ISOLATION OF *Bacillus thuringiensis* AND ITS EFFECT AGAINST *Galleria mellonella*

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This is to certify that **Miss Jyoti Limbu** has completed this dissertation work entitled **"Isolation of *Bacillus thuringiensis* and Its Effect against *Galleria mellonella*"** as a partial fulfillment of the requirement of M.Sc. Degree in Microbiology **(Agriculture)** under my supervision. To our knowledge, this work has not been submitted for any other degree.

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ABSTRACT

The greater wax moth (GWM) *galleria mellonella* is a ubiquitous pest of the honey bee. The damage caused by *G. mellonella* is severe in tropical and sub tropical regions, and is believed to be one of the contributing factors to the decline in both feral and wild honey bee population. An integrated pest management (IPM) is required to control the GWM, within which biological controls play a key role. *Bacillus thuringiensis* is a gram positive rod shaped bacterium which has an insecticidal property. Hence, the main objective of this study was to isolate *Bacillus thuringiensis* and its effect against *Galleria mellonella* under laboratory conditions.

100 soil samples were collected from different areas of Itahari, Tarhara, Dharan and Vedetar. Out of 100 soil samples, 30 Bt isolates were obtained which were tested against GWM larvae. Three different concentration of crystal/spore mixture (1mg/ml, 500µg/ml, and 250µg/ml) was maintained. Mortality was observed after the four days of inoculation. Among 30 isolates, only four isolates showed an insecticidal activity. *B. thuringiensis* isolated from Tarhara showed the 57.78% efficacy which was much more than other isolates. The relation between treatment and control was statistically significant ($p<0.05$). The mean difference was significant at 0.05 levels on both bacterial death and survival. The result concluded that, there BT could be safe microbial agent in managing GWM.

Key words: Entomopathogen, *Bacillus thuringiensis*, *Galleria mellonella*, efficacy, bioassay

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LIST OF ABBREVIATIONS

BT	:	*Bacillus thuringiensis*
BD	:	Bacterial death
NA	:	Nutrient Agar
LB	:	Lauria Berteni
NB	:	Nutrient Broth
LC	:	Lethal concentration
GWM	:	Greater wax moth

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CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1Background

Bacillus thuringiensis (BT) is an insecticidal bacterium producing crystalline proteins called delta-endotoxins (δ-endotoxin) during its stationary phase of its growth. Bt was originally discovered from diseased silkworm (*Bombyx mori*) by Shigetane Ishiwatari in 1902.These δ -endotoxins are toxic to a great number of insects and turns Bt into a valuable tool to be used in the Insect Pest Management (IPM) (Valicente et al 2010).Some of crystal proteins are toxic to a high number of insect species of the orders lepidoptera, Diptera and Coleoptera, in addition to a few Hemiptera (MacIntosh et al1990). When orally ingested by insects, this crystal protein is solubilized in the midgut, forming proteins called δ-endotoxins. The toxicity of these crystals to the insects is determined by the presence of the specific receptors in the midgut epithelium (Bravo et al 2007) and Nematoda (Wei et al 2003).

The optimal condition for the Cry toxin to grow and sporulate is in the insect's alkaline gut. This facilitates the ability to infect insect guts. The primary action of Cry proteins is to lyse midgut epithelial cells through insertion into the target membrane and form pores. Once ingested, crystals are solubilized in the alkaline environment of midgut lumen and activated by host proteases (Brar et al 2007).*B.thuringiensis* is indigenous to many environments including soil (Martin and Travers, 1989), insect cadavers (Cadavos et al 2001), stored product dust (Hongyu et al 2000), leaves of plants (Smith et al 199l).Moreover, *B. thuringiensis* has been isolated from marine sediments (Maeda et al 2000), and aquatic environments (Iriarte et al2000).However, the normal habitat of the organism is soil. The organism grows naturally as a saprophyte, feeding on dead-organic matter, therefore, the spores of *B. thuringiensis* persist in soil and vegetative growth occurs when nutrients are available. Because of this, *B. thuringiensis* can also be found in dead insects (Tuba 2002).

B. thuringiensis produce one or more crystalline inclusion (parasporal crystal) bodies during the sporulation of its growth cycle and these can be seen under the phase contrast microscope. Several terminologies are used for the crystalline inclusions, for example, insecticidal crystal proteins (ICPs), cry toxins or δ-endotoxin (Tuba 2002).Meadows analyzed three prevailing hypothetical niches of *B. thuringiensis* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism (Medows et al 1997).

More than 50,000 strains isolated from several environments such as insects, stored products, plants, soil and marine environments are currently recognized (Sanchis et al 1996).However, investigators have shown that these *B. thuringiensis* strains varied in their pesticidal activities (Sharma 1994; Leyns et al 1995; Khyami-Horani et al 1996; Carneiro et al 1998; Al-Banna and Khyami-Horani, 2004). The main target pest of *B. thuringiensis* insecticides include various lepidopterous (butterfly), dipterous (flies and mosquitoes), and individual coelopterous (Beetle) species. Some strains have also been found to kill off nematodes (Edward et al 1998).

Wax moth control is a comprehensive problem particularly in warm climates, the best practices is to make the colony stronger and healthier because weak colonies increases the wax moth population rapidly (Sanford, 1985).The greater wax moth continues to be a global challenge to the bee health and the beekeeping industry and the rather scanty research attention it has received compared to other global bee pests and parasites (Paul 2017). The damage caused by *G. mellonella* larvae is severe in tropical and sub-tropical regions, and is believed to be one of the contributing factors to the decline in both feral and wild honeybee populations. Previously, the pest was considered a nuisance in honeybee colonies; therefore, most studies have focused on the pest as a model for in vivo studies of toxicology and pathogenicity (Miechal 2010).

The use of chemical insecticides to control insect pests has increased over the years and has generated resistance to chemicals. Microbial pesticides may become feasible and reliable alternative to control insects. Crop species are

infested by many species of insect and pathogens resulting in heavy losses in yield. Products from biological sources are emerging as a new crop protection strategy. Bt. *(Bacillus thruingiensis*) consists of the largest and most widely commercialized bio-pesticides category. Various formulations of *Bacillus thuringiensis* have been used to control the greater wax moth(Williams 1976).

The concept of Bio-intensive IPM involving biocontrol agents and other non-chemical approaches has emerged as a viable alternative forming a part of organic farming system. One of the main advantages of microbial control agent is that they can replace, at least in part, some of the most hazardous chemical insecticides. The uses of these safer and biodegradable biological control agents also have a number of ecological advantages. The products produced using non-chemical inputs are termed as organic products. Organically produced agricultural commodities are sold at a premium price both in domestic as well as overseas market. There are several families of entomopathogenic bacteria such as Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococaceae (Tanada1993). Bacteria belonging to family Bacillaceae possess wide range of insecticidal activity; from which, *B. thuringiensis* (BT) is the most widely used and successful microbial pesticide.

In the Recent past the population of both feral and wild Honeybees has been declined. This has spurred anxiety against apiculturists, scientists and general public due to threat it poses to global food and nutritional security given by honey bees to food production and feeding the global population. Honey bee is attacked by many pests pest including (Ants, termites, beetles, wasps and moths). The most serious and noises insect pest of beekeeping is moths, namely the greater wax moth (*Galleria mellonella*) (El-Niweiri et al 2005).

The Greater Wax Moth (*Galleria Mellonella)* is worldwide serious pest of honey bee. It burrows into the edge of unsealed cells with pollen, bee brood and honey through to the midrib of honeybee comb. The greater wax moth chewed their way down to the midrib of the comb (tunnels), this tunneling destroys the wax cell of the comb and also they can cause dead to bee brood (Adam et al 2017). The damage caused by this pest is severe in tropical and

sub tropical regions and it considered as the major contributing factor in decreasing the honeybee population (Kwadha et al 2017).

It is difficult to treat honey-bee comb against infestation by wax moths in bee hives because control measures must not harm the bees (Burges and Bailey 1967). Several chemical and non chemical method have been developed to control GWM in stored honeybee comb including freezing, heating and CO2. The chemical include fumigants, such as p-dichlorobenzene, acetic acid, calcium cyanide, ethylene dibromide, methyl bromide and phosphine. Despite of all these efforts, safe and low cost wax moth control is needed especially for commercial beekeepers in tropical and sub tropical regions. The safest and the cheapest way to control of wax moth is a biological control (Basedow et al 2012). Microbial insecticide such as *Bacillus thuringiensis*, attacks moth larvae almost exclusively without harming bees or leaving toxic residues dangerous to human (Burges and Bailey 1967). Treatment with *Bacillus thuringiensis*, used as a suspension sprayed onto the combs and its effect on the larvae of the wax moth last for several weeks (Sanford, 1985).

The most important active components in *Bacillus thuringiensis* bacterium are the crystal proteins. The endotoxin produced by *Bacillus thuringiensis* is specific for some lepidopterous larvae such as GWM. Death of larvae is occurred due to septicemia between days to several weeks depends on size and dose of bacteria (Burges and Bailey 1967). Hence the main aim of this research was to perform isolation of *Bacillus thuringiensis* and its effect against *Galleria mellonella*.

1.2 Objectives

1.2.1General objective:

Isolation of *Bacillus thuringiensis* and its effect against *Galleria mellonella*.

1.2.2 Specific objectives:

1. To isolate, identify and characterize *Bacillus thuringiensis* isolated from different altitude of Koshi Zone.
2. To determine the insecticidal effect of *B. thuringiensis* against greater wax moth (*Galleria mellonella).*
3. To extract the crystal spore and determine LC_{50} dose.

CHAPTER II

LITERATURE REVIEW

2.1 *Bacillus thuringiensis*

Bacillus thuringiensis is a ubiquitous gram–positive, rod shaped, endospore forming bacteria which produces a crystal during the stationary phase of its growth cycle. ***B. thuringiensis*** was initially characterized as an insect pathogen (depending on insect) to the parasporal crystals. This observation led to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera. There are more recent reports of *B. thuringiensis* isolates active against other insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against nematodes, mites, and protozoa. *B. thuringiensis* is already a useful alternative or supplement to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control. It also a key source of genes for transgenic expression to provide pest resistance in plants (Schnef et al 1998).

2.1.1 Ecology and Prevalence

B. thuringeinsis is mainly a soil bacterium living as both saprophytic and parasitic (Glazer and Nikaido, 1992). *Bacillus thuringiensis* seems to be indigenous to many environments. Strains have been isolated worldwide from many habitats, including soil, insects, dead insects, sericulture environments, forests and cultivated soils, stored products dust, and coniferous and deciduous leaves. Because of the economic importance of *B. thuringiensis*, many researchers have investigated the distribution of *B. thuringiensis*in various geographical regions and in different sources (Schnepf et al 1998).

Meadows (1993) suggested that there are three hypothetical niches of *B. thuringiensis* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism. Many *Bacillus thuringiensis* strain obtained from diverse environment show no insecticidal activity. Maeda et al

(2000) found that the 6 stains of *B. thuringiensis* obtained from marine environments of Japan exhibit no insecticidal activities.

The remarkable diversity of *B. thuringiensis* strains and toxins is due at least in part to a high degree of genetic plasticity. Most *B. thuringiensis* toxin genes appear to reside on plasmids (Gonalez et al 1981).The diversity in flagellar H-antigen agglutination reaction is one indication of enormous genetic diversity against genetic diversity among *B.thuringiensis* isolates (Schnepf et al 1998).

2.1.2 Morphology and Physiology

Bacillus thuringiensis forms white colonies which spread out and expand over the plate very quickly. *B. thuringiensis* has dimensions ranging from 3-5 µm in length and 1-1.2 µm in width. The spore of the organism are ellipsoidal, unswollen and lie in the sub terminal position in the cell. The best criteria of distinguish *Bacillus thuringiensis* from other Bacillus species is the presence of parasporal crystal inclusion. Two types of δ-endotoxin are produced by Bt strains. They are named *Cry* and *Cyt* proteins. Each insecticidal crystal protein is the product of a single gene. The genes synthesize these endotoxins are often located on large, transmissible plasmids. *Cry* and *Cyt* proteins differ structurally. The most important feature of these proteins is their pathogenicity to insects and each crystal protein has its distinct host range. The number and type of δ -endotoxins produced determine the bioactivity of a Bt strain (Crickmore et al 1995). Based on the amino acid homology, over 300 *cry* genes have been classified into 47 groups and 22 *cyt* genes have been divided into two classes (Web 2005).

There are five crystal morphologies: bipyramidal crystal related to cry 1 proteins; cuboidal crystal related to *cry* 2 proteins; flat- square crystals, typical of *cry* 3 proteins; and bar shaped inclusion related to cry4D proteins (Lopez-meza and Ibarra, 1996; Subedi and Bhattrai,2002).

2.1.3. History of *Bacillus thuringiensis*

Bacillus thuringiensis was first isolated by Japnease Biologist Ishiwata in 1901 while investigating the cause of sitto disease that was killing silkworm. This common soil bacterium is found in grain dust from silos and other grain storage facilities. Ernst Berliner isolated the spore forming bacteria from Mediterranean flour moth cadaver in 1991. He named it *Bacillus thuringiensis.*

Researchers, Hanny, Fitz-James and Angus found that the main insecticidal activity against lepidepteron insectwas due to parasporal crystal in 1956.Another Canadian worker T. Angus (1954-1956) confirmed the work of Aoki and Chigasaki as well as Hannay's suggestions of relationship between pathogenicity and the crystals (Neppl 2000).

Bacillus thuringiensis was only known to be effective against the larva of the insect *lepidoptere* (caterpillars) until the late 1970's. Israeli researchers Goldberg and Margalit discovered a new subspecies *Bt israelensis*, toxic to larvae of certain flies (order Diptera). Bti products have become important tool in the control of mosquitoes and other flies. Subsequent discoveries have revealed BT subspecies and strains toxic to beetles (coleptera), ticks, nematodes and other groups of pests (Michael 2002). There are thousands of strains of BT exist. Each strain has its own insecticidal crystal protein, or δ-endotoxin, which is incoded by a single gene on a plasmid in the bacterium. *B. thuringiensis* toxins are biodegradable and do not persist in the environment (Neppl 2000).

The rapid development of recombinant DNA techniques and the advance molecular biology transformed knowledge and understanding of the genetics of BT toxin expression and mode of action. In 1980, molecular biologists Ernest Schnepf and Helen Whiteley first cloned a BT toxin gene, for which they later determined the complete sequence of DNA. This allowed the construction in the laboratory of synthetic version of gene, opening the door to creating new BT strains with higher potency, broader spectrum of activity, and higher production efficiency than previously available. It also pointed towards the insertion of Bttoxin gene into the DNA of plants which hinder them to plant feeding insects. The widespread adoption of transgenic BT cotton and

has been shown to the significant reduction in overall uses of pesticide on these crop (Michael 2002).

2.1.4 Classification and Nomenclature

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

The *cry* proteins constitute the largest group of insecticidal proteins produce by species of *Bacillus*. To date, 73 different types of cry proteins (cry 1 to cry 73) has classified by Bt toxin nomenclature committee including three domain and ETX_MTX2 family proteins showing toxicity against lepidopterans, colepterons, hemipterans, dipterans, nematodes(human and animal parasites and free living; *Rhabditida*) snails.

There are 45 different serotypes of *B thuringiensis* have been classified as 58 serovars of *Bacillus thuringiensis* have been cloned, sequenced and named *cry* and *cyt* genes (Bravo et al 1998). To date, over 100 *cry* gene sequences have been determined and classified in 22 groups and different subgroups with regard to their amino acid similarity. The proteins toxic for lepidopteran insects belong to the *Cry1, Cry9*, and *Cry2* groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the *Cry1B* and *Cry1I* proteins, which have dual activity. The *Cry5, Cry12, Cry13*, and *Cry14* proteins are nematicidal, and the *Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19*, and *Cyt* proteins are toxic for dipterans insects (Bravo 1997).

2.1.5 Formulation of *Bacillus thuringiensis* preparants

Insecticidal BT preparants were first commercialized in France in the late 1930s (Lambert et al 1992). BT has been one of the most consistent and significant biopesticide for use on crops as an insecticidal spray.Commercially available *B. thuringiens* preparants contain both spore and toxic crystal protein (delta endotoxin). Spore and crystals obtained from fermentation are mixed with the additives including wetting agents, sticker's sunscreen and synergist in the production process (Burges and Jones, 1999).

It is expected that the rapid loss of crystal toxin of *B. thuringiensis* is UV inactivation. Many approaches such as chromophores to shield Btpreparants against sunlight (Dunkle and Shasha, 1989; Cohen et al 1998) and enhancing the melanin producing mutants of the organism enhances the UV resistance and insecticidal activity (Patel et al 1996). Melanin is a natural pigment that is easily biodegradable in the natural. It has been reported that it absorbs radiation consequently provides photo protection to Bt. Therefore, melanin is considered to appear in the new formulation for prolonging the toxicity of BT product under field conditions and UV irradiation (Zang et al 2016)

Sensitivity of BT spores to UV-B radiation depends upon their culture age. 48 h of culture is thought to be the maximal resistance to UV-B, and 24 h approach its maximal larvicidal activity (Zang et al 2016)

Knowledge of Insect feeding behavior is a fundamental requirement for the development, optimization and utilization of biopesticides (Navon et al 2000).

2.1.6 *Bacillus thuringiensis* genome

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

2.1.7. Toxin structure

To date, the structures of three crystal proteins - *Cry3A, Cry1Aa*, and *Cyt2A* have been solved by X-ray crystallography. *Cry3A* and *Cry1Aa* have about 36% amino acid sequence identity. This similarity is reflected in their three-dimensional structures; the corresponding domains can virtually be superimposed. *Cyt2A*, however, shows less than 20% amino acid sequence identity with *Cry1Aa* and *Cry3A*, and a similar alignment score would be obtained if the *Cyt2A* sequence were randomized. The structure of *Cyt2A* is radically different from the other two structures. The structures of *Cry1Aa*, *Cry3A*, and *Cyt2A* are compared in Fig. 1. The *Cyt* toxins, unlike the *Cry*δ endotoxins, are able to lyse a wide range of cell type's in-vitro. *Cyt2A* consists of a single domain in which two outer layers of α-helix wrap around a mixed β-sheet. *Cyt1A* is believed to have a similar structure. *Cry3A* and *Cry1Aa*, in contrast to *Cyt2A*, both possess three domains. Domain I consists of a bundle of seven antiparallel α-helices in 10 which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called βprism fold (330, 343). Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology (Schnepf et al 1998).

The component of crystal protein toxic to lepidoptera larva is 130KDa which upon cleavage in the insect yields the functional (insecticidal) proteins of lower molecular weight; very often the crystal formed is an assemblage of many proteins (Crickmoreet al 1998).

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2.1.8 Mode of action of *Bacillus thuringiensis*

The *cry* protein of *B. thuringiensis* constitutes a smaller, distinct group of crystal proteins with insecticidal activity against several dipteran larvae, particularly mosquitos and black flies (Ben 2014). The primary classification of BT strains are based on their H flagella antigenic determinants (Dumanoir, 1999). Cry protein lyses the midgut epithelial cells of target insects by forming pores which is the primary action of cry protein (Aronsen and Shai, 2001). Bt cry protein acts as a bonafide ion channel in lipid bilayers and the midgut of epithelium (Schnep et al 1998).Crystals are formed as protoxins by Bt. to become active; a susceptible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, midgut proteases convert the protoxins into active toxins. The active toxin binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). As a result, pore formation leads to gut paralysis. Finally, insect larvae stop feeding and die from lethal septicaemia (Aransonet al1986).

Serine proteases such as chymotrypsin, thermolysin, elestase are important in both solubilization and activation of protoxins (Yang and Davies, 1971). Besides these digestive proteases, a novel DNase from an insect has been found to act synergistically with the crystal protein and to convert it to the active DNA-free toxin in the larval gut (Clairmontet al 1998).

Spores are known to synergize the insecticidal activity of crystals when tested against insects. This may be related to the invasion of haemocoel through the ulcerated midgut, and the subsequent development of septicaemia (Li et al 1987). The efficiency and potency of Cry toxins to control insects could be increased by the addition of enzyme chitinase in *BT* preparations. The chitinase acts on the peritrophic membrane which is composed of a network of chitin and proteins (Smirnoff, 1973). This enzyme hydrolyses the β-1, 4 linkages in chitin so it may disrupt the peritrophic membrane by creating holes and facilitates the contact between δ-endotoxins and membrane receptors in the mid gut epithelium (Regevet al 1996). Some factors such as pH, enzymes,

peritrophic membrane, enzyme detoxification, and antimicrobial characteristics of gastric juice of insect gut make insects resistant to the toxin (Davidson 1992).

2.1.9 Development of *Bacillus thuringiensis* preparants

The first evidence of resistance developing in the field against *B. thuringiensis* deltaendotoxins was published in 1985. Low levels of resistance were found in *Plodiainter punctella*, the Indianmeal moth, in storage bins of *B. thuringiensis*-treated grain. The *B. thuringiensis* resistance problem became greater when the first reports of high resistance to *B. thuringiensis* toxins in the field came in 1990 from Hawaii, Florida, and New York in the United States. The species found to be losing susceptibility to *B. thuringiensis* toxin was *Plutella xylostella*, and the diamondback moth. Resistance in *P. xylostella* was detected in several other countries, including Japan, China, the Philippines, and Thailand. Malaysia also reported *B. thuringiensis* resistance in the diamondback moth in 1990. Thus far *P. xylostella* is still the only insect species in 14 which very considerable resistance has been found to develop outside of the laboratory (Schnepf et al 1998).

Eleven species have developed resistance to various strains of *B. thuringiensis* toxin in the laboratory but not in the field: *Ostrinianubilalis* (the European corn borer), *Heliothis virescens* (the tobacco budworm), *Pectinophora gossypiella* (the pink bollworm moth), *Culexquinquefasciatus* (the mosquito), *Caudracautella* (the almond moth), *Chrysomelascripta* (the cottonwood leaf beetle), *Spodopteraexigua* (the beet armyworm), *Spodopteralittoralis* (the Egyptian cotton leaf worm), *Arctiacaja*(the gardentiger moth), *L. decemlineata* (the Colorado potato beetle), and *Aedesaegypti* (the yellow fever mosquito) (Schnepf et al 1998).

2.1.10 Mechanism of resistance

The mechanisms underlying *B. thuringiensis* resistance are found in the sequential steps in the proposed mode of action of *B. thuringiensis* toxins. Following the ingestion of *B. thuringiensis* crystals by susceptible insects, protoxins are solubilized and hydrolyzed by gut m-proteinases to an active

toxin form. Activated cry proteins pass through the peritrophic membrane and bind to proteins in the brush border membrane of midgut epithelial cells. Toxin binding is followed by events that lead to cell lysis and disintegration of the brush border membrane, and eventually insect death. Any change in insect gut physiology that affects one or more steps in this process could prevent toxicity and lead to the development of resistant pest populations (Li et al 2004). Most studies have focused on two steps in the mode of action proteolytic activation of protoxin and binding of active toxin to receptors. Reduced binding of Cry proteins to midgut receptors has been associated with resistance in several strains of *Plodiainterpunctella, Plutellaxylostella, Heliothisvirescens, Spodopteraexigua,* and*Leptinotarsadecemlineata* (Li et al 2004).

However, a loss of toxin binding was not associated with resistance to *B. thuringiensis* in several insect. Therefore, other mechanisms of resistance may be operating in these 15 insects. Serine proteinases, such as trypsin, chymotrypsin, and elastase, are important in both the solubilization and activation of *B. thuringiensis* protoxins. In some insects, changes in these proteinases have been associated with resistance to *B. thuringiensis* toxin. A strain of *P. interpunctella* resistant to *B. thuringiensis* subsp. *Entomocidus* HD198 processed *B. thuringiensis* protoxin at a slower rate than the parental susceptible strain. This resistant strain lacked a major gut proteinase involved in activation of *B. thuringiensis* protoxin, and the proteinase mechanism was responsible for about 90% of the total resistance to cry1Ab (Li et al 2004).

Enzymes from a strain of *H. virescens* resistant to *B. thuringiensis* subsp. *kurstaki* HD 73 were reported to activate the protoxin more slowly and degrade the toxin faster than enzymes from a susceptible strain. In *Spodopteralittoralis,* increases in the specific activity of gut proteinases were associated with the loss of sensitivity to *Cry1C* due to an increase in thedegradation of active toxin. Aminopeptidase is an exopeptidase and a marker for membrane proteins. It is localized to the brush border membrane of midgut epithelial cells and is involved in the digestion of peptides and amino acid transport. A membrane-bound glycosyl-phosphatidylinositol

anchored aminopeptidase N has been reported to bind *Cry1Ac* toxins in several different insects. Previously, a strain of *B. thuringiensis*-resistant *Ostrinianubilalis*, selected for resistance to *B. thuringiensis* subsp. *kurstaki* (HD-1), was described with lower trypsin-like proteinase activity compared to the parental susceptible strain, but the reduced trypsin-like proteinase activity was in the soluble fraction of gut proteinases in the resistant strain. Soluble proteinases from the resistant strain incubated with *Cry1Ab*protoxin resulted in lower amounts of an active toxin fragment relative to incubations with proteinases from the susceptible strain. This reduction in toxin activation may account for lower susceptibility to *B. thuringiensis* toxins by resistant *O. nubilalis* larvae (Li et al 2004).

2.2.1 The Greater Wax Moth, *Galleria mellonella*

Introduction

Galleria mellonella, the greater wax moth (GWM) is a moth of the family pyralidae. *G. Mellonella* found throughout the world. It is one of two species of wax moths, with other being lesser wax moth. Wax moth larvae are very destructive and can quickly destroy stored beeswax combs. They tunnel and chew through combs, particularly combs that have contained brood and pollen. An assessment of the economic impact of GWM at the global scale is still lacking. However, losses attributed to *G. mellonella* infestation in the southern United States were estimated to be approximately $3 and $4 million in 1973 and 1976, respectively, which approximately represent 3.9% and 5.1% of the profit in the respective years (Turker et al 1963).The destructive nature of the pest is attributed to its high reproductive potential and rapid development time (Kwadha et al 2017).

The greater wax moth *galleria mellonella* is extensively used as a laboratory test animal for basic studies in many disciplines (physiology, biochemistry, toxicology, pathology etc). In both laboratory and field studies its egg, larval and pupal stages are often used as a host or prey for rearing parasitic and predaceous insects (Dadd 1960).

Systematic position

Systematic position of *Galleria mellonella* proposed by Linnaeus (1758) is given in table.

Order	Lepidoptera
Family	Pyralidae
Sub family	Galleriinae
genus	*Galleria*

2.2.1.2Morphology

Adult moths are pale brown to grey, usually about 20 mm long. The grey wings are often mottled and appear as "roof" or "boat" shaped when folded over the body. Egg: Wax moth eggs vary in size, with an average length and width of 0.478 mm and 0.394 mm, respectively. The egg is of spheroidal shape with interspersed wavy lines which gives it a rough texture (Ellis et al 2013).Larvae are creamy white, but turn grey on reaching their fully grown size of up to 28 mm in length. Fully developed larvae spin silky cocoons that may be found in a mass of webbing in the comb, and on the frames and internal surfaces of the hive. Larvae may form small canoe shaped depressions in the wooden hive components in which to spin their cocoons. Larvae can also bore through the wooden bars of frames. After spinning the cocoon, the larvae commence the pupal stage which lasts about 14 days when temperatures are high but as long as 2 months during cooler temperatures (Smita et al 2009)

.2.2.1.3 Life cycle

The greater wax moth is a typical holo metabolous insect and develops through four distinct life stages, namely, egg, larva, pupa, and adult. The duration taken by the moth to complete its life cycle varies from weeks to months and is affected by both biotic (intra-and interspecific) and abiotic factors(Gulathi et al 2004). Oviposition begins a fairly short time after emergence and mating of females.Femalemoths lay eggs in clusters of 50–150

in tiny cracks or crevices inside the hive which minimizes egg detection and enhances larval survival (Charriere1997). Eggs take between 3–30 days before hatching into larvae (Williams 1997).Upon hatching, wax moth larvae are approximately 1–3 mm in length and 0.12–0.15 mm in diameter (Smith 1965). They remain in the larval stage anywhere between 28 days to 6 months, during which they undergo eight to 10 molting stages. At last instar, the larvae spins cocoon of silk for itself and enters the pupal stage (Paul 2017). They remain in the larval stage between 28 days to six months, during which they undergo eight to ten molting stages. Pupae start off as a brownish white, but gradually darken to a dark brown color just before adults are ready to emerge (Warren et al 1964).Adult moths are brown gray and range from 10 to 18 mm in length. Females live for an average of 12 days; males live for an average of 21 days (Warren et al 1964).

2.2.1.4 *Bacillus thuringiensis* to control greater wax moth

Controlling the GWM in live honeybee colonies has been challenging. With the development of new techniques in the field of biotechnology, there was the potential of making a breakthrough using *Bacillus thuringiensis.* The bacterium *Bacillus thuringiensis* is a microorganism that is harmless to people, honey bees and the environment, and has also been used to kill young wax moth larvae (Doug Somerville 2007).The majority of bioassays of *B thuringiensis* with larvae of the greater wax moth, *Galleria mellonella* have employed synthetic diets or foundation wax both for the administration of pathogen and also food for the experimental insects (Rogers et al 1966). The pathogenicity of the *B. thuringiensis* depends both on the viability of the spores and on the toxicity of the crystals of protein toxin (Burges et al 19760); consequently potency can be evaluated only by bioassay.

CHAPTER–III

MATERIALS AND METHOD

3.1 Materials

Materials and media used in thesis work are listed in appendix I.

3.2 Methods

3.2.1 Study site

The study was carried out in Central Campus of Technology Hattisar Dharan and Regional Agricultural Research Station (RARS) Tarhara from October 2018 to March 2019.

3.2.2 Sample Collection

Soil sample was collected from different altitudes ranging from Itahari, Tarhara, Dharan and Vedetar. Soil sample was collected from 3-5 cm depth and collected in sterile plastic bags and transported into the laboratory at 4 ^{0}C.

3.2.3 Isolation of *Bacillus thuringiensis*

B. thuringiensis was isolated by Acetate selection method as described by Travers et al (1987). One gram of soil sample was taken in a sterile conical flask containing one milliliter of 0.25M sodium acetate (pH 6.8) and 9 ml of Lauria Bertani (LB) broth. The broth was incubated at 37ºC for 24 hrs. After incubation 0.1 ml of sample was taken and spread on nutrient agar. Plates were incubated at 37ºC for 24hrs. Bt like colonies were sub cultured on nutrient agar for further identification.

3.2.4 Identification of *Bacillus thuringiensis*

The organisms were identified according to the (Bergey's Manual of Systematic Bacteriology, Volume 2; 1986).

3.2.4.1 Microscopic examination

a. Gram Staining

A thin smear of fresh culture was prepared in clean grease free slide and heat fixed. Smear was flooded with crystal violet for one minute and washed with distilled water. The slide was flooded with grams iodine for 45 seconds and washed with distilled water. Then it was flooded with 95% ethyl alcohol (decolorize) for 10 seconds. The smear was flooded with safranin (counter stain) for one minutes and washed with distilled water. Excess water was removed by blotting paper, air dried and observed under microscope.

b. Spore staining

A thin smear of culture was prepared on clean glass slide. The smear was flooded with malachite green and steamed over boiling water bath for five minutes, keeping the smear moist by adding dye as required. Slide was washed with distilled water and counterstained with safranin for 30 seconds. It was washed with tap water, air dried and observed under microscope.

c. Crystal staining

A thin film of Five days old culture is made on clean, grease free glass slide. The smear was air dried and heat fixed at 110ºC for 10 minutes. The smear was stained with 0.25% coomassie brilliant blue for 3 minutes and washed with distilled water and observed under microscope. Crystals were stained blue.

3.2.4.2 Biochemical Characteristics

For the study of biochemical characteristics of the organisms, the pure colonies were transferred into Nutrients broth (Appendix-II) and incubated at 37ºC for 24 hours. Then

The isolates from Nutrient broth were used to inoculate respective biochemical media.

a. Catalase test

Small amount of bacterial colony was picked from nutrient agar plates and transferred in a clean, sterilized glass slide. A drop of 3% H_2O_2 was placed in a slide and mixed with the help of sterile glass rod. Production of gas bubbles indicated the positive test.

b. Oxidase test

Whatmann no.1 filter paper soaked with1% tetra methyl –p phenylenediaminedihydrochloride was taken. Paper was moistened with sterile distilled water. Colony to be tested was picked up with the help of sterile glass rod and smeared over the moist area. Development of an intensedeep purple blue indicated the positive reaction.

c. Citrate utilization test

Simmons's citrate agar slant was prepared. Organism was streaked on the slant and incubated at 37ºC for 24 hrs. Positive tests were confirmed by development of intense blue colour and growth of the organisms on the slant.

d. Sugar utilization test

For this test, basal medium consisting of peptone, sodium chloride, phenol red was incorporated with carbohydrate at the concentration of 1 %.(w/v). Durham's tube was inserted upside down in the test tube. The tube was filed initially with the solution. Then the solution was autoclaved. After this isolates were inoculated in the tubes and incubated at 37ºC for 24 hours. Positive test indicated by changing colour from pink to yellow and production of gas bubbles in Durham's tube. Sugars used were glucose, mannitol and sucrose.

3.2.4.3. Hydrolysis test

a. Gelatin hydrolysis test

Test organism was stabbed on the tube containing nutrient gelatin medium and incubated along with uninoculated medium at 37ºC for two weeks. Tubes were removed daily from the incubator and placed in refrigerator for 15-20 minutes

every to check the gelatin liquefaction. Positive test showed complete or partial liquefaction even after exposure to cold temperature.

b. Casein hydrolysis test

Solidified skim milk agar plates were divided into four sectors with each being streaked with individual isolate. Plates were incubated at 37ºC for 24 hrs. Clear zone around the colony after flooding with mercuric chloride solution indicated the positive test.

c. Starch hydrolysis test

Solidified starch agar plates were divided into four sectors and inoculated with the isolates and incubated for 37ºC for 24 hours. Positive test showed clear zone around the colonies after flooded with grams iodine solution.

d. Lecithinase activity

Organism was streakedEgg yolk emulsion medium and incubated at 37ºC for 24 hours. The presence of opalescence around the colony indicates positive test.

3.2.4.5 Antibiotic sensitivity test

The Antibiotic susceptibility test was done by Kirby Bauer Disk Diffusion Assay.24 hour's old organism was swabbed with the help of sterile cotton swab on sterile Muller Hinton agar plate. Then, antibiotic discs were placed on the plate with sterile forceps. In this test streptomycin, tetracycline, ampicillin, and amoxicillin were used. *Bacillus thuringiensis* is susceptible to streptomycin, tetracycline, and resistant to ampicillin (Zing R et al 2013)

Maintenance of culture

A loopful of bacterial culture was taken and transferred to nutrient agar slant. Pure culture of *Bacillus thuringiensis* slants was stored at 4ºC.

3.3 Rearing of greater Wax Moth *Galleria mellonella*

The greater wax moth *Glleria melllonella* was reared in the laboratory according to Mohamed and Coppel (1983).

The experimental population of *Galleria mellonella* was reared at 26.5ºC and fed on artificial diet. It comprises following ingredients.

Deionized, preboiled, water,	100ml
Honey, commercial brand,	150ml
Bee wax,	3g
Cholesterol,	1g
Multivitamin supplement	4ml
Maize flour	100gm
Milk powder	100 gm

Deionized water was heated to 80ºc and honey, glycerine, beewax and cholesterol were mixed separately. When all the ingredients were in solution it was allowed to cool at 50ºc and multivitamin supplement was added. Maize flour was taken in a container and the above solution was added and mixed well. The resulting mixture was loose, friable consistency. The medium (diet) was stored at room temperature in a tightly closed container.

Adult wax moths were kept into 1litre mason jars where the mating took place. Folded sheets of wax with paper clips where the moth deposited eggs. The eggs were remove and transferred into the 500ml mason jar with 150 gm of medium and incubated at 28.5ºc in the dark. After 10-15 days second instar larvae were appeared. After 8- 10 days larvae were transferred into 1liter Mason jar with175gram of medium. Within 5-10 days third instar larvae became available which were used for bioassay.

3.4 Preparation of mass culture of *Bacillus thuringiensis*

Lauria Berteni (LB) broth was used for the preparation of mass culture of *Bacillus thuringiensis.*

Preparation of spore/protein mixture for bioassay

Pure culture of Bt was inoculated in sterile nutrient broth and incubated in water bath shaker at 25ºc for 4 days. Spores were harvested by centrifugation

at 10000 rpm for 15 minutes. Pellets were washed twice with distilled water. Cell disruption process was done by lophilization. Lyophilized culture was dried in laminar air flow.Resulting dry powder was assayed for biological activity (Hammounel et al., 2010). It was stored at -20^{o}c until use.

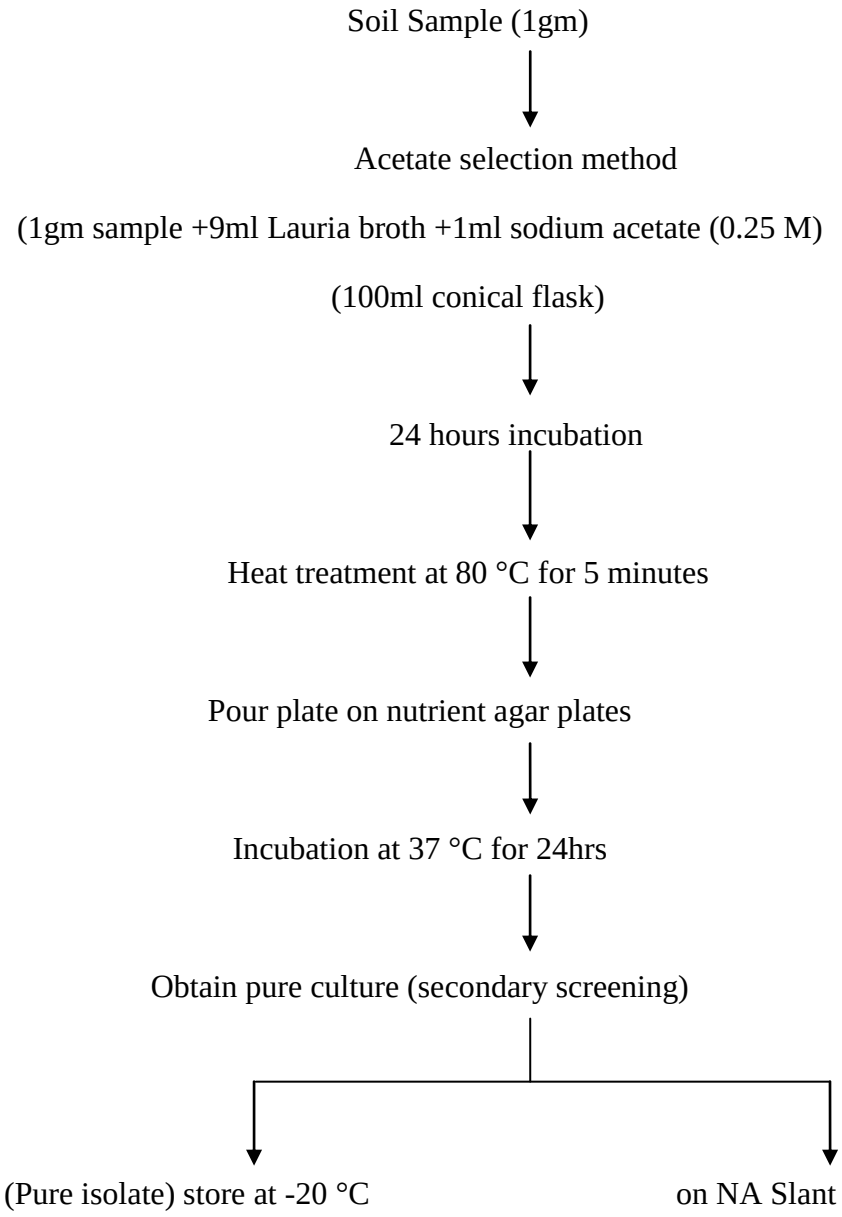
3.4.1 Bioassay

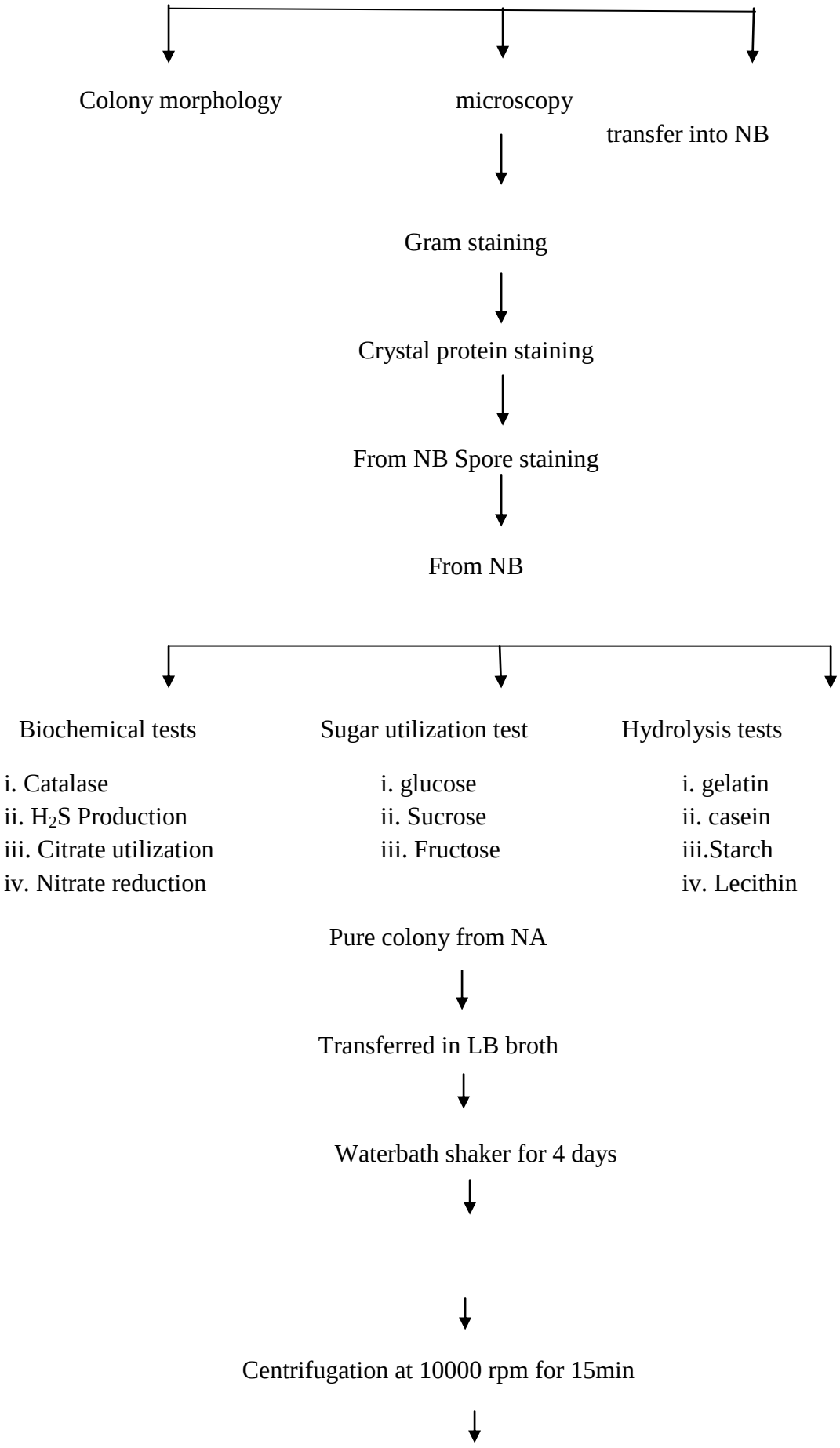
Bioassay was performed according to (Ramarao N.et al 201). *Bacillus thuringiensis* preparation (spore/protein mixture) in the form of powder was used for bioassay. Three different concentrations (1mg/ml, 500µg/ml and 250µg/ml) of spore/protein mixture were prepared in phosphate Saline (PBS: 1M KH2PO4, 1M K2HPO4, 5M NaCl, pH-7.2).Third instar larvae were used for bioassay. 0.5ml of each concentration was mixed in 1gram of larval food and placed in disposable plastic cups (30mm diameter).For each dose three replicates were prepared with 5 larvae in each replicate.As control, the artificial diet was supplemented with sterile 0.5ml distilled water. The cups were covered with muslin cloth and wrapped with rubber band. Total 45 larvae per each isolate were used. Larvae was put into an empty box for 2 hours to starve.Dead larvae were inert and turned black.Mortality was recorded after four days until pupation.

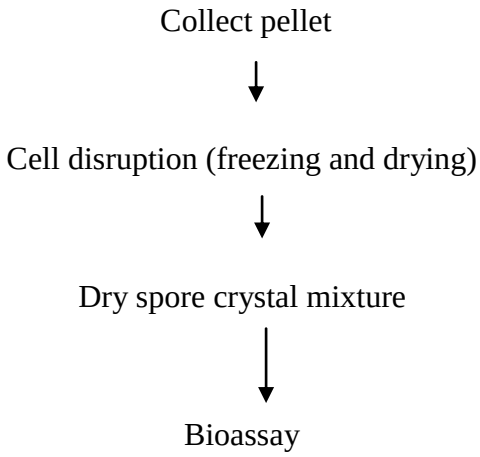
3.6 Data analysis

Data recorded from bioassay was documented and tabulated. The data were statistically analyzed by SPSS version 16.Statistical significance was established when p value was less than equal to 0.05.

Flow chart for the study




Colony morphology
microscopy
transfer into NB
Gram staining
Crystal protein staining
From NB Spore staining
From NB
Biochemical tests
Sugar utilization test
Hydrolysis tests
i. Catalase
ii. H2S Production
iii. Citrate utilization
iv. Nitrate reduction
i. glucose
ii. Sucrose
iii. Fructose
i. gelatin
ii. casein
iii.Starch
iv. Lecithin
Pure colony from NA
Transferred in LB broth
Waterbath shaker for 4 days
Centrifugation at 10000 rpm for 15min


Collect pellet
Cell disruption (freezing and drying)
Dry spore crystal mixture
Bioassay

CHAPTER IV

RESULTS

The study was conducted in Central Campus of Technology, Hattisar Dharan and RARS Tarhara. 100 Soil samples were collected and Isolation was done by acetate selection method. Identified BT isolates were used to study their insecticidal properties against greater wax moth (*Galleria mellonella).*

4.1 Sample Size:

Soil sample was collected from the different area of Itahari, Tarhara, Dharan and Vedetar with increasing altitude. 25 samples from each place were collected. The figure **4.1** shows the sample population.

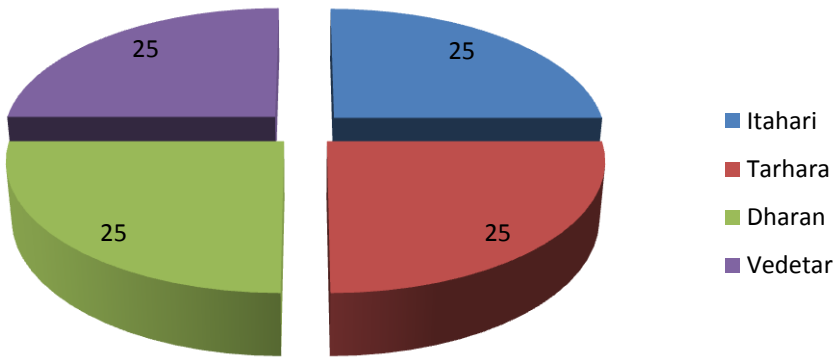


Fig 4.1: Soil sample size for isolation of *Bacillus thuringiensis*

4.2 Distribution of *Bacillus thuringiensis* in soil. .

Soil sample was collected from different altitudes ranging from Itahari to Vedetar. 25 soil samples from each place were collected. Out of 100 samples analyzed 30 isolates of Bacillus *thuringiensis* was obtained. The number of isolates from Itahari, Tarhara, Dharan and Vedetar were 7, 9, 8 and 6 respectively. The distribution of BT in soil is shown by Figure 4.2

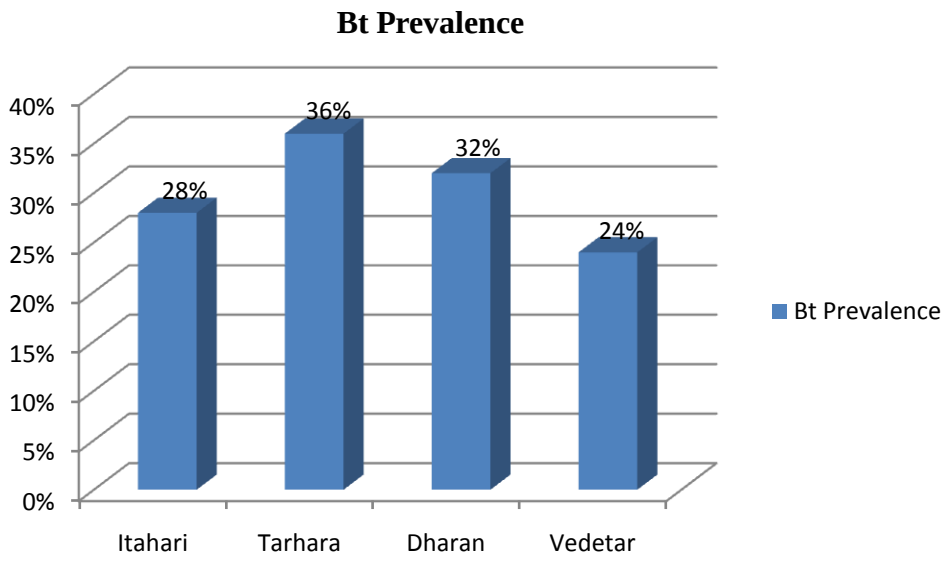
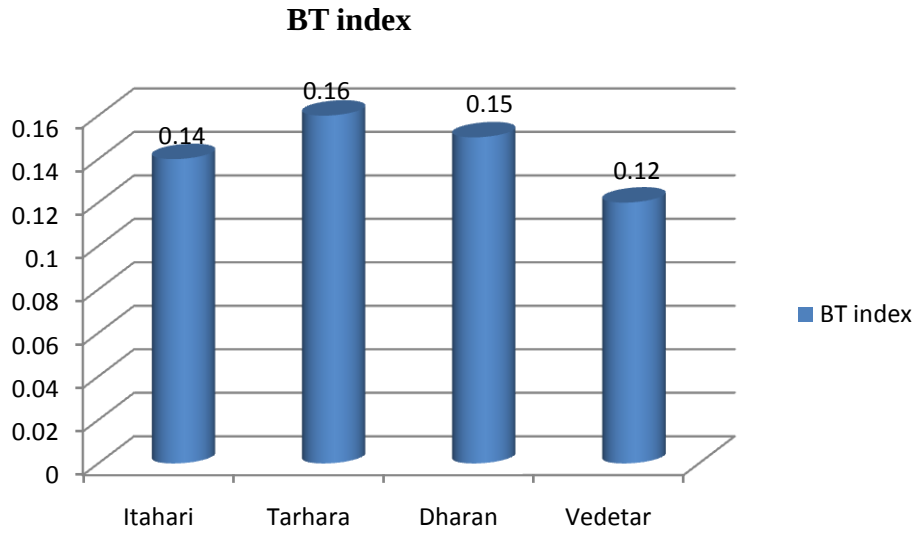


Fig 4.2 BT isolates from sampling area

4.3 BT index

BT index was calculated according to (Analia and Flavia, 2012). It was calculate as the number of BT isolates obtained divided by the number of colonies examined.



4.4 Dead and live record of Bioassay.

Three different concentrations (1mg/ml, 500µg/ml, and 250µg/ml) of spore/crystal were maintained for Bioassay. Dead and live record of bioassay was recorded every day until pupation. The dead and live bioassay in shown in Table 4.3.1

4.4.1 Dead and live record of Bioassay at 1mg per ml of toxin

Sample	Replication	Day 4		Day 5		Day 6		Day 7		Day 8		Total Death	p value
		L	D	L	D	L	D	L	D	L	D		
	R1	3	2	2	1	2	0	0	2	-	-	5	
NS1	R2	4	1	3	1	3	1	1	1	-	-	4	
	R3	3	2	2	1	1	1	1	0	-	-	5	
	R1	5	0	4	1	3	1	3	0	2	1	3	
S8	R2	5	0	5	0	4	1	3	1	2	1	3	
	R3	5	0	5	0	4	1	3	1	2	1	3	
	R1	5	0	5	0	4	1	3	1	1	2	4	0.010
SS1	R2	5	0	5	0	5	0	4	1	3	1	2	
	R3	5	0	5	0	4	1	3	1	2	1	3	
	R1	5	0	5	0	4	1	3	1	2	1	3	
VD5	R2	5	0	5	0	4	1	3	1	1	2	4	
	R3	5	0	5	0	5	0	3	2	2	1	3	
	R1	5	0	5	0	5	0	5	0	5	0	0	
Control	R2	5	0	5	0	5	0	5	0	5	0	0	
	R3	5	O	5	0	4	1	4	0	4	0	1	

L=LIVE, D=DEATH

Table 4.3.1 Dead and live record of Bioassay at 1mg per ml of toxin

4.4.2 Dead and live record of Bioassay at 500 µg per ml of toxin.

Dead and live record of Bioassay at 500 µg per ml of toxin is shown in Table 4.4.2

Sample	Replication	Day 4		Day 5		Day 6		Day 7		Day 8		Total Death	p value
		L	D	L	D	L	D	L	D	L	D		
NS1	R1	5	0	5	0	4	1	3	1	2	1	3	
	R2	5	0	5	0	4	1	3	1	2	1	3	
	R3	5	0	3	2	2	1	2	0	2	0	3	
S8	R1	5	0	5	0	4	1	4	o	4	0	1	
	R2	5	0	5	0	5	0	4	1	3	1	2	
	R3	5	0	5	0	4	1	3	1	2	0	2	0.020
SS1	R1	5	0	5	0	4	1	4	0	3	1	2	
	R2	5	0	5	0	5	0	4	1	4	0	1	
	R3	5	o	5	0	4	1	4	0	4	0	1	
VD5	R1	5	0	4	1	4	0	4	0	4	0	1	
	R2	5	0	5	0	3	2	3	2	3	0	2	
	R3	4	1	4	0	3	1	3	0	3	0	1	

L=LIVE, D=DEATH

Table 4.4.2: Dead and live record of Bioassay at 500 µg per ml of toxin

4.4.3: Dead and live record of Bioassay at 250 µg per ml of toxin.

Dead and live record of bioassay at 250 µg per ml of toxin is shown in Table 4.4.3

Sample	Rep	Day 4		Day 5		Day 6		Day 7		Day 8		Total Death	p value
		L	D	L	D	L	D	L	D	L	D		
NS1	R1	5	0	5	0	4	1	3	1	3	0	2	
	R2	5	0	5	0	4	1	4	0	4	0	1	
	R3	5	0	5	0	4	1	4	1	4	0	2	
S8	R1	5	0	5	0	5	0	4	1	4	0	1	
	R2	5	0	5	0	4	1	4	1	4	0	1	0.202
	R3	5	0	5	0	5	0	4	1	4	0	1	
SS1	R1	5	0	5	0	5	0	4	1	4	0	1	
	R2	5	0	5	0	4	1	4	0	4	0	1	
	R3	5	0	5	0	5	0	5	0	5	0	0	
VD5	R1	5	0	5	0	5	0	4	1	4	0	1	
	R2	5	0	5	0	5	0	5	0	5	0	0	
	R3	5	0	5	0	5	0	4	1	4	0	1	

L=LIVE D=DEATH

Table 4.4.3: Dead and live record of Bioassay at 250 µg per ml of toxin.

4.5 BD% and S % Record of Bioassay.

The BD% and S% was calculated from bioassay record. BD% was found to be greater in NS1 (57.78%) whereas SS1 showed least BD% (31.11). In control survival rate was found to be greater (97%). Detail information for this study is presented in Table 4.4

Treatment	Death%	Survival %	p value
Control	0.00	97	
NS1	57.78	42.22	
S8	35.56	64.44	0.00
SS1	31.11	68.89	
VD5	35.56	64.44	

Table 4.5 BD during Bioassay

4.6 Efficacy of BT isolates

Efficacy % of BT isolates were calculated by modified Abbott method (Abbott 1987). In this study NS1 exhibited greater efficacy against GWM (57.78%) whereas SS1 exhibited least efficacy (31.11%) The efficacy of BT isolates are shown in Table 4.5

Efficacy % of BT isolates

Treatment	Death% by Bt	% survival	Efficacy%	p value
Control	0.00	97	-	-
NS1	57.78	42.22	57.78	0.58
S8	35.56	64.44	35.56	0.45
SS1	31.11	68.89	31.11	0.00
VD5	35.56	64.44	35.56	0.04

Table 4.6: Efficacy of BT isolates

4.7 LC50 of *B. thuringiensis*

The dose mortality response of *Galleria mellonella* at different concentrations of spore/ crystal mixture of *B. thuringiensis* isolates the LC50 value was calculated and is shown in Table 4.6

Bt isolate	Treatment	LC_{50} value µg/ml
NS1	Greater wax moth	388.29
S8		416.20
SS1		463.15
VD5		476.63

Table 4.7: LC50 of *B. thuringiensis*

CHAPTER V

DISCUSSION AND CONCLUSION

5.1. DISCUSSION

Bacillus thuringiensis is Gram-positive, spore forming bacteria that synthesize a large diversity of crystal proteins (Cry and Cyt) during sporulation. Some of these are toxic for a wide range of insects belonging to the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera and Homoptera as well as being active against nematodes, mites and protozoa (Jhonson et al 1998; Schnepf et al., 1998). This characteristics has resulted in *B. thuringiensis* being the most widely used bacterium over the last 50 years for biological control of pests and vectors of disease and its safety to non target insects, birds and mammals has been well demonstrated (Seigel 2001; Jensen et al, 2002).

Several collections of *B. thuringiensis* strains from different regions of the world have been recently characterized. These strains have been isolated from diverse microhabitats such as soil, plant leaves, dead insects and stored grains, as well as aquatic environments such as marine sediments mangroves and fresh water. Characterization of these collections has revealed the great variability and diversity of *B. thuringiensis* in nature (Ben-Dov et al 1997).

Our study was conducted in Central Campus of Technology, Hattisar Dharan and RARS Tarhara from February 2019. 100 Soil samples were collected from all four geographical regions of koshi zone Nepal.25 soil samples were obtained each from Itahari, Tarhara, Dharan and vedetar according to increasing altitude from Itahari (104 m) to vedetar (1420 m). Soil samples were collected by scraping off soil with a sterile spatula 2 to 5 cm below the soil and collected in a sterile polythene bag. Soil samples were transferred to the Microbiology laboratory at 4°C. Isolation of *Bacillus thuringiensis* was done by acetate selection method as described by Travers et al (1987). Identified Bt isolates were used to study their insecticidal properties against greater wax moth (*Galleria mellonella)*. Nepal has a heterogeneous climate that gives unique geographical features and abundant biological resources to study ecological distribution of the organism in soil. The objective of this work was

to isolate Bacillus *thuringiensis* from different areas of different altitudes of Koshi zone and its efficacy against greater wax moth (*Galleria mellonella).*

B. *thuringiensis* isolates were obtained from soil samples of different areas of various altitudes where none of the areas had been previously treated with isolation of *B.thuringiensi*. After acetate selection (Travers et al 1987) of 100 soil samples from 4 different areas (Itahari, Tarhara, Dharan and vedetar), 30 Bt isolates were obtained. Among 30 isolates only 4 isolates showed an insecticidal activity against greater wax moth *Galleria mellonella.*

The screening for local BT isolates was carried out by the acetate enrichment method described by Travers et al. (1987). These authors claimed that germination of BT spores is selectively inhibited in the presence of sodium acetate, whereas most other spore formers are germinated. Selection of BT was attempted by eliminating germinated cells through 15 min of heat treatment at 80 °C. We observed only marginal selection by acetate over other Bt (Bt index = 0.3), and that is consistent with a previous study reported by Bernhard et al. (1997).In earlier reports varying values of Bt index were frequently reported in several studies which ranged from 0.009 to 0.380 in soil samples of India (Thaphan et al. 2008), 0.034 to 0.055 in samples of Western Ghats, India (Ramalakshmi and Udayasuriyan 2010) and 0.2 to 0.5 in sample from New Zealand (Chilcott and Wigley 1993). In other earlier reports frequency for isolation of BT from soil samples varied, ranging from 3 to 85% (Wang et al. 2003) and (Shishir et al. 2014) reported 0.86 Bt index in their samples from Bangladesh. In our study BT index was ranging from0.2 to 0.3.Higher Bt index was found in Dharan (0.3) and least Bt index was found in Vedetar which is 0.2.

Variable percentage of *B. thuringiensis* was found depending on their origin, 28% in Itahari, 36% in Tarhara, 32% in Dharan and 24% in Vedetar. Rana P. et al (2002) only 0.022% of Bt were obtained from soil samples from far western, mid western, western, central and eastern regions of Nepal which is relatively less in comparison to this study. Ohba et al. (2002) reported that the frequency of Bt-positive soil samples averaged between 9.5% and 16.9% in the oceanic islands of Japan, which are in agreement with the general

percentages obtained from this study. The general percentages of *B. thuringiensis* obtained from samples of Asia, and Central and south Africa (94%), Europe (84%), USA (60%), and New Zealand (56%) (Meadows 1993).Nevertheless, these percentages were obtained not only from soil sample but also from the samples from other sources such as insects, silos and mills, which may be more successful source of *Bacillus thuringiensis* (Zang et al 2000). Higher BT index was found in Tarhara which is 0.16 followed by Dharan and Itahari (0.15 and 0.14) respectively .Least BT index was found in Vedetar which was 0.12.

Vilas-Bo and Manoel (2004) suggested the BT index may be an outcome of the biotic environmental factor, e.g., the vegetal top, the type of insect commonly found in the area, or microorganism in the soil, besides, abiotic factors such as the nutrient availability, texture, pH, temperature and humidity. These factors could be the reason behind variation of Bt index in our study zones.

The Greater Wax Moth, (*Galleria mellonella*) is a worldwide serious honey bee pest, especially in warm climate regions of the world (Calvert, 1982; Hachiro and Knox, 2000). Larvae are mostly found in old combs of honey bees, feeding on beeswax, wax residues of honey, insect skin and pollen (Hachiro and Knox, 2000). The larvae of the wax moth cause considerable damage to unattended combs by bees and to combs in storage (Caron, 1992). Adults and larvae are capable of transferring serious bee diseases, such as foulbrood (Charriere and Imdorf, 1999).The greater wax moth, Galleria mellonella Linnaeus has been known as a serious pest against Honey bee in tropical and subtropical regions (kwadha et al 2017).

The Bioassay of Bt against Greater wax moth was performed for both qualitative and quantitative study. A series of bioassays were performed by providing the food contaminating with the spores and crystals. Spores and crystals were both included in the suspensions because they produce a higher level of mortality than either crystals or spores alone (Crickmore 2006). Qualitative bioassay results showed only 0.13% of local BT isolates were toxic to the tested *Galleria melllonella* larva. Chilcott and Wigley (1993)

showed that the percentage of isolates obtained from soil with toxicity against lepidopteran larvae ranged from 37% to 88%. Similarly, Iriarte et al. (1998) reported that most of the BT isolates showed insecticidal activity (above 25% mortality) against some lepidopteran species. In our study BT isolates showed 31% to 57% mortality which is consistent with the previous reports.

B. thuringiensis isolated from Tarhara showed high degree of mortality which is 57.78% followed by Dharan and vedetar (35.56% and 31.11%) respectively. Three different concentration of spore/ protein mixture (1mg/ml, 500µg/ml, and 250µg/ml) were used for bioassay. Among all isolates NS1 was the most potent which showed 57.57% efficacy followed by S8, SS1 which is 35.56%. *B. thuringiensis* isolated from Vedetar showed least efficacy which is 31.11%. This difference may be due to ecological environment which was not highly selective as of high altitude region and also may be due to various agricultural and industrial practices, which could not be explained from the present study. The Bioassay of Bt protein at concentration of 1mg/ml with mortality of Greater wax moth was statistically significant ($p<0.05$). Similar statistical significance was observed even with BT protein mixture with concentration of 500µg/ml. The Bioassay with 250µg/ml was not statistically significant ($p>0.05$).LC 50 value of the tested isolates against *Galleria mellonella* larvae varied from 388.29 to 476.63µg/ml. The toxicity of *Bacillus thuringiensis* depends upon the size and abundance of crystal protein found in the bacteria (Rana et al 2002).

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The use of chemical insecticides to control insect pests has increased over the years and has generated resistance to chemicals. Microbial pesticides such as *B. thuringiensis* may become feasible and reliable alternative to control insects.

This study shows that the soil is a very important source of *B. thuringiensis.* From this result it is concluded that *B. thuringiensis* is a safe microbial agent for Controlling greater wax moth *Galleria mellonella* and its use in the development of bio insecticides to control insect pests.

RECOMMENDATIONS

1. Insect pests are major limiting factor in the agriculture commodity so they must be controlled by *Bacillus thuringiensis* as Bio pesticides.

2. Confirmation of *Bacillus thuringiensis* must be done through PCR.

3. Mass production of *Bacillus thuringiensis* must be done.

4 Evaluation of Bt must be done on farmer's field.

5. Bt must be tested in different species for virulence and efficacy.

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Photograph No.: 1 Bacillus thuringiensis culture

Photograph No.: 2.Crystal protein staining

Photograph No.: 3 Adult greater wax moth

Photograph No.: 4. Greater wax moth larva

Photograph No.: 5 working in lab

APPENDIX -I

Materials and equipments
List of the materials
Glass wares

Petri plates
Beaker
Conical flasks
Eppendrufftube
Glass slides
Micro pipettes
Measuring cylinder
Micro pipette tips
Glass rod
Test tubes

Miscellaneous

Inoculating loop
Test tube rack
Gloves
Forceps
Labeling sticker
Bunsen burner
Cotton swabs
Marker
Match box

Equipments

Autoclave
Microscope
Hot air oven
Water bath shaker
Laminar airflow
Incubator
Refrigerator
Centrifuge
Digital balance

Reagents/strains

Saffranin
Lysol
Alcohol
Coomassic brilliant blue R-250
Crystal violet

Antibiotis

Streptomycin
Ampicillin
Tetracycline
Amoxicillin

Culture media

Agar powder
Beef extract
Nutrient broth
Lauria Berteni broth
Peptone
Nutrient agar
Typtone

Biochemical media

Casein
Fructose
Starch
Simmon's Citrate Agar
Glucose
Gelatin
Sucrose

APPENDIX - II

I.Culture Media

1. LauriaBertani Broth

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Final pH (at 25°C)	6.8 ±0.2

2. LauriaBertani Agar

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	6.8 ±0.2

3. Nutrient Broth

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Final pH	4

4. Nutrient Agar

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	7.4 ±0.2

II. Biochemical Media

1. Nitrate Broth

Ingredients	(Grams/Litre)
Beef extract	3.0
Peptone	5.0
Potassium Nitrate	1.0
Sodium thiosulphate	0.025
Final pH at (25°C)	7.3±0.2

2. Carbohydrate fermentation medium

Ingredients	(Grams/Litre)
Peptone	10.0
Sodium chloride	5.0
Phenol red	0.018
Glucose-	1%
Sucrose-	1%
Fructose	1%
Carbohydrates	
Final pH at (25°C)	7.2±0.2

3. Hydrolysis Agar Media

i. Casein Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Casein	1.0
Final pH at (25°C)	7.2±0.2

ii. Egg yolk agar

Ingredients	(Grams/Litre)
Nutrient Agar	200 ml
Egg-yolk emulsion	8.0 ml

iii. Gelatin Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Gelatin	1.0
Final pH at (25°C)	7.2±0.2

iv. Starch Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Starch	1.0
Final pH at (25°C)	7.2±0

APPENDIX - III

The efficacy % was calculated by using Abbotto's Formula given below.

$$\text{Efficacy \%} = \frac{\text{survival untreated} - \text{survival treated}}{\text{Survival untreated}} \times 100$$

Survival untreated=97

NS1Survival treated=22.22

S8 survival treated=35.56

SS1 survival treated=35.56

VD5 survival treated=64.44

So,

$$\text{Efficacy of NS1} = \frac{97-22.22}{97} \times 100$$

$$=77.09\%$$

$$\text{Efficacy of VD5} = \frac{97-64.44}{97} \times 100$$

$$=33.56\%$$

$$\text{Efficacy of S8} = \frac{97-35.56}{97} \times 100$$

$$=35.56$$

$$\text{Efficacy of SS1} = \frac{97-35.56}{97} \times 100$$

=63

APPENDIX -IV

BT index was calculated according to (Analia and Flavia, 2012).

$$\text{BT index} = \frac{\text{No of BT isolates obtained}}{\text{No of colonies examined}}$$

$$\text{BT index of Itahari} = \frac{7}{50} = 0.14$$

$$\text{BT index of Tarhara} = \frac{9}{56} = 0.16$$

$$\text{BT index of Dharan} = \frac{8}{53} = 0.15$$

$$\text{BT index of Vedetar} = \frac{6}{50} = 0.12$$

APPENDIX- V

Statistical Analysis

Treatment (1mg/ml) -Mortality

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.000[a]	5	.010
Likelihood Ratio	15.012	5	.010
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .20.

H_0- There is no significant difference in Treatment with mortality

H_1- There is significant difference in Treatment with mortality

p=0.10 i.e p<0.05, Test statistics is Significant so we Reject Null Hypothesis and accept Alternative Hypothesis.

Treatment(500µg/ml) and Mortality

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.792[a]	3	.020
Likelihood Ratio	9.605	3	.022
N of Valid Cases	15		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is .40.

There is significant difference in Treatment and mortality.

Treatment (250µg/ml) and Mortality

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.194[a]	2	.202
Likelihood Ratio	3.188	2	.203
N of Valid Cases	15		

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is .40.

There is no significant difference in treatment (250µg/ml) with mortality.

Univariate Analysis of Variance- ANOVA

Tests of Between-Subjects Effects

Dependent Variable: Mortality

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	38.889[a]	2	19.444	31.048	.000
Intercept	160.444	1	160.444	256.194	.000
Treatment	38.889	2	19.444	31.048	.000
Error	20.667	33	.626		
Total	220.000	36			
Corrected Total	59.556	35			

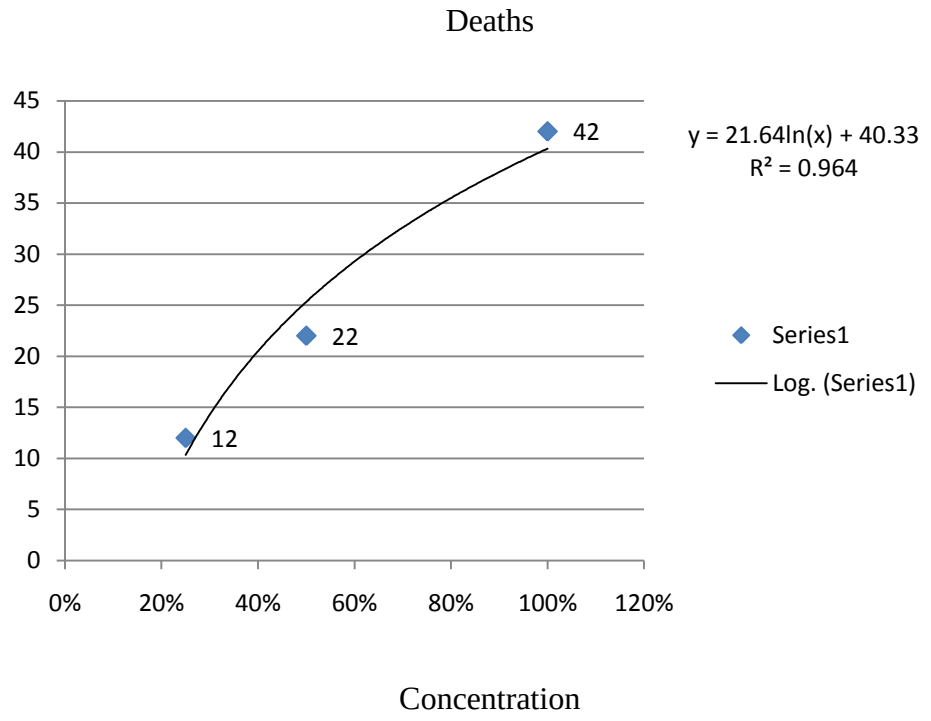
a. R Squared = .653 (Adjusted R Squared = .632)

H_0- There is no significant difference in mortality with three different Treatments

H_1- There is significant difference in mortality with three different Treatments

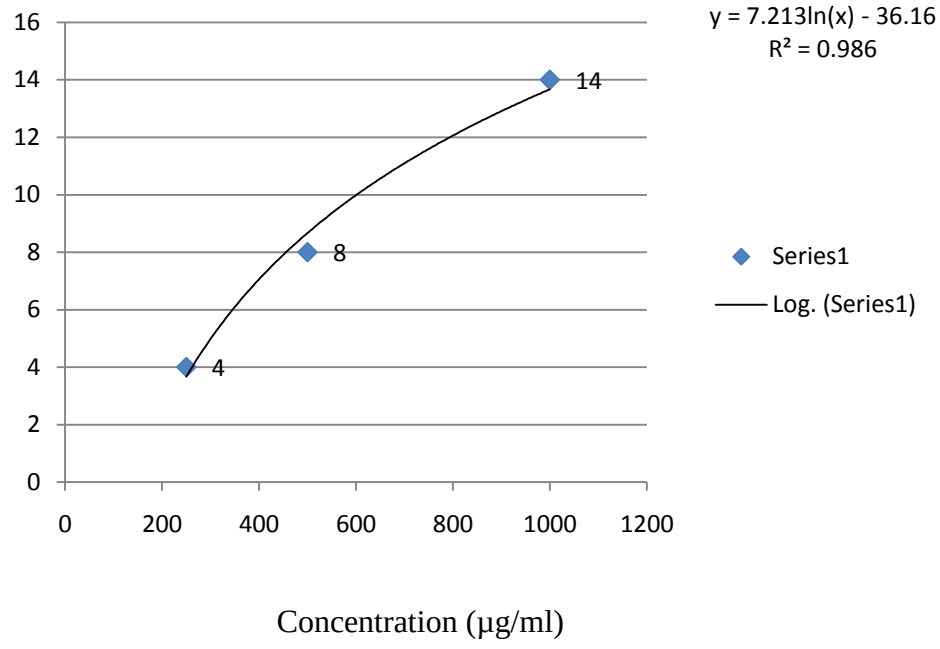
p=0.00 i.e. p<0.05, Test statistics is Significant so we Reject Null Hypothesis and accept Alternative Hypothesis.

Result-There is significant difference in Mortality by three different concentrations of treatments.



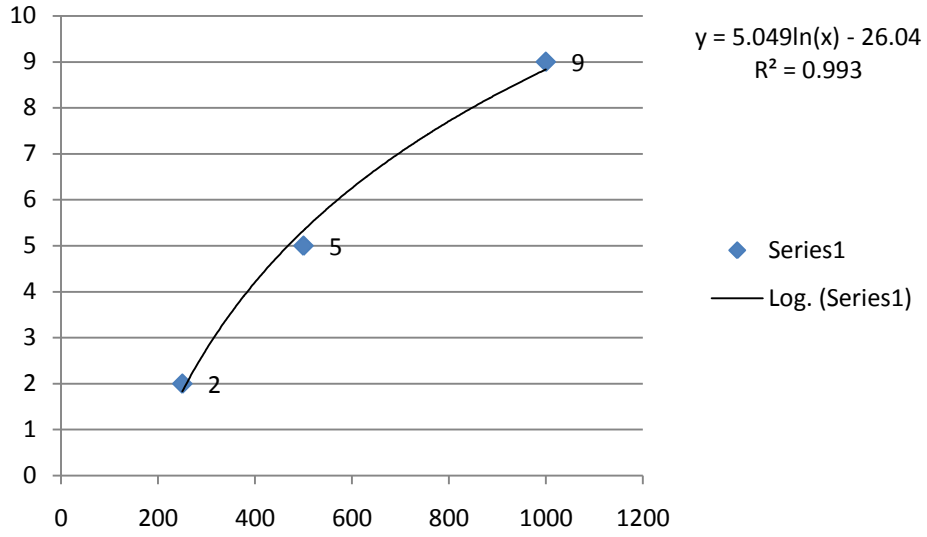
By calculation,LC_{50}(Lethal concentration $_{50}$) = 412

ISOLATE CODE: NS1



LC_{50}= 388.29 µg/ml

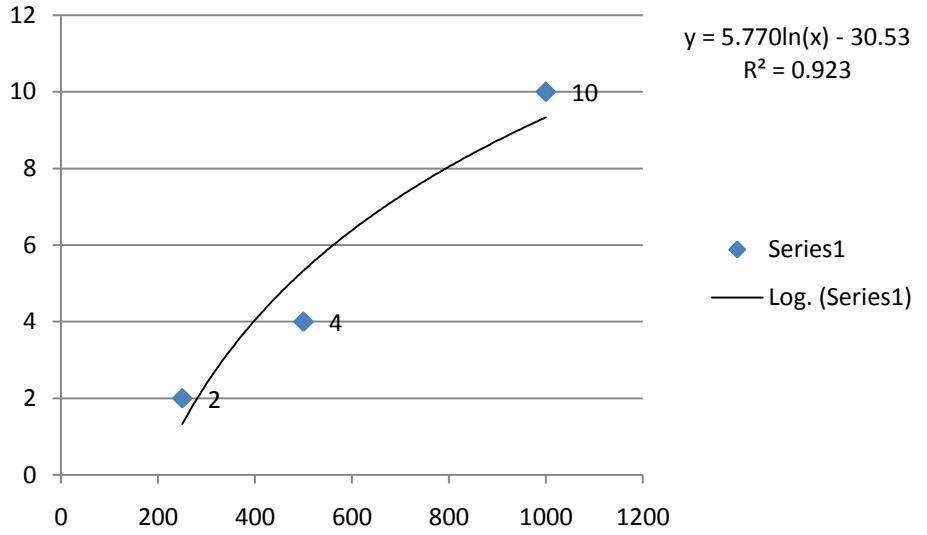
ISOLATE CODE: S8



Concentration (µg/ml)

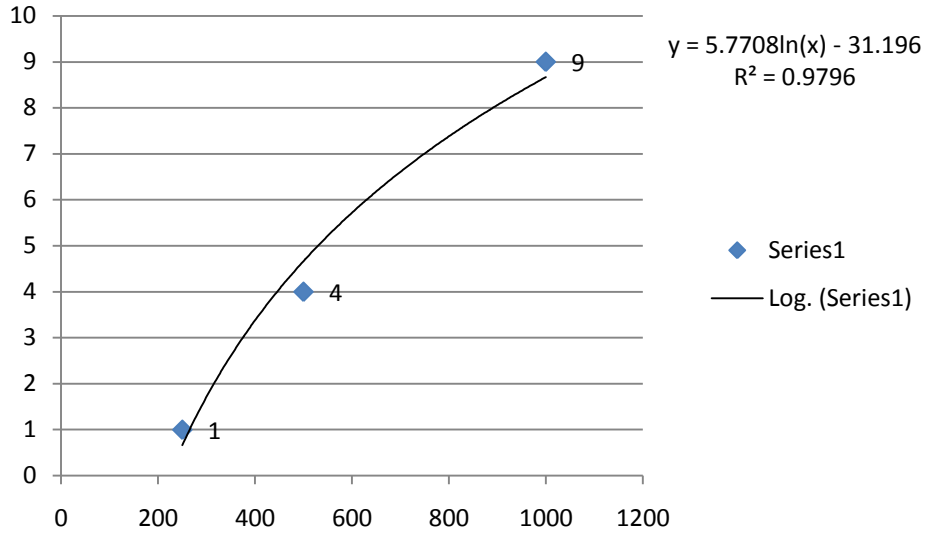
LC_{50}= 416.20 µg/ml

ISOLATE CODE: SS1



LC_{50}= 463.15 µg/ml

ISOLATE CODE: vd1



LC_{50}= 476.63 µg/ml