# 4. MATERIALS AND METHODS

## 4.1 Periodic survey of tea plantations:

For collection of sporadic pest specimens (lepidopteran), different tea plantations of Darjeeling foothills as well as its adjoining plains, Terai and the Dooars regions were extensively surveyed. The tea gardens selected for survey were Sepoydhura T.E, Makaibari T.E, Ambotia T.E, Soureni T.E, Singel T.E, Goomtee T.E, Sungma T. E, Seeyok T.E and Singtam T. E of lower elevation of Darjeeling hills (Fig. 4.1; Fig. 4.4A), MatigaraT.E., Dagapur T.E., Nischintapur T.E., Mohorgong T. E., New Chamta T. E., Simulbari T.E., Panighata T.E., Trihana T. E., Kamalpur T.E., Atal T.E., Naxalbari T. E., Gungaram T.E., Gayaganga T. E. and Kamla T.E. from the Foothills or Terai region (Fig. 4.2; Fig. 4.4B), Bagracote T.E., Chalsa T.E., Samsing T.E., Nagrakata T.E., Grassmore T.E., Binnaguri T.E., Hantapara T.E., Madhu T.E., Kalchini T.E., Chuapara T.E., Bhatkhawa T.E., Kumargram T.E. and Sankosh T.E. from the Plains or Dooars region (Fig.4.3; Fig. 4.4C)

## 4.2 Collection of dead and moribund larvae:

Moribund and dead pest larvae were collected from natural populations occurring in tea gardens and those dying in laboratory reared population. After proper surface sterilization, dead larvae (from tea garden as well as laboratory reared) were stored in double distilled water at -20°C for extraction and study of pathogenic bacteria in future.

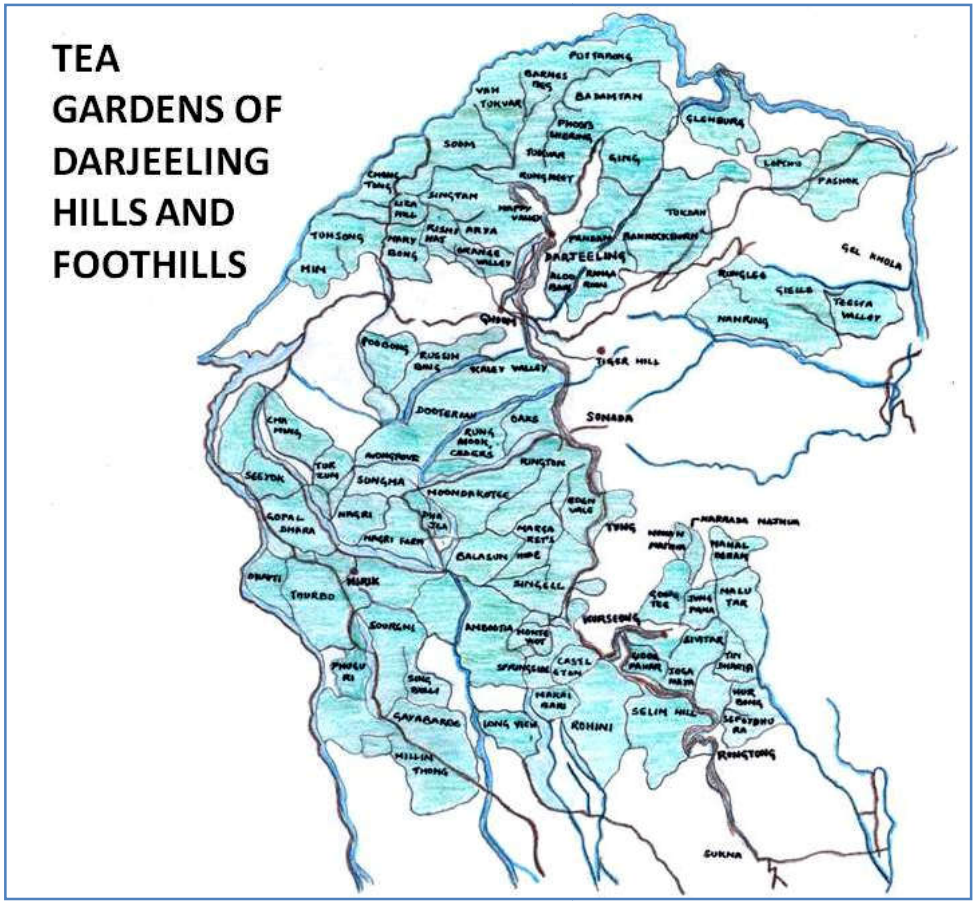



**Fig. 4.1: Map showing tea gardens of Darjeeling hills and foothills.**

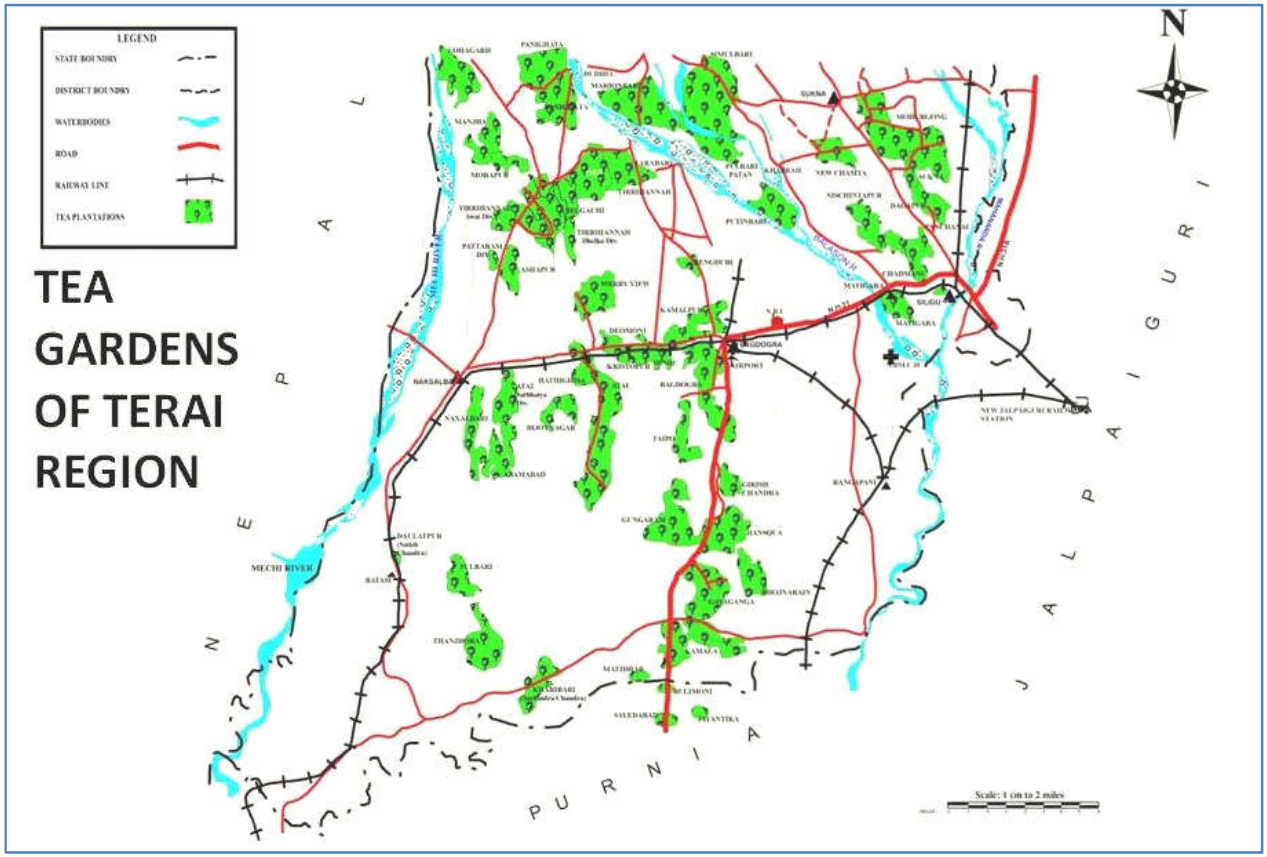



**Fig. 4.2: Map showing tea gardens of the Terai region of Darjeeling district.**

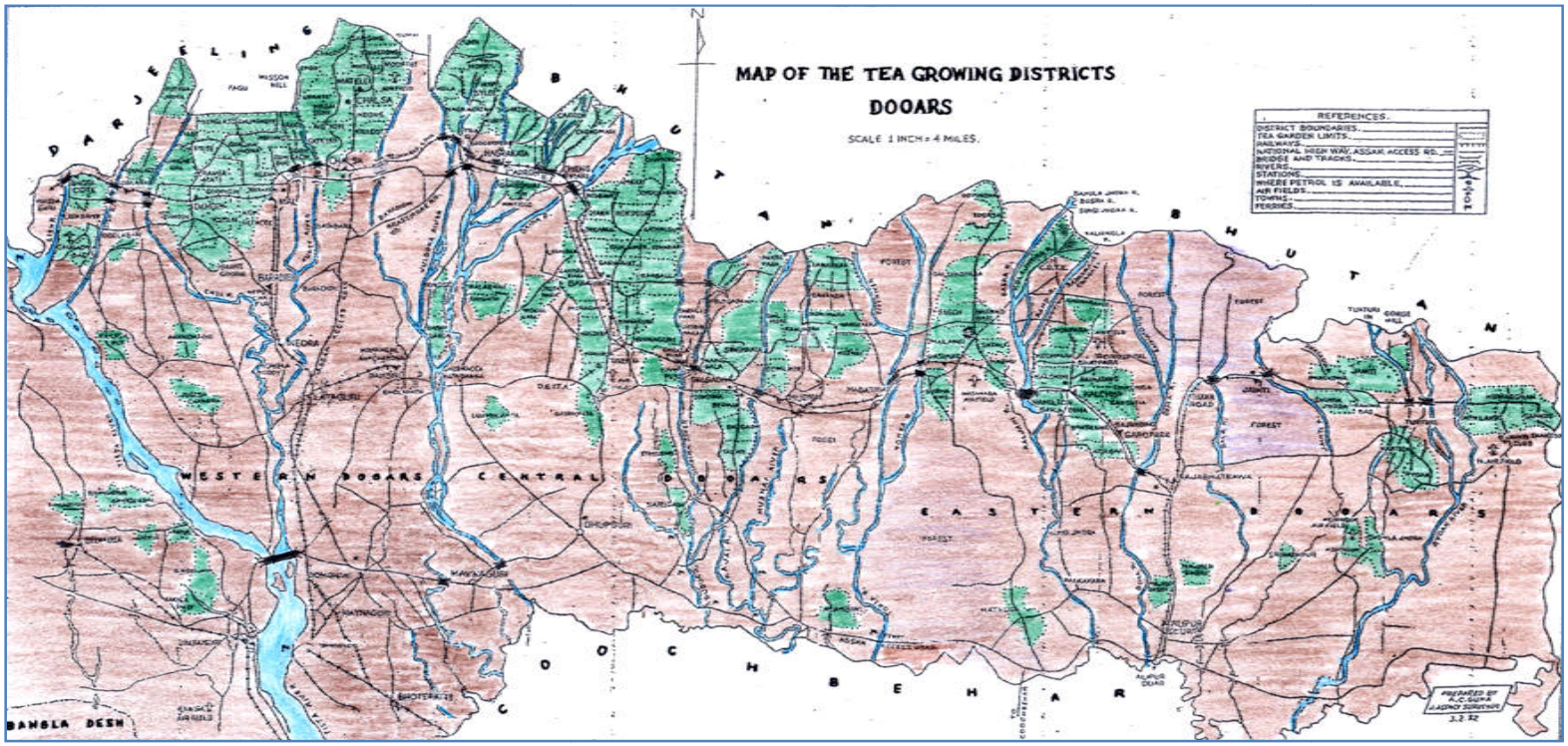



**Fig. 4.3: Map showing the tea gardens of the Dooars region of Jalpaiguri and Alipurduar districts.**

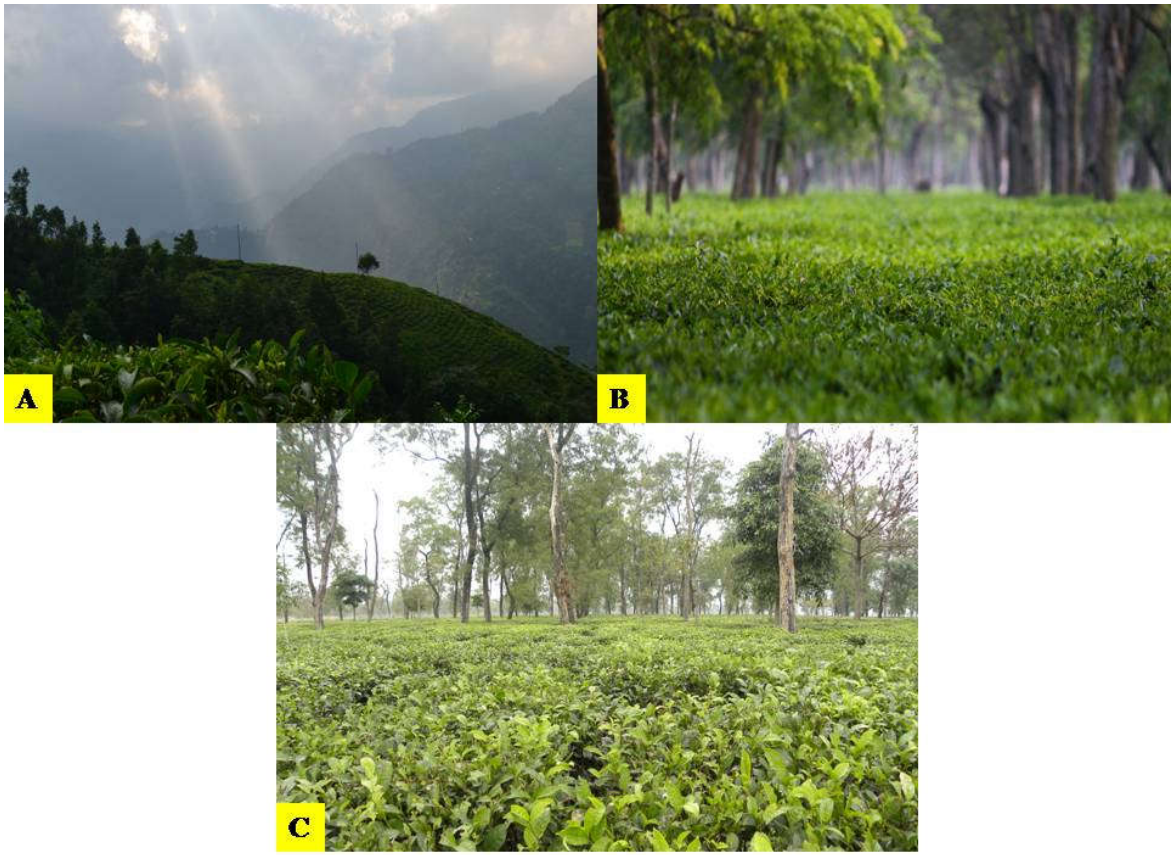



**Fig. 4.4: Tea gardens of A) Darjeeling hill region (Soureni T.E), B) Terai region (Kamalpur T.E) and C) Dooars region (Kalchini T.E).**

## 4.3 Rearing of insect

Larvae and moth of lepidopteran pests collected from different tea plantations were reared in laboratory in aseptic conditions for one generation. Fresh tea twigs/leaves of Tocklai vegetative clone (TV-25) from the experimental tea garden organically maintained at the Campus University of North Bengal was supplied as food.

## 4.4 Isolation of entomopathogenic bacteria:

For isolation of bacteria, dead larvae were dipped in 70% alcohol for two seconds for surface sterilization, washed three times with autoclaved double distilled water and stored at -20°C following Lacey and Brooks (1997). The stored dead larvae were thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cheese cloth. The filtrate was centrifuged at 3000 rpm for 30 minutes. The supernatant was discarded and from the precipitate containing bacterial suspension, serial dilutions ($10^{-1}$ to $10^{-7}$) were made in sterile double distilled water. 100 µl of different dilutions were plated on Nutrient agar medium (0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl and Distilled water, pH 7.4 at 25°C) by 'streak-plate inoculation method' incubated at 30°C in bacteriological incubator. Three replications for each dilution were taken for plating. After 24 hours, flat colonies of chalky white round smooth or fried egg appearance were picked up from mixed culture and again plated on to T3 medium (Tryptone 0.3%, Tryptose 0.2%, Yeast extract 1.5%, MnCl .0005%, 0.05 M Sodium phosphate and Distilled water, pH 6.8) and incubated at 30°C until lysis. All the selected colonies were purified using single colony isolation and spotted on to a master plate. The infectivity was determined following Koch's postulates by infecting healthy first instar larvae

with this isolated bacterium. After proving the Koch's postulates (Koch 1876, Black 1996, Fredericks and Relman 1996, Martin et al. 2008) the bacterial viability was checked by inoculating in new agar media.

## 4.5 Screening and identification of entomopathogenic bacteria from tea pests

### 4.5.1. Morphological characteristics:

#### 4.5.1.1 Microscopic study

- **Gram's staining**

Overnight grown culture was smeared on a slide, air dried and then heat fixed. The cells were stained with crystal violet stain for about one minute and then washed with distilled water for a few seconds using a wash bottle. The slides were then trated with Gram's iodine for about one minute followed by 95% alcohol for 10 seconds and washed with distilled water. The slides were counter stained with safranin for one minute and washed in gentle stream of water. The slides were air dried and observed under oil immersion objective (100X) of a phase contrast microscope (Olympus- CX31). Those bacterial cell appearing as purple violet were referred to as Gram-positive and those appearing pink were described as Gram-negative (Fig. 4.5A) (Murray et al., 1999).

- **Crystal protein staining**

The isolates were grown in LB broth at 37°C under constant shaking. Overgrown culture (i.e. after 3-4 days) was taken, smeared on a slide, air dried and then heat fixed. The slides were stained with Coomassie blue for about 3 minutes and then washed in a gentle stream of distilled water. The slides were air dried and then

observed under oil immersion objective lense of a phase contrast microscope (Fig. 4.5B) (Sharif and Alaeddinoĝlu 1988).

- **Endospore staining**

Over grown culture was taken, smeared uniformly on a glass slide, air dried and then heat fixed. The slides were covered with a piece of blotting paper, flooded with a few drops of Malachite green (Sigma) and then steamed in water bath for 10 minutes. The blotting paper was removed and the slides were washed with a gentle stream of distilled water. The slides were counter stained with safranin for 30 seconds and washed with distilled water. The slides were air dried and observed under oil immersion objective for presence of green endospores. Once stained the endospores do not readily decolorize and appear green within red cells (Fig. 4.5C) (Hussey and Zayaitz 2007).

### 4.5.1.2 Motility test

Motility of the bacteria was observed by Hanging drop method of Cappuccino and Sherman (1996). A clean coverslip containing a drop of liquid culture was covered by a depression slide (Petroleum jelly was applied as ring on the depression slide). The slide was turned quickly so that the drop continues to adhere to the inner surface of the coverslip. The slides were observed under 40X of phase contrast microscope for motility assessment. Commercially available *Bacillus thuringiensis kurstaki* (*Btk*) was used as control for comparative study which is used as biopesticides in many organic gardens.

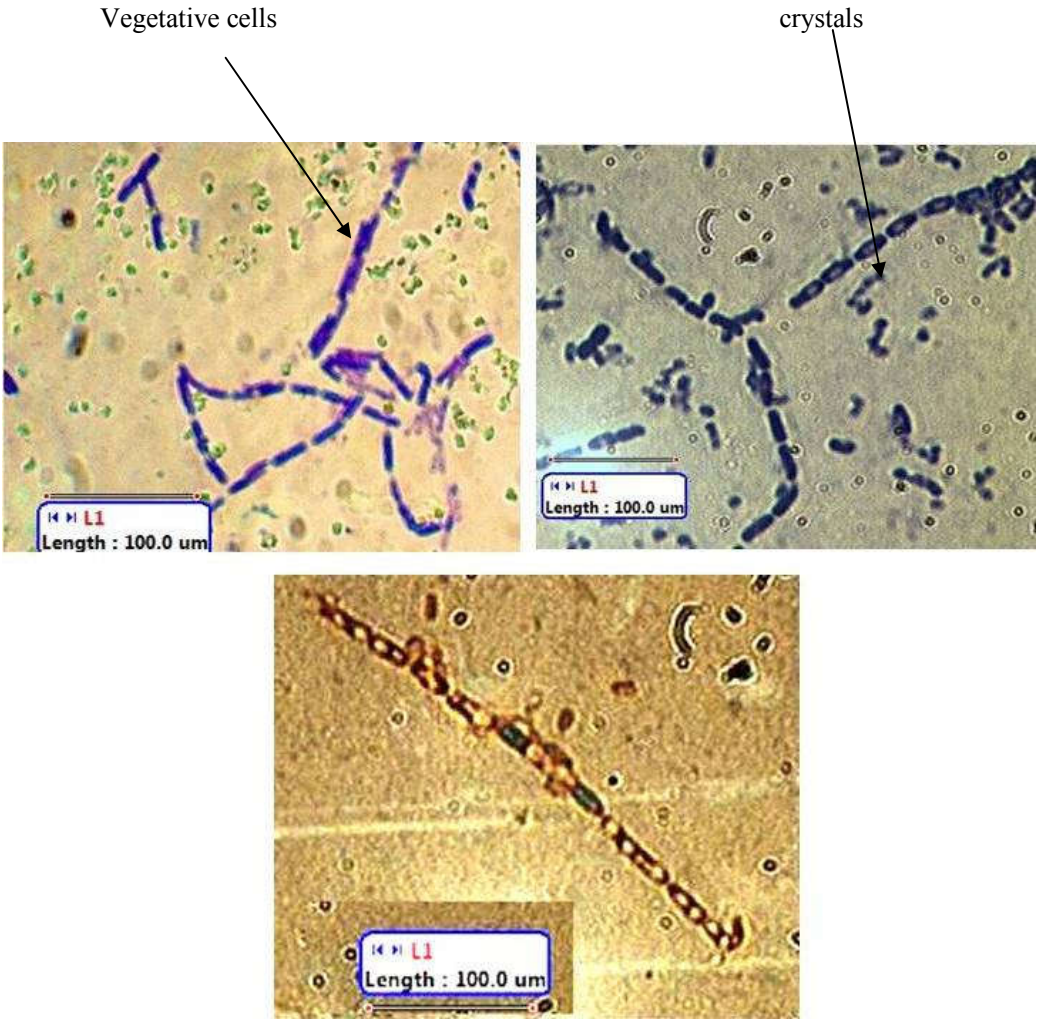



**Fig. 4.5A: Gram's staining; B. Crystal protein staining C. Endospore staining of bacteria.**

## 4.5.2 Biochemical characteristics:

### Using API kit

Biochemical analyses like Indole, Voges-Proskour, Methyl red, Citrate utilization, Esculin hydrolysis, Lysine Decarboxylase, Ornithin Decarboxylase, $H_2S$ production, Nitrate reduction, fermentation of different carbohydrates, Urease tests were performed using Biochemical testing kit (KB003, Himedia) with *Btk* as reference. This kit consists of medium for 24 biochemical tests and separate disc for oxidase test. 1-3 well isolated colonies were picked and a homogenous suspension was prepared in 2-3 ml distilled water.The density of the suspension was measured as 0.1 OD at 630 nm 200 µl of this suspension was transferred into each well of the test systems. To prevent any contact with air, the wells were filled up with mineral oil. Then the panels were incubated for 18-24 hrs at 37°C (Alsina and Blanch 1994). Oxidase test was done by picking up a single colony and rubbing it on the oxidase disc. Colour change was observed within 60 seconds. The biochemical tests and the results are summarized in the Table 4.1.

In addition to the above tests, the isolates were also characterized using three different biochemical tests for identification of entomopathogenic *Bacillus* sp. following the methods of Garrity (2001) and Aneja (2003) with sight modifications.

**Table 4.1: Details of biochemical tests used for identifying bacterial strains.**

| Strip I | Result Interpretation chart | | | | | |
|---|---|---|---|---|---|---|
| **No.** | **Test** | **Reagents to be added after incubation** | **Principle** | **Original colour of the medium** | **Positive reaction** | **Negative reaction** |
| 1 | ONPG | — | Detects β – galactosidase activity | Colourless | Yellow | Colourless |
| 2 | Lysine utilization | — | Detects Lysine decarboxylation | Olive green to Light Purple | Purple / Dark Purple | Yellow |
| 3 | Ornithine utilization | — | Detects Ornithine decarboxylation | Olive green to Light Purple | Purple / Dark Purple | Yellow |
| 4 | Urease | — | Detects Urease activity | Orangish yellow | Pink | Orangish yellow |
| 5 | Phenylalanine Deamination | 2-3 drops of TDA reagent | Detects Phenylalanine deamination activity | Colourless | Green | Colourless |
| 6 | Nitrate reduction | 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Napthylamine | Detects Nitrate reduction | Colourless | Pinkish Red | Colourless |
| 7 | $H_2S$ production | — | Detects $H_2S$ production | Orangish yellow | Black | Orangish yellow |
| 8 | Citrate utilization | — | Detects capability of organism to utilize citrate as a sole carbon source | Green | Blue | Green |
| 9 | Voges Proskauer's | 1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B | Detects acetoin production | Colourless / Light Yellow | Pinkish red | Colourless/ slight copper |
| 10 | Methyl red | 1-2 drops of Methyl red reagent | Detects acid production | Colourless | Red | Yellowish- orange |
| 11 | Indole | 1-2 drops of Kovac's red reagent | Detects deamination of tryptophan | Colourless | Pinkish Red | Colourless |
| 12 | Malonate utilization | — | Detects capability of organism to utilize sodium malonate as a sole carbon source | Light green | Blue | Light green |

| Strip II | Result Interpretation chart | | | | |
|---|---|---|---|---|---|
| **No.** | **Test** | **Principle** | **Original colour of the medium** | **Positive reaction** | **Negative reaction** |
| 13 | Esculin hydrolysis | Esculin hydrolysis | Cream | Black | Cream |
| 14 | Arabinose | Arabinose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 15 | Xylose | Xylose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 16 | Adonitol | Adonitol utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 17 | Rhamnose | Rhamnose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 18 | Cellobiose | Cellobiose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 19 | Melibiose | Melibiose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 20 | Saccharose | Saccharose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 21 | Raffinose | Raffinose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 22 | Trehalose | Trehalose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 23 | Glucose | Glucose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 24 | Lactose | Lactose utilization | Pinkish Red / Red | Yellow | Red / Pink |
| 25 | Oxidase | Done on Oxidase disc separately. Detects cytochrome oxidase production. | Colourless | Deep purple within 10 seconds | White/ Purple after 60 seconds |

- **Starch hydrolysis**

Starch hydrolysis test was carried out following the methods of Cowan and Steel (2004). Bacterial cultures were streaked on sterile Starch agar medium plate. The plates were incubated for 72 h at 30ºC. Later, the surfaces of the plates were flooded with iodine solution. The plates were examined for the starch hydrolysis around the line of the growth of organism. *E. Coli* inoculated starch agar medium plate was maintained as control (Fig. 4.6).

- **Casein hydrolysis**

Casein hydrolysis test was performed according to the method described by Cowan and Steel (2004). Bacterial cultures were streaked on sterile skim milk agar medium plate. The plates were incubated for 48 h at 30ºC. The plates were examined for clear area around the line of growth. *E.coli* inoculated skimmed milk agar medium plate was maintained as control (Fig. 4.7).

- **Catalase test**

Catalase test was performed following the method of Thiery and Frachon (1997). Nutrient agar slants were inoculated with the bacterial cultures. The cultures were incubated at 30ºC for 24 h. The tube was held at an angle and 3-4 drops of Hydrogen peroxide was dropped to flow over the growth of each slant. An uninoculated tube was kept for control. The culture was observed for appearance or absence of effervescence (Fig. 4.8).

### 4.5.3 Anaerobic growth

Anaerobic agar medium was distributed into culture tubes up to 7.5 cm and sterilized by autoclaving at 121$^{o}$C temperature and 15 pounds per square inch pressure for 20 minutes. The tubes were inoculated with a small (outside diameter 1.5 mm) loopful of

2 days old culture on nutrient agar medium by stabbing up to the bottom of the column. The tubes were incubated at 30$^{o}$C for 7 days and observed for growth along the length of the stab (anaerobic). An uninoculated tube was kept for control for the test (Claus and Berkeley 1986) (Fig. 4.9).

### 4.5.4 Similarity Matrix

The pair wise similarity based on matching biochemical result between pairs of different *Bt* strains was evaluated using the Dice Coefficient (SD) formula (Dice 1945).

**SD =2a/ (n1+n2)**

Where: a = the number of matching biochemical test result common between a pair of profiles, n1 and n2 = the total number of biochemical tests, respectively, in the first and second profiles.

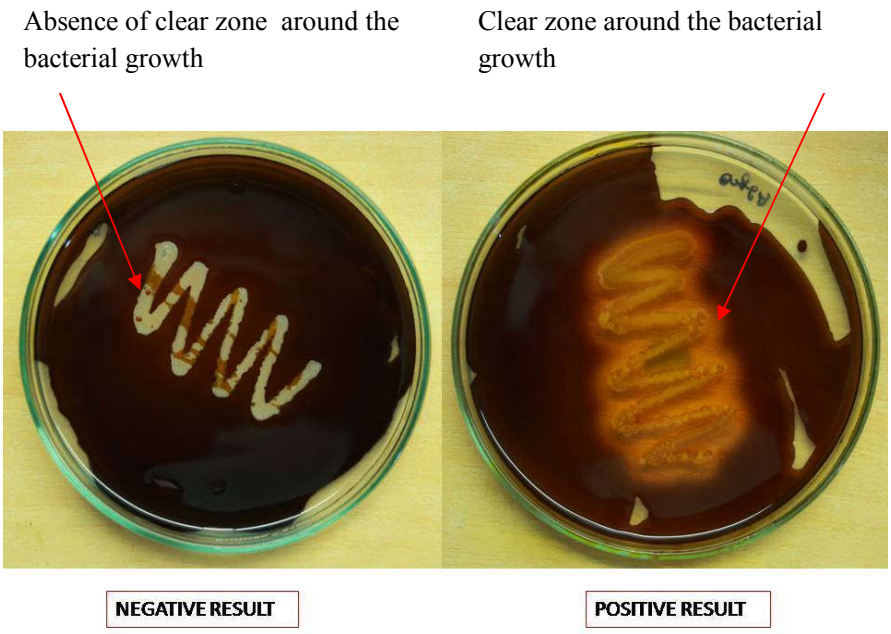



**Fig. 4.6: Plates showing negative and positive starch hydrolysis result.**

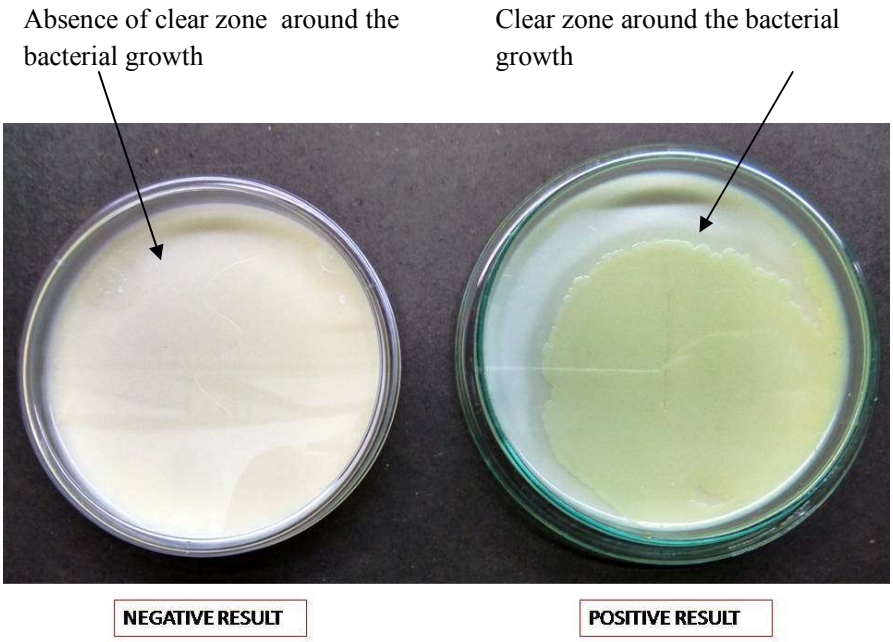


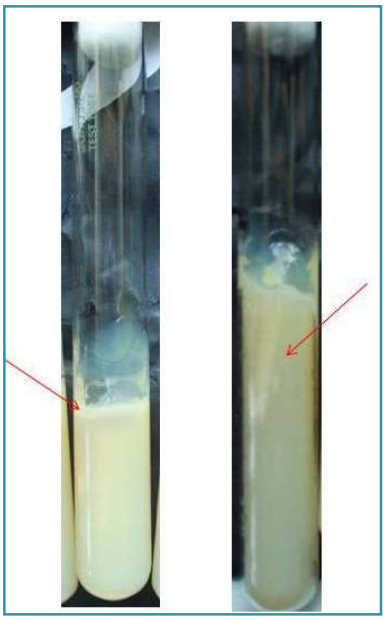

**Fig. 4.7: Plates showing negative and positive casein hydrolysis result.**

Absence of effervescence

Presence of effervescence

**Fig. 4.8: Slants showing positive catalase test.**

No growth along the length of the stab



Growth along the length of the stab

**Fig. 4.9: Slants showing positive anaerobic growth of bacterium.**

## 4.5.5 Physiological characteristics

### 4.5.5.1 Quantitative analysis of bacteria from larvae

Dead or moribund larvae were surface sterilized with 70% alcohol. The suspension obtained from each putrefied larvae were mixed separately in 5ml of sterilized phosphate buffer solution (PBS, pH 7.4) and filtered twice through two layers of cheese cloth to remove debris (Poinar and Thomas 1978). The suspension was diluted to $10^{-8}$ (Christine and Ted 1992). Each suspension was plated on nutrient agar and incubated at 30$^{o}$C for 24 hours. After incubation, the total number of bacteria in larvae was determined by counting the number of colonies on the plates, which were inoculated with diluted bacterial suspension.

### 4.5.5.2 Doubling time or Generation time

In this procedure, growth of the bacterial strain was determined by turbidimetric method of Cappuccino and Sherman (1996). The O.D. was measured at 540 nm every 30 minutes interval using a UV-Vis spectrophotometer (Rayleigh UV-2601). Finally log of O.D. and time interval were plotted on a graph paper (Fig. 4.10) and from this the generation time (doubling time) was determined using the graphical method. *Btk* was used as control for comparison. The generation time (GT) was calculated as follows:-

GT= t (OD at 0.8)- t (OD at 0.4)

### 4.5.5.3 Determination of colony forming unit/ ml

The number of organisms per ml of original culture was calculated by multiplying the number of colonies counted by the dilution factor (Cappuccino and Sherman 1996).

**Number of cells per ml= number of colonies X dilution factor**

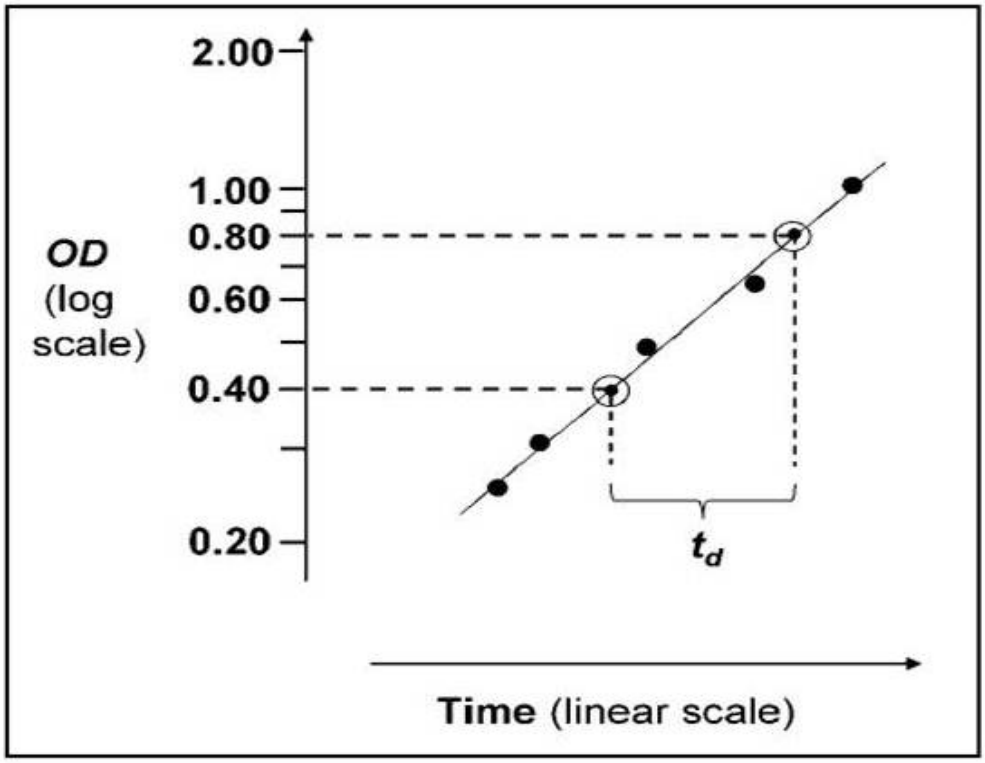



**Fig. 4.10: Graph showing the generation time calculation.**

## 4.5.6 SDS-PAGE analysis

### 4.5.6.1 Crystal protein of the bactera

The crystal protein was harvested by the method of Patel et al. (2009). Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. Sporulated culture of each isolate was pelleted down at 8000 rpm for 3 min and re-suspended in 50 µl sterile distilled water. 5 µl of 1 (N) NaOH was added after vortexing, and incubated for 5 min. 15 µl of Laemmli's buffer [4% SDS, 20% Glycerol, 10% 2-Mercaptoethanol, 0.004% Bromphenol Blue, 0.125 (M) Tris-HCl pH 6.8] was added and boiled for 2 min. The mixture was centrifuged at 8000 rpm for 3 min 20 µl sample was loaded per well of SDS-PAGE (10% Polyacrylamide gel, 100:1 Acrylamide/ Bisacrylamide ratio) as described by Laemmli (1970) and Quesada-Moraga et al. (2004). Gels were stained with staining solution (50% (v/v) ethanol, 10% (v/v) acetic acid and 0.1% (w/v) Coomassie brilliant blue R-250) for 40 min, and destained in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. The bands were analysed using gel documentation system (Spectroline TVD 1000R/F).

### 4.5.6.2 Quantitative and qualitative analysis of whole cellular protein of the bacteria

The bacterium was cultured on Luria-Bertani (LB) agar for 24 hours at 37°C and the protein was extracted using 1% lysozyme solution and lysis buffer comprising 4% SDS, 20% glycerol, 2% 2-marcaptaethanol, 0.125 (M) Tris-HCL pH 6.8 and MiliQ water (Costas 1992). The extracted protein of each strain was divided into two parts. The first part was used for the quantitative protein analysis (total protein estimation) using UV- Visual spectrophotometer (Rayleigh UV-2601). The second part was used for SDS-PAGE analysis.

- **Quantitative analysis by Spectrophotometer:**

The total protein in each tested strains was measured spectrophotometrically following the method of Haggag and Yousef (2010). About 0.02 ml of protein solution was added to 1ml of reagent (1) [32 mM potassium sodium tartrate; 200 mM sodium hydroxide; 30 mM potassium iodide, and 12 mM copper sulphate]. For preparation of standard sample, 0.02 ml of standard Bovine serum albumin (BSA) was mixed with 1 ml of reagent (1). The samples were mixed well by vortex and left for 30 min at room temperature. The absorbance of sample (As) and that of standard (A std.) were measured against reagent blank at 545 nm. The protein concentration was calculated according to the standard equation.

- **SDS-PAGE analysis of whole cellular protein of bacteria**

The second part of cellular protein was used for qualitative analysis using SDS-PAGE. Gels were stained with 50% ethanol, 10% acetic acid and 0.1% (w/v) Coomassie brilliant blue R-250 for 40 min and destained in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. The bands were analysed using gel documentation system (Spectroline TVD 1000R/F).

- **Plasmids of the isolates**

were purified by alkaline lysis method of Ehrt and Schnappinger (2003) with some modifications. 2 ml of overnight culture grown in LB broth at 30ºC was pelleted and re-suspended in 100µl of TE buffer [(40 mM Tris-HCL, 2 mM EDTA (pH 7.9)]. To it 200µl of lysis solution [3% SDS, 15% sucrose, 50 mM Tris-hydroxide, (pH 12.5)] was added and incubated for 30 min at 60ºC. Then 2µl of proteinase K was added, mixed gently and incubated for another 90 min at 37ºC. 1 ml of phenol (Tris saturated) was added to each tube vortexed and centrifuged (SIGMA 3K30) at 8000

rpm for 2 min. The upper aqueous phase was collected in a separate tube and 1ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture and the tubes were inverted carefully several times and centrifuged at 8000 rpm for 2 min. The upper aqueous phase was collected in a separate tube and equal volume of chloroform was added to each tube and inverted several times and centrifuged at 8000 rpm for 2 min. The upper aqueous phase was collected in fresh tubes and $1/10^{th}$ the volume of supernatant, 3M sodium acetate (pH 5.2) was added to each tube. Then 0.8 volume of isopropanol was added to the supernatant mixed throughly and kept at RT for 1hr. The samples were then centrifuged at 13000 rpm for 15 min at 4°C and the supernatant harbouring plasmid DNA was separated carefully. The supernatant was discarded and 500 µl cold 70% ethanol was added to each tubes. The mixture was centrifuged at 10000 rpm for 5 min at 4ºC. The DNA was air dried and dissolved in 50 µl TE [(1 M Tris-HCl, 0.5 M EDTA (pH 8.0)] and sotred at 4ºC for future use. Plasmids of the isolates were then electrophoresed in 0.8% agarose gel prepared in 1X TAE [(40mM Tris, 20mM Acetate, 1mM EDTA (pH 8.6)] buffer containing 0.5 µg/ml of ethidium bromide. *Hind* III and *Eco R*I double digested λ DNA was used as the size marker. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

## 4.6 Bioassay of bacterial isolates and determination of $LC_{50}$ value and $LT_{50}$ value

The efficacy of the entomopathogenic bacteria was determined by bioassay. Extraction of spore-crystal was done following the method of Shishir et al. (2012). Isolates with crystal bodies was cultured in 20ml of $T_3$ liquid medium and incubated for 7 days at 30 °C with regular shaking manually. Aliquots of 1.5 ml liquid culture

was then centrifuged at 5000 rpm for 15 min. Pellet (spore and crystal mixture) was washed twice with 1 ml sterile distilled water by centrifuging at 5000 rpm for 15 min. Next weight of pellet was taken and re-suspended in 1 ml of distilled water. The suspension was kept at 4°C for future use. Bioassay was done after the procedure of Dulmage et al. (1970). Different concentrations (100, 300, 500, 750 and 1000 μg/ml) of crude samples (spores and crystal) were used in the $LC_{50}$ (median lethal concentration) and $LT_{50}$ (median lethal time) bioassay by spreading these uniformly on tea leaves offered as food according to the procedure of Unnamalai and Vaithilingam (1995). For conducting bioassay ninety second instar larvae from laboratory culture were used for each bacterial concentration. The experiment was set up in 3 replicates each containing 30 caterpillars (Fig. 4.11). Sterile distilled water applied leaves were used as control. The mortality was observed at an interval of 24 hrs from the day of inoculation (1$^{st}$ day feeding up to 9$^{th}$ day). The mortality caused by each concentration was corrected for the control rate of mortality in each assay by using Abbott's formula (Abbott 1925). The corrected per cent mortality was calculated using Abbots formula, as follows:

$$\textbf{Corrected mortality (\%)} = \frac{X-Y}{X} \times 100$$

X = % live insects in control,; Y = % live insects in treatment.

Data were subjected to probit analysis (Finney and Tattersfield 1952) and median lethal concentration ($LC_{50}$) value was calculated from the regression equation.

Median lethal time ($LT_{50}$) value was also determined simultaneously following the method of Biever and Hostetter (1971):

$$\mathbf{LT_{50} = a + e\,(c-b)/D}$$

Where, a = the number of hours from the initiation of the test until the reading made just before the 50% value was recorded, b = the total number of larvae dead at the reading just before 50% value was recorded, c = 50% of the total number tested, D= the number of larvae dying in 24 hr period during which the 50% mortality was reached, and e = the number of hours between mortality counts.

### 4.6.1 Cross infectivity testing on mulberry silkworm

The cross infectivity of isolated entomopathogenic bacteria was tested by exposing beneficial Lepidopteran, like silkworm (*Bombyx mori*) separately. As the sericulture industry is running side by side with the tea industry in North Bengal so it is necessary to determine the toxicity/infectivity of the bacterial isolate to silk worms before trying the isolates in the field. For testing the infectivity in the laboratory condition multivoltine silkworms were taken (Fig. 4.12). Disease free layings (DFLs) were collected from West Bengal Sericulture Department situated at Matigara, Siliguri. Newly hatched neonates were fed with fresh and sterilized mulberry leaf collected from University of North Bengal campus and from West Bengal Sericulture Department, Matigara. The second instar larvae were taken for the cross infectivity test. Crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were prepared in distilled water. Ninety second instar caterpillars were taken for each treatment with varying concentrations of bacteria (Three replicates each containing 30 caterpillars). Similarly 90 caterpillars were taken for control experiment. Number of mortality was counted every 24 hours from the day of inoculation, ($1^{st}$ day feeding up to $9^{th}$ day).


**Fig. 4.11: Bioassay setup in laboratory.**

**Fig. 4.12: Silk worm (*Bombyx mori*) larvae feeding mulberry leaves.**

## 4.7 Genomic DNA extraction for PCR analysis

The genomic DNA was isolated following the methods of (Sambrook et al. 1989, Juárez-Pérez et al. 1997, Sambrook and Russell 2001) with some modifications. 1.5 ml of overnight grown culture was centrifuged at 8000 rpm at room temperature for 5 min and the pellet was collected by discarding the supernatant. The pellet was resuspended in 567 µl TE (10mM Tris-HCl pH 8.0 and 1mM EDTA pH 8.0). 3µl Proteinase K (20 mg/ml) and 30µl 10% SDS were then added to the suspension. The mixture was incubated for 1 hr at 37ºC which turned the solution clear and viscous. To this 100 µl of 5 M NaCl was added and mixed well. Finally 80µl CTAB/NaCl solution was added and the mixture was incubated for the second time at 60ºC for 10 min. An equal volume (750µl) of Tris-saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10000 rpm for 15 min at room temperature. The upper aqueous phase was transferred to a new tube and equal volume (750µl) of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 10000 rpm for 10 min. the upper aqueous phase was transferred to a new tube and 450µl of isopropanol was added and mixed properly until a clump of DNA is visible. The mixture was centrifuged at 10000 rpm for 5 min at 4ºC, the pellet was washed with 1 ml of 70% ethanol for 30 sec and centrifused at 10000 rpm for 5 min at 4ºC. Finally the pellet (DNA) was air dried and dissolved in 30-50 µl TE buffer and sotred at 4ºC for future use. Genomic DNA of the isolates were then electrophoresed in 1% agarose gel prepared in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide. *Hind* III and *Eco R*I double digested λ DNA was used as the size marker. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

### 4.7.1 Quantification of DNA

Amount of DNA was calculated by using the following formulae (Sambrook and Russell 2001).

1 O. D. at 260 nm = 50 µg of double stranded DNA/ ml

Amount of double stranded DNA = (X) x 50/2

Where, X = absorbance of the sample at 260 nm

Purity of the total DNA isolated was also tested by using spectrophotometer based on $^{A}280/260$ and also checking the integrity of the DNA by running in 1% agarose gel. The extracted DNA was used for amplification of specific sequence by Polymerase chain reaction (PCR).

### 4.7.2 PCR amplification for 16s rRNA sequence

PCR was performed with Universal 16S rRNA gene specific primer pair (Table 4.2). The PCR was set up in 0.2 ml PCR tubes. The components of the reaction mixture have been shown in the Table 4.3. The contents were mixed followed by a brief spin. PCR was carried out in a 96 well thermal cycler (Eppendorf) with the following cycling program: lead heat at 105°C, initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 30 s, and primer extension 72 °C for 7 min. The product was stored at 4°C. The 16S rRNA sequence of the purified PCR product was determined using an Applied Biosystems model 3130 DNA sequencer and the ABI PRISM cycle sequencing kit. The sequence obtained were aligned using MEGA 5 and compared with available sequences from NCBI GeneBank using BLAST program (Altschul et al. 1997).

## 4.8 Identification of *cry* gene

Colony PCR was done using rapid cell lysate as the DNA samples after the method of Apaydin et al. (2005) and Valicente and Lana (2010). *Bacillus* strains were grown on Luria agar plate for 12h. A loopful of cells was transferred to the following ml of sterile distilled water and frozen at -20° C for 20 min. The mixture was transferred to boiling water for 10 min to lysate the cells. The resulting cell lysate was briefly spun for 10 sec at 10000 rpm. 2µl of this suspension was added as template to specific primers for *cry* genes (Table 4.4). The reaction mixture for PCR has been shown in the Table 4.5.

### 4.8.1 PCR amplification of *cry*1, *cry* 2 and *cry* 9 genes.

The PCR was carried out for the amplification of *cry* 1, *cry* 2 and *cry* 9 genes using the primer sets (Table 4.4) separately. All the ingredients (Table 4.5) were mixed followed by a brief spin. PCR was carried out in a 96 well thermal cycler (Eppendorf) with the following cycling program: lead heat at 105°C, initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 49 °C for 45s for *cry* 1, 54°C for *cry* 2 and 61°C for *cry* 9 and primer extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The product was stored at 4°C.

### 4.8.2 Agarose gel electrophoresis

The PCR products were seperated in 1% agarose gel prepared in 1x TAE buffer. About 5µl of the PCR amplified products were mixed with 1µl of 6x loading dye (0.48% SDS,0.03% Xylene Cyanol, 0.03% Bromophenol Blue) and loaded into the wells of agarose gel containing 0.5 µg/ml ethidium bromide (Sambrook et al. 1989 ).

A 100 bp step up ladder was used as size marker. The electrophoresis was carried out with a constant voltage of 70 Volts in 1x TAE until the dye front migrated to nearly ¾th of the gel length. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

## 4.9 Statistical analysis

Quantitative data were subjected to standard statistical analysis. Software packages such as SSP and Origin 4.0. MEGA5 were used for sequence alignment. For bioassay, cross infectivity, results were determined with the help of probit analysis. MS-EXCEL was used in all the experiments.

**Table 4.2: Primer pair used for amplification of 16S rRNA gene.**

| Primer pair | Sequence (5' to 3')[a] | Size of amplicon (bp) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|
| Universal 16S rRNA gene specific primer pair | AGA GTT TGA TCC TGG CTC AG<br>ACG GCT ACC TTG TTA CGA CTT | 1500 | 50 | (Weisburg et al. 1991) |

a The top primer is the sense primer and the bottom primer is the antisense primer.

**Table 4.3: Reaction mixture for PCR for 16S rRNA gene.**

| Sl. | No Reaction mixture | |
|---|---|---|
| 1 | 10 X *Taq* Buffer (2mM) | 3.0µl |
| 2 | $MgCl_2$ | 1.5 µl |
| 2 | dNTPs mix (1mM) | 2.0µl |
| 3 | Forward primer (-) 20 pmoles | 1.0µl |
| 4 | Reverse primer, (+)20 pmoles | 1.0µl |
| 5 | *Taq* DNA polymerase(1U/µl) | 0.20µl |
| 6 | Template DNA (200 ng/µl) | 2.0µl |
| 7 | Milli Q water | 15.67µl |
| | Total | 25.0µl |

**Table 4.4: Primers for *cry* 1, *cry* 2 and *cry* 9 genes.**

| **Gene** | **Sequence** (5' to 3')[a] | **Expected product size (bp)** | **Reference** |
|---|---|---|---|
| ***cry* 1** | CATGATTCATGCGGCAGATAAAC<br>TTGTGACACTTCTGCTTCCCATT | **277** | (Ben-Dov et al. 1997) |
| ***cry* 2** | GTTATTCTTAATGCAGATGAATGGG<br>CGGATAAAATAATCTGGGAAATAGT | **1500** | (Sauka et al. 2005) |
| ***cry* 9** | CGGTGTTACTATTAGCGAGGGCGG<br>GTTTGAGCCGCTTCACAGCAATCC | **354** | (Ben-Dov et al. 1997) |

a The top primer is the sense primer and the bottom primer is the antisense primer.

**Table 4.5: Reaction mixture for PCR for *cry* genes.**

| Sl. | No Reaction mixture | |
|---|---|---|
| 1 | 10 X *Taq* Buffer (2mM) | 2.0µl |
| 2 | $MgCl_2$ | 2.4 µl |
| 2 | dNTPs mix (1mM) | 4.0µl |
| 3 | Forward primer (-) 20 pmoles | 2.0µl |
| 4 | Reverse primer, (+)20 pmoles | 2.0µl |
| 5 | *Taq* DNA polymerase(1U/µl) | 0.5µl |
| 6 | Template DNA | 5.0µl |
| 7 | Milli Q water | 7.1µl |
| | Total | 25.0µl |