If template and primers are mismatched, particularly at the 3' end of the primer, amplification would be reduced or eliminated.

Bravo *et al.* (1997) identified that the strains with PCR products of size other than those of predicted ones are candidates for harbouring putative novel *cry* genes. Some of the isolates yielded two bands upon amplification by PCR, with *cry* gene primer. This may be owing to the fact that the strains may harbour one or more genes related to *cry* family.

The preliminary screening by universal primers saves effort by sorting the strains for specific screening, which then produce a PCR product with a unique size for each *cry* gene. Strains with unique PCR product profiles can be easily characterized by performing additional PCR with specific primers.

In the present study, *B. thuringiensis* was isolated from different environments belonging to the Western Ghat regions of Kerala. The isolates were characterized at morphological, biochemical and molecular levels. The insecticidal activity of the isolates was assessed against the major lepidopteran pest on cucurbitaceous vegetables, the pumpkin caterpillar (*Diaphania indica*). Molecular characterization of the isolates were done by PCR. Primers were designed to amplify *cry*1A gene from *B. thuringiensis* isolates. Profiling of *cry*1 and *cry*4 genes in the isolates were carried out. *cry*1 and *cry*4 fragments from the most efficient isolates were cloned and sequenced. Further investigations are required for the amplification of full length *cry* genes from *B. thuringiensis* isolates and their expression. Emphasis must be laid on the identification and isolation of novel *cry/vip* genes from bacterial strains which will be useful in pyramiding of different resistant genes. These can be utilized for developing transgenic crops, with increased resistance to insect attack.

Summary

SUMMARY

The study on 'Cloning of genes encoding insecticidal proteins (*cry/vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005- 2007. The objective of the study was to characterize the *cry* genes from *B. thuringiensis* isolated from Western Ghats of Kerala. The salient features of the study are summarized below.

1. *B. thuringiensis* strains were isolated from three districts, namely Kottayam, Kozhikode and Ernakulam, coming under the Western Ghat region of Kerala.
2. Single colonies of pure cultures were stab inoculated in LBA media in cryostorage vials and maintained under refrigerated conditions.
3. Cultural and morphological characters of the isolates were studied on LBA media, with HD1 as the reference strain. All the isolates produced creamy white, puffy colonies. Form of the colonies varied from circular to irregular, with flat elevation. Margins of the colonies varied from entire to undulate.
4. All isolates were Gram positive as indicated by blue colour upon Gram staining and by the absence of viscous threads after KOH treatment. The cells were rod-shaped, blue in colour and arranged in a chain-like manner. All the isolates were capable of forming endospores.
5. Crystal protein staining with Coomassie brilliant blue revealed the presence of dark bluish crystal protein inclusions. The shape of crystal protein inclusions varied from irregular, spherical, triangular and bipyramidal for different isolates. Four isolates viz., KY1, KY5, KK7 and KK8 isolates produced composite type of crystal proteins. Out of the 11 isolates analyzed, eight produced spherical crystal proteins.

6. Biochemical characterization of the isolates was done using seven-different biochemical tests. All the isolates gave a negative response to KOH, gelatin hydrolysis and Voges- Proskauer test
7. In case of urea hydrolysis, six isolates namely KY1, KY5, KY6, KK7, KK8, EM11 and the reference strain HD1 degraded urea, indicating the presence of urease. Isolates KY2, KY3, KY4, KK9 and EM10 gave negative reaction for urease test. All the isolates except KK9 indicated positive reaction for hydrolysis of starch.
8. Six isolates, namely KY1, KY2, KY4, KY5, KY6 and EM11 along with the reference strain HD1 gave a positive response for the hydrolysis of esculin. The other five isolates namely KY3, KK7, KK8, KK9 and EM10 gave negative reaction.
9. All the isolates including reference strain HD1 produced a clear zone around the growth, which indicated a positive reaction for lecithin.
10. The insecticidal activity of *Bacillus thuringiensis* isolates were assessed by diet contamination method on pumpkin caterpillar, by incorporating crude crystal protein preparations into it.
11. Statistical analysis of daily mortality was carried out using Kendall's Coefficient of Concordance based on mortality on 5^{th} and 8^{th} days. Ranking of the isolates based on larval mortality on 5^{th} day gave the highest score to reference strain HD1. Among the native isolates, KK7 KK8, KK9 and KY2 were on par.
12. The isolates were ranked based on number of days taken for fifty per cent mortality; HD1 was the most efficient which took only two days to reach fifty per cent mortality. Among the native isolates, KY2 was the most efficient one, which took three days to reach fifty per cent mortality. Isolates KK7, KK8, KK9, KY5, KY4 and KY3 took three days to achieve fifty per cent mortality.
13. Total DNA was isolated from *Bacillus thuringiensis* isolates following the procedure of Sambrook and Russel (2001). The quantity of DNA in

the sample varied from 1.337µg/ml to 20.132µg/ml. The OD_{260}/OD_{280} ranged between 1.78 and 1.97 indicating the good quality of DNA.

14. One pair each of gene specific primer was designed based on the homology within the conserved regions of *cry* genes present in different species of *Bacillus thuringiensis*. Primers had a melting temperature of 66^0C and sequence length of 20 to 22 bp.
15. Profiling of *cry*1 and *cry*4 genes from *B. thuringienis* isolates was done using universal *cry*1 and *cry*4 primers. Amplification with universal *cry*1 gene primer was obtained for eight isolates KY2, KY3, KY5, KY6, KK7 KY8, KY9 and EM11 along with the reference strain HD1. Amplicon size of the fragment obtained was approximately 250bp. No amplification was obtained for isolates KY1, KY4 and EM10. To confirm the gene sequence, the amplicons were eluted and cloned.
16. Amplification for *cry*4 gene was obtained for isolates KY1 and EM10. Single band of 575bp was obtained for isolate KY1. For isolate EM10 three bands of size 531, 942 and 1245bp were obtained.
17. PCR of all the isolates found to be positive in *cry*1 gene profiling, was done with *cry*1A primer. Amplification was obtained in two isolates KY3 and KK7. The amplicon obtained from the isolate KY3 was sequenced.
18. The amplified gene fragments obtained with the isolate KY5, EM11 and EM10 were eluted, cloned into pGEMT vector and competent *E.coli* cells were transformed with the ligated product. A combination of blue and white colonies was obtained after overnight incubation confirming successful transformation. High recombination efficiency (63% to 70%) was observed for the three amplicons cloned. The amplicons obtained with universal *cry*4 primer in the isolate KY1 and with *cry*1A primer in the isolate KY3 were subjected to sequencing, after purifying the PCR product.
19. Presence of insert was checked by PCR amplification of the cloned insert. Single amplified bands exactly similar to the genomic DNA

amplification were obtained in plasmids of white colonies. Plasmids isolated from blue colony could not produce any amplification.

20. The cloned insert of *cry*1 and *cry*4 genes were sequenced using T7 universal primer. Vector screening of the sequences were done to detect the regions showing similarity to vectors.
21. Theoretical analysis of the sequence using blastn and blastp progammes showed 100 per cent identity with *cry*1 and *cry*4 genes present in different species of *B. thuringiensis.*
22. Phylogenic tree was constructed with cry4em10 and other five sequences showing maximum homology with this. Restriction analysis revealed distribution pattern of cleavage sites of different restriction enzymes.
23. The secondary structure prediction of the sequences were done using SOPMA programme and it showed that random coils as the major structural components. The amino acid and nucleic acid composition of the cloned sequences were analysed using AASTAT and TACG tools available in Biology Workbench.
24. Gene prediction analysis carried out using the software GENSCAN indicated the presence and location of exons in the sequences. *cry*4em10 had an initial exon with 465 bp length. *cry*4ky1 possessed a terminal exon having a length of 224 bases. *cry*1Aky3 had a terminal exon of 440bp length.
25. The 'Motif Scan' of amino acid sequence of *cry*4em10 revealed similarity to tyrosine kinase region. Functional aspects of domains discovered through 'InterProscan' revealed anaphylatoxin/fibulin, thiolase and ferredoxin- iron sulphur binding sites and EGF like regions.
26. Kyte and Doolittle hydropathy plot analysis revealed the absence of transmembrane regions in the deduced amino acid sequences of all the *cry* genes. All the sequences were in general rich in hydrophilic aminoacids.
27. In order to amplify the variable region of *cry*1 gene having fragment size of 1500 to 1600bp, PCR was carried out using *cry*1 family primers

designed by Juarez-Perez *et al.* (1997). Amplification was obtained in isolates KY4 and KK9.

28. Future research may be concentrated on cloning of novel and full length cry genes from *B. thuringiensis*, which can be expressed in transgenic crops as a means to combat resistance development in insect populations.

Appendices

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* Originals not seen

Annexures

ANNEXURE I

Composition of different media used in the study

1. Luria Bertani (LB) broth

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
pH adjusted to	-	7± 0.2
Distilled water	-	1 l

2. Luria Bertani agar medium

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
Agar	-	20g
pH adjusted to	-	7± 0.2
Distilled water	-	1 l

3. T3 medium

Tryptone	-	3 g
Tryptose	-	2 g
Yeast extract	-	1.5 g
Sodium phosphate	-	1.2 g
pH adjusted to	-	6.8
Distilled water	-	1 l

4. T3 agar medium

Tryptone	-	3 g
Tryptose	-	2 g

Yeast extract	-	1.5 g
Sodium phosphate	-	1.2 g
Agar	-	20 g
pH adjusted to	-	6.8
Distilled water	-	1 l

5. Nutrient gelatin medium

Peptone	-	5 g
Beef extract	-	3 g
Gelatin	-	120 g
pH adjusted to	-	6.8
Distilled water	-	1 l

6. Urea agar medium

Peptone	-	1 g
Glucose	-	1 g
Sodium chloride	-	5 g
Monopotassium phosphate	-	2 g
Phenol red (1.2%)	-	10 ml
Agar	-	20 g
pH adjusted to	-	6.8
Distilled water	-	1 l

(40% urea to a final concentration of 50ml/l after autoclaving).

7. Nutrient agar medium

Peptone	-	5 g
Beef extract	-	3 g
Agar	-	15 g
pH adjusted to	-	7

(Two egg yolks to be added into the medium under sterile conditions, after autoclaving, when the temperature is around 40 to 50^0C).

8. Starch agar medium

Peptone	-	5 g
Beef extract	-	3 g
Soluble starch	-	2 g
Agar	-	15 g
pH adjusted to	-	7
Distilled water	-	1 l

9. Voges Proskauer medium

Peptone	-	5 g
D- glucose	-	8 g
Disodium phosphate-		5 g
pH adjusted to	-	6.9
Distilled water	-	1 l

10. SOC medium

Tryptone	-	2 g
Yeast extract	-	0.5 g
1M NaCl	-	1 ml
1M KCl	-	0.25 ml
2M Mg^{+2} stock	-	1 ml
2M glucose	-	1 ml
pH adjusted to	-	6.9

Both Mg^{+2} stock and glucose should be filter sterilized before adding to the media.

11. Artificial medium for rearing pumpkin caterpillar

Sorbic acid	-	1.30 g
Methyl para benzoate	-	1.0 g
Cholestrol	-	0.5g
Ascorbic acid	-	4.0 g
Casein	-	15.0 g
Sucrose	-	30.0g
Chickpea flour	-	30.0g
Wheat bran	-	50.0g
Yeast	-	10.0g
Pumpkin	-	100.0g
Formaldehyde(40%)	-	1ml
Abdec drops	-	2ml
Vegetable oil	-	1ml
Vit. E (200mg)	-	1 capsule
Agar agar	-	10g
Distilled water	-	740ml

100g pumpkin is cooked in 300ml water

Yeast is dissolved in 50ml water

Vit. E is dissolved in 50ml water

ANNEXURE II

I. Reagents used for Gram staining

1. Crystal violet/ Gram stain

Solution A

Crystal violet (90% dye content)	-	2 g
Ethyl alcohol (95%)	-	20 ml

Solution B

Ammonium oxalate	-	0.8 g
Distilled water	-	80 ml

Mix solution A & B

2. Gram's iodine

Iodine	-	1 g
Potassium iodide	-	2 g
Distilled water	-	300 ml

3. Ethyl alcohol

Ethyl alcohol (100%)	-	95 ml
Distilled water	-	5 ml

4. Safranine

Safranine O	-	0.25 ml
Ethyl alcohol	-	10 ml
Distilled water	-	100 ml

II. Reagents used for endospore staining

1. Amidoblack (1.5%)

Amidoblack	-	1.5 g
Acetic acid	-	10 ml
Methanol	-	50 ml
Distilled water	-	40 ml

2. Carbol fuchsin (1%)

III. Reagent used for crystal protein staining

Coomassie Brilliant Blue (CBB)

CBB powder	-	0.25 g
Glacial acetic acid	-	7 ml
Absolute alcohol	-	50 ml
Distilled water	-	43 ml

IV. Reagent used for VP test

1. Barrit's reagent A

Alpha naphthol	-	5 g
Absolute ethanol	-	95 ml

2. Barrit's reagent B

Potassium hydroxide	-	40 g
Creatine	-	0.3 g
Distilled water	-	100 ml

ANNEXURE III

I. Reagent used for crystal protein harvesting

1. TE Buffer

10Mm Tris HCl (pH 8.0)	-	10 ml
1Mm EDTA(pH 8.0)	-	5 ml
Distilled water	-	1 l

Autoclaved and stored at room temperature

2. PMSF(Phenyl Methyl Sulfonyl Flouride)

PMSF	-	1.7 g
Propanol	-	100 ml

3. 0.5M NaCl

I1. Reagent used for DNA isolation

1. 10mM Tris Cl

1M Tris Cl (pH -8.0)	-	1ml
Distilled water	-	100 ml

2. 100mM NaCl

1M NaCl	-	10 ml
Distilled water	-	100 ml

3. TE buffer

(Tris Cl- 50mM; EDTA	-	20mM)
Tris Cl - .05 M (pH -8.0)	-	0.394 g
.02M EDTA (pH -8.0)	-	0.372 g
Distilled water	-	100 ml

4. Lyzozyme stock

Lyzozyme	-	50 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 50 mg lyzozyme in 1 ml water and was stored under refrigerated conditions.

5. RNase stock

RNase	-	10 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 10 mg RNase in 1 ml water and was stored under refrigerated conditions at -20^{0}C.

6. 2 % SDS in TE buffer

SDS	-	2 g
TE buffer	-	100 ml

7. Proteinase K

Proteinase K	-	20 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 20 mg Proteinase K in 1 ml water and was stored under refrigerated conditions at -20^{0}C.

8. Phenol: chloroform(1:1 v/v)

To 1 part of Tris saturated phenol, 1 part of chloroform was added.

9. Chloroform: isoamylalcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamylalcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10. 3M sodium acetate

Sodium acetate	-	20.412 g
Distilled water	-	50 ml

11. Chilled isopropanol

12. 70% ethylacohol

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

ANNEXURE IV

Buffers and dyes used in gel electrophoresis

1. 6x Loading/ tracking dye

Bromophenol blue	-	0.25%
Xylene cyanol	-	0.25%
Glycerol	-	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature

ANNEXURE V

I. Reagent used for competent cell preparation

1. Solution A

   Ice- cold 100mM $CaCl_2$

II. Reagents used for plasmid isolation

1. Solution I (Resuspension buffer)

Glucose	-	50mM
Tris	-	25mM
EDTA	-	10mM
pH	-	8.0

2. Solution II (Lysis buffer)

NaOH	-	0.2 M
SDS	-	1 %

3. Solution III

CH_3COOK	-	5M
pH	-	5.5

CLONING OF GENES ENCODING INSECTICIDAL PROTEINS (*cry* / *vip* genes) OF *Bacillus thuringiensis* FROM WESTERN GHATS OF KERALA

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The study entitled 'Cloning of genes encoding insecticidal proteins (*cry/vip* genes) of *Bacillus thuringiensis* from Western Ghats of Kerala' was carried out in the Molecular Biology Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005- 2007. The crystal protein genes (*cry/vip*) of *B. thuringiensis* possess insecticidal activity against larvae of insect orders Lepidoptera, Diptera and Coleoptera. In the present study, an attempt was made to isolate and clone *cry* genes of *B. thuringiensis* from the Western Ghats of Kerala.

Bacillus thuringiensis strains were isolated from soil samples collected from different locations of the Western Ghats of Kerala. The pure colonies obtained were stab inoculated and stored under refrigerated conditions. Variability among the isolates were studied by various cultural, morphological and biochemical tests. The insecticidal activity of the isolates was determined by bioassay against the major lepidopteran pest of cucurbitaceous vegetables, the pumpkin caterpillar.

The information on *cry*1A gene sequences of different species of *Bacillus thuringiensis* available in the public domain NCBI was collected and subjected to multiple sequence alignment to detect conserved boxes of the gene among species. Based on the data, one pair of gene specific primer was designed for amplification of partial *cry1*A gene fragment of about 800bp in *B. thuringiensis* isolates.

Total DNA was isolated from the *B. thuringiensis* strains of Western Ghats of Kerala. Profiling of *cry1* and *cry*4 genes of bacterial isolates were done using universal primers for *cry*1 and *cry*4. Amplification was obtained with *cry*1 gene for seven isolates and with *cry*4 gene primer for two isolates. The amplicons

obtained with universal *cry1* primer from two isolates and with *cry*4 primer from one isolate were used for cloning.

The amplicons obtained with *cry*1 and *cry*4 primers were eluted, cloned in pGEMT vector and transformed into competent cells. High level of recombination was observed on blue-white screening. Recombination of the insert was confirmed by PCR of the plasmid isolated from white colonies. The cloned fragments were sequenced. The amplicon obtained with *cry*4 primer in the second isolate was sequenced after purifying the PCR product.

The *cry*1ky5 and *cry1*em11 sequences when subjected to Blast search revealed significant levels of homology with *cry*1 genes reported from other *B. thuringiensis* strains deposited in the public domain. The *cry*4em10 sequence when subjected to Blast search, showed high level of similarity with *cry*4 genes from *B. thuringiensis.* The *cry*4ky1 sequence showed similarity with *cry* genes of different species of *B. thuringiensis.* The sequences were also subjected to various sequence analysis using bioinformatics tools which include ORF finder, SOPMA, GENSCAN, AASTAT and TCAG tools of Biology Workbench and Interproscan.

PCR of all the isolates found positive in *cry*1 gene profiling, was done with *cry*1A primer designed during this study. Amplification was obtained with *cry*1A primer in two isolates. The amplicon obtained in one isolate was subjected to sequencing after purifying the PCR product. The *cry*1Aky3 sequence showed similarity with other *cry* genes of *Bacillus thuringiensis* present in the NCBI databank. 1500 bp long variable region of *cry*1 was amplified in two isolates using specific primers.

Future research works should be focused on the isolation of *B. thuringiensis* from completely undisturbed ecological niches. Novelty of *cry* genes can be detected by restriction digestion of the genes. Characterization of novel full-length *cry* genes and its expression in transgenic crops will help to develop resistant varieties thereby reducing insecticide applications and resistance development in insect pest populations.