CHAPTER-1

1. INTRODUCTION

Over half a century of synthetic pesticide applications had led to the emergence and spread of resistance in agricultural pest and vectors of human diseases and to the environmental degradation. The very properties that made these chemicals useful - long residual actions and toxicity to a wide spectrum of organisms - have brought about serious problems. An urgent need has thus emerged for environment friendly pesticides to reduce contamination and the likelihood of insect resistance (Ben-Dov *et al.*, 1997) Human attempts at insect control have changed over time from natural methods to Synthetic chemical control and again now looking to natural methods as it is safe and free from pollution and other undesirable side affects (Neppl, 2000)

One such alternative is the use of microbial insecticides – insecticides that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non target animals and humans is extremely low. Compared to other commonly used insecticides they are safe for both the pesticides user and consumers of treated crops (Neppl, 2000). The soil bacterium *B. thuringiensis* fulfills the requisites of a microbiological control agent against agricultural pest and vectors of diseases that lead to it's wide spread commercial applications (Ben-Dov *et al.*,1999).

The main target pest of *B. thuringiensis* insecticides include various lepidopterous (butterfly), dipterous (flies and mosquitoes), and individual coleopterons (Beatle) species. Some strains have also been found to kill off nematodes (Edward *et al.*,1988) .Conventional *B. thuringiensis* preparations such as those register in Germany but also worldwide are mostly derived from the highly potent strain *B. thuringiensis var. kurstaki* HD1, which was isolated in sixties (Dulmage,1970).

Nepal being an agriculture based country is no exception regarding these problems. Research work shows that the use of pesticides in Nepal is increasing in total amounts. The generally used chemical pesticides in Nepal are organochlorine such as

dichlorobiphenyl trichloroethane (DDT), hexachlorocyclohexane, Malathion, organophosphates, carbamates pyrethroids, fungicides as well as rodenticides. These pesticides are non-biodegradable, which have long term toxicity, accumulate in the food chain and pollute the environment. Surface run off from agricultural lands contaminates nearly all the rivers, lakes, ponds, wells etc. Pesticides residues in water may reach human through drinking water. It may also reach human through vegetables, fruits etc, if they are not properly cleaned. DDT residue has been detected in many food items including rice, wheat, meat, vegetables and milk in Nepal. Some of these products show the residual level above the safety limits of Food and Agricultural Organization (FAO) /World Health Organization (WHO). The Central Food Research Laboratory, Kathmandu has also detected pesticides residues in many food samples: among the sample examined 87.5% were the milk sample that were tested and found to contain DDT (Bhattarai, 2002). WHO has estimated that one million pesticides poisoning cases occur every year causing around 20,000 deaths per year globally. Pesticide poison may lead to severe human diseases like asthma and skin disorders, enlargement of liver, cancer, reproductive problems, tumors, and spontaneous abortion psychological problems and even to some extent degeneration of nerves often resulting in paralysis. Dinitrophenols are toxic to liver, kidney and nervous system (Subedi, 1999)

The characterizations of the strains from different niches provide useful information on the ecological patterns of distribution of *B. thuringiensis* and opportunities for the selection of strains to develop novel bioinsecticidal products.

Insect bioassay and rocket immunoelectrophoresis are currently used to detect and measure the levels of crystal insect toxin produced by *B. thuringiensis*. Because insect bioassays are time consuming and cumbersome and require relatively large amounts of material, they are impractical, especially for environmental applications. Although more sensitive than bioassay, rocket immunoelectrophoresis also requires a considerable amount of antigen (at least 10 mg/ml), so that the utility of the method is probably restricted to quality control for commercial fermentations and other industrial processes. (Wie *et al.*, 1982)

ELISA (Enzyme-Linked Immunosorbent Assay) is an immunoassay utilizing enzyme conjugated antibodies, with antigen or antibodies bound to a solid support. The assay measures changes in enzyme activities proportional to the antigen or antibody concentrations involved in the underlying immune reactions. It is a highly versatile and sensitive analytical procedure for qualitative or quantitative determination of antibodies and almost any kind of antigens. The method makes use of the very high capacity of antibodies to discriminate between different epitopes. Provided antibodies of high specificity and affinity are available, the detection limits of the assay may be well below 1 ng/mL. (Perlmann and Perlmann, 2001) Hence ELISA can be effectively used to study cross reactivity of a given type of antigen. Identical antigens possess 100% crossreactivity with the given antisera and non identical ones don't show any degree of crossreactivity. Thus the diversity of the given antigen and hence organisms in a given complex population can be studied by their cross reactivities among themselves. Feeling the needs to study crystal protein diversity of *B. thuringiensis* strains, Indirect ELISA procedure was optimized in this study.

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To study the immunocrossreactivity of δ-endotoxin, the crystal protein from *Bacillus thuringeinsis* isolates from Khumbu Base Camp of Everest Region (Sagarmatha National Park and Phereche soil samples).

2.2 SPECIFIC OBJECTIVES

i. To isolate, identify and characterize *B. thuringeinsis* from soil samples of Khumbu regions.
ii. To extract crystal proteins from *B. thuringeinsis* isolates.
iii. To identify and purify the crystal protein having characteristic molecular mass (Mr) using SDS PAGE and Native PAGE.
iv. To test the efficiency of the crystal protein against mosquito larva (*Culex* spp)
v. To raise polyclonal antibody against purified crystal protein.
vi. To optimize ELISA (Enzyme Linked Immunosorbent Assay) for crystal protein detection using the polyclonal antibody.
vii. To study immunocrossreactivity of crystal proteins using polyclonal antisera.
viii. To confirm delta endotoxin in crude protein preparation by Western Blotting

CHAPTER-III

3. LITERATURE REVIEW

3. 1. *BACILLUS THURINGIENSIS*

Bacillus thuringiensis is a ubiquitous gram positive, spore forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. *B thuringiensis* was initially characterized as insect pathogen, and its insecticidal activity was attributed largely or completely (depending upon the insects) to the parasporal crystals. This observation leads to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the order Lepidoptera, Diptera, and Coleoptera. There are more recent reports of *B. thuringiensis* isolates active against other insects of order (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against Nematodes, mites and protozoa. *B. thuringiensis* is already a useful alternative or supplement to the synthetic chemical pesticide application in commercial agriculture for pest management and mosquito control. It is also a key source of genes for transgenic expression to provide pest resistant in plants (Schnepf *et al.*, 1998)

3.2 ECOLOGY AND PREVALENCE OF *B. THURINGIENSIS*

B. thuringiensis seems to be indigenous to many environments. Strains have been isolated worldwide from many habitats, including soil, insects, dead insects, sericulture environments, forests and cultivated soils, stored products dust, and coniferous and deciduous leaves. Because of the economic importance of *B. thuringiensis*, many researchers have investigated the distribution of *B. thuringiensis* in various geographical regions and in different sources (Schnepf *et al.*, 1998).

The diversity in flagellar H-antigen agglutination reaction is one indication of enormous genetic diversity among *B.thuringiensis* isolates. The Pasteur Institute has catalogued 55 different flagellar serotypes and 8 non-flagellar biotypes (Schnepf *et al.*, 1998).

3.3 MORPHOLOGY AND PHYSIOLOGY OF *B. THURINGIENSIS*

B. thuringiensis has dimensions ranging from 3-5 um in length and 1-1.2 um in width. It is an entomopathogenic, spore forming *Bacillus* whose spore is ellipsoidal to cylindrical in shape with a thin spore wall enclosed by two envelops, the endo- and exosporium. Spore are central to terminal in position without distentention of the mother cell, and inclusion body inside the cell *B. thuringiensis* produces crystalline parasporal protein body, rarely two or three, in the cell during the phase of spore formation. The protein body stains like other cell material and is found outside the exosporium and separates readily from the liberated spore. *B. thuringiensis* shows the presence of flagellar but absence of capsules. Motility could be seen in some but not in all strains (Subedi, 1999 and Bhattarai, 2002).

Different strains of *B. thuringiensis* shows a slight variation in their physiological characteristics such as production of acetoin, lecithinase, proteolytic enzymes, urease and formation of sucrose and salicin. These properties have been utilized for the recognition of strains; their association with particular serotypes has diminished as the number of isolates increases (Subedi, 1999 and Bhattarai, 2002). *B. thuringiensis* is able to ferment carbohydrates to produce acid from sugars like sucrose, glucose, mannose, salicin, esculin etc but do not produce gas. *B. thuringiensis* is unable to produce acid from sugars like arabinose, xylose and mannitol. It can as well as grow in aerobic and anaerobic conditions provide sufficient nutrients (Subedi, 1999 and Bhattarai, 2002).

Chemical tests shows that *B. thuringiensis* is able to reduce nitrate to nitrite or free nitrogen. It is catalase positive test. It can utilize citrate as a sole source of carbon metabolism. Most strains gives VP test and urease positive (Subedi, 1999 and Bhattarai, 2002). *B. thuringiensis* can hydrolyze gelatin, starch and skimmed milk and lecithin. Sporulation and crystal production occur best with vigorous aeration of liquid cultures or on solid media where the surface growth is exposed to atmospheric oxygen (Subedi, 1999 and Bhattarai, 2002). *B.thuringiensis* is unable to grow at 5ºC. However 11-89% is able to grow at 10ºC. It grows fully at various temperatures from 30-40ºC (Bergey's manual of systematic bacteriology, Vol-2, 1986) but unable to grow at and above 50ºC. *B.thuringiensis* shows pH ranging towards the neutral condition is more preferable for the

production of crystal proteins. Delta endotoxin can be stored at 4ºC (Subedi, 1999 and Bhattarai, 2002).

3.4 HISTORICAL DEVELOPMENT OF *B. THURINGIENSIS*

This common soil bacterium, most abundantly found in grain dust from silos and other grain storage facilities, was discovered first in Japan in 1901 by Ishawata and then in 1911 in Germany by Berliner. Chehsire and Cheyne, 1885, studied foul wood disease of the honeybee and Ishwata 1902 isolated a spore forming aerobic bacteria from the diseased silkworm. In 1915 Berliner isolated *Bacillus* from the diseased larvae of the Mediterranean flour moth Anagasta (ephestia) in Thuringin; Germany. Later, Mates (1927) isolated the *Bacillus* from the same insect host which Berliner had found earlier. A Canadian worker Hannay while studying sporulation in *B. thuringiensis* observed the inclusion bodies besides the spores, as Berliner. He suggested that these inclusions might be involved in the insect disease caused by these bacteria. Another Canadian worker T. Angus (1954-1956) confirmed the work of Aoki and Chigasaki as well as Hannay's suggestions of relationship between pathogenicity and the crystals (Neppl, 2000).

Subsequently there are thousands of strains of *B. thuringiensis* exist. Each strain produces its own unique insecticidal crystal protein, or -endotoxin, which is encoded by a single gene on a plasmid in the bacterium. *B. thuringiensis* toxins are biodegradable and do not persist in the environment (Neppl, 2000). *Bacillus thuringiensis* first became available as a commercial insecticide in France in 1938 and in the 1950s entered commercial use in the United States. . In 1987 came the first reports of insertion of genes encoding for *B. thuringiensis* -endotoxins into plants. The first transgenic plants to express *B. thuringiensis* toxins were tobacco and tomato plants. Although using *B. thuringiensis* in the form of transgenic crops is now very common, the more traditional spray form of *B. thuringiensis* is still widely used (Neppl, 2000).

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3.5 CLASSIFICATION AND NOMENCLATURE OF *B. THURINGIENSIS*

Since the first cloning of an insecticidal crystal protein gene from *Bacillus thuringiensis* many other such genes have been isolated. Initially, each newly characterized gene or protein received an arbitrary designation from its discoverers: *icp*, *cry*, *kurhd1,* Bta, bt1, bt2 etc.type B and type C, and 4.5 kb, 5.3 kb, and 6.6 k*B.* The first systematic attempt to organize the genetic nomenclature relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes. The *cry* I genes encoded proteins toxic to lepidopterans; *cry*II genes encoded proteins toxic to both lepidopterans and dipterans; *cry*III genes encoded proteins toxic to coleopterans; and *cry*IV genes encoded proteins toxic to dipterans alone (Crickmore *et al.*, 1998)

This system provided a useful framework for classifying the ever-expanding set of known genes. Inconsistencies existed in the original scheme, however, due to attempts to accommodate genes that were highly homologous to known genes but did not encode a toxin with a similar insecticidal spectrum. The *cry*IIB gene, for example, received a place in the lepidopteran dipteran class with *cry*IIA, even though toxicity against dipterans could not be demonstrated for the toxin designated CryIIB. Other anomalies arose after the nomenclature was established. The protein named CryIC, for example, was reported to be toxic to both dipterans and lepidopterans, while the protein designated CryIB was reported to be toxic to both lepidopterans and coleopterans. Because the nomenclature system provided no central committee or database to maintain standardization, new genes encoding a diverse set of proteins without a common insecticidal activity each received the name *cry*V, based on the next available Roman numeral (Crickmore *et al.*, 1998). The known *cry* and Cyt gene sequences with revised nomenclature assignments and Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins as purposed by Crickmore *et al.*, 1998 were given in the Appendix-XI.

According to Bravo *et al.*, 1998, currently 45 different serotypes of *Bacillus thuringiensis* have been classified as 58 serovars. Many Cry protein genes have been cloned, sequenced, and named *cry* and Cyt genes. To date, over 100 *cry* gene sequences have been determined and classified in 22 groups and different subgroups with regard to their amino acid similarity. The proteins toxic for lepidopteran insects belong to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and

Cry8 proteins as well as the Cry1B and Cry1I proteins, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematicidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipterans insects (Bravo, 1997)

3.6 *B. THURINGIENSIS* GENOME

B. thuringiensis strains have a genome size of 2.4 to 5.7 million bp. Most *B. thuringiensis* isolates have several extrachromosomal elements, some of them circular and others linear. The proteins comprising the parasporal crystal are encoded by large plasmids. Sequences hybridizing to *cry* gene probes occur among *B. thuringiensis* chromosomes as well, although it is unclear to what degree these chromosomal homolog contribute to production of the crystal (Schnepf *et al.*, 1998).

3.7 TOXIN STRUCTURE

To date, the structures of three crystal proteins—Cry3A, Cry1Aa, and Cyt2A have been solved by X-ray crystallography. Cry3A and Cry1Aa have about 36% amino acid sequence identity. This similarity is reflected in their three-dimensional structures; the corresponding domains can virtually be superimposed. Cyt2A, however, shows less than 20% amino acid sequence identity with Cry1Aa and Cry3A, and a similar alignment score would be obtained if the Cyt2A sequence were randomized. The structure of Cyt2A is radically different from the other two structures. The structures of Cry1Aa, Cry3A, and Cyt2A are compared in Fig. 1. The Cyt toxins, unlike the Cry -endotoxins, are able to lyse a wide range of cell types in vitro. Cyt2A consists of a single domain in which two outer layers of -helix wrap around a mixed -sheet. Cyt1A is believed to have a similar structure. Cry3A and Cry1Aa, in contrast to Cyt2A, both possess three domains. Domain I consists of a bundle of seven antiparallel -helices in which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel -sheets joined in a typical “Greek key” topology, arranged in a so-called -prism fold (330, 343). Domain III consists of two twisted, antiparallel -sheets forming a -sandwich with a “jelly roll” topology (Schnepf *et al.*, 1998).

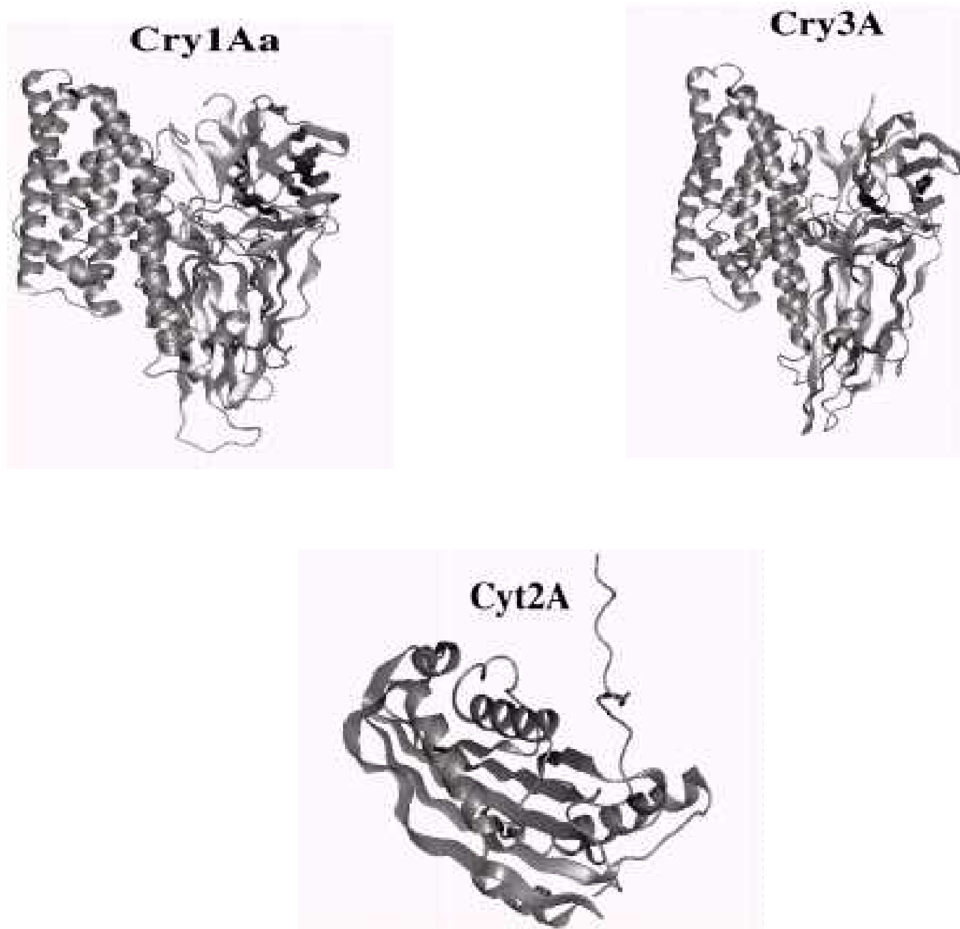

Figure 1: Three dimensional structures of Cry1A, Cry3A and Cry2A

3.8 MECHANISM OF ACTION OF TOXIN (INSECTICIDAL ACTIVITY)

The mechanism of action of *B. thuringiensis* crystal proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry-toxin to mid gut receptors and insertion of the toxin into the apical membrane to create ion channels or pores (Schnepf *et al.*, 1998).After solubilization, many protoxins must be processed by insect midgut proteases to become activated toxins. The major proteases of the lepidopteran insect midgut are trypsin-like or chymotrypsin-like. The Cry1A protoxins are digested to a 65-kDa toxin protein in a processive manner starting at the C terminus and proceeding toward the 55- to 65-kDa toxic core. The carboxy terminal end of the protoxin, which initially appears to be wound

around the toxin in an escargot-like manner, is clipped off processively in 10-kDa sections during processing of the protoxin. DNA is intimately associated with the crystal and appears to play a role in proteolytic processing. The mature Cry1A toxin is cleaved at R28 at the amino-terminal end; Cry1Ac, at least, is cleaved at K623 on the carboxy-terminal end. Two stages of processing have been detected for Cry1Ia with trypsin or *Ostrinia nubilalis* midgut proteases: a fully toxic intermediate, with an N terminus at protoxin residue 45 and a C terminus at residue 655 or 659, is further processed to a partially toxic core, with an N terminus clipped to residue 156 (Schnepf *et al.*, 1998).

Activated *cry* toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvilli of susceptible insects. Binding is a two-stage process involving reversible and irreversible steps. The latter steps may involve a tight binding between the toxin and receptor, insertion of the toxin into the apical membrane, or both. It has been generally assumed that irreversible binding is exclusively associated with membrane insertion. The truncated Cry1Ab molecules containing only domains II and III can still bind to midgut receptors, but only reversibly, supports the notion that irreversible binding requires the insertion of domain I. Tight binding of Cry1Aa and Cry1Ab to purified *Manduca sexta* aminopeptidase N (APN) has been observed, and Cry1Ac may also show some degree of irreversible binding to M. sexta APN. There are likewise indications of irreversible binding for Cry1Ac to purified Lymantria dispar APN. Finally, Vadlamudi *et al.* calculated similar binding constants when toxin bound to brush border membrane vesicles (BBMV) and to nitrocellulose-immobilized receptor (i.e., a ligand blot). In M. sexta, the Cry1Ab receptor is believed to be a cadherin- like 210-kDa membrane protein, while the Cry1Ac and Cry1C receptors have been identified as APN proteins with molecular masses of 120 and 106 kDa, respectively. Incorporation of purified 120-kDa APN into planar lipid bilayers catalyzed channel formation by Cry1Aa, Cry1Ac, and Cry1C. There is also some evidence that domain II from either Cry1Ab or Cry1Ac can promote binding to the larger protein, while domain III of Cry1Ac promotes binding to the presumed APN. Alkaline phosphatase has also been proposed to be a Cry1Ac receptor. In *Heliothis virescens*, three aminopeptidases bound to Cry1Ac on toxin affinity

columns. One of them, a 170-kDa APN, bound Cry1Aa, Cry1Ab, and Cry1Ac, but not Cry1C or Cry1E. *N*-Acetylgalactosamine inhibited the binding of Cry1Ac but not that of Cry1Aa or Cry1A*B.* The three Cry1A toxins each recognized a high-affinity and a low-affinity binding site on this 170-kDa APN. In gypsy moth (L. dispar), the Cry1Ac receptor also seems to be APN, while Cry1Aa and Cry1Ab bind to a 210-kDa brush border membrane vesicle (BBMV) protein. In *Plutella xylostella* and *Bombyx mori* as well, APN appears to function as a Cry1Ac binding protein. An *M. sexta* gene encoding a Cry1Ab-binding APN has also been cloned, as has its *P. xylostella* homolog. Insertion into the apical membrane of the columnar epithelial cells follows the initial receptor-mediated binding, rendering the toxin insensitive to proteases and monoclonal antibodies and inducing ion channels or nonspecific pores in the target membrane (Schnepf *et al.*, 1998). The nature of the ion channel or pore-forming activity of *cry* toxins in the insect is still controversial. It is alternatively described as a large lytic pore that is not specific for particular ions or as an ion-specific channel that disrupts the membrane potential but does not necessarily lyse midgut epithelial cells (Schnepf *et al.*, 1998).

3.9 DEVELOPMENT OF RESISTANCE TO *B. THURINGIENSIS*

The first evidence of resistance developing in the field against *B. thuringiensis* delta-endotoxins was published in 1985. Low levels of resistance were found in *Plodia interpunctella*, the Indianmeal moth, in storage bins of *B. thuringiensis*-treated grain. The *B. thuringiensis* resistance problem became greater when the first reports of high resistance to *B. thuringiensis* toxins in the field came in 1990 from Hawaii, Florida, and New York in the United States. The species found to be losing susceptibility to *B. thuringiensis* toxin was *Plutella xylostella*, and the diamondback moth. Resistance in *P. xylostella* was detected in several other countries, including Japan, China, the Philippines, and Thailand. Malaysia also reported *B. thuringiensis* resistance in the diamondback moth in 1990. Thus far *P. xylostella* is still the only insect species in which very considerable resistance has been found to develop outside of the laboratory (Schnepf *et al.*, 1998).

Eleven species have developed resistance to various strains of *B. thuringiensis* toxin in the laboratory but not in the field: *Ostrinia nubilalis* (the European corn borer), *Heliothis virescens* (the tobacco budworm), *Pectinophora gossypiella* (the pink bollworm moth), *Culex quinquefasciatus* (the mosquito), *Caudra cautella* (the almond moth), *Chrysomela scripta* (the cottonwood leaf beetle), *Spodoptera exigua* (the beet armyworm), *Spodoptera littoralis* (the Egyptian cotton leaf worm), *Trichoplusia ni* (the tiger moth), *L. decemlineata* (the Colorado potato beetle), and *Aedes aegypti* (the yellow fever mosquito) (Schnepf *et al.*, 1998).

3.10 MECHANISM OF RESISTANCE

The mechanisms underlying *B. thuringiensis* resistance are found in the sequential steps in the proposed mode of action of *B. thuringiensis* toxins. Following the ingestion of *B. thuringiensis* crystals by susceptible insects, protoxins are solubilized and hydrolyzed by gut m-proteinases to an active toxin form. Activated *cry* proteins pass through the peritrophic membrane and bind to proteins in the brush border membrane of midgut epithelial cells. Toxin binding is followed by events that lead to cell lysis and disintegration of the brush border membrane, and eventually insect death. Any change in insect gut physiology that affects one or more steps in this process could prevent toxicity and lead to the development of resistant pest populations (Li *et al.*, 2004). Most studies have focused on two steps in the mode of action: proteolytic activation of protoxin and binding of active toxin to receptors. Reduced binding of Cry proteins to midgut receptors has been associated with resistance in several strains of *Plodia interpunctella, Plutella xylostella*, *Heliothis virescens*, *Spodoptera exigua*, and *Leptinotarsa decemlineata* (Li *et al.*, 2004)

However, a loss of toxin binding was not associated with resistance to *B. thuringiensis* in several insect. Therefore, other mechanisms of resistance may be operating in these insects. Serine proteinases, such as trypsin, chymotrypsin, and elastase, are important in both the solubilization and activation of *B. thuringiensis* protoxins. In some insects, changes in these proteinases have been associated with resistance to *B. thuringiensis* toxin. A strain of *P. interpunctella* resistant to *B. thuringiensis* subsp. *Entomocidus* HD-

198 processed *B. thuringiensis* protoxin at a slower rate than the parental susceptible strain. This resistant strain lacked a major gut proteinase involved in activation of *B. thuringiensis* protoxin, and the proteinase mechanism was responsible for about 90% of the total resistance to *cry*1Ab (Li *et al.*, 2004).

Enzymes from a strain of *H. virescens* resistant to *B. thuringiensis* subsp. *kurstaki* HD-73 were reported to activate the protoxin more slowly and degrade the toxin faster than enzymes from a susceptible strain. In *Spodoptera littoralis*, increases in the specific activity of gut proteinases were associated with the loss of sensitivity to Cry1C due to an increase in the degradation of active toxin. Aminopeptidase is an exopeptidase and a marker for membrane proteins. It is localized to the brush border membrane of midgut epithelial cells and is involved in the digestion of peptides and amino acid transport. A membrane-bound glycosyl-phosphatidylinositol anchored aminopeptidase N has been reported to bind Cry1Ac toxins in several different insects. Previously, a strain of *B. thuringiensis*-resistant *Ostrinia nubilalis*, selected for resistance to *B. thuringiensis* subsp. *kurstaki* (HD-1), was described with lower trypsin-like proteinase activity compared to the parental susceptible strain, but the reduced trypsin-like proteinase activity was in the soluble fraction of gut proteinases in the resistant strain. Soluble proteinases from the resistant strain incubated with Cry1Ab protoxin resulted in lower amounts of an active toxin fragment relative to incubations with proteinases from the susceptible strain. This reduction in toxin activation may account for lower susceptibility to *B. thuringiensis* toxins by resistant *O. nubilalis* larvae (Li *et al.*, 2004).

3.11 PROTEIN EXTRACTION

Most of the proteins and enzymes studied in the early days of protein chemistry isolated from extra cellular fluids. The reason for this is not just because it was easy to obtain the raw materials but because extra cellular proteins are most stable often as a result disulphide cross links and they tend to be small molecules but those protein present inside the cell are less stable and need more techniques for study. Cell disruption is the first step in any analytical process in which the contents of cell are to be released and extracted. (Khatri, 2002)

3.12 PROTEIN PURIFICATION

Several attempts have been made to take advantage of the enormous resolution power of this analytical system to purify proteins. The basic objective is to isolate particular protein of interest from other contaminating proteins to study structure and properties. Electroelution is an efficient method for recovery of proteins from PAGE gels; the subsequent steps reduce their yield. Several techniques have been reported to improve it; however, they require a series of procedures, preparative systems, or special equipment not always available in nonspecialized biochemical laboratories. (Khatri, 2002)

3.13 PROTEIN ASSAY

There has been an increase in the number of colorimetric assay techniques for the determination of protein concentration over the past 20 years. This has resulted in a perceived increase in sensitivity and accuracy with the advent of new techniques. The techniques include Bradford assay, Lowry assay, the bicinchoninic acid assay and the biuret assay. (Sapan *et al.*, 1999)

3.13.1 CB dye-binding assay (Bradford assay)

The use of the metachromatic response observed on the binding of CB to proteins for the determination of protein concentration was popularized by Bradford in1976. The binding of CB dyes to proteins was first studied by Fazekas de St. Groth *et al.* in 1963. (Sapan *et al.*, 1999)

The ease and high sensitivity of the CB protein assay have driven its extensive use for the determination of protein concentration in a wide variety of protein samples. The assay is based on the binding of the dye to the protein(s), which results in a dye–protein complex with increased molar absorbance. The assay is performed at acid pH, at which the dye is protonated and absorbs at 465 nm in solution; on binding to the protein, there is a metachromatic response with the development of a species absorbing at approx. 595 nm, where the unprotonated species would absorb. (Sapan *et al.*, 1999)

3.14 ELECTROPHORESIS

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique (Khatri, 2002).

Gel electrophoresis is a technique in which charged molecules, such as protein or DNA, are separated according to physical properties as they are forced through a gel by an electrical current. Proteins are commonly separated using polyacrylamide gel electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. PAGE can be used as a preparative tool to obtain a pure protein sample, or as an analytical tool to provide information on the mass, charge, purity or presence of a protein. Several forms of PAGE exist and can provide different types of information about the protein(s). Nondenaturing PAGE, also called native PAGE, separates proteins according to their mass: charge ratio. SDS-PAGE, the most widely used electrophoresis technique, separates proteins primarily by mass (Khatri, 2002).

3.14.1 NONDENATURING PAGE

Nondenaturing system is used to separate intact proteins, especially oligomeric proteins, by a nondestructive means for later assessment of biological activity. On nondenaturing system, protein separation depends on a combination of differences in molecular size and shape as well as charge. Separation by size is accomplished by varying the pore size of the acrylamide polymer as a function of both the concentration of the acrylamide (range about 3-30%, w/v) and the amount of cross-linker used. In general, the higher the acrylamide concentration, the smaller the protein that remains in gel: this can be counteracted by decreasing the amount of cross-linker used, which in turn increases the degree of gel swelling during standard staining and washing procedures. Separation by charge in nondenaturing gel systems is permitted because the protein separation can be

performed at any pH between 3 and 11 to allow for maximal charge differences neighboring protein species. These include the molecular weight of the stability in the range of pH 4 to 9. Once these parameters are known optimal resolution can be obtained by varying certain components within a single gel system. For example the initial choice of the gel system for separation of acidic protein of molecular weight 20,000 might be a system operating at an alkaline pH i.e.9 with an acylamide concentration of 12-15 %(Jakoby, 1984).

It should be noted that biologically active proteins such as enzymes often require specials electrophoresis conditions in order to retain activity after separation. For example the heat lability of the some enzymes requires that electrophoresis be conducted most conveniently in a refrigerated room or if necessary using circulating water of 0-4C in the cooling jacket of the slab gel apparatus. In addition ammonium persulphate an agent commonly used in gel polymerization reaction often interferes with enzymes activity after elution from the gel. For this reason the photo activated polymerizing agent riboflavin can also be used instead of ammonium persulphate in the preparation of the stacking gel. For the various systems if ammonium persulphate affects enzyme activity then preelectrophoresis can be used. Dithioithreitol (40-100 m*M*) or 2-mercaptoethanol (upto 1 *M*) may be included in the sample buffer to reduce some disulfide linkages, although detergent denaturing is often necessary for complete disulfide reduction (Jakoby, 1984).

3.14.2 SDS- PAGE OF PROTEINS

Acrylamide mixed with bisacrylamide forms a cross-linked polymer network when the polymerizing agent, ammonium persulfate, is added (Figure 1). The ammonium persulfate produces free radicals faster in the presence of TEMED (N, N, N, N'-tetramethylenediamine). The size of the pores created in the gel is inversely related to the amount of acrylamide used. Gels with a low percentage of acrylamide are typically used to resolve large proteins and high percentage gels are used to resolve small proteins.

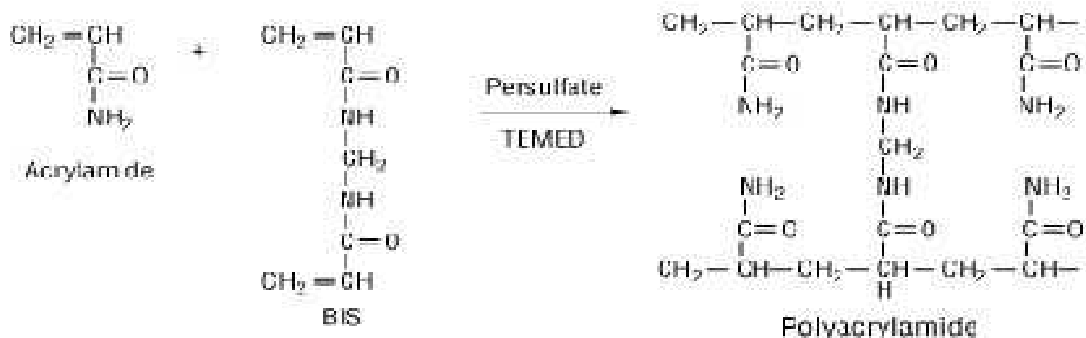

Figure 2. Polymerization and cross-linking of acrylamide.

However, polyacrylamide of sufficient stability will always be so dense, that friction influences the migration of proteins in the gel. The electrophoretic mobility of a protein is determined mostly by its net charge per unit mass at the given pH, but is inversely proportional to its frictional coefficient in the gel, determined by the proteins size and shape. In the denaturing, reductive variant of PAGE, SDS-PAGE all differences between the proteins in charge per unit mass has been eliminated by the SDS (sodium dodecylsulphate) and the proteins migrate solely according to subunit size. The charged SDS molecules bind all along the polypetide chain, giving the chain equal charge per unit length. Thus, the denatured polypeptide chains are separated electrophoretically only according to their subunit length. The polyacrylamide gels may be cast as i fixed concentration gel (e.g. 10%) or as a gradient gel (e.g. 7-15 %). There is also a choice between continuous and discontinuous buffer systems. If the same buffer ions are present throughout sample, gel and electrophoresis buffer the electrophoresis is referred to as continuous buffer PAGE. In discontinuous buffer PAGE the separation is usually preceded by a stacking of the sample to a narrow band in a low-porosity stacking gel. (Khatri, 2002)

The high pore size stacking gel is to concentrate the protein sample into a sharp band before it inters to the main separating gel. This is achieved by utilizing differences in the ionic strength and pH between the electrophoresis buffer and the stacking gel. The band sharpening effect relies on the fact that negatively charged glycine ions (in running buffer) have a lower electrophoretic mobility than do the protein-SDS complex which in turn has lower mobility than the chloride ion of the loaded buffer and the stacking gel. When current is switched on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical current. The glycinate ions can only move at the same speed as Cl^- ion if they are in the region of the higher field strength. Field strength is inversely proportional to conductivity which is proportional to conc. The result is that three species of interest adjust their concentrations so that [Cl^-] > [protein-SDS] > [glycinate ions]. There is only a small quantity of protein-SDS complexes so they concentrate in a very tight bond between glycinate and Cl^- boundaries. Once the glycinate reaches the separating gel it becomes more fully ionized in higher pH environment and its mobility increases. Thus the interface between the glycinate and Cl^- leaves behind the protein-SDS complexes which are left to electrophoresis at their own rates. The negatively charged protein SDS complexes now continue to move towards the anode and because they have the same charge per unit length they travel into the separating gel under the applied electric field with the same mobility .However as they pass through the separating gel the protein separate owing to the molecular sieving properties of the gel. (Khatri, 2002) Detection of the separated protein bands may be done in several ways. Staining for protein with Coomassie Brilliant Blue G-250 is a standard procedure detecting 0.1 to 1µg per band (depending on band dimensions). Coomassie Brilliant Blue G- 250 can also be used and by using dilute staining solution, destaining can be omitted. An increase in detection sensitivity of approximately a factor 100 can be obtained with silver staining.

3.14.2.1 Determination of Molecular Weight

This is done by SDS-PAGE of proteins known molecular weight along with the proteins to be characterized. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide and its *Rf* value. The *Rf* is calculated as the ratio

of the distance migrated by the molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight by electrophoresis *(M*r) is to plot a standard curve of distance migrated vs. log10MW for known samples, and read off the log*Mr* of the sample after measuring distance migrated on the same gel. (Khatri, 2002)

3.15 IMMUNOLOGY

A substance which can Induce a detectable immune response is known as an antigen immune response is expressed either in the form of activated cells (cell mediated immune response) an d or as antibodies (humoral response). (Talwar and Gupta, 1997)

Antibody formation consists of two phases an induction and production phase. Specific information for the anamnestic response is stored in the lymphoid cells. The magnitude of immune response depends on various factors namely

i. Choice and strain of Animals
ii. Form and dose of the antigen
iii. Adjuvant
iv. Route of immunization
v. Immunization schedule.

i. Choice of strain of animals

Rabbits, goats, sheep and horses are generally employed for production of antibodies. (Talwar and Gupta, 1997)

ii. Form and dose of antigen

Antigens may be of several types, soluble and particulate proteins, viruses sub cellular particulates and entire complex cells such as tumors cells and bacteria. (Talwar and Gupta, 1997) Soluble antigens when injected are readily diluted out, catabolized and hence stimulated poor immune response. To overcome this problem, primary immunizing dose of soluble antigen should therefore be given with adjuvant and by a route of immunization in such cases can be subcutaneous (SC), intramuscular (IM) or intradermal

(ID). Materials to be injected should be kept sterile to avoid immunogenic degradation. Soluble materials can be made sterile by passing through Millipore s discs having a pore size of 0.45 or 0.22 μ. Particulate antigens can be obtained in reasonably sterile form by operating under sterile conditions. Preservatives that do not alter antigenicity, such as 0.5% phenol or merthionate (0.01%) can be used. Proteins can usually be given a wide range (1 μg to 500 μg/animal) without inducing high or low dose tolerance. (Talwar and Gupta, 1997)

iii. Preparation of Emulsion

In Syringe Method, appropriate dose of the antigen to be used is dissolved or suspended in physiological saline. Protein antigens upto 100 mg/ml usually produce emulsions. To a volume of IFA or CFA in a clean, dry, sterile wide mouth small beaker or bottle add vigorously an equal volume of antigen solution. The dispersion of thee water phase is increased by repeatedly taking up the emulsion into the syringe and the ejecting becomes more viscous. Large amounts of emulsion can be prepared in mechanical emulsifiers. (Talwar and Gupta, 1997)

iv. Route of immunization

An antigen can be administered into animal body by a variety of routes. The main consideration while selecting a route is form and nature of the antigen. Intramuscular is one of the most frequently adopted routes of immunization and suitable for alum precipitated and adsorbed antigens. The antigen is deposited in the muscular layer e.g. thigh muscles. The needle is inserted from the rear at right angles to the skin surface at a point half way the femur, so that its point lies within the muscle. The inoculation is then made, needle withdrawn and the site gently massaged. (Talwar and Gupta, 1997)

v. Immunization schedule

After complete dose of immunization animals should be bled to test the serum for

antibody production. Rabbits can be bled from the marginal vein of the ear. About 10 to 50 ml of blood can be collected in a single bleed from a healthy rabbit repeatedly, several times at an assistant with its head protruding over the edge of the table. Downward pressure is applied at the back with one hand and holds the neck region with other. Lateral margin of the ear is dry shaved and cleaned with 70% ethanol. To have a good look at vein ear may be transmitted by placing a table lamp beneath it. Vein if not prominent can be dilated either by rubbing the ear with fingers or cotton swab moistened with xylene. (Talwar and Gupta, 1997)

3.16 SEPARATION AND PRESERVATION OF SERUM OR PLASMA

After collecting the blood it is allowed to clot at room temperature. A thin glass rod is moved gently along the side of the tube to retract the clot. During collection and retraction of the cot, care should be taken not to hemolyze the blood as it beads to the degradation of immunoglobulin by the action of enzymes. As a rule of thumb, recovery of the serum should be about one half of the blood volume. After 30 min to 2 hour free serum is collected by a Pasteur pipette in a centrifuge tube. The tubes with remaining clot are left overnight in cold for a final collection of serum. Blood cells are removed by centrifugation at 4°C for 20 min at 2500 rpm and top layer of the clear serum is collected by means of Pasteur pipette. (Talwar and Gupta, 1997)

Storage of serum; Before storing the serum it should be decomplemented by incubation at 56°C for 30 min. Decomplemented serum can be stored for long time by freezing at -20°C. To avoid repeated freeze and thaw the samples to be stored are aliquot in small volumes of 0.1 to 0.5 ml. (Talwar and Gupta, 1997)

3.17 IMMUNODIFFUSION (ID)

The immune system of vertebrates responds to exposure to antigens, i.e. foreign ('non-self') molecules, for example proteins or polysaccharides, by forming antibodies. Antibodies are immunoglobulin equipped with recognition sites for the fine structures (epitopes) of the antigens that stimulated their formation. The antibodies formed against any specific antigens can be detected by many techniques. One of the mostly used

techniques is immunodiffusion test. (Perlmann and Perlmann, 2001)

3.17.1 Ouchterlony immunodiffusion tests

Immunodiffusion (ID) is a technique used for detecting antigen-antibody reactions. Immunodiffusion tests are based on the theory of double diffusion which was originally described by Oudin in 1948 and Ouchterlony in 1949. Soluble antibodies and antigens are placed in separate wells that have been cut into a gel, and allowed to diffuse outward in the medium. Precipitate lines will appear in the medium where the antigens and antibodies have combined at the same relative concentrations. . The antibody is divalent, and displays two reactive sites capable of combining with the antigen in the ID gel (IgM molecules, which have a higher valence, are generally too large to diffuse well in ID gels and are not considered in this context). The antigen may have any number of determinant groups (sites where the antibody binds). However, as a general rule, molecules with fewer than 4 determinants are not active in the immunodiffusion test. Extensive crosslinking must occur between antigen and antibody in order for the immune complexes to precipitate within the gel matrix.

When multivalent antigens combine with divalent antibodies in solution, 3-dimensional lattices are formed which aggregate and precipitate. The amount of precipitate varies with the proportion of antigen and antibody. At equivalent or optimal proportions almost all the antigen and antibody precipitate together. This proportion forms the most rapid precipitin format. If there is an excess of antibody, the complexes formed with the antigen are insoluble. When there is an excess of antigen, the precipitate has a tendency to dissolve due to the formation of soluble complexes. If this occurs the concentration of antibody should be increased. The precipitate of antigen-antibody complexes can be visualized in gels or can be stained.

3.18 ELISA

ELISA, enzyme-linked immunosorbent assay is a highly versatile and sensitive analytical procedure for qualitative or quantitative determination of antibodies and almost any kind

of antigens. Three principal types of ELISA are generally recognized: direct ELISA, indirect ELISA, and sandwich ELISA ((Perlmann and Perlmann, 2001).

3.18.1 Indirect ELISA to determine specific antibodies

Solid-phase immobilization of antigens or antibodies is achieved by linking them, either through adsorption or covalently, to a suitable matrix. Such immobilization makes use of the capacity of various plastics (e.g. polyvinylchloride or polystyrene) to adsorb proteins as monomolecular surface layers without significantly altering their immunological properties. The reactants are usually adsorbed onto the wells of a 96-well microtitration plate of polystyrene, an adsorption characterized by strong hydrophobic binding and slow dissociation rates. After coating with antigen or antibody, the residual protein-binding capacity of the solid matrix is blocked by exposing it to an excess of unrelated protein such as gelatin or bovine serum albumin. Coating of the microtitration plates is followed by addition of the test solution. For antibody assay, the test solution may be a serum containing an unknown concentration of antibodies against a solid-phase antigen. Then follows addition of antiglobulin–enzyme conjugate and a substrate generating a coloured product when hydrolysed. The resulting change in colour may be recorded visually or spectrophotometrically to determine the antibody concentration. Of the many different enzymes suitable for ELISA, alkaline phosphatase, β-galactosidase or horseradish peroxidase are most commonly used. (Perlmann and Perlmann, 2001)

Before testing, the ELISA conditions must always be optimized which depends on different factors such as ELISA plate, antigen dilution, antibody dilution, enzyme system (enzyme conjugated second antibody) substrate used etc. Horseradish peroxidase, one of the most widely used enzyme system, is a 40 kDa protein that catalyzes the oxidation of substrates by hydrogen peroxide, resulting in a colored or fluorescent product or the release of light as a byproduct. HRP functions optimally at a near-neutral pH and can be inhibited by cyanides, sulfides and azides (acidic conditions). TMB (3, 3’, 5, 5’-tetramethylbenzidine) is the most common chromogenic substrate for use in ELISA with peroxidase systems. TMB is a noncarcinogenic analog of benzidine, which is used as a substrate for enzyme immunoassays using horseradish peroxidase conjugated antibodies

and molecular probes. This novel formulation of TMB employs a non-hazardous, non-toxic solvent system that permits the use of buffers that are more appropriate to the function of the TMB dye reaction. This system develops a blue reaction product when it is reacted with peroxidase conjugates. This can then be read visually at 370 nm and 620 to 650 nm.When the reaction is stopped, the color turns from blue to yellow and is read at 450 nm (Schneider *et al.*, 2004).

3.19 WESTERN BLOTTING

Western blotting (immunoblotting) is a system for the immunological detection of specific proteins that have been transferred from a polyacrylamide gel to an immobilizing membrane Proteins transferred to immobilizing filter membranes, such as nitrocellulose, are well suited to high-sensitivity solid-phase immunoassay detection techniques. There are several clear advantages to utilizing membrane-immobilized proteins, the main one being that a near replica of the protein zones in the separation gel can be obtained (western blot). This method is ideally suited to the study of specific proteins in biological extracts; it is also relatively fast and extremely cost effective since only minute amounts of immunological reagents are used. This protocol describes the electrophoretic transfer (electroblotting) of proteins from the separation gel to the membrane followed by detection using peroxidase conjugated secondary antibodies with the chromogen. (Whitehouse and Lovegrove, 2005)

3.20 NUMERICAL INDEX OF THE DISCRIMINATORY ABILITY OF TYPING METHOD

Typing systems are used to define specific characteristics of the object under study. The Procedures are specific for different phenotypic or genetic parameters and can be general (i.e, applicable to any microbial species) or species or genus specific (Belkum *et al.*, 2001). Epidemiological typing of bacterial strains can be carried by a variety of techniques, including serotyping, biotyping, bacteriophage and bacteriocin typing and genotyping. When microbial typing methods are compared, the three main characteristics that need to be considered are its typeability, reproducibility, and discriminatory power

(Hunter and Gaston, 1988). The typeability of a method is the proportion of the population of distinct strains that can be assigned a type marker by that method (Hunter, 1990). The reproducibility of a typing method is the proportion of strains that are typed the same on repeat testing, preferably after a period of a few months (Hunter, 1990).

The discriminatory power of a typing method is its ability to distinguish between unrelated strains. It is determined by the number of types defined by the test method and the relative frequencies of these types (Hunter and Gaston, 1988). The discriminatory power of a method is an estimate of its ability to differentiate between two unrelated strains. Gaston and Hunter, 1988 have suggested that discriminatory power can be defined mathematically as the probability that the two strains chosen at random from a population of unrelated strains will be distinguished by that typing method. This probability can be calculated by Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat. This probability, numerical index of discriminatory power (D) is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where s is the number of types, x_j is the number of population members falling into the jth type, and N is the size of the population (Hunter and Gaston, 1988 and Hunter, 1990). Thus, a D value of 1.0 would indicate that typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, an index of 0.0 would indicate that all members of a strain population were of an identical type. An index of 0.50 would mean that if one strain was chosen at random from a strain population, there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first (Hunter, 1990). This equation can be applied both to a direct comparison of the discriminating power of typing methods and to analysis of the discriminating power of combined typing schemes (Hunter and Gaston, 1988). The acceptable level of discrimination will depend on a number of factors, but an index of greater than 0.90 would seem to be desirable if the typing results are to be interpreted with confidence (Hunter and Gaston, 1988)

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 MATERIALS

The materials used in this thesis work were given in Appendix-I.

4.2 METHODOLOGY

4.2.1 SAMPLE COLLECTION AND TRANSPORT

The soil samples were collected from the Sagarmatha national park and Phereche region of Khumbu base region and were transported to RESEARCH LABORATORY FOR AGRICULTURAL BIOCHEMISTRY AND BIOTECHNOLOGY (**RLABB**) where the study was carried out from March 2005 to December 2005 in joint collaboration with Central Department of Microbiology, Tribhuvan University, Kirtipur.

4.2.2 ISOLATION OF *BACILLUS THURINGIENSIS*

Bacillus thuringiensis were isolated by Acetate selection method (Travers *et al.*, 1987). The soil samples (1gm) were taken in a sterile 100 ml conical flask containing one milliliter of 0.25M sodium acetate (pH 6.8) and 9 ml of Lauria Bertani (LB) broth. The broth was incubated on the water bath shaker at 30°C for 4 hours and then heat treated at 80°C for 5 mins in hot water bath. One millilitre of the sample was dispensed in a sterile petriplate in which 20 ml of molten Lauria agar media was poured, shaken well and left till the media solidified and then incubated overnight in water bath shaker at 30°C.

4.2.3 IDENTIFICATION

The organism were identified by standard microbiological techniques including colonial characteristics, morphological characteristics and biochemical characteristics (Bergey's Manual of Systematic Bacteriology, Volume 2, 1986)

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4.2.4 INSECT BIOASSAY

Mosquito and their larva were collected from the ditches in local area of Bode, Bhaktapur. The larva was identified as *Culex* spp at the Central Department of Zoology, Tribhuvan University, Kirtipur.

The bacterial isolates were first grown in conical flasks containing BHI Broth (pH 7.2) at 30°C for 3-7 days. Then hundred milliliters of seasonal water with 0.3 ml of 5% brewer's yeast were taken in each jar bottles and were sterilized. In each bottle 10 mosquito larva and 5 ml of sporulated bacterial suspension is mixed into the presterilized bottle and was kept at room temperature for 7 days. The mortality rate was recorded till 7 days. Each sample was processed for duplicate assay.

4.2.5 PRODUCTION AND PURIFICATION OF *B. THURINGIENSIS* CRYSTAL PROTEINS

B. thuringiensis strains were incubated till autolysis. Spores and crystals were separated by centrifugation at 10,000 × *g* for 20 min and then washed four times with phosphate buffer of pH 7.0. The pellet was finally suspended in carbonate buffer (50mM sodium carbonate, 10mM β-mercaptoethanol and 1mM EDTA with PH-10.5) for overnight with constant shaking.

The crystal protein was purified from other protein by Native PAGE. After gel electrophoresis, bands were cut out using a clean scalpel. The excised gel pieces were placed in clean microcentrifuge tubes. One milliliter of elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) was added. The gel pieces were crushed using a clean pestle and incubated overnight in a rotary shaker at 30°C. The supernatant (crystal protein) was collected by centrifuging at 10,000 × *g* for 10 minutes.

4.2.6 PROTEIN DETERMINATION AND PROTEIN PROFILING

Protein content in the supernatant was determined by Bradford assay (1976). An aliquot of the supernatant was tested for the presence of protein by SDS-PAGE (Laemmli, 1970).

4.2.7 LABORATORY ANIMAL

A pair of New Zealand white rabbits weighing 2.5-3Kg of 2-3 months (not immunized before) was obtained from National FMD control section Budhanilkantha (Khoreto Bibhag). The rabbits were tested for parasites and found free of parasites and any diseases.

4.2.8 IMMUNIZATION

A suspension of purified crystal protein was centrifuged at 10,000 x g for 30 min at 5°C. The pellet was resuspended in saline to a concentration equivalent to 1.0 mg/ml. The suspension was emulsified in an equal volume of Freund's complete adjuvant (Difco). Each rabbit was injected with 1.25 ml (500 mg of protein) of the emulsified crystals by the intramuscular route. The booster injections with incomplete adjuvant were given after each 14 days. The animals were bled 7 days after third booster dose. Polyclonal antisera were pooled and decomplemented by incubation at 56°C for 30 min. Aliquots of antisera (0.1 to 0.5 ml) were stored at -20°C.

4.2.9 IMMUNODIFFUSION TEST

The presence of polyclonal antibody was confirmed in 1% agarose in Phosphate buffer of pH 7.2 by Ouchterlony double diffusion method (Talwar and Gupta, 1997). Undiluted and diluted antisera (*viz*. 1:10, 1:100, 1:1000 and 1:10000) were tested against antigen preparation (1mg/ml) incubating for 72 hours at 4°C in moist chambers. After washing several hours with 0.15M NaCl.bands were visualized with Amido Black stain. Finally, undiluted antisera were tested against S_1, S_{2a}, S_4, S_5, S_{10}, P_2, P_6 and P_{10} antigens.

4.2.10 OPTIMIZATION OF INDIRECT – ELISA

Cobalt irradiated polystyrene microplates (Nune ImmuplatesI-Nunc, Denmark} were used as solid phase. Titration was done as Trottier et al., (1992) and Voller et al., (1979). The polyclonal antisera and conjugate (anti rabbit IgG conjugated with Horse Radish Peroxidase (HRP)) were optimized against the S_6 antigen (6-8 µg coated per well) for Indirect Enzyme-linked immunosorbent assay (Ind-ELISA). Following the optimized protocol, the antisera were tested against all the endotoxins from respective *B. thuringiensis* isolates for immunocrossreactivity.

4.2.11 WESTERN BLOTTING

The presence of antibodies was confirmed by Western Blotting (Talwar and Gupta, 1997). The purified whole crystal protein was resolved into its component bands by SDS-PAGE and the resolved bands were transferred onto Nytran membrane (nitrocellulose) using transfer buffer (pH 8.3). After blocking and washing, bands were visualized using TMB as substrate (Schneider *et al*., 2004).

CHATPTER-V

5. RESULTS

5.1. ISOLATION AND IDENTIFICATION OF *B.THURINGIENSIS*

All soil samples were collected from the Sagarmatha National Park and Phereche region of the Khumbu region. All the samples were proceeded for acetate selection method for the isolation of *B.thuringiensis* as described by Martin and Travers, 1989. The colonies with various morphologies were picked up randomly and transferred to Brain Heart Infusion agar (BHI) plate for pure culture. These colonies were then transferred to Nutrient agar (NA) plates and then preceded for colonial morphological, cultural and biochemical characterization. All the characteristics were compared with the standards described by Bergey's Manual of Systematic Bacteriology, Volume 2, 1986.

5.2 COLONIAL MORPHOLOGY

Out of 52 Phereche soil samples, 63 different isolates were isolated and colony characteristics of each isolate were observed Table 1 and out of 39 different soil samples from SNP, 46 different isolates were isolated and colonial characteristics of each were observed Table 2.

Table 1: Colony Characteristics of the Phereche isolates

Codes of isolates	Colour	Configu-ration	Margin	Consis-tency	Opacity	Elevation
P_1	Creamy yellow	Irregular	Smooth	Mucoid	Opaque	Convex
P_2	White creamy	Irregular	Smooth	Mucoid	Opaque	Flat
P_3	Creamy White	Branching	Irregular	Mucoid	Opaque	Convex
P_{4a}	Creamy white	Irregular	Smooth	Mucoid	Opaque	Convex
P_{4b}	Yellowish	Irregular	Smooth	Mucoid	Opaque	Flat
P_5	White creamy	Irregular	Smooth	Mucoid	Opaque	Flat
P_6	Creamy yellowish	Irregular	Irregular	Mucoid	Opaque	Flat
P_7	White creamy	Irregular	Irregular	Mucoid	Opaque	Flat
P_{8a}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{8b}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{9a}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex

P_{9b}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{10}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{11a}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{11b}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{12a}	White creamy	Irregular	Irregular	Mucoid	Opaque	Flat
P_{12b}	White creamy	Irregular	Irregular	Mucoid	Opaque	Flat
P_{12c}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{13}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{14}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{15}	White	Branching	Irregular	Dry	Opaque	Flat
P_{16a}	White		Smooth	Dry	Opaque	Flat
P_{16b}	White	Branching	Irregular	Dry	Opaque	Flat
P_{17a}	White creamy	Irregular	Irregular	Dry	Opaque	Raised
P_{17b}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
P_{18}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
P_{19}	Creamy	Swarming	Irregular	Mucoid	Opaque	Convex
P_{20}	Creamy	Swarming	Irregular	Mucoid	Opaque	Convex
P_{21}	Creamy		Irregular	Mucoid	Opaque	Convex
P_{22}	Creamy white	Swarming	Irregular	Mucoid	Opaque	Flat
P_{23}	Yellowish white	Irregular	Smooth	Mucoid	Opaque	Flat
P_{24}	White	Swarming	Smooth	Mucoid	Opaque	Flat
P_{25}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{26}	White	Smooth	Irregular	Dry	Opaque	Flat
P_{27}	White	Smooth	Irregular	Dry	Opaque	Flat
P_{28}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
P_{29}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
P_{30}	White	Smooth	Irregular	Dry	Opaque	Flat
P_{31}	White	Smooth	Irregular	Dry	Opaque	Flat
P_{32}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{33}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{34}	Creamy yellowish	Irregular	Irregular	Mucoid	Opaque	Flat
P_{35}	Creamy yellowish	Irregular	Irregular	Mucoid	Opaque	Flat
P_{36}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{37}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{38}	White	Smooth	Irregular	Dry	Opaque	Flat
P_{39}	Creamy	irregular	Smooth	Mucoid	Opaque	Convex
P_{40}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
P_{41}	Watery dull white	Smooth	Irregular	Mucoid	Opaque	Raised
P_{42}	Creamy	Irregular	Irregular	Mucoid	Opaque	Raised
P_{43}	Watery dull white	Smooth	Irregular	Mucoid	Opaque	Raised
P_{44}	Watery dull white	Smooth	Serrate	Mucoid	Opaque	Raised
P_{45}	Watery dull white	Smooth	Serrate	Mucoid	Opaque	Raised
P_{46}	Creamy	Smooth	Irregular	Dry	Opaque	Convex
P_{47}	Creamy	Smooth	Serrate	Mucoid	Opaque	Convex
P_{48a}	Watery dull white	Smooth	Irregular	Mucoid	Transpar	Raised

					ent	
P_{48b}	Watery dull white	Smooth	Serrate	Mucoid	Transpar ent	Raised
P_{49}	Watery dull white	Smooth	Serrate	Mucoid	Transpar ent	Raised
P_{50}	Watery dull white	Smooth	Irregular	Mucoid	Transpar ent	Raised
P_{51a}	Watery dull white	Smooth	Irregular	Mucoid	Transpar ent	Raised
P_{51b}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
P_{52}	Watery	Smooth	Serrate	Mucoid	Opaque	Raised
P_{53}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex

Table 2: Colony characteristics of SNP isolates

Codes of Isolates	Colour	Configu-ration	Margin	Consis-tency	Opacity	Elevation
S_{1}	Creamy	swarming	Smooth	Mucoid	Opaque	convex
S_{2a}	Creamy	swarming	Irregular	Mucoid	Opaque	Convex
S_{2b}	White	Swarming	Irregular	Dry	Opaque	Flat
S_{3}	Yellowish	Round	Smooth	Mucoid	Opaque	Flat
S_{4}	Yellowish	Irregular	Smooth	Mucoid	Opaque	Flat
S_{5}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{6}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{7}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{8}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{9}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{10}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{11}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{12}	Creamy	irregular	Smooth	Mucoid	Opaque	Convex
S_{13}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{14}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{15a}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{15b}	Dull white	Branching	Irregular	Dry	Opaque	Flat
S_{16}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{17}	Watery	Smooth	Serrate	Mucoid	Opaque	Convex
S_{18}	Creamy	irregular	Irregular	Dry	Opaque	Convex
S_{19}	Creamy	Branching	Irregular	Mucoid	Opaque	Convex
S_{20}	Creamy	irregular	Irregular	Mucoid	Opaque	Convex
S_{21}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{22}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{23}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{24}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{25}	Creamy	Irregular	Smooth	Mucoid	Opaque	Convex
S_{26}	Watery dull white	Smooth	Irregular	Mucoid	Transparent	Raised

S_{27a}	Watery dull white	Smooth	Smooth	Mucoid	Opaque	Raised
S_{27b}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{28a}	Creamy	Branching	Irregular	Mucoid	Opaque	Convex
S_{28b}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{29}	Creamy white	Smooth	Irregular	Mucoid	Opaque	Convex
S_{30}	Creamy white	Smooth	Irregular	Mucoid	Opaque	Convex
S_{31}	Milky white	Smooth	Smooth	Mucoid	Opaque	Flat
S_{32}	Creamy	irregular	Irregular	Mucoid	Opaque	Convex
S_{33}	Creamy	irregular	Irregular	Mucoid	Opaque	Convex
S_{34}	Milky white	Smooth	Irregular	Mucoid	Opaque	Flat
S_{35a}	Milky white	Smooth	Smooth	Mucoid	Opaque	Flat
S_{35b}	Creamy white	Smooth	Irregular	Mucoid	Opaque	Convex
S_{36}	Creamy white	Smooth	Smooth	Mucoid	Opaque	Convex
S_{37}	Creamy	irregular	Irregular	Mucoid	Opaque	Convex
S_{38a}	Creamy	Irregular	Irregular	Mucoid	Opaque	Convex
S_{38b}	White	Smooth	Irregular	Dry	Opaque	Convex
S_{39}	Creamy white	Smooth	Irregular	Mucoid	Opaque	Convex
S_{41}	Creamy white	Smooth	Irregular	Mucoid	Opaque	Convex

5.3. MORPHOLOGICAL CHARACTERISTICS

All the isolates were found to Gram positive spore forming rods. Out of 109 isolates 86 were found to have crystal protein (Table 3).

Table 3: Crystal staining of *B. thuringiensis* isolates.

Sampling site	**Crystal Staining**	**Isolates**
Phereche (63)	+ve (46)	P_1, P_2, P_3, P_5, P_6, P_7, P_{9a}, P_{10}, P_{12a}, P_{12b}, P_{13}, P_{14}, P_{15}, P_{17a}, P_{18}, P_{21}, P_{22}, P_{24}, P_{25}, P_{26}, P_{27}, P_{28}, P_{29}, P_{30}, P_{31}, P_{32}, P_{33}, P_{34}, P_{35}, P_{37}, P_{38}, P_{39}, P_{40}, P_{41}, P_{42}, P_{43}, P_{44}, P_{45}, P_{46}, P_{47}, P_{48a}, P_{49}, P_{50}, P_{51b}, P_{52}, P_{53}
	-ve (17)	P_{4a}, P_{4b}, P_{8a}, P_{8b}, P_{9b}, P_{11a}, P_{11b}, P_{12c}, P_{16a}, P_{16b}, P_{17b}, P_{19}, P_{20}, P_{23}, P_{36}, P_{48b}, P_{51a}
Sagarmatha National Park (46)	+ve (40)	S_1, S2a, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10}, S_{13}, S_{14}, S_{15a}, S_{15b}, S_{16}, S_{18}, S_{20}, S_{21}, S_{22}, S_{23}, S_{24}, S_{25}, S_{26}, S_{27a}, S_{27b}, S_{28a}, S_{28b}, S_{30}, S_{31}, S_{32}, S_{33}, S_{34}, S_{35a}, S_{35b}, S_{36}, S_{36}, S_{37}, S_{38a}, S_{38b}, S_{39}, S_{41}

	-ve (6)	S_{2b}, S_{11}, S_{12}, S_{17}, S_{19}, S_{29}

5.4 BIOCHEMICAL CHARACTERIZATION OF *B. THURIENGIENSIS* ISOLATES

Table 4: Biochemical tests of the crystal positive B. *thuringiensis* isolates.

Isolates	P_{1}	P_{2}	P_{3}	P_{5}	P_{6}	P_{7}	P_{9a}	P_{10}	P_{12a}	P_{12b}	P_{13}	P_{14}	P_{15}	P_{17a}	P_{18}	P_{21}	P_{22}	P_{24}	P_{25}	P_{26}
Biochemical tests																				
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	+	-	+
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SIM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	-	+	-	+	-	-	+	+	+	-	+	+	+	+	-	-
O/F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
MR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-
Sugar utilization tests																				
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	+	-	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+	+
Sucrose	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+	+	+	-	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+
Hydrolysis Tests																				
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

contd…………..

Isolates	P_{31}	P_{32}	P_{33}	P_{34}	P_{35}	P_{37}	P_{38}	P_{39}	P_{40}	P_{41}	P_{42}	P_{43}	P_{44}	P_{45}	P_{46}	P_{47}	P_{48a}	P_{49}	P_{50}
Biochemical tests																			
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SIM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
O/F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
MR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Sugar utilization tests																			

Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

solates	S_1	S_{2a}	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{13}	S_{14}	S_{15a}	S_{15b}	S_{16}	S_{18}	S_{20}	S_{21}	S_{22}	S_{23}	S_{24}	S_{25}	S_2
Biochemical tests																							

Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Hydrolysis Tests																				
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Tween 20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

Contd….

atalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
xidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
itrate	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	+	-	+	+	-	+	+	+
itrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
otility	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	-	-	+	+	+
/F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+
Sugar utilization tests																							
lucose	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+
ructose	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+
altose	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ucrose	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+	+
alicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rabinose	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
annitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis Tests																							
tarch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
elatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ween 20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
asein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Isolates	S_{27a}	S_{27b}	S_{28a}	S_{28b}	S_{30}	S_{31}	S_{32}	S_{33}	S_{34}	S_{35a}	S_{35b}	S_{36}	S_{37}	S_{38a}	S_{3}
Biochemical tests															
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SIM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+
O/F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
MR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+
Sugar utilization tests															
Glucose	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Sucrose	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Hydrolysis Tests															
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

contd……….

5.5 INSECT BIOASSAY

Insecticidal activities of sample S_6 were found to be highest among six SNP isolates and that of sample P_2 were highest among four Phereche isolates (Table 5)

Table 5: Insecticidal activities of *B. thuringiensis* endotoxins using Stationary phase culture

S.No.	Samples	Sets	No. of Deaths out of 10 mosquito larva			Total deaths out of 20 larva	Percent [a]
			24 hrs	48 hrs	72 hrs		
1	Control	I	0	0	0	0	0
		II	0	0	0		
2	S_1	I	3	6	8	14	70
		II	4	6	6		
3	S_{2a}	I	3	5	5	12	60
		II	6	6	7		
4	S_4	I	2	3	3	8	40
		II	1	4	5		
5	S_5	I	8	8	9	18	95
		II	7	8	9		
6	**S_6**	**I**	**8**	**9**	**10**	**20**	**100**
		II	**9**	**10**	**10**		
7	S_{10}	I	3	3	5	14	70
		II	5	6	9		
8	P_1	I	2	4	5	12	60
		II	2	5	7		
9	P_2	I	4	7	7	17	85
		II	5	8	10		
10	P_6	I	2	4	4	10	50
		II	5	5	6		
11	P_{10}	I	3	4	4	9	45
		II	4	4	5		

a =final percentage of death of larva after 72 hours

5.6 PROTEIN DETERMINATION

Table 6: Determination of crude Crystal Protein concentration from SNP and Phereche

S.No	Protein sample	Conc. perml (mg/ml)	S.No	Protein sample	Conc. per ml (mg/ml)
1	S_1	0.021	44	P_5	0.512
2	S_{2a}	0.084	45	P_6	0.443
3	S_3	0.317	46	P_7	0.204
4	S_4	0.021	47	P_{9a}	0.512
5	S_5	0.072	48	P_{10}	0.707
6	S_6	1.411	49	P_{12a}	0.128
7	S_7	0.952	50	P_{12b}	0.204
8	S_8	0.512	51	P_{13}	0.185
9	S_9	0.317	52	P_{14}	0.418
10	S_{10}	1.028	53	P_{15}	0.292
11	S_{13}	0.493	54	P_{17a}	0.273
12	S_{14}	0.040	55	P_{18}	0.430
13	S_{15a}	0.751	56	P_{21}	0.122
14	S_{15b}	0.065	57	P_{22}	0.072
15	S_{16}	0.021	58	P_{24}	0.210
16	S_{18}	0.040	59	P_{26}	0.160
17	S_{20}	0.606	60	P_{27}	0.091
18	S_{21}	0.147	61	P_{28}	0.204
19	S_{22}	0.084	62	P_{29}	0.192
20	S_{23}	0.210	63	P_{30}	0.091
21	S_{24}	0.028	64	P_{31}	0.040
22	S_{25}	0.047	65	P_{32}	0.462
23	S_{26}	0.015	66	P_{33}	0.575
24	S_{27a}	0.128	67	P_{34}	0.072
25	S_{27b}	0.122	68	P_{35}	0.109
26	S_{28a}	0.795	69	P_{36}	0.147
27	S_{28b}	0.135	70	P_{37}	0.449
28	S_{30}	0.109	71	P_{38}	0.443
29	S_{31}	0.940	72	P_{39}	0.084
30	S_{32}	0.965	73	P_{40}	0.040
31	S_{33}	0.474	74	P_{41}	0.575
32	S_{34}	0.713	75	P_{42}	0.147
33	S_{35a}	0.713	76	P_{43}	0.204
34	S_{35b}	0.474	77	P_{44}	0.021
35	S_{36}	0.506	78	P_{45}	0.040
36	S_{37}	0.587	79	P_{46}	0.468
37	S_{38a}	0.399	80	P_{47}	0.581
38	S_{38b}	0.499	81	P_{48a}	0.512

39	S_{39}	0.562	82	P_{49}	0.040
40	S_{41}	0.084	83	P_{50}	0.078
41	P_{1}	0.028	84	P_{51b}	0.166
42	P_{2}	0.474	85	P_{52}	0.216
43	P_{3}	0.204	86	P_{53}	0.531

5.7 SDS-PAGE.

Using 12% acrylamide separating gel, the SDS-PAGE of crystal protein from isolate S_6 revealed five different bands of molecular weight ranging from 40KD to110KD (Table 7).

Table 7: SDS-PAGE band profiles of crystal proteins from isolate S_6

Sample	Bands[a]	Rf values[b]	Molecular Weight(KD)
S_6	First	0.14	107
	Second	0.23	83
	Third	0.30	71
	Fourth	0.38	58
	Fifth	0.52	40

a= reading from loading well onwards
b= ratio of distance moved by the band to that of tracking dye

5.7 INSECT BIOASSAY USING PURIFIED CRYSTAL PROTEIN

Insecticidal activity of S_6 endotoxin (3oμg/ml), partially purified from autolysed *B. thuringiensis*, S_6 broth by alkaline solution method as described elsewhere and purified by Native PAGE was found to be 100 %(10/10) efficient (Pang, 1994).(Table 8)

Table 8: Insecticidal activity of purified endotoxins from *B. thuringiensis* S_6

S.No.	Crystal protein[a]	No. of deaths out of 10 mosquito larva[b]			Percent [c]
		24 hours	48 hours	72 hours	
1	Control	0	0	0	0
2	**S_6**	**8**	**10**	**10**	**100**

a= partially purified by alkaline solution method and major band eluted from Native PAGE
b= average percentage of two sets of corresponding assay
c=final percentage of death of larva after 72 hours.

5.8 OUCHTERLONY DOUBLE DIFFUSION METHOD

The presence of polyclonal antibody in the serum bled 7 days after third booster dose with incomplete adjuvant was confirmed in 1% agarose in Phosphate buffer of pH 7.2 by Ouchterlony double diffusion method (Talwar and Gupta, 1997). The precipitin lines were observed with undiluted and diluted antisera (*viz*. 1:10, 1:100) against antigen

preparation (1 mg/ml).Similar precipitin lines were observed with S_1, S_{2a}, S_4, S_5, S_{10}, P_2, P_6 and P_{10} antigens.

5.9 ELISA OPTIMIZATION

Using 6-8 µg of crystal protein coated microtitre plate per well, the optimal dilutions of the first (antisera) and second (conjugate) antibodies were found to be 1000 and 2000 folds respectively (Trottier *et al.*, 1992). (Table 9). The average cut-off value for colour observation was determined at OD_{450} = 0.045 (Figure)

Table 9: Optimization of polyclonal antisera against crystal protein[a] and conjugate

Serial number	1st antibody (antisera)	2nd antibody (conjugate)	OD_{450} [b]
1. (Blank)	1:10	1:1000	0.000
2. (negative control)[c]	1:10	1:1000	0.001
3.	1:10	1:1000	0.396
		1:2000	0.094
		1:10,000	0.002
4.	1:100	1:1000	0.190
		1:2000	0.072
		1:10,000	0.002
5.	**1:1000**	1:1000	0.068
		1:2000	**0.045**
		1:10,000	0.001
6.	1:10,000	1:1000	0.007
		1:2000	0.010
		1:10,000	0.001

a= the microtitre plates were coated with 6-8 µg of crystal protein per well except the blank ones.
b= Average value of the three sets of corresponding assay

c= Preimmunized serum

5.9. IMMMUNOCROSSREACTIVITY

Table 10: Crossreactivity [a] of *B.thuringiensis* crystal proteins from SNP soil samples with polyclonal antiserum against S_6-crystal protein

S.No.	Crystal proteins from SNP isolates	OD_{450} [b]	Cross reactivity
1	Positive control (S_6)	0.044	100.00
2	Negative control	0.000	00.00
3	S_1	0.034	77.27
4	S_{2a}	0.034	77.27
5	S_3	0.034	77.27
6	S_4	0.016	36.36
7	S_5	0.013	29.55
8	S_7	0.042	95.45
9	S_8	0.015	34.09
10	S_9	0.016	36.36
11	S_{10}	0.037	84.09
12	S_{13}	0.013	29.55
13	S_{14}	0.014	31.82
14	S_{15a}	0.031	70.45
15	S_{15b}	0.013	29.55
16	S_{16}	0.009	20.45
17	S_{18}	0.012	27.27
18	S_{20}	0.034	77.27
19	S_{21}	0.0098	22.27
20	S_{22}	0.014	31.82
21	S_{23}	0.016	36.36
22	S_{24}	0.014	31.82
23	S_{25}	0.013	29.55
24	S_{26}	0.013	29.55
25	S_{27a}	0.013	29.55
26	S_{27b}	0.014	31.82
27	S_{28a}	0.013	29.55
28	S_{28b}	0.013	29.55
29	S_{30}	0.017	38.64
30	S_{31}	0.038	86.36
31	S_{32}	0.036	81.82
32	S_{33}	0.037	84.09
33	S_{34}	0.009	20.45
34	S_{35a}	0.011	25.00
35	S_{35b}	0.034	77.27
36	S_{36}	0.039	88.64
37	S_{37}	0.039	88.64
38	S_{38a}	0.012	27.27
39	S_{38b}	0.009	20.45
40	S_{39}	0.008	18.18
41	S_{41}	0.034	77.27

a= Ratio of test absorbance to that of S_6 as 100%

b= Average absorbance value of the two sets of corresponding assay

Table 11: Cross reactivity [a] of *B.thuringiensis* crystal proteins from Phereche soil samples with polyclonal antiserum against S_6-crystal protein

S.No.	Crystal protein from Phereche isolates)	OD_{450} [b]	Cross reactivity
1	Positive control(S_6)	0.044	100.00
2	Negative control	0.000	00.00
3	P_1	0.011	25.00
4	P_2	0.041	93.18
5	P_3	0.012	27.27
6	P_5	0.012	27.27
7	P_6	0.009	20.45
8	P_7	0.010	22.73
9	P_{9a}	0.013	29.55
10	P_{10}	0.039	88.64
11	P_{12a}	0.009	20.45
12	P_{12b}	0.006	13.64
13	P_{13}	0.012	27.27
14	P_{14}	0.013	29.55
15	P_{15}	0.009	20.45
16	P_{17a}	0.013	29.55
17	P_{18}	0.012	27.27
18	P_{21}	0.010	22.73
19	P_{22}	0.010	22.73
20	P_{24}	0.016	36.36
21	P_{26}	0.013	29.55
22	P_{27}	0.012	27.27
23	P_{28}	0.011	25.00
24	P_{29}	0.036	81.82
25	P_{30}	0.039	88.64
26	P_{31}	0.040	90.91
27	P_{32}	0.014	31.82
28	P_{33}	0.013	29.55
29	P_{34}	0.013	29.55
30	P_{35}	0.013	29.55
31	P_{36}	0.014	31.82
32	P_{37}	0.013	29.55
33	P_{38}	0.013	29.55
34	P_{39}	0.009	20.45
35	P_{40}	0.007	15.91
36	P_{41}	0.007	15.91
37	P_{42}	0.011	25.00
38	P_{43}	0.010	22.73

39	P_{44}	0.013	29.55
40	P_{45}	0.014	31.82
41	P_{46}	0.009	20.45
42	P_{47}	0.009	20.45
43	P_{48a}	0.016	36.36
44	P_{49}	0.015	34.09
45	P_{50}	0.011	25.00
46	P_{51b}	0.015	34.09
47	P_{52}	0.012	27.27
48	P_{53}	0.038	86.36

a= Ratio of test absorbance to that of S_6 as 100%
b= Average absorbance value of the two sets of corresponding assay

Of the total 86 endotoxin producing isolates, 31 (36.05%) corresponding endotoxins were 25-30% crossreactive with the polyclonal antisera raised against the S_6 endotoxin. Similarly, 6 (6.97%) were 75-80% and 85-90% crossreactive each and 4 (4.65%) were 80-85% crossreactive. Only 3 (3.49%) were more than 90% crossreactive. (Table 12)

Table 12: Percentage of *B. thuringiensis* endotoxins immunocrossreactive with that of S_6

Range[a] of immunocrossreactivity	**Frequency of crossreactive endotoxins**	**Percentage**
10-15	1	01.16
15-20	3	03.49
20-25	14	16.28
25-30	31	36.05
30-35	10	11.63
35-40	7	08.14
40-70	0	00.00
70-75	1	01.16
75-80	6	06.97
80-85	4	04.65
85-90	6	06.97
90-95	2	02.33
95-100	1	01.16
Total	**86**	**100**

a= the immunocrsossrectivity range is considered with D=0.92 and individual value of each is given in Table 13

5.10 DISCRIMINATORY INDEX VALUE (D)

Numbers of isolates (N) = 86
Number of different polymorphic types (S) = 22

Table 13: Calculation of Discriminatory index value (D)

No. of population	% cross - reactivity[a]	Isolates giving similar % immunocross reactivity	Number
n_1	13.64	P_{12b}	1
n_2	15.91	P_{40},P_{41}	2
n_3	18.18	S_{39}	1
n_4	20.45	S_{16},S_{34},S_{38b},P_6,P_{12a},P_{15},P_{39},P_{46},P_{47}	9
n_5	22.27	S_{21},P_7,P_{21},P_{22},P_{43}	5
n_6	25.00	S_{35a},P_1,P_{28},P_{42},P_{50}	5
n_7	27.27	S_{17},S_{38a},P_3,P_5,P_{13},P_{18},P_{27},P_{52}	8
n_8	29.55	S_5,S_{13},S_{25},S_{26},S_{27a},S_{28a},S_{28b},P_9,P_{14},P_{17a} P_{26},P_{33},P_{34},P_{35},P_{37},P_{38},P_{44}	17
n_9	31.82	S_{14},S_{22},S_{24},S_{27b},P_{32},P_{36},P_{45}	7
n_{10}	34.09	S_8,P_{49},P_{51b}	3
n_{11}	36.36	S_4,S_9,S_{15b},S_{23},P_{24},P_{48a}	6
n_{12}	38.64	S_{30}	1
n_{13}	70.45	S_{15a}	1
n_{14}	77.27	S_1,S_{2a},S_3,S_{20},S_{35b},S_{41}	6
n_{15}	81.82	S_{32},P_{29}	2
n_{16}	84.09	S_{10},S_{33}	2
n_{17}	86.36	S_{31},P_{53}	2
n_{18}	88.64	S_{36},S_{37},P_{10},P_{30}	4
n_{19}	90.91	P_{31}	1
n_{20}	93.18	P_2	1
n_{21}	95.45	S_7	1
n_{22}	100.00	S_6	1
Total Number(N)			**86**

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

$$\text{Now, } D = 1 - \frac{1\,(2x6(6-1) + 17(17-1) + 3(3-1) + 4x2(2-1) + 7(7-1) + 8(8-1) + 9(9-1) + 5(5-1) + 4(4-1))}{86(86-1)}$$

Hence, D= 0.926

5.10. WESTERN BLOT.

The antibody raised against the crystal protein was finally confirmed by Western Blot (Talwar and Gupta, 1997).

CHAPTER - VI

6. DISSCUSSION

All the soil samples were collected from high altitude mountain area; Khumbu base camp, the Everest region of Nepal and processed in Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB). Out of 63 Phereche isolates, 46% were creamy and convex, all others were white (17%), watery-dull white (14%), and white creamy (14%), and occasionally creamy yellowish (5%) and yellowish (2%) were observed. Out of 46 SNP isolates, creamy colored colonies have highest percentage (63%) and yellowish colored colonies were not observed. Most of the Phereche and SNP isolates were convex and mucoid and occasionally flat were also observed. Almost all the isolates were opaque and mucoid. Most of the isolates were irregular, some were smooth and few were swarming (Table 1 and Table 2). All the isolates were found to contain characteristic Gram positive rod on Gram staining. Crystal staining was performed for all 108 isolates, out of which 86 (79.63%) were found to contain crystal protein (Table 3)

Biochemical tests were performed only for the crystal producing isolates. All the crystal forming *B thuringiensis* isolates were found to be catalase, oxidase, nitrate, indole and VP positive and were fermentative. Out of 86 were capable of utilizing citrate, 72% motile and 86% were glucose, fructose utilizer while 86% were not capable of utilizing maltose. All the isolates utilized salicin while none were capable of utilizing mannitol, but 78% urease positive and 95% arabinose negative. All the isolates were capable of hydrolysing starch, gelatin, tween 20 and casein (Table 4 and Table 5)

The most prominent characteristic of *B. thuringiensis* is the intracellular production of an insecticidal δ-endotoxin in the form of a proteinaceous crystal during sporulation. The majority of *B. thuringiensis* strains are toxic to larvae of the order Lepidoptera. (Huber-Lukac *et al.*, 1986). Of the total 86 *B. thuringiensis* six SNP isolates (viz: S_1, S_{2a}, S_4, S_5, S_6 and S_{10}) and four Phereche isolates (P_1, P_2, P_6 and P_{10}) were randomly selected for preliminary bioassay using *Culex* spp. of mosquito larva as target species. Cultures were

grown to the stationary phase, the suitable phase for the sporulation and production of δ endotoxin (Drobniewski *et al.*, 1989). The assay was set in duplicate to read reproducible results. Each set was set with 10 larva and 100 ml of sterilized water with 0.3 ml of 5% brewer's yeast. Five milliliter of *B. thuringiensis* culture of stationary phase was added in each and allowed to stand for upto three days (Bhattarai, 2002) .The number of deaths in each set was recorded for one, two and three days each so as to know the efficiency of individual activities of the respective isolates. Of the 10 isolates tested against the mosquito larva, S_6 was the most effective one (100% efficient within three days). Most of them were highly efficient to kill the larva, ranging 50% to 100% (Table 5 and Table 8).

The crystal proteins from the autolysed cultures (Huber-Lukac *et al.*,1986) were purified in alkaline solution (50mM sodium carbonate, 10mM β-mercaptoethanol and 1mM EDTA) having pH 10.5. Protein crystals were separated from spores and cell debris by a two-phase system and contaminating proteases were inactivated by 1 mM DMSO (instead 1 mM diisopropylfluorophosphate) and 1 mM EDTA (Delafield *et al.*, 1968). The dissolved protein was washed in Phosphate buffer (pH 7.2) with Tween 20 (0.05%) instead Triton X100 by Delafield et al, 1968. The dissolved crystal protein was neutralized (Gill et al 1987), concentrated using 10% TCA and quantitated by Bradford Assay (Bradford, 1976) so as to feed the proteins in SDS-PAGE. About 8-12μg (20 μl) of protein in Blue juice (Appendix VI) was loaded per well and electrophoresed (Pfannenstiel *et al.*, 1986) for two hours to resolve the proteins. Five bands were observed for S_6 preparation having molecular weight of 40, 58, 71, 83 and 107 KD using BSA (66KD), casein (22KD) and lysozyme (14 KD) as markers. Pfannenstiel et al (1986) demonstrated the importance of inhibiting proteases during purification and solubilization of *Bacillus* toxins. During isolation of the *B. thuringiensis* subsp. *israelensis* toxin, characteristic discrete protein bands migrating at 135, 94, 72, and 65 kilodaltons were not found, though some (at least three distinct ones) of the crystal toxins were found to posses activities in multiple protease-resistant domains (Pfannenstiel *et al.*, 1986).

To determine which proteins are responsible for the biological activities, the proteins must be separated under non-denaturing conditions (Pfannenstiel *et al.*, 1986).The crystal protein preparation from S_6 was eletrophoresed in non denaturing gel (Native PAGE) and

the major band was selected and extracted from the gel. Gel slices were grinded in a minimum volume of phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) (Pfannenstiel *et al.*, 1986) and centrifuged. The crystal protein from the supernatant was concentrated using 10% TCA. Separate preparations were mixed and the protein (30µg/ml per assay as used by Pang, 1994) was again tested for insect bioassay. The purified proteins were found to be highly effective against the mosquito larva (Table 8) indicating the active fractions.

Polyclonal antisera (anti-crystal protein antibodies) were raised in a pair of New Zealand white rabbits using the purified crystal protein emulsified in an equal volume of Freund's Complete Adjuvant (Pfannenstiel *et al.*, 1986; Khatri *et al.*, 2002; Huber-Lukac *et al.*, 1986; Pang, 1994; Delafield *et al.*, 1968; Armstrong *et al.*, 1985, Wie *et al.*, 1982). Using 1% agarose gel in phosphate buffer (pH 7.2) at 4 C the antigen antibody reaction was demonstrated by Ouchterlony Assay (Talwar and Gupta, 1997). A series dilution of antisera was prepared and undiluted fraction showed clean precipitin lines in the gel directing the equivalence concentrations of the antibodies against the antigen used. The procedure was repeated to test the crossreactivity of the polyclonal antisera against purified crystal proteins from SNP and Phereche isolates (Figure 10 and Table 11). All the preparations showed cross reactivity with the polyclonal antibodies indicating the similarities of antigenic determinants among the crystal proteins assayed. Though Ouchterlony test is simple to interpret, it shows low sensitivity for reproducible data. Hence, ELISA was developed and subsequently optimized for antibodies concentration to the antisera.

A 96-well polystyrene microtitre plate was coated with the 6-8 µg (Trottier *et al.*, 1992) purified crystal protein as known antigen to detect the corresponding antibody in antiserum to develop Indirect-ELISA. The optimal dilution was determined by using chequerboard titration (Trottier *et al.*, 1992, Voller *et al.*, 1979). All sera were diluted in 0.02M PBS coating 0.05% tween 20 and 0.30M NaCl and applied at 100µl per well (Trottier *et al.*, 1992). The use of NaCl in the diluent PBS was suggested by Tijssen *et al.* (1985) to achieve higher immunological specificity. A series of dilutions of antiserum (*viz.* 1:10, 1:100, 1:1000 and 1:10,000) were titrated as first antibody against a series of

dilution of conjugate (anti-rabbit IgG conjugated with Horse Radish Peroxidase (HRP) antibody. (*viz.* 1:1000, 1:2000 and 1:10,000). The concentrations of antibodies were indirectly assayed by respective degree of color development after adding TMB as substrate in the wells. ELISA reader was used to read absorbance characteristic at 450 nm (Schneider *et al*., 2004). Simultaneously the visible color change as average cut-off value was observed to be OD_{450} = 0.045. Thus, the first and second antibody concentrations were defined 1:1000 and 1:2000 as optimal. Below OD_{450} = 0.045, there was not clearly visible change in color to naked eyes (Table 9). Trottier *et al.* (1992) choose the optimal dilution which demonstrated a maximum positive to negative ratio between positive and negative serum controls so as to minimize background coloring to distinguish the positive test with negative control using minimum concentration of polyclonal antibody and conjugates. Between each ELISA step, plates were washed five times with a microplate washer (Trottier *et al.*, 1992) in order to prevent the false positive result.

The optimized ELISA protocol was used to study the cross-reactivity among the total 86 isolates using the polyclonal antiserum raised against the S_6 endotoxin. According to Hunter, (1990) and Hunter and Gaston, (1988) the indirect ELISA was assessed to have discriminatory index (D) value of 0.92 with N=86, S=22. Hence the Ind-ELISA method was found to discriminate the isolates into 13 groups with 92% confidence. Considering D=0.92, the ranges of immunocrossreactivities were tabulated as shown in table (12). Most of the isolates were 20-40% cross-reactive, with highest 31 (36.05%) comprising 25-30% cross-reactive. Second to which, 14(16.28%) comprising 20-25% crossreactive. Interestingly, not a single frequency of crossreactive endotoxin was found in the range of 40-70%. Above 70%, 6(6.97%) were 75-80% and 85-90% crossreactive each and 4(4.65%) were 80-85% cross reactive. Above 90%, only 3(3.49%) were found to be crossreactive .The results clearly showed that *B. thuirngiensis* isolates from Khumbu base region were highly diverse for their crystal protein antigenicity.

Considering the specificities of monoclonal antibodies produced by hybridoma technology to detect the specific antigen (Huber-Lukac *et al.*, 1986; Matsuyama *et al*., 1995), Huber-Lukac (1986) characterized monoclonal antibodies to a crystal protein of *B. thutringiensis* subsp. *kurstaki*. Huber-Lukac (1986) aseptically removed spleen after 4

days of final immunization for hybridization. Two separate fusions were performed; 2x 10^8 spleen cells from mice in each series were fused with 3 x 10^7 Sp2/0-Agl4 mouse myeloma cells by using 50% polyethylene glycol 1500. The fused cells were dispensed at a density of 2.5 x 10^6 cells per well into 24-well tissue culture plates and cultured as previously described. Hybridomas secreting specific antibodies were cloned by limiting dilutions; 0.3 hybridoma cell per well was distributed into a 96-well microtiter plate together with 2 x 10^5 spleen cells used as feeder cells.

Hybridoma cell lines secreting specific antibodies against the crystal protein of the bacteria were detected by indirect enzyme-linked immunosorbent assay (ELISA) by the method of Voller *et al.* (1979) and Trottier et al., (1992). Cross-reaction assays with protoxins of different *B. thuringiensis* subspecies were also performed by indirect ELISA Ten monoclonal antibodies were produced against a κ-1-type crystal protein of *Bacillus thuringiensis* subsp. *kurstaki.* Eight of the antibodies belonged to the immunoglobulin G1 (IgG1) subclass, with pI values ranging from 5.5 to 8.6, one could be assigned to the IgG2b subclass, and one could be assigned to the IgM class. Competitive antibody-binding assays and analysis of antibody specificity indicated that the 10 antibodies recognized at least nine distinct antigenic determinants. Their studies also disclosed that crystal proteins from strains of the same subspecies can exhibit substantial differences in antigenic structure. (Huber-Lukac *et al.*, 1986)

The SDS-PAGE (12%, 1.5 mm thick and 12X8 cm) gel was cut half and one part was used as reference gel to locate the protein bands with 0.1% amido black lOB in methanol-glacial acetic acid-water (45:10:45) for 30 min (instead 5 min) and distained in 10% Glacial acetic acid (Drobniewski *et al.*,1989). For immunoblot analysis, the proteins from the second half of the cut gel were transferred electrophoretically to nitrocellulose membrane (Armstrong et al, 1985; Drobniewski *et al.*, 1989; Towbin *et al.*, 1979; Talwar and Gupta, 1997). The electroblotted proteins were probed first with the polyclonal antibodies raised in the rabbits against the purified S_6 crystal protein and then with goat anti-rabbit IgG-alkaline phosphatase (Gill et al 1987; Drobniewski *et al.*,1989; Armstrong et al, 1985; Gupta and Talwar, 1997).

CHATPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

1. Out of 109 *B. thuringiensis* isolates from the Khumbu Base Camp of the Everest region, 86 were found to be crystal protein producers. Only crystal producing *B. thuringiensis* isolates were processed further.

2. Among the 10 randomly selected *B. thuringiensis* isolates for Insect Bioassay, the insecticidal activities of sample S_6 were found to be highest among six SNP isolates and that of sample P_2 were highest among four Phereche isolates.

3. Of the 10 isolates, the endotoxin from S_6 sample (most efficient one in insect bioassay) was as reference strain.

4. Using 12% acrylamide separating gel, the SDS-PAGE of purified crystal protein from isolate S_6 revealed five different bands of molecular weight ranging from 40KD to 110KD

5. The purified crystal protein from isolate S_6 was immunized into a pair of New Zealand white rabbits intramuscularly

6. The presence of polyclonal antibody in the serum bled 7 days after third booster dose with incomplete adjuvant was confirmed in 1% agarose in Phosphate buffer of pH 7.2 by Ouchterlony double diffusion method (Talwar and Gupta, 1997).

7. The precipitin lines were observed with undiluted and diluted antisera (*viz.* 1:10, 1:100).

8. The polyclonal antisera (first antibody) and conjugate (second antibody) were optimized against 6-8 μg of crystal protein coated microtitre plate per well, the

optimal dilutions of the first (antisera) and second (conjugate) antibodies were found to be 1000 and 2000 folds respectively.

9. Of the total 86 endotoxin producing isolates, 31 (36.05%) corresponding endotoxins were 25-30% crossreactive with the polyclonal antisera raised against the S_6 endotoxin. Similarly, 6 (6.97%) were 75-80% and 85-90% crossreactive each and 4 (4.65%) were 80-85% crossreactive. Only 3 (3.49%) were more than 90% crossreactive.

10. The antibody raised against the crystal protein was finally confirmed by Western Blot.

7.2 RECOMMENDATION

- Endotoxin from *B .thuringiensis* isolates should be highly purified using advanced techniques such as Column chromatography, Density gradient centrifugation.

- Monoclonal antibodies should be raised for typical insecticidal crystal protein to study the immunocrossreactivity among *B.thuringiensis* endotoxins with high specificity and reproducibility.

- *B. thuringiensis* produces different crystal proteins that are insecticidal to different insects .Each of these should be studied developing IND-ELISA separately

CHAPTER VIII

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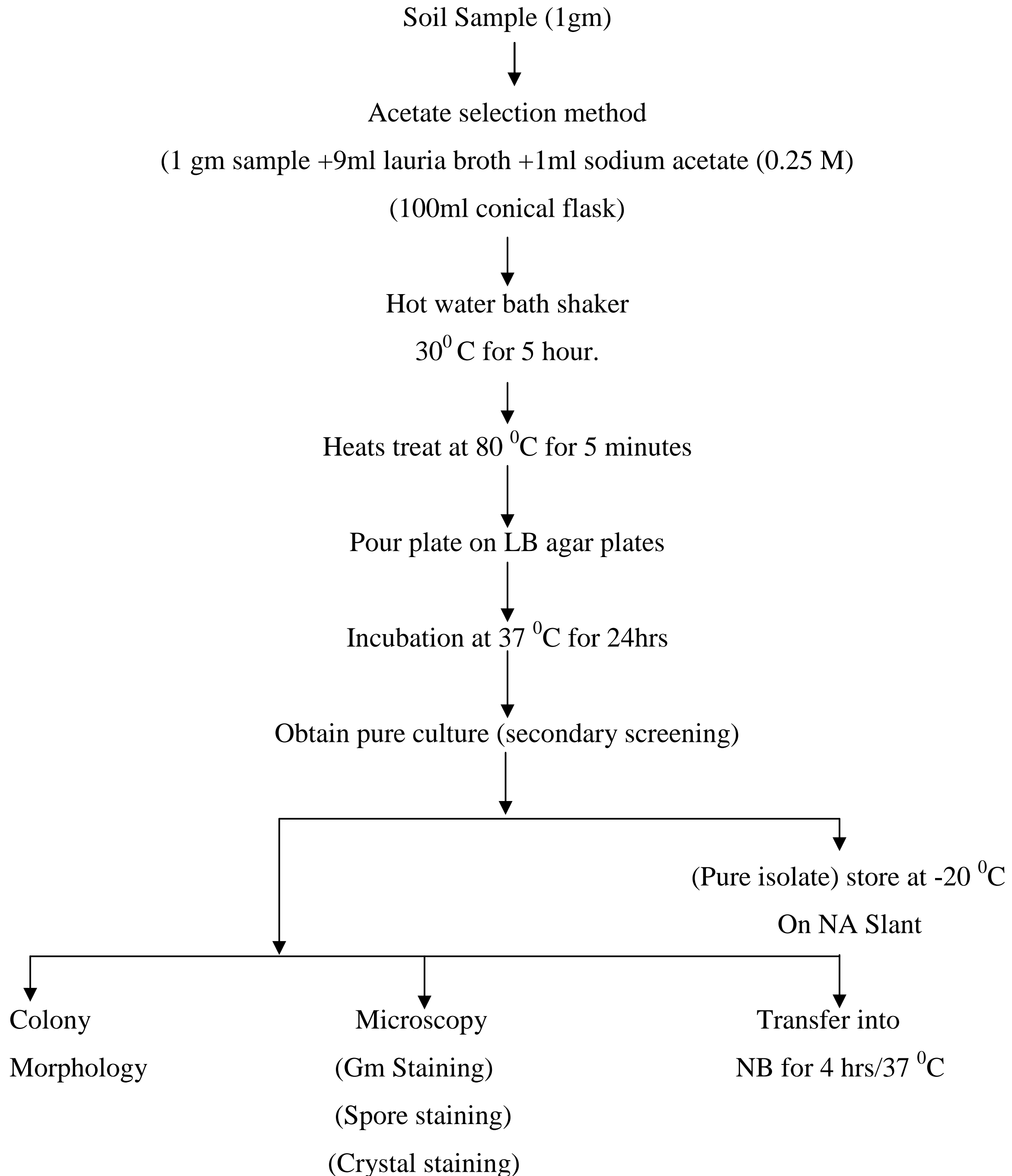
http//info.med.yale.edu

Figure 3: Flow chart for the of *B. thuringiensis* isolation (Travers et al, 1987)



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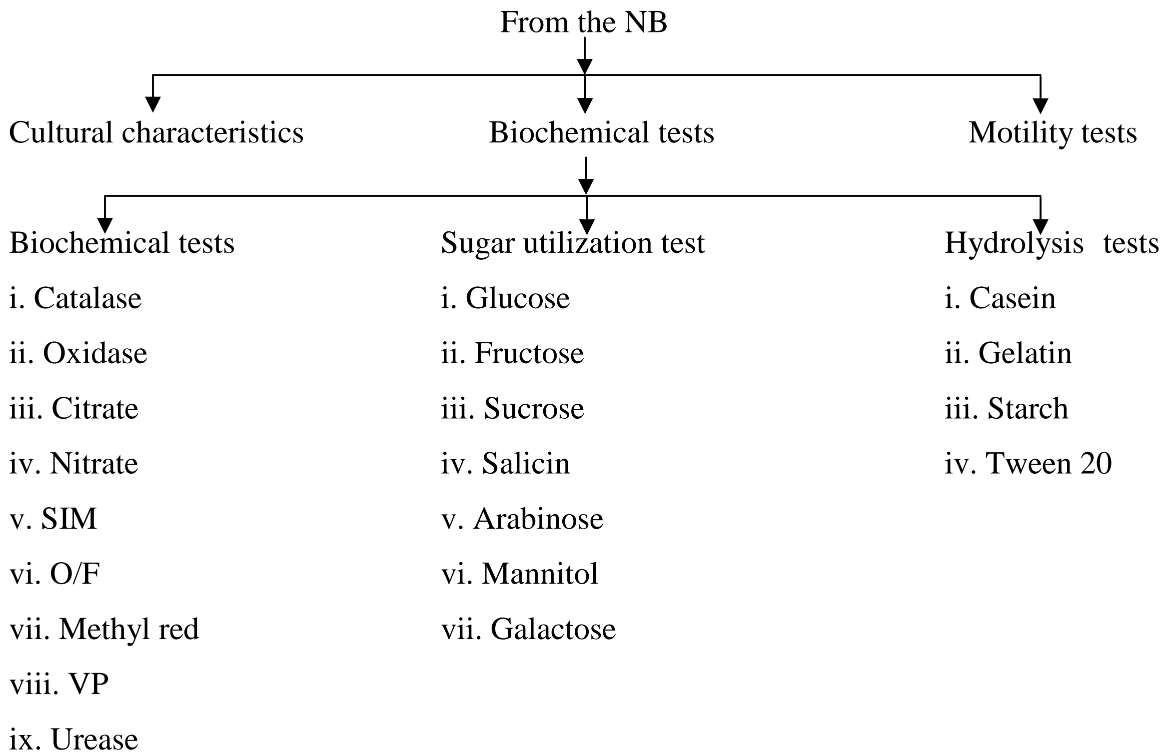

Figure 4: Flow chart for immunization schedule (Pang, 1994)

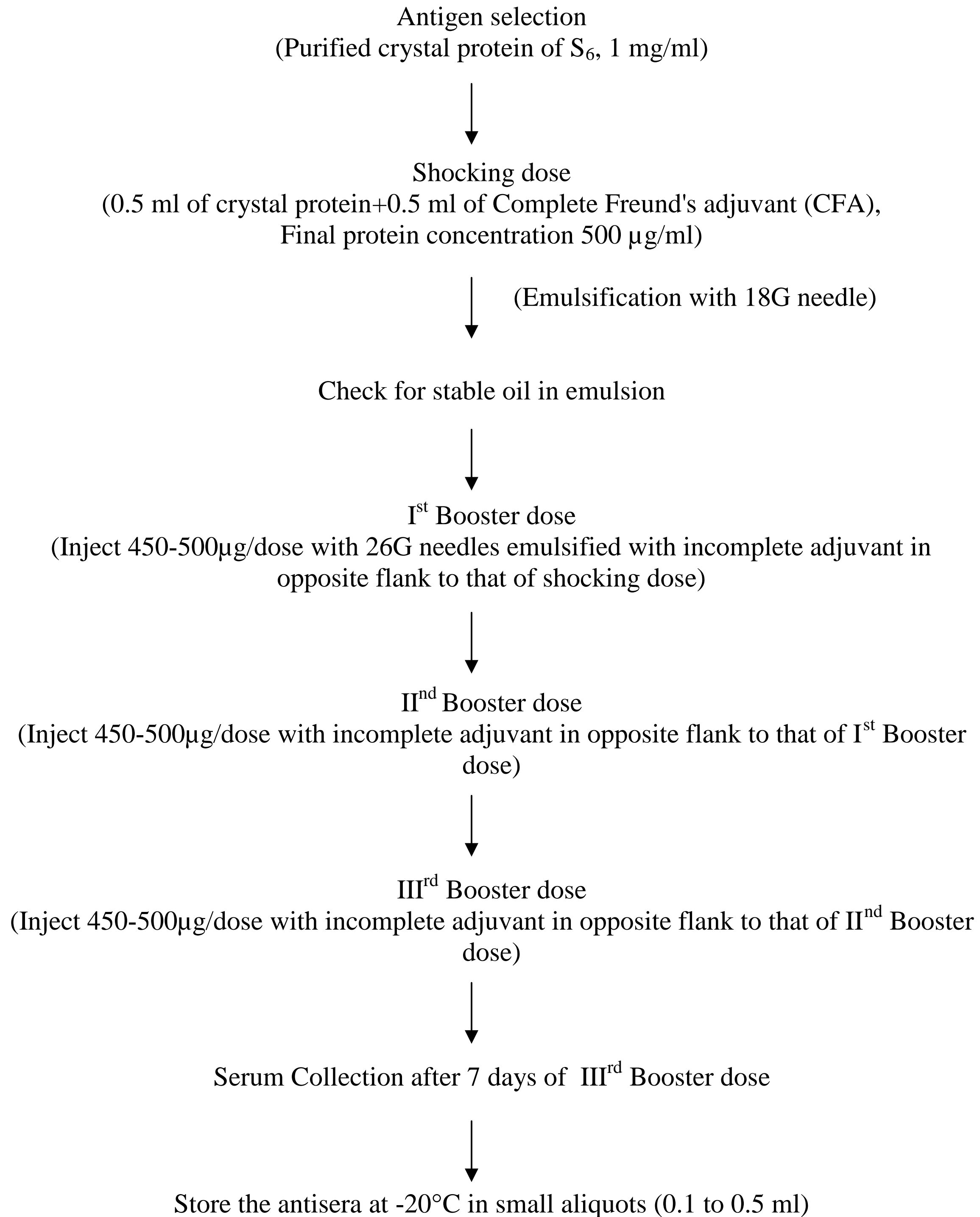

Figure 5: Flow chart for delta endotoxins immunocrossreactivity of *B. thuringiensis* isolates by Ind-ELISA [Trottier et al., (1992) and Voller et al., (1979)]

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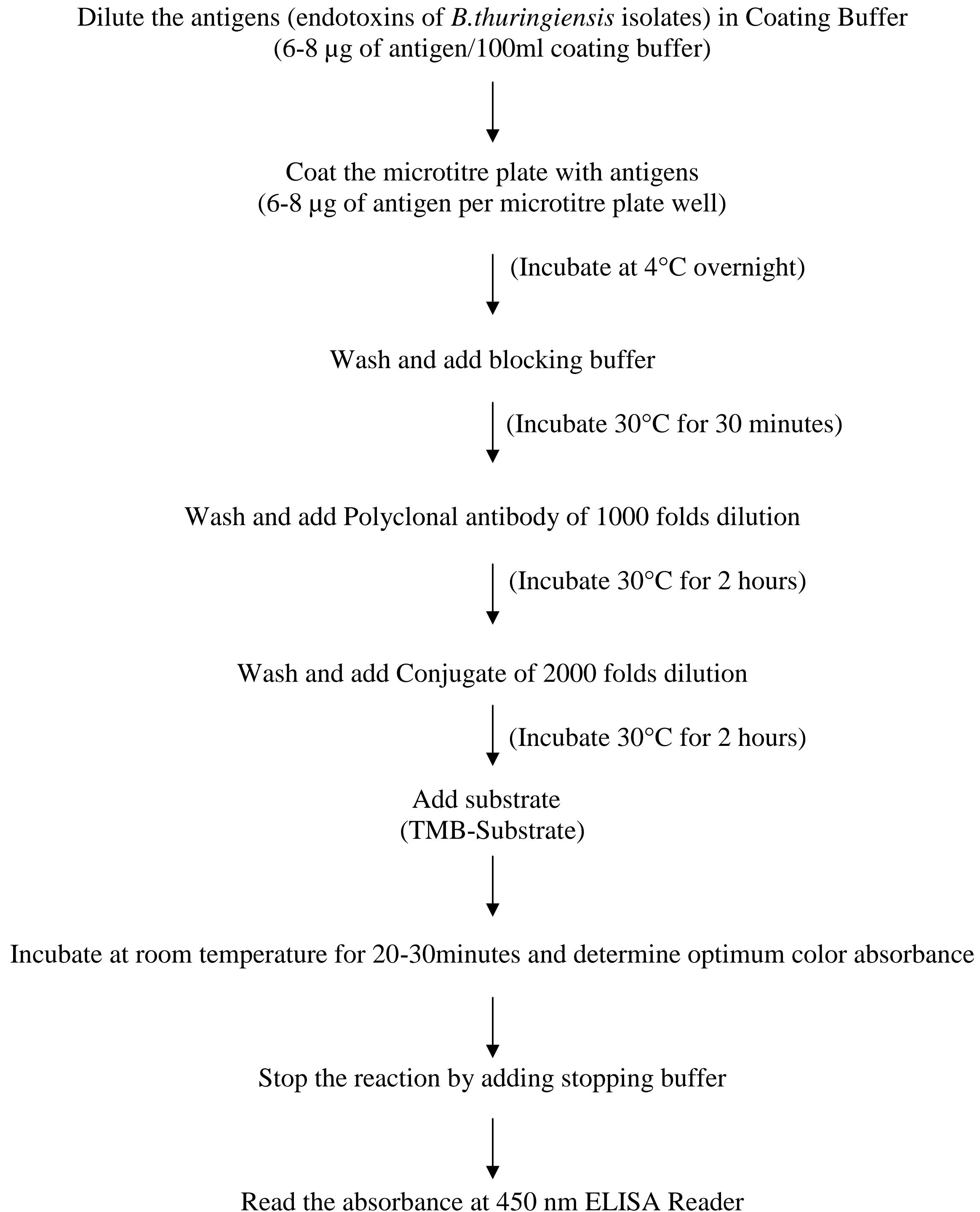
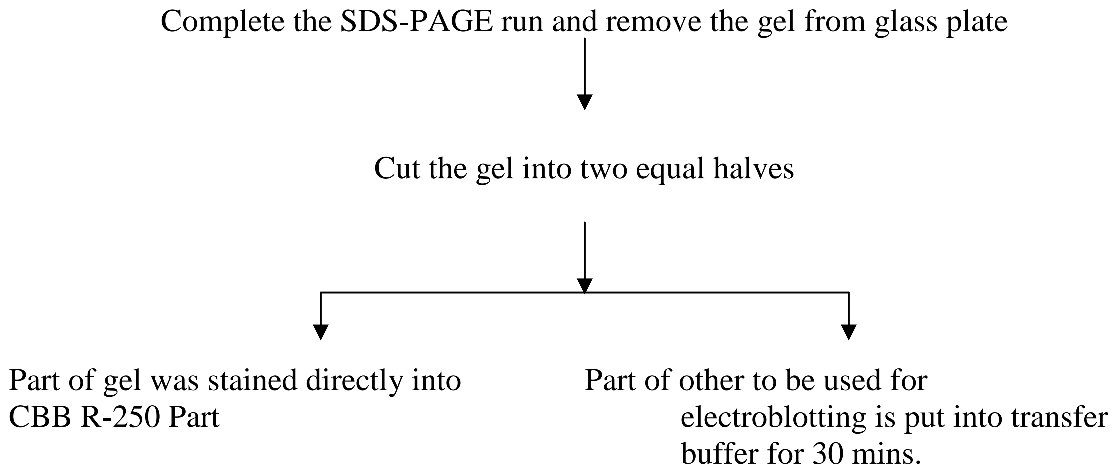
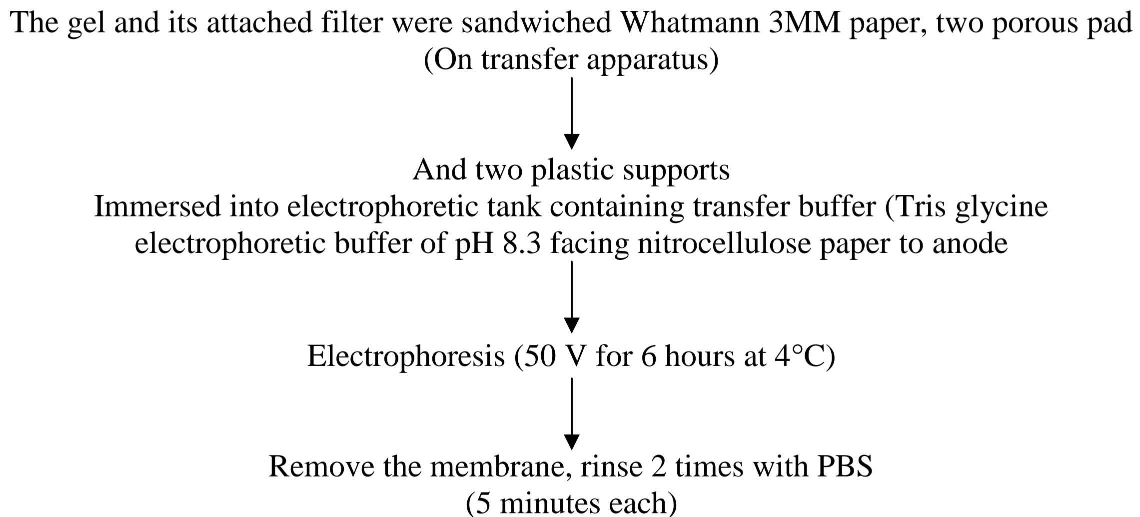


Figure 6: Flow chart for Western Blotting (Talwar and Gupta, 1997)

1. SDS-PAGE



2. BLOTTING



contd……

3. IMMUNOASSAY

Block the binding sites for immunoglobulin on the nitrocellulose paper
(With 2% BSA)

Incubate by gentle shaking for 45 minutes at room temperature

Wash 2 times with PBS/T and 1 time with PBS for 5 minutes

Binding of the primary antibody (1:1000) to the target

Incubate overnight at 4°C temperatures

Wash 3 times with PBS /T and incubate for 10 mins into 250 ml of 150mM NaCl
And 50 Mm Tris HCl (ph 7.5)

Incubate of the nitrocellulose filter paper with Second antibody (conjugated with peroxidase 1:2000) for 2 hours at room temperature.

5 X washing with PBS/T and NaCl

Add chromogenic substrate (TMB)
Incubate at room temperature for 20-30 mins and observe for color band reaction

Stop the reaction by soaking the filter and distilled water.

Dry and store at dark Place take the photograph for permanent record.

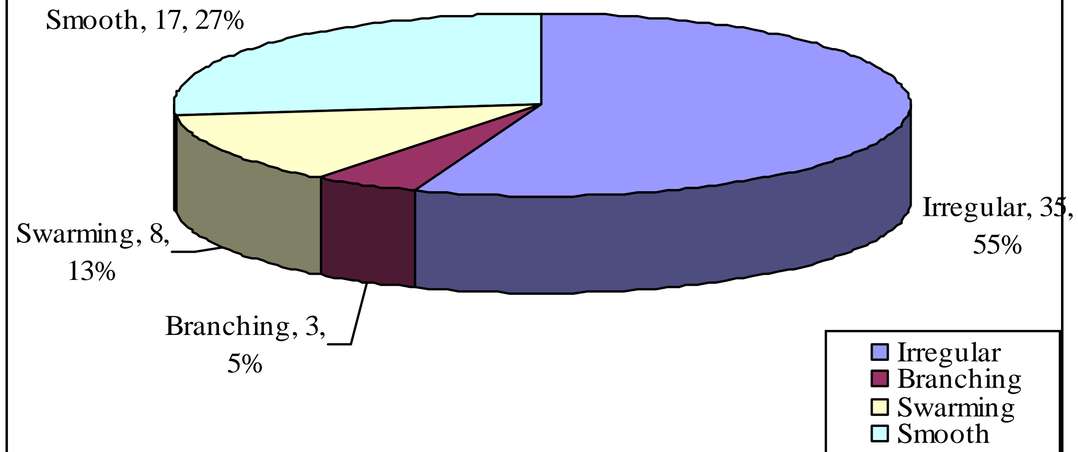


Figure 7: Colony Configuration of the Phereche Isolates
Smooth, 17, 27%
Swarming, 8, 13%
Branching, 3, 5%
Irregular, 35, 55%
Irregular
Branching
Swarming
Smooth

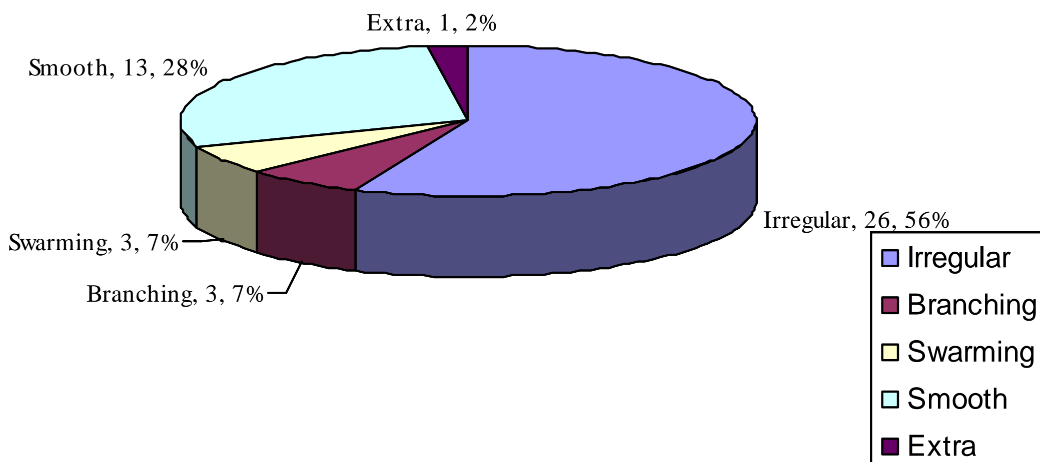


Figure 8: Colony Configuration of the SNP Isolates
Extra, 1, 2%
Smooth, 13, 28%
Swarming, 3, 7%
Branching, 3, 7%
Irregular, 26, 56%
Irregular
Branching
Swarming
Smooth
Extra

Figure 9: Opacity (Phereche isolates)

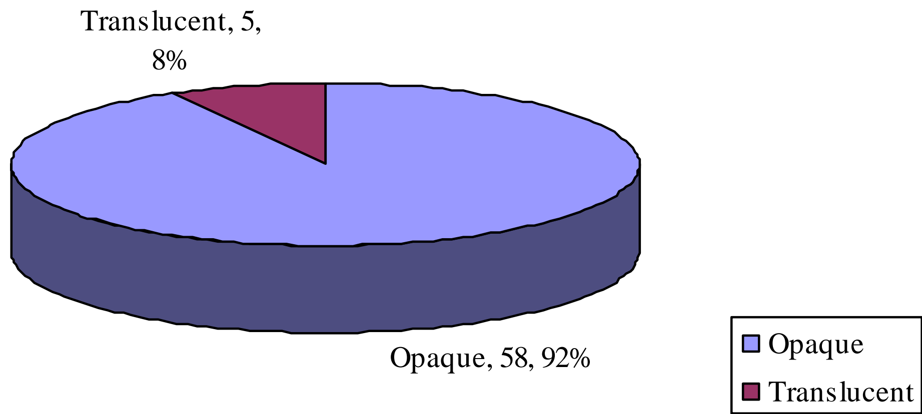


Figure 10: Opacity (SNP isolates)

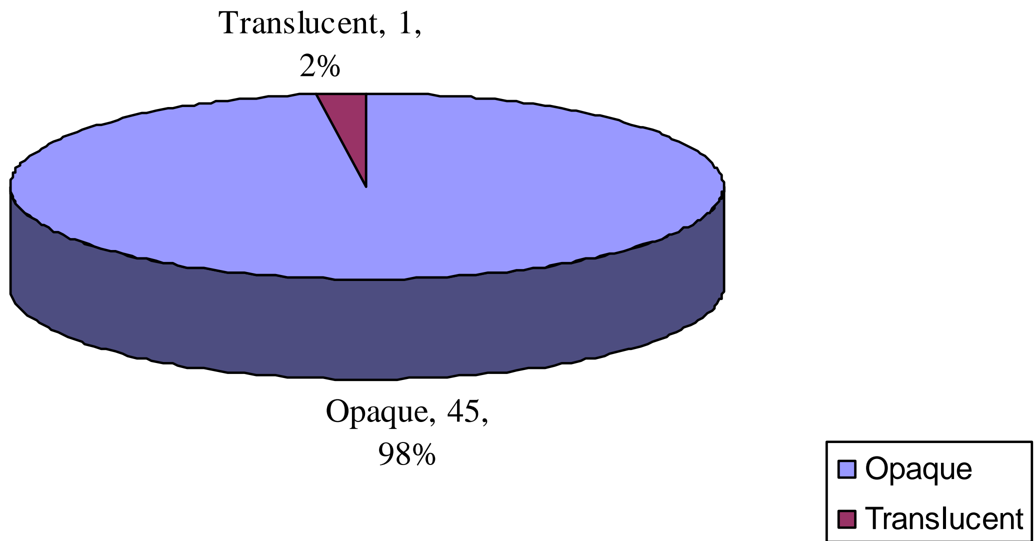


Figure 11: Consistency (phereche isolates)

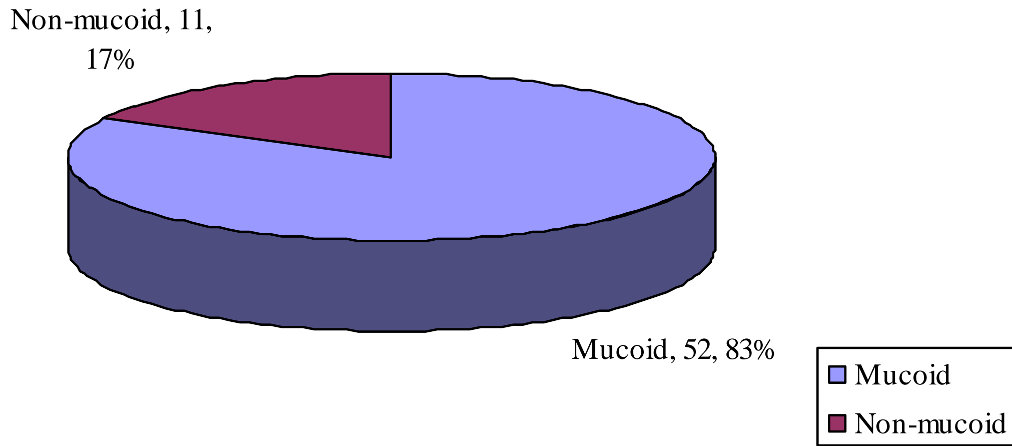


Figure 12: Consistency (SNP isolates)

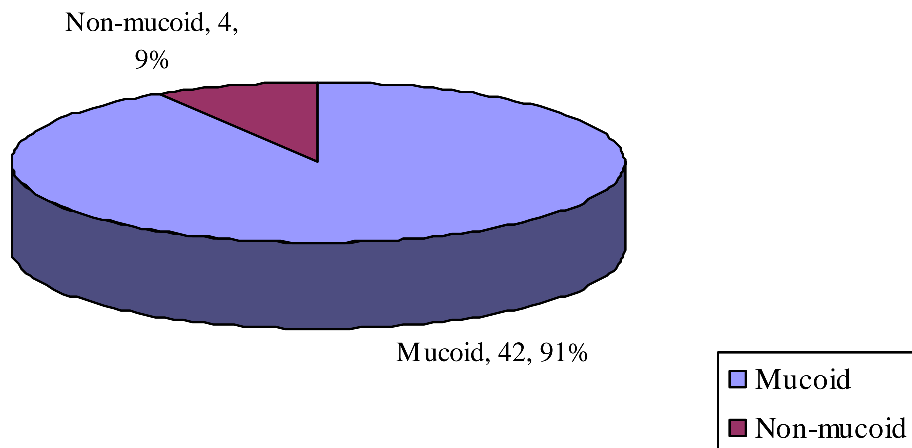


Figure 13: Elevation (Phereche isolates)

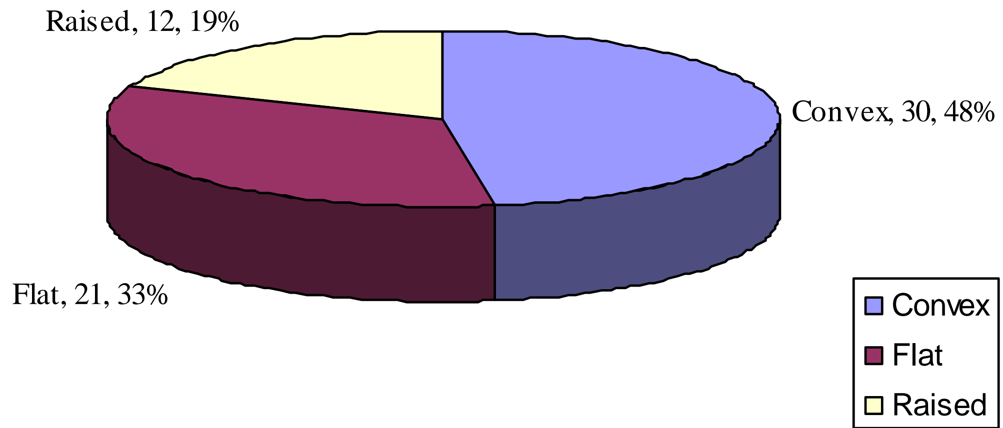


Figure 14: Elevation (SNP isolates)

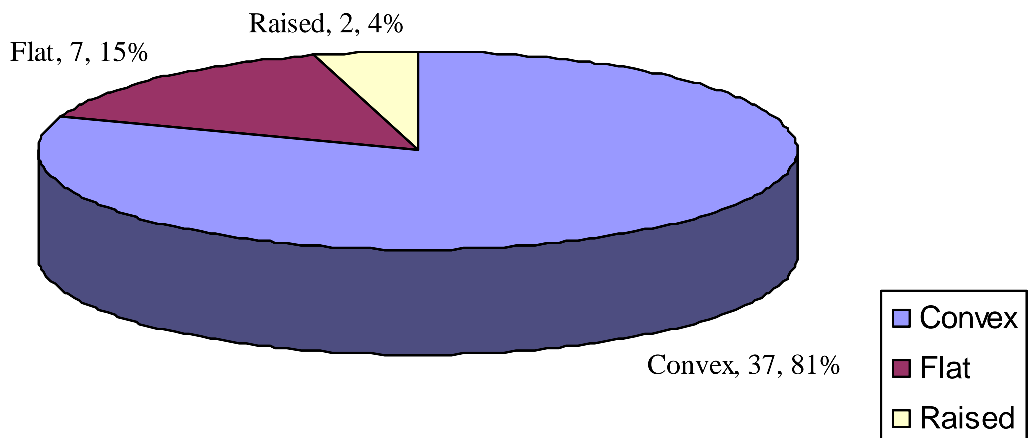


Figure 15: Color (Phereche isoletes)

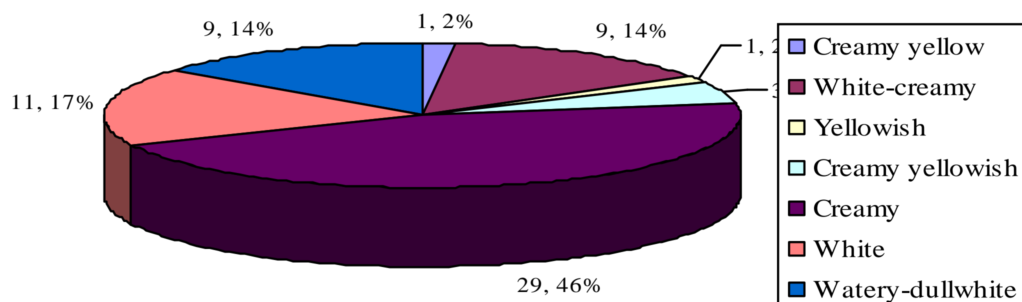
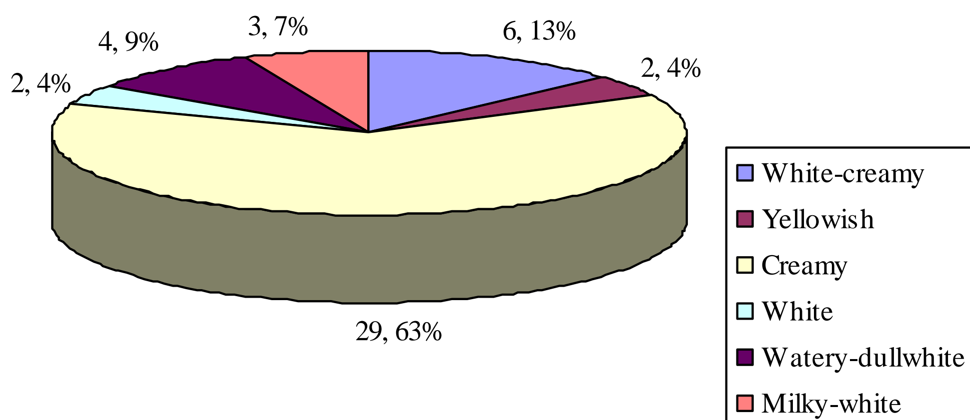


Figure 16: Color (SNP isolates)



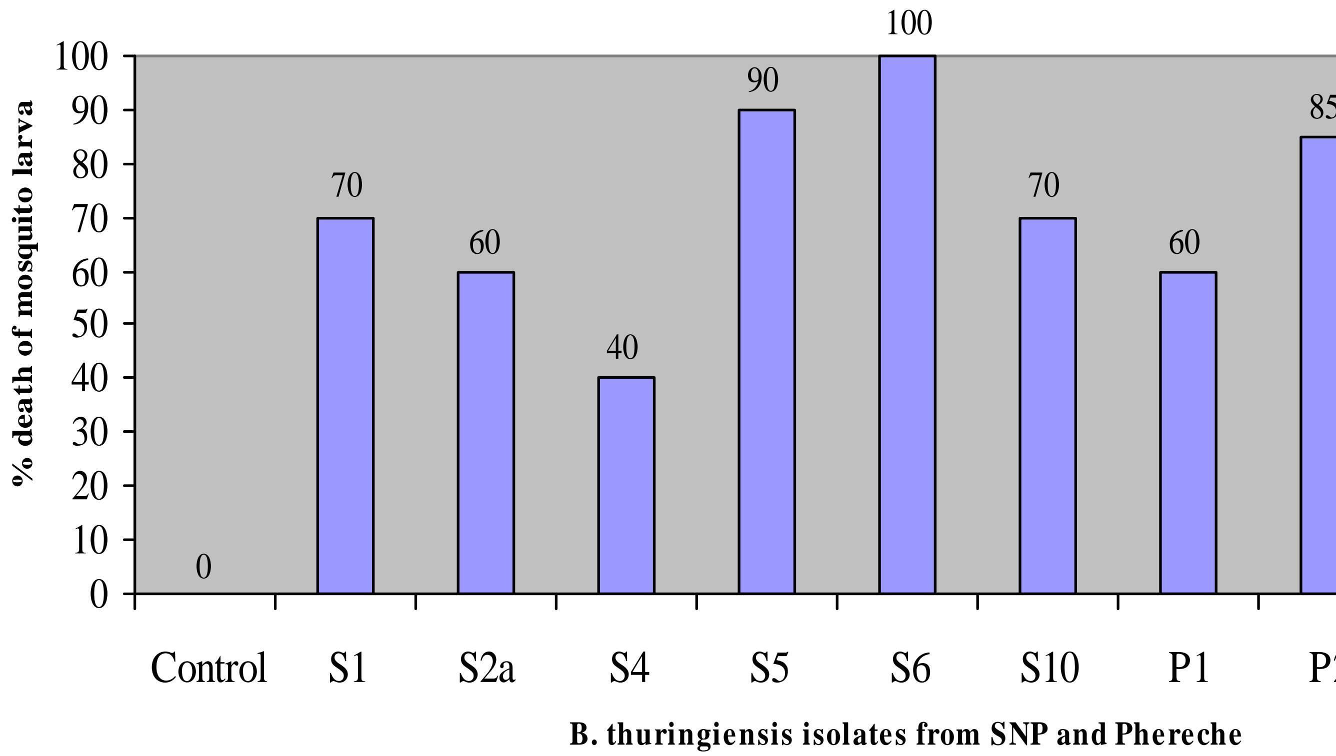


Figure17: Insect Bioassay of SNP and Phereche B. thuringiensis
% death of mosquito larva
100
90
80
70
60
50
40
30
20
10
0
0
70
60
40
90
100
70
60
Control
S1
S2a
S4
S5
S6
S10
P1
B. thuringiensis isolates from SNP and Phereche

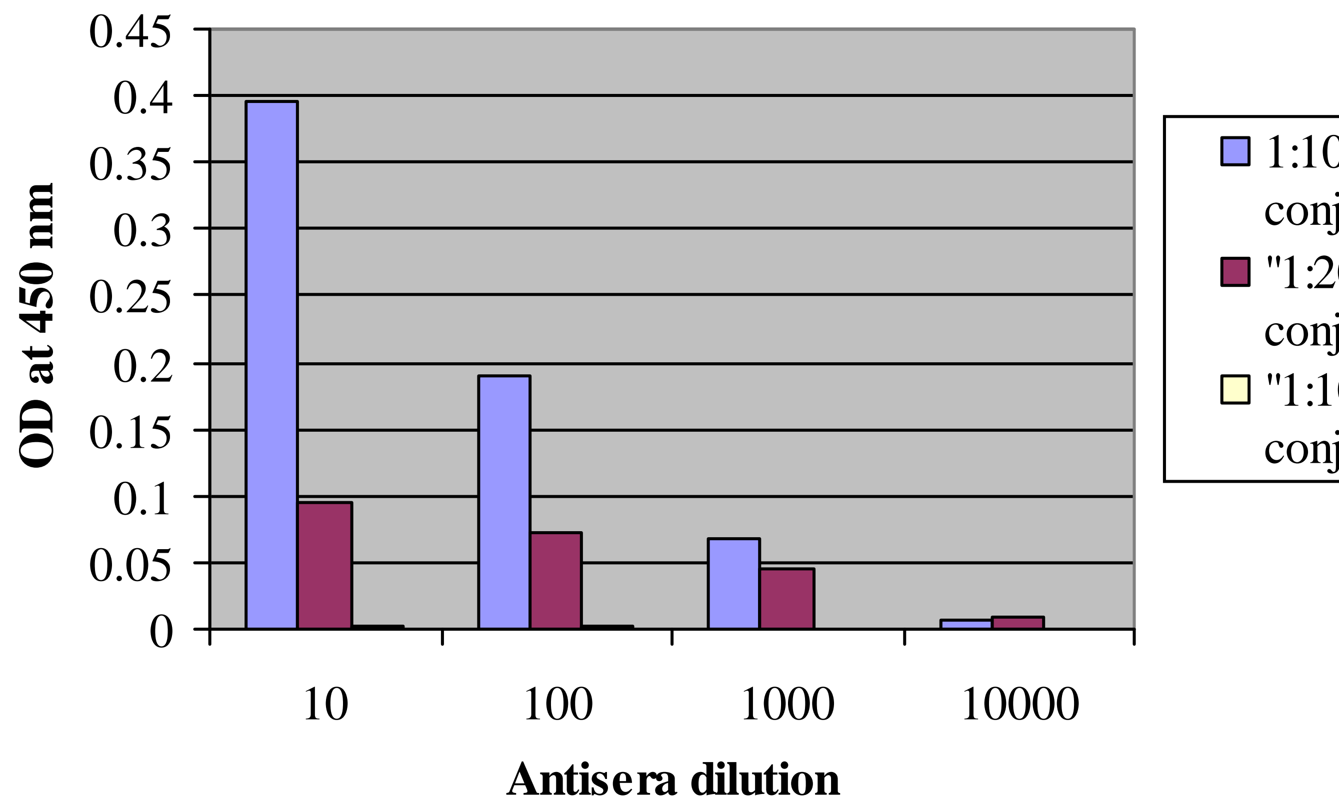


Figure 18:-Optimization of polyclonal antisera against
protein and conjugate
OD at 450 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
10
100
1000
10000
Antisera dilution

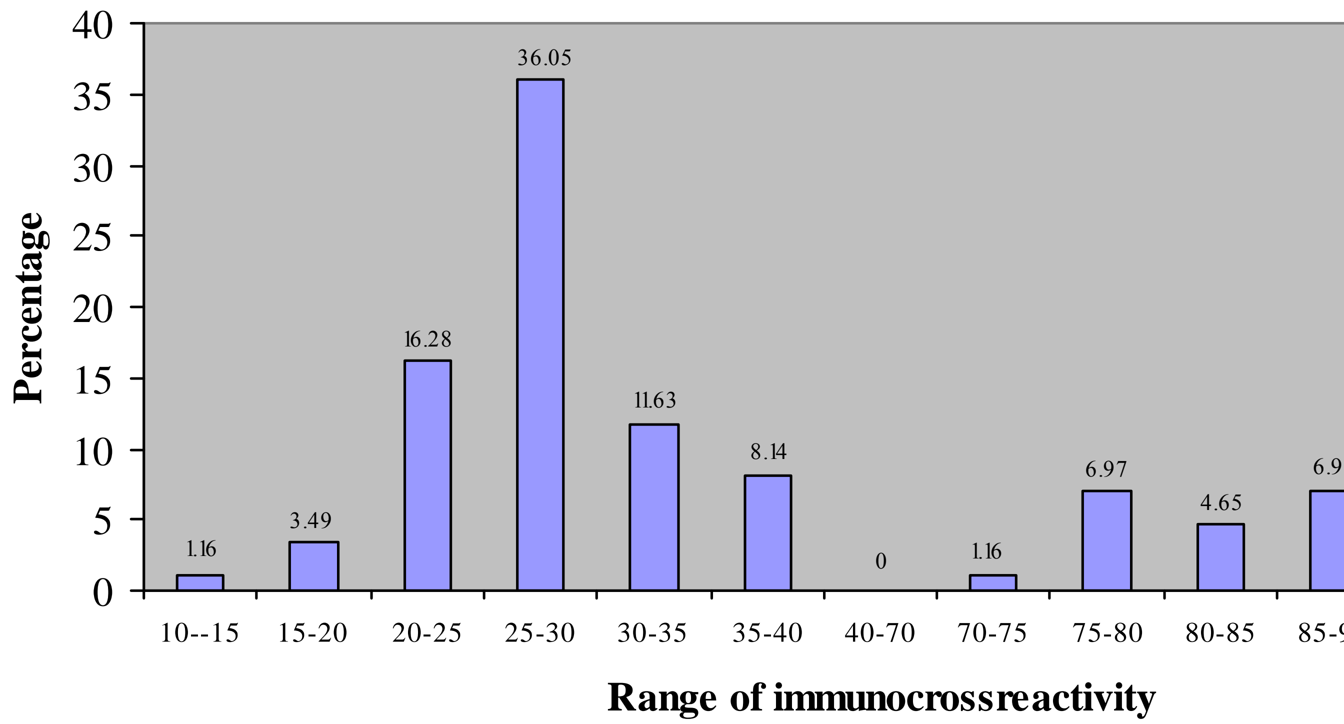


Figure 19:- Percentage of immunocrossreactive B thuringiensis endo
frequencies
Percentage
40
35
30
25
20
15
10
5
0
1.16
3.49
16.28
36.05
11.63
8.14
0
1.16
6.97
4.65
6.9
10--15
15-20
20-25
25-30
30-35
35-40
40-70
70-75
75-80
80-85
85-9
Range of immunocrossreactivity