



Isolation of *Bacillus thuringiensis* and its Insecticidal Effect against *Galleria mellonella*

^aJyoti Limbu, ^aBijay Kumar Shrestha, ^aJenish Shakya, ^bSabin Bahadur Khatri, ^aHemanta Khanal*

^aDepartment of Microbiology, Central Campus of Technology, Tribhuvan University, Dharan, Nepal

^bDepartment of Food Technology, Central Campus of Technology, Dharan, Nepal

*Corresponding email: khanal.hemanta@gmail.com

Abstract

Bacillus thuringiensis (Bt) synthesizes a large diversity of crystal proteins (Cry and Cyt) during sporulation which exhibit insecticidal activity against insects and protozoa. The main aim of this study was to isolate *Bacillus thuringiensis* and study its insecticidal effect against *Galleria mellonella*. Soil samples from four different geographical locations of Koshi Zone viz. Itahari, Tarhara, Dharan and Vedetar of Eastern Nepal were collected. The isolation of Bt was done by acetate selection method. The insect bioassay of Bt isolates were performed against greater wax moth (*G. mellonella*) by feeding the third instar larvae by extracted crystal spores with three different concentrations. The overall distribution of Bt from the study sample was found to be 30% (30/100). Bt was isolated from all four geographical locations with higher incidence; 9 (36%) in Tarhara region followed by Dharan (32%), Itahari (28%) and Vedetar (24%). However, the incidence of Bt with potent insecticidal activity against *G. mellonella* was reported to be 4% (4/100). The insecticidal activity of isolated Bt between test and control groups was found to be statistically significant ($p < 0.05$). LC₅₀ value of Bt from Tarhara (Tar1) was 388.29 µg/mL, Dharan; Drn8 and Drn1 was 416.20 µg/mL and 463.15 µg/mL respectively and from Vedetar (Vd5) was 476.63 µg/mL. In overall study the Bt isolated from Tarhara (Tar1) region exhibited greater incidence, Bt index, efficacy and effective level of LC₅₀ against greater wax moth. Native Bt strains isolated from soil of Eastern Nepal possess effective insecticidal activity and hence can be used as biocontrol agent in controlling honeycomb pest like *G. mellonella*.

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1. Introduction

Biological control is the most valuable tool of Integrated Pest Management (IPM) which involves the use of biological agents such as parasitoids, predators and entomopathogen like fungi, bacteria, virus nematodes etc. to control pests (Chandler et al., 2011). Bacterial biopesticide has significant attention in pest control (Sarwar, 2015). *Bacillus thuringiensis* (Bt) is the most commonly used and successful entomopathogenic bacterium (Jurat-Fuentes & Jackson, 2012).

Bt is a gram positive, rod shaped, insecticidal bacterium which produces crystal protein called δ -endotoxin during stationary phase of its growth (Kumar et al., 1996). The insecticidal property of Bt against great number of insects leads Bt into an important tool to be used in the insect pest

management. The crystal protein produced by Bt is toxic against large number of insect species of orders Lepidoptera, Diptera, Coleoptera and few Hemiptera (MacIntosh et al., 1990). When crystal protein is ingested orally by insects, it solubilizes in the midgut which causes cell lysis and disruption of midgut epithelium leading to death. However, the presence of specific receptor in the midgut determines the toxicity of crystal protein (Bravo et al., 2007).

B. thuringiensis with different potentialities and different insecticidal effects have been observed in various part of the world. The eastern region of Nepal has a heterogeneous climate with unique geographical features and abundant biological resources (Carpenter & Zomer, 1996). Bt isolated from this region may help in finding new cry genes with potential insecticidal activity against the greater wax moth (*G. mellonella*)

which is a worldwide serious honey bee pest in tropical and subtropical regions of world (Shimanuki & Knox, 2000, Kwadha et al., 2017). PCR analysis of Bt with universal primer is specific for detection of *cry1* and *cry2* class specific genes (Aly, 2007). Lethal Concentration 50 (LC50) is the amount of a substance required to kills 50% of a test animals exposed after a single dose. The main aim of this study was to isolate *Bacillus thuringiensis* from different areas of Koshi Zone and study its insecticidal efficacy against larva of greater wax moth (*G. mellonella*). This study was also aimed to determine Lethal Concentration 50 (LC50) of extracted crystal spore against the larva of moth.

2. Materials and Methods

2.1 Research design

This study was carried out in Central Campus of Technology Hattisar Dharan and Regional Agricultural Research Station (RARS) Tarhara from October 2018 to March 2019. Soil samples were collected from different altitudes ranging from Itahari (374 ft.), Tarhara (418 ft.), Dharan (1272 ft.) and Vedetar (5140 ft.) of Koshi Zone, Nepal. From each geographical region 25 different soil samples were collected and in total 100 different soil samples from four regions were collected. Soil samples weighing 10gm was collected in sterile plastic bags from 3-5cm depth and transported into the laboratory at 4°C. Materials used in this experiment were Sodium acetate (HiMedia, India), Lauria Bertani (LB) broth (HiMedia, India), Nutrient Agar (HiMedia, India), Coomassie brilliant blue (HiMedia, India), Phosphate buffer saline (HiMedia, India), Grams staining reagents (HiMedia, India), MR-VP medium and reagents (HiMedia, India), Indole medium (HiMedia, India), Citrate agar medium (HiMedia, India), Gelatin agar medium (HiMedia, India), Starch agar medium (HiMedia, India), Egg yolk agar medium (HiMedia, India), Blood agar medium (HiMedia, India), Carbohydrate fermentation broth (HiMedia, India), SIM medium (HiMedia, India), Triple sugar iron agar (HiMedia, India).

2.2 Isolation and identification of *Bacillus thuringiensis*

B. thuringiensis was isolated by Acetate selection method as described by Travers et al. (1987) with slight modification. One gram of soil sample was taken in a sterile conical flask containing 1mL of 0.25M sodium acetate (pH 6.8) and 9mL of Lauria Bertani (LB) broth (HiMedia, India). The broth was incubated for

overnight at 30°C. After incubation the broth was heated at 100°C for 5 minute. After heat treatment, 0.1mL of sample was taken and spread on nutrient agar (HiMedia, India) and plates were incubated at 30°C for overnight. Bt like colonies white, large, nearly circular with fine irregular margins and may be glossy, less glossy or rough were selected and further sub cultured on nutrient agar (HiMedia, India). Bt was further identified by routine microbiological tests like Gram's staining and other biochemical tests like indole test, MR test, VP test, citrate test, starch test, gelatin test, beta haemolysis test, sucrose, fructose, mannitol, lactose fermentation test, motility test and lecithinase test after incubation of the culture in the respective biochemical test media (Sneath, 1986). For presence of parasporal crystal staining the bacterial culture incubated for 72 hours in a nutrient broth was used. Smear was stained with 0.25% coomassie brilliant blue for 3 minutes and washed with distilled water and observed under light microscope at oil immersion (Muniady et al., 2011). The bacteria were identified as *Bacillus thuringiensis* based on colony morphology, Gram's staining, biochemical test and parasporal body staining. In this study *B. thuringiensis* DOR Bt-1 strain was used as a positive control.

2.3 Rearing of greater Wax Moth *G. mellonella*

The greater wax moth *G. mellonella* was obtained from Department of Entomology, Regional Agricultural Research Station (RARS) Tarhara and reared in the laboratory according to Mohamed and Coppel, (1983). Artificial diet for moth comprised following ingredients:- Deionized, preboiled water – 100mL, Honey, commercial brand – 150mL, Bee wax – 3gm, Cholesterol – 1gm, Multivitamin supplement – 4mL, Maize flour – 100gm, Milk powder – 100gm.

Adult wax moths were kept into 1litre mason jars where the mating took place. Folded sheets of wax with paper clips were placed where the moth deposited eggs. The eggs were removed and transferred into the 500mL Mason jar with 150gm of medium and incubated at 28.5°C in the dark. After 10-15 days second instar larvae appeared. After 8-10 days larvae were transferred into 1liter Mason jar with 175gram of medium. Within 5-10 days third instar larvae became available which were used for insect bioassay.

2.4 Preparation of mass culture of *Bacillus thuringiensis*

The mass culture of Bt was prepared as described

by Ammouneh et al. (2011). Lauria Berteni (LB) broth was used for the preparation of mass culture of Bt. Pure culture of Bt was inoculated in sterile LB broth and incubated in water bath shaker at 25°C for 4 days. Spores were harvested by centrifugation at 10000 rpm for 15 minutes. Pellets were washed twice with sterile distilled water. Cell disruption process was done by lyophilization. Lyophilized powder was dried in laminar air flow and later it was stored at -20°C until use.

2.5 Insect bioassay

Insect Bioassay was performed according to Ammouneh et al. (2011). Bt preparation (spore/protein mixture) in the form of powder was used for bioassay. Three different concentrations (1000µg/mL, 500µg/mL and 250µg/mL) of spore/protein mixture were prepared in phosphate buffer saline (PBS: 1M KH₂PO₄, 1M K₂HPO₄, 5M NaCl, pH-7.2). Third instar larvae were used for the bioassay. About 0.5mL of each concentration was mixed with 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 a negative control, the artificial diet was supplemented with sterile 0.5mL distilled water. The cups were covered with muslin cloth and wrapped with rubber band. Hence in total, 45 larvae for each Bt isolate were taken under study. Larvae were put into an empty box for 2 hours to starve before releasing them for treatment. Dead and live record of bioassay was recorded every day until pupation.

2.6 Data analysis

Data recorded from bioassay was documented and tabulated in MS-EXCEL 2010. The statistical analysis for calculating LC₅₀ values of each treatment were performed according to Stephan (1977) from concentration-mortality curves. The data were statistically analyzed by SPSS version 16.0 windows software. Statistical significance was established when p value was less than equal to 0.05 with 95% confidence interval.

3. Results and Discussion

3.1 Distribution of Bt in soil

The overall distribution of Bt from the study sample was found to be 30% (30/100). The number of Bt isolates from Itahari, Tarhara, Dharan and Vedetar were 7 (28%), 9 (36%), 8 (32%) and 6 (24%)

respectively (Fig. 1).

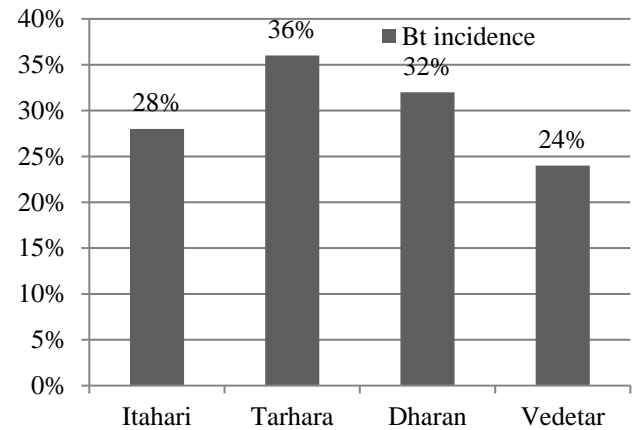


Fig. 1: Bt isolates from sampling area

3.2 Bt index

Bt index is defined as the ratio of number of identified Bt colonies to the total number of spore-forming bacilli colonies. Bt was calculated according to Alvarez & del Valle Loto, (2012). The highest Bt index was found to be 0.16 from Tarhara and least index of 0.12 from Vedetar (Fig. 2).

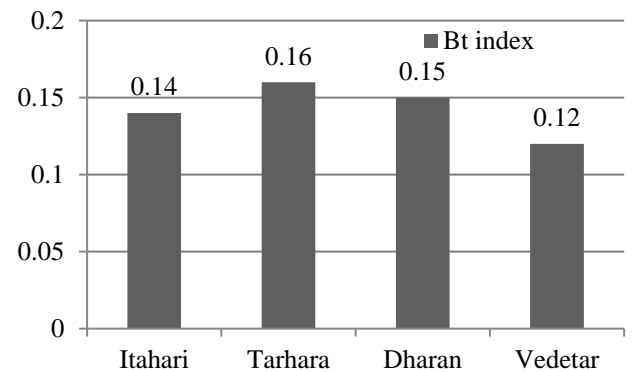


Fig. 2: Bt index

3.3 Distribution of Potent Bt isolates

Bt isolates were obtained from soil samples of different areas of various altitudes where none of these areas had been previously treated with isolation of Bt. After acetate selection (Travers et al., 1987) of 100 soil samples from 4 different areas (Itahari, Tarhara, Dharan and Vedetar), 30 (30%) Bt isolates were obtained. Among 30 isolates only 4 (4%) isolates showed an insecticidal activity against greater wax moth (*G. mellonella*) and remaining 26 (96%) were negative. Potent isolates were recovered from all study areas except from Itahari; the isolate Tar1 was isolated from Tarhara, Drn8 and Drn1 from Dharan and Vd5 from Vedetar (Fig. 3).

Table 1: Insect Bioassay. For each concentration treatment 15 larvae were taken under study that required 45 larvae for study in all three concentrations. Cn-Concentrations. Tar1-Tarhara1, Drn8-Dharan8, Drn1-Dharan1, Vd5-Vedetar5

Treatment	Total larva used in all 3 Cn	Total Deaths at 1000 µg/mL	p value	Total Deaths at 500 µg/mL	p value	Total Deaths at 250µg/mL	p value
Positive Control	45	14		12		8	
Negative Control	45	0		0		0	
Tar1	45	14		9		5	
Drn8	45	9		5		3	
Drn1	45	9	P<0.05	4	P<0.05	2	P>0.05
Vd5	45	10		4		2	

Table 2: Efficacy % of Bt isolates

Treatment	Efficacy %	% survival	% Mortality by all Bt isolates	p value
Positive control	75.55	24.45	-	-
Negative control	0.00	100.00	-	-
Tar1	62.22	37.78		
Drn8	37.78	62.22		
Drn1	33.33	66.67	42.22 %	P<0.05
Vd5	35.56	64.44		

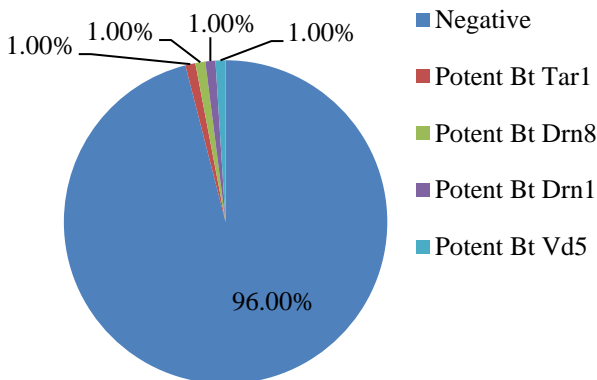


Fig. 3: Distribution of Potent Bt isolates

3.4 Insect bioassay

Three different concentrations (1000µg/mL, 500µg/mL and 250µg/mL) of spore/crystal were maintained for insect Bioassay. Dead and live record of bioassay was recorded every day until pupation (Table 1).

3.5 Efficacy of Bt isolates

Efficacy % of Bt isolates were calculated by modified Abbott, (1925). In this study Tar1 exhibited greater efficacy against greater wax moth (62.22%) whereas Drn1 exhibited least efficacy (33.33%) (Table 2).

3.6 LC50 of Bt

The dose mortality response of *G. mellonella* at different concentrations of spore/crystal mixture of Bt isolates LC50 value was calculated (Table 3).

Table 3: LC50 of Bt

Bt isolate	Tar1	Drn8	Drn1	Vd5
LC50 value	388.29	416.20	463.15	476.63

*All measurements in µg/mL

The entomopathogenic activity of *Bacillus thuringiensis* has made it widely used successful biopesticide. Since, Nepal is an agriculture country the use of this biopesticide in agriculture will be ecofriendly and effective approach. 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 et al. (2002) reported only 0.022% of Bt isolates 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. Nepal has a wide diversity in ecosystem and biological resources that forms the bedrock for variation in the distribution of beneficial microorganism in soil. The isolated native Bt is considered as the indigenous microflora of the soil. However, its commercial use in developing nation like Nepal in pest management has rarely being applied.

Selection of Bt was attempted by eliminating germinated cells through 5 min of heat treatment at 100°C. This modification was effective for selective

isolation of *Bacillus thuringiensis* from other bacillus species. This experiment with modification was based on the fact that acetate inhibits the germination of Bt spores allowing it to resist heat treatment which will allow for its selective isolation from other spore forming and non-spore forming organisms which will germinate on medium during incubation but would be killed in heat treatment (Rana et al., 2002). 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). Ramalakshmi & Udayasuriyan (2010) reported 0.034 to 0.055 Bt index in samples of Western Ghats, India. Bt index of 0.2 to 0.5 was observed in sample from New Zealand (Chilcott & Wigley, 1993). Shishir et al. (2014) reported 0.86 Bt index in their samples from Bangladesh. The studies reported diversity in Bt index in different areas across the world. In this study, higher Bt index was found in Tarhara which was 0.16 followed by Dharan and Itahari with 0.15 and 0.14 respectively. Least Bt index was found in Vedetar which was 0.12. Vilas-Bo & Lemos (2004) suggested the Bt index may be an outcome of the biotic environmental factor, e.g., the vegetation type, 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 these study Zones.

In this study 30 isolates from 100 soil samples were confirmed to be *B. thuringiensis* based on staining, biochemical tests and coomassie brilliant blue staining (CBBS) technique although the morphology of the bacteria differed from the sample site. In this study the coomassie brilliant blue staining (CBBS) of 72 hours old culture revealed centrally located parasporal body. The parasporal body (crystal protein) is made of protein compound which can be well stained by coomassie brilliant blue as dark blue color where the spore remain unstained and the vegetative cell gets light blue stain (Ammons et al., 2002). The presence of crystal protein in Bt isolates from our sample site indicates that soil of Eastern Nepal is rich in diversity of potent Bt strains.

The greater wax moth, *G. mellonella* Linnaeus has been identified as a serious pest against Honey bee. Larvae are mostly found in old combs of honey bees, feeding on beeswax, wax residues of honey, insect skin and pollen (Shimanuki & Knox, 1991). The larvae of

the wax moth cause considerable damage to unattended combs by bees and to combs in storage (Caron, 1992). In this study 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 produced a higher level of mortality than either crystals or spores alone (Crickmore, 2006). Qualitative bioassay results showed 42.22% of mortality by all Bt isolates tested to *Galleria mellonella* larva. Chilcott & 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 this study Bt isolates showed 33.33% to 62.22% mortality which is consistent with the previous reports. Bt isolated from Tarhara showed high degree of mortality which indicates that soil of Tarhara area harbors potent strains of Bt. The soil of Tarhara which has been used for organic agricultural practices might have contributed in ecological maintenance of native strains of potent Bt. The insecticidal activity of isolated Bt among test and control groups was found to be statistically significant ($p < 0.05$).

Three different concentration of spore/protein mixture (1000 μ g/mL, 500 μ g/mL, and 250 μ g/mL) were used for bioassay. Among all isolates Tar1 was the most potent which showed 62.22% efficacy followed by Drn8 and Vd5 which is 37.78% and 35.56% respectively. *B. thuringiensis* isolated from Drn1 showed least efficacy which was 33.33%. 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 1000 μ g/mL reported significant mortality of greater wax moth which was statistically significant ($p < 0.05$). Similar statistical significance was observed even with Bt protein mixture with concentration of 500 μ g/mL. However, the insect bioassay with 250 μ g/mL was not statistically significant with test and control group ($p > 0.05$). There was statistical difference in mortality of greater wax moth by isolated Bt at three different concentrations

($p < 0.05$). Hence, it is reported that the level of toxins also determined the mortality of insect. LC50 value of the tested isolates against *G. mellonella* larvae varied from 388.29 to 476.63 µg/mL. It is reported that, toxicity of *Bacillus thuringiensis* depends upon the size and abundance of crystal protein found in the bacteria (Rana et al., 2002). So, this might be the reason behind various toxicity levels among different isolates. In overall analysis the Bt isolated from Tarhara exhibited greater incidence, Bt index, efficacy against greater wax moth and effective level of LC50. Further studies are needed to understand the role of ecological environment and soil composition that harbors potent Bt strains from soil of Tarhara region of Koshi Nepal.

4. Conclusions

The study revealed that the soil of eastern Nepal holds wide diversity of native Bt strains. The diversity in cry protein content and insecticidal activity might have relationship with geographical location and environment. From this study it is also concluded that *B. thuringiensis* is a safe microbial agent for controlling greater wax moth (*G. mellonella*) and it can be used in the development of bio insecticides to control pests. The mass production, formulation and rational use of Bt as biopesticide can assist in eliminating agricultural pests and hence can prevent the environmental hazards caused by use of chemical pesticides.

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Conflicts of Interest

The authors declare no conflicts of interest.

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