MOLECULAR CHARACTERISATION OF NATIVE *Bt* ISOLATES AND LABORATORY EVALUATION OF THEIR NANO-FORMULATIONS ON *Helicoverpa armigera* (Hub.)

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B. Sc. (Hons.) Ag.

MASTER OF SCIENCE IN AGRICULTURE
(ENTOMOLOGY)

ANGRAU

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BY

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B. Sc. (Hons.) Ag.

**THESIS SUBMITTED TO THE
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
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FOR THE AWARD OF THE DEGREE OF**

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CHAIRPERSON: Dr. D. V. SAI RAM KUMAR

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2022

DECLARATION

I, **Ms. Bodapati Anusha** hereby declare that the thesis entitled **"Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)"** submitted to **Acharya N. G. Ranga Agricultural University** for the degree of **Master of Science in Agriculture** in the major field of **Entomology** is the result of original research work done by me. I also declare that any material in the thesis has not been published earlier.

Place : **(BODAPATI AUSHA)**
Date : **I.D. No. BAM-20-38**

CERTIFICATE

Ms. Bodapati Anusha has satisfactorily prosecuted the course of research and the thesis entitled **"Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any university.

Place:
Date:

(D. V. SAI RAM KUMAR)
Chairperson

CERTIFICATE

This is to certify that the thesis entitled **"Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)"** submitted in partial fulfilment of the requirements for the degree of Master of Science in Agriculture in the major field of **Entomology** of the Acharya N. G. Ranga Agricultural University, Guntur, is a record of the bonafide original research work carried out by **Ms. Bodapati Anusha** under our guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

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With ever regardful memories………

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Place:

Date: ***(Bodapati Anusha)***

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LIST OF ABBREVIATIONS AND SYMBOLS

%	:	Per cent
&	:	And
±	:	Plus or minus
/	:	Per
@	:	At the rate of
<	:	Less than
=	:	Is equal to
>	:	Greater than
$	:	Dollar
˚C	:	Degree celsius
µl	:	Microliter
µg	:	Microgram
ANOVA	:	Analysis of Variance
BOD	:	Biological Oxygen Demand
bp	:	Base pair
Bt	:	*Bacillus thuringiensis*
CABI	:	Centre for Agriculture and Bioscience International
CD	:	Critical Difference
CFU	:	Colony Forming Units
CV	:	Coefficient of Variance
DNA	:	Deoxy ribo Nucleic Acid
DNTPs	:	Deoxynucleotide Triphosphates
e.g.,	:	For example
et al.,	:	And others
etc.	:	and so on
FAO	:	Food and Agriculture Organisation
Fig.	:	Figure

g	:	Gram
hpi	:	Hours post infection
h	:	Hours
I	:	Isolate
i.e.	:	That is
IPM	:	Integrated Pest Management
IU	:	International Unit
LC	:	Lethal concentration
LSD	:	Least Significant Difference
LT	:	Lethal Time
mg	:	Milligram
ml	:	Millilitre
No.	:	Number
NP’s	:	Nanoparticles
NS	:	Non-Significant
NTSYS	:	Numerical Taxonomy and Multivariate Analysis System
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplification of Polymorphic DNA
Rep- PCR	:	Repetitive Extragenic Palindromic Sequence
RFLP	:	Restriction Fragment Length Polymorphism
RH	:	Relative humidity
rpm	:	Revolutions per minute
SDS-PAGE	:	Soduim Dodecyl Sulphate Poly Acrylamide Gel Electrophorosis
sec	:	seconds
SEm	:	Standard Error of mean
SEM	:	Scanning Electron Microscopy
Taq	:	*Thermus acquaticus*

UPGMA	:	Unweighted Pair Group Method with Arithmetic Mean
UV	:	Ultra violet
viz.,	:	Namely
Zn	:	Zinc

ABSTRACT

Name of the Author	:	**Bodapati Anusha**
I. D. No.	:	**BAM/2020-38**
Title of the thesis	:	**Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)**
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The present study on "Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)" was conducted at Department of Entomology, Agricultural College, Bapatla during the period 2021-22.

Molecular diversity studies of seven *Bt* isolates including a reference strain HD1 was done using 10 RAPD decamer primers (OPA-02, OPA-05, OPA-07, OPA-09, OPA-10, OPA-12, OPA-18, OPC-19, OPK-14 and OPN-05). All the primers showed polymorphism. The polymorphic information content of primers ranged from 00.388 (OPA-09) to 0.478 (OPA-12). The dendrogram constructed using NTSYSpc 2.02i revealed that the seven isolates fell into two different clusters comprising of three (Isolates 49, HD1 and 55) and four isolates (Isolates 16, 52, 493 and 51) respectively. PCoA constructed using PAST software revealed that isolate 16 and 52 showed close genetic relationship and also isolate 49 and HD1 were found to show close genetic relationship among themselves and higher divergence with the remaining isolates. Crystal protein profiling of *Bt* isolates done using SDS-PAGE revealed the presence of band at 130-140 kDa indicating presence of Cry1 protein in isolates-16 and HD1, Vip3 protein of 92kDa in isolate- 49 & 493 and Cry2 protein of 65-70 kDa in isolates- 51, 52 & 55 were observed.

To know the bioefficacy of native *Bt* isolates *viz.*, isolate 16, 49, 51, 52, 55 and 493 along with a reference strain HD1 were grown on nano enriched media and bioassay was conducted on the third instar larvae of *H. armigera*. Mortalities were observed from 48h after treatment for isolates 16, 493 and HD1 and maximum mortality was observed after 72h of treatment. Mean per cent mortalities ranged from 21.67 to 96.67 for all the isolates with NPs. For *Bt* grown on CaO based nano enriched media, the mortalities ranged from 25 to 96.67% and for MgO and ZnO based nano enriched media, the mortalities ranged from 23.33 to 95% and 21.67 to 93.33%, respectively.

To confirm the insecticidal activity of seven native *Bt* isolates including a reference strain HD1, bioassay was conducted on the third instar larvae of *H. armigera* using diet incorporation method. Mean per cent mortality of native isolates ranged between 23.33 and 91.67 and the LC_{50} values were between 2.78×10^7 and 7.73×10^{11} CFU ml^{-1} and LT_{50} in the range of 104.62 to 140.57 hpi at a dose of 1×10^{12}. The highest mortality of 91.67 per cent was observed in HD1 with LC_{50} and LT_{50} values of 2.78×10^7 CFU ml^{-1} and 104.62 hpi, respectively. Among the native isolates the lethal action with reference to LT_{50} was recorded by isolate 493 followed by isolate I-16, I-51, I-49 and I-55 with 119.95, 130.78, 136.51 and 136.53 hpi respectively and the LC_{50} of 1.75×10^9, 1.09×10^{10}, 2.90×10^{10} and 5.04×10^{10} respectively. LC_{90} values were in the range of 5.89×10^{11}(HD1) to 5.75×10^{16}(isolate- 52) and LT_{90} values were in the range of 162.47 hpi (HD1) to 245.93 hpi (isolate-55). Bioassay studies revealed that, the reference strain HD1 (96.67%) was more effective than the nanobased formulations of native isolates although, CaO based nano formulation of 493 (91.67%) was more effective among the native isolates.

Chapter – I

Introduction

Chapter I

INTRODUCTION

Integrated pest management (IPM) involves integration of all the available strategies to control the pests that damage crops (FAO, 2021). But, in the current plant protection scenario, with the indiscriminate and over-use of insecticides, several indirect deleterious effects are witnessed on the environment and also on non-target organisms, along with some direct effects like insecticidal resistance development, which is considered as major issue in IPM. So, to prevail over the problems caused by these chemical insecticides, there is an imminent need to shift from chemical control to biological control.

Biological control is an important component of Integrated Pest Management. It helps in lowering the pest population by using natural enemies. Among biological control methods, use of pathogenic microbes to control insect pests plays a key vital role (Tanada, 1959), resembling to any insecticide, hence highly favored by the farming community. Among these microbial agents, entomo-pathogenic bacteria play a major role in pest control. Bacteria belonging to the genus *Bacillus* have wide spread use in pest management. Thus, they have considerable market share in the global bio-pesticide market.

In particular, the bacterium, *Bacillus thuringiensis* being ubiquitous, gram positive, aerobic, soil borne, spore forming, facultative, crystalliferous pathogen is highly specific in causing acute infections to insects. It has been sold technically across several countries, since the late 1950's, and until now. *Bt* is a widely accepted bio-insecticide among the farming communities, containing different insecticidal proteins namely Cry, Vip and Cyt (Bravo *et al.*, 2017). δ-*cry* genes of many *Bt* strains produce crystal toxin proteins that have insecticidal action (Schnepf *et al*., 1998). Upon ingestion from the crystal, the active toxin is generated in the insect gut, through solubilization and proteolytic activity by specific proteases from high molecular weight protoxin proteins (Bravo *et al*., 2011). This active mature toxin binds to specific receptors of midgut epithelium leading to pore formation followed by lysis of epithelial cells and death of the organism (Schnepf *et al*., 1998).

Different *cry* genes are carried by the bacterium, resulting in the production of different Cry proteins. So, the research into novel *cry* genes with broad spectrum of activity is an ongoing effort worldwide (Baxter *et al.*, 2011). These *cry* genes are classified depending on the sequence similarities (Charan *et al*., 2013). The results observed from nucleotide sequencing of these genes provide a basis for identification of different strains of bacteria. This information promises to contribute to increased efforts directed at the improvement of these insecticidal activities through molecular manipulations.

In order to characterize the genetic heterogenity of native *Bt* isolates, the retrieved *Bt* isolates from various locations were subjected to RAPD (Randomly amplified polymorphic DNA) marker-based analysis. RAPD is quicker, less labor-intensive and mainly avoids the need of pure DNA, just a small quantity of template DNA is sufficient for amplification reaction (Sikora *et al.,* 1997). Despite of many techniques to study the genetic relationship among the isolates, RAPD being the easy and reliable one, is used to study the diversity between the isolates (Williams *et al*., 1990). RAPD analysis was also found to generate characteristic fingerprints of various bacterial strains, and is one of the most successful molecular biology technique to study the genetic diversity among the *Bt* isolates and was in practice all around the world (Kumar *et al.,* 2010).

Bt has a very long and safe history that is used as a biopesticide and this has directed research into the nature of the toxin moiety that is central to mortality of target organisms. The efficacy of the insecticidal properties of *Bt* can be increased by means of nanotechnological methods. *Bt* as nanoparticles in the insect gut will have more pronounced quick mortality and thus effectively drives new avenues for plant protection. Currently, this line of research needs to be explored in many ways, so as to promote the utilization of novel nano-based formulation. Nano-based particles of bio-pesticides provide larger specific surface area, thereby enhancing the affinity to the target site of action. It has an added advantage, as it penetrates readily into the cells and selectively get accumulated in various types of cells. They also travel along the neurons and the blood vessels provoking oxidative stress in a short span of time.

Bt maintained on nano-enriched media was found to cause higher mortality in the insects compared to those maintained on normal media. In order to enhance the bio efficacy of *Bt* isolates, the mineral salts such as Zn, Ca, Mg *etc*., were added to the media before inoculation (Valicente *et al*., 2010). Generally, the bioefficacy of *Bt* in causing mortality of lepidopteran larvae depends on the number of colony forming units (CFU) in *Bt*. Addition of minerals such as Zn, Ca and Mg was found to enhance the number of CFU in *Bt*, that leads to increase in the bio efficacy of *Bt* isolates (Gayathri, 2019).

Nanoparticles are molecular aggregates formed by the alteration of molecular and atomic properties of the basic element. NP's of these minerals (Ca, Mg and Zn) increases CFU in *Bt* thereby decreasing the dose of biopesticides (Gayathri *et al.,* 2018).

Bt was found to be highly virulent against lepidopteran pests that are known to cause huge loss to the farmers. *Helicoverpa armigera*, a polyphagous lepidopteran pest of tropical origin, is known to cause severe damage to crops such as Cotton, Chick pea, Tomato, Maize *etc*., (Denlinger, 1986). Monetary losses due to *H. armigera* result from direct reduction of crop yields and also from the expense incurred to control, mainly the cost that farmers afford for the insecticides. In India, *H.armigera* was found to commonly destroy half of the yield of the pulse crops, where the losses were estimated around $US 300 million per annum (CABI, 2020).

To determine the insecticidal activity of native *Bt* isolates, bioassays were conducted in the laboratory on various insect pests all around the world. *Bt* is known to produce virulence factors during both vegetative and stationary phases, which leads to insecticidal activity (Chapple *et al*., 2000 and Hansen and Salamitou, 2000).

In this context, the present investigations were carried out to characterize different strains or isolates of *Bt* by DNA finger printing to get to know the genetic similarity among the native isolates and conducting bioassay studies against *H.armigera* with native *Bt* isolates along with *Bt* isolates maintained on nano mineral enriched media at laboratory level with the below objectives.

Objectives of Investigation:

1. Molecular characterisation of native *Bt* isolates with DNA finger printing by RAPD-PCR.
2. To develop nano-scale particles with native *Bt* isolates.
3. To evaluate *Bt* isolates on pod borer, *H. armigera* (Hub.) in laboratory.

Chapter – II

Review of Literature

Chapter II

REVIEW OF LITERATURE

The studies on '**Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)**' were reviewed in this chapter. This chapter has the information on the molecular characterisation of bacterial isolates for genetic diversity studies of the isolates, that were collected from different locations. It also includes the impact of different *Bt* strains and their nano formulations on the mortality of *H. armigera* in laboratory. Studies conducted across the globe on entomopathogens was extreme, however the research on nanobased biopesticides and nanobased insecticides were scanty in India and was started recently. The purpose of this review was to identify the extent of research conducted on entomopathogens and also the gaps in documented information on understanding the microbial management of *H. armigera*. Relevant published literature is presented in different heads accordingly.

2.1 MOLECULAR CHARACTERISATION OF NATIVE *Bt* ISOLATES WITH DNA FINGER PRINTING BY RAPD-PCR.

Malkawi *et al.* (1999) recovered 16 isolates of *B. thuringiensis* from different Jordanian habitats. The isolates were studied for variability at molecular level using RAPD. The total genomic DNA of the isolates along with three reference strains were amplified with decamer primers. Electrophoretic analysis revealed presence of polymorphism among the isolates. Using Cluster analysis, a dendrogram was constructed, which revealed that 15 isolates fell into one cluster with six sub- groups showing regional variation among isolates.

Gupta *et al*. (2001) investigated 16 *Xanthomonas oryzae* isolates from various geographical regions in India, as well as 12 isolates from the Philippines, using seven polymorphic RAPD primers *viz.*, OPA-03, OPA-04, OPA-10, OPA-11, OPK-7, OPK12 and OPK-17. The RAPD primers produced simple, specific and repeatable fingerprinting patterns, demonstrating that RAPD markers can be used to distinguish *X. oryzae* isolates. Primers PJEL1 and PJEL2, used in insertion sequence IS1112-based PCR, also generated specific and reproducible fingerprint patterns for the same set of the *X. oryzae* isolates. For the same collection of *X. oryzae* isolates, the IS -112 based PCR produced unique and repeatable fingerprinting patterns. Based on RAPD-PCR (seven primers) and

IS 1112 PCR (two primers) data, 16 out of 18 *X. oryzae* isolates were categorised into five separate clusters and two isolates from the Philippines were loosely associated with them at a similarity of 0.57. RAPD-PCR and IS1112-PCR were found to be beneficial in detecting genetic variation among isolates.

Pattanayak *et al*. (2001) used 19 random decamer primers to perform RAPD fingerprinting of 21 *B. thuringiensis* serovars representing distinct serotypes. From 13 of the 19 primers, 172 polymorphic fragments ranging in size from 161-2789 bp were amplified. Pair-wise genetic similarity study indicated relatively low similarity values among *Bt* serovars, ranging from 3-68 per cent, indicating substantial genetic divergence. In the dendrogram, 19 *Bt* serovars formed two major clusters, while the remaining two formed single clusters. The genetic relatedness of serovars and serotypes was discovered through clustering of *Bt* strains.

Babalola *et al*. (2002) extracted rhizosphere bacteria such as *Pseudomonas* spp., *Enterobacter sakazakii* and *Klebsiella oxytoca* from maize and sorghum rhizospheres and used the RAPD approach to look for genetic variation. The three bacteria were amplified using three primers (OPA-09, OPA-10, OPA-16), their DNA fingerprinting patterns were noticeably different. The three primers produced a total of 68 bands, 62 of which were variable. In each isolate, the number of polymorphic RAPD loci ranged from 1 to 13. *E. sakazakii* and *K. oxytoca* were the most closely related among the three species, according to cluster analysis.

Anjali *et al*. (2003) used various PCR-based DNA fingerprinting techniques for bacterial strain identification and discrimination. Fourteen *Salmonella* strains were grouped into nine groups, with the greatest number of strains (five) in one category. In the Box PCR method, primers with highly conserved repetitive sequences created six separate groups that all had a shared band. As a result, serotype *Typhimurium* may be grouped together using Box PCR. When compared to the other two approaches, the results revealed that RAPD had a strong discriminating power for *Salmonella* isolates.

Bradic *et al*. (2003) isolated nine native *Sinorhiziobium meliloti* strains from alfalfa nodules collected from several field areas in Croatia, including three reference strains, for genetic characterisation using PCR-RFLP of the 16s rDNA, rep-PCR and RAPD-PCR. The results of the 16s rDNA PCR-RFLP revealed that the majority of isolates (77%) had two reference strains 469 and 528 and indigenous strain C16 that was distinct from each other.

Levy *et al*. (2005) used a PCR based RAPD method to identify genetic markers in *B. anthracis* strains. 25 differential genetic markers were identified which divided the strains into five different groups. Three selected RAPD-markers were cloned and sequenced. The five RAPD-derived genotypes could be defined by integration of these three markers. They opined that classification and differentiation of *B. anthracis* isolates by genetic markers play an important role in anthrax research.

Sadder *et al*. (2006) isolated seven Jordanian *Bt* strains, which have been analysed for toxicity against important pests and also differentiated through serotyping. The *Bt* isolates were analysed at the molecular level using RAPD markers. Five more international strains were incorporated in the analysis. The RAPD markers showed high polymorphism among the isolates tested. However, the data did not align completely with earlier serotyping for most isolates. Therefore, it is recommended to engage several analyses (*e.g*., biochemical and molecular) when classifying newly surveyed *Bt* isolates in the world. The applied RAPD technology was very useful, fast and informative in differentiating *Bt* strains similar to related studies of *Bt* (Hansen *et al*., 1998; Gaviria and Priest, 2003).

Sijapati *et al*. (2008) focused on optimising RAPD reactions and cycling conditions to generate DNA fingerprints of 10 isolated Nepalese *Bt* strains. The strains were obtained at various altitudes ranging from roughly 70 metres to 5050 metres above sea level. 24 primers out of 100 examined produced DNA fingerprints, revealing the genetic diversity among Nepalese *Bt* isolates.

Rai *et al*. (2009) recovered *B. thuringiensis* from soil samples collected from various districts in Vidharbha, Maharashtra and India, using the sodium acetate selection procedure reported by Travers *et al*., 1987. Also all isolates were examined for *cry* gene amplification, as well as several biochemical parameters specified by Sneath (1986) and morphological characterisation of crystal protein using the Smirnoff staining method (1962). A total of 30 RAPD primers were tested for polymorphism with purified DNA and DNA acquired from whole cells initially. Primers such as OPA-1, OPA-11, OPA-19 and OPA13 produced discriminating patterns as well as amplification patterns that were extremely comparable across isolates with the same serology and strains that were poisonous to *Bombyx mori*. *B. thuringiensis* subsp. *kurstaki* H3abc was distinguished from the three *B. cereus* strains examined using RAPD analysis. OPA-1 distinguished all serotypes with repeatable findings.

Sahukhal *et al*. (2009) isolated *Bt* strains from soil samples collected from Nepal. The strains were tested using 100 decamer RAPD primers by RAPD- PCR. Electrophoretic analysis revealed that higher polymorphic bands were found at 700- 900 bp followed by 400-700 and 1200- 1600 bp. Discriminatory capacity was found to be 0.9901. Isolates from high altitude region were found be rich in genomic polymorphism.

Chaves *et al*. (2010) used three distinct random primers to amplify the genomic DNA fingerprints of 40 serovars. The primers 2 and 3 were determined to have the best discrimination power among the primers tested. A dendrogram was created based on the discrimination, revealing that the serovars were separated into two groups, showing substantial genetic divergence.

Kumar *et al*. (2010) had isolated 70 *B. thuringiensis* from soil samples collected from cotton fields. These isolates were characterized by RAPD markers to determine their genetic diversity pattern based on their source of origin. The random decamer primers used for amplification, which generated a total of 1935 fragments; of these 1865 were polymorphic and 68 monomorphic. The primers OPA03, OPA08, OPD14, OPD19, OPD20, OPE17 and OPD19 produced 100% polymorphic fragments, whereas primers OPC06, OPC20 and OPD17 produced 20, 31 and 17 monomorphic fragments, respectively. The dendrogram constructed using RAPD banding pattern revealed that 70 isolates fell into two separate clusters, cluster I and cluster II, including 26 and 44 *B. thuringiensis* isolates, respectively. These two main clusters were further divided into four subclusters at 150 and 80 per cent similarity index. All primers showed amplification and indicated good diversity of *B. thuringiensis* isolates and revealed the strain differences.

Jawahar *et al*. (2013) investigated the genetic diversity of 32 *B. thuringiensis* strains obtained from different soil samples across India along with seven reference samples. A total of 7 RAPD markers were employed to study the molecular diversity. The dendrogram was constructed using NTSYS 2.2, which revealed that on a similarity level of 25 per cent, eight primary groups emerged. It was discovered that the isolates from the same region had similar levels of diversity. The findings aided in determining the strain diversity.

Ravi Charan *et al*. (2013) isolated 8 *Bt* strains (*Bt*-3, 34, 41, 52, 55, 112, 116, 127) from soil samples collected from Andhra Pradesh and Karnataka, India. The isolates were characterized with REP- PCR (Repetitive Extragenic Palindromic Sequence) to identify the closely related relationship among the isolates. The REP- PCR profile of these isolates showed *Bt*- 52 and *Bt*- 127 formed separate cluster in phylogenetic tree.

Sabir *et al*. (2013) aimed to identify 12 native *Bt* strains using morphological, physiological, biochemical and molecular approaches. The RAPD banding profiles showed the presence of polymorphism among the strains studied. The data obtained was used to discriminate the studied *Bt* isolates.

Da Silva and Valicente (2013) determined the genetic diversity of 65 strains of *Bt* using REP- PCR. Based on the repetitive sequences, the BOX primer was most informative with 26 fragments, followed by ERIC (19) and REP (10), generating a total of 55 fragments. The dendogram showed that ten groups were formed when 45% was the average distance of the population: group 1 with 41.5 per cent of the isolates, 33.8 per cent of the isolates were distributed in other groups and 24.6 per cent did not form distinct group. 53.2 per cent of the isolates from Embrapa were found to be in the group 1 and 29.8 per cent of the isolates were distributed in other groups. *Bt* strains from USDA and Institute Pasteur showed more variability.

Chettri and Tamang (2015) collected 39 samples and 43 samples of Tungrymbai and Bekang, naturally fermented soybean foods from different regions of Meghalaya and Mizoram, respectively. These foods were analysed for microbial load. A total of 428 isolates of *Bacillus* were isolated from Tungrymbai (211) and Bekang (217). Molecular characterization using ARDRA, ITS-PCR and RAPD- PCR techniques were done and it was identified that *Bacillus* isolated from Tungrymbai were identified as *B. licheniformis* (25.5%), *B. pumilus* (19.5%) and *B. subtilis* (55%) and species of *Bacillus* from Bekang were *B. brevis* (2%), *B. circulans* (7.5%), *B. coagulans* (6.5%), *B. licheniformis* (16.5%), *B. pumilus* (9.1%), *B. sphaericus* (4.6%), *B. subtilis* (51.8%) and *Lysinibacillus fusiformis* (2%). It was found that the most dominant bacterium was *B. subtilis* in both the products.

Shishir *et al.* (2015) investigated the genetic diversity of 177 *Bt* strains from Bangladesh, using a decamer AGCTCAGCCA to perform RAPD-PCR analysis. The binary matrices were derived from the dendrogram, which was used to categorize the strains into 15 genomic categories. The presence of *cry* 1 gene subgroups was also determined, with *cry*1Aa and *cry*1Ca genes being the most common (21.74%).

Avsar *et al*. (2017) identified a total of 31 isolates as *Bacillus* sp. based on the morphological and the physiological properties. Molecular characterization was done using RAPD- PCR with M13-10, OPL 3 primers, ERIC primers and P11/P13 SSCP. The dendrogram revealed that the isolates were grouped into two major clusters at similarity levels of 5 per cent, 2 per cent, 38 per cent and 15 per cent (or) above.

Khowal *et al*. (2017) isolated arsenic resistant bacteria from soil. RAPD-PCR technique was used to identify the genetic similarity between the arsenic resistant isolates. RAPD-PCR primer OPA-02 amplifies at 0.5 Kb DNA band specific to *B. pumilus* 3ZZZ strain and 0.75 Kb DNA band specific to *B. subtilis* 3PP strain.

Imam and Imam (2018) evaluated genetic variation of an isolate of *B. thuringeinsis* extracted from soil and a reference (Agrin, commercial product of *Bacillus*) using RAPD-PCR, to differentiate between them using random primers. Phylogenetic analysis of the isolates revealed that the *Bt* isolate has closer genetic similarity with the reference commercial product.

Bahuguna *et al*. (2020) tested organic agricultural materials and isolated seven distinct bacterial colonies (A-1, B-1, C-1, D-1, E-1, E-2 & E-3). These isolates were processed for sequential identification utilizing *Bacillus* agar. PCR analysis was also performed with RAPD primers and species-specific primers. All the isolates were proven to be gram (+ve) and spore forming on *Bacillus* agar. RAPD-PCR and SS-PCR discriminated and provided evidence for D-1 as *B thuringeinsis* and E-1, E-2, E-3 as *B. licheniformis,* respectively.

Elhameed *et al*. (2020) screened 22 bacterial isolates to know the cellulolytic activity. Among these 22 isolates, 5 isolates were selected with higher cellulase activity. Genetic distances among the selected strains were determined by phylogeny analysis using RAPD-PCR. Based on that similarity among the isolates was revealed. The reports revealed similarity between the morphological characterization and the genetic distances.

Koche *et al*. (2020) isolated Plant Growth Promoting Rhizobacterial strains belonging to fluorescent *Pseudomonas* from the rhizosphere of citrus in Vidarbha region of Maharashtra, India. Based on the morphological and physiological characters, the strains were recognised as *P. flourescens*, *P. putida* and *P. aurigonosa*. Genotyping of these *P. flourscenes* strains was done using 16 RAPD primers. Among the primers used 6 primers (OPA-16, OPB-18, OPC-15, OPC-19, OPG-5, OPG-10) showed polymorphism. Similarity co-efficient between each pair of accessions was used to construct a dendrogram using the Unweighted Paired Group Method with Arithmetic Average (UPGMA). Cluster analysis carried out based on the similarity data generated from the 8 isolates using 6 primers accounted for a total of 296 polymorphic DNA bands.

The various *P. fluorescens* isolates were divided into two major classes. Two of the isolates *i.e.* Pf_{15} and Pf_{14} formed one group. Second class consisted of different subgroups with Pf_9, Pf_6 and Pf_{19}, Pf_{18} and Pf_{20} and Pf_1. The similarity among these isolates ranged from 0.158 to 0.044. Genetic variability exists among the various isolates of *Pseudomonas* depending on the geographical locations.

Adesetan *et al*. (2020) extracted genomic DNA from *B. cereus* isolated from some related foods. Genotypic profiling of *B. cereus* was performed with RAPD-PCR using 13 OPR- 13 primer. The gel was examined and the binary data was recorded from the gels based on the presence/ absence of bands in individual lanes. The scatter diagram of scored bands was constructed using NTSYS software which revealed grouping of the isolates. The dendrogram generated for the RAPD profile showed that all the strains were closely related, with a similarity coefficient of 70 per cent.

2.1.1 Characterisation of Crystal Proteins of Native *Bt* Isolates

Chilcott and Wigley (1993) reported that the isolates producing 50, 42 and 40 kDa proteins were found to be nontoxic to Lepidoptera.

Arrieta *et al*. (2004) indicated that *B. thuringeinsis* isolates collected from Costa Rican coffee plantations producing Cry1 proteins with molecular weights between 130 and 150 kDa in size, Cry2 proteins between 65 and 70 kDa, Cry3 with 75 kDa, Cry7 and Cry8 with 130 kDa, Cry9 with 130-140 kDa, Cry22 with 76 kDa, Cry34 and Cry37 with 14 kDa showed insecticidal action against Coffee berry borer, *Hypothenemus hampei.*

Arretia and Espinoza (2006) used SDS-PAGE to analyse 146 *Bt* strains and found a number of electrophoretic patterns with Cry proteins, molecular weights ranging from 20 to 160 kDa.

Gough *et al*. (2005) collected *B. thuringeinsis* strains from various Australian soil samples that produced protein bands of 130 and 28 kDa.

Kati *et al.* (2007) reported the use of SDS-PAGE for protein profiling of *B. thuringeinsis* isolates producing proteins of ~135 kDa and ~65 kDa size corresponding to Cry1 and Cry2 proteins.

Liu *et al*. (2009) discovered a novel *B. thuringeinsis Bt*11 strain from soil samples in China, which exhibited polypeptides ranging from 20 to 130 kDa, with 35, 80 and 130 kDa proteins being the primary components.

Out of 70 *Bt* strains collected from Western ghats of Tamil Nadu and analysed by SDS-PAGE, 17 strains (24.2%) showed two major polypeptide bands with molecular weights in the range of 135 and 65 kDa (Ramalakshmi and Udayasuriyan., 2010).

Zheng *et al.* (2010) discovered a *B. thuringeinsis* isolate, JF 19-2 in soil samples from Western China that contained insecticidal crystal protein of 70 kDa.

Diverse electrophoretic patterns in Cry proteins with molecular weights ranging from 20 to 160 kDa have been reported earlier (Mahadeva Swamy *et al.*, 2013)

Rajashekhar (2017) reported that protein profile showed the presence of 20 to 245 kDa protein bands in pre-solubilized form and bands of 18 to 110 kDa in solubilized form.

Based on the protein profile of *Bt* strains, proteins were categorized into 3 groups *viz.*, group I (18-60 kDa), group II (65-105 kDa) and group III (110-245 kDa). SDS-PAGE analysis of the spore-crystal mixtures of indigenous *B. thuringeinsis* strains showed molecular weights ranging from 150 to 28 kDa (Reyaz *et al*., 2017).

The isolates *Bt*MA-64 and *Bt*MA-194 produced well defined bands with molecular weights of 100- 150 kDa range, but the isolates *Bt*MA-104, *Bt*MA- 251, *Bt*MA-410, and *Bt*MA-450 produced proteins with less molecular weights (Lobo *et al.*, 2018).

Nair *et al*. (2018) harvested the spore-crystal mixture from the *Bt* isolates of Qatar and analysed through SDS-PAGE analysis.

2.2 DEVELOPMENT OF NANO-SCALE PARTICLES WITH NATIVE *Bt* ISOLATES

2.2.1 Synthesis of Ca Nanomaterial

The calcium ions were known to maintain the cell structure, transport, motility and cell differentiation processes such as spore formation, heterocyst formation and fruiting body development (Dominguez., 2004).

Palanivelu and Rubankumar (2013) used a sol-gel technique at 75 °C to make hydroxyapatite (HAP) NPs, which were used in biomedical applications such as bone deficiencies, bone augmentation and implant covering. The functional groups, microstructure and phase analysis of the synthesised HAP were investigated using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and scanning electron microscopy (SEM). The XRD spectra of dried HAP precursor indicated that crystallite size was less than 45 nm and the FTIR spectrum confirmed the functional groups of phosphate (P_3O_4) and hydroxyl (O-H). SEM photos of HAP revealed that it was in the shape of a platelet with a size of 30 to 75 nm.

Bhoopathi and Rubankumar (2014) used calcium nitrate tetra hydrate and di-ammonium hydrogen phosphate as calcium and phosphorus precursors, respectively, in a sol-gel process to make HAP. For HAP sol manufacture, double distilled water was employed as a diluting media and ammonia was used to alter the pH. XRD, SEM and FTIR were used to determine the phase content, morphology and types of bonds present in synthesised HAP (Ca/P=1.67).

Hariharan *et al*. (2014) used chitosan as a precursor to synthesize calcium carbonate NPs from cockle shells using a precipitation technique. SEM, XRD, UV Visible and FTIR Spectroscopy were used to characterise the synthesised calcium carbonates. Cockle shells were discovered to be a viable supply of calcium carbonate, as opposed to the materials used, which were both naturally occurring and by-products of the sea food industry. The methods used were both cost-effective and eco-friendly.

Prabhavathi *et al*. (2014) synthesized MgO and CaO nanoparticles using simple sol-gel method and reagents and the characterization of MgO and CaO using FTIR, X-ray diffraction and scanning electron microscopy (SEM).

Mallik *et al*. (2015) used sol-gel synthesis to make calcium titanate (CT) powders from an equimolar solution of calcium oxide, ethanol and titanium (IV) isopropoxide. XRD was used to study the phase analysis and morphology of powder particles, while TEM with an energy dispersive X-ray spectrometer was used to evaluate the composition and size of powder particles (EDS). After drying at 100 ˚C for 24 hours, XRD verified the presence of phase pure crystalline $CaTiO_3$, while TEM investigation revealed $CaTiO_3$ particles with diameters of about 13 nm and some agglomerated particles of 20-30 nm. Finally, they came to the conclusion that the reported sol-gel synthesis was a novel way for producing nano $CaTiO_3$ particles at lower temperatures than any previous method.

Habte *et al*. (2019) used sol-gel process to create calcium oxide nanoparticles from eggshell. $CaCl_2$ solution was made by dissolving raw eggshell in HCl, adding NaOH to the solution dropwise to agitate the $Ca(OH)_2$ gel and then drying the gel at 900 °C for 1 hour. Scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), X-Ray fluorescence (XRF) and X-ray diffraction were used to analyse the produced nanoparticle (XRD). The FE-SEM pictures of calcium oxide nanoparticles revealed that they had a nearly spherical morphology. The nanoparticles ranged in size from 50 to 198 nm.

2.2.2 Synthesis of Mg Nanomaterial

Wahab *et al*. (2007) used the sol-gel approach to successfully produce cubic shaped MgO NPs at room temperature using magnesium nitrate and sodium hydroxide. At 300 and 500 ˚C, hydrated magnesium oxide NPs were annealed in the air. The produced NPs had high crystallinity and were pure magnesium oxide periclase phase with (200) orientation, according to X-ray diffraction patterns. The average diameters of the generated NPs were in the range of 50-70 nm, according to morphological analysis using field emission scanning electron microscopy (FESEM). FTIR spectroscopy was used to examine the powder composition, and the results confirmed that the brucite phase magnesium hydroxide was converted to MgO periclase phase at 300 ˚C. The phase change of the produced MgO NPs occurred at 280-300 ˚C, according to thermogravimetric measurements.

Camtakan *et al*. (2011) produced MgO by precipitating hydroxide and then thermally decomposing the hydroxide. XRD, SEM and multipoint Brunauer, Emmett and Teller were used to describe MgO. The relative impact of test parameters such as adsorbate concentration, pH, contact duration and temperature on MgO for U (VI) ion adsorption performance was investigated and the optimal sorption efficiency was determined to be 87.61±3.10 per cent. With NH_4Cl and $NaNO_3$ solutions comprising 0.1 M, desorption efficiency was determined to be 100 per cent in a single step.

Athar *et al*. (2012) synthesized cubic MgO nanoparticles with good shape, limited surface area characteristics and mono dispersity utilising a non-aqueous sol-gel technique. Analytical techniques such as TEM, SEM, XRD, TGA/DTA, FTIR and Raman spectroscopy were used to characterise the nanoparticle. The average particle size was found to be 43 nm, with a surface area of 2.81 m^2/g. With strong crystallinity and well-defined uniform morphologies, the non-aqueous method allows for a better understanding of the reaction mechanism at a molecular level.

Athar (2013) used magnesium acetate tetrahydrate and potassium hydroxide in a stoichiometric ratio manufactured ultra-pure and colloidal MgO nano powders with well-defined shape at ambient temperature and the synthesized particles were characterized.

Mg^{2+}, the divalent cation is vital for the stabilization of ribosomes and membranes, for neutralization of nucleic acids, and also as a cofactor in a variety of enzyme mediated reactions (Groisman *et al*., 2013).

Suresh *et al*. (2014) successfully synthesised magnesium oxide NPs from *Nephelium lappaceum* L. peels as a natural ligation agent. The crystallinity and spherical morphology of the biosynthesized NPs were revealed by XRD and SEM. The particle size was determined to be 60-70 nm using XRD and SEM examination. Particle size analysis revealed that produced MgO powders had a particle size of around 100 nm. XRD, SEM-Energy Dispersion X-ray analysis and PSA analysis were used to validate the successful production of MgO NPs.

Agarwal *et al*. (2015) employed magnesium nitrate and sodium hydroxide as starting materials for the liquid phase approach of producing MgO NPs. The XRD study revealed that the 1:1molar ratio significantly increased the nanocrystalline size of MgO. Using an Arrhenius plot, the average crystalline size of the MgO NPs was found to be 25.91 nm and the activation energy of the MgO NPs was determined to be 0.49 eV or 48.196 KJ/mol, with the I/V characteristics being linear and obeying the Ohms law in the operating temperature range (50-350 °C).

According to Wagh *et al*. (2015), the sol-gel procedure for the synthesis of oxide base materials was currently garnering attention because of its low-cost, low-temperature technique that provides for fine product control. Magnesium chloride was employed as a precursor in the manufacture of MgO powder using the sol-gel process, which involved hydrolysis and condensation. XRD and SEM techniques were used to characterise the produced powder after it was burned at various temperatures.

Maurya and Bhatia (2017) used the precursors citric acid ($C_2O_4H_2$) and magnesium chloride to make MgO nanoparticles utilising the microwave aided Sol gel synthesis method ($MgCl_2.6H_2O$). It's a straightforward, unique, and cost-effective technique. SEM, TEM and X-ray diffraction were used to analyse the structure, morphology, and crystalline phase of magnesium oxide nanocrystals (XRD). FTIR and UV-visible techniques were used to determine the presence of functional groups and optical characteristics.

Sutapa *et al*. (2018) dissolved magnesium acetate and oxalic acid in methanol to make MgO nanoparticles, which were then annealed. Fourier Transform Infrared Radiation (FTIR) were used to characterise functional groups. Infrared spectroscopy, XRD (X-ray Diffraction) crystal profile analysis and SEM morphology. The FTIR and XRD data revealed that magnesium acetate was transformed to magnesium oxalate (precursor), which was subsequently annealed into MgO nanoparticles. The results of the XRD and SEM analysis show that magnesium complexes have changed. The solid layer formed by the Mg polymer complex network was transformed into a cubic structure.

2.2.3 Synthesis of Zn Nanomaterial

Vafaeea and Sasani (2007) first synthesized 3–4 nm ZnO NPs in a spherical form using TEA (triethanolamine) as a surfactant to prepare ZnO NPs. The best concentration of each component was adjusted by comparing between different absorption spectra of the soil. The best sol with respect to its optical properties was analyzed by luminescence spectroscopy. TEM microscopic images and electron diffraction images of these particles were obtained to represent the morphology and crystal phase of the particles, respectively. The experimental results show that the zinc oxide NPs prepared by this method have higher luminescence spectra than other methods.

Singh *et al*. (2011) prepared ZnO NPs *via.* 2 different routes (i) *via* sol- gel method (ii) by solid state reaction method, under same ambient conditions by keeping all the parameters same. The properties of both the particles were analysed. It was found that the NPs prepared *via*., Sol- gel process were comparatively highly crystalline.

Zinc, the metal ion, was found to be involved in many important biological processes and are very important for the survival of all organisms. They are found ubiquitously in all living organisms, exclusively as main constituents of proteins, including storage proteins enzymes, and transcription factors (Hood and Skaar, 2012).

Robina *et al*. (2013) also synthesized ZnO NPs with particle size less than 50nm by simple sol-gel method. These NPs were used as a source layer for the extraction of electrons in hetero junction organic solar cells. Zinc acetate was used as a precursor material in this case. X-ray powder diffraction, Ellipsometry and SEM were used to study the crystal structure, optical properties and surface morphology of the synthesized NPs, respectively. The presence of (100), (002) and (101) planes in the XRD cells clearly indicates that ZnO has a wurtzite structure under the aggregate conditions. The surface morphology studied by SEM showed that the NPs were spherical with size less than 100 nm. Large-scale development of these NPs is also observed with a uniform size distribution. The transmission values decreased significantly with increasing pH from 2-

9. A refractive index of about 1.5 was observed at 350 nm for all samples except the synthesized sample with pH 9.

Homayoonfal *et al*. (2014) synthesized ZnO nanoparticles by adopting an industrial method, where the NPs were synthesized continuously by Spray Pyrolysis method. The properties of synthesized NPs and the effect of NPs on performance of polymeric materials was analysed. The analysis revealed that the application of NPs can develop and enhance the usuability of polymeric materials.

Brintha and Ajitha (2015) prepared ZnO NPs by aqueous method, sol-gel method and hydrothermal method. The synthesized particles characterized by XRD, SEM, EDX and UV. X-ray diffraction studies showed that the synthesized ZnO NPs had wurtzite structure and grain sizes ranging from 13 to 18 nm. SEM study showed that the surface morphology of ZnO NPs was spherical during hydrothermal and changed in floral arrangement in aqueous solution and sol-gel process. The visible UV spectra of the NPs showed a blue shift compared with the spectrum of the general sample.

Ghorbani *et al*. (2015) used a simple technique for the synthesis of ZnO NPs by direct precipitation method using Zn nitrate as zinc source and KOH as precipitating agent. The compound that precipitated was calcined and characterized by UV- vis, TEM and DLS analysis which showed that the particle size were in the range of 30+15 nm.

Jurablu *et al*. (2015) produced ZnO nano powders by the sol-gel method from an ethanol solution of zinc sulphate heptahydrate in the presence of diethylene glycol surfactant. The detailed structural and microstructural investigations were carried out using XRD, high resolution transmission electron microscopy (HRTEM), FESEM, FTIR and UV-Vis spectrophotometer. XRD pattern showed that the zinc oxide NPs exhibited hexagonal wurtzite structure. The average particle size of ZnO was achieved around 28 nm as estimated by XRD technique and direct HRTEM observation. Surface morphology studies by SEM and TEM described spherical particles with cluster formation.

Deepak and Sheela (2016) performed the synthesis of ZnO NPs by gel sol method and characterized by XRD, visible UV spectroscopy and FESEM. XRD was used to know the size of the particles and FESEM images were used to determine its morphology. pH 9 gave better morphology and good crystallization properties. They observed a morphological change by varying its solvent content.

Hasnidawani *et al*. (2016) synthesized zinc oxide nanostructures by the most practical method using the sol-gel method due to its simplicity and ability to control particle size morphology through systematic tracking response parameters. Dehydrated zinc acetate (Zn $(CH_3COO)_2.2H_2O$) as precursor and ethanol (CH_2COOH) were used as solvent, sodium hydroxide (NaOH) and distilled water were used as the intermediate. ZnO NPs were characterized using XRD, EDX, FESEM and a nanoparticle analyzer. The EDX characterization results showed that the ZnO NPNs have good purity (zinc content 55.38%; oxygen content 44.62%) and the resulting XRD spectrum showed oxygen peaks and that of zinc, indicating high crystallized nature. FESEM micrographs showed that the synthesized ZnO has a rod structure.

Supraja *et al*. (2016) characterized ZnO NPs using UV-visible spectroscopy analysis and recorded local surface plasmon resonance (LSPR) at 230 nm. FTIR analysis revealed that the primary and secondary amine groups associated with proteins present in the bark extract were responsible for the reduction and stabilization of *Bt* Zn NPs. The morphology and crystal phase of the NPs were determined by TEM. The hydrodynamic diameter (20.3 nm) and positive zeta potential (4.8 mV) were measured by dynamic light scattering technique.

Haque *et al*. (2020) adopted two different methods for the synthesis of zinc oxide Nanoparticles. The first method was the synthesis through sol- gel method and the other was the biosynthesis method using the leaf extracts of *Azadirachta indica* (Neem). The performances of both the variants were analysed. The comparison between the variants, revealed that the biosynthesis method was more effective than the sol- gel method.

2.2.4 Development of Nano-scale Particles with Native *Bt* Isolates

Najitha Banu *et al.* (2014) worked on biosynthesis of silver nanoparticles using *B. thuringiensis*. The *Bt*-Ag nanoparticles were characterized by using UV-visible spectrophotometer followed by SEM and energy-dispersive X-ray (EDX) spectroscopy.

Murthy *et al.* (2014) generated nanoparticles from technical powder of *B. thuringiensis var. kurstaki* for improving efficacy of insecticidal properties of *Bt*. The *Bt* nanoparticles were characterized by particle sizing and zeta potential through Light scattering, electron microscopy and heat viable spore count determination.

Vimala Devi and Vineela (2015) worked on the bio-efficacy of *B. thuringiensis* through particle size reduction and formulated suspension concentrate. This *Bt* was characterized by DLS (dynamic light scattering) to determine particle size.

Gayathri *et al*. (2018) synthesized nanoparticles of ZnO, CaO, MgO & FeO using sol- gel method. The synthesized particles were characterized by UV–Visible Spectrum, FTIR Analysis, XRD Analysis, Particle Size and Zeta Potential Analysis and TEM.

Rao *et al*. (2018) synthesized nano-$Mg(OH)_2$ (magnesium hydroxide nanoparticles, MHNPs) to control the loss of the Cry1Ac protein and delivered protein to *H. armigera*. They could successfully load the Cry1Ac protein onto MHNPs through electrostatic adsorption. These Cry1Ac-loaded MHNPs could remain on the surface of cotton leaves, resulting in enhanced adhesion of Cry1Ac protein by 59.50 per cent and increased pest mortality by 75.0 per cent.

2.2.5 Evaluation of Nano formulations of *B. thuringeinsis* on Insects

Vineela *et al.* (2017) worked on the bioefficacy of *B. thuringiensis var. kurstaki* against *Spodoptera litura* by particle size reduction. Unmilled and milled *Bt* powders were evaluated through bioassays at 27 ± 1^0C against third instar *S. litura* larvae by leaf disc technique using castor leaves. Results obtained showed that all the milled powders gave higher mortality of *S. litura* larvae in comparison to the unmilled *Bt* powder.

Malaikozhundan *et al.* (2017) worked on the synthesis and evaluation of *B. thuringiensis* coated zinc oxide nanoparticles (*Bt*-ZnO NPs) on *C. maculatus.* These biologically characterized *Bt*-ZnO nanoparticles were characterized by UV-Vis spectroscopy, XRD, FTIR, TEM and Zeta potential. Results suggest that, *Bt*-ZnO nanoparticles were effective against *C. maculatus* and could be used as nano biopesticides in the control of pests.

Gayathri *et al.* (2018) worked on evaluation of nano based biopesticides against *S. litura* on groundnut. Laboratory evaluation of nano-based *B. thuringeinsis* was done against *S. litura* larvae and experiment was done for the comparision on the effectiveness of *Bt* treated with CaO, MgO, FeO and ZnO. The results obtained were similar in both the cases of CaO and MgO at 20ppm and was found effective in control of *H. armigera*.

Iqbal *et al*. (2022) discovered the insecticidal potential of zinc oxide (ZnO) nanoparticles synthesised with *B. thuringiensis* on *Musca domestica*. The biological features of *Musca domestica* were tested for acute toxicity and sublethal effects of ZnO nanoparticles generated in the culture supernatant of *B. thuringiensis* ser. *israelensis* (*Bti*) as a reducing agent.

2.3 EVALUATION OF *Bt* ISOLATES ON POD BORER, *H. armigera* (HUB.) IN LABORATORY

2.3.1 Evaluation of *B. thuringeinsis* on Insects

The symptoms of *Bt* infection in *H. armigera*, according to Majumdar *et al*. (1955), were the normal green colour changing to pink, the body becoming mushy and lymph and the dorsal blood vessel becoming apparent. After 48 hours of therapy, the symptoms manifested and the larvae perished after 72 hours.

Daoust and Roome (1974) conducted laboratory bioassay experiments with *Bt* (Biotrol BTB - 183) formulations. These investigations provided a solid foundation for comparison with field trials and the development of a standard testing protocol. The final bioassay research for *B. thuringiensis* required a range value (bracket value) of 1806 to 66223 spores/mm^2.

In the late 1960's, a more potent strain of *B. thuringiensis* (HD-1) was found as *B. thuringiensis* var. *kurstaki*, which was 20 to 200 times more active than *B. thuringeinsis* var. *thuringiensis*. These results sparked a lot of academic and business interest in using *B. thuringiensis* to control *Heliothis* spp. (Dulmage, 1981).

Gaikwad *et al*. (1988) reported that the mortality (LC_{50}) of 2nd, 3rd, 4th, and 5th instar larvae of *H. armigera* treated with Delfin WG was reduced as the day interval increased.

Purohit and Deshpande (1991) assayed third instar larvae of *H. armigera* with *B. thuringiensis* var *kurstaki* in laboratory and reported that maximum larval mortality (90%) takes place after 96 h of treatment.

Navon, (1993) had given that the products based on the HD-1 isolates of the *kurstaki* sub species were commercially used for the control of lepidopteran pests in forestry and agriculture.

Shenmar *et al*. (1996) conducted a laboratory study and showed that the two formulations of *B. thuringiensis* (Delfin and Dipel 8 L) were found to be more effective against the larvae of *H. armigera* than NPV.

In the treatment of pod borer in pigeon pea, Kulkarni (1999) compared the efficiency of microbial pesticides such as *B. thuringiensis*, NPV and *N. releyi* to synthetic herbicides. None of the microbes were able to prove their superiority. On the basis of larva reduction, pod and seed damage and yield. *B. thuringiensis* was determined to be superior than chemicals, followed by NPV and *N. releyi*.

Pharindera (2000) reported that *B. thuringiensis* caused mortality in larvae after 48 hours of inoculation, with 100% *H. armigera* mortality on the fourth day at two higher dosages of 800 and 1600 μg/ml.

Polanczyk *et al*. (2003) used the diet incorporation method to test the pathogenicity of 58 native isolates on second instar larvae of the fall armyworm at a concentration of 3107 cells/ml. After therapy, mortality was tracked up to seven days. *Bt morrisoni* strain was the most virulent of all the isolates, with an LC_{50} value of 8.6106 cells/ml and 80 per cent mortality rate.

Martnez *et al*. (2004) characterised the HU4-2 strain of *Bt* sub sp. *aizawai*, which contained eight different *cry* genes and was found to have a broad spectrum of activity against lepidopterans, including *H. armigera*, *S. exigua*, *S. littoralis* and *S. frugiperda*, as well as being moderately toxic to two mosquito species *Culex pipiens* and *Aedes aegypti*.

Prasad *et al*. (2006) reported that the silica containing formulation was more effective compared to commercial formulation Halt 5 per cent WP. Thirty two recipes of 7 per cent WDP formulation of *B. thuringiensis kurstaki* (HD-1) were prepared by using eight carriers, two dispersing agents and two wetting agents. The LC_{50} values ranged from 0.025 to 0.154 per cent against neonates of *H. armigera*.

When carboxy methyl cellulose was employed as an adjuvant in the formulation of bio-pesticide, Singh *et al*. (2007) investigated the life of *Bt* cells up to 15 days after spray and discovered that the per cent survival of *Bt* spores after 15 days was around 25-34 per cent. When *H. armigera* larvae were fed with treated leaves from the field, the commercial formulation Dipel had a 33.1 per cent mean spore survival rate and a 61.5 per cent mean per cent mortality rate after 9 days of spraying.

Kashyap and Amla (2007) evaluated four *B. thuringiensis kurstaki* strains (HD1, Dipel, HD73 and HD1 Dipel) for their toxicity against *H. armigera* and *S. litura*. The LC_{50} for *S. litura* was determined as 0.11 μg of HD73, 0.027 μg of HD1, 0.20 μg of Dipel and 0.018 μg of HD1 Dipel, whereas LC_{50} for *H. armigera* was calculated to be 0.04 μg of HD73, 0.031 μg of HD1, 0.011 μg of Dipel and 0.008 μg of HD1 Dipel.

Khanna *et al*. (2009) compared the effectiveness of native *Bt* isolates from Punjab soils to a standard *Bt* formulation Halt 5 wp in chickpea. During the first spray, *Btc* 5 (local isolate) exhibited 4.55 larvae/m^2, which was very low compared to the untreated control (7.41 larvae/m^2) and 4.33 larvae/m^2 during the second spray compared to the untreated control (7.83 larvae/m^2). The larvae counts were taken 72 hours after spray from three randomly selected 1 m^2 sites in each replication of a treatment.

Lalitha *et al*. (2011) compared the bio-efficacy of 28 native isolates against *H. armigera* in field bean to a reference strain HD1. Out of all the native isolates studied, HD1 demonstrated 85.42 per cent larva decrease after seven days of spraying, with the least amount of pod damage. Solid-based formulations of the same isolates tested yielded the maximum yield and had the least pod damage.

Lalitha and Muralikrishna (2012) tested 114 native *Bt* strains obtained from soil samples collected from various districts of Andhra Pradesh against I^{st} and III^{rd} instar larvae of *S. litura* in laboratory by leaf dip bioassays at $3.2x10^5$ CFU ml^{-1} and mortality ranged from 10%- 93.33 per cent. The HD1 reference strain had the highest mortality (93.33%), which was comparable to the native *Bt* isolates, 375 (90%) and 416 (86.67%).

Malik *et al*. (2013) used the diet incorporation approach to test the efficacy of local *Bt* isolates against *H. armigera* and *E. vittella*. HW 4.4 and INS 2.25 were the most effective isolates against *H. armigera*, with LC_{50} values of 9 ng/mg artificial diet. Local *Bt* isolates HFZ 11.3, MR 19.1, and MG 2.6 showed a LC_{50} value of 2 ng/mg of artificial diet against *E. vittella*.

The combined effect of *B. thuringiensis* (*Bt*) and *B. subtilis* (*Bs*) in an artificial diet on *H. armigera* was studied by Chandrasekaran *et al*. (2015). *Bt* and *Bs* spore concentrations were examined at $1{\times}10^2$, $1{\times}10^4$, $1{\times}10^6$, $1{\times}10^8$ and $1{\times}10^{12}$ spores ml^{-1}. At $1{\times}10^{12}$ spores ml^{-1}, the LC_{50} values of the combined *B. thuringiensis* and *B. subtilis* were significantly lower (3.19%) than those of *B. thuringiensis* alone (4.19%) and *B. subtilis* alone (4.81%).

Lone *et al*. (2017) estimated LC_{50} of spore-crystal mixtures of *Bt*-JK12, 17, 22, 48 and 72 against second instar larvae of *H. armigera*. It was observed to be 184.62, 275.39, 256.29 and 259.93 µg ml^{-1}, respectively. It was observed that *B. thuringiensis* isolate JK12 exhibited higher toxicity against *H. armigera* than that of *B. thuringiensis* HD1, hence can be commercially exploited to control insect pest for sustainable crop production.

Pavani (2019) investigated native *Bt* isolates from Guntur district soils. The leaf dip bioassay with native *Bt* isolates -49, 51, 52, 55 and HD_1 against third instar *S. litura* yielded 61.67, 66.67, 58.33, 60.00 and 90.00 per cent mortality, respectively. The *vip* 3, *cry* 2, *cry* 9, *cry* 9 and *cry* 1 genes were found in these isolates, according to their molecular characterization.

Harika (2020) tested the native *Bt* isolates against third instar larvae of *S. frugiperda*. The leaf dip bioassay against *S. litura* with native *Bt* isolates, isolate- 49, 51, 52, 55, 493 along with the reference HD1 was done. The data recorded revealed mortality percentages between 56.57-91.67. The highest mortality was recorded in the reference strain *i.e*., HD1 and among the wild types highest mortality was observed in the isolate 493 (86.67%).

Anusha (2021) tested the native *Bt* isolates (*Bt*- 16, 49, 51, 52, 55, 493 & HD1) against third instar larvae of *Maruca vitrata*. The flower dip bioassay was carried out to evaluate the efficacy of the native isolates against the test insect. Results obtained from the native isolates revealed that the mortality percentages were between 28.33 to 90.00 per cent. It was observed that the highest mortality was observed in the reference strain HD1 *i.e*., 93.33 per cent.

Chapter – III

Material and Methods

Chapter- III

MATERIAL AND METHODS

The research on "**Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)**" was carried out during 2021-22. Investigations were carried out in the Department of Entomology, Agricultural College, Bapatla. The details of the material used and methods followed were presented in this chapter.

3.1 MOLECULAR CHARACTERISATION OF NATIVE *Bt* ISOLATES WITH DNA FINGER PRINTING BY RAPD-PCR

Seven bacterial isolates were selected from the repository of Department of Entomology, Agricultural College, Bapatla, which were previously collected from cotton grown field soil samples. The study on genetic diversity among the Bacillus isolates was conducted at the Central instrumentation cell, Agricultural College, Bapatla. Genomic DNA was extracted for the collected isolates of *Bt* by modified CTAB method (William and Copeland, 2012). Using Nanodrop, the integrity and concentration of isolated DNA were assessed. Total genomic DNA was isolated and dissolved in 1X TE buffer before being kept at 4 ^{o}C. The isolated DNA was employed as a template for RAPD-PCR, and the PCR amplified products were analysed using Agarose Gel Electrophoresis. The genetic diversity among the isolates was investigated using data collected from polymorphic bands and a dendrogram was constructed.

3.1.1 Preparation of Buffer Stock Solutions

Required stock solutions of buffers needed for the analysis were prepared as per the procedure mentioned below.

$$\text{Molarity} = \left\{ \frac{\text{Weight}}{\text{Mol. Weight}} \times \frac{1000}{\text{required volume}} \right\}$$

1 M Tris, pH 8: To make 1 M Tris of 100 ml, 12.114 g of Tris base (Mol. Wt = 121.14) was dissolved in 100 ml of distilled water. The pH of the buffer was then adjusted to 8 with concentrated HCl.

0.5 M Ethylene Diamine Tetra Acetic Acid (EDTA), pH 8: To prepare 100 ml of 0.5 M EDTA, 18.612 g of EDTA (Mol. Wt = 372.24) was dissolved in 100 ml of distilled water. During stirring, NaOH pellets were added to improve EDTA solubility and adjusted the buffer pH to 8.

5 M NaCl: To make 5 M NaCl of 250 ml, 73.05 g of NaCl (Mol. Wt = 58.44) was dissolved in 250 ml of distilled water.

50X Tris Acetate EDTA (TAE): To make a 50X TAE buffer of 500 ml, 121.14 g of Tris, 50 ml of 0.5 M EDTA, and 28.5 ml of glacial acetic acid were mixed and the volume was increased to 500 ml using distilled water.

TE buffer: In a 100 ml Duran bottle, 1 ml of 1 M Tris and 0.2 ml of 0.5 M EDTA were added, followed by 98.8 ml of distilled water to make the solution to 100 ml.

CTAB/NaCl: After dissolving 4.1 g of NaCl in 80 ml of distilled water, 10 g of CTAB was slowly added while heating (65 $^{\circ}$C) and stirring. Finally, using distilled water, the volume was adjusted to 100 ml.

10 mM Tris (pH 8.0): 1 M Tris, (pH 8.0) was diluted in nuclease-free water (1:100) *i.e.*, by adding 1 ml of 1 M Tris pH 8 to 99 ml of nuclease-free water.

All the prepared buffers were autoclaved and stored for future use.

10% SDS solution: 10 g of SDS was dissolved in 80 ml of distilled water. To dissolve SDS quickly, solution was heated upto 60 ºC using a water bath. As the contents fully dissolved, the volume of the solution was made up to 100 ml using distilled water and the prepared SDS solution was utilized for further use without autoclaving.

Lysozyme stock solution: 100 mg solid lysozyme was dissolved in 1 ml of 10 mM Tris (pH 8.0) by pipetting several times with a 1 ml pipette.

3.1.2 Protocol for Isolation of Bacterial DNA

Respective bacterial isolates grown in T_3 broth were used for DNA isolation.

1. The culture solution was shaken for few seconds and 1.5 ml of the bacterial suspension was pipetted out in appropriate centrifuge tube.
2. The sample was centrifuged at 10,000 rpm for 5 min and the supernatant was discarded.
3. The pellet was resuspended in lysozyme buffer of 590 µl volume, mixed well and incubated at 37 ºC for 30 min.

4. After incubation, 40 µl of 10 per cent SDS solution and 4 µl of proteinase K (20 mg/ml) was added in the solution and incubated at 56 ºC for 1-3 h.
5. Now 100 µl of 5 M NaCl and 100 µl of CTAB/NaCl were added, mixed well and incubated at 65 ºC for 10 min.
6. Later, 0.5 ml of chloroform: isoamyl alcohol solution (24:1) was added and mixed well in vortex.
7. This mixture was centrifuged at maximum speed for 10 min at room temperature.
8. The upper phase was carefully transferred into a fresh tube
9. Then, 0.5 ml of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added, mixed well and centrifuged at max speed for 10 min at room temperature.
10. Again, the supernatant was transferred into a fresh tube and mixed with 0.5 ml of chloroform: isoamyl alcohol (24:1) properly and centrifuged at max speed for 10 min at room temperature.
11. The upper aqueous phase containing thick material was transferred to a fresh tube and added with 0.6 ml volume of isopropanol and incubated at -20 ºC for 2h and centrifuged at max speed for 15 min at 4 ºC.
12. At this stage, the supernatant was discarded and 500 µl of 70 per cent alcohol was added and mixed properly.
13. The solution was spinned at max speed for 5 min.
14. Ethanol was decanted and the pellet was air dried at room temperature
15. The obtained pellet was dissolved in 20 µl of 1X TE buffer and checked in 1.4 per cent agarose gel.
16. The DNA concentration and purity was checked in Nanodrop system further for sufficient quantity.

3.1.3 Quantification of the genomic DNA

As it was crucial to know exact quantity of DNA present in the isolated sample volume before carrying out the experiment on further characterization or cloning of DNA. Concentration can be accurately measured by Nanodrop. After checking the concentration of the DNA, based on the intensity, DNA samples were diluted and stored at -20 ˚C for further PCR analysis.

3.1.4 PCR Amplification Using RAPD Primers

The composition of the reaction mixture (20 µl) used in PCR for DNA amplification of the respective bacterial isolate was as follows and the reaction mixture was standardized and samples were run in thermal cycler for amplification (HIMEDIA, Prima-96 ™). The RAPD primers utilized in the present study are represented in Table 3.1. and the PCR components and programme is given in the Table 3.2 and Table 3.3.

Table 3.1. Details of Random Primers used in RAPD- PCR

S. No	RAPD Primers	Primer Sequence	GC%	T_m value
1	OPA-02	5’ TGCCGAGCTG 3’	70	37 ºC
2	OPA-05	5’ AGGGGTCTTG 3’	60	37 ºC
3	OPA-07	5’ GAAACGGGTG 3’	60	37 ºC
4	OPA-09	5’ GGGTAACGCC 3’	70	37 ºC
5	OPA-10	5’ GTGATCGCAG 3’	60	37 ºC
6	OPA-12	5’ TCGGCGATAG 3’	60	37 ºC
7	OPA-18	5’ AGGTGACCGT 3’	60	37 ºC
8	OPC-19	5’ GTTGCCAGCC 3’	70	37 ºC
9	OPK-14	5’ CCCGCTACAC 3’	70	37 ºC
10	OPN-05	5’ ACTGAACGCC 3’	60	37 ºC

Table 3.2. Reaction mixture used for RAPD-PCR (20 µl)

S. No.	Components	Quantity (µl)
1	10X PCR buffer with $MgCl_2$ (Thermo Scientific)	3
2	10 mM dNTPs’ (Thermo Scientific)	2
3	Molecular grade water	7.3
4	5U *Taq* polymerase (Thermo Scientific)	0.5
5	Random primer	1.2
6.	Template DNA	6
	TOTAL	20

Table 3.3. RAPD-PCR Programme for amplification of bacterial DNA

S. No.	Steps in PCR	Temperature
1	Initial denaturation	94 ºC for 5 min
2	Denaturation	94 ºC for 1 min
3	Annealing	37 ºC for 1 min
4	Primer extension	72 ºC for 2 min
5	No. of cycles	45
6	Final extension	72 ºC for 8 min

The PCR product was checked for on 1.4 per cent agarose gel and visualized in gel documentation system (SYNGENE, Gene flash, U. K.).

3.1.5 Preparation of Gel and Loading of DNA Samples

Agarose gel solution was prepared by taking 1.4 g of agarose in 100 ml of 1X TAE buffer in a conical flask and heated in a micro wave until agarose dissolved completely and clear solution was obtained. 2.5-3 µl of ethidium bromide was added to luke warm solution and poured into casting tray after placing the Teflon comb. After solidification of gel the comb was gently removed and the gel cast was placed in horizontal electrophoresis unit.

PCR amplified product of 5 µl was mixed with loading dye (2.5 µl) (Novagen, U.S.A) and was loaded in the wells of 100 bp ladder was loaded in the first well along with the samples for knowing the size of test samples. The gel was run in horizontal electrophoresis unit (Genaxy, India) at 80 V and 60 mA for 1 h and visualized in gel documentation system (SYNGENE, Gene flash, U. K.).

3.1.6 Isolation of spore- crystal mixture from indigenous *Bt* isolates

To obtain the spore crystal mixture from each isolate, a single colony of *Bt* culture from T_3 agar plate was inoculated into the culture tubes containing 5 ml of T_3 broth and kept for overnight incubation in a shaking incubator (Orbitek, Scigenics) maintained at 30 ^{o}C and 200 rpm. From the overnight grown cultures, 1 per cent inoculum (250 µl) was added to the 250 ml conical flask containing 25 ml of T_3 broth in a shaking incubator maintained at 30 ^{o}C, 200 rpm for 48-60 h. The growth and lysis of the bacterial cells was checked using the phase contrast microscope after 48 h. After confirming at least 90% cells lysis, culture was centrifuged at 4 ^{o}C for 10 min at 10,000 rpm and resulting pellet was suspended in 25 ml of ice-cold Tris-EDTA buffer [Tris 10 mM, EDTA 1Mm, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)] and washed once with 25 ml of ice- cold 0.5 M NaCl and centrifuged for 10 min at 10,000 rpm, followed by 2 washes with 25 ml Tris-EDTA buffer with 0.5 mM PMSF at the same speed and time (Ramalakshmi and Udayasuriyan, 2010). Finally, the pellet was suspended in 500 µl of sterile distilled water containing 10 µl of 1 mM PMSF and stored at -20 ^{o}C as aliquots of 50 µl for later use.

3.1.7 SDS–PAGE Analysis

Protein profiling was done using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), with spore crystal mixtures from all the *Bt* samples with and without solubilization with NaOH buffer (Navya *et al*., 2022). SDS – PAGE was performed by following the standard protocol (Laemmli, 1970), using 10 per cent separating gel and 4 per cent stacking gel. Reagents required for SDS–PAGE analysis are given in annexure III.

The gel template plates were cleaned thoroughly with water and wiped with 70 per cent ethanol. The plates were assembled by placing appropriate spacers on both sides and sealed with 0.8 per cent agarose at both sides and bottom. For separating gel (10%), acrylamide mix was prepared according to the composition (Annexure III) and used. After pouring separating gel, ethanol was added on top to get smooth interface between the stacking and separating gel. After the polymerization, the alcohol layer was removed by using Whatman filter paper No. 1. Above the separating gel, stacking gel (4%) was poured, comb was placed and allowed to polymerize for 20-30 min. After polymerization, comb was carefully removed for loading the samples into the wells. The sample was prepared in the ratio of 4:11:15 (spore crystal mixture, distilled water and 1X loading dye) and kept in boiling water for 1 minute 45 seconds. The gel was initially run at current of 20 mA till the dye front reached the separating gel and the current supply was increased to 30 mA. After the completion of electrophoresis (dye front reaching the bottom of separating gel), gel unit was dismantled and gel was removed carefully for staining with 0.133 per cent Coomassie Brilliant Blue R250 for 1 hour. After staining, gel was rinsed with distilled water and destaining was done until the background become colorless and gel was documented. The molecular mass of the proteins was compared with protein ladder covering a wide range molecular weight from 10-315 kDa.

3.1.8 Statistical Analysis

The RAPD profile was visualized as bands in the gel documentation system. The bands were manually scored '1' for the presence and '0' for the absence of the band. Only clear and unambiguous bands were taken into consideration and the bands were not scored if they were faint or diffused, as such fragments possess poor reproducibility. The band sizes were determined by comparing with the 100bp DNA ladder. The binary data was analysed for the construction of similarity matrix and the dendrogram using NTSYSpc.2.02i software (Rohlf, 1998).

3.2 TO DEVELOP NANO-SCALE PARTICLES WITH NATIVE *Bt* ISOLATES.

3.2.1 Synthesis of Nanoparticles

The nanoparticles selected in this investigation *viz*,. oxides of Ca, Mg and Zn were prepared by using sol-gel method.

3.2.1.1 Synthesis of CaO NP's:

Nanoscale CaO particles were prepared using sol-gel method. In this method, 1per cent of calcium nitrate (tetrahydrate purified) was mixed with 0.05 per cent of sodium citrate tri-basic dehydrate (extra pure) and stirred at 60 ^{o}C for 3 hours. Then, the solution was filtered using filter paper (Whatman No.1) and calcined at 1000 °C for 6 h.

3.2.1.2 Synthesis of MgO NP's:

Magnesium oxide NP's were prepared by sol-gel method using magnesium nitrate and sodium hydroxide at room temperature. Then, the solution was filtered using filter paper (Whatman No.1) and hydrated Magnesium Oxide NP's were dried at 500 °C.

3.2.1.3 Synthesis of ZnO nanoparticles:

Oxalate decomposition technique was used to prepare ZnO nanoparticulates. Equimolar (0.2M) solutions of zinc acetate and oxalic acid mixed to prepare zinc oxalate. The precipitate so formed as a result of mixing zinc acetate and oxalic acid was collected and thoroughly rinsed with double deionized water (DI-water) and allowed to dry in the air at room temperature. Then the oxalate made into fine powder form and decomposed in the air by keeping it in a pre-heated muffle furnace for 45 min at 500 °C (Prasad *et al*., 2012).

3.2.2 Preparation of Nanoparticulate Solutions:

Oxide NP's of Ca, Mg and Zn at 20 ppm concentration of nanoparticulate solutions were prepared by adding the respective volumes of distilled water.

The prepared nanoparticulate solutions of oxides of Zn, Ca and Mg at 20 ppm in 1:9 ratio (1ml of nanoparticulate solution to 9ml of T_3 media) was added to the T_3 media before sterilization to study the catalytic activity of nanomaterials on *B. thuringiensis*.

3.2.3 Inoculation of *Bt* to the Nanomaterial based Media:

From *Bt* strains one loopful of bacteria was inoculated into 1 ml of T_3 broth and incubated at 25 °C over night. The culture was mixed into 9 ml of sterile water, from this 1ml was taken out and mixed into 10 ml of sterile distilled water, like wise 10^{-12} serial dilutions were made. From this 0.1 ml was taken out by using micro pipette and dropped into petri plate and spread with L rod and wrapped with polythene film under Laminar Air Flow chamber. These plates were kept for incubation at 28±1 °C for 24 h.

3.3 To evaluate *Bt* isolates on pod borer, *H. armigera* (Hub.) in laboratory.

3.3.1 Mass Rearing of *H. armigera*

Mass rearing of *H. armigera* was carried out in the Department of Entomology, Bapatla at 25±1°C, 70±5.0 per cent relative humidity (RH) and light : dark as 16:8 h.

3.3.1.1 Rearing of *H. armigera*

The initial nucleus culture of the test insect was obtained from ICRISAT during the egg stage and emerged neonate larvae of pod borer were reared on the artificial diet (Sharma *et al*., 2014) in the laboratory conditions at room temperature (27± 2 ºC) with 70 - 80 per cent RH.

The test insect was reared in multicavity trays which were cleaned with a glass cleaning solution (conc./diluted potassium dichromate + conc. sulphuric acid). Subsequently, the glassware was sterilized by swabbing with two per cent formaldehyde and by exposing to UV-light chamber for two or three hours before use.

The egg masses collected from the ICRISAT were initially surface sterilized with 0.2 per cent sodium hypochlorite and kept for hatching in incubator. The newly emerged neonate larvae were maintained on artificial diet. Every two days, the larvae were given fresh diet and used trays were cleaned, sterilized regularly so as to maintain absolute hygiene. As the larvae grew larger *i.e.*, from the third instar onwards, the larva were maintained individually in the multicavity trays, to avoid cannibalism. The diet was changed daily as the larva grew larger (Plate 3.1).

Two to three days after pupation, the pupae were taken out and kept for adult emergence in oviposition cage, which was lined inside with black paper. A cotton swab dipped in 10 per cent honey solution fortified with vitamin-E was made to hang in the cage to feed the newly emerged adults. The egg masses collected daily were surface sterilized. After hatching, the larvae were reared to different instars for further laboratory studies. Intermittently, larvae collected from the field were also added to the culture to maintain the vigour.

3.3.1.2 Preparation of Artificial diet

Artificial diet was prepared as suggested by Sharma *et al*., (2014). The composition of artificial diet for 400 ml is given in Table 3.4. Equipment's required for artificial diet preparation were cleaned and sterilized with one per cent sodium hypochlorite and later washed in distilled water, including containers and plastic Jars.

To prepare 400ml of artificial diet the composition was divided into three fractions:

Fraction A: All the powdered ingredients from Fraction A (Table 3.4) were mixed in a clean container. Distilled water was boiled and cooled to 60 ^{0}C, and then mixed with the pre-mixed ingredients using a blender for one min.

Fraction B: Agar powder was weighed in a separate container and then cold distilled water was added in a separate pan. It was boiled and cooled to 60 °C and all the ingredients of fraction B were added to fraction A and then blended for three min.

Fraction C: Add 10 per cent formalin, streptomycin sulphate, multivitamin capsules and a capsule of 400 mg vitamin E to mixture of fraction A and B.

Table 3.4. Composition of Chickpea flour based ICRISAT diet for mass rearing of *H. armigera*

S. No.	Ingredients	Composition
Fraction A		
1	Chickpea flour	100 g
2	Sorbic acid	1.0 g
3	Ascorbic acid	1.67 g
4	Methyl-p- hydroxybenzoate	1.5 g
5	Yeast	16.0 g
6	Distilled water	200ml
Fraction B		
7	Agar	5.8 g
8	Distilled water	200ml
Fraction C		
9	Formaldehyde 10%	10 ml
10	*Streptomycin sulphate	0.2 g
11	Multivitamin capsules	1
12	Vitamin E capsules	2

*Streptomycin Sulphate was included in the ICRISAT diet in order to avoid contamination

Then the prepared diet was poured into the trays, air dried and sterilized in laminar air flow for 10 min. Certain diet ingredients such as chickpea flour was autoclaved before use to prevent bacterial spoilage. Finally, the trays were stored at 4 to 8 °C for a week.

3.3.2 Bioassay with Native *B. thuringiensis* Isolates and their Nano-formulations against Third Instar larvae of *H. armigera*

3.3.2.1 Maintenance of *Bt* Cultures

Six lepidopteran specific native *B. thuringiensis* isolates (*Bt*- 16, 49, 51, 52, 55 and 493) along with a reference strain HD1 that were selected from the repository of insect pathology lab at Agricultural College, Bapatla were used in the present study. For maintaining *Bt* cultures, T_3 medium was used. The glassware and the T_3 medium used for sub culturing of *B. thuringiensis* cultures were sterilized in an autoclave at 121 ^{0}C at 15 psi for 15 min. Later the molten agar medium was poured in sterile Petri plates under aseptic conditions in the laminar airflow chamber that was initially sterilized through ultra-violet radiation for about 20 min and cleaned with ethanol before the inoculation work. The *Bt* isolates were streaked on to the media plates and were kept for incubation for 24 hours for the growth of *Bt* colonies. Simultaneously a reference strain, HD1 was also inoculated and maintained on the same selective medium.

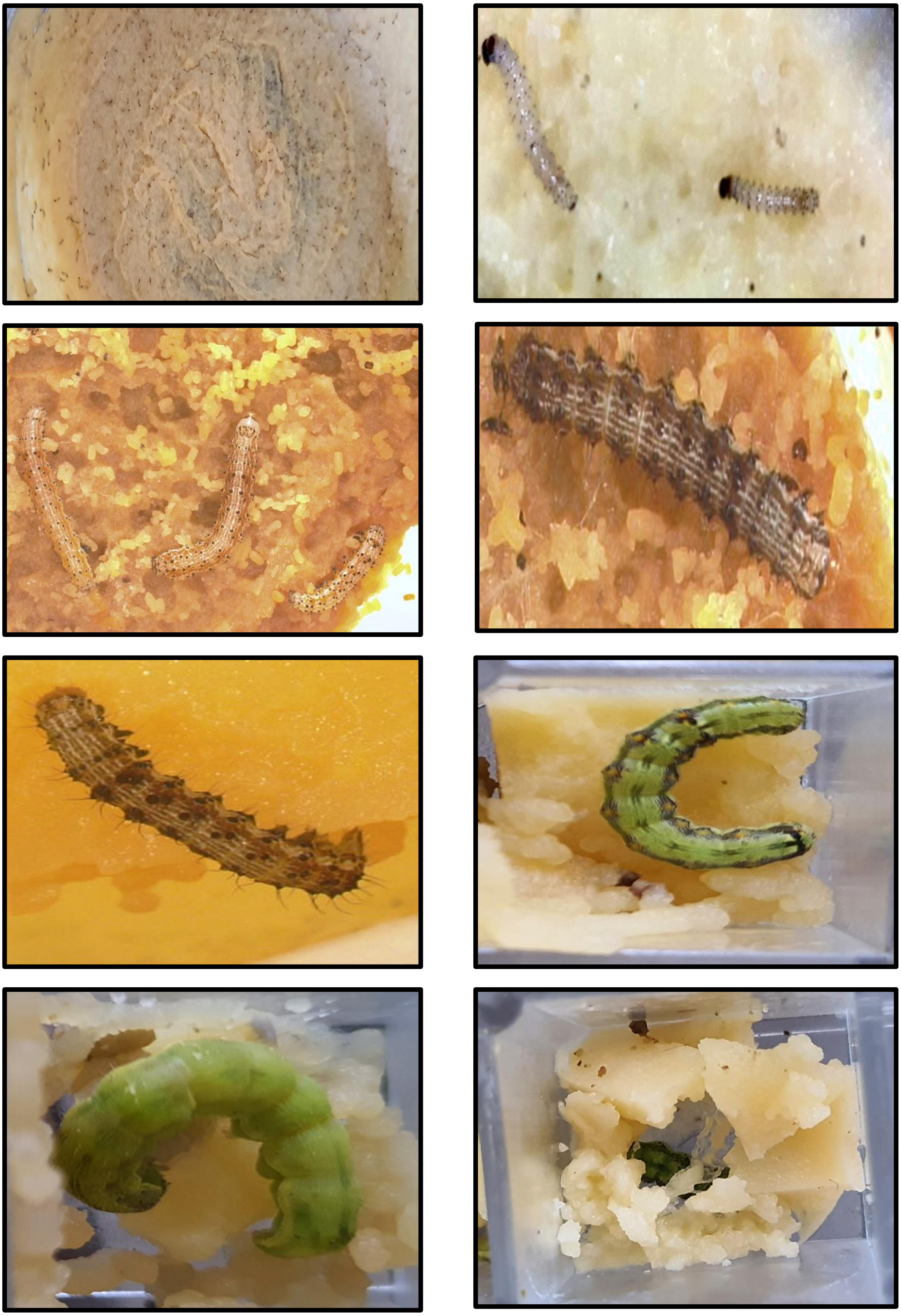


Plate 3.1. Different instars of *H. armigera* larvae on ICRISAT based semi- synthetic diet

3.3.2.2 Preparation of Different Concentrations of *B. thuringiensis* Isolates

Different concentrations of each isolate of native and nano formulated *B. thuringiensis* were prepared at 10^{-1} to 10^{-12} concentrations by serial dilution technique and from each dilution, 100 µl aliquot was spread on T_3 medium petri plates with "L" rod and incubated at 37 ºC for 24 h. After 24 h, the colony count was taken based on the following standard formula.

$$\text{Number of colonies per milliliter of sample} = \frac{\text{Number of colonies}}{\text{The aliquot taken x Dilution factor}}$$

After colony counting, required concentrations (Table 3.5.) were prepared by using the following formula

$$N_1V_1 = N_2V_2$$

Where,

V_1 was volume of the broth

N_1 was known concentration of broth

V_2 required volume

N_2 was required concentration.

Table 3.5. Concentrations of native and nano-formulated *Bt* isolates used for bioassay treatments on *H. armigera*

Treatments	**Concentration (CFU ml^{-1})**
T_1	1×10^{8}
T_2	1×10^{9}
T_3	1×10^{10}
T_4	1×10^{11}
T_5	1×10^{12}
T_6	Tween 80 (0.1%)
T_7	Control

3.3.2.3 Diet Incorporation Bioassay Method:

To confirm the insecticidal activity of native isolates and nano formulations of *B. thuringiensis,* a diet incorporation bioassay experiment was carried out along with reference strain *B. thuringiensis* sub sp. *kurstaki* (HD1) (Plate 3.2).

Developmental responses of *H. armigera* larvae were measured by performing bioassays on artificial diet into which *Bt* toxin had been incorporated. The diet was then poured into 50-well plates with untreated diet used as the control. All the *Bt* cultures at the concentrations mentioned in the Table 3.5 were used in the bioassay. The diet was poured as a thin layer into 50 celled multi cavity trays, with approximately 4 ml per well with a surface area of 3.14 cm^2 . One pre-starved (4 h) third instar larva was released in each well. A total of 60 larvae were used for each concentration @ 20 larvae per replication. These trays were kept in a rearing chamber at 25±1 °C, 70±5.0 per cent relative humidity (RH) and with light: dark as 16:8 hours. The observation on mortality were recorded at 24, 48, 72, 96 and 120 h after treatment (Vimala Devi and Vineela, 2015). In addition, an untreated check was also maintained in order to get corrected mortality. The per cent mortality was corrected as per Abbott's formula (1925) using the standard formula (Chandrasekaran *et al*., 2015).

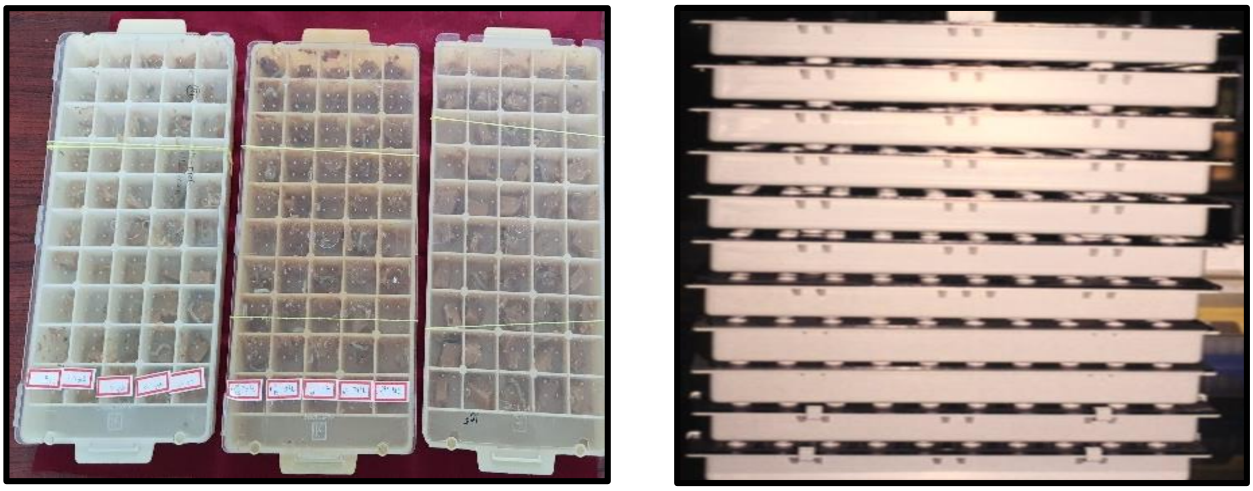
$$\text{Corrected larval mortality }(\%) = \frac{(\%\text{Mortality in treatment} - \%\text{Mortality in control})}{(100 - \%\text{Mortality in control})} \times 100$$

3.3.3 Statistical Analysis

The data generated from the laboratory bioassay experiments were subjected to statistical analysis by Completely Randomized Design (CRD). After suitable transformation, data was subjected to analysis of variance (ANOVA) and means were compared and subjected to probit analysis for calculation of LD_{50} and LT_{50} (Finney, 1971) using Statistical Package for Social Sciences (SPSS) ver.16.0 software.

a. Broth inoculated with *Bt*

b. Serial dilutions of *Bt*



C. Larvae kept for bioassay

Plate 3.2. Laboratory based *Bt* bioassay setup on *H. armigera*

Chapter – IV

Results and Discussion

Chapter IV

RESULTS AND DISCUSSION

The present investigation on '**Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)'** was conducted during 2021-22 at the Agricultural College, Bapatla. The isolates utilised in this research were collected from the repository of Insect pathology laboratory of Agricultural college, Bapatla. The results pertaining to the present studies are presented in this chapter.

4.1 MOLECULAR CHARACTERISATION OF NATIVE *Bt* ISOLATES WITH DNA FINGER PRINTING BY RAPD-PCR

4.1.1 RAPD DNA Fingerprinting of Native *Bt* Isolates

Under this study, seven *Bt* isolates including the reference strain HD1 (Plate 4.1) were taken and DNA was extracted and amplified using RAPD- PCR with 10 different decamer primers (Plate 4.2 to Plate 4.11). The purity and concentrations of DNA isolated is given in the Table 4.1.

Among the ten primers studied, seven primers namely OPA-02, OPA-05, OPA-07, OPA-09, OPA-10, OPK-14 and OPC-19 showed 100 per cent polymorphism. Mathimaran (2001) also studied RAPD characterization to know the genetic variability in *Glomus mossae* isolated from various agro climatic regions of Karnataka and observed 58.33% polymorphism among the isolates.

In the RAPD analysis a total of 123 amplicons were produced in all the primers. Number of amplicons produced by each primer ranged from 8(OPA-05) to 17(OPA-18) with a mean value of 12.3. Number of polymorphic amplicons obtained by each primer ranged from 8 (OPA-05) to 16 (OPA-18) with a mean value of 11.9. The number of monomorphic bands produced by each primer ranged from 0 in case of OPA-02, OPA-05, OPA-07, OPA-09, OPA-10, OPC-19, OPK-14 to 2 in OPA-12 and OPN-05. The per cent polymorphism ranged from 83.33 (OPN-05) to 100 (OPA-02, OPA-05, OPA- 07, OPA-09, OPA-10, OPC-19 and OPK-14) with a mean per cent of 96.32 (Table 4.2).

Similarly, Dharmender *et al* (2008) isolated 70 *Bt* isolates from various soil samples collected from different cotton fields. These *Bt* isolates were characterized by 7 RAPD markers to understand the genetic variation among the isolates depending on the source of origin. A total of 1935 amplicons were generated from 7 markers. Out of total amplicons obtained, 1865 were found to be polymorphic and 68 fragments were found to be monomorphic. Primers OPA- 03, OPA- 08, OPD- 14, OPD-19, OPD-20, OPE- 17 and OPE- 19 generated 100% polymorphic bands. The dendrogram constructed from RAPD data revealed that 70 isolates fell into two different clusters showing genetic divergence among the isolates

Similar line of research was also carried out by Adesetan *et al* (2020), wherein *B. cereus* isolates were conducted to genotypic profiling by RAPD-PCR using the primer OPR- 13 and constructed the dendrogram using banding pattern. From the findings, it was depicted that the isolates were related closely with a similarity index of 70 per cent.

4.1.2 Genetic Similarity Matrix and Cluster Analysis of Seven *Bacillus thuringeinsis* Isolates

A high level of genetic diversity was revealed when cluster analysis was carried out with *Bacillus thuringeinsis* isolates based on similarity matrix (Table 4.3). A dendrogram constructed based on the cluster analysis revealed that the seven isolates clustered at a linkage coefficient of 0.60 on the dendrogram with isolate-16 and isolate-55 spanning the extremes. It was observed from the dendrogram that the seven isolates fell into two major clusters I and II (Fig.4.1). Of the two major clusters observed, 4 isolates formed as cluster I and the remaining 3 isolates formed the cluster II. Further, the cluster I divided into two groups cluster Ia comprising of 3 isolates (Isolates 16, 52 and 493) and cluster Ib with single isolate *i.e.,* Isolate 51. The major cluster II was again divided into 2 subclusters IIa comprising of two isolates (Isolates 49 and HD1) and IIb with only one isolate *i.e.,* Isolate 55.

Jaccard's similarity index of *Bt* isolates was studied and represented in Table 4.3. The highest similarity index obtained was 0.71 and the lowest similarity obtained was 0.50. The highest genomic similarity was present between isolate-49 and HD1 strain of *Bacillus thuringeinsis* and the minimum similarity was obtained between isolate- 51 and HD1.

Remaining isolates were found to be present between these two ranges of maximum and minimum values.

Table 4.1. Details on purity and concentrations of Isolated DNA from the *Bt* isolates

Sl. No	Isolate	Purity (260/280nm)	Concentration (mg/ml)
1	I-16	1.942	320.77
2	I-49	1.807	128.11
3	I-51	1.886	110.27
4	I-52	1.827	133.61
5	I-55	1.850	152.61
6	I-493	1.775	114.46
7	HD1	1.827	121.22

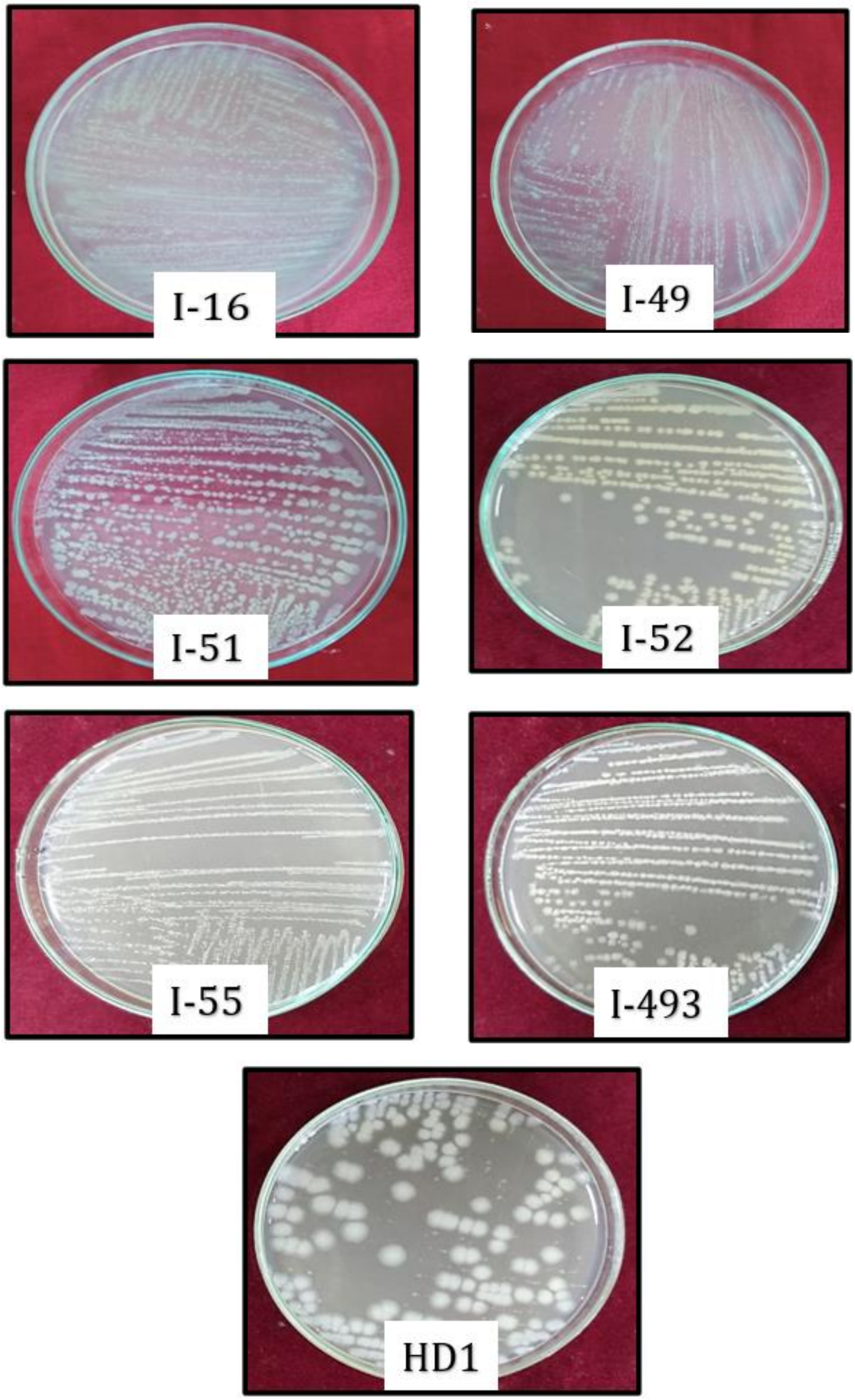


Plate 4.1. **Purified colonies of *Bacillus thuringeinsis* maintained on T_3 selective medium**

Table 4.2. Genetic variation showing polymorphism among *B. thuringeinsis* isolates

Sr. No	Primer	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	polymorphism %	PIC
1	OPA- 02	9	9	0	100	0.39
2	OPA- 05	8	8	0	100	0.42
3	OPA- 07	11	11	0	100	0.47
4	OPA- 09	11	11	0	100	0.39
5	OPA- 10	15	15	0	100	0.46
6	OPA- 12	14	12	2	85.71	0.48
7	OPA- 18	17	16	1	94.12	0.42
8	OPC- 19	14	14	0	100	0.47
9	OPK- 14	12	12	0	100	0.42
10	OPN- 05	12	10	2	83.33	0.45
Total		**123**	**119**	**5**	**-**	**4.37**
Mean		**12.3**	**11.9**	**0.5**	**96.32**	**0.44**
Range		**8-17**	**8-16**	**0-2**	**83.3-100**	**0.39-0.48**

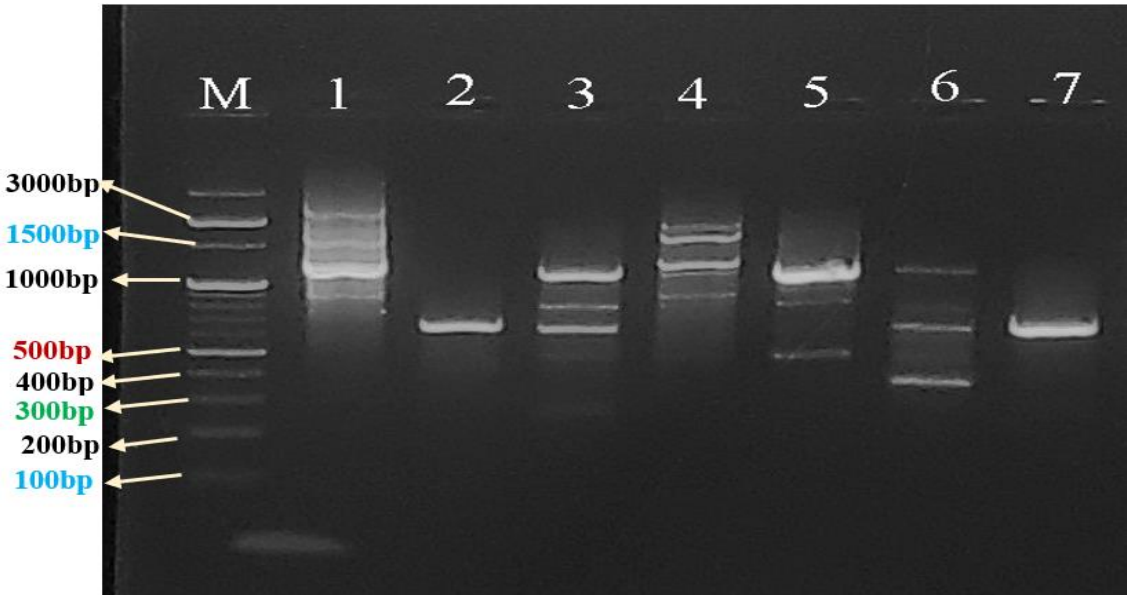


Plate 4.2. **DNA amplification of native *Bt* isolates using OPA- 02 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

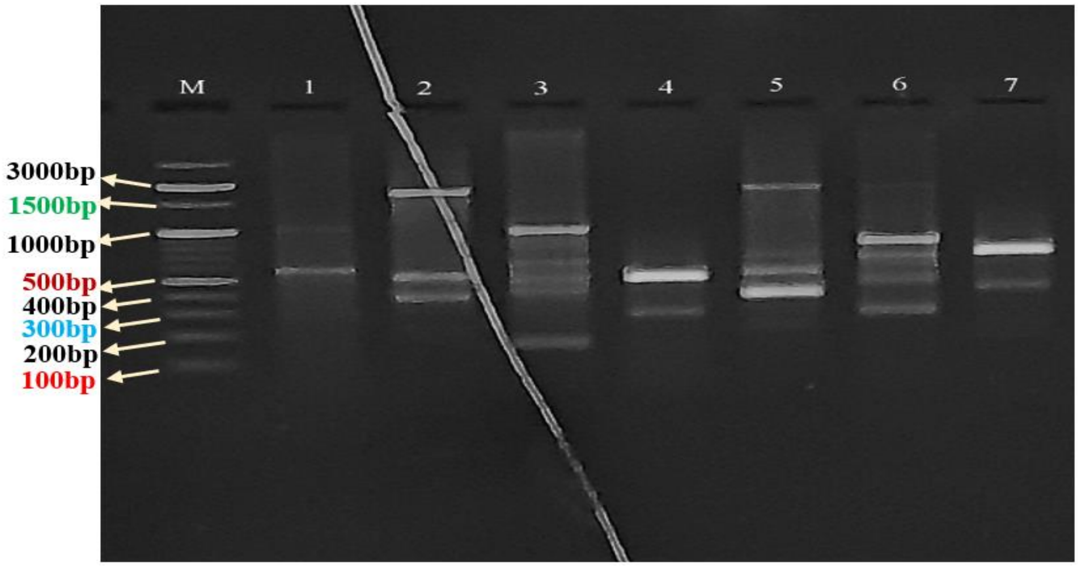


Plate 4.3. **DNA amplification of native Bt isolates using OPA- 05 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

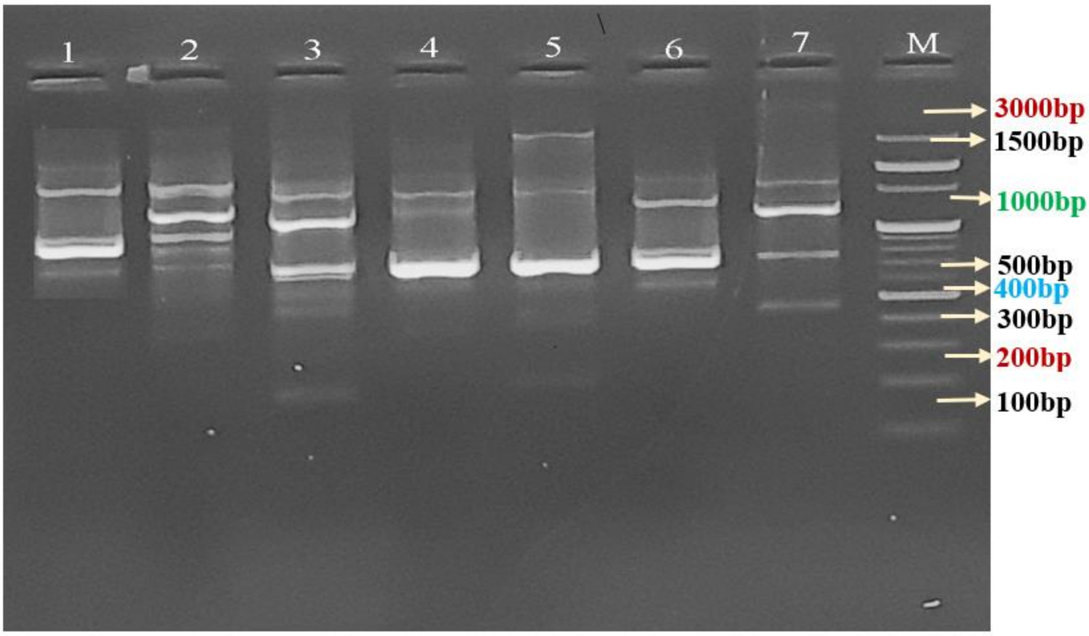


Plate 4.4. **DNA amplification of native Bt isolates using OPA- 07 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

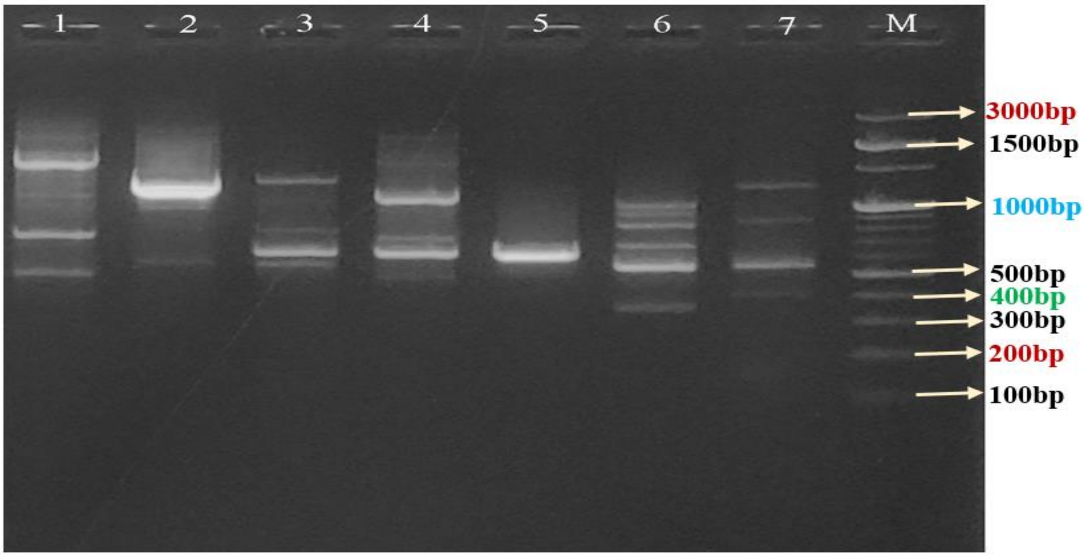


Plate 4.5. **DNA amplification of native Bt isolates using OPA- 09 random primer in agarose gel electrophoresis (1.4%)(Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

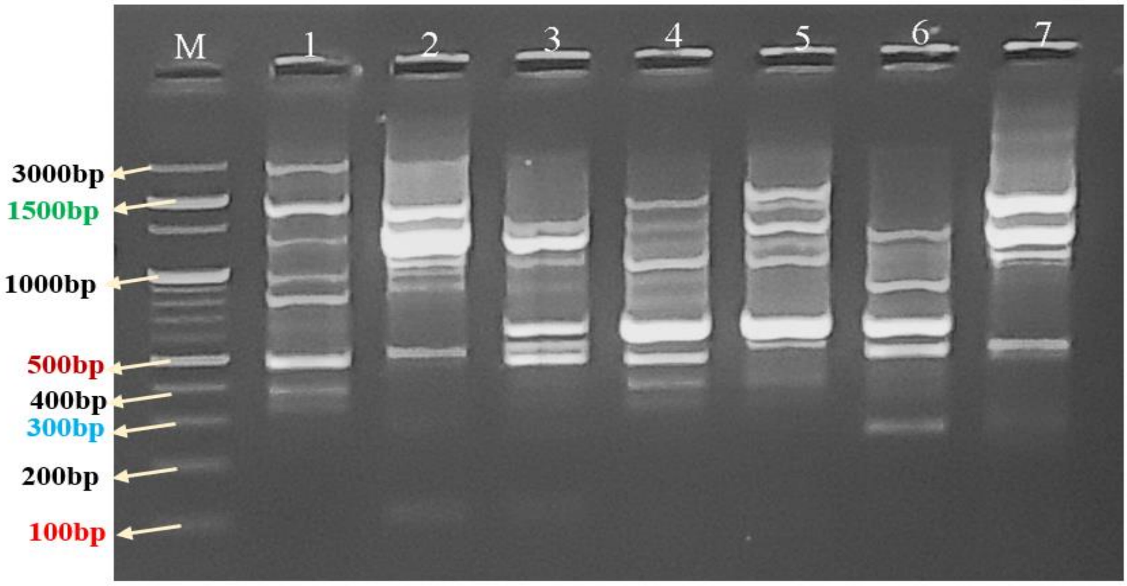


Plate 4.6. **DNA amplification of native Bt isolates using OPA- 10 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

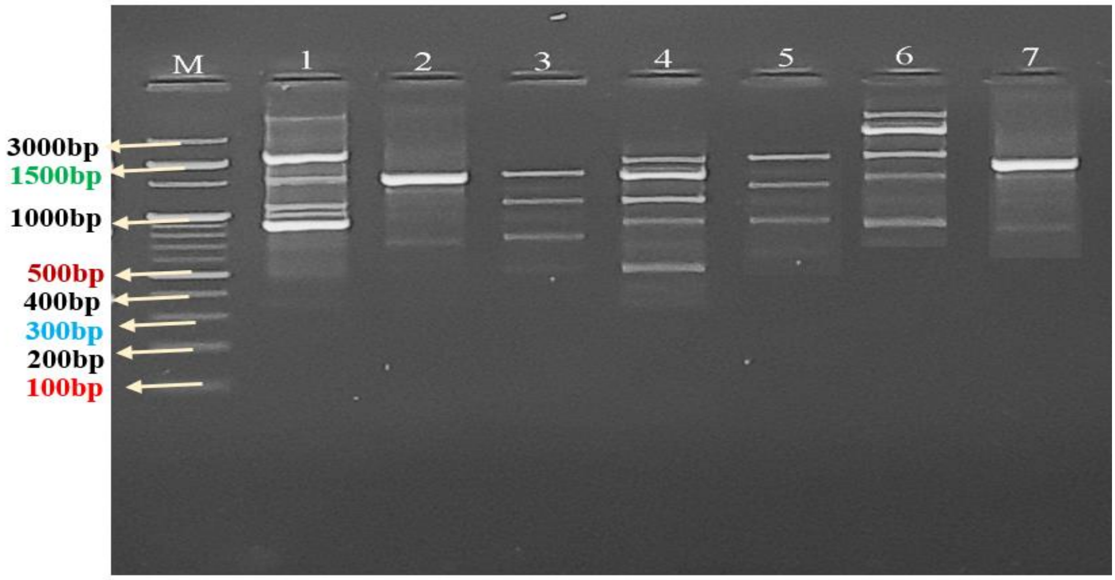


Plate 4.7. **DNA amplification of native Bt isolates using OPA- 12 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

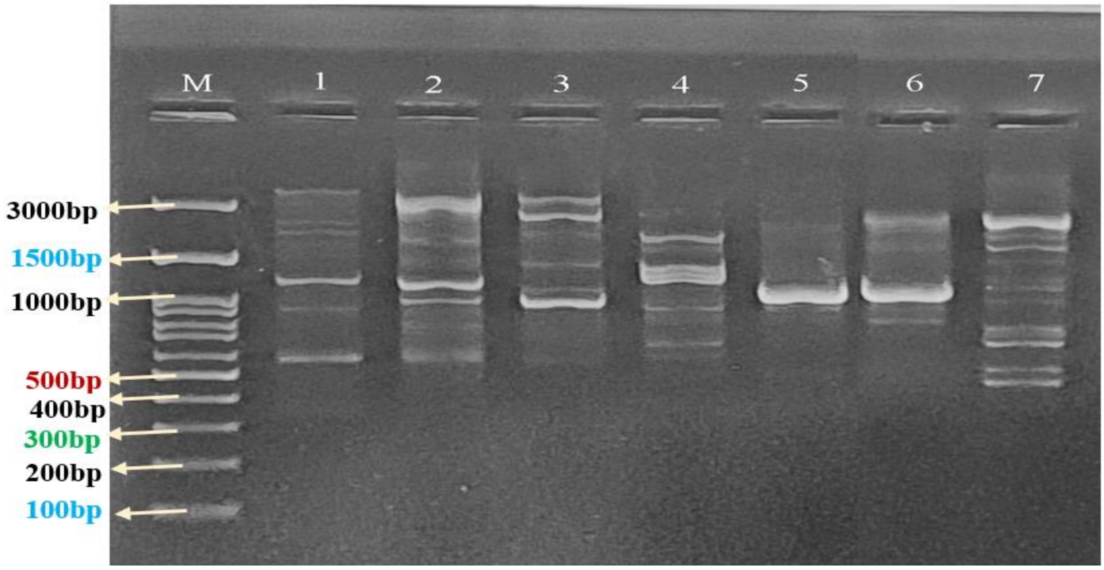


Plate 4.8. **DNA amplification of native *Bt* isolates using OPA- 18 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

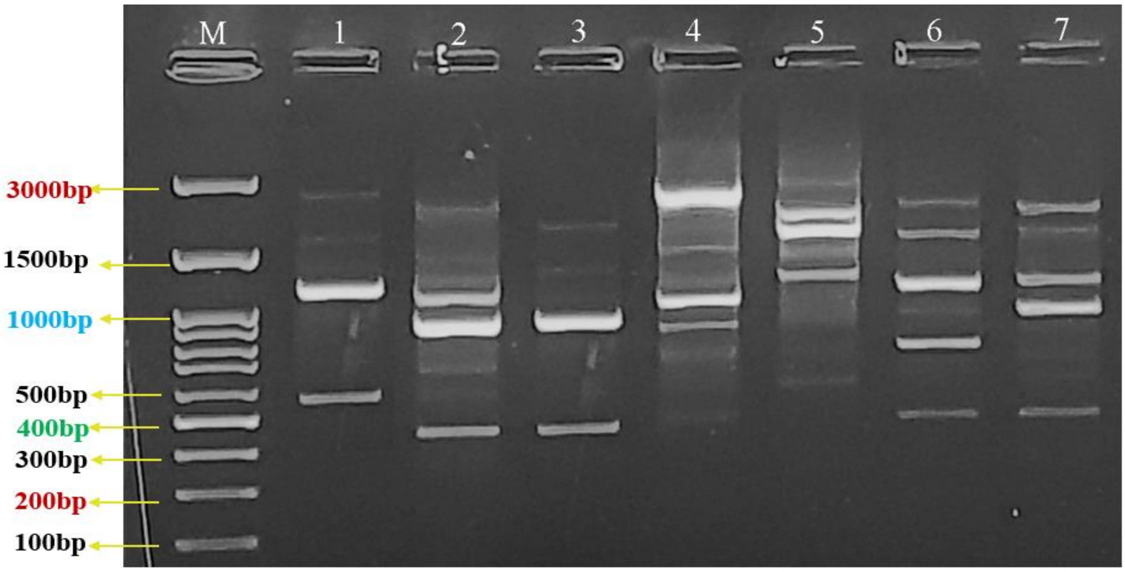


Plate 4.9. **DNA amplification of native *Bt* isolates using OPC- 19 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

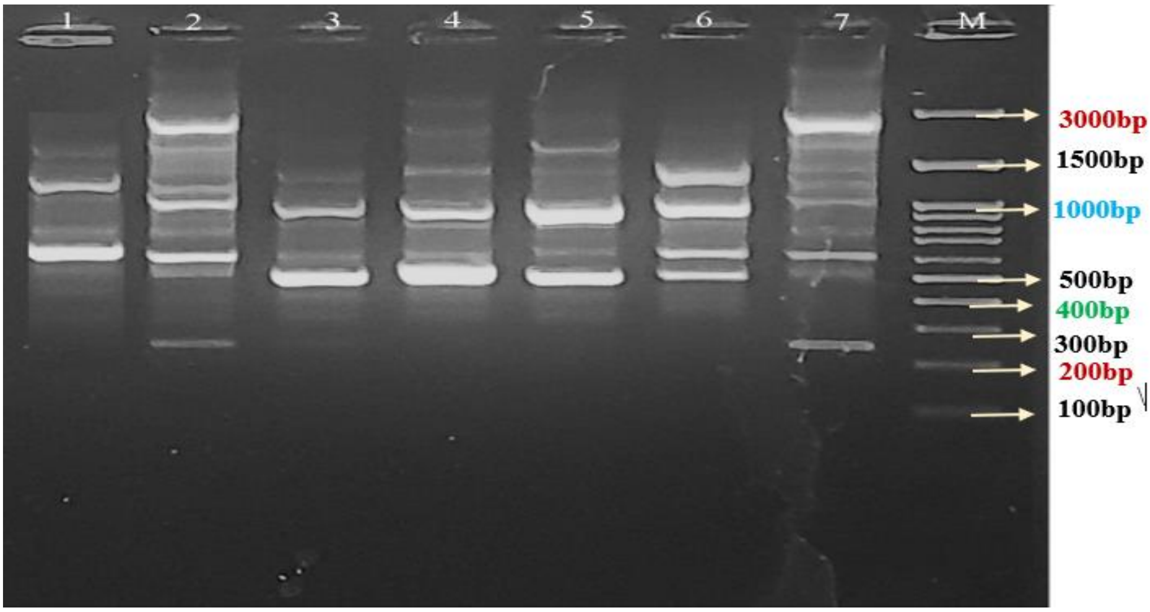


Plate 4.10. **DNA amplification of native *Bt* isolates using OPK- 14 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

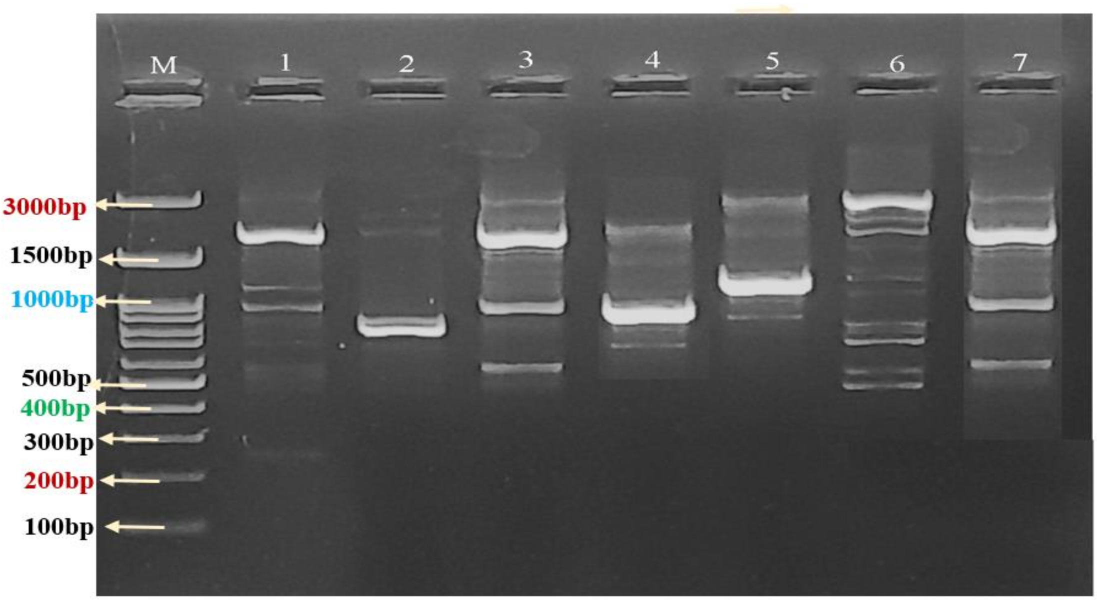


Plate 4.11. **DNA amplification of native *Bt* isolates using OPN-05 random primer in agarose gel electrophoresis (1.4%) (Lanes (1-7) left to right: Marker (M) 100 bp ladder, I-16, I-49, I-51, I-52, I-55, I-493, HD1)**

Table 4.3. Genetic similarity matrix of seven *Bacillus thuringeinsis* isolates

	I16	**I49**	**I51**	**I52**	**I55**	**I493**	**HD1**
I16	1.00						
I49	0.56	1.00					
I51	0.65	0.60	1.00				
I52	0.69	0.62	0.64	1.00			
I55	0.62	0.66	0.57	0.67	1.00		
I493	0.63	0.53	0.58	0.69	0.57	1.00	
HD1	0.62	0.71	0.50	0.65	0.63	0.66	1.00

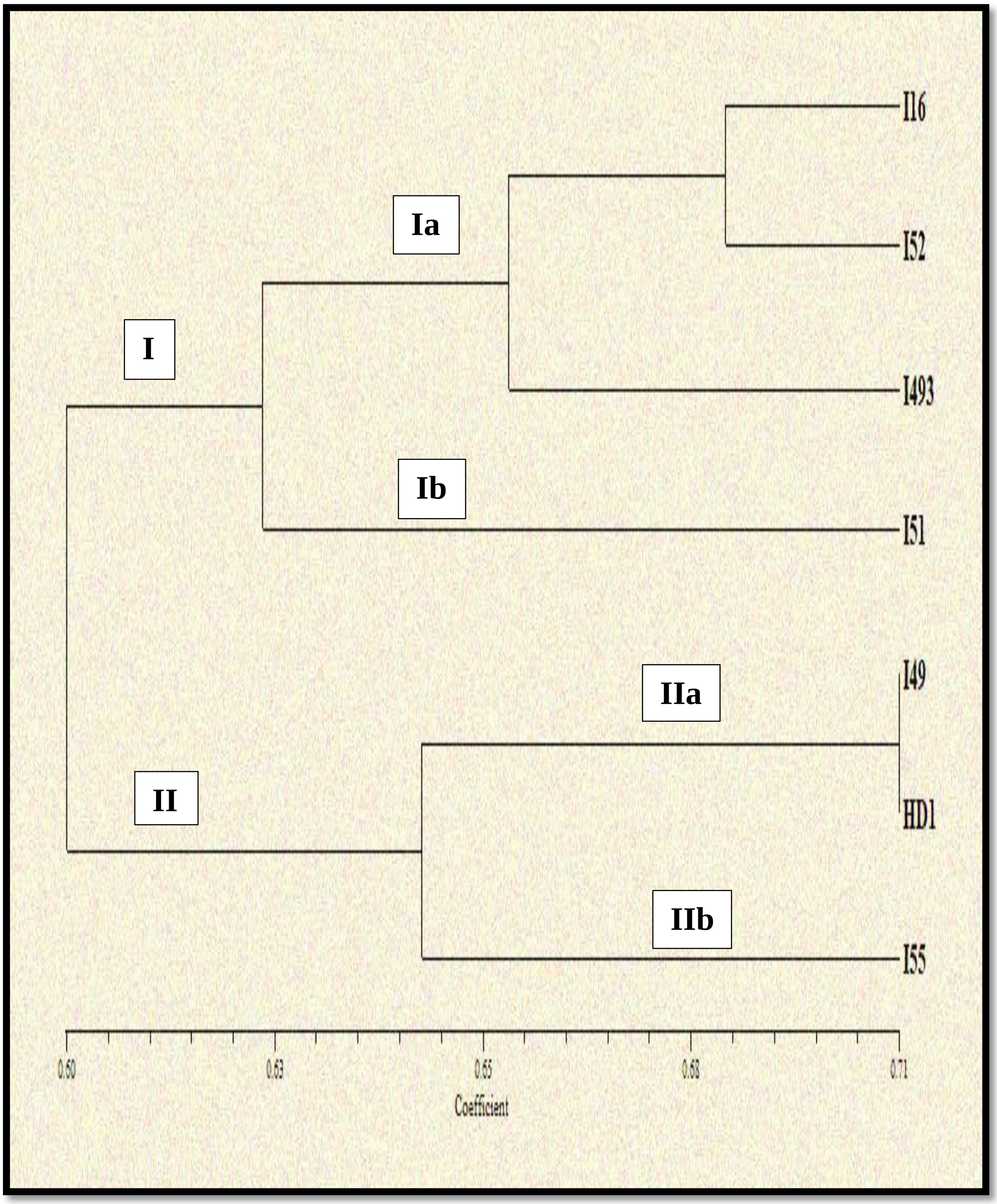


Fig. 4.1. Dendrogram constructed based on Jaccard's similarity coefficient of *Bacillus thuringeinsis* isolates based on RAPD amplification profile

Kundan kumar (2010) conducted a study on *Bt* isolates extracted from the soil samples. The genetic divergence among the strains was studied using RAPD decamer primers. The binary data obtained from the banding pattern was analysed for construction of similarity matrix and cluster analysis using NYSYS software. Close similarity among the strains was observed between the strains *Bt* 18 and *Bt* 19. The *Bt* strains were observed to fall into two major groups using cluster analysis.

4.1.3 Principal Coordinate Analysis (PCoA)

PCoA was performed, since it being the most explorative method that reduces complexity of multivariate data and also to know the genetic diversity among the native *Bt* isolates (Fig. 4.2). Two principal coordinates were included, where, all the isolates were found to be within the eclipse. Isolate 16 and 52 were found closer to the coordinate 1 (X-axis) showing close genetic relationship between the isolates, whereas, isolate 493 was found closer to coordinate 2 (Y-axis). While, HD1 and isolate 49 were far away from coordinate 2 showing close genetic relationship with each other and high divergence with the other isolates.

These results were in agreement with Petkar *et al*. (2019) who performed PCoA for 99 *Fusarium oxysporum* isolates from 4 different regions of SE United States and were named as GA1, GA2, GA3 and FL. It was reported that the GA isolates belonged to three regions clustered together as they were similar to each other, whereas the population from Florida (FL) fell into separate cluster indicating that it was more diverse to the remaining isolates.

4.1.4 Characterisation of Crystal Proteins of native *Bt* Isolates

Protein profiling of *Bacillus thuringeinsis* was done with SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) using spore crystal mixture that is prepared from all the seven isolates including the reference strain HD1. SDS-PAGE results revealed several banding patterns among the isolates (Plate 4.12). Proteins of various molecular weights ranging from 40 kDa to 140 kDa in size was observed among the isolates. Among the isolates, isolate 16 and HD1 were found to produce band at ~130 kDa, revealing the presence of Cry1 protein in the isolates. Isolates 49 and 493 were found to produce band at ~91 kDa indicating the presence of Vip3 protein. Isolate 51, 52 and 55 was observed to produce band at a range of 65-70 kDa that shows the presence of Cry2 protein.

Similar work was done by Mukhija and Khanna (2018), who had analysed the molecular weight of Cry proteins with spore-crystal mixture using SDS-PAGE by comparing with the known protein marker. The results obtained existence of various banding patterns ranging from 10 kDa to 115 kDa.

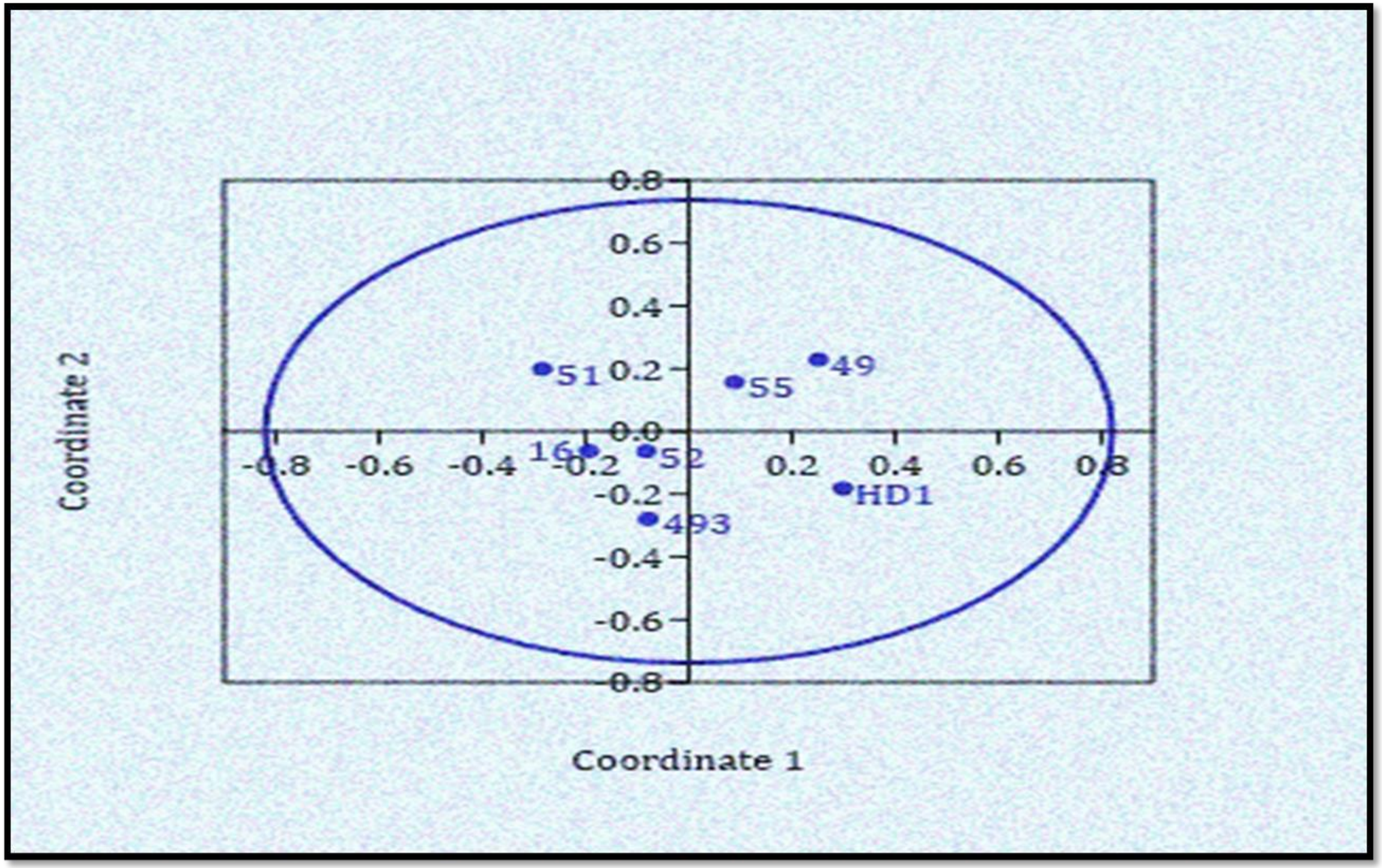
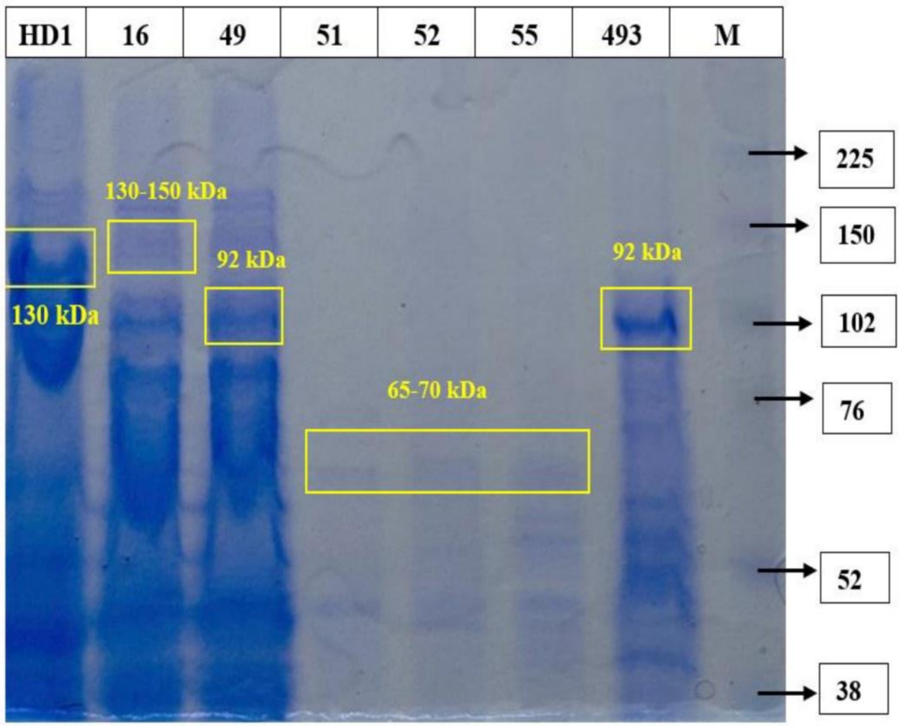
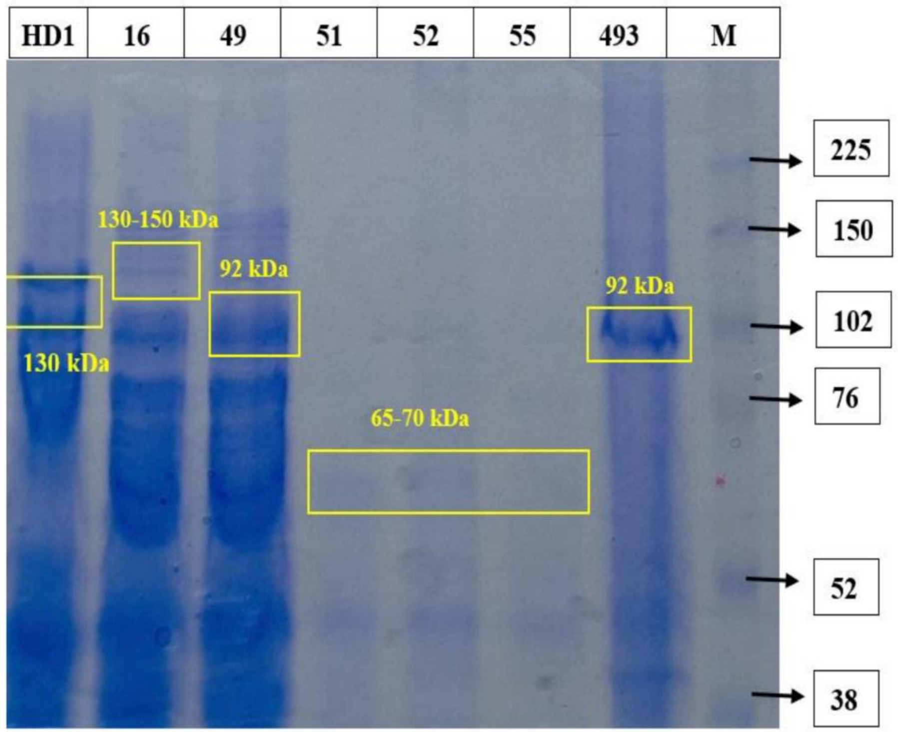


Fig. 4.2. Principle Coordinate Analysis (PCoA) of native *Bt* isolates based on RAPD amplification profile



a. Without alkali solubilization



b. With alkali solubilization

Plate 4.12. Crystal protein profiling of *Bacillus thuringiensis* isolates

4.2 TO DEVELOP NANO-SCALE PARTICLES WITH NATIVE *Bt* ISOLATES

The aim of the present study was to evaluate the influence of *Bt* isolates grown on the nano mineral enriched media against *H.armigera*.

4.2.1. Evaluation of *Bt* grown on Nano mineral enriched Media against *H. armigera*

The synthesis process of nanoscale mineral particles (NP's) proposed in the current study was given in chapter-3 (Material and methods).

NP's of CaO, MgO and ZnO at 20 ppm were incorporated into the T_3 media. *Bt* maintained on nano enriched mineral media was tested against 3^{rd} instar larvae of *H. armigera*.

It was observed that the mortality of *H. armigera* occured from 48h after treatment for the isolates 16, 493 and HD1, whereas, for the other isolates, the mortalities were observed from 72h after treatment, and the mortalities were found to be highest after 72h after treatment in almost all the isolates treated with CaO, MgO and ZnO NP's (Table 4.4, 4.7 and 4.10). Similar outcome was obtained by Vineela *et al*. (2017), who conducted bioassay to know the bioefficacy of *Bt* formulated through particle size reduction on *S. litura*, where the mortalities were observed from 48h after treatment with the isolate.

The mortalities of *H. armigera* was recorded from 48 hrs after infection and range from 25 to 96.67% with *Bt* maintained on CaO enriched media (Table 4.4), while it was in the range of 23.33 to 95% in MgO based isolates (Table 4.7) and in the ZnO based isolates, mortalities were in the range of 21.67 to 93.33% (Table 4.10). The results were in agreement with Gayathri *et al*., (2018) who conducted bioassays with *Bt* isolate grown on nano enriched mineral media incorporated with CaO, FeO, Mgo and ZnO nanoparticulates. The mortalities were observed to be 96.67% with CaO based media, 96.66 per cent with MgO and 86.67 per cent with ZnO based media at 20, 50 and 10ppm, respectively.

The highest mortality was observed in the reference strain HD1 for all the three NP's. Among the three NP's, CaO based *Bt*- HD1 was found to be highly effective against *H. armigera* and was found to record maximum mortality of 96.67 per cent, followed by MgO based *Bt*- HD1 of 95 per cent and ZnO based *Bt*- HD1 was found to record mortality of 93.33 per cent.

Among the native isolates, Isolate- 493 was found to be the best one, where the mortalities observed were in the same trend as observed in the reference strain, HD1 *i.e.*, CaO based *Bt*- 493 was found to record maximum mortality of 91.67 per cent , followed by MgO and ZnO based *Bt*- 493 of and 90.00 and 88.33 per cent respectively, at the concentration of 1× 10^{12} CFU ml^{-1}. The next best isolate was found to be isolate- 16. Among the NP's it was observed that MgO based *Bt*- 16 was found to record highest mortality of 86.67 per cent, followed by CaO and ZnO based *Bt*- 16 of 83.33 and 81.67 per cent at a concentration of 1× 10^{12} CFU ml^{-1}. The least effective isolate was observed to be isolate- 49 for CaO based *Bt* as it recorded the lowest mortality percentage of 65.00 per cent, for MgO based *Bt* it was observed to record 63.33 per cent for the isolates 49 and 52 and for ZnO based *Bt* the mortality was recorded to be 56.67 per cent with isolate 52.

Similar findings were reported by Valicente *et al*. (2010), who studied the influence of $MnSO_4$, $MgSO_4$, $FeSO_4$ and $ZnSO_4$ mineral salts, by incorporating the mineral salts into LBA media at concentrations of 0.02g, 0.03g and 0.002g respectively, which resulted in increase of number of spores of *Bt* i.e., 2 x 10^8 cells ml^{-1}, when compared to control. The bioassay conducted against first instar *S.frugiperda* using *Bt* maintained on mineral salts enriched media showed higher efficacy causing 60 per cent mortality.

4.2.1.1. Evaluation of *Bt* grown on CaO based nano enriched media

In the present investigation, native *Bt* isolates were grown on CaO based nano enriched mineral media along with the reference strain, and these isolates were used in bioassay studies against the 3rd instar larvae of *H. armigera* (Table 4.4).

The mean per cent mortality was found to range from 25.00-96.67 at various concentrations observed ($1×10^8$ to $1×10^{12}$ CFU ml^{-1}). The mean per cent mortality was found to be increasing with the increase in the concentration. No mortality was found in both the control and the treatment with Tween- 80 only.

Among all the isolates, HD1 was found to show maximum mortality at all the concentrations and the mortality ranged from 58.33 (at $1×10^8$) to 96.67 per cent (at $1×10^{12}$ CFU ml^{-1}). Among the six native isolates, the highest mortality rate was observed in *Bt*- 493 with a mortality per cent of 91.67. The next best isolate was found to be *Bt*- 16 at a concentration of $1×10^{12}$ CFU ml^{-1}, where the mortality observed was 83.33%. These were followed by isolates 52, 51, 55 and 49 where the mortalities were recorded to be 75, 66.67, 66.67 and 65 per cent respectively at 1×10^{12} CFU ml^{-1}. The least effective isolate among all the isolates was observed to be isolate 49.

Among the seven isolates tested, the LC_{50} values ranged from 3.95×10^{7} to 5.68×10^{10} CFU ml^{-1} . The lowest LC_{50} was 3.95×10^{7} observed in the reference strain HD1. The highest median lethal value was recorded by I-52 (5.68×10^{10} CFU ml^{-1}) and was found to be minimal effective of all isolates tested. The values of chi-square (χ^2), regression equation, LC_{50} values and fiducial limits pertaining to the tested *Bacillus thuringeinsis* isolates were presented in the Table 4.5. The lethal concentration response plotted against the probit mortality was given in Fig. 4.3.

For the seven isolates used, the LT_{50} values ranged from 81.22 to 135.43 hpi. The lowest LT_{50} observed in reference strain HD1 *i.e.*, 81.22 hpi showing quick mortality among all the isolates tested. Among the native isolates it was observed to be 83.08 hpi for isolate 493 and the highest LT_{50} was observed to be 135.43 hpi for isolate 52. The LT_{50} values of all the isolates were given in Table 4.6. The lethal time response plotted against the probit mortality was given in Fig. 4.4.

4.2.1.2 Evaluation of *Bt* grown on MgO based nano enriched media

In the experiment conducted to know the bioefficacy of *Bt* isolates maintained on MgO based nano enriched mineral media against 3rd instar larvae of *H. armigera*, the mean per cent mortality ranged from 23.33 to 95 at different concentrations (1×10^{8} to 1×10^{12} CFU ml^{-1}). It was recorded that mean per cent mortality increased with increase in concentration (Table 4.7).

Among all the isolates, HD1 was found to record maximum mortality *i.e.*, 58.33 at 1×10^{8} to 95.00% at 1×10^{12} CFU ml^{-1} at all the concentrations. Among the native isolates, the highest mortality rate was observed in *Bt*- 493 with a mortality per cent of 90.00. The next isolate with maximum mortality was found to be *Bt*- 16 at a concentration of 1×10^{12} CFU ml^{-1} *i.e.*, 86.67 per cent and are followed by isolates 51 and 55, where the mortalities were recorded to be 68.33 and 66.67 per cent respectively at 1×10^{12} CFU ml^{-1}. The least effective treatment among all the isolates was observed to be isolate 49 and 52 at 1×10^{12} CFU ml^{-1} with 63.33 per cent mortality.

The LC_{50} values for the seven isolates grown on MgO based nano enriched media were in the range of 3.09×10^{7} to 4.16×10^{10} CFU ml^{-1}. The lowest lethal value was 3.09×10^{7} observed in the reference strain HD1. The highest median lethal value was recorded by the I-49 (4.16×10^{10} CFU ml^{-1}) and was found to be least effective of all isolates tested. The values of chi-square (χ^2), regression equation, LC_{50} values and fiducial limits pertaining to the tested *B. thuringeinsis* isolates were presented in the Table 4.8. The lethal concentration response plotted against the probit mortality was given in Fig. 4.5.

The LT_{50} values for the isolates were in the range of 82.80 to 128.78 hpi. Among all the isolates tested, the least LT_{50} value was observed in the reference strain HD1 *i.e.*, 82.80 hpi. Among the native isolates the least LT_{50} was observed for isolate 493 (85.70 hpi) showing quick mortality. The highest LT_{50} was observed for the isolate 49 *i.e.*, 128.78 hpi. The LT_{50} values of the seven isolates was mentioned in Table 4.9. The lethal time response plotted against the probit mortality was given in Fig. 4.6.

The results were in acceptance with Rao *et al*. (2018), who had synthesized magnesium hydroxide nanoparticles (MHNPs) in order to avoid the loss of Cry1Ac protein, and the protein was delivered to *H. armigera.* The Cry1Ac protein was successfully loaded onto MHNPs, which remained on the leaf surface of cotton, resulted in increased adhesion of protein and mortality of *H. armigera* by 75 per cent.

4.2.1.3 Evaluation of *Bt* grown on ZnO based nano enriched media

In the work done to know the bioefficacy of native *Bt* isolates grown on ZnO based nano enriched mineral media against *H. armigera*, the mean per cent mortalities ranged from 21.67 - 93.33 per cent at various concentrations of 1×10^8 to 1×10^{12} CFU ml^{-1}. Morphomimitic aberrations were observed in *H. armigera* treated with ZnO based *Bt* isolate 16 and 493 (Plate 4.13). Observations revealed that mean per cent mortalities increased with increase in concentrations (Table 4.10).

Among all the isolates tested, the reference strain HD1 recorded maximum mortality at a concentration of 1×10^{12} CFU ml^{-1} *i.e*., 93.33 per cent. Among the native isolates, isolate 493 was found to cause highest mortality at a concentration of 1×10^{12} CFU ml^{-1} *i.e*., 88.33 per cent, followed by isolate 493, isolates 16, 51, 55 and 49 recorded mortalities of 81.67, 70.00, 63.33 and 61.67 per cent at a concentration of 1×10^{12} CFU ml^{-1}. The least mortality was observed in the treatment with isolate 52 with mortality of 56.67 per cent.

The LC_{50} values for the seven isolates maintained on ZnO based nano enriched media were in the range of 3.86×10^7 to 1.23×10^{11} CFU ml^{-1}. The lowest lethal value was 3.86×10^7 observed in the reference strain HD1. The highest median lethal value was recorded by the I-55 (1.23×10^{11} CFU ml^{-1}) and was found to be minimal effective among the isolates tested. The values of chi-square (χ^2), regression equation, LC_{50} values and fiducial limits pertaining to the tested *Bacillus thuringeinsis* isolates were presented in the Table 4.11. The lethal concentration response plotted against the probit mortality was given in Fig. 4.7.

Among the seven isolates tested, the LT_{50} values were in the range of 83.52 to 140.57 hpi. The least LT_{50} value was observed in HD1 showing faster mortality i.e., 83.52 hpi. Among the native isolates, the least lethal time value was found to be in isolate 493 *i.e.,* 86.75 hpi and the highest value was observed for the isolate 52 *i.e.*, 140.57 hpi. The LT_{50} values for the isolates were given in the Table 4.12. The lethal time response plotted against the probit mortality was given in Fig. 4.8.

The results were in accordance with Vielkind *et al*. (2013), who had observed the growth inhibition in *Pseudomonas putida* by increasing ZnO nanoparticulates to 500 mg/L. The reasons mentioned were (i) production of ROS (Reactive Oxygen Species), (ii) disruption of bacteria cell wall as a result of interaction of NP's (iii) induced antibacterial effects between the cells (iv) cytoplasmic accumulation of NP's.

a. **Larval- Pupal intermediaries**

b. Malformed pupae

c. Pupal- Adult intermediaries

Plate 4.13. Morphomimitic insect growth aberrations observed in *H. armigera* treated with nano based ZnO *Bt* isolates at a concentartion of 1×10^{12} CFU ml^{-1}

Table 4.4. Mean per cent mortality of third instar larvae of *H. armigera* with *Bt* isolates grown on nano based CaO mineral enriched medium at 48 hrs after treatment

Concentration/ isolate	HD1	I-16	I-49	I-51	I-52	I-55	I-493
1×10^{8} CFU ml^{-1}	58.33 (49.80)^{hijk}	41.67 (40.20)^{no}	25.00 (30.00)^{r}	36.67 (37.26)^{opq}	25.00 (31.07)^{qr}	28.33 (32.14)^{pqr}	50.00 (45.00)^{klmn}
1×10^{9} CFU ml^{-1}	68.33 (55.77)^{fgh}	50.00 (45.00)^{klmn}	35.00 (36.27)^{opqr}	41.67 (40.20)^{no}	31.67 (36.27)^{opqr}	38.33 (38.24)^{op}	58.33 (49.80)^{hijk}
1×10^{10} CFU ml^{-1}	80.00 (63.55)^{cde}	63.33 (52.74)^{ghij}	43.33 (41.16)^{mno}	55.00 (47.87)^{ijkl}	43.33 (41.16)^{mno}	45.00 (42.13)^{lmno}	73.33 (58.93)^{efg}
1×10^{11} CFU ml^{-1}	88.33 (70.12)^{bc}	73.33 (58.93)^{efg}	58.33 (49.80)^{hijk}	58.33 (54.75)^{fgh}	66.67 (46.91)^{jklm}	53.33 (46.91)^{jklm}	80.00 (63.43)^{de}
1×10^{12} CFU ml^{-1}	96.67 (81.39)^{a}	83.33 (65.95)^{cd}	65.00 (53.73)^{fghi}	66.67 (60.00)^{def}	75.00 (50.77)^{hijk}	66.67 (53.73)^{fghi}	91.67 (73.40)^{b}
Tween80- 0.1 %		0.00 %					
Control		0.00 %					
		Isolates		**Concentrations**		**Isolate*concentrations**	
SEm ±		0.741		0.633		1.653	
CD (p=0.05)		1.48		1.25		3.30	
CV (%)		4.07					

* Mean of three replications (N=20) ; Mean values followed by same alphabet in a column are not significantly different by Tukey HSD (P=0.05)

Table 4.5. Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based CaO mineral enriched medium

Isolate	Chi-square (χ^2)	Regression equation	*LD_{50} (CFU ml^{-1})	Fiducial limit (CFU ml^{-1})
I-HD1	1.318	Y= -2.821 + 0.371 X	3.95×10^7	(5.93×10^6)-(1.31×10^8)
I-16	0.296	Y= -2.751 + 0.312 X	6.98×10^8	(1.75×10^8)-(1.85×10^9)
I-49	0.445	Y= - 3.201 + 0.317X	2.91×10^{10}	(1.12×10^{10})-(9.17×10^{10})
I-51	0.649	Y= - 2.814 + 0.326X	3.12×10^9	(9.52×10^8)-(8.26×10^9)
I-52	0.095	Y= - 3.212 + 0.306X	5.68×10^{10}	(1.75×10^{10})-(3.00×10^{11})
I-55	0.285	Y= - 2.617 + 0.257X	2.89×10^{10}	(9.96×10^9)-(1.18×10^{11})
I-493	1.327	Y = - 2.846 + 0.351 X	1.48×10^8	(2.86×10^7)- (4.35×10^8)

* Mean of three replications (N=20)

Table 4.6. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based CaO mineral enriched media at 1×10^{12} CFU ml^{-1}

Isolate	Chi-square (χ^2)	Regression equation	*LT_{50} (hpi)	Fiducial limit (hpi)
I-HD1	10.135	Y= - 11.143 + 5.714 X	81.22	72.77 – 89.05
I-16	13.314	Y= - 12.142 + 5.714 X	94.84	83.12 – 106.58
I-49	12.596	Y= - 8.369 + 4.015 X	121.41	106.39 – 142.75
I-51	8.470	Y= - 9.627 + 4.698 X	112.01	101.71 – 123.82
I-52	7.496	Y= - 8.454 + 3.966 X	135.43	126.46 – 146.89
I-55	9.507	Y= - 8.817 + 4.230 X	121.42	108.79 – 138.15
I-493	14.374	Y= - 13.346 + 6.529 X	83.08	72.00 – 93.34

* Mean of three replications (N=20)

Table 4.7. Mean per cent mortality of third instar larvae of *H. armigera* with *Bt* isolates grown on nano based MgO mineral enriched medium at 48 hrs after treatment

Concentration/ isolate	HD1	I-16	I-49	I-51	I-52	I-55	I-493
1×10^{8} CFU ml^{-1}	58.33 (49.80)^{ijkl}	43.33 (41.16)^{opq}	23.33 (28.86)^{t}	35.00 (36.27)^{qrs}	26.67 (31.07)st	26.67 (31.07)st	48.33 (44.04)^{mno}
1×10^{9} CFU ml^{-1}	68.33 (55.77)^{fgh}	51.67 (45.96)^{lmno}	31.67 (34.23)^{rs}	38.33 (38.24)^{pqr}	35.00 (36.27)^{qrs}	38.33 (38.24)^{pqr}	58.33 (49.80)^{ijkl}
1×10^{10} CFU ml^{-1}	80.00 (63.43)^{cd}	63.33 (52.74)^{ghijk}	43.33 (41.16)^{opq}	51.67 (45.96)^{lmno}	43.33 (41.16)^{opq}	46.67 (43.09)^{nop}	71.67 (57.86)^{efg}
1×10^{11} CFU ml^{-1}	86.67 (68.66)^{bc}	75.00 (60.00)^{def}	56.67 (48.84)^{jklm}	60.00 (50.77)^{hijkl}	55.00 (47.87)^{klmn}	55.00 (47.87)^{klmn}	78.33 (62.29)^{de}
1×10^{12} CFU ml^{-1}	95.00 (77.08)^{a}	86.67 (68.66)^{bc}	63.33 (52.74)^{ghijk}	68.33 (55.77)^{fgh}	63.33 (53.73)^{ghij}	66.67 (54.75)^{fghi}	90.00 (71.57)^{b}
Tween80- 0.1 %		0.00 %					
Control		0.00 %					
		Isolates		**Concentrations**		**Isolate*concentrations**	
SEm ±		0.593		0.501		1.321	
CD (p=0.05)		1.17		0.99		2.63	
CV (%)		3.27					

* Mean of three replications (N=20) ; Mean values followed by same alphabet in a column are not significantly different by Tukey HSD (P=0.05)

Table 4.8. Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based MgO mineral enriched medium

Isolate	Chi-square (χ^2)	Regression equation	*LD_{50} (CFU ml^{-1})	Fiducial limit (CFU ml^{-1})
I-HD1	0.706	Y= -2.531 + 0.338 X	3.09×10^7	(3.43×10^6)-(1.17×10^8)
I-16	0.914	Y= -2.740 + 0.313 X	5.49×10^8	(1.42×10^8)-(1.42×10^9)
I-49	0.400	Y= - 2.956 + 0.278 X	4.16×10^{10}	(1.62×10^{10})-(1.36×10^{10})
I-51	0.671	Y= - 2.260 + 0.228 X	8.36×10^9	(2.43×10^9)-(2.74×10^{10})
I-52	0.083	Y= - 2.582 + 0.244 X	3.82×10^{10}	(1.32×10^{10})-(1.50×10^{11})
I-55	0.275	Y= - 2.611 + 0.252 X	2.25×10^{10}	(8.03×10^9)-(7.49×10^{11})
I-493	0.845	Y = - 2.599 + 0.316 X	1.72×10^8	(3.19×10^7)- (5.16×10^8)

* Mean of three replications (N=20)

Table 4.9. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based MgO mineral enriched medium at 1×10^{12} CFU ml^{-1}

Isolate	Chi-square (χ^2)	Regression equation	*LT_{50} (hpi)	Fiducial limit (hpi)
I-HD1	10.102	Y= - 10.870 + 5.667 X	82.80	74.05 – 90.97
I-16	3.980	Y= - 11.643 + 5.714 X	100.18	95.03 – 105.42
I-49	8.433	Y= - 8.574 + 4.064 X	128.78	115.56 – 147.62
I-51	8.149	Y= - 9.113 + 4.374 X	121.10	109.70 – 135.66
I-52	9.312	Y= - 8.565 + 4.081 X	125.49	112.11 – 144.17
I-55	7.990	Y= - 8.856 + 4.223 X	125.63	117.55 – 133.92
I-493	12.561	Y= - 11.143 + 5.714 X	85.70	75.22 – 95.59

* Mean of three replications (N=20)

Table 4.10. Mean per cent mortality of third instar larvae of *H. armigera* with *Bt* isolates grown on nano based ZnO mineral enriched medium at 72 hrs after treatment

Concentration/ isolate	HD1	I-16	I-49	I-51	I-52	I-55	I-493
1×10^{8} CFU ml^{-1}	56.67 (48.84)^{ijklm}	41.67 (40.20)^{pqr}	21.67 (27.71)^{u}	35.00 (36.27)^{rst}	28.33 (32.14)^{tu}	28.83 (32.14)^{tu}	46.67 (43.09)^{nop}
1×10^{9} CFU ml^{-1}	66.67 (54.75)^{efgh}	48.33 (44.04)^{mnop}	33.33 (35.25)^{rst}	40.00 (39.23)^{pqrs}	31.67 (34.23)st	36.67 (37.26)^{qrst}	55.00 (47.87)^{jklmn}
1×10^{10} CFU ml^{-1}	78.33 (62.29)^{cd}	65.00 (53.73)^{fghi}	41.67 (40.20)^{pqr}	53.33 (46.91)^{klmno}	40.00 (39.23)^{pqrs}	45.00 (42.13)^{opq}	71.67 (57.86)^{def}
1×10^{11} CFU ml^{-1}	86.67 (68.66)^{b}	71.67 (57.86)^{def}	56.67 (48.84)^{ijklm}	61.67 (51.76)^{ghijk}	51.67 (45.96)^{lmno}	51.67 (45.96)^{lmno}	75.00 (60.00)^{cde}
1×10^{12} CFU ml^{-1}	93.33 (75.24)^{a}	81.67 (64.69)^{bc}	61.67 (51.76)^{ghijk}	70.00 (56.79)^{efg}	56.67 (49.80)^{hijkl}	63.33 (52.74)^{fghij}	88.33 (70.12)^{ab}
Tween80- 0.1 %		0.00 %					
Control		0.00 %					
		Isolates		**Concentrations**		**Isolate*concentrations**	
SEm ±		0.612		0.521		1.371	
CD (p=0.05)		1.22		1.03		2.72	
CV (%)		3.46					

* Mean of three replications (N=20) ; Mean values followed by same alphabet in a column are not significantly different by Tukey HSD (P=0.05)

Table 4.11. Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based ZnO mineral enriched medium

Isolate	Chi-square (χ^2)	Regression equation	*LD_{50} (CFU ml^{-1})	Fiducial limit (CFU ml^{-1})
I-HD1	0.209	Y= -3.217 + 0.416 X	3.86×10^{7}	(4.49×10^{6})-(1.44×10^{8})
I-16	0.796	Y = - 2.758 + 0.312 X	7.39×10^{8}	(1.75×10^{8})- (2.03×10^{9})
I-49	0.871	Y= - 2.939 + 0.275 X	4.93×10^{10}	(1.88×10^{10})-(1.70×10^{10})
I-51	0.438	Y= - 2.320 + 0.237 X	5.97×10^{9}	(1.74×10^{9})-(1.81×10^{10})
I-52	0.635	Y= - 2.227 + 0.201 X	1.23×10^{11}	(3.18×10^{10})-(1.17×10^{12})
I-55	0.210	Y= - 2.340 + 0.221 X	3.95×10^{10}	(1.26×10^{10})-(1.88×10^{11})
I-493	1.804	Y = - 3.017 + 0.351 X	2.44×10^{8}	(4.72×10^{7})- (7.17×10^{8})

* Mean of three replications (N=20)

Table 4.12. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based ZnO mineral enriched media at 1×10^{12} CFU ml^{-1}

Isolate	Chi-square (χ^2)	Regression equation	*LT_{50} (hpi)	Fiducial limit (hpi)
I-HD1	12.489	Y= - 11.142 + 5.714 X	83.52	73.37 – 92.99
I-16	13.544	Y= - 12.143 + 5.714 X	96.41	84.29 – 108.80
I-49	11.475	Y= - 8.203 + 3.895 X	127.55	112.01 – 151.26
I-51	10.446	Y= - 8.832 + 4.264 X	117.86	105.10 – 134.28
I-52	8.563	Y= - 8.063 + 3.754 X	140.57	124.38 – 167.47
I-55	10.765	Y= - 8.320 + 3.957 X	126.62	111.84 – 148.45
I-493	19.024	Y= - 11.643 + 5.714 X	86.75	73.14 – 99.50

* Mean of three replications (N=20)

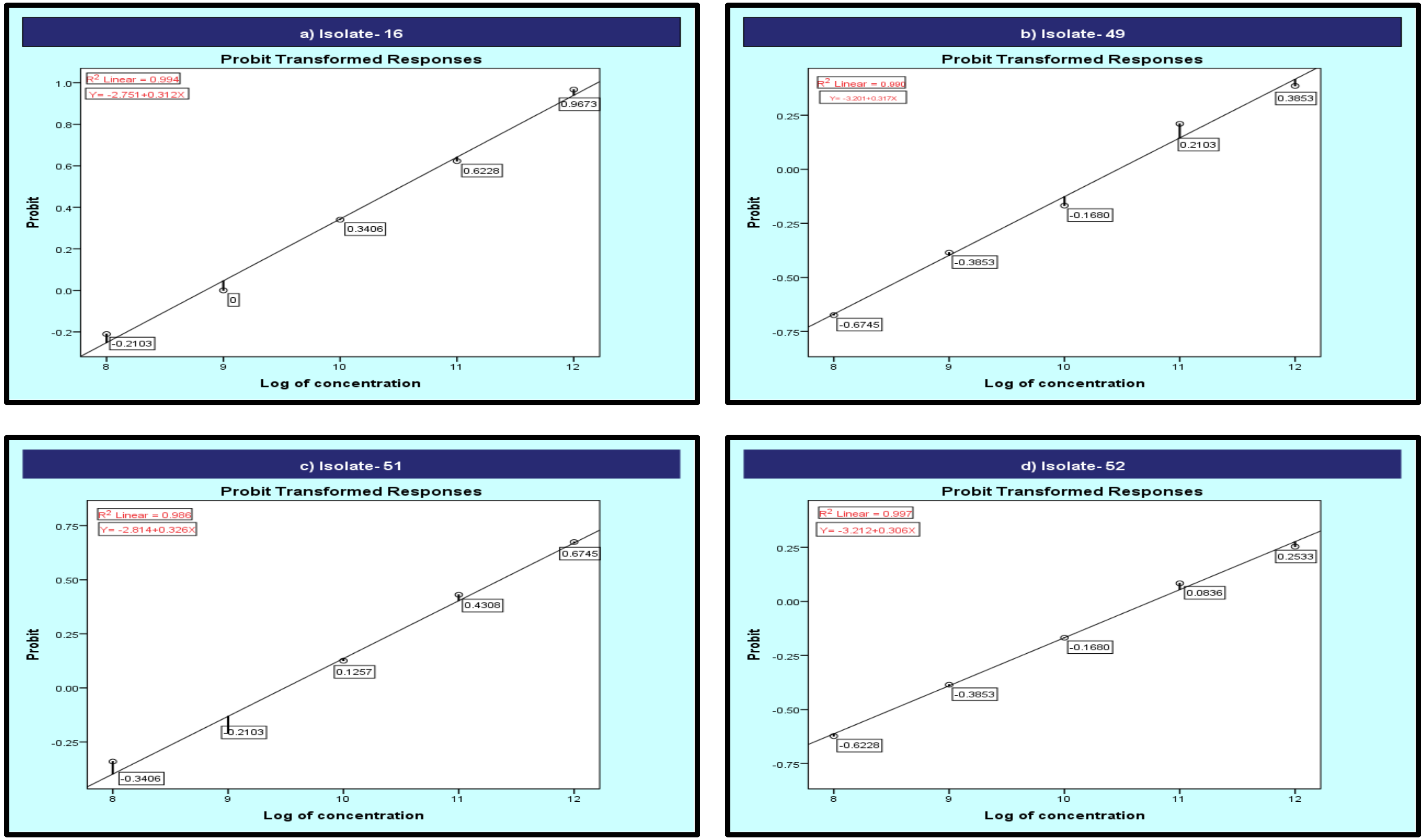
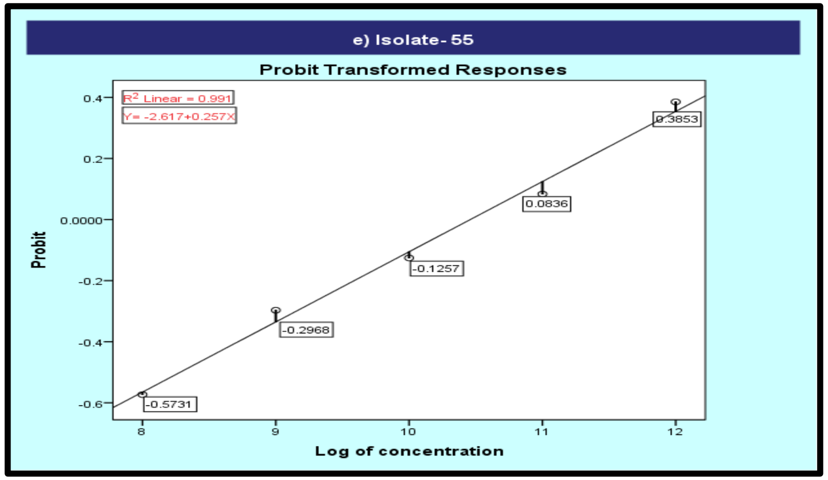
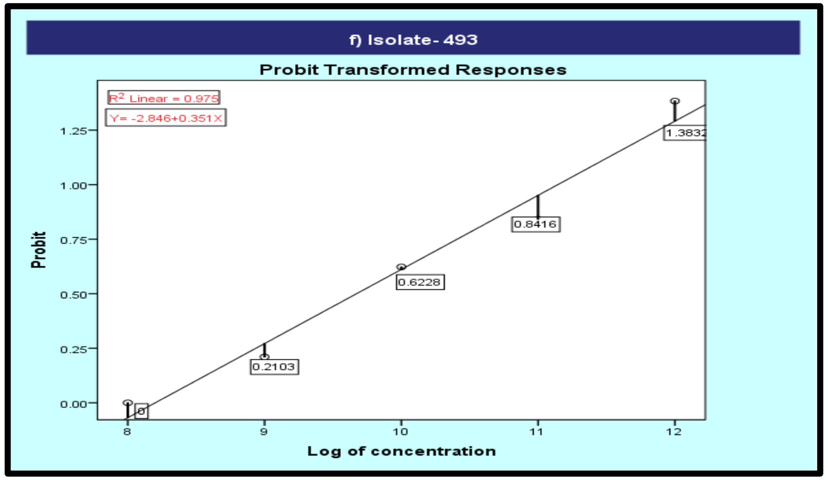


Fig. 4.3. **Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based CaO mineral enriched medium**


e) Isolate- 55
Probit Transformed Responses
R^2 Linear = 0.991
Y= -2.617+0.257X
Probit
Log of concentration
-0.5731
-0.2968
-0.1257
0.0836
0.3853


f) Isolate- 493
Probit Transformed Responses
Y= -2.846+0.351X
Probit
Log of concentration
0.2103
0.6228
0.8416

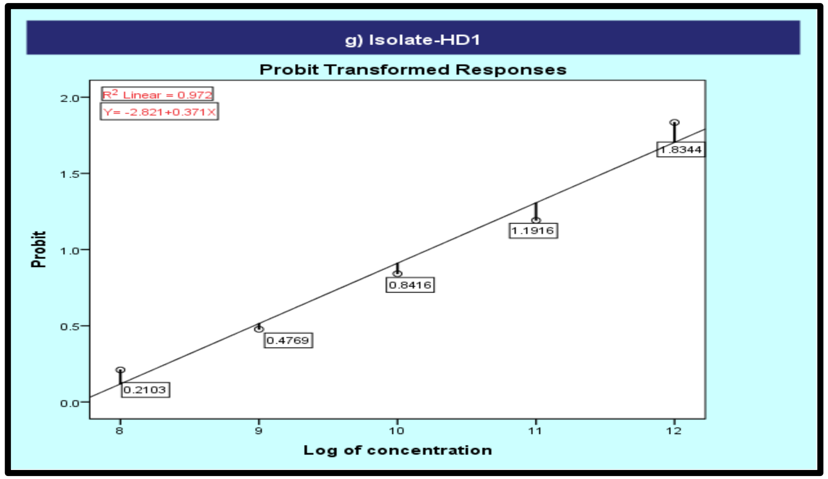

g) Isolate-HD1
Probit Transformed Responses
R^2 Linear = 0.972
Y= -2.821+0.371X
Probit
Log of concentration
0.2103
0.4769
0.8416
1.1916
1.8344

Fig. 4.3. (Cont.)

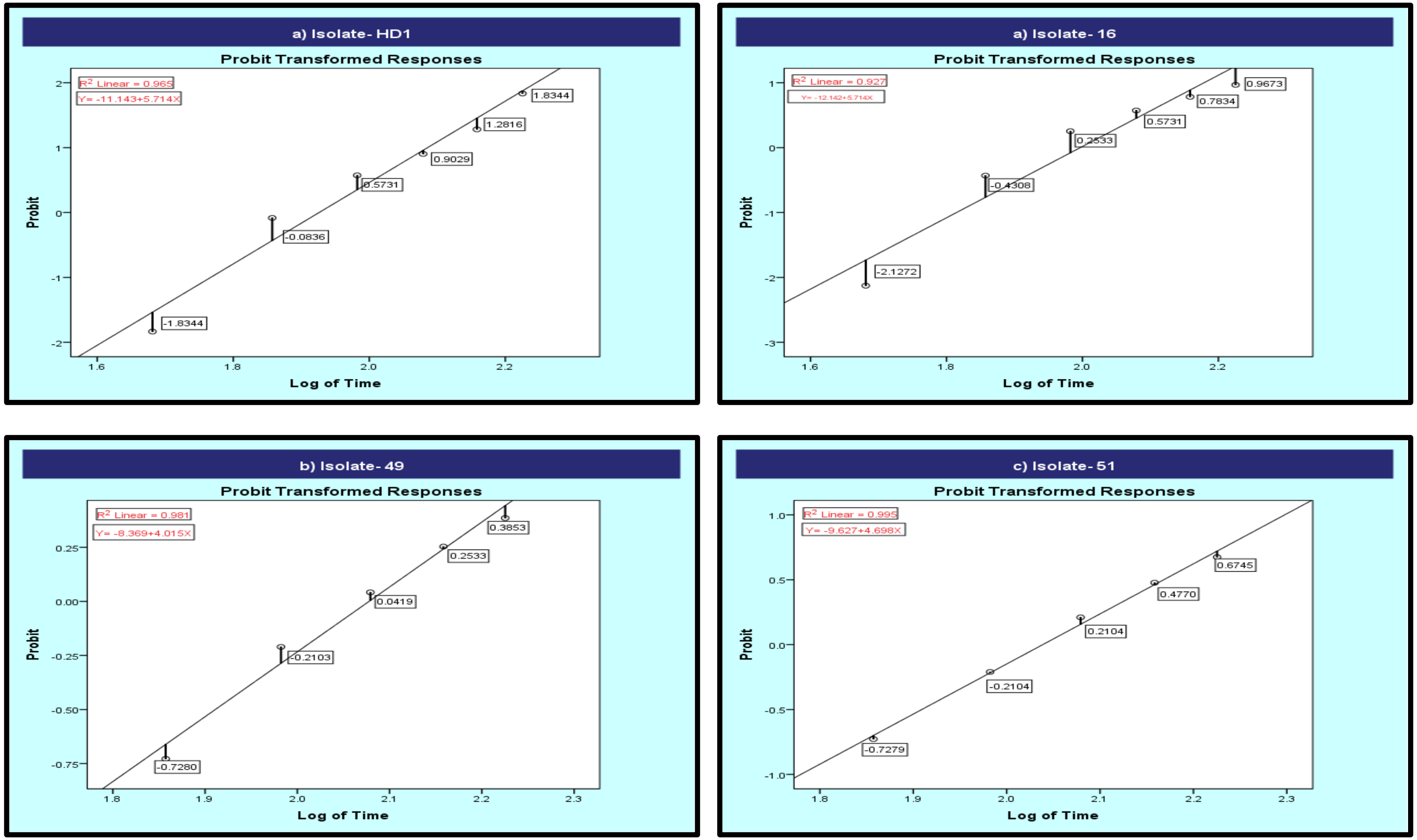


Fig. 4.4. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with native isolates of *Bt* grown on nano based CaO mineral enriched medium at 1× 10^{12} CFU ml^{-1}

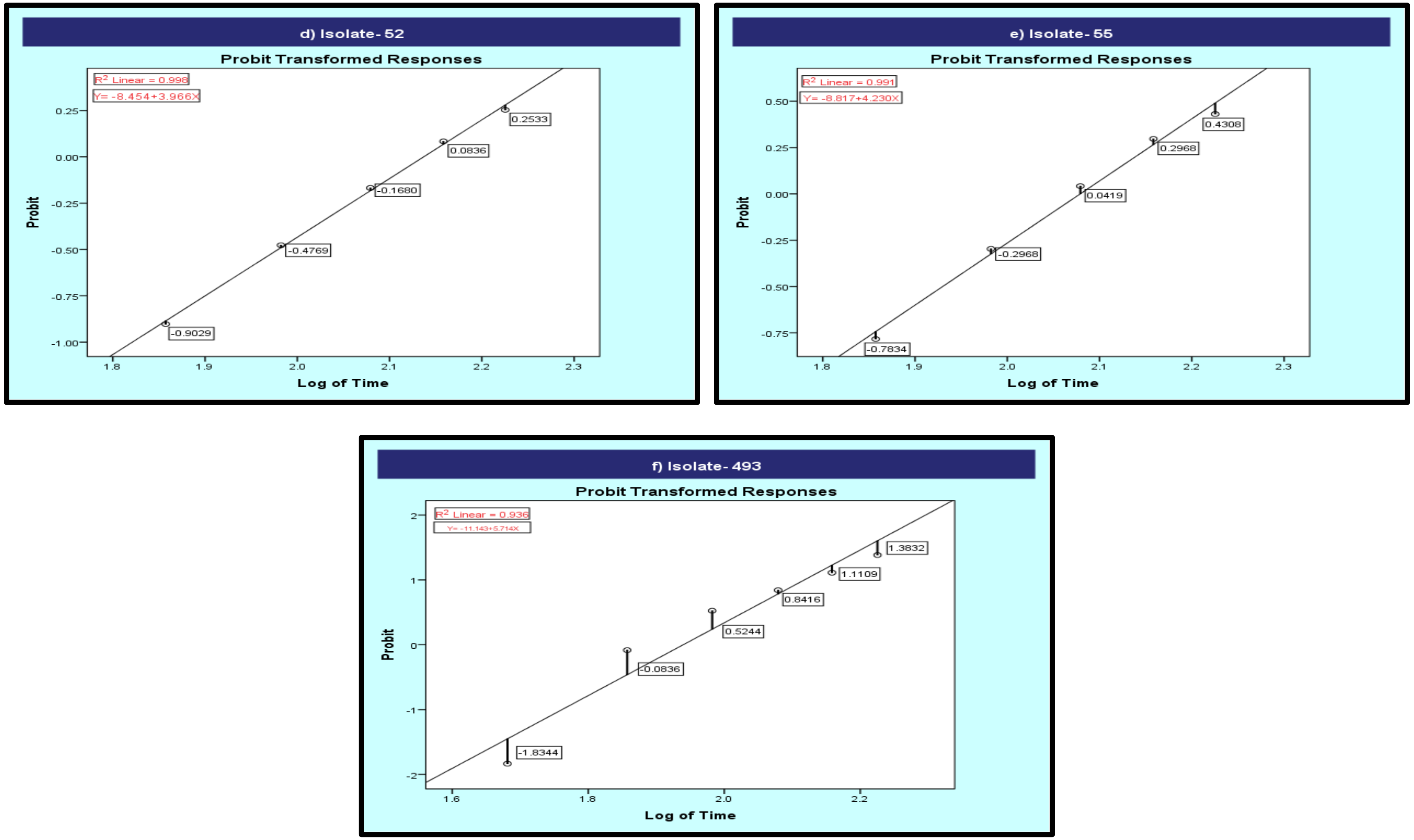

d) Isolate- 52
Probit Transformed Responses
R2 Linear = 0.998
Y= -8.454+3.966X
0.2533
0.0836
-0.1680
-0.4769
-0.9029
Probit
Log of Time
e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.991
Y= -8.817+4.230X
0.4308
0.2968
0.0419
-0.2968
-0.7834
Probit
Log of Time
f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.936
Y= -11.143+5.714X
1.3832
1.1109
0.8416
0.5244
-0.0836
-1.8344
Probit
Log of Time

Fig. 4.4. (Cont.)

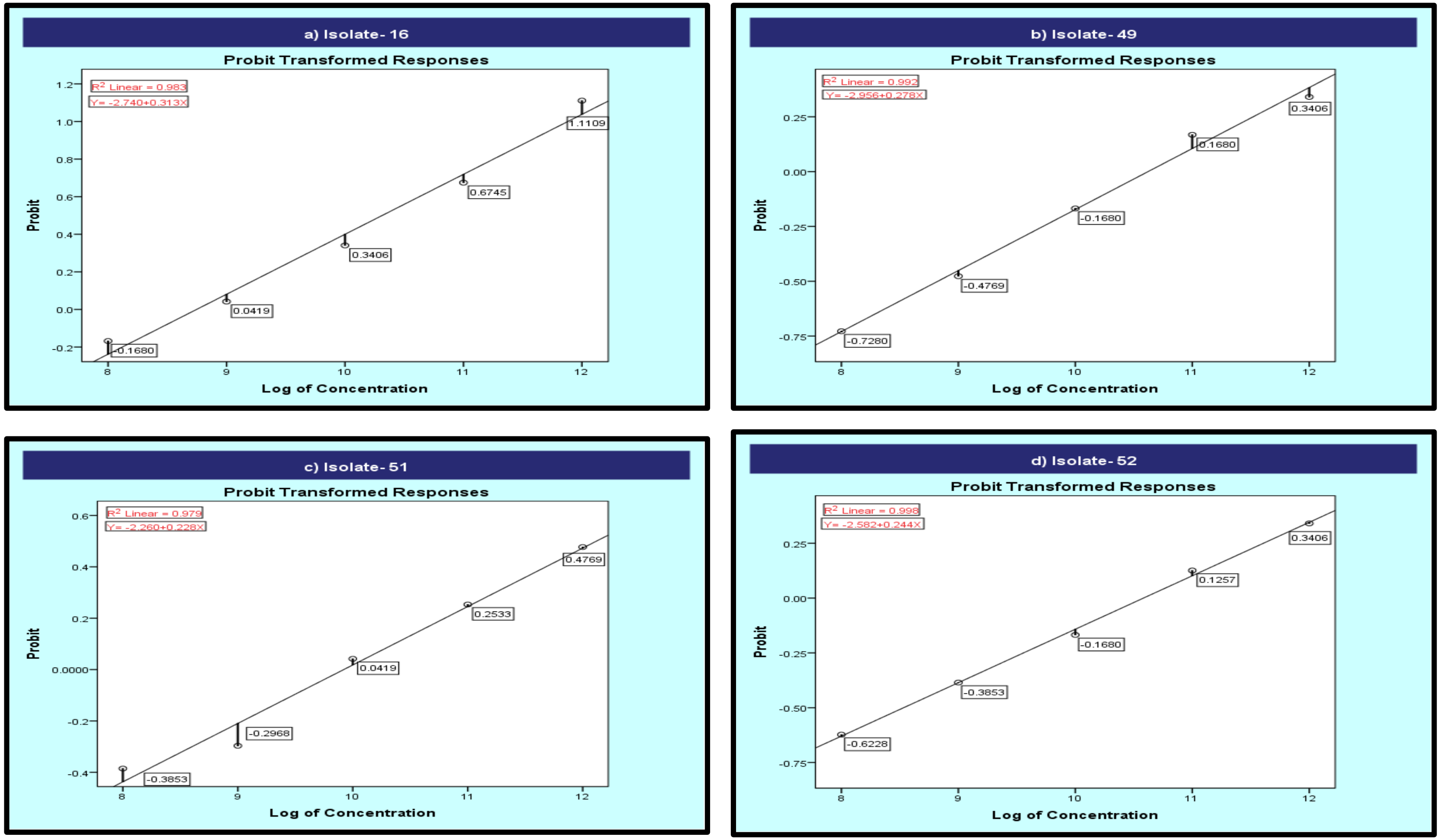


Fig. 4.5. Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with native isolates of *Bt* grown on nano based MgO mineral enriched medium

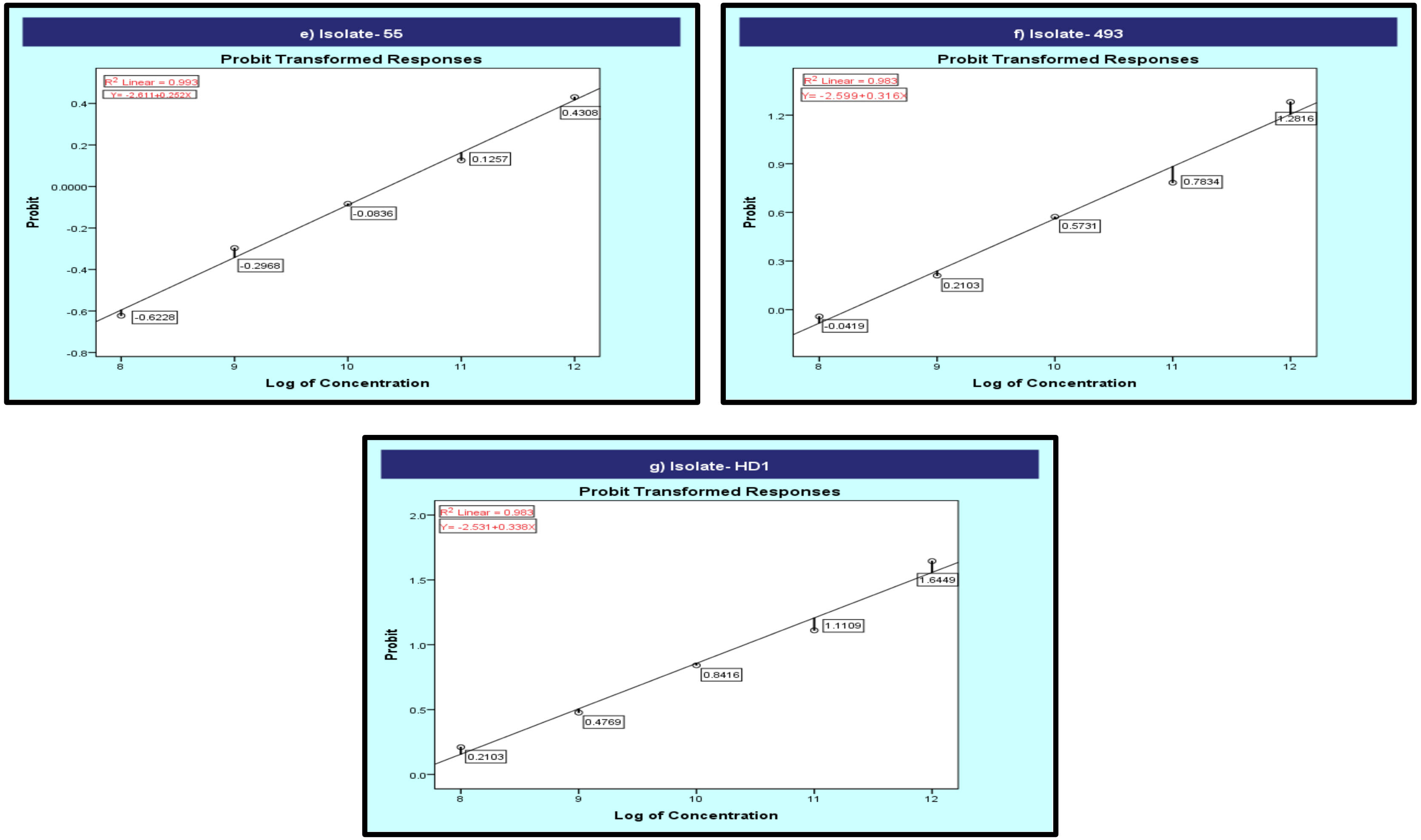

e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.993
Y= -2.611+0.252X
Probit
Log of Concentration
0.4308
0.1257
-0.0836
-0.2968
-0.6228
f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.983
Y= -2.599+0.316X
Probit
Log of Concentration
1.2816
0.7834
0.5731
0.2103
-0.0419
g) Isolate- HD1
Probit Transformed Responses
R2 Linear = 0.983
Y= -2.531+0.338X
Probit
Log of Concentration
1.6449
1.1109
0.8416
0.4769
0.2103

Fig. 4.5. (Cont.)

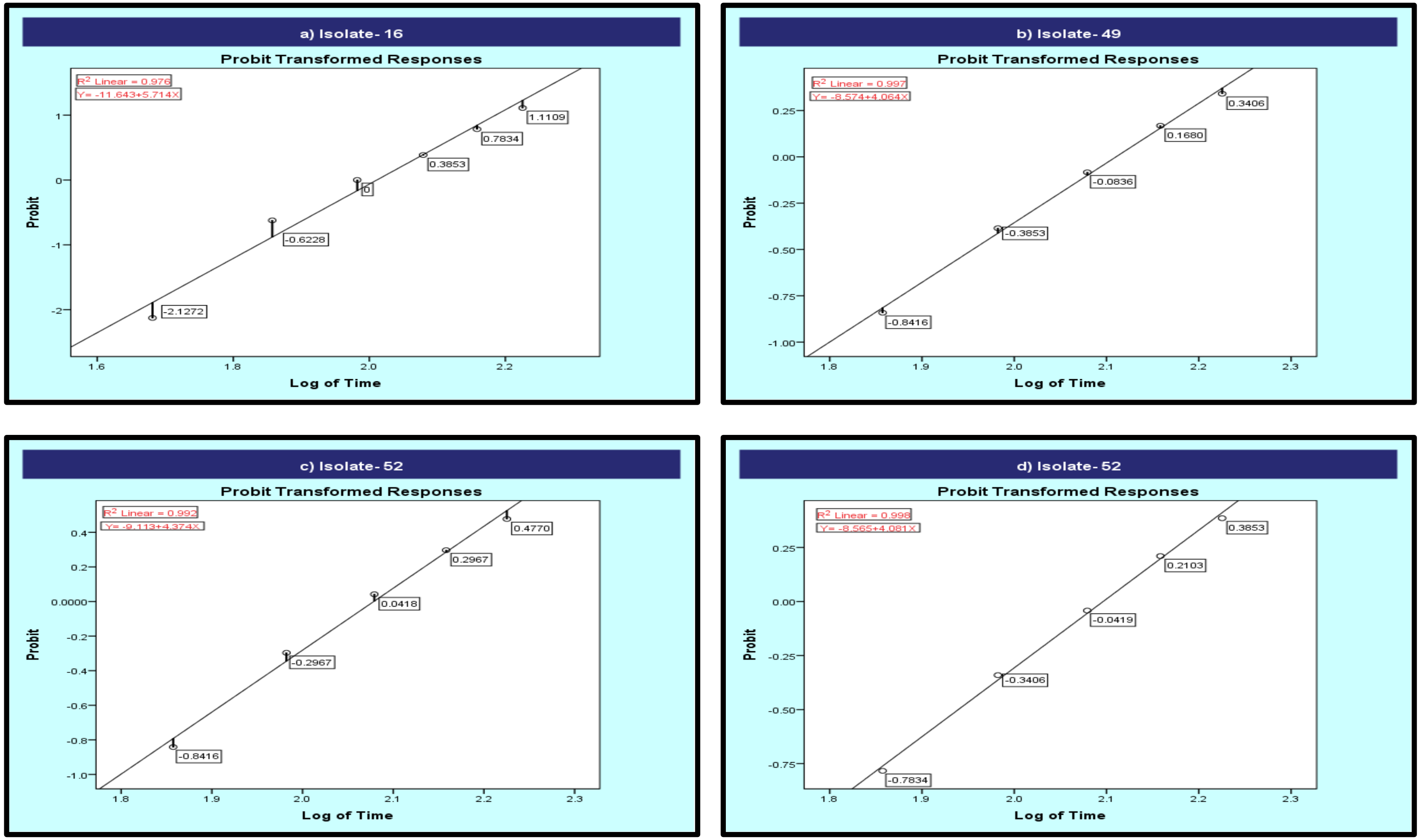
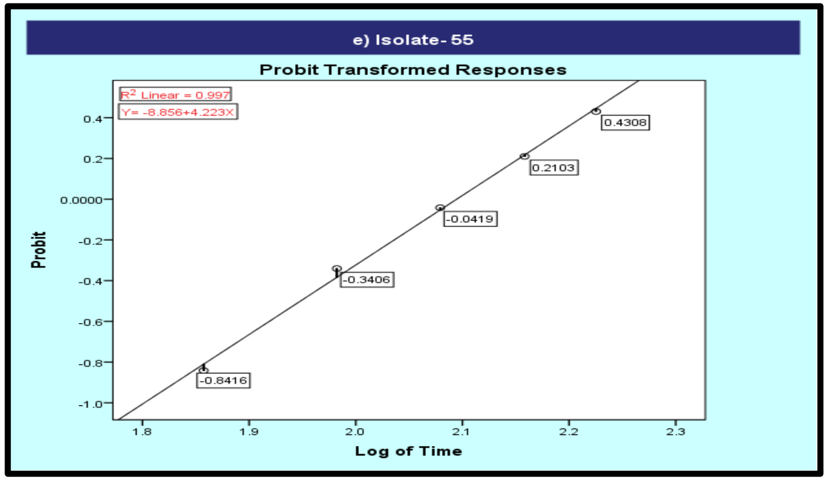
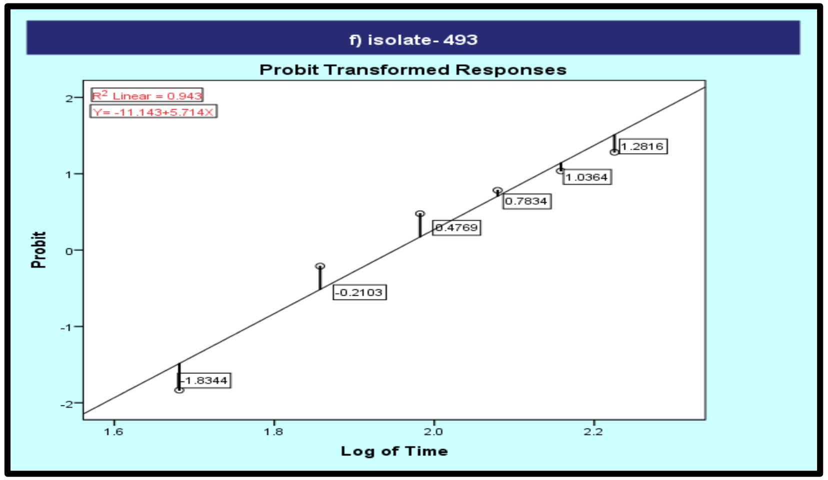


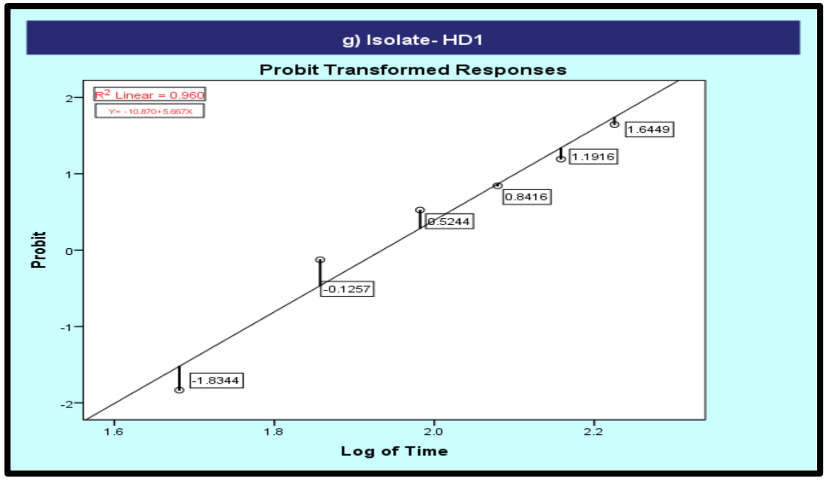
Fig. 4.6. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with native isolates of *Bt* grown on nano based MgO mineral enriched medium at 1× 10^{12} CFU ml^{-1}



e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.997
Y= -8.856+4.223X
0.4308
0.2103
-0.0419
-0.3406
-0.8416
0.4
0.2
0.0000
-0.2
-0.4
-0.6
-0.8
-1.0
Probit
1.8
1.9
2.0
2.1
2.2
2.3
Log of Time



f) isolate- 493
Probit Transformed Responses
R2 Linear = 0.943
Y= -11.143+5.714X
1.2816
1.0364
0.7834
0.4769
-0.2103
-1.8344
2
1
0
-1
-2
Probit
1.6
1.8
2.0
2.2
Log of Time



g) Isolate- HD1
Probit Transformed Responses
R2 Linear = 0.960
Y= -10.870+5.667X
1.6449
1.1916
0.8416
0.5244
-0.1257
-1.8344
2
1
0
-1
-2
Probit
1.6
1.8
2.0
2.2
Log of Time

Fig. 4.6. (Cont.)

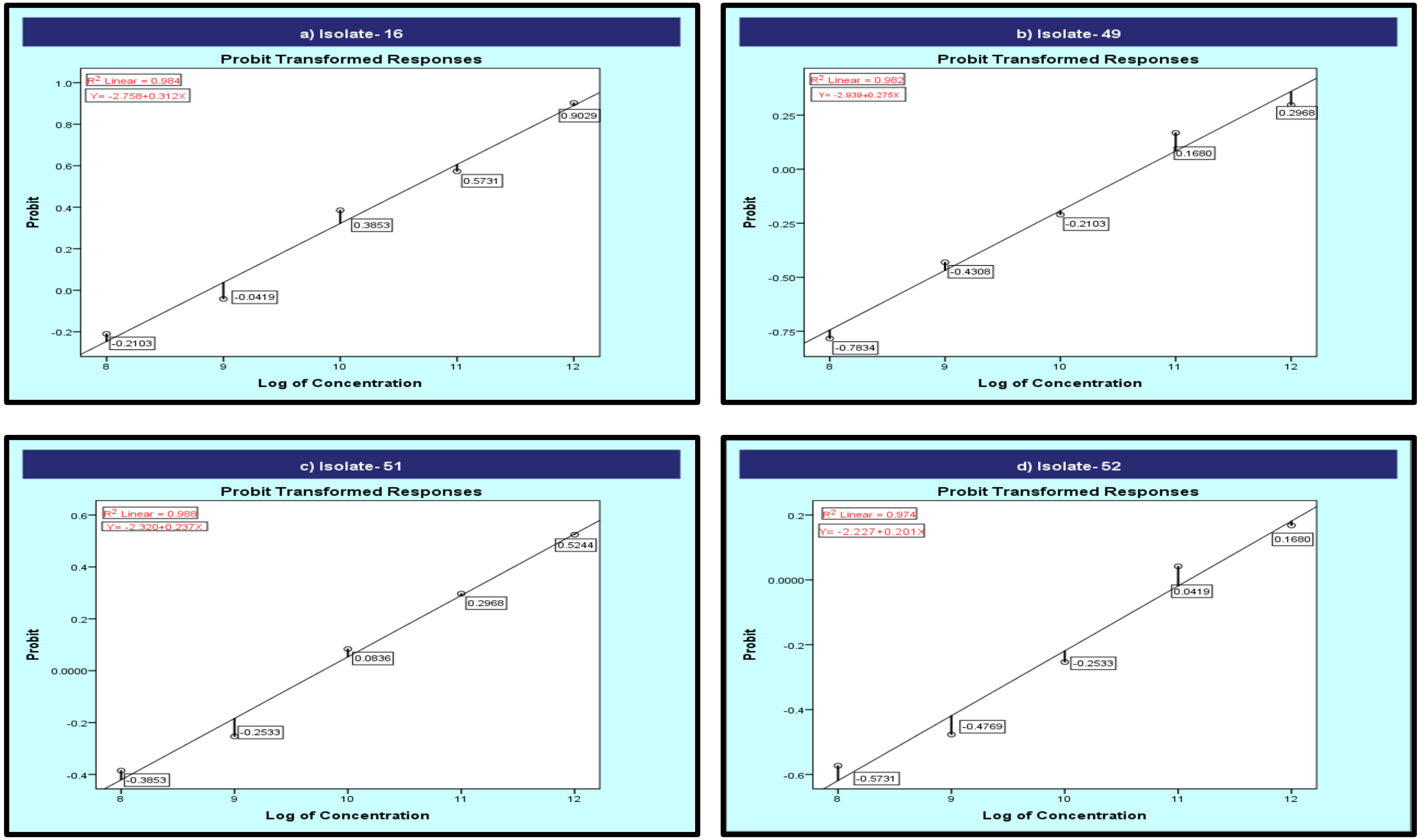


Fig. 4.7. **Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based ZnO mineral enriched medium**

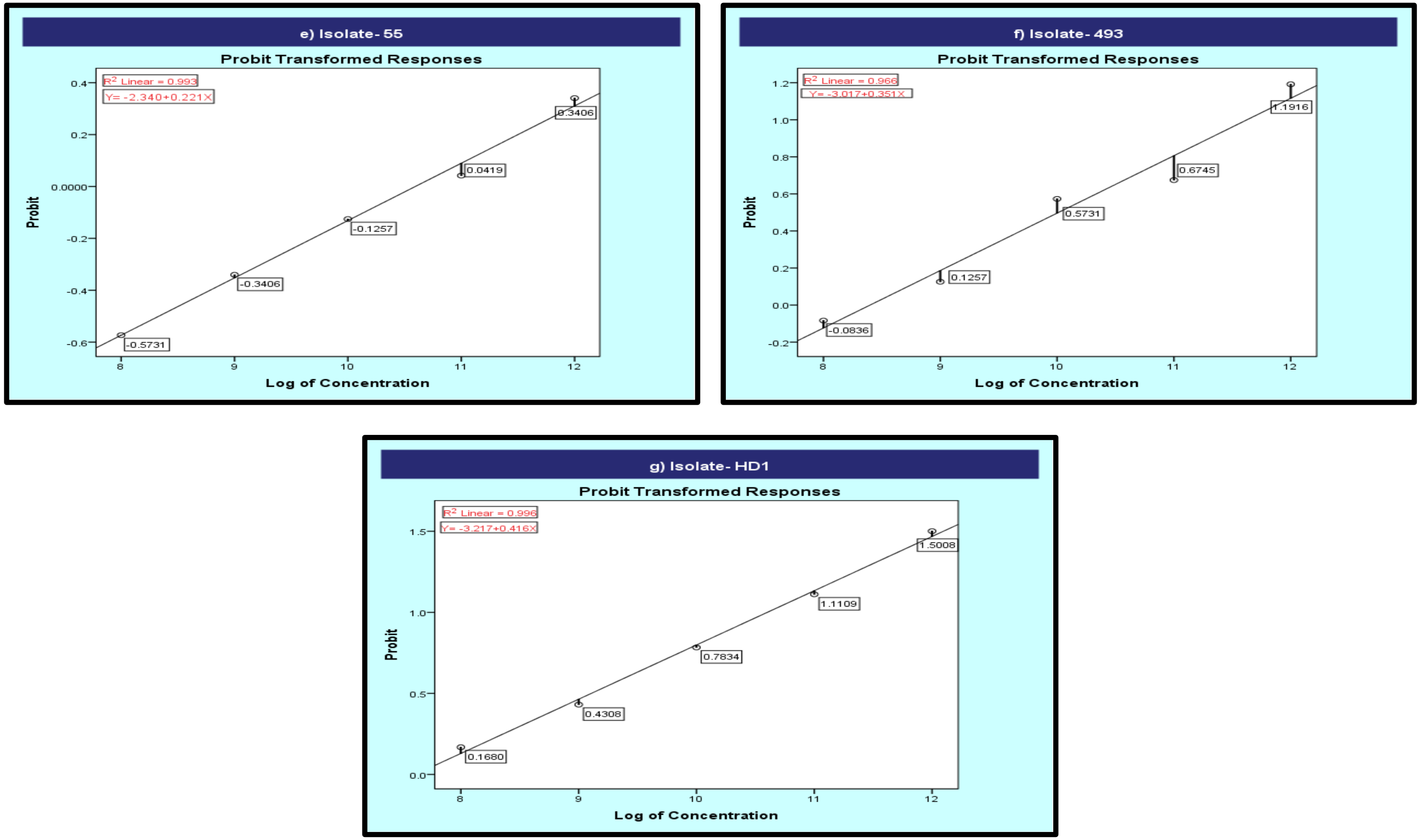

e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.993
Y= -2.340+0.221X
0.3406
0.0419
-0.1257
-0.3406
-0.5731
Probit
Log of Concentration
f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.966
Y= -3.017+0.351X
1.1916
0.6745
0.5731
0.1257
-0.0836
Probit
Log of Concentration
g) Isolate- HD1
Probit Transformed Responses
R2 Linear = 0.996
Y= -3.217+0.416X
1.5008
1.1109
0.7834
0.4308
0.1680
Probit
Log of Concentration

Fig. 4.7. (Cont.)

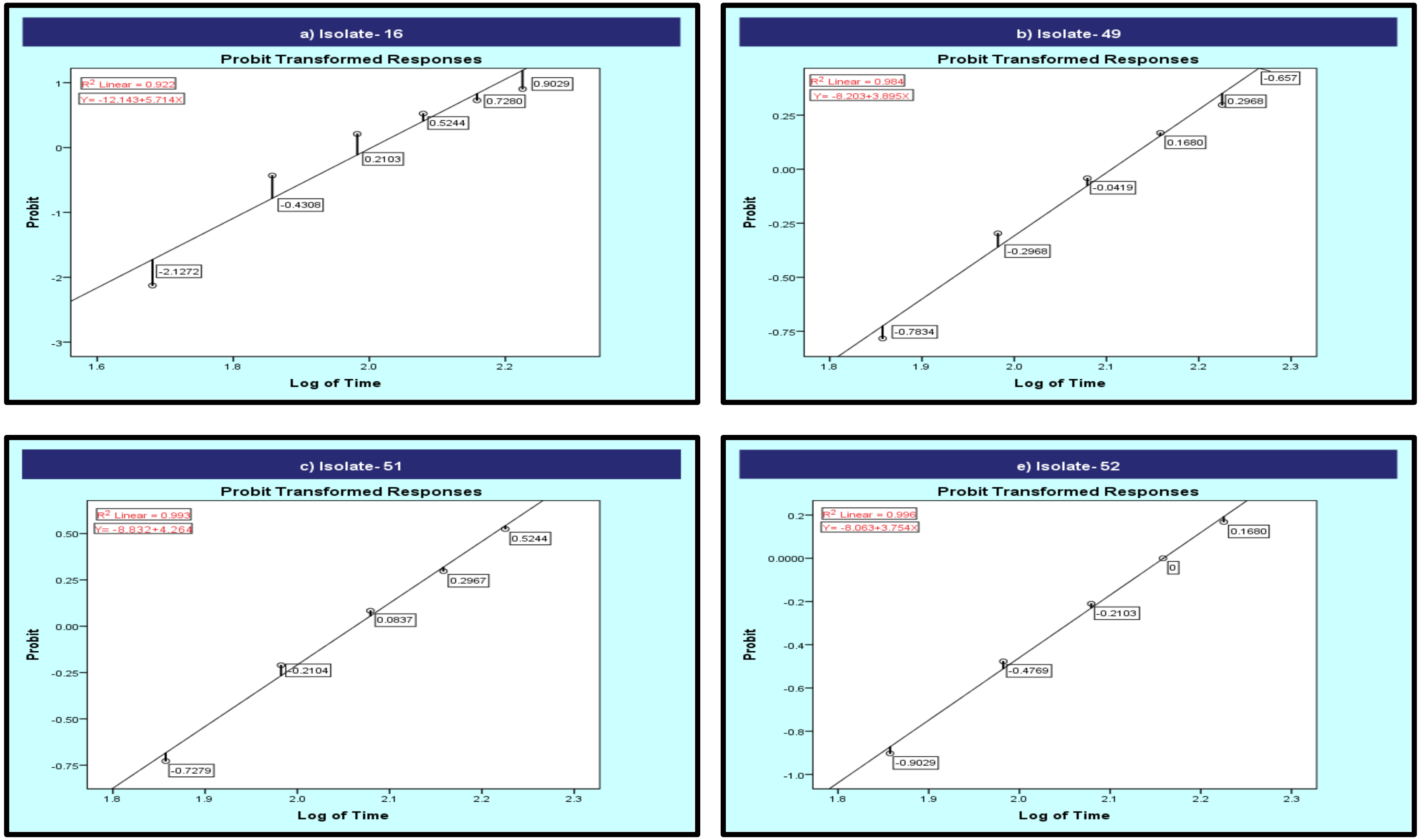
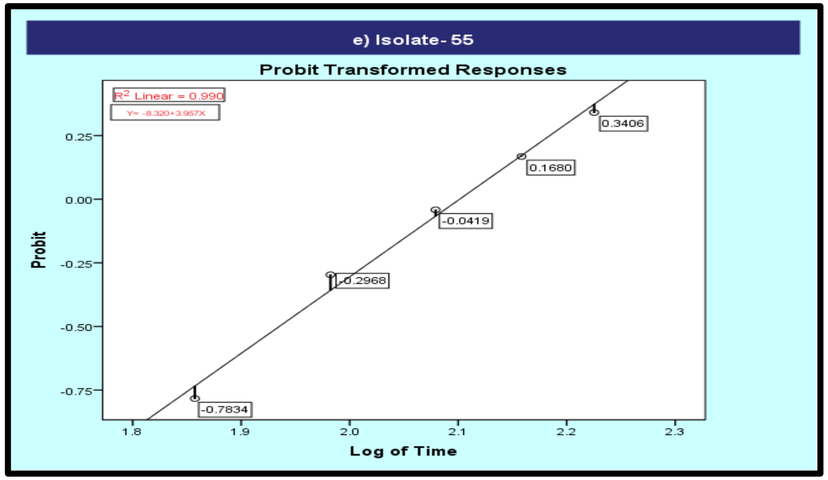
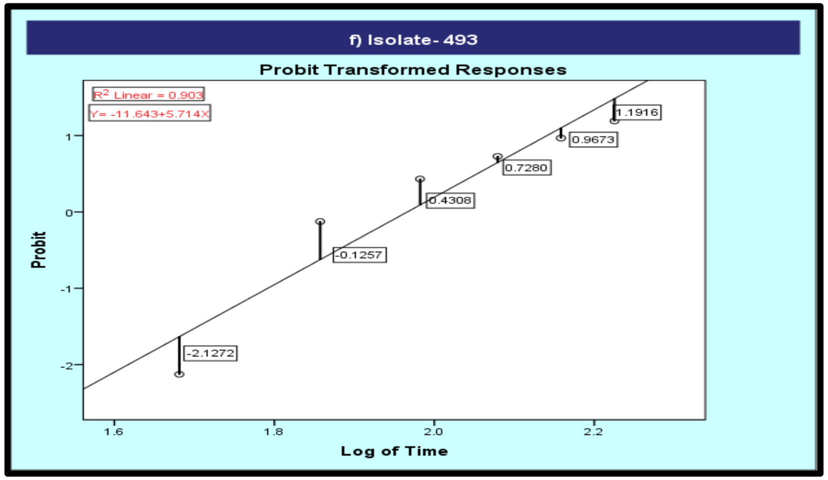


Fig. 4.8. **Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates grown on nano based ZnO mineral enriched medium at 1× 10^{12} CFU ml^{-1}**


e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.990
Y= -8.320+3.957X
0.25
0.00
-0.25
-0.50
-0.75
Probit
0.3406
0.1680
-0.0419
-0.2968
-0.7834
1.8
1.9
2.0
2.1
2.2
2.3
Log of Time


f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.903
Y= -11.643+5.714X
1
0
-1
-2
Probit
1.1916
0.9673
0.7280
0.4308
-0.1257
-2.1272
1.6
1.8
2.0
2.2
Log of Time

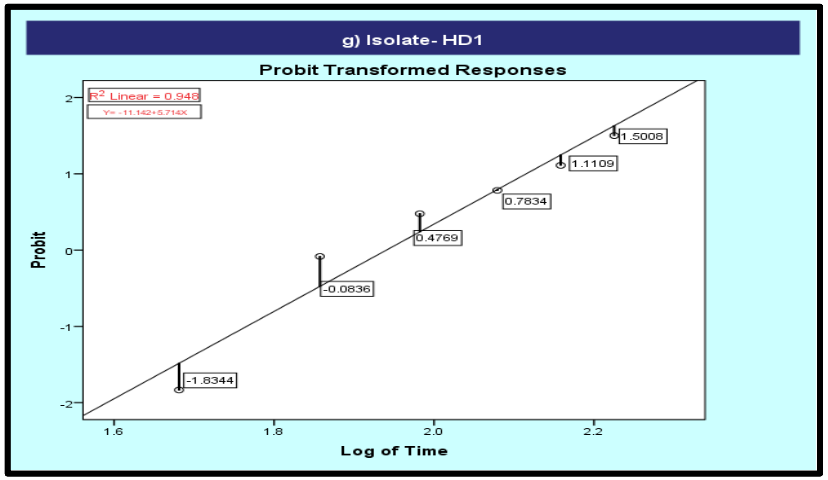

g) Isolate- HD1
Probit Transformed Responses
R2 Linear = 0.948
Y= -11.142+5.714X
2
1
0
-1
-2
Probit
1.5008
1.1109
0.7834
0.4769
-0.0836
-1.8344
1.6
1.8
2.0
2.2
Log of Time

Fig. 4.8. (Cont.)

4.3 TO EVALUATE *Bt* ISOLATES ON POD BORER, *H. armigera* (Hub.) IN LABORATORY

In the present investigation, seven *B. thuringeinsis* isolates including the standard reference strain *B. thuringeinsis* var. *kurstaki* (*Btk*-HD1) were subjected to bioassay against third instar larvae of *H. armigera*. Different life stages of *H. armigera* were shown in Plate 4.14.

The mean per cent larval mortality was observed to range between 23.33 to 91.67 at various concentrations tested *i.e.,* 1×10^{8} to 1×10^{12} CFU ml^{-1}. It was observed that the highest mortalities were recorded at a concentration of 1×10^{12} CFU ml^{-1}. Also, the mean per cent larval mortalities increased with increase in the concentration. Mortality was observed to be zero in both the control treatment and the treatment with Tween- 80. Among the seven isolates tested, it was observed that the highest mortality was recorded in the reference strain *Btk* HD1 at all the concentrations. The mortality in reference strain HD1 ranged from 56.67 to 91.67 per cent (1×10^{8} to 1×10^{12} CFU ml^{-1}). The *Bt* infected larvae was shown in Plate 4.15.

It was observed that among the native isolates tested, *Bt* isolate- 493 recorded highest mortality, and was on par with the reference strain at concentrations 1×10^{10} and 1×10^{12} CFU ml^{-1} concentrations with mean mortality of 70.00 and 88.33% respectively for *Bt* isolate- 493 and 76.67 and 91.67 per cent for reference strain. Among the native isolates, after 493, the next effective isolates were isolate- 16, isolate- 51, isolate- 55 and isolate- 49, which showed highest mortalities at 1×10^{12} CFU ml^{-1} concentration and were on par with each other at all concentrations except for the isolate 16 at 1×10^{12} CFU ml^{-1} and also at 1×10^{10} CFU ml^{-1} . The mean per cent mortality was recorded to be 78.33, 66.67, 63.33 and 61.67 per cent respectively, at 1×10^{12} CFU ml^{-1}.

The lowest mean per cent larval mortality was recorded in the isolate- 52, that ranged from 26.67 to 58.33 for 1×10^{8} to 1×10^{12} CFU ml^{-1} concentration. The mortality of *Bt* isolate- 52 was observed to be on par with isolates 51 and 55 at all the concentrations.

From the present investigation, it was evident that the mean per cent mortality with larvae increased with increase in the concentration of *Bt i.e*., increase in number of CFU, revealing a positive correlation with dose applied. The mean per cent mortalities at various concentrations for all the *Bt* isolates tested were given in Table. 4.13.

Similar work was carried out by Anusha(2021), where the same native *Bacillus thuringeinsis* isolates were used in the present study *i.e.,* I- 16, 49, 51, 52, 55, 493 along with reference strain HD1, but were tested against 3rd instar *M. vitrata* larvae using flower dip bioassay method. The mortalities recorded were 81.67, 60.00, 70.00, 65.00, 68.33 and 90.99 per cent respectively.

Similarly, Harika (2020) tested the same native *Bt* isolates that are used in the current study (I- 49, 51, 52, 55, 493 and HD1) using leaf disc bioassay against 3rd instar *Spodoptera frugiperda* and the mortalities were observed to be 60.00, 66.67, 56.67, 63.33, 86.67 and 91.67 per cent respectively.

4.3.1 Lethal Concentration (LC_{50} & LC_{90})

Among seven isolates, LC_{50} values ranged from 2.78×10^{7} to 7.73×10^{10} CFU ml^{-1}. The lowest median lethal concentration was 2.78×10^{7} CFU ml^{-1} and was observed in the reference strain HD1. Among the six native *Bt* isolates the least LC_{50} value was observed in the I-493 with 2.30×10^{8} CFU ml^{-1} followed by I-16, I-51, I-55 and I-49 with median lethal concentration of 1.75×10^{9}, 1.09×10^{10}, 2.90×10^{10} and 5.04×10^{10} respectively. The highest median lethal concentration was recorded by the I-52 (7.73×10^{10} CFU ml^{-1}) and was therefore found to be least effective of all the native *Bt* isolates tested.

Among seven isolates, LC_{90} values ranged from 5.89×10^{11} to 5.75×10^{16} CFU ml^{-1}. The lowest LC_{90} was 5.89×10^{11} CFU ml^{-1} and was observed in the reference strain HD1. Among the six native *Bt* isolates the least LC_{90} value was observed in the I-493 with 3.49×10^{12} CFU ml^{-1} followed by I-16, I-51, I-49 and I-55 with LC_{90} of 1.42×10^{14}, 3.59×10^{15}, 5.28×10^{15} and 1.13×10^{16} respectively. The highest median lethal concentration was recorded by the I-52 (5.57×10^{16} CFU ml^{-1}) and was therefore found to be minimal effective of all the native *Bt* isolates tested.

The values of chi-square (χ^2), regression equation, LC_{50} & LC_{90} values and fiducial limits pertaining to the tested *B. thuringeinsis* isolates were presented in the Table 4.14. The regression equation clearly depicted that mortality of larvae increased with increase in the dose. The chi-square values showed no heterogeneity of the test population, which indicated the goodness of fit. Lethal concentration response was plotted against probit mortality in Fig.4.9.

The results were in acceptance with Lone *et al*. (2017) who estimated the Median lethal concentration (LC_{50}) of *Bt*-JK12, 17, 22 and 48 against second instar larvae of *H. armigera*. The LC_{50} values were observed to be 184.62, 275.39, 256.29 and 259.93 µg ml^{-1}, respectively. It was observed that *B. thuringiensis* isolate JK12 exhibited higher toxicity against *H. armigera* than that of *B. thuringiensis* HD1, hence can be commercially exploited to control insect pest for sustainable crop productio

Table 4.13. Mean per cent mortality of third instar larvae of *H. armigera* with native isolates of *Bt*

Concentration/ isolate	HD1	I-16	I-49	I-51	I-52	I-55	I-493
1×10^{8} CFU ml^{-1}	56.67 (48.84)^{hijk}	38.33 (38.24)^{no}	23.33 (28.86)^{r}	33.33 (35.25)^{opq}	26.67 (31.07)^{qr}	28.33 (32.14)^{pqr}	46.67 (43.09)^{lmn}
1×10^{9} CFU ml^{-1}	68.33 (55.77)^{ef}	46.67 (43.09)^{lmn}	35.00 (36.27)^{opq}	38.33 (38.24)^{no}	33.33 (35.25)^{opq}	36.67 (37.26)^{op}	56.67 (48.84)^{hijk}
1×10^{10} CFU ml^{-1}	76.67 (61.14)^{de}	58.33 (49.80)^{ghij}	41.67 (40.20)^{mno}	48.33 (44.04)^{klm}	41.67 (40.20)^{mno}	46.67 (43.09)^{lmn}	70.00 (56.79)^{def}
1×10^{11} CFU ml^{-1}	85.00 (67.21)^{bc}	65.00 (57.73)^{fgh}	55.00 (47.87)^{ijkl}	61.67 (51.76)^{fghij}	53.33 (46.91)^{jkl}	55.00 (47.87)^{ijkl}	76.67 (61.14)^{de}
1×10^{12} CFU ml^{-1}	91.67 (73.41)^{a}	78.33 (62.29)^{cd}	61.67 (51.76)^{fghij}	66.67 (54.75)^{fg}	58.33 (49.80)^{ghij}	63.33 (52.74)^{efghi}	88.33 (70.12)^{ab}
Tween80- 0.1 %		0.00 %					
Control		0.00 %					
		Isolates		**Concentrations**		**Isolate*concentrations**	
SEm ±		0.620		0.521		1.380	
CD (p=0.05)		1.23		1.04		2.75	
CV (%)		3.52					

* Mean of three replications (N=20); values in paranthesis are arc sine transformed; Mean values followed by same alphabet in a column are not significantly different by Tukeys HSD(P=0.05)

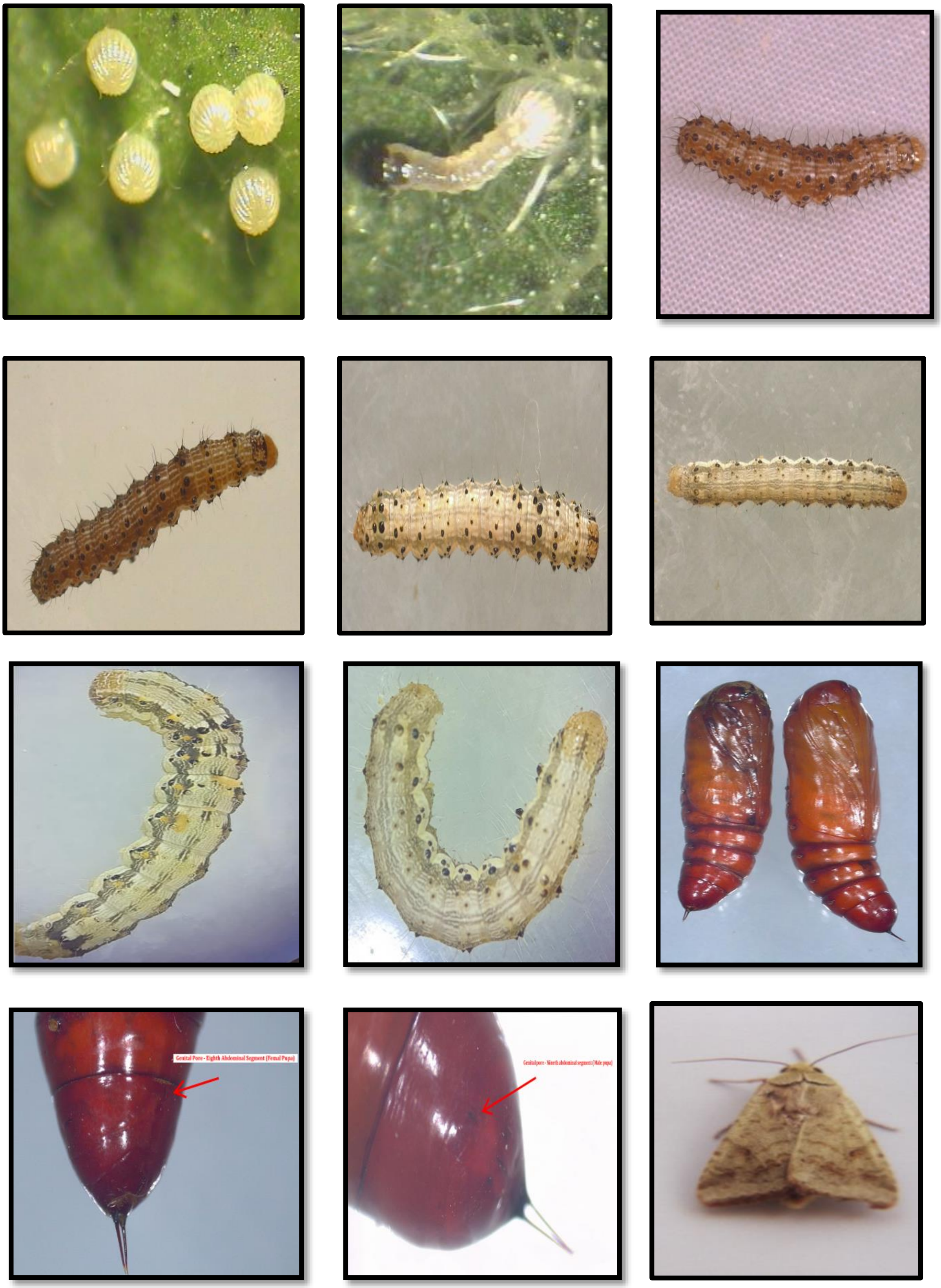


Plate 4.14. Different life stages of *Helicoverpa armigera*

Plate 4.15. ***Bacillus thuringeinsis*** **infected larvae of** ***H. armigera***

4.3.2 Lethal Time (LT_{50} & LT_{90})

The LT_{50} & LT_{90} values of the seven *Bt* isolates were evaluated for a uniform highest dose of 1×10^{12} CFU ml^{-1} against 3rd instar larvae of *H. armigera* (Table 4.15). A lethal time response was plotted against probit mortality in the Fig. 4.10.

The median lethal time values of the *Bt* isolates tested ranged from 104.62 to 140.57 hours. Among the *Bt* isolates tested, it was found that the reference strain HD1 was found to kill the larvae faster comparatively and achieved 50 per cent mortality in the larvae at 104.62 hours. Median lethal time values of six native isolates ranged from 113.99 to 140.57 hours. Among the six native *Bt* isolates, I-493 has achieved the fastest mortality on 3rd instar larvae of *H. armigera* with 113.99 hpi, followed by I-16, I-51, I-49 and I-55 with 119.95, 130.78, 136.51 and 136.53 hpi respectively. Among the native *Bt* isolates tested the *Bt* isolate-52 showed slower kill with fifty per cent mortality at 140.57 hours.

The LT_{90} values of the *Bt* isolates tested ranged from 162.47 to 245.93 hours. Among the *Bt* isolates tested, it was found that the reference strain HD1 was found to kill the larvae faster comparatively and achieved 90 per cent mortality in the larvae at 162.47 hours. LT_{90} values of six native isolates ranged from 178.54 to 245.93 hours. Among the six native *Bt* isolates, I-493 has achieved the fastest mortality on 3rd instar larvae of *H. armigera* with 178.54 hpi, followed by I-16, I-51, I-52 and I-49 with 195.03, 226.32, 226.34 and 245.35 hpi, respectively. Among the native *Bt* isolates tested the *Bt* isolate-55 showed the slower kill with ninety per cent mortality at 245.93 hours.

The outcome was in confirmity with the work done by Murali Krishna *et al.* (2018) who given that the LT_{50} values of four isolates, F468, F493, N30 & N115 were 74.28, 78.52, 95.70 & 88.68 h, respectively, for the isolates tested against *S. litura*.

Table 4.14. Median Lethal Concentration response at 50 per cent mortality of third instar larvae of *H. armigera* with native isolates of *Bt*

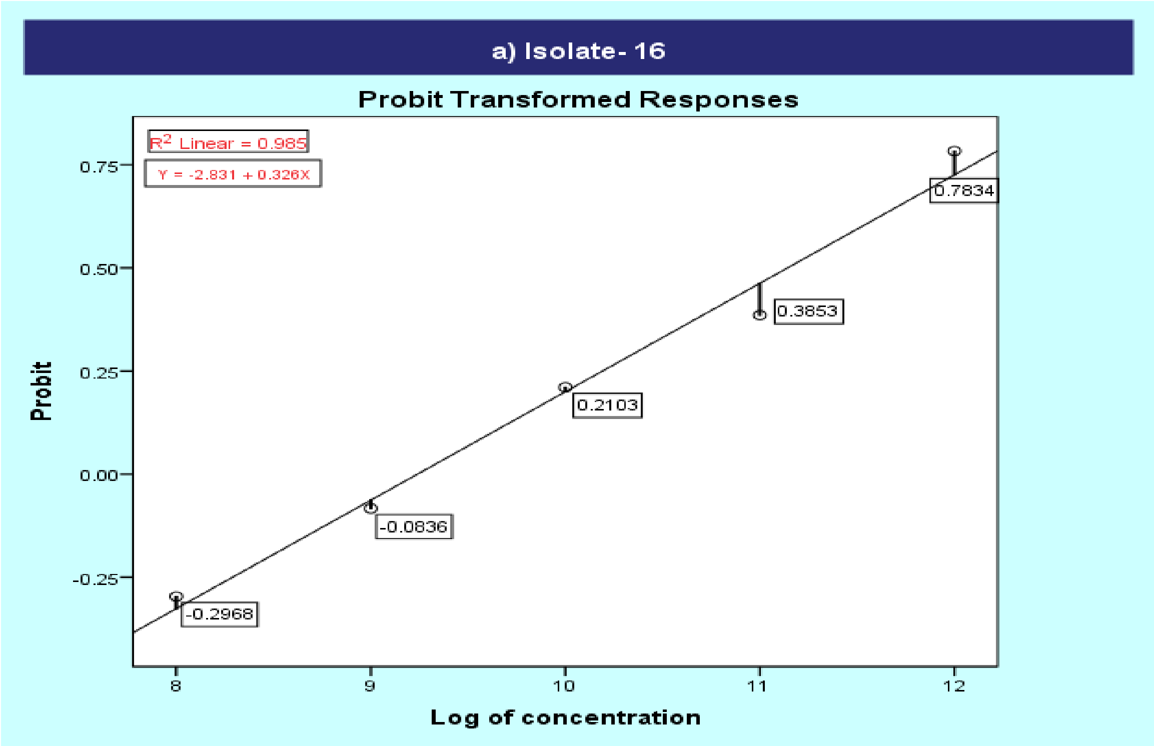
Isolate	Chi-square (χ^2)	Regression equation	*LD_{50} (CFU ml^{-1})	Fiducial limit (CFU ml^{-1})
I-HD1	0.107	Y= -2.812 + 0.353 X	2.78×10^7	(2.09×10^6)-(1.24×10^8)
I-16	0.614	Y= -2.831 + 0.326 X	1.75×10^9	(4.50×10^8)-(4.81×10^9)
I-49	0.513	Y= - 2.732 + 0.255X	5.04×10^{10}	(1.80×10^{10})-(1.96×10^{11})
I-51	0.736	Y= - 2.331 + 0.232X	1.09×10^{10}	(3.37×10^9)-(3.60×10^{10})
I-52	0.329	Y= - 2.756 + 0.254X	7.73×10^{10}	(2.30×10^{10})-(4.74×10^{11})
I-55	0.047	Y= - 2.613 + 0.251X	2.90×10^{10}	(9.38×10^9)-(1.18×10^{11})
I-493	0.591	Y = - 2.562 + 0.306 X	2.30×10^8	(4.40×10^7)- (6.78×10^8)

* Mean of three replications (N=20)

Table 4.15. Median Lethal Time response at 50 per cent mortality of third instar larvae of *H. armigera* with native isolates of *Bt* at 1×10^{12} CFU ml^{-1}

Isolate	Chi-square (χ^2)	Regression equation	*LT_{50} (hpi) at 1×10^{12} CFU ml^{-1}	Fiducial limit (hpi)
I-HD1	1.904	Y= - 13.539 + 6.704 X	104.62	100.07 – 109.18
I-16	2.072	Y= - 12.621 + 6.071 X	119.95	114.58 – 125.73
I-49	5.023	Y= - 11.070 + 5.099 X	136.51	129.10 – 145.63
I-51	4.213	Y= - 11.389 + 5.381 X	130.78	124.24 – 138.45
I-52	6.631	Y= - 10.025 + 4.667 X	140.57	132.22 – 151.25
I-55	4.288	Y= - 11.760 + 5.014 X	136.53	129.08 – 145.70
I-493	1.877	Y= - 13.526 + 6.576 X	113.99	109.13 – 119.05

* Mean of three replications (N=20)



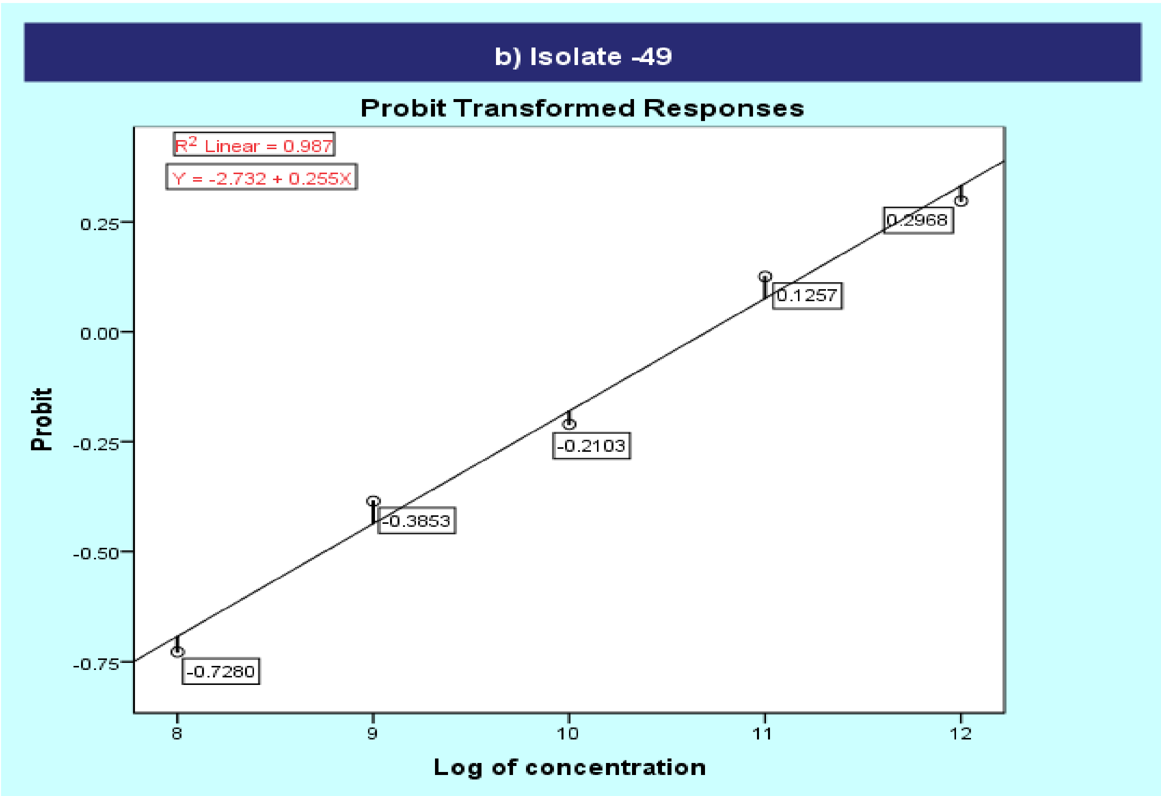
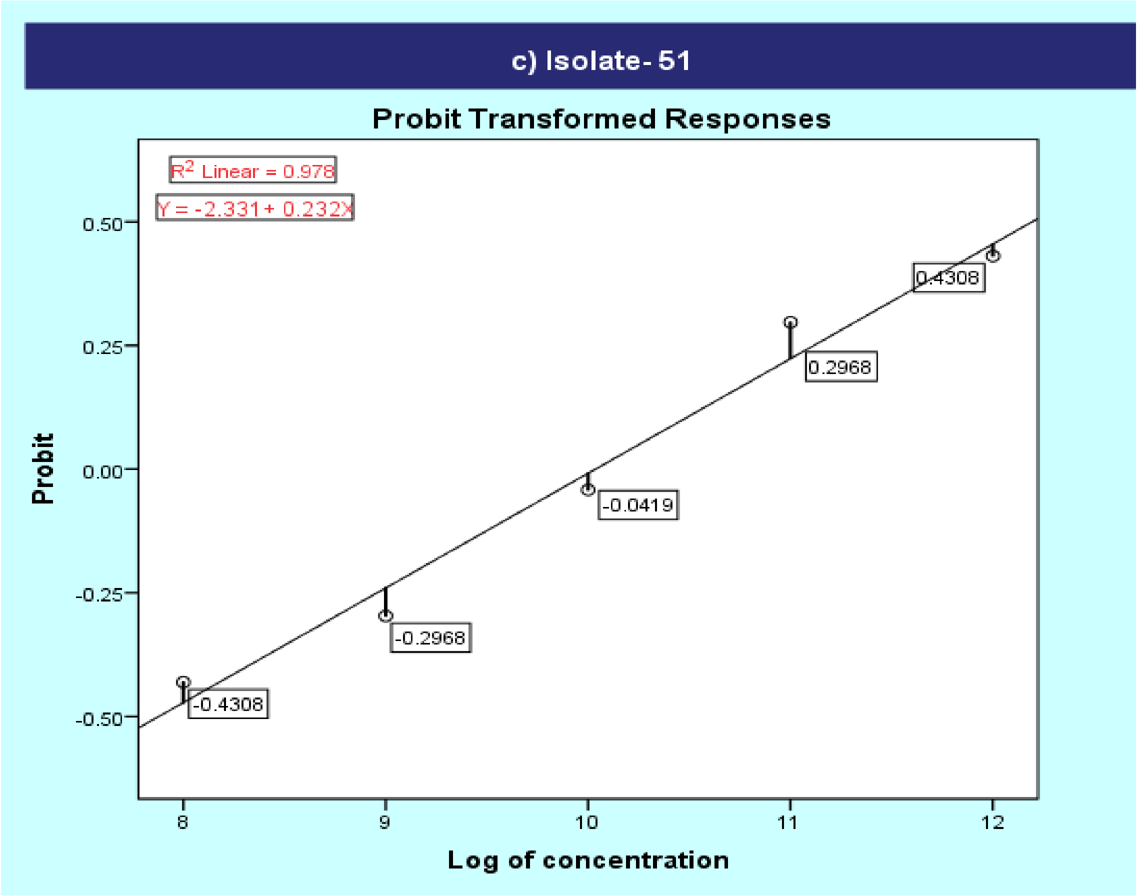


Fig. 4.9. **Median Lethal Concentration mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates**

Fig. 4.9. (Cont.)


c) Isolate- 51
Probit Transformed Responses
R2 Linear = 0.978
Y = -2.331+ 0.232X
0.50
0.25
0.00
-0.25
-0.50
Probit
0.4308
0.2968
-0.0419
-0.2968
-0.4308
8
9
10
11
12
Log of concentration

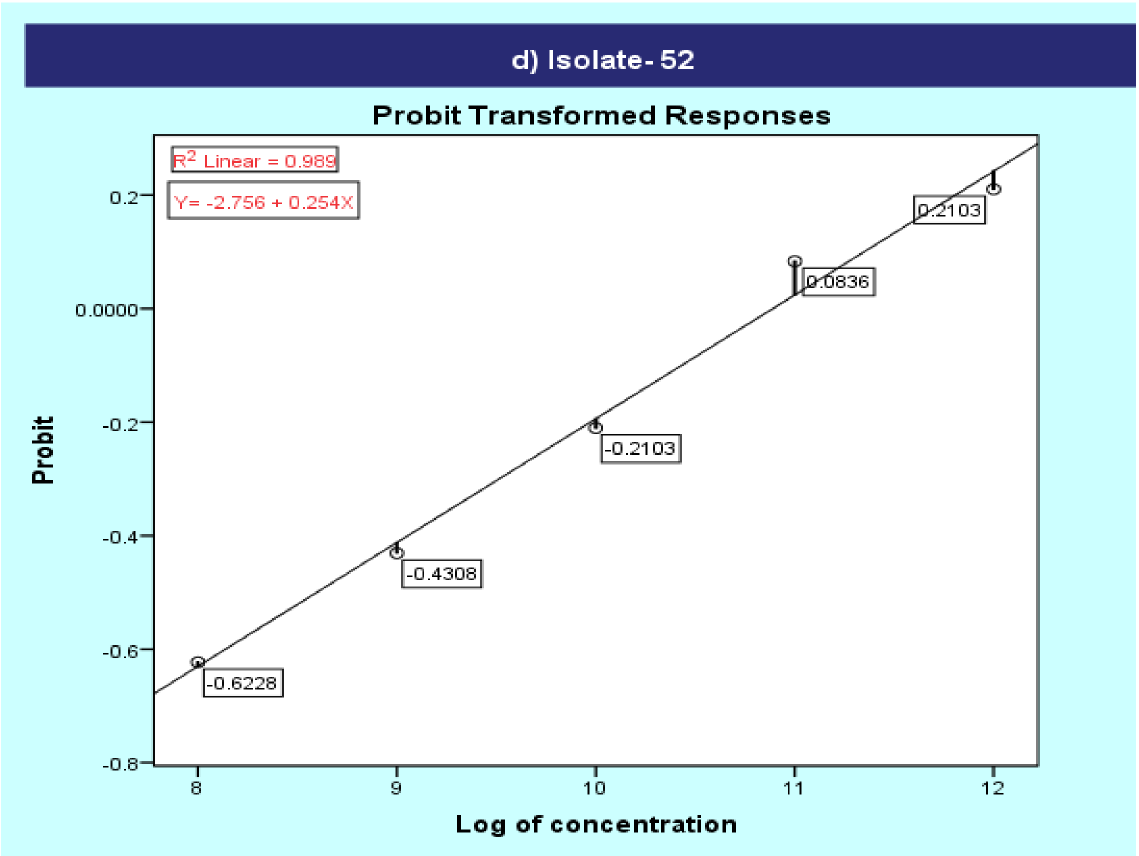
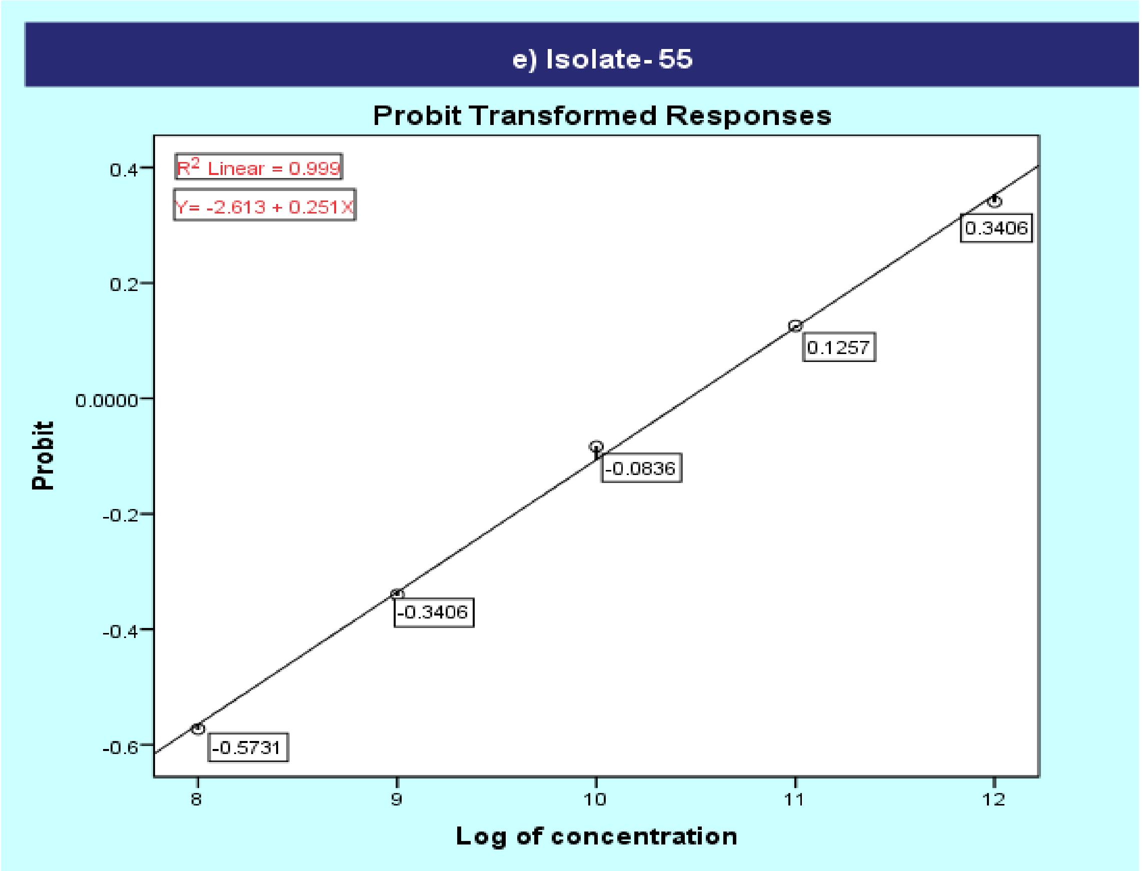
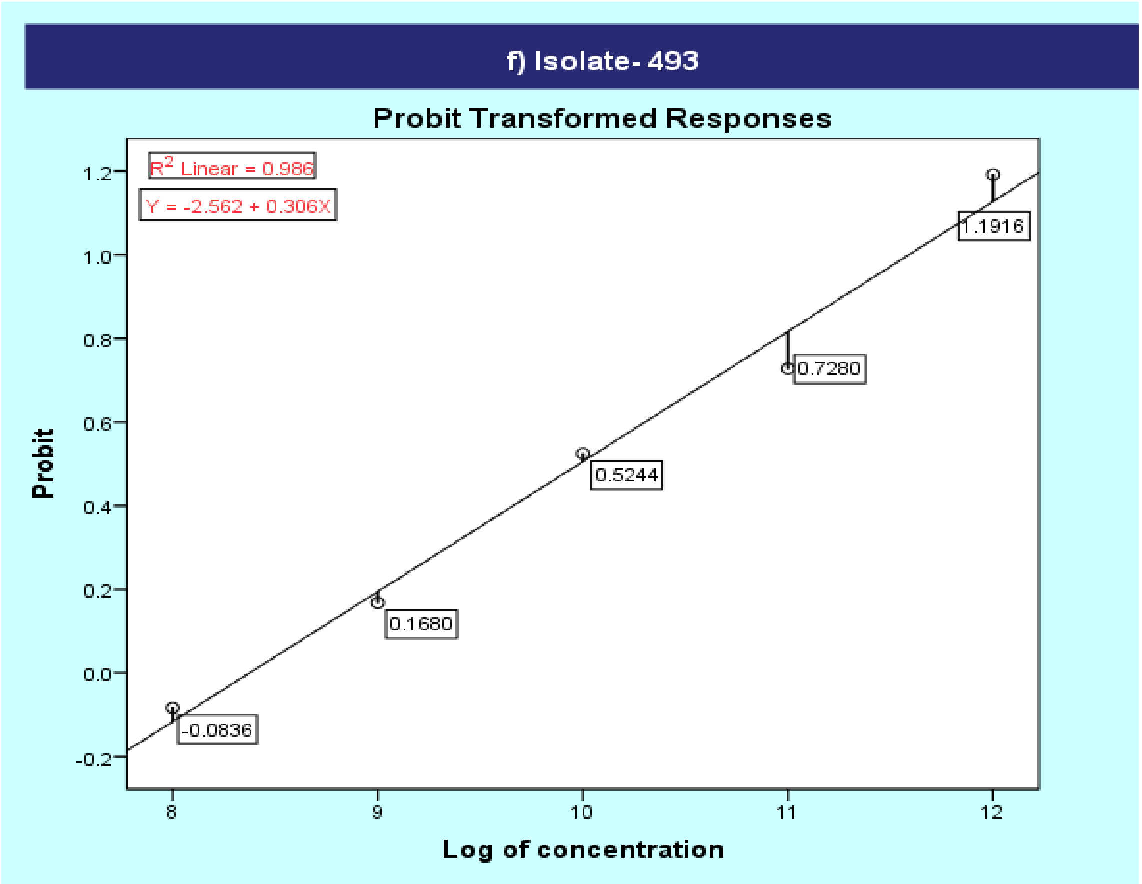

d) Isolate- 52
Probit Transformed Responses
R2 Linear = 0.989
Y= -2.756 + 0.254X
0.2
0.0000
-0.2
-0.4
-0.6
-0.8
Probit
0.2103
0.0836
-0.2103
-0.4308
-0.6228
8
9
10
11
12
Log of concentration

Fig. 4.9. (Cont.)



e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.999
Y= -2.613 + 0.251X
0.3406
0.1257
-0.0836
-0.3406
-0.5731
Probit
Log of concentration



f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.986
Y = -2.562 + 0.306X
1.1916
0.7280
0.5244
0.1680
-0.0836
Probit
Log of concentration

Fig. 4.9. (Cont.)

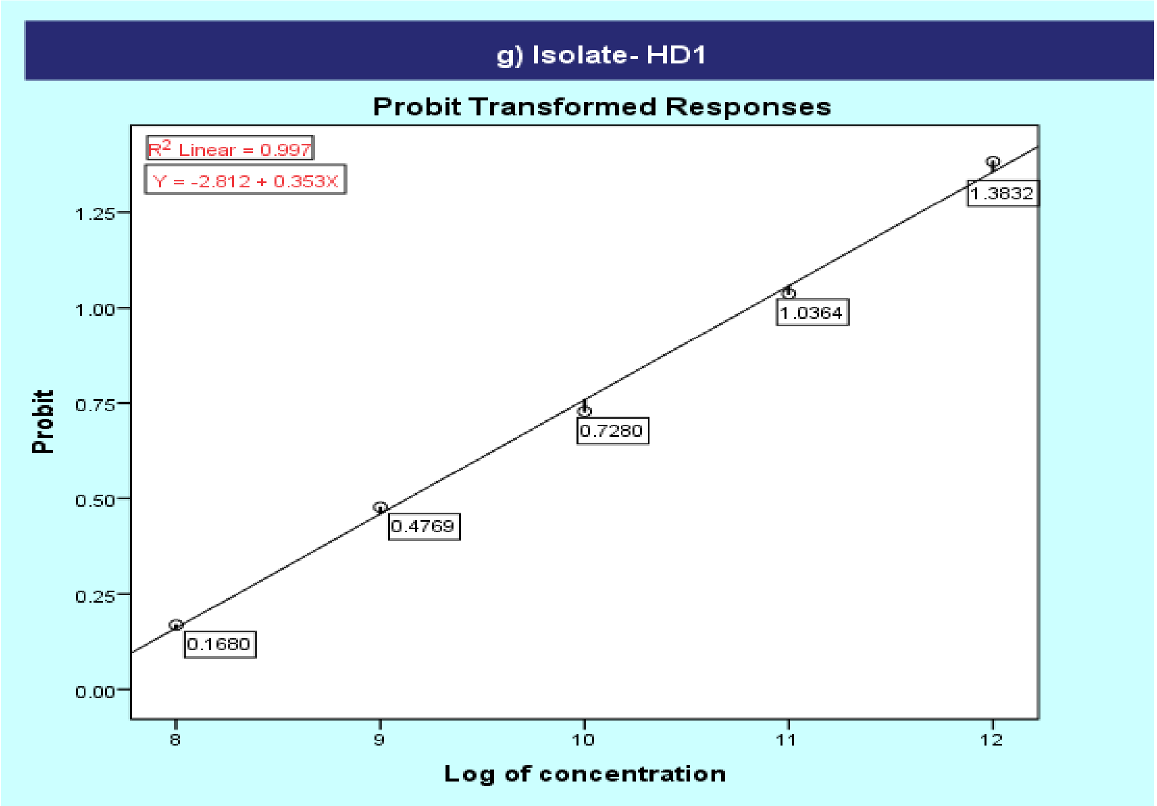


Fig. 4.9. (Cont.)

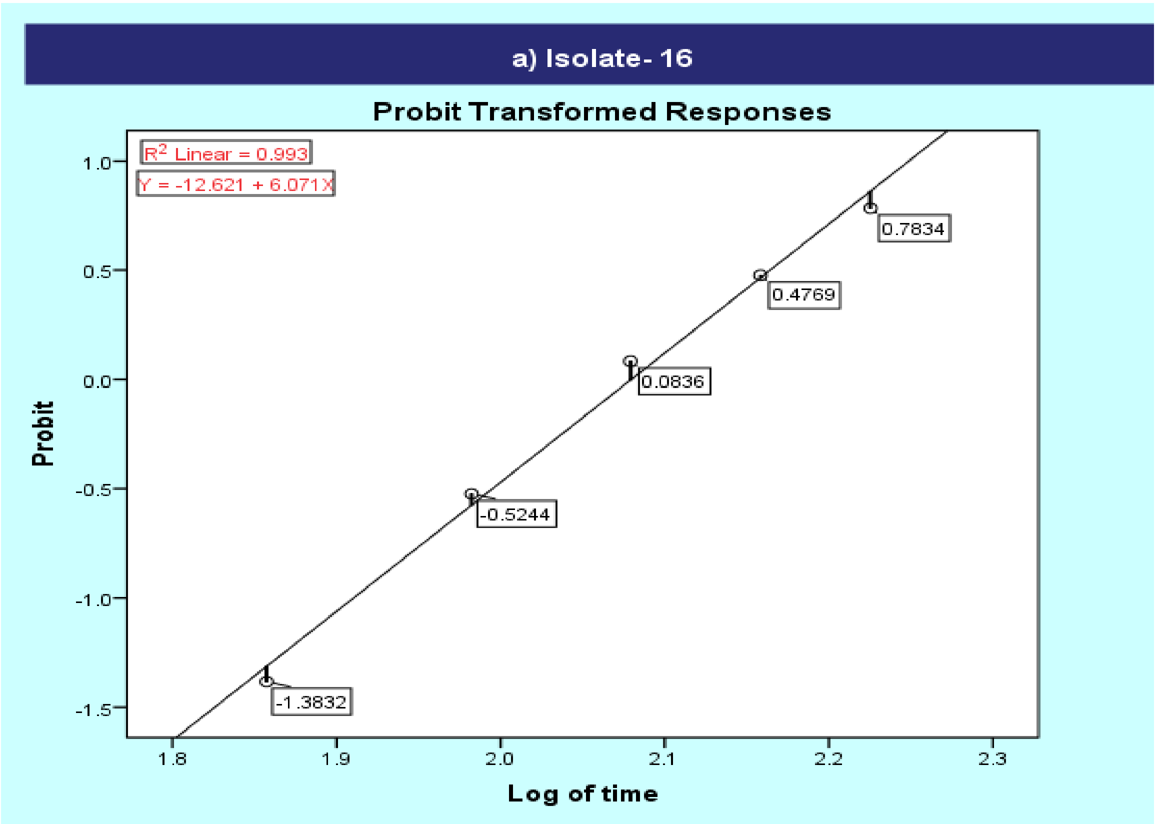
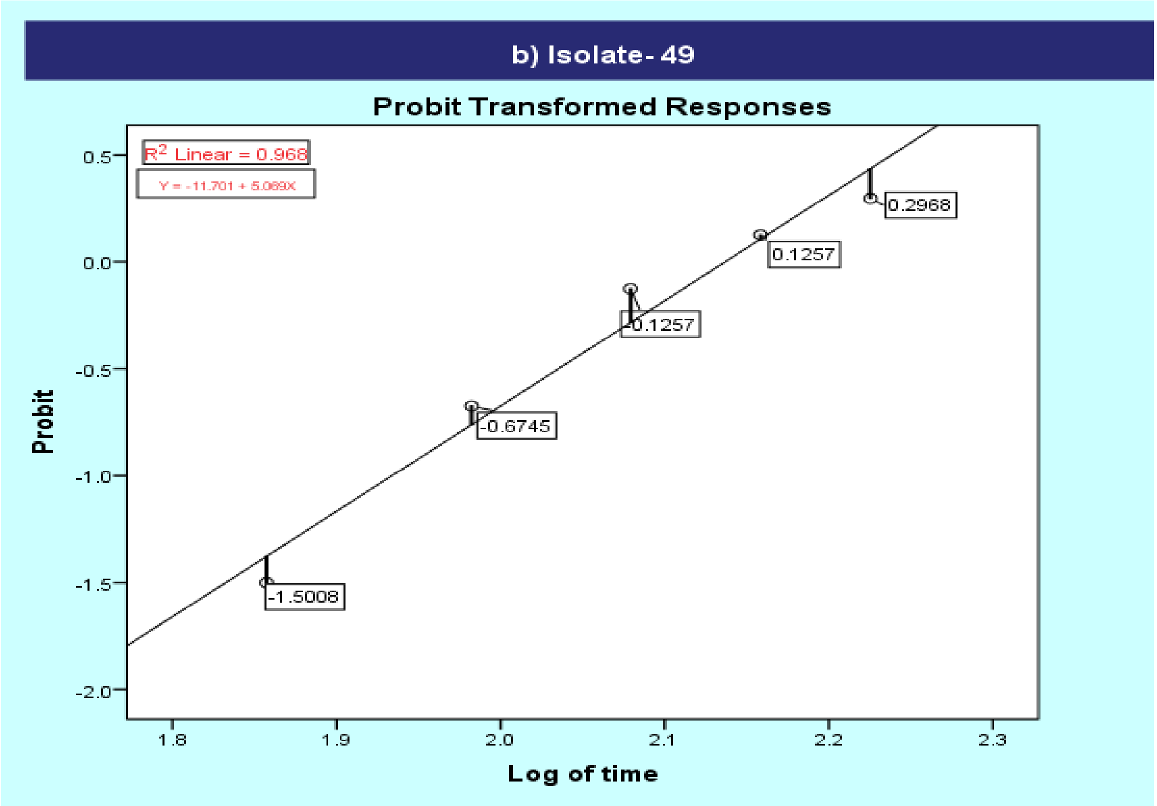


Fig. 4.10. Median Lethal Time mortality response of 3rd instar larvae of *H. armigera* with *Bt* isolates at 1× 10^{12} CFU ml^{-1}


b) Isolate- 49
Probit Transformed Responses
R² Linear = 0.968
Y = -11.701 + 5.069X
0.2968
0.1257
-0.1257
-0.6745
-1.5008
Probit
0.5
0.0
-0.5
-1.0
-1.5
-2.0
1.8
1.9
2.0
2.1
2.2
2.3
Log of time

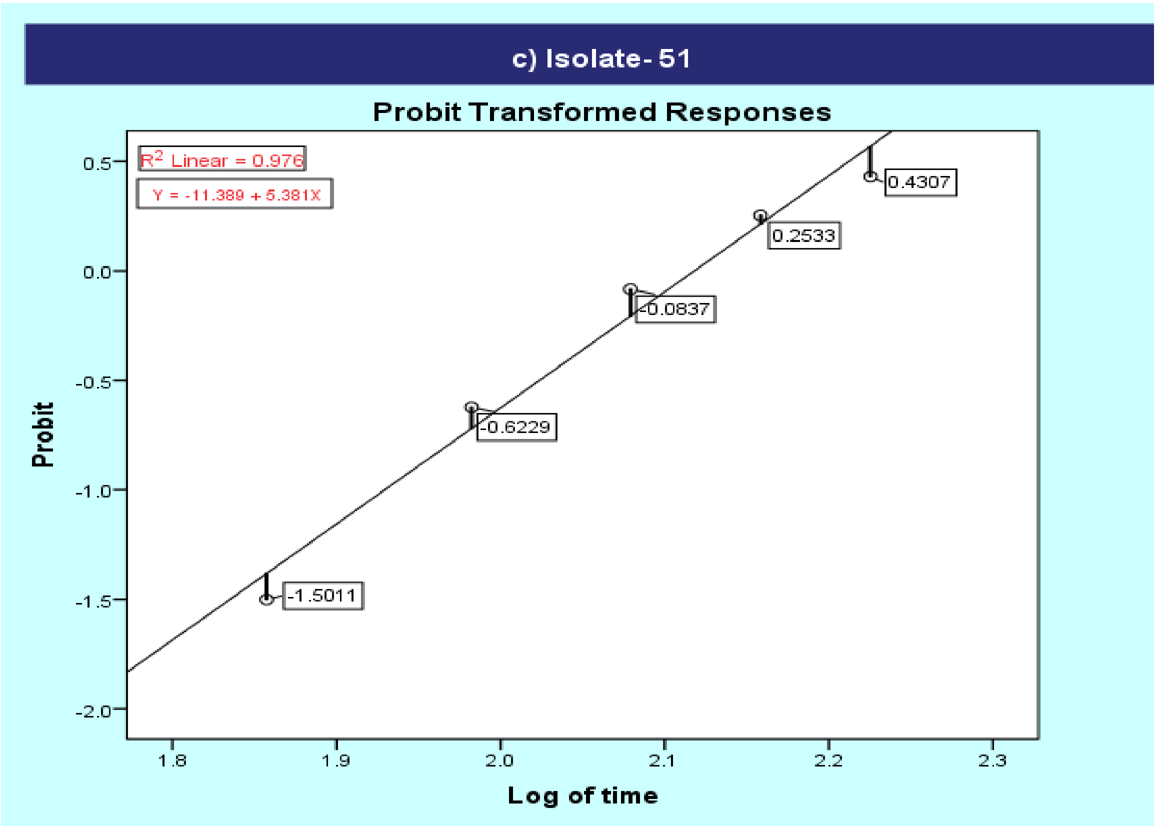
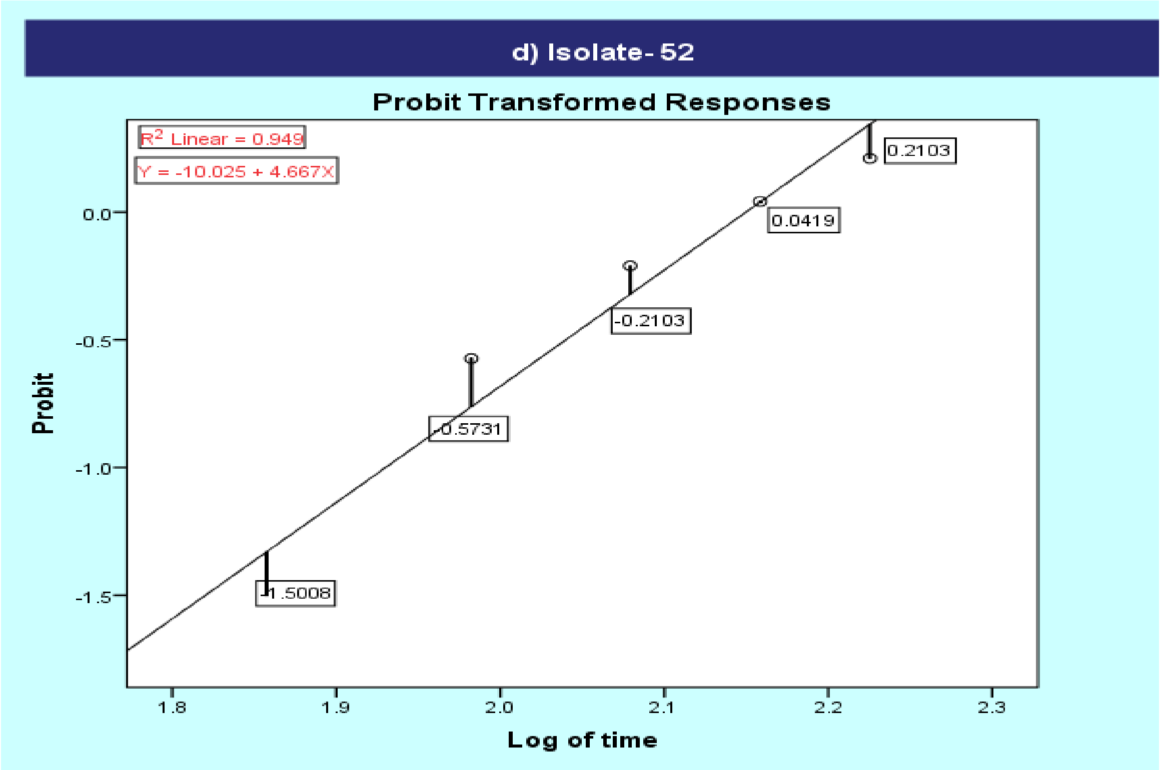
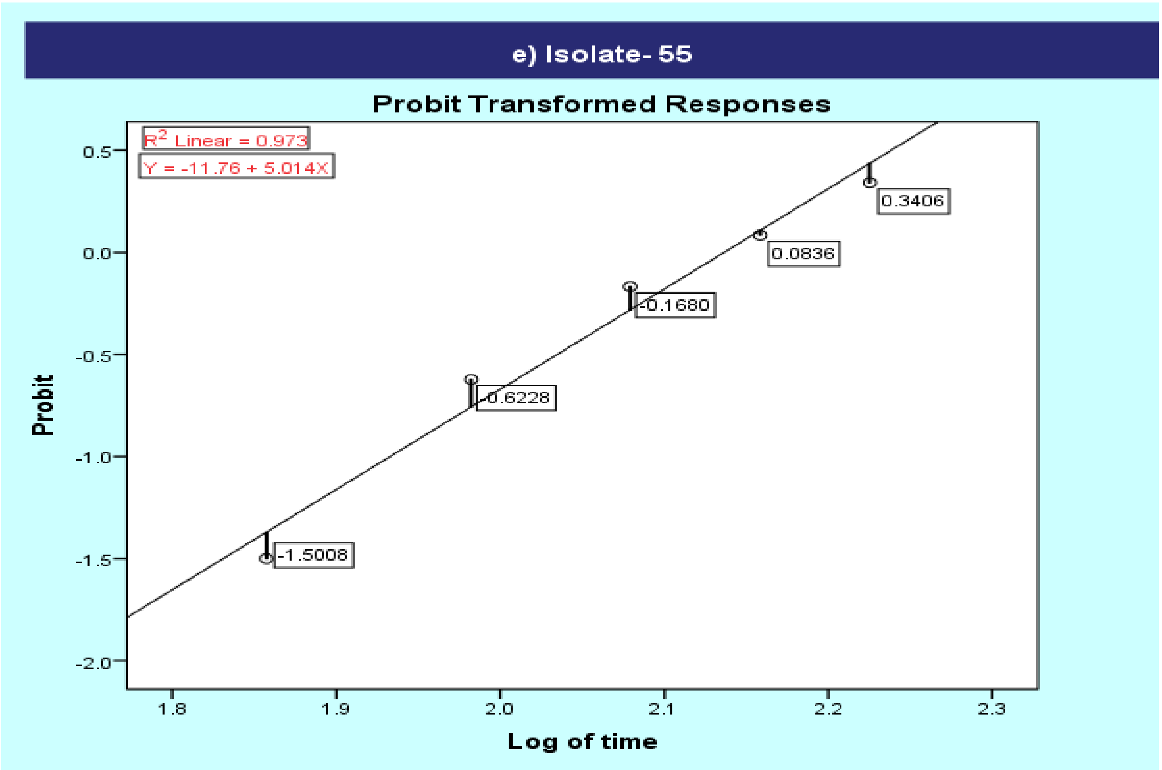

c) Isolate- 51
Probit Transformed Responses
R² Linear = 0.976
Y = -11.389 + 5.381X
0.4307
0.2533
-0.0837
-0.6229
-1.5011
Probit
0.5
0.0
-0.5
-1.0
-1.5
-2.0
1.8
1.9
2.0
2.1
2.2
2.3
Log of time

Fig. 4.10. (Cont.)

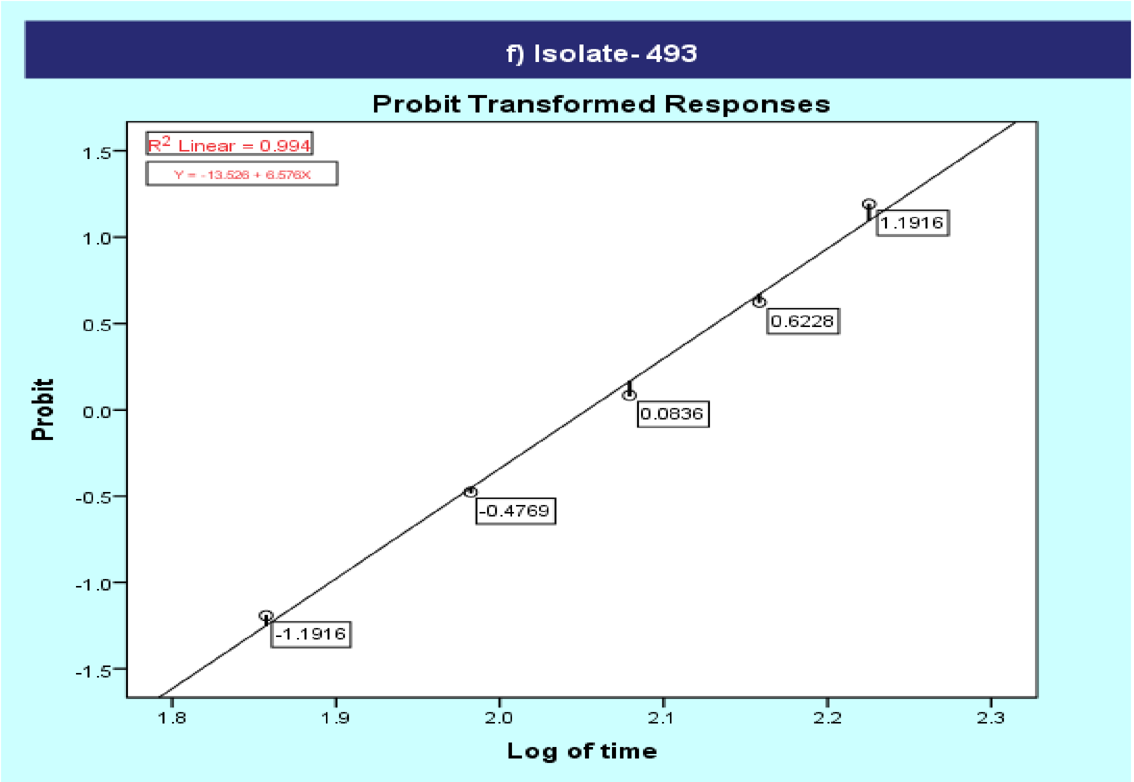


d) Isolate- 52
Probit Transformed Responses
R2 Linear = 0.949
Y = -10.025 + 4.667X
0.2103
0.0419
-0.2103
-0.5731
-1.5008
0.0
-0.5
-1.0
-1.5
Probit
1.8
1.9
2.0
2.1
2.2
2.3
Log of time



e) Isolate- 55
Probit Transformed Responses
R2 Linear = 0.973
Y = -11.76 + 5.014X
0.3406
0.0836
-0.1680
-0.6228
-1.5008
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Probit
1.8
1.9
2.0
2.1
2.2
2.3
Log of time

Fig. 4.10. (Cont.)


f) Isolate- 493
Probit Transformed Responses
R2 Linear = 0.994
Y = -13.526 + 6.576X
1.1916
0.6228
0.0836
-0.4769
-1.1916
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Probit
1.8
1.9
2.0
2.1
2.2
2.3
Log of time

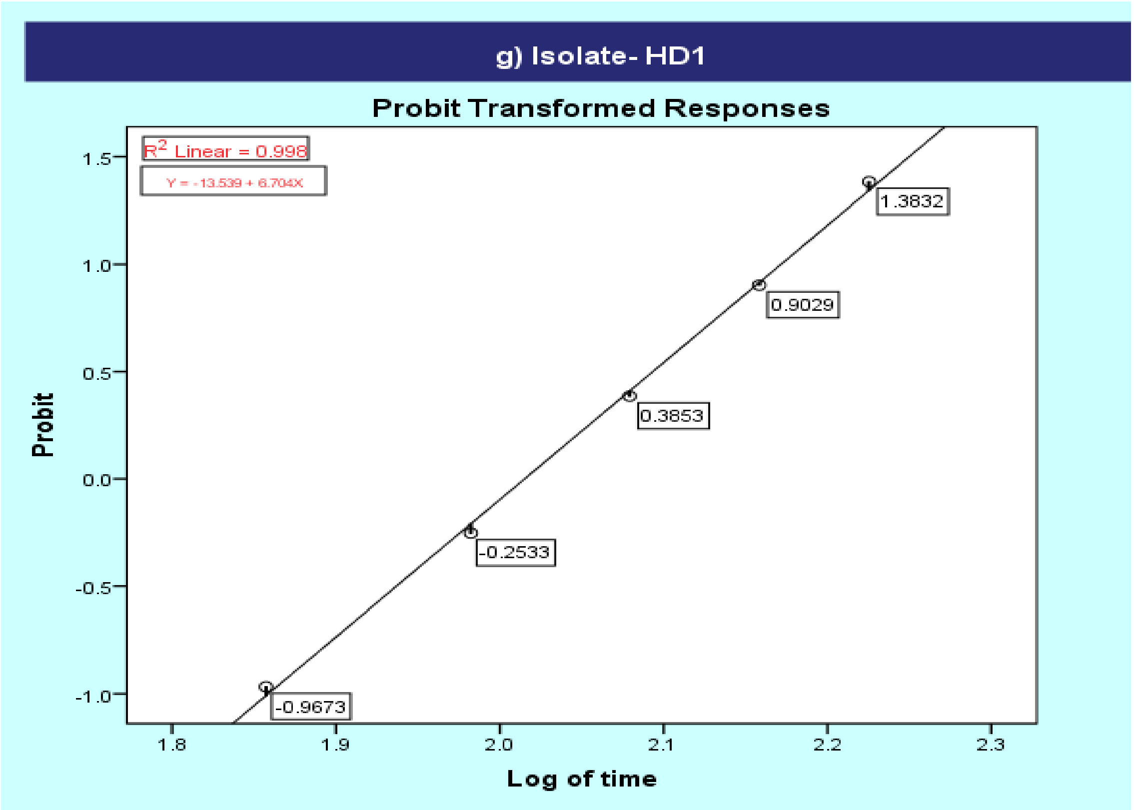

g) Isolate- HD1
Probit Transformed Responses
R2 Linear = 0.998
Y = -13.539 + 6.704X
1.3832
0.9029
0.3853
-0.2533
-0.9673
1.5
1.0
0.5
0.0
-0.5
-1.0
Probit
1.8
1.9
2.0
2.1
2.2
2.3
Log of time

Fig. 4.10. (Cont.)

Chapter – V

Summary and Conclusions

Chapter V

SUMMARY AND CONCLUSIONS

The present investigation on '**Molecular characterisation of native *Bt* isolates and laboratory evaluation of their nano-formulations on *Helicoverpa armigera* (Hub.)'** was conducted during 2021-22 at Agricultural College, Bapatla. The results pertaining to the above study was summarized below.

Biological diversity is variability among the organisms. The diversity is basis for the continuous evolution and maintains the life sustaining systems in biosphere. An experiment conducted using RAPD analysis revealed the genetic relationship among the seven *Bt* isolates. A total of 10 decamer primers were used in the genetic diversity studies of the *Bt* isolates. A total of 123 amplicons were observed from all the 10 primers. Among the total amplicons observed, 95.93 per cent were found to be polymorphic and 4.07 per cent was found to be monomorphic. From the gel images, binary data was scored and was normalized.

Dendrogram was constructed using the binary data derived from the gel images. The seven isolates fell into two separate clusters I and II. From the dendrogram, it was observed that the isolate 16, 51, 52 and 493 fell in cluster I , where as, the remaining isolates 49, 55 and HD1 fell into the cluster II. Similarity index matrix for the isolates revealed that the highest similarity was observed between the isolate 49 and HD1 with a similarity index of 0.71 and the least similarity index was observed between isolate 51 and HD1 with a similarity index of 0.50.

PCoA was done for the isolates to know the genetic variation among the *Bt* isolates. It was observed that all the isolates were found to be within the eclipse. Isolates 16 and 52 were observed to be found closer to coordinate 1 revealing close genetic relationship between the isolates, while, HD1 and isolate 49 was found to be far away from coordinate 2 revealing close relation between these isolates and high divergence with these remaining isolates.

Crystal protein profiling of *Bt* isolates was done using SDS-PAGE. The results revealed the presence of band at 130-140 kDa indicating presence of Cry1 protein in isolates-16 and in the refernce strain (HD1), band was observed at 92kDa indicating the presence of Vip3 protein in isolate- 49 & 493 and Cry2 protein of 65-70 kDa was observed in isolates- 51, 52 and 55 were observed.

To evaluate the efficacy of *Bt* isolates that were grown on nano mineral enriched media, where CaO, MgO and ZnO nanoparticles at 20ppm were incorporated into the media. Among the isolates tested for their efficacy against 3rd instar larvae of *H. armigera*, *Bt* isolates grown on CaO based nano mineral enriched media was found to be more effective compared to the *Bt* isolates grown on MgO based nano mineral enriched media. Mortalities were observed from 48h after treatment for the isolates maintained on CaO and MgO enriched media. The maximum mortality was observed at 72h after treatment for the isolates.

The mortalities ranged from 21.33 to 96.67 per cent for the isolates. Among the isolates grown on CaO based nano mineral enriched media, the mortalities ranged from 25 to 96.67 per cent. For the isolates maintained on MgO based nano mineral enriched media, the mortalities ranged from 23.33 to 95 per cent and for ZnO based nano mineral enriched media, the mortalities were found to fall between 21.67 to 93.33 per cent.

Among all the isolates, the highest mortality was observed in the reference strain, HD1 for all the three nanoparticulates *i.e.*, 96.67, 95 and 93.33 per cent for *Bt* maintained on CaO, MgO and ZnO based nano mineral enriched media, respectively. Among the native isolates, isolate 493 recorded highest mortalities. The highest mortality was observed in *Bt* grown on CaO followed by MgO and ZnO based nano enriched media *i.e.,* 91.67, 90.00 and 88.33 per cent, respectively. The lowest mortality was recorded in the isolate 49 for CaO based nano mineral enriched media *i.e.,* 65 per cent and for MgO based *Bt* the lowest mortality was recorded for the isolates 49 and 52 *i.e.,* 63.33 per cent and for ZnO based *Bt*, the lowest mortality was observed in Isolate 52 *i.e.,* 56.67 per cent.

The LC_{50} values for the *Bt* isolates maintained on CaO based nano mineral enriched media was found to be 3.95×10^{7} to 5.68×10^{10} CFU ml^{-1} and for MgO and ZnO based *Bt* isolates the LC_{50} values were in the range of 3.09×10^{7} to 4.16×10^{10} CFU ml^{-1} and 3.86×10^{7} to 1.23×10^{11} CFU ml^{-1}, respectively. The LT_{50} values for the *Bt* isolates maintained on CaO based nano mineral enriched media was found to be 81.22 to 135.43 hpi and for MgO and ZnO based *Bt* isolates the LT_{50} values were in the range of 82.80 to 128.78 hpi and 83.52 to 140.57 hpi, respectively.

The diet incorporation bioassay experiments were conducted against the 3rd instar larvae of *H. armigera*; the *Bt* isolates recorded mortalities that ranged from 23.33 to 91.67 per cent. The mortality was found to be recorded highest with HD1 strain (91.67%) and

The diet incorporation bioassay experiments were conducted against the 3rd instar larvae of *H. armigera*; the *Bt* isolates recorded mortalities that ranged from 23.33 to 91.67 per cent. The mortality was found to be recorded highest with HD1 strain (91.67%) and the least mortality was observed in the isolate 52, (58.33%). All the seven isolates exerted mortality greater than 50 per cent at a concentration of 1×10^{12}.

The LC_{50} was determined using the mortality of 50 per cent population of *H. armigera* with *Bt* isolates and was recorded from 2.78×10^{7} to 7.73×10^{10} CFU ml^{-1}. The LC_{50} for the reference strain, HD1 was recorded to be the least (2.78×10^{7} CFU ml^{-1}). The highest LC_{50} was recorded by the isolate 52 (7.73×10^{10} CFU ml^{-1}). Among the native isolates the lowest LC_{50} value was recorded by isolate 493 (2.30×10^{8} CFU ml^{-1}).

The LT_{50} values were also determined and was found to range from 104.62 to 140.57 hpi at uniform highest dose of 1×10^{12} CFU ml^{-1} among the *Bt* isolates tested. Among the *Bt* isolates LT_{50} value of HD1 was recorded to be the least and was found to kill the larvae faster *i.e.,* 104.62 hpi. Among the native isolates the LT_{50} was found to be lowest in isolate 493 and was recorded to be 113.99 hpi. The highest LT_{50} was found to be recorded for the isolate 52 and was determined as 140.57 hpi and was found to kill the larvae lately. LC_{90} values were in the range of 5.89×10^{11} (HD1) to 5.75×10^{16} (isolate- 52) and LT_{90} values were in the range of 162.47 hpi (HD1) to 245.93 hpi (isolate-55).

A comparision among the native isolates and nano based formulations showed that the reference strain HD1 was more effective and, CaO based nano formulation of 493 was found more effective among the native isolates against *H. armigera*.

CONCLUSIONS

- RAPD analysis was done for the seven *Bt* isolates including the reference strain, HD1 to determine the genetic diversity between the existing isolates. A total of 123 bands were observed for all the 10 decamer primers used in the RAPD analysis. 95.93 per cent of them were polymorphic and 4.07 per cent was found to be monomorphic.

- Dendrogram constructed based on the scored data, revealed that the seven isolates fell into two separate clusters with the isolates 16 and 55 being the extremes. Isolates 49, 55 and HD1 fell into one cluster, while the isolates 16, 51, 52 and 493 fell in the other cluster.

- Similarity matrix showed that the highest similarity was present between isolate 49 and HD1 with a similarity index of 0.71 and the least similarity was observed between isolate 51 and HD1 with a similarity index of 0.50.

- PCoA constructed for the isolates revealed that all the isolates were found within the eclipse. Isolates 16 and 52 were found to be closer to Coordinate 1 showing close genetic relationship, while, HD1 and isolate 49 were found to be far away from Coordinate 2, showing greater divergence from the remaining isolates.

- Protein profiling done for the *Bt* isolates revealed the presence of Cry1 (isolates 16 and HD1), Vip3 (isolates 49 and 493) and Cry2 (Isolates 51, 52 and 55) proteins.

- The highest mortality was observed for the *Bt* isolates maintained on CaO based nano enriched media among all the three NP's tested (CaO, MgO and ZnO).

- The mortalities ranged between 25 to 96.67% for the isolates grown on CaO based nano enriched media against 3rd instar *H. armigera* larvae. The LC_{50} and LT_{50} values ranged between 3.95×10^{7} to 5.68×10^{10} CFU ml^{-1} and 81.22 to 135.43 hpi, respectively.

- The mortalities were between 23.33 to 95% for the isolates maintained on MgO based nano enriched media against 3rd instar larvae. The LC_{50} and LT_{50} values ranged between 3.09×10^{7} to 4.16×10^{10} CFU ml^{-1} and 82.80 to 128.78 hpi respectively.

- The lowest mortalities were recorded in the isolates maintained on ZnO based nano enriched media among all the NP's tested.

- The mortalities were in the range of 21.33 to 93.33% for ZnO NP's based isolates and the LC_{50} and LT_{50} were in the range of 3.86×10^7 to 1.23×10^{11} CFU ml^{-1} and 83.52 to 140.57 hpi respectively.

- Among the nano-based formulations, MgO based HD1 formulation and CaO based 493 nano formulation was more effective.

- All the seven *Bt* isolates including the reference strain HD1 tested in the laboratory were found to show mortality of more than 50 per cent on *H. armigera* larvae. Among the native *Bt* isolates used in addition to reference strain HD1, native isolates 493 and 16 were superior with 88.33 and 78.33 per cent mortality, respectively.

- The LC_{50} and LT_{50} were recorded lowest by the reference strain, HD1 (2.78×10^7 CFU ml^{-1}, 104.62 hpi), among the native isolates, isolate 493 recorded lowest LC_{50} and LT_{50} values (2.30×10^8 CFU $ml^{-1}$and 113.99 hpi). The LC_{90} values ranged between 5.89×10^{11} (HD1) and 5.75×10^{16} (isolate- 52). LT_{90} values ranged 162.47 hpi (HD1) and 245.93 hpi (isolate-55).

- Among the isolates tested, the reference strain HD1 was more effective and for CaO based nano formulations of 493 was more effective than native isolates agaiast *H. armigera*.

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Note: The pattern of Literature Cited presented above is in accordance with the guidelines for thesis presentation, Acharya N. G. Ranga Agricultural University, Lam, Guntur.

Appendices

APPENDIX - I

A) 1X TAE buffer

2 ml of 50X TAE buffer was added to 100 ml distilled water. For 1 litre of 50X TAE buffer following were required.

Tris base	-	242.0 g.
Glacial acetic acid	-	57.1 ml
0.5 M EDTA	-	100 ml
Distilled Water	-	840 ml
pH	-	8.0

APPENDIX - II

A) T_3 medium (Travers *et al*., 1987)

Tryptose	- 2.0 g.
Tryptone	- 3.0 g.
Yeast extract	- 1.5 g.
Magnesium chloride	- 0.005 g.
Agar	- 15.0 g.
Distilled water	- 1000 ml
pH	- 6.8

Sodium phosphate solution : 17.799 g of Sodium phosphate in 50 ml distilled water

B) T_3 broth

Tryptose	- 2.0 g.
Tryptone	- 3.0 g.
Yeast extract	- 1.5 g.
Magnesium chloride	- 0.005g.
Distilled water	- 1000ml
pH	- 6.8

Sodium phosphate solution : 17.799 gm of Sodium phosphate in 50 ml distilled water

C) Comassie brilliant blue solution

Alcohol	- 50ml
Comassie brilliant blue powder	- 0.25g.
Glacial acetic acid	- 7ml
Distilled water	- 43ml

APPENDIX – III

BUFFERS AND SOLUTIONS

SDS-PAGE

Reagents

Solution A : 29 g of Acrylamide and 1 gram of Bis-acrylamide were dissolved in 100 ml of water, filtered and stored at 4 °C in a brown bottle

Solution B : Tris-SDS, 1.5M (pH 8.8) stored at 4°C (Separating gel)

Solution C : Tris- SDS, 0.5M (pH 6.8) stored at 4°C

(Stacking gel)

Tris-SDS pH 8.8 (1.5M Tris SDS, pH 8.8-100 ml)

Take 18.2 g of Tris with 50 ml of distilled water and adjust the pH to 8.8 with concentrated HCl then add 0.4 g of SDS and make up the volume 100 ml.

Tris-SDS pH 6.8 (0.5M Tris SDS, pH 6.8-100 ml)

Take 6.055 g of Tris with 50ml of distilled water and adjust the pH to 6.8 with concentrated HCI then add 0.4 g of SDS and make up to the volume 100 ml.

Ammonium persulphate (APS) 10%

0.1 g of APS is dissolved in 1 ml of distilled water and vortexed completely. It is prepared freshly.

TEMED

It was used as such and stored at 4°C.

Sample loading buffer (4X)

Tris-SDS, 0.25 M (pH 6.8) was added with 8 per cent SDS, 40 per cent glycerol, 20 per cent β mercaptoethanol, 0.5 per cent bromophenol blue and dispensed into aliquots for use, stored at room temperature.

Electrode buffer (10X)

Tris-base (0.25 M) was added with glycine (1.92 M) and 1 per cent SDS and stored at room temperature. IX electrode buffer was used for running the gel.

Staining solution

250 mg of Coomassie Brilliant Blue R 250 was dissolved in 40 ml methanol followed by addition of 10 ml of glacial acetic acid and then volume was made up to 100 ml with distilled water.

Destaining solution

It contains glacial acetic acid, methanol and distilled water in the ration of 1:4:5 v/v.

Composition of Separating gel and stacking gel

Composition	**Separating gel (10%)**	**Stacking gel (4%)**
Water	6.15 ml	3.5 ml
Solution A	4.95 ml	0.833 ml
Solution B	3.75 ml	-
Solution C	-	0.633 ml
APS (10%)	0.15 ml	0.050 ml
TEMED	0.009 ml	0.005 ml
Total	15.001 ml	5.02 ml