Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.20, 2016

Isolation and Characterization of Mosquitocidal Bacillus Thuringensis, Analysis of Its Crystal Proteins and Plasmid

WILDA ONYANCHA
PG AND RESEARCH DEPARTMENT OF MICROBIOLOGY, K.S.RANGASAMY COLLEGE OF ARTS AND SCIENCE

1.INTRODUCTION

Today, India ranks second worldwide in farm output. Agriculture sector employs nearly 58% of India's workforce and constitutes 22% of total Indian economy. With sowing and building up at a rapid pace for the majority of crops, crop production is also anticipated to increase by 2.5 % during the next financial years to come. The growth in the GDP of agriculture and allied sectors is anticipated at 3.2% during the financial year 2009, as stated by the Centre for Monitoring Indian Economy (CMIE). India is the largest producer in the world of milk, cashew nuts, coconuts, tea, ginger, turmeric and black pepper (Agribusiness in India, 2009). Our country is the second largest producer of wheat, rice, sugar, cotton, silk, peanuts and the third largest producer of tobacco. India is the largest fruit producer, accounting for 10% of the world fruit production. It is the leading producer of bananas, sapotas and mangoes (Indian agriculture ,2008)

Out of 750,000 identified species , about 10,000 species of insects are important as pest throughout the world. Over 1,800 species of weeds out of the known 30,000 cause serious economic loss. About 15,000 species of nematodes produce more than 1,500 serious deleterious effects on plants. Over 1,00,000 species of pests destroy food which could be food for 135 million people. The word pest has no biological meaning. Pests are organisms that diminish the value of resources in which we are interested. In India, crops are affected by over 200 major pests, 100 plant diseases, hundreds of weeds and other pests like nematodes, harmful birds, rodents and the like. About 4,800 million rats cause havoc in India. Approximately, 30% of Indian crop yield potential is being lost due to insects, disease and weeds which in terms of quantity would mean 30 million tones of food grain. The value of total loss has been placed at Rs 50,000 million, represents about 18% of the gross national agriculture production. (Manncsa, 2009).

An insecticide is a pesticide used against insects in all developmental forms. Insecticides include ovicides and larvicides used against the eggs and larvae of insects. Insecticides are widely used in agriculture, medicine, industry and household. The use of insecticides is believed to be one of the key features behind the increase in agriculture productivity in the last century. Nearly all types of insecticides have the potential to considerably change the ecosystem. Many of the insecticides are toxic to human body and others are intense in food chain. So far, use of synthetic chemical pesticides had been the widely used approach for reducing the estimated 45% gross crop loss due to pests and diseases, amounting to around Rs. 290 billion per annum. More and more quantities of chemicals are used for agricultural intensification to feed an ever growing population. In fact, the pest induced loss is on the rise despite increasing usage of pesticides. Fortunately, realization of the negative effects of these chemicals on nature and natural resources like pollution, pesticide residue, pesticide resistance etc, have forced many to shift focus on to more reliable, sustainable and environment friendly agents of pest control, the biopesticides. In spite of the claimed efficacy, their use, however, has remained very low due to a number of socio-economic, technological and institutional constraints. Nonetheless, rise in income levels due to a growing economy coupled with increasing awareness of health related effects of chemical pesticides has increased the demand of organic food. In view of this demand and the government's efforts to mitigate climate change, biopesticides are going to play an important role in future pest management programmes. (Sinha and Biswas, 2008).

A pesticide that is of biological origin i.e., viruses, bacteria, pheromones, plant or animal compounds is known as biopesticide. Or simply origin of the active ingredient of a biopesticide is natural not synthetic. They are highly specific affecting only the targeted pest or closely related pests and do not harm humans or beneficial organisms while chemical pesticides are broad spectrum and known to affect non-target organisms including predators and parasites as well as humans. The striking feature of biopesticides is environment friendliness and easy biodegradability, thereby resulting in lower pesticide residues and largely avoiding pollution problems associated with chemical pesticides. Further, use of biopesticides as a component of Integrated Pest Management (IPM) programs can greatly decrease the use of conventional (chemical) pesticides, while achieving almost the same level of crop yield. However, effective use of biopesticides demands understanding of a great deal about managing pests especially by the end users. In terms of production and commercialization also biopesticides have an edge over chemical pesticides like low research expenditure, faster rate of product development as well as flexible registration process (Sinha and Biswas, 2008).

Biopesticides represent only 2.89% (as on 2005) of the overall pesticide market in India and is expected to exhibit an annual growth rate of about 2.3% in the coming years. In India, so far only 12 types of biopesticides

have been registered under the Insecticide Act, 1968 . Neem based pesticides, *Bacillus thuringensis*, NPV and *Trichoderma* are the major biopesticides produced and used in India .Whereas more than 190 synthetics are registered for use as chemical pesticides. Most of the biopesticides find use in public health, except a few that are used in agriculture. Besides, i) transgenic plants and ii) beneficial organisms called bio-agents: are used for pest management in India. Consumption of biopesticides has increased from 219 metric tons in 1996-97 to 683 metric tons in 2000-01, and about 85% of the biopesticides used are neem based products. Consumption of chemical pesticides has significantly fallen from 56,114 MT to 43,584 MT during the same period (_Sinha and _Biswas, 2008).

Bacillus thuringiensis is a ubiquitous, gram-positive and spore-forming bacterium. During sporulation, it produces intracellular crystal proteins (cry proteins), which are toxic to insects. *Bacillus thuringiensis* (Bt) is a well known and widely studied bacterium which is known for its use in pest management. Because of its insecticidal activity, it has been used for nearly fifty years to control certain insect species among the orders Lepidoptera, Coloeptera, and Diptera. *B. thuringiensis* is closely related to *B.cereus*, a soil bacterium, and *B. anthracis*, the cause of anthrax: the three organisms differ mainly in their plasmids. Like other members of the genus, all three are aerobes capable of producing endospores. (Madigan and Martinko , 2005). Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins (called crystal proteins or Cry proteins), which are encoded by *cry* genes. In most strains of *B. thuringiensis* the *cry* genes are found within the bacterial plasmid. (Stahly *et al* ., 1978).

The genetic diversity of *B. thuringiensis* strains shows differences according to the regions where they were isolated. Thus, each habitat may contain novel *B. thuringiensis* strains, which have some toxic effects on target spectra of insects. Today, Bt is the most successful commercial xenobiotic with its worldwide application. When compared with the chemical pesticides, Bt has the advantages of being biologically degradable, selectively active on pests and less likely to cause resistance. Safety of Bt formulations for humans, beneficial animals and plants explains the replacement of chemical pesticides in many countries with these environmentally friendly pest control agents.

B. thuringiensis was first isolated by the Japanese scientist Ishiwata (1901) from silkworm larvae, *Bombyx mori*, exhibiting sotto disease. After 10 years, Berliner (1911) isolated the same Gram (+), spore-forming, rod shaped soil bacterium from diseased flour moth larvae, *Anagasta kuehniella*, in the Thuringia region of the Germany and named it as *Bacillus thuringiensis*.

In the early 1930s, Bt was used against *Ostrinia nubilis*, the European corn borer. The first commercial product was available in 1938 in France, with the trade name Sporeine (Weiser, 1986). It was Bt subspecies *kurstaki* that was used for the control of the insect (Lepidopteran) pests in agriculture and forestry (Lhy and Ebersold, 1981). New commercial products arrived in 1980s after the discovery of subspecies israelensis in 1976 (Goldberg and Margalit, 1977). Bt *israelensis*, being a Diptera active subspecies, opened the gate for black fly and mosquito larvae control. Dulmage discovered more active *B. thuringiensis* var. *kurstaki* (HD1), which was commercialized in the USA as Dipel .The demand of *B. thuringiensis* based insecticides in agriculture sector declined, in the mid 1970s, because of more effective chemical pesticides. In the 1980s, *B. thuringiensis* research was stimulated by progress in biotechnology. First, Schnepf and Whiteley (1981) cloned a crystal toxin gene from *B. thuringiensis* subsp. k*urstaki* into *E.coli*, since then much research has been performed to improve target spectra and to find out more infectious strains of *B. thuringiensis.*

The larvicidal activity of Bt is attributed to parasporal crystals that it produces. Because of the crystalline structure of these crystals, they are called as crystal proteins (Cry) or delta endotoxins. The genes encoding for the crystal proteins are named as cry genes, and their common characteristic is the expression of the genes during the stationary phase. Therefore, the crystal proteins accumulate in the mother cell and are released upon completion of sporulation. Also a cytolytic protein, cytolysin (Cyt), is found in the crystal inclusions of the Diptera active strains.

Bt can be isolated from several different sources and different environments (Bernard, 1986; Martin and Travers, 1989; Smith and Couche, 1991). The natural strains can be classified according to their biochemical properties and flagellar (H) antigens (de Barjac and Fracon, 1990). To determine the pathotype of the strains, this kind of a classification is not appropriate; instead delta endotoxin composition is the criterion that determines the range of insect pests on which the organism is active.

The insecticidal crystal proteins of Bt are known to be encoded by large plasmids and cry gene sequences homologues to the ones in plasmids also occur in chromosomes (Gonzalez *et al*., 1981; Kamdar *et al*., 1983, Honigman *et a*l., 1986; Carlson and Kolsto, 1993).

The cry genes have been introduced into several plants. The first plant to express these genes was tobacco (Vaeck *et a*l., 1987; Barton *et al*., 1987). Later on, potato, cotton, rice, corn were transformed and at 1996, transgenic potato, cotton and corn carrying cry genes were sold to farmers (Schnepf *et al*., 1981). As these crops provide effective control of major insect pests, farmers rapidly adopted the Bt-crops to provide higher yields (Betz *et al*., 2000).

Apart from transgenic Bt-plants, Bt formulations themselves are also very important in plant protection. These formulations are also used for the black fly and mosquito control. The formulations can include wetting agents, stickers, sunscreeners, synergists and phagostimulants (Navon, 2000).

Hence the present investigation is aimed to study the following objectives :

i. Screening of novel isolates of *Bacillus thuringiensis* and testing their insecticidal property.
ii. Extraction of crystal proteins, protein profiling using SDS-PAGE and analysis plasmid DNA analysis of . *Bacillus thuringiensis* isolates.

2. REVIEW OF LITRATURE

Human attempts on insect control have been changed over time from natural methods to synthetic chemical control and now again the trend changed to natural methods. While synthetic insecticides are presumably safer and less persistent than those used in the past, such as DDT, they are still a cause for concern. Long-term exposure to modern synthetic insecticides has been associated with cancer, liver damage, immunotoxicity, birth defects, and reproductive problems in humans and other animals. This is why development of biologically natural methods of insect control, or biopesticides, is preferential today. (Kegley and Wise, 1998)

Bacillus thuringiensis as insecticide

Bacillus thuringiensis (Bt) first became available as a commercial insecticide in France in 1938 and in the 1950s entered commercial use in the United States. For many years, Bt primarily came in the form of a spray to be applied to crops. The non-persistent nature of the insecticide necessitated many reapplications during early use (van Frankenhuyzen, 1993).

In the 1980s, commercial interest in Bt grew very rapidly as many popular synthetic insecticides became ineffective due to insect resistance, or became unusable due to environmental restrictions, and as the field of genetic engineering grew. In 1987 came the first reports of insertion of genes encoding for Bt delta-endotoxins into plants. The first transgenic plants to express Bt toxins were tobacco and tomato plants (van Frankenhuyzen, 1993). The first Bt plant-pesticide, Bt field corn, was registered with the United States Environmental Protection Agency in 1995 (USEPA, 1999b). Today, major Bt transgenic crops also include corn, cotton, potatoes, and rice. The engineering of plants to express Bt delta-endotoxins has been especially helpful against pests that attack parts of the plant that are usually not well-protected by conventional insecticide application. A prime example of this is protection against *Ostrinia nubilalis*, the European corn borer. Larvae of this lepidopteran bore into the stalk of a corn plant and destroy its structural integrity. In the stalk, the pest is relatively safe from pesticide application. With toxins engineered into the plant, *O. nubilalis* is exposed and its damage becomes easier to control (Ely, 1993). Overall, because of benefits such as these, Bt has become a major presence in agriculture. In 1997, Bt cotton, corn, and potatoes covered nearly 10 million acres of land in the United States alone. These crops have also been commercialized and are in wide use in Canada, Japan, Mexico, Argentina, and Australia (Frutos et al., 1999). While using Bt in the form of transgenic crops is now very common, the more traditional spray form of Bt is still widely used (Liu and Tabashnik, 1997).

General Characteristics of *Bacillus thuringiensis*

B. thuringiensis is a member of the genus *Bacillus* and like the other members of the taxon has the ability to form endospores that are resistant to inactivation by heat,desiccation and organic solvents. The spore formation of the organism varies from terminal to sub terminal in sporangia that are not swollen, therefore, *B. thuringiensis* resembles other *Bacillus* species in morphology and shape (Stahly *et al.,* 1991). The organism is a gram-positive and facultative anaerobe. The shape of the cells of the organism is rod. The width of the rod varies 3-5µm in size when grown in standard liquid media. The most distinguishing feature of *B. thuringiensis* is the presence of a parasporal crystal body that is near to the spore, outside the exosporangium during the endospore formation (Andrews *et al*., 1985; Andrews *et al*., 1987; Bulla *et al*.,1995).

Ecology and Prevalence of *Bacillus thuringiensis*

B.thuringiensis is indigenous to many environments including soil (Martin and Travers, 1989; Bernard *et al*., 1997), insect cadavers (Corazzi *et al*., 1991; Kaelin *et al*.,1994; Itaqou-Apoyolo *et al*., 1995; Lopez-Meza and Ibarra, 1996; Cadavos *et al*., 2001),stored product dust (Chambers *et al*., 1991; Meadows *et al*., 1992; Hongyu *et al*., 2000),leaves of plants (Smith and Couche, 1991; Bel *et al*., 1997; Mizuki *et al*., 1999), and aquatic environments (Iriarte *et al*., 2000; Ichimatsu *et al*., 2000). Moreover, *B.thuringiensis* has recently been isolated from marine sediments (Maeda *et al*., 2000),and also from the soils of Antarctica (Forsty and Logan, 2000). Thus, it is obvious that *B. thuringiensis* is widespread in nature. However, the normal habitat of the organism is soil. The organism grows naturally as a saprophyte, feeding on dead-organic matter, therefore, the spores of *B. thuringiensis* persist in soil and vegetative growth occurs when nutrients are available. Because of this, *B. thuringiensis* can also be found in dead insects.

Meadows (1993) suggested three prevailing hypothetical niches of *B. thuringiensis* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism. However, the true role of the bacteria is not clear. The insecticidal activity of *B. thuringiensis* are rare in nature. For instance, Iriarte *et al.*

(2000) reported that there is no relationship between mosquito breeding sites and pathogenic action level of *B. thuringiensis* in the surveyed aquatic habitats. However, another study suggests that habitats with a high density of insect mortality were originated by the pathogenic action of this bacterium (Itoqou-Apoyolo *et al*., 1995).

Morphological Properties of *Bacillus thuringiensis*

Colony morphology can help to distinguish *B. thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough clonies, which spread out and an expand over the plate very quickly. *B. thuringiensis* strains have unswollen and ellipsoidal spores that are in the subterminal position. The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *B. thuringiensis* from other closely related *Bacillus* species. The morphology, size, and number of parasporal inclusions may vary among *B. thuringiensis* strains.

However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 proteins (Aronson *et al*., 1976); cuboidal inclusions related to Cry 2 proteins and usually associated with bipyramidal crystals (Ohba and Aizawi, 1986); amorphous and composite crystals releated to Cry 4 and Cyt proteins (Federici *et al*., 1990); and flat, square crystals, related to Cry 3 proteins (Hernstadt *et al*., 1986 ; Lopez-Meza and Ibarra, 1996). Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains. Bernard *et al*. (1997) isolated 5303 *B. thuringiensis* from 80 different countries and 2793 of them were classified according to their crystal shape. They reported that the proportion with bipyramidal shaped crystals was 45.9%, while 14 % were spherical and 4 % rectangular.

Insecticidal Crystal Proteins of *Bacillus thuringiensis*

The crystal proteins of *B. thuringiensis* show host specificity. For this reason,each type of Cry protein can be toxic to one or more specific insect species. Because of this specific toxicity, they do not affect many beneficial insects, plants and animals including humans. The specificity of these insecticidal crystal proteins (ICPs) derives from their mode of action (Adang, 1991; Gill *et al*., 1992). The parasporal crystals of *B. thuringiensis* contain the ICPs in the form of protoxins. After ingestion of parasporal crystals by the susceptible insect, the crystals are dissolved in alkaline conditions (pH 10-12) in the insect mid-gut, generating 130 to 135 kDa protein chains called protoxin. These proteins are then processed to the actual toxic fragments of 60-65 kDa by the gut proteases (Gill *et al*., 1992; Höfte and Whiteley, 1989; Knowles, 1994). Finally, these activated toxins bind to specific receptors present in the larval mid-gut epithelia. The activated toxin binding to thespecific receptors on the cell membrane creates ion channels or pores. The pore formation causes osmotic shock. As a result of this process, the cell membrane lyses, paralysis occurs and consequently, the insect stops feeding and dies from starvation (Knowles, 1994).

Application of Cry Proteins for the pest control

Bacillus thuringiensis is at present considered to be the prevailing form of biologically produced pest control, and is commonly referred to simply as Bt (Smith *et al*, 1996). Back in 1995, worldwide sales of *Bt* reached $90 million (Smith *et al*, 1996), prompting the motion towards a natural alternative to hazardous synthetic pesticides. Although time consuming, it has become well recognized that Cry-based pesticides generally have low costs for development and registration. Astoundingly the cost of Bt pesticides is estimated at 1/40th that of a comparable novel synthetic chemical pesticide (Becker & Margalit, 1993). These pesticides are based primarily on the strain *Bacillus thuringiensis* HD-1 subsp. *kurstaki* (Dulmage *et al*, 1970), which produces CrylAa, CrylAb, CrylAc, and Cry2Aa toxins. The huge success that was achieved by these projects were reflected in results throughout the forestry world, encompassing more than
one pest species.

Bacillus thuringiensis subsp. *israelensis* has become one of the most effective and potent biological pesticides in attempts to combat mosquitoes and blackflies, insect pests capable of spreading fatal human diseases. Mosquitocidal activity has been identified through tests conducted with Cry2Aa, CrylAb and Cry1Ca . Many new uncharacterized isolates containing uncharacterized *cry* genes have also been shown to display mosquitocidal activity (Ragni *et a*l, 1996).

Various Pathogenic Factors of *Bacillus thuringiensis*

Certain strains of *B. thuringiensis* produce extracellular compounds, which might contribute to virulence. These extracellular compounds include phospholipases, β-exotoxins, proteases, chitinases and vegetative insecticidal proteins (VIPs) (Zhang *et al*., 1993; Levinson, 1990; Estruch, 1996; Lövgren, 1990; Schnepf *et al*., 1998). *B. thuringiensis* also produces antibiotic compounds having antifungal activity (Stabb *et al*., 1994). However, the cry toxins are more effective than these extracelluler compounds and allow the development of the bacteria in dead or weakened insect larvae. Some strains of *B. thuringiensis* produce a low molecular weight, heat stable toxin called β-exotoxin, which has a nucleotide-like structure. Because of its nucleotide like structure it inhibits the activity of DNA-dependent RNA polymerase of both bacterial and mammalian cells (Glazer and Nikaido, 1995).

B. thuringiensis strains also produce a protease, which is called inhibitor A. This protein attacks and selectively destroys cecropins and attacins which are antibacterial proteins in insect. As a result of this, the

defense response of the insect collapses. The protease activity is specific, because it attacks an open hydrophobic region near the C-terminus of the cecropin and it does not attacks the globular proteins (Dalhambar and Steiner, 1984). Other important insecticidal proteins, unrelated to Cry proteins, are vegetative insecticidal proteins (VIPs). These proteins are produced by some strains of *B.thuringiensis* during vegetative growth. These VIPs do not form parasporal crystals and are secreted from the cell. For this reason, they are not included in the Cry protein nomenclature. For example, the VIP 1A gene encodes a 100 kDa protein which is processed from its N-terminus. This processing produces an 80 kDa product, which has been shown to be toxic to western corn root warp larvae (Schnepf, 1998).

The *cry* Genes

The genes coding for the insecticidal crystal proteins are normally associated with plasmid of large molecular mass (Gonzales and Carlton, 1980). Many Cry protein genes have been cloned, sequenced, and named *cry* and *cyt* genes. To date, over 100 *cry* gene sequences have been organized into 32 groups and different subgroups on the basis of their nucleotide similarities and range of specificity (Crickmore *et al*., 1998; Bravo *et al.,* 1998). For example, the proteins toxic for lepidopteran insects belong to the Cry 1, Cry 9, and Cry 2 groups. The toxins against coleopteran insects are the Cry 3, Cry 7, and Cry 8 proteins and Cry1Ia1, which is a subgroup of Cry 1 proteins. The Cry 5, Cry 12, Cry 13 and Cry 14 proteins are nematocidal, and the Cry 2Aa1, which is a subgroup of Cry 2 proteins, Cry 4, Cry 10, Cry 11, Cry 16, Cry 17, Cry 19, and Cyt proteins are toxic to dipteran insects. Each of the *B. thuringiensis* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal protein. Transfer of plasmids among *B. thuringiensis* strains is the main mechanism for generating diversity in toxin genes (Thomas *et al*., 2001).

It has been known since the 1980s that the crystal toxins are encoded by genes on plasmids of *Bacillus thuringiensis*. There can be 5 or 6 different plasmids in a single *Bacillus thuringiensis* strain, ranging in size from 0.5-6.7 Kbp, and these plasmids can encode different toxin genes. Most δ -endotoxin genes are found on large plasmids which are either self transmissible or can be co-transferred from a donor to a receptor strain in a conjugation process, so there are a potentially wide variety of strains with different combinations of crystal toxins (Bernhard *et al*, 1997)

The development of Bt pesticides

Bt produces such a large range of toxins which exhibit different targetspecificity, it is necessary to carefully select the effect which is desired to insure non targeted insects are not eradicated. A cassette of desirable genes can be engineered to produce a cocktail of protoxins would by far be more beneficial than making use of isolated protoxin with potential damaging results (Wu *et al*, 1994). Such gene cocktails have been successfully produced through introduction of desired genes into to a host cell by means of conjugation-like systems (Gonza´lez *et al*, 1982) and electroporation technology (Belliveau & Trevors, 1989). Shuttle vectors have also been employed to transfer plasmid replicons containing cloned *cry* genes into host Bt cells to introduce additional genes, thus upgrading the cells natural artillery (Gonza´lez *et al*, 1982). Understanding of the nature of *cry* gene expression has led to the production of many beneficial pesticides and with knowledge of transformation and genetic manipulation, a foundation has been laid for potentially more effective biopesticides.

3. MATERIALS AND METHODS

Sample collection

Soil samples were collected from different agricultural and non- agricultural fields at 15 locations of Mizoram of India. Top layer of soil (about 1 cm) was taken. From each location, five to seven samples each of about 10 g were collected from different spots. Each samples were mixed thoroughly and put in polythene packets and transferred to lab for further processing.(Ohba and Aizawa, 1986).

Isolation of *Bacillus thuringiensis*

Processing of Soil samples:

All soil samples were transported to the laboratory in sterile 200-ml glass bottles. The soil stock was sampled into 2g of soil with 10ml of saline solution in a centrifuge tube (Travers *et al*, 1987). The samples were heat shocked at 80°C for 10 minutes to eliminate all bacteria incapable of producing endospores. Since it is known that *Bacillus thuringiensis* produces spores, it was safe to assume that if it was present in the soil, it would be in our heat treated sample (Travers *et al*, 1987).

Inoculation onto Nutrient agar plates:

The samples were diluted 5 fold to eliminate the amount of humic material within the samples and to reduce the overall colony forming units within each sample. The diluted sample were cultured on nutrient agar and observed. Those colonies resembling the smooth round shape, and earthy colour of *Bacillus* strains were selected for further testing.

Once the sporulating bacteria were isolated, they were plated on nutrient agar plates for 24 hours at 30°C in order to give the spores chance to germinate on media with adequate nutrients and at optimal temperature (Travers *et al*, 1987). This media however offers favorable growth conditions for a wide range of

bacteria as well as *Bacillus thuringiensis*. In order to compensate for this a series of selection tests were further employed to isolate *Bacillus thuringiensis* from the range of bacteria present in the crude soil sample population (Travers *et al*, 1987).

Characterization of *Bacillus thuringiensis* Isolates

Morphological Characterization

Gram staining :

A very small inoculum of bacteria was smeared onto a clean slide using an inoculation loop. The sample was diluted with a drop of sterile water and allowed to air dry. Then specimen was heat-fixed by passing the slide through an open flame. Gram staining procedure was performed as per the standard protocol . Slides were observed under light microscopy under oil immersion (Provine & Gardner, 1974; Bergey's Manual of Systematic Bacteriology, 1986).

Endospore stain (Schaeffer-Fulton staining method):

A small inoculum of bacteria was smeared onto a clean slide using an inoculation loop and diluted with a drop of sterile water. Schaeffer-Fulton staining method was performed as per the standard protocol .Once the slides had dried, the specimens were observed under a compound microscope with oil immersion (Mormak & Casida, 1985; Bergey's Manual of Systematic Bacteriology, 1986).

Biochemical Characterization

Catalase test :

The test involved adding hydrogen peroxide to each sample of bacteria . Catalase test performed as per the standard protocol. The presence of bubbles indicated the ability to break down hydrogen peroxide into water and oxygen (Bergey's Manual of Systematic Bacteriology, 1986); (Reagents and Tests, in Bailey & Scott's Diagnostic Microbiology, 1978).

Indole test:

Catalase test performed as per the standard protocol. A positive reaction indicated by the presence of cherry red colour ring. (Bergey's Manual of Systematic Bacteriology, 1986)

Voges– proskauer test:

VP test performed as per the standard protocol. The change of surface colour to pink in 10 -15 min indicated the positive for VP test. . (Bergey's Manual of Systematic Bacteriology, 1986)

Nitrate Reduction test:

Nitrate Reduction test performed as per the standard protocol. A positive reaction indicted by the red colour to the addition of zinc dust while negative reaction colorless. (Reagents and Tests, in Bailey & Scott's Diagnostic Microbiology, 1978).

Starch hydrolysis test:

The test organism was inoculated on to the starch agar plates. The plates were incubated at 35 ˚C for 24 – 48 hrs. After incubation the plates were flooded with iodine solution. A clear zone around the growth indicates hydrolysis and un hydrolysed area seen blue colour.

D- mannitol test:

D- mannitol test performed as per the standard protocol.(Bergey's Manual of Systematic Bacteriology, 1986). No change in colour of medium indicate negative for fermentation of D- mannitol . *B .thuringiensis* seldom utilizes D-mannitol.

Larvicidal assay activity against mosquitoes larvae

Collection of Mosquito larvae:

Mosquito (Diptera) larvae were collected from the ditches and stagnant water pits in local area around Thokavadi, Tiruchengode, for insect bioassay. The larvae were identified as *Culex* spp.

Larvicidal assay :

Bioassays of the isolated cultures of *B..thuringiensis* were essentially carried out as bacterial dilutions (1:10 to 1:1000000) were placed in small cups, in duplicates, along with 10-third-instar mosquito larvae in each cups. Appropriate controls were kept simultaneously using distilled water instead of cultures. The cups were, then, covered with muslin and kept at room temperature 27 ± 2ºC. The mortality percentage was recorded by counting the number of living larvae and corrected by using appropriate control and applying the following Abbott's formula:

Cm % = [1 – (n in T after treatment /n in Co after treatment)] * 100

Where,

Cm is corrected mortality, n is Insect population, T is treated, Co is control (Abbott, 1925). The bacterial cultures exhibiting significant activities against mosquito larvae were kept on slants and stored at 4ºC for further characterization.

Protein estimation

Protein content estimation in effective cultures was carried out as per the standard protocol (Lowry *et al* ,1951). Protein concentrations were measured with bovine serum albumin as the standard

Spore crystal mixture (SCM) Extraction

In order to obtain SCM , selected isolates was grown in nutrient agar medium (5 days, 30ºC) until complete lysis. SCM were suspended in 1ml of ice-cold 1mol/L NaCl and centrifuged (10000rpm, 5min), the pellet was suspended in distilled water . The presence and morphology of spore crystals confirmed by smearing onto slides and staining with Coomassie brilliant blue, observed under light microscope (100X) (Sharif *et al*, 1988)

Crystal Protein Extraction

A slightly modified procedure of Armelle (1991) was used for protein extraction.

10 mL of 48 h bacterial cultures were centrifuged at 4 000 rpm for 10 min. The pellet was resuspended in 500 µL of 1 M NaCl. This mixture was transferred into an Eppendorf tube and centrifuged at 7000 rpm for 7 min. The pellet was resuspended in 250 µL of TE buffer and centrifuged at 7000 rpm for 7 min. Then, the pellet was suspended in 250 µL of dH2O and centrifuged at 7000 rpm for 7 min. After discarding the supernatant, 150 µL of 10 mg/mL lysozyme solution in TE buffer was added and the suspension was incubated at 37°C for 30 min. 25 µL of 10% SDS solution was added into the suspension which was then Vortexed for 30 sec. It was centrifuged at 6000 rpm for 10 min and 100 µL of 0.2% SDS solution was added to the pellet. For denaturation, 60 µL of gel loading buffer was added to 30 µL of this mixture in another Eppendorf tube. Finally, this sample was incubated at 90°C in a water bath for 7 min for three times and placed on ice until it cools. The final native and denatured samples were stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed by the standard method (Laemmli ,1970) by using 10% running and 4% stacking gels, and the gels were stained with 0.4% Coomassie blue. The molecular mass of proteins was determined by using protein standards .The proteins were separated by using a vertical polyacrylamide gel apparatus. Electrophoresis was run at 20 mA at the beginning when the samples reached to the separating gel, then the current was increased to 40 mA and the electrophoresis was continued until the samples reached to the end of the gel.

Isolation and analysis of plasmid DNA.

B. thuringiensis was grown in LB broth, and plasmid DNA was prepared by using
a modification of the method of Crosa and Falkow . Briefly, the pellet of a 1.5-ml culture was lysed with 0.1 TE buffer (50 mM Tris, 20 mM EDTA [pH 8.5]) containing 2 mg of lysozyme per ml, 0.5 ml of 20% SDS solution, and 1.0ml of a 5 mg/ml solution of protease in TE buffer. After being mixed by gentle inversions, the cell suspension was incubated at 37°C for 30 min. Subsequently, 0.2 ml of 3 N NaOH was added to the suspension and mixed gently for 3 min. The suspension was neutralized by addition of 0.6 ml of 2 M Tris-HCl (pH 7.0) and mixed gently. 5 M NaCl was then added, and the suspension was mixed by inversions, placed on ice for 15 min, and then centrifuged at 12,000 x g for 15 min. The supernatant was transferred into a fresh centrifuge tube, and 2 volumes of ice-cold ethanol were added. The tube was kept at -20°C for 15 min and then centrifuged at 12,000 x g for 15 min. The supernatant was discarded, and the pellet was dried by inverting the tube over a paper towel for a few minutes. The pellet was dissolved in 50 µl of TE buffer and kept at -20°C until used. Plasmid DNA was analyzed by electrophoresis on 0.6% horizontal agarose slab gels (Crosa and Falkow ,1981)

4. RESULTS

Sample collection and Isolation of *Bacillus thuringiensis*

Fifteen samples comprising soil from agricultural and non agricultural lands were collected from parts of Mizoram and further processed in laboratory.

Totally 15 *Bacillus thuringiensis* isolates obtained colony morphology were observed as rough,white, yellow and spreaded over the plate and their colony count on nutrient agar was found to be more.(Table 1)

Table 1: Showing the colony count of *Bacillus thuringiensis* isolates

S.No	Isolate	Location	Dilution	CFU/ml
1	Isolate 1	Tanhril(teak plantation)	10^4	1.80 X 10^6
2	Isolate 2	Ramrikawn(garden)	10^4	1.45X 10^6
3	Isolate 3	Zotlong (banana plantation)	10^4	1.30 X 10^6
4	Isolate 4	Luangmual (common)	10^4	1.40X 10^6
5	Isolate 5	Kanan(common)	10^4	0.95X 10^7
6	Isolate 6	Dawrpuai(common)	10^4	0.85X 10^7
7	Isolate 7	Zonuam (common)	10^4	0.95X 10^6
8	Isolate 8	Chawnpui(common)	10^4	0.66 X 10^6
9	Isolate 9	Lengpui (common)	10^4	1.23 X 10^6
10	Isolate 10	Tanhril(garden)	10^4	1.44 X 10^6
11	Isolate 11	Tanhril(banana plantation)	10^4	1.61 X 10^6
12	Isolate 12	Zotlong(garden)	10^4	1.87 X 10^6
13	Isolate 13	Zonuam(plantation)	10^4	1.79 X 10^6
14	Isolate 14	Kanan (garden)	10^4	1.06X 10^6

Morphological Characterization

The morphological characteristics of *B.thuringiensis* was observed by microscopic examinations after gram staining and spore staining . The isolates was found to be gram positive ,rod-shaped , having ellipsoidal and oval spores located in sub terminal and terminal regions of un swollen mother cells and crystals are observed as polymorphic. The characteristics of cells, spores and crystals of *B.thuringiensis* isolates are given in (Table 2) (Plate 1,2)

Table 2: Showing morphological characteristics of *B.thuringiensis isolates*

S.No	Isolate	Vegetative cell	Spore	Crystal
1	Isolate 1	Gram positive rods	Terminal oval spores	Polymorphic
2	Isolate 2	Gram positive rods	Sub terminal oval spores	Polymorphic
3	Isolate 3	Gram positive rods	Terminal oval spores	Polymorphic
4	Isolate 4	Gram positive rods	Terminal oval spores	Spherical and oval
5	Isolate 5	Gram positive rods	Sub terminal oval spores	Polymorphic
6	Isolate 6	Gram positive rods	Sub terminal oval spores	Polymorphic
7	Isolate 7	Gram positive rods	Terminal oval spores	Polymorphic
8	Isolate 8	Gram positive rods	Sub terminal oval spores	Ellipsoidal
9	Isolate 9	Gram positive rods	Sub terminal oval spores	Polymorphic
10	Isolate 10	Gram positive rods	Terminal oval spores	Polymorphic
11	Isolate 11	Gram positive rods	Sub terminal oval spores	Ellipsoidal
12	Isolate 12	Gram positive rods	Terminal oval spores	Polymorphic
13	Isolate 13	Gram positive rods	Terminal oval spores	Polymorphic
14	Isolate 14	Gram positive rods	Terminal oval spores	Polymorphic

The size of the vegetative cells and spores were measured using micrometry and their values are given in (Table 3, 4)

Table 3: Showing the size of vegetative cells

S.No	Isolate	Length (µm)#		Breadth(µm)#	
		Mean ± SE	Range	Mean ± SE	Range
1	Isolate1	4.89 ± 2.24	2.72 – 5.44	2.72 ± 1.22	2.70 – 2.72
2	Isolate2	4.62 ± 2.12	2.72 –5.44	2.74 ± 1.21	1.65 – 2.72
3	Isolate3	4.24 ± 1.90	3.33 – 4.44	2.21 ± 0.99	2.10 – 2.22
4	Isolate4	4.68 ± 2.10	4.44 – 5.55	2.48 ± 1.11	2.22 – 3.33
5	Isolate5	4.64 ± 1.80	2.72 – 5.44	2.44 ± 1.12	1.36– 2.72
6	Isolate6	4.64 ± 1.80	2.72 – 5.44	2.72 ± 1.22	2.70 – 2.72
7	Isolate7	4.24 ± 1.90	3.33 – 4.44	2.21 ± 0.99	2.10 – 2.22
8	Isolate8	4.89 ± 2.24	2.72 – 5.44	2.20 ± 1.00	2.10 – 2.22
9	Isolate9	4.68 ± 2.10	4.44 – 5.55	2.44 ± 1.12	1.36– 2.72
10	Isolate10	4.62 ± 2.12	2.72 –5.44	2.50 ± 1.12	1.36– 2.72
11	Isolate11	4.68 ± 2.10	4.44 – 5.55	2.21 ± 0.99	2.10 – 2.22
12	Isolate12	4.24 ± 1.90	3.33 – 4.44	2.48 ± 1.11	2.22 – 3.33
13	Isolate13	4.24 ± 1.90	3.33 – 4.44	2.74 ± 1.21	1.65 – 2.72
14	Isolate14	4.62 ± 2.12	2.72 –5.44	2.20 ± 1.00	2.10 – 2.22

Table 4: Showing the size of spores

S.No	Isolate	Diameter (µm)#	
		Mean ± SE	Range
1	Isolate1	1.84 ± 0.82	1.00 – 2.00
2	Isolate2	1.67 ± 0.74	1.00 – 2.00
3	Isolate3	1.84 ± 0.82	1.00 – 2.00
4	Isolate4	1.48 ± 0.66	1.00 – 2.00
5	Isolate5	1.67 ± 0.74	1.00 – 2.00
6	Isolate6	1.84 ± 0.82	1.00 – 2.00
7	Isolate7	2.53 ± 1.13	2.00 – 1.00
8	Isolate8	2.67 ± 0.74	2.00 – 1.00
9	Isolate9	1.48 ± 0.66	1.00 – 2.00
10	Isolate10	1.67 ± 0.74	1.00 – 2.00
11	Isolate11	1.67 ± 0.74	1.00 – 2.00

12	Isolate12	1.84 ± 0.82	1.00 – 2.00
13	Isolate13	4.24 ± 1.90	3.33 – 4.44
14	Isolate14	1.48 ± 0.66	1.00 – 2.00

- Mean of 5 observations and SE - Standard Error

Biochemical characteristerization

The biochemical were performed for further identification. The *B.thuringiensis* isolates were shown positive results for catalase, methyl red, voges proskaeur and nitrate and negative reaction for D-mannitol tests (Table 5). All the isolates hydrolyzed starch by forming clear zone around the colonies (Table 6) (Plate3). All isolates are salt tolerant shown growth in agar plates with 5% NaCl.

S.NO	ISOLATES	Catalase	Methyl red	Voges proskaeur	Nitrate	D-mannitol
1	Isolate 1	+	+	+	+	-
2	Isolate 2	+	+	+	+	-
3	Isolate 3	+	+	+	+	-
4	Isolate 4	+	+	+	+	-
5	Isolate 5	+	+	+	+	-
6	Isolate 6	+	+	+	+	-
7	Isolate 7	+	+	+	+	-
8	Isolate 8	+	+	+	+	-
9	Isolate 9	+	+	+	+	-
10	Isolate 10	+	+	+	+	-
11	Isolate 11	+	+	+	+	-
12	Isolate 12	+	+	+	+	-
13	Isolate 13	+	+	+	+	-
14	Isolate 14	+	+	+	+	-

Table 3 : + positive results and – negative results

Table 4 : Showing Starch hydrolysis zones of *B.thuringiensis* isolates

S.No	Isolate	Zone size found around colones (mm)
1	Isolate 1	5
2	Isolate 2	6
3	Isolate 3	8
4	Isolate 4	7
5	Isolate 5	4.5
6	Isolate 6	7.5
7	Isolate 7	5
8	Isolate 8	6
9	Isolate 9	6.5
10	Isolate 10	7
11	Isolate 11	5.5
12	Isolate 12	8
13	Isolate 13	4
14	Isolate 14	7

Larvicidal activity

Study of mortality rate of mosquito's larvae made, all the test isolates done the larvicidal activity but in the differantial levels, isolates 1,2, 4, and 7 showed high toxicity (larval mortality above 60% in 24hrs in 1 : 10 dilution, all other isolates too showed the mortality rate below 60%. (table 6, 7 and8) (plate 4)

Mortality data analysed, corrected value obtained by using Abbott's formula. And it was found to be above 80% for isolate 2,3,4 and 9 and highy toxicity shown at less time (table 9)

Table 6 : Showing 24 hours larvicidal activity (*Culex* spp) mortality rate at different dilutions of Culture

S.No	NAME	1:10	1:100	1:1000	1:10000	1:100000	1:1000000
1	Isolate 1	5	5	2	1	-	-
2	Isolate 2	6	6	5	5	3	-
3	Isolate 3	7	6	6	5	2	2
4	Isolate 4	6	5	3	1	1	-
5	Isolate 5	4	5	4	2	-	-
6	Isolate 6	5	3	3	3	1	1
7	Isolate 7	3	2	1	1	2	1
8	Isolate 8	5	5	2	1	-	-
9	Isolate 9	6	4	2	1	2	1
10	Isolate 10	4	2	2	1	-	-
11	Isolate 11	5	2	3	2	-	-
12	Isolate 12	4	2	1	1	-	-
13	Isolate 13	3	2	2	1	1	1
14	Isolate 14	5	2	2	1	1	-

Table 7 : Showing 48 hours larvicidal activity (*Culex* spp) mortality rate at different dilutions of Culture.

S.No	NAME	1:10	1:100	1:1000	1:10000	1:100000	1:1000000
1	Isolate 1	6	5	2	2	-	-
2	Isolate 2	9	8	8	8	6	3
3	Isolate 3	9	8	9	8	7	5
4	Isolate 4	8	5	3	1	1	1
5	Isolate 5	8	5	6	6	3	-
6	Isolate 6	8	8	8	3	3	-
7	Isolate 7	3	2	1	1	2	1
8	Isolate 8	7	8	4	2	1	1
9	Isolate 9	9	6	7	6	4	2
10	Isolate 10	7	4	4	4	3	3
11	Isolate 11	7	6	6	6	3	3
12	Isolate 12	6	3	2	2	1	1
13	Isolate 13	5	4	3	2	2	2
14	Isolate 14	5	3	3	2	2	2

Table 8 : Showing 72 hours larvicidal activity (*Culex* spp) mortality rate at different dilutions of Culture.

S.No	NAME	1:10	1:100	1:1000	1:10000	1:100000	1:1000000
1	Isolate 1	7	6	3	2	1	-
2	Isolate 2	10	9	9	9	9	7
3	Isolate 3	10	9	9	10	8	8
4	Isolate 4	10	6	3	2	4	1
5	Isolate 5	10	8	6	6	5	2
6	Isolate 6	9	9	9	5	3	-
7	Isolate 7	5	3	1	1	2	1
8	Isolate 8	9	9	6	4	2	2
9	Isolate 9	10	9	9	8	7	6
10	Isolate 10	9	8	5	5	4	4
11	Isolate 11	9	8	7	6	4	3
12	Isolate 12	8	5	5	3	3	3
13	Isolate 13	7	6	4	3	2	2
14	Isolate 14	7	6	5	5	3	3

Table 8 : Showing corrected mortality rate obtained using Abbot's formula

S.No	NAME	Corrected Mortality Rate (%)
1	Isolate 1	60.0
2	Isolate 2	83.3
3	Isolate 3	86.6
4	Isolate 4	80.0
5	Isolate 5	73.3
6	Isolate 6	73.3
7	Isolate 7	36.0
8	Isolate 8	70.0
9	Isolate 9	83.3
10	Isolate 10	73.3
11	Isolate 11	70.0
12	Isolate 12	60.0
13	Isolate 13	50.0
14	Isolate 14	56.6

Protein estimation

Total protein content of isolates is estimated for all samples it showed protein concentration ranges from 44µg/ ml to 106µg/ ml. (table 10).

Table 10 : Showing corrected mortality rate obtained using Abbot's formula

S.No	Standard	OD value	Sample	OD value	Proteins estimated (µg/ml)
1	S1	0.014	S1	0.035	44
2	S2	0.028	S2	0.057	68
3	S3	0.048	S3	0.074	90
4	S4	0.062	S4	0.086	106
5	S5	0.082			

Spore crystal mixture extraction

Spore crystal mixture after extraction were observed under light microscope after Coomassie brilliant blue staining (plate 5).

SDS PAGE

The protein profile for effective isolates was obtained using SDS-PAGE with an objective to find the variation in the protein banding pattern. It was found there is not much variation in the protein band pattern of effective isolates 2, 3, 4 and 9 all are above 120 kDa (plate 6)

Plasmid analysis

The plasmids from the effective isolates 2,3,4 and 9 all analyzed for plasmid homology , they showed the unique band pattern above 16 kbp and other band below 16 kbp. (plate 7)

5. DISCUSSION

Bacillus thuringiensis are commonly found in the soil , it is the natural habitat of the organisms, their spores persist in soil (Martin and Travers, 1989). So, fifteen soil samples are collected from agricultural and non agricultural areas in the study and thirteen isolates were isolated from these samples. Occurrence of *B.thuringiensis* in soil and population size is relatively higher than earlier reports (Martin and Travers, 1989)

Bacteria were grown in the nutrient agar medium at 37˚C for 2-3days. Sporulating cultures were obtained .Further obtained isolates underwent gram staining and spore staining then observed under the microscope for observing the specific morphology of cells, spores and parasporal crystals. Several morphological forms of parasporal crystals were categorized (Bernard *et al*, 2007). The isolates are found to be gram positive rods with blunt ends and oval spores were located in terminal and sub terminal regions, crystals are polymorphic most are oval-spherical in nature.

Genus Bacillus is recognized as gram positive rods, producing catalase and being aerobic organisms. Identification of *B.thuringiensis* isolates made on the basis of morphology and biochemical characteristics. However, culturing in medium along with sodium acetate excludes the contamination other than *B.thuringiensis* .Organisms positive for catalase and VP tests belong to group I of *Bacillus sp.* (Sneath ,1986). Production of crystals and non swollen sporangium identified the organisms as *B.thuringiensis* (Thiery and Frachon , 1997).

In present study serves as a model for entamocidal activity of *B.thuringiensis* using Mosquito larvae (*Culex spp*) as representative of dipteran insects. The high mortality rate of larvae within the time interval lower than 25 hours observed on the tests with entopathogenic bacteria *B.thuringiensis*. The organism produces the intracellular crystals , which are toxic to insects brings about collapse in the nervous systems, loss in ability to fluctuate , asphyxia and ends with death. Body paralysis is initially noted with in 24 hours with evident structural

disorganization of the intestinal epithelium showing secretion of vesicles and irregularly disposed brush border (Knowles *et al.,* 2005).

SDS-PAGE analysis were performed to determine the protein profiles of crystal proteins of effective *B.thuringiensis* isolates . Here the test isolates protein showed 20-130 kDa , multiple bands. The heavy protein bands not much varied are obtained in the lanes 2,3,4 and 9 due to high toxicity of isolates than others. How ever it is likely that the 130 – 140 kDa proteins are responsible for larvicidal activity , because the proteins of this class are the protoxins in most insecticidal strains of *B.thuringiensis.* (Hofte and Whiteley ,1989).

Isolation of plasmids from the effective *B.thuringiensis* isolates were performed and analysed for their plasmid homology . The test isolates 2,3,4 and 9 showed the unique plasmid band pattern above 16 kbp and it may be responsible for their effectiveness.Since sofar it was described that the cry genes are located on large plasmids (Lereceus,1993)

6. SUMMARY

In this study to find out the *Bacillus thuringiensis* isolates having high insecticidal activity and characterizing them. The agricultural and non agricultural soil samples were collected from the different parts of Mizoram ,these samples were processed in laboratory , plated on to agar plates and colonies were isolated. Further identification of *B.thuringiensis* isolates were made by morphological and biochemical characterization Fourteen isolates was found to be Gram positive rods with terminal and sub terminal un swollen spores , produces polymorphic crystals. Larvicidal activity of *B.thuringiensis* was tested against *Culex* mosquitos' larvae. The isolates 2,3,4 and 9 showed high mortality rate more than other isolates, they showed 60% mortality in 24 hrs further more exposure they showed mortality rate above 80% in less exposure time. The effective *B.thuringiensis* isolates proteins were studied by SDS-PAGE , they showed heavy band ranges between 40 to 130 kDa. The plasmid were isolated and analyzed for highly toxic isolates 2,3,4 and 9 , they showed unique homology of bands were clearly observed, this may be reason for their effectiveness. Further by knowing the unique nature of *B.thuringiensis* isolates and more effective Bio- insecticidal crystals can be developed.

7. REFERENCES

Abbott WS (1925). A method of computing effectiveness of insecticide. *Journal of Economic Entomoology.* 18.

Adang, M. J., (1991).*Bacillus thuringiensis* insecticidal crystal proteins: gene structure, action and utilization.. 1991; in *Biotechnology for Biological Control of Pest and Vectors*, edited by K. Maramorosh, (CRC Press, Boca Raton Fla), 3-24.

Agribusiness in India , retrieved on December 2009,UK India Business Council.

Andrews, R. E., Jr. R. M. Faust, H. Wabiko, K. C. Raymond, and L. B. Bulla, (1987) .The biotechnology of *Bacillus thuringiensis"*, CRC Critical Reviews of Biotechnology, 6, 163-232.

Andrews, R. E., M. M. Bibilops, and L. A. Bulla, (1985), Protease activation of the Entomocidal protoxin of *Bacillus thuringiensis* subsp. *Kurstaki*., *Applied Environ. Microbiol.*, 50, 737-742. 265.

Armelle, D. (1991). Detection by in vivo recombination shows that the 28- Kilodalton cytolytic polypeptide from Bacillus thuringiensis subsp. Israelensis is not essential for mosquitocidal activity. *Journal of Bacteriology.* 21, 3374-3381.

Aronson, A.I., and P. Fritz-James, (1976),.Structures and morphogenesis of the bacterial pore coat., *Bacteriology Review.,* 40, 360-402

Barton, K. A., Whiteley, H. R., Yang, N. S. (1987). *Bacillus thuringiensis* Delta-endotoxin expressed in transgenic Nicotiana tabacum provides Resistance to lepidopteran insects. Plant Physiol. 85, 1103-1109.

Becker, B. And J. Margalit. (1993). Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies, p. 147-170. *In* P. F. Entwistle, J. S. Cory, J. S.Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: Theory and practice. Wiley & Sons, Chichester, England.

Bel, Y., F. Granedo, T. M. Alberola, M. J. Martinez-Sebastian, and J. Ferrė,. (1997), Distribution, frequency and diversity of *Bacillus thuringiensis* in olive tree environments in Spain.., *Systematic of Applied Microbiology,* 20, 652- 658.

Belliveau, B. H., and J. H. Trevors. (1989). Transformation of *Bacillus cereus v*egetative cells by electroporation. *Applied Environmental Microbiology*. 55:1649-1652.

Bergey's manual of systematic bacteriology (1986)- Volume 2. Baltimore, London, Los Angeles, Sidney: Williams and Wilkins, 965-1599.

Berliner, E. (1911). Ober die Schlaffsucht der Mehlmottenraupe. Z. Gesamte Getreidewes. (Berlin), 3, 63-70.

Bernhard, K. (1986). Studies on the delta-endotoxin of *Bacillus thuringiensis* Var. *Tenebrionis*. FEMS *Microbiology Lett*. 33, 261-265.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P. And Burges, H.G. (1997). Natural isolates of worldwide Distribution, and activity against insect pests. *Journal of Invertebrate Pathology*. 70, 59-68.

Bernhard, K., P. Jarrett , M. Meadows, J. Butt, D. J. Ellis, G. M. Roberts, S.Pauli, P.Rodgers, and H. D. Burges, (1997),.Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests., *Journal of invertebrate pathology*, 70, 59-68.

Betz, F. S., Hammond, B. G., Fuchs, R. L. (2000). Safety and adventages of *Bacillus thuringiensis*- protected plants to control insect pests. *Regul. Toxicol. Pharm*. 32, 156-173.

Bikramjit Sinha and Indranil Biswas (2008) Potential of Biopesticide in Indian Agriculture vis- a-vis Rural Development, India, Science and Technology.

Bravo, A., S. Sarabia, L. Lopez, H. Ontiveros, C. Abarca, A. Ortiz, M. Ortiz, L. Lina, F.J. Villalobos, G. Pena, M.-E. Nunez-Valdez, M. Soberon, R. Quintero, (1998),.Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection.. *Appl. Environ. Microbiol.,*64, 4965-4972.

Bulla, L. A., Jr. R. M. Faust, R. Andrews, and N. Goodman, (1985).Insecticidal bacilli., in *The Moleculer Biology of the Bacilli, vol 2*, (Academic Press, Inc., New York,1985), 185-209.

Cadavos, C. F. G., R. N. Fonseca, J. Q. Chaves, L. Rabinovitch, C. J. P. C. Araújo-Coutinho, (2001),.Identification of entomopathogenic *Bacillus* isolated from *Simulium* (Diptera, Simuliidae) larvae and adults.., *Mem Inst Oswaldo Cruz, Rio de Janerio,* 96, 1017-1021.

Carlson, C. R. & Kolsto, A. B. (1993). A complete physical map of a *Bacillus thuringiensis* chromosome. *J. Bacteriol*. 175, 1053-1060.

Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. G. Koziel. (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl. Environ. Microbiol.* 57:3057- 3061

Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. M. Johnson, and C. Gawron-Burke, (1991), Isolation and characterization of a novel insecticidal crystal proteingene from *Bacillus thuringiensis* subsp. *Aizawai*., *Journal of Bacteriology,*173, 3966-3976.

Crickmore, N., D.R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean, (1998),. Revison of nomenclature for the *Bacillus thuringiensis p*esticidal crystal proteins.., *Microbiol. Mol. Biol. Rev.,* 62, (1998), 807-813.

Crosa, J. H., and S. Falkow. (1981). Plasmids,. In P.Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester,W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology,Washington, D.C. 266-282

Dalhambar, G., and H. Steiner, (1984),.Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of Antibacterial proteins in insects.. *Eur. J. Biochem.,* 139, 247-252.

De Barjac, H. & Fracon, E. (1990). Classification *of Bacillus thuringiensis* Strains. *Entomophaga,* 35, 233-240.

Dulmage, H. T. (1970). Insecticidal activity of HD-1, a new isolate of *Bacillus Thuringiensis* var. *Alesti*. J. Invertebr. Pathol. 15:232-239.

Ely, S. (1993). The engineering of plants to express *Bacillus thuringiensis* delta-endotoxins. In: Bacillus thuringiensis, An Experimental Biopesticide: Theory and Practice, Entwistle, P. F., Cory, J. S., Bailey, M. J., and Higgs, S., Eds., John Wiley & Sons, Chichester, UK, 105-124.

Estruch, J. J., G. W. Warren, M. A. Mullins, G. Y. Nye, J. A. Craig, and M. G. Koziel, (1996),.Vip3, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteraninsects.., *Proceeding National Academic Science USA,* 93, 5389-5394.

Forsty, G., and N: A: Logan, (2000),. Isolation of *Bacillus thuringiensis* from Northern ictoria Land, Antartica.., *Letters in Applied Microbiology*, 30, 263-266.

Frutos, R., Rang, C., and Royer, M. (1999). Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. *Critical Reviews in Biotechnology* 19: 227-27

Gill, S. S., E. A Cowles, and P. V. Pietranto, (1992),. The mode of action of *Bacillus Thuringiensis* endotoxins..,

Annu. Rev. Entomol., 37, 615.636.

Glazer, A. N., H. Nikaido, (1995) "Microbial insecticides", in *Microbial Biotechnology Fundamentals of Applied Microbiology,* (W.H. Freeman and Company, New York), 209-229.

Goldberg, L. H. & Margalit, J. (1977). A bacterial spore demonstrating rapid Larvicidal activity against Anopheles sergentii, Uranotenia unguiculata, Culex Univitatus, Aedes aegypti and Culex pipiens. Mosquito News, 37, 355-358.

Gonzales, J. M., and B. C. Carlton, (1980),.Patterns of plasmid DNA in crystalliferous strains of *B.thuringiensis... Plasmid,* 3, 92-98.

Gonzalez, J. M., Dulmage, H. T., Carlton, B. C. (1981). Correlation between Specific plasmids and delta-endotoxin production in *Bacillus thuringiensis. Plasmid.* 5, 351-365.

González, J. M., Jr., Brown, B. J., and Carlton, B. C. (1981). Transfer of *Bacillus thuringiensis* plasmids coding for -endotoxin among strains of *B. thuringiensis* and *B. Cereus*. *Proc. Natl. Acad. Sci*. USA 79:6951-6955.

Herrnstand, C., G.G. Soares, E.R. Wilcox, and D.I. Edwards, (1986), .A new strain of *Bacillus thuringiensis* with activity against coleopteran insects., *Bio/Technology,* 4, 305-308.

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

Hongyu, Z., Y. Ziniu, D. Wangxi, (2000),.Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehauses in China.., *Crop Protection,* 19, 449-454.

Honigman, A., Pazerini, G. N., Yawets, A., Oron, U., Schuster, S., Broza, M.,Snek, B. (1986). *Gene*. 42, 69-77.

Ichimatsu,T., E. Mizuki, K. Nishimura, T. Akao, and H. Saitoh,. (2000), Ocurance of *Bacillusthuringiensis* in fresh waters of Japan.., *Current Microbiology,* 40, 217-220.

Indian agriculture , Retrieved on- February (2008) ,Agribusiness Information Centre .

Iriarte, J., V. C. Dumanoir, Y. Bel, M. Porcar, M. D. Ferrandis, M.-M. Lecadet, J. Ferré, (2000)., " Characterization of *Bacillus thuringiensis* ser. Balearica Serotype H48) and ser. Navarrensis (Serotype H50): two novel serovars in Spain.", *Current Microbiology*, 40, 17-22

Ishiwata, S. (1901). On a kind of severe flacherie (sotto disease). Dainihon Sanshi Kaiho, 114, 1-5.

Itoqou-Apoyolo, C., L. Drift, J. M. Vassal, H. Debarjac, J. P. Bossy, F. Leclant, and R.Frutos, (1995),. Isolation multiple subspecies of *Bacillus thuringiensis* from a Population of the Europian sunflower moth, *Homoesoma nebulella..*, *Applied and Environmental Microbiolgy,* 61, 4343-4347.

Kaelin, P., Morel, P. And Gadani, F. (1994). Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F.). *Appl. Environ. Microbiol*.60:19-25

Kamdar, H. & Jayaraman, K. (1983). Biochem. Biophy. Res. Commun. 110, 477-482.

Kegley, S.E. and Wise, L.J. (1998). Pesticides in fruit and vegetables. Sausalito, CA: University Science Books.

Knowles,B. H., (1994),.Mechanism of action of *Bacillus thuringiensis* insecticidal detaendotoxins.. *Adv. Insect. Physiol,* 24, 27-308.

Laemmli, U. K., (1970).,Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4 ,*Nature* 227, 680 – 685

Lereculus D.,Delecluse, (1993),. Diversity of *Bacillus thuringiensis* txins and genes,: *Bacillus thuringiensis,* an Environmental Biopesticide : Theory and Practice. Chichester – John Wiley, New York .37-70.

Levinson, B. L., (1990). High performance liquid charamotography analysis of two Exotoxins produced by some *Bacillus thuringiensis* strains., in A*nalytical Chemistry of Bacillus thuringiensis*, edited by L. A. Hickle and W. L. Fitch, (American Chemical Society, Washington, D.C.), 115-136.

Lhy, P. & Ebersold, H. R. (1981),. *Bacillus thuringiensis* delta endotoxin: Histopathology and molecular mode of action. In: Pathogenesis of Invertebrate Microbial Diseases (Ed. E. W. Davidson) Osmun Publ. Totowa, NJ .235-267.

Liu, Y. B. And Tabashnik, B. E.(1997). Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis*. Proc. R. Soc. Lond B 264:605-10.

Lopez-Meza, J. E., J. E. Ibarra, (1996),.Characterization of a novel strain of *Bacillus Thuringiensis..*, *Applied and Environmental Microbiolgy*, 62, 1306- 1310.

Lövgren, A, M.-Y. Zang, A. Engstöm, G. Dalhammar, and R. Landen, (1990),. Molecular characterization of

immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis..*, *Moleculer Microbiology,* 4, 2137-2146.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ , (1951),. Protein measurement with the folin phenol reagent. ., *J Biol Chem; 193:265-75.*

Madigan M; Martinko J (editors). (2005). Brock Biology of Microorganisms*, 11th ed., Prentice Hall.*

Maeda, M. E. Mizuki, Y. Nakamura, T. Hatano, and M. Ohba, (2000),. Recovery of *Bacillus thuringiensis* from marine sediments of Japan.., *Current Microbiology,* 40, , 418-422.

Manncsa ,(June 11, 2009),.Pesticides in Agriculture, *Agropedia.* 265

Martin, P. A. & Travers, R. S. (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 55, 2437-2442.

Meadows, M.P., D. J. Ellis, J. Butt, P. Jarret, and H. D. Burges,.(1992),. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill.., *Applied and Environmental Microbiolgy,* 58, 1344-1350.

Mizuki,E., T. Ichimatsu, S.-H. Hwang, Y. S. Park, H. Saitoh, K. Higuchi, and M. Ohba,(1999),. Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants in Japan.., *Journal of applied microbiology,* 86, 979-984.

Mormak, D. A. And Casida, L. E. Jr. (1985). Study of *Bacillus subtilis* .Endospores in Soil by Use of a Modified Endospore Stain.1985. Appl Environ Microbiol. 49(6): 1356–1360.

Navon, A. (2000). *Bacillus thuringiensis* insecticides in crop protection - Reality and prospects. Crop Prot. 19, 669-676.

Ohba, M., and K. Aizawa, (1986),. Distribution of *Bacillus thuringiensis* in soils of Japan.., *J.Invertebrate. Pathol*, 47, 277-282.

Provine, H. And Gardner, P. (1974). The Gram's stained smear and its interpretation. *Hosp. Pract.* 9, 85-91.

Ragni, A., Thiéry. I. And Delécluse. A. (1996). Characterization of six highly Mosquitocidal *Bacillus thuringiensis* strains that do not belong to the H-14 Serotype. *Curr. Microbiol*. 32:48-54.

Reagents and Tests, (1978) in *Bailey & Scott's Diagnostic Microbiology.*

Schnepf, H. E. And H. R. Whiteley, (1981),.Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*., *Proceeding of the National Academy of Sciences USA,* 57, 2893-2897.

Sharif F.A., Alaeddnoglu N.G (1998).A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis , Journal of Industrial Microbiology*.3, 227-229

Smith, G. P., Merrick, J. D., Bone, E. J. And Ellar, D. J. (1996). Mosquitocidal Activity of the cryic -endotoxin from *Bacillus thuringiensis* subsp. *Aizawai*. *Appl. Environ. Microbiol.* 62:680-684

Smith, R. A., and G. A. Couche, (1991),.The phylloplane as a source of *Bacillus thuringiensi* variants.., *Applied and Environmental Microbiology*, 57, 311-315.

Sneath, P. H. A. (1986),. Nomenclature of bacteria. *Biological Nomenclature Today*,. Edited by W. D. L. Ride & T. Youne`s. Oxford: IRL Press. 36–48

Stabb, E. V., L. M. Jcobson, and J. Handelsman, (1994),.Zwittermycin A-producing strains of *B. Cereus* from diverse soils.., *Appl. Environ. Microbiol.,* 60, 4404-4412.

Stahly DP, Dingman DW, Bulla LA, Jr, Aronson AI. , (1978),. Possible origin and function of the parasporal crystal in *Bacillus thuringiensis*. *Biochem Biophys Res Commun.* 84(3):581–588.

Stahly, D. P., Andrews, R. E., Yousten, A. A. (1992). The Genus *Bacillus*- Insect Pathogens. In: The Prokaryotes 2nd Ed. (Eds. A. Ballows, H. G. Traer,M. Dworkin, W. Harder, K. Schleier) Vol.1. Springe- Verlang, New York., 1713.

Stahly, D. P., R. E. Andrews, A. A. Yousten, (1991), .The genus *Bacillus*- õnsect pathogens., in *The Prokaryotes 2nd eddition,* edited by A. Ballows, (Springer Verlag, New York), 1097-1745.

Thiery, I. And E. Frachon. (1997). Identification, isolation, culture and preservation of enthomopathogenic bacteria, *In* A.L.*Bacillus* sp. *Biologia*, Bratislava, 55, 283-287.

Thomas, D. J., A. W. Morgan, J. M. Whipps, and J. R. Saunders, (2001),.Plasmid transfer Between *Bacillus thuringiensis* subsp. *Israelensis* strains in labrotory culture, river water, and dipteran larvae.., *Appl. Environ. Microbiol,* 67, 330-338.

Travers, R.S., Martin, P.A. and Reicheferfer, C.F. (1987). Selective process for efficient isolation of soil *Bacillus*

spp. Applied and Environmental Microbiology.53, 1263-1266.

Vaeck, M., Reynaerts, A., Hote, H., Jansens, S., De Beukeleer, M., Dean, C., Zabeau, M., Van Montagu, M., Leemans, J. (1987),. Transgenic plants protected from insect attack. Nature, 328, 33-37.

Van Frankenhuyzen, K. (1993),. The challenge of *Bacillus thuringiensis*. In: *Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice, Entwistle, P. E., Cory, J. S., Bailey, M. J., and Higgs, S., Eds., John Wiley & Sons, Chichester, UK, 1-35.

Weiser, J. (1986). Impact of *Bacillus thuringiensis* on applied entomology in Eastern Europe and in the Soviet Union. In: Mitteilungen aus der Biologischen Bundesansalt f・ Land- und Fostwirschaft Berlin-dahlem (Eds. A Krieg and M. Huger), Paul Parey, Berlin. 233, 37-50.

Wu, D., Johnson, J. J. And Federici, B. A. (1994). Synergism of mosquitocidal toxicity between cyta and cryivd proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. *Mol. Microbiol*. 13:965-972.

Zhang, M.-Y., A. Lövgren, M. G. Low, and R. Landen, (1993),. Characterization of an a Virulent pleiotropic mutant of the insect pathogen *Bacillus thuringiensis*: Reduced expression of flagellin and phospholipases.., *Infections and Immunity,*61, 4947-4954.