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Pathogenicity of two strains of *Bacillus* infecting the lepidopteran tea pests, *Buzura suppressaria* and *Eterusia magnifica* in Darjeeling foothill region

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Abstract

Bacillus sp. BS01 and *Bacillus* sp. RS01 were isolated from diseased caterpillars of leaf feeding pests of tea, *Buzura suppressaria* and *Eterusia magnifica*, respectively from the Darjeeling foothill region. They were studied for their morphological, biochemical and physiological parameters and compared with the *Bacillus thuringiensis kurstaki* (*Btk*) strain used as commercial microbial pesticide. Analysis based on polyphasic approach such as growth phase, biochemical tests, whole body protein, crystal protein profiles along with bioassay (i.e. LC_{50} and LT_{50} values) of the bacterial isolates established these as two different strains of *Bacillus* sp. They differed in biochemical testing, growth phase, whole body protein profile, crystal protein profile, trypsinization of crystal protein and LC_{50} and LT_{50} values. These two strains were also distinct from commercial microbial pesticide *Btk*. The biochemical characteristics in particular the whole body and crystal protein profiles of the two *Bacillus* strains showed marked difference from those of *Btk*. The generation time of *Btk* was lower than either of the two isolated *Bacillus* strains. These new strains isolated from the host insects open up the possibility of their future use as microbial pesticide after further development in their formulation and determining their safety standards.

Keywords: Buzura suppressaria, Bacillus sp. BS01, *Bacillus* sp. RS01, *Camellia sinensis*, Darjeeling, *Eterusia magnifica*, Tea

Introduction

Tea is the main agro-industry of Darjeeling hills and its adjoining plains of the Dooars, Terai and the North-East region of India. But the plantation of this region is severely damaged by attacks of lepidopteran tea pests. *Buzura suppressaria* commonly called as looper and *Eterusia magnifica* called as red slug caterpillar cause a substantial loss by defoliating the crop both quality and quantity of tea. To control the attack of these tea pests, the present practice is largely based on the use of synthetic pesticides, with some back lashes such as environmental pollution, human health hazard, resistance in pests (Sarker and Mukhopadhyay, 2006) and MRL problems affecting export of tea. In view of this, efforts are being made to evolve alternative strategies of management of these pests through application of microbial bioagents. Development of microbial pesticides would greatly help production of export quality tea through organic farming.

Strains of *Bacillus* sp. BS01 and *Bacillus* sp. RS01 were isolated from two of the lepidopteran pests of tea that showed natural diseased condition in plantation. Growth rate, biochemical testing, vegetative protein profile, crystal protein profile, trypsinization of crystal and bioassay i.e. LC_{50} and LT_{50} values of the two strains proved them to be similar to *Bacillus thuringiensis* but differing in their strains or subspecies characters. They also differed from *Bacillus thuringiensis kurstaki*, which is commonly sprayed in garden with marginal control of these pests.

The present study was aimed to isolate and characterize the naturally occurring bacterial pathogens of *B. suppressaria* and *E. magnifica* and to know their potential as biopesticide through determination of their median lethal concentration and time. *Bacillus thuringiensis kurstaki* was used as a reference for comparison.

Materials and Methods

Bacterial strains were isolated from larvae of *B. suppressaria* and *E. magnifica*. For isolation of bacteria, the larvae dying of disease were taken for surface sterilization with 70% alcohol and then washed three

times with sterilized double distilled water. Then they were stored in double distilled water within sterilized eppendorf tube. The eppendorf was wrapped with parafilm and stored at -20°C (Lacey and Brooks, 1997). The stored dead larvae were taken and thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3000 rpm for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was taken for pure culture isolation by 'dilution streak method' in nutrient agar medium. The infectivity were determined following Koch's postulates by infecting healthy first instar larvae with these two bacteria. After proving the Koch's postulates the viability was checked by inoculating in newly prepared agar medium at weekly intervals.

Characterization of the strain BS01 and RS01

Morphological characteristics

Cell, spore shape and structure of crystal protein, colony texture, motility observed for the two bacterial isolates and the same were compared with *Bacillus thuringiensis kurstaki* used as reference.

Biochemical characteristics

Several biochemical tests 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 Biochemcial testing kit (KB003) (Himedia) with *Btk* as reference.

Physiological characteristics

Doubling or Generation time (Cappuccino and Sherman, 1996)

Growth of the bacterium was determined by turbidimetric method. The O.D. value was taken at 540 nm in spectrophotometer at every 30 minutes interval. Finally optical density and time interval were plotted on a graph paper and from this the generation time (doubling time) was determined. *Btk* was taken as reference.

SDS-PAGE profile of crystal protein

Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. The crystal was harvested in high pH buffer of sodium carbonate and 2-marcaptoethanol after Kranthi (2005) with slight modification [2-Marcaptoethanol was used instead of Dithiothreitol (DTT)]. This crystal protein was taken for SDS-PAGE analysis.

Trypsinization of crystal proteins

Aliquotes (100µl) of solubilized crystal proteins of the bacterial strains were subjected to trypsin treatment at 37°C in a 10:1 (vol/vol) soluble protein/trypsin ratio with 1 mg of trypsin per ml dissolved in sterilized double distilled water. After two hours incubation, samples were analyzed by SDS-PAGE (Lopez-Meza, 1996).

SDS-PAGE analysis of vegetative protein of the bacterium

For SDS-PAGE analysis of vegetative protein of Gram positive bacteria, the bacteria were cultured on Luria-Bertani (LB) agar for 24h at 37°C and the proteins were extracted using 1% lysozyme solution and lysis buffer containing 4% SDS, 20% glycerol, 2% 2-marcaptoethanol, 70% Tris-HCL, pH 6.8, and 4% deionized water and analyzed by SDS-PAGE according to the method described by Costas (1992). Gels were fixed in trichloroacetic acid and were stained with Brilliant blue G-250 (Neuhoff *et al.*, 1988; Konecka *et al.*, 2006).

Bioassay

Crude spore crystal mixture (100, 300, 500, 750 and 1000 µg/ml) of the bacteria isolated from *B. suppressaria* and *E. magnifica* respectively were used for bioassay after the method of Unnamalai and Sekar, (1995). Leaves dipped in different concentrations were offered as food. Second instar larvae (n=100) were used for each treatment (concentration). Sterile distilled water was used in control. The mortality was observed at an interval of 24 h from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula. Data were subjected to probit analysis after Finney (1954), and median lethal concentration (LC_{50}) value was calculated from the regression equation. Value of median lethal time (LT_{50}) was also determined simultaneously following the method of Biever and Hostetter (1971).

Results and Discussion

Characterization of the two bacterial isolates (BS01 and RS01)

All the morphological characteristics like vegetative body structure, spore-shape, motility, colony texture, crystal protein shape of the isolated bacterial strains BS01 from *B. suppressaria* and RS01 from *E. magnifica* were found to be similar with that of *Bacillus thuringiensis kurstaki*. BS01 and RS01 showed all the characteristics of genus *Bacillus* which included rod shaped vegetative body, endospore formation, gram

positivity, facultative anaerobic nature, catalase positivity, acid production from glucose and motility (Sneath, 1986).

Characteristics of the two *Bacillus* strains (BS01 and RS01) showed same biochemical properties except in nitrate reduction test where BS01 showed negative reaction and RS01 showed positive reaction. However, these two strains showed much difference in their biochemical tests, when compared with the results of *Btk* (Table 1).

Table 1. Comparative account of biochemical tests of BS01, RS01 and *Btk*

Biochemical Characteristics	*Btk*	BS01	RS01
ONPG	+	-	-
Lysine decarboxylase	+	+	+
Ornithin decarboxylase	+	+	+
Urease	-	+	+
Phenyl alanine Deamination	-	-	-
Nitrate reduction	+	-	+
H_2S production	-	-	-
Citrate utilization	-	-	-
V-P TEST	+	+	+
Methyl red	-	-	-
Indole	-	-	-
Malonate	-	-	-
Esculin hydrolysis	-	-	-
Arabinose	+	-	-
Xylose	+	-	-
Adonitol	-	-	-
Rhamnose	-	-	-
Cellobiose	+	-	-
Melibiose	+	-	-
Saccharose	+	-	-
Raffinose	-	-	-
Trehalose	+	+	+
Glucose	+	+	+
Lactose	+	-	-
Oxidase	-	+	+
Sorbitol	-	-	-

Growth phase or determination of doubling time

The generation or doubling time of the two strains was much different. The doubling time was 60 min in case of BS01 and 120 min in case of RS01. The *Btk* which was used as reference had totally different doubling time from the above two strains.

SDS-PAGE profile of crystal protein (trypsin untreated)

When protein composition was analyzed by SDS-PAGE, crystals of BS01 showed difference in banding pattern with RS01. Bands of molecular weight 53 and 49 kDa were present in RS01 strain but were absent in BS01 strain. In BS01 strain a 52 kDa molecular weight protein band was present which was absent in RS01 strain. The BS01 strain differed from *Btk* in 42 kDa position whereas RS01 differed in 52 and 42 kDa banding position. So, a major difference in banding pattern was found between BS01 and RS01 and *Btk* (Fig. 1).

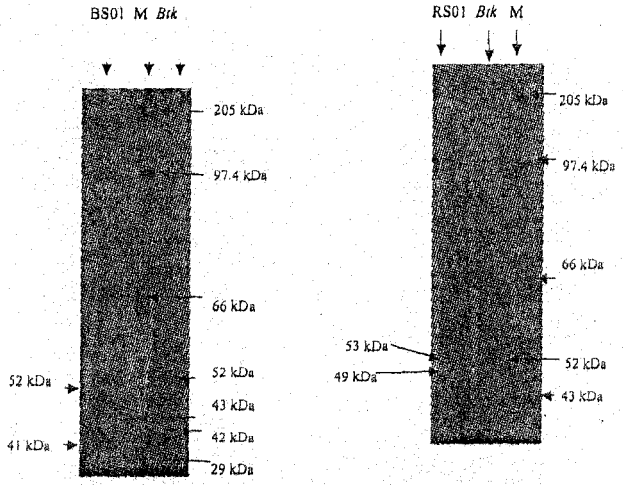

Fig. 1. SDS-PAGE profile of crystal protein of BS01, RS01 and *Btk*

Trypsinization of crystal protein

The presence of any putative peptide fragment resistant to tryptic digestion in the BS01 and RS01 strains crystals were verified. After solubilization the crystals were treated with trypsin. Trypsin digestion revealed the trypsin resistant peptides. The expected molecular masses of which on SDS-PAGE gel were 49, 44 and 38 kDa in case of BS01 and in case of RS01 strain these were 51 and 43 kDa molecular weight peptides (Fig. 2). Whereas in case of *Btk* it was 49, 44 and 40 kDa. So differences in banding pattern were found between BS01 and RS01 which also differed from *Btk*. This experimental result revealed that 49, 44 and 38 kDa molecular weight peptides were the toxic components of BS01 and 51 and 38 kDa of RS01 strains, respectively.

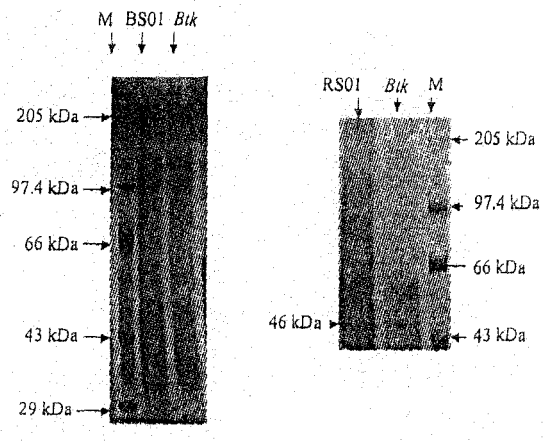

Fig. 2. Trypsinization of crystal protein of BS01, RS01 and *Btk*

Whole cell protein profile

Difference in banding pattern of whole body protein profile was found between *Btk* and BS01 strains. Three major protein bands (43, 34 and 30 kDa) were present in BS01 strain which were absent in *Btk*. On the other hand two major protein bands (44 and 31 kDa) were present in *Btk* which were absent in BS01 strain. In case of RS01 strain, 44 kDa protein band was present which was totally absent in BS01 strain but present in *Btk*. So, major differences at cellular protein level present between BS01, RS01 and *Btk* strains (Fig. 3).

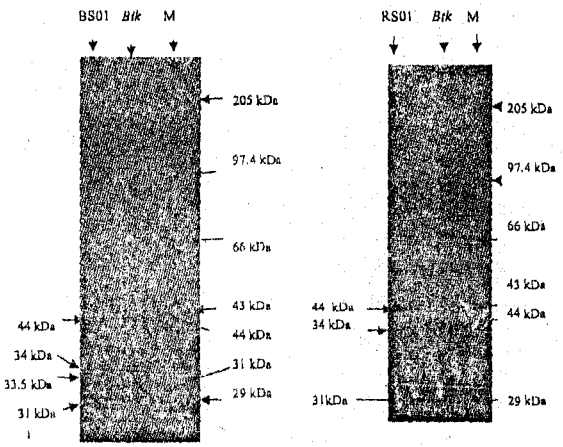

Fig. 3. SDS-PAGE profile of vegetative protein of BS01, RS01 and *Btk*

Bioassay

The mortality of second instar *B. suppressaria* caterpillars varied from 81 to 3% within 9 days. The LC_{50} value was found to be 446.7 µg/ml with fiducial limits 407.96 µg/ml (lower limit) 485.44 µg/ml (upper limit). The LT_{50} values were found to be 6.19 days for 1000 µg/ml, 6.5 days for 750 µg/ml and 8.92 days for 500 µg/ml concentrations. In case of RS01 strain the LC_{50} value was found to be 458.2 µg/ml with fiducial limits 457.955 µg/ml (lower limit) and 458.245 µg/ml (upper limit). The LT_{50} values were found to be 5.6 days for 1000µg/ml, 5.69 days for 750µg/ml and 6.19 days for 500µg/ml concentrations. On the other hand in case of *Btk* which was used against *B. suppressaria* the LC_{50} value was found to be 524.8µg/ml with 462.655µg/ml (lower limit) 586.945µg/ml (upper limit). The LT_{50} values were found to be 6.35 days for 1000µg/ml and 8.91 days for 750µg/ml. The LC_{50} value of *Btk* which was used against *E. magnifica* was found to be 416.9µg/ml with 416.76µg/ml (lower limit) and 417.034µg/ml (upper limit). The LT_{50} values were found to be 6.67 days for 1000µg/ml, 6.93 days for 750µg/ml and 7.54 days for 500 µg/ml concentrations (Table 2).

The two *Bacillus* strains BS01 and RS01 isolated from *B. suppressaria* and *E. magnifica* showed typical characteristics of *Bacillus thuringiensis* especially in their vegetative body structure and crystal production during sporulation. As crystals are the typical distinguishing characteristics of *Bt* (Heimple and Angus, 1958; Bai *et al.*, 2002), the isolates were identified as *Bt* strains on the basis of the crystal formation (Bai *et al.*, 2002; Brussock and Currier, 1990). However, on biochemical testing of ONPG, Lysine decarboxylase, Ornithin decarboxylase, Urease, Nitrate reduction, V-P, acid production from glucose etc. they showed difference among themselves (BS01 and RS01) and also with *Bacillus thuringiensis kurstaki*, the latter being used as microbial pesticide against lepidopteran pests. The growth pattern that is the generation time was found to be different among the two isolates and also with the commercial formulation of *Btk*. The crystal protein profile was found to be different amongst two isolates also *Btk*. Further the trypsinized crystal proteins also differed among the strains. The vegetative protein profile in SDS-PAGE was found to be decisively different among the two strains and also from *Btk*.

In case of bioassay, these two strains were found to be highly toxic to their respective hosts from which they had been isolated. Due to their close median lethal concentration values (LC_{50}) which were not much different from the commercially used *Btk*, developing these new strains into commercial pesticides, for controlling of *B. suppressaria* and *E. magnifica* caterpillars offers great potential. The LT_{50} values of these strains were also comparatively lower than the commercially used bacterium, *Btk*. The possibility of finding cross infectivity of these strains to other harmful lepidopteran pests is still open and bioassay against other insect pests is under way.

Report of development of insect resistance to *Btk* has stimulated new research to find additional *Bt* strains and other microbes that have specific spectrum against certain insects (Bai *et al.*, 2002; Mc. Gaughey, 1985; Monnerat *et al.*, 2000; Salama and Abdel-Razek, 2000).

Bacillus sp. was reported earlier (De *et al.*, 2006) from *B. suppressaria* and a detail account has already been furnished in this report. But as no *Bacillus* strain has so far been reported from *E. magnifica* of sub Himalayan tea plantation, the isolated strain (*Bacillus* sp. RS01) is a new report. Based on the distinguishing

Table 2. Comparative account of LC_{50} and LT_{50} values of BS01, RS01 and *Btk*.

Name of bacterium	Mortality (%)	LC_{50} with Fiducial limits	LT_{50}	Hetero-geneity	Regression
Btk tested on *B. suppressaria*	75%for 1000 µg/ml 60%for 750 µg/ml 48%for 500 µg/ml 18%for 300 µg/ml 17%for 100 µg/ml	524.8µg/ml With 462.655µg/ml (Lower limit) 586.945µg/ml (Upper limit)	6.35 for 1000µg/ml 8.91 for 750µg/ml	$\chi^2(5)$=120 for 1000µg/ml $\chi^2(5)$=85.7143 for for 750 µg/ml $\chi^2(5)$=63.1579 for 500 µg/ml $\chi^2(5)$=19.7802 for 300 µg/ml $\chi^2(5)$=18.5792 for100µg/ml	Y=1.669X+2.253
BS01 tested on *B. suppressaria*	81% for 1000 µg/ml 59% for 750 µg/ml54 % for 500 µg/ml 6% for 300 µg/ml 3% for 100 µg/ml	446.7 µg/ml With 407.96 µg/ml (Lower limit) 485.44 µg/ml (Upper limit)	6.19 for 1000µg/ml 6.5 for 750µg/ml 8.92 for 500µg/ml	$\chi^2(5)$=3.0457 for 100 µg/ml $\chi^2(5)$=6.1856 for 300 µg/ml $\chi^2(5)$=73.9726 for 500 µg/ml $\chi^2(5)$=83.6879 for 750 µg/ml $\chi^2(5)$=136.1345 for 1000 µg/ml	Y=3.33X-21.3
Btk tested on *E. magnifica*	87% for 1000 µg/ml 83%for 750 µg/ml 77%for 500 µg/ml 21%for 300 µg/ml 20%for 100 µg/ml	416.9µg/ml With 416.76µg/ml (Lower limit) 417.034µg/ml (Upper limit)	6.67 for 1000µg/ml 6.93 for 750µg/ml 7.54 for 500 µg/ml	$\chi^2(5)$=118.6868 for 1000 µg/ml $\chi^2(5)$=107.1048 for 750 µg/ml $\chi^2(5)$=91.3234 for 500 µg/ml $\chi^2(5)$=4.6192 for 300 µg/ml $\chi^2(5)$=3.9216 for 100 µg/ml	Y=2.61X-8.81
RS01 tested on *E. magnifica*	78% for 1000 µg/ml 72% for 750 µg/ml 61 % for 500 µg/ml 21% for 300 µg/ml 20% for 100 µg/ml	458.2 µg/ml With 457.955 µg/ml (Lower limit) 458.245 µg/ml (Upper limit)	5.6 for 1000µg/ml 5.69 for 750µg/ml 6.19 for 500µg/ml	$\chi^2(5)$= 93.8312 for 1000 µg/ml $\chi^2(5)$=79.4543 for 750 µg/ml $\chi^2(5)$=56.7966 for 500 µg/ml $\chi^2(5)$=4.6192 for 300 µg/ml $\chi^2(5)$=3.9216 for 100 µg/ml	Y=2.16X-4.16

characters and the killing efficacy, the *Bacillus* sp. BS01 and *Bacillus* sp. RS01 hold a promise to be developed in future as microbial pesticides.

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Corrigendum:

Trypsinization of crystal protein:

The presence of any putative peptide fragment resistant to tryptic digestion in the BS01 and RS01 strains crystals were verified. After solubilization the crystals were treated with trypsin. Trypsin digestion revealed the trypsin resistant peptides. It was found that no difference in banding pattern was there between BS01 and *Btk*. But a 46 kDa molecular weight protein band was there in RS01 strain which was totally absent in *Btk* (Fig. 2). So difference in banding pattern were found between BS01 and RS01 which also differed from *Btk*

Whole cell protein profile:

Difference in banding pattern of whole body protein profile was found between *Btk* and BS01 strains. Two major protein bands (44 and 31 kDa) were present both in *Btk* and BS01 strain. But the difference was found in 33.5 and 34 kDa molecular weight protein bands which were present only in BS01 strain but absent in *Btk*. On the other hand one protein band of 31 kDa present in *Btk*, was absent in RS01 strain. Instead in RS01 strain a 34 kDa protein band was present which was totally absent in *Btk*. So, major differences were evident between RS01 and *Btk* strain (Fig. 3).

Short Scientific Report

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A report on the naturally occurr ng pathogenic bacteria of the lepidopteran tea pest, *Buzura suppressaria* (Lepidoptera: Geometridae), from Darje(ling foothills and plains

(Manuscript Received: 27-02-06; Revise : 17-05-06, Accepted: 26-04-07)

Keywords: Buzura suppressaria, Camellia sinensis, bacterial pa ıogen, Darjeeling

The looper caterpillar, *Buzura suppressaria* Guenee (Le ıidoptera: Geometridae), attacks and defoliates mat ıre tea, *Camellia sinensis* (L.) O. Kuntze causing heavy crop loss (up to 40%) (Banerjee, 1993; Anonymous, 1994). A bacterial pathogen caused mo tality of the populations of 2nd and the 3rd brood of *B. suppressaria* (Das, 1965). In recent years, the looper act vities have greatly increased due to its greater tole rance to chemical pesticides of organophosphate and syn thetic pyrethroids (Sannigrahi and Talukdar, 2003; Sarker and Mukhopadhyay, 2006). In view of this, efforts are being made to evolve alternate strategies for the management of this pest through application of microbial bio agents.

B. thuringiensis formulations have been applied eff ectively against tea pests, such as *Caloptilia theivora* an l *Cydia leucostoma* (Unnamalai and Sekar, 1995) and *B. suppressaria* (Das and Barua, 1990). It has also being su ccessfully used in controlling the tea totricid ca erpillars (Kariya, 1977) and other lepidopteran pests (M uraleedharan and Selvasundaram, 2002).

The present study was aimed to isolate and ch aracterize the naturally occurring bacterial pathogen of *B. suppressaria* up to genus level and then conduct bi ıassay to determine the lethal concentration (LC_{50}) and le hal time (LT_{50}).

200-250 larvae of *B. suppressaria* were collected on T ıcklai vegetative clone, TV-25, from mature tea pl antations about 20 years old of Darjeeling foothills and it adjoining plains. These were reared in laboratory for tw o generations at 27 ± 2 °C; 72 ± 2 % RH with a p otoperiod (L: D) of 13:11 hrs. in aseptic conditions. Fresh tea twigs (TV-25) from the experimental tea garden maintained on North Bengal University campus was supplied as food. The two-day old second instar larvae (0.015 g) were used for bioassay test.

For isolation of bacteria, dead larvae were collected during February-March and putrefied for 5 days with autoclaved double distilled water in a sterile glass container. Whole body was thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3000 rpm for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was stored in autoclaved eppendorf tube with double distilled water at –20 °C (Lacey and Brooks, 1997). Pure culture of the bacterium was isolated from mixed populations by dilution streak method in nutrient agar medium. Viability was checked by inoculating in new agar medium at weekly intervals. To characterize the bacterium up to genus level some experiments were conducted. Gram staining followed by Catalase assay and Voges-Proskour (V-P) reaction were performed with the bacterial isolate after the method of Thiery and Frachon (1997). Subsequently its anaerobic growth was also observed (Sneath, 1986). To find out whether spores were present in the body of bacterium, a staining procedure was applied with 0.5% Basic fuchsin. Growth phase of the isolate (*Bacillus* sp.) and *Bacillus thuringiensis* kurstaki (from IARI, New Delhi) was compared and their doubling time was determined after Cappucino and Sherman (1996).

Extraction and bioassay of crude spore mixture was done after the procedure of Dulmage *et al.* (1970) with some modifications. Different concentrations of crude

samples (spores) (500, 750 and 1000 µg/ml) 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 Sekar (1995). Twenty second instar larvae per concentration @ three replicates were fed and sterile distilled water was used in control. The mortality was observed at an interval of 24 hrs from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula (Abbott, 1925). Data were subjected to probit analysis (Finney, 1954) 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):

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

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.

The larvae of infected *B. suppressaria* turned brownish with putrification and liquefactions of tissue. The bacterium isolated from such cadavers showed characteristic colony formation, growth pattern, and sporulation. Further, the catalase assay, V-P reaction and anaerobic growth results favoured the identity of the bacterium as one close to *Bacillus thuringiensis*. However, the bacterium differed from the commonly occurring lepidopteran strain *B. thuringiensis* kurstaki by a faster growth rate (Fig. 1) and its pathogenicity (Per. Comm.). The doubling time of the newly identified *Bacillus* sp. was 60 min (approx.) whereas for *B. thuringiensis* kurstaki was about 90 min. Due to the bacterial infection the mortality of *B. suppressaria* in the natural population varied from 40-60% in different seasons i.e. during pre-monsoon (March- June), monsoon (July- October) and post-monsoon (November- February) (Fig. 2) with an average mortality of about 50%. It is reported that bacterial disease takes a heavy toll of 2nd and 3rd broods of *B. suppressaria* between May and August (Das, 1965). A formulation of isolate (*Bacillus* sp.) and its application in various doses with food proved the bacterial pathogen to be effective in causing dose dependant mortality of *B. suppressaria* caterpillars. Percentage mortality of *B. suppressaria*, when infected with different concentration of crude spore mixture of the isolate (*Bacillus* sp.), ranged from 41.66 to 80 in first 9 days (Table 1). The median lethal concentration (LC_{50}) was found to be 727.8 µg/ml and median lethal time (LT_{50}) was to be 8.05 days in case of 750 µg/ml spore concentration to 5.93 days in 1000 µg/ml spore concentration (Table 2). The lowest concentration of 500 µg/ml was not found effective in larvae as the caterpillars continued up to pupation.

Use of bacteria, especially *B. thuringiensis*, is well established as microbial pesticide for different tea pests (Kariya, 1977; Barbora, 1995). Efficacy study of different strains of *B. thuringiensis* along with determination of LC_{50} values has been done for tea leaf eating caterpillars, *Caloptilia theivora* (LC_{50} 0.296 µg/ml with max. conc. of crude sample 0.300 µg/cm²) and *Cydia leucostoma* (LC_{50} 0.221 µg/ml with max. conc. of crude sample 0.300 µg/cm²) (Unnamalai and Sekar, 1995). As proper identity and bioassay of the naturally occurring bacterium (*Bacillus* sp.) of *B. suppressaria* in question was so far not known, the present investigation substantiates to some

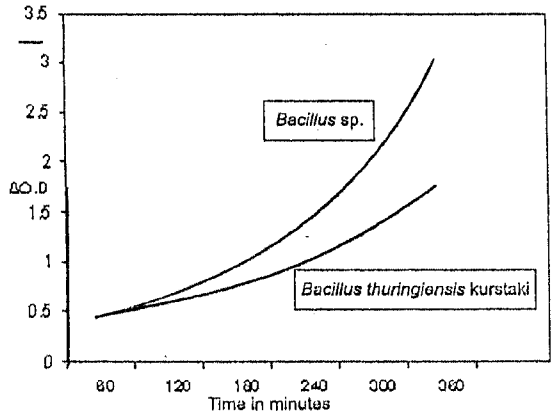

Fig. 1. Comparison of growth curves at exponential phase of the newly identified *Bacillus* sp. and *Bacillus thuringiensis* kurstaki.

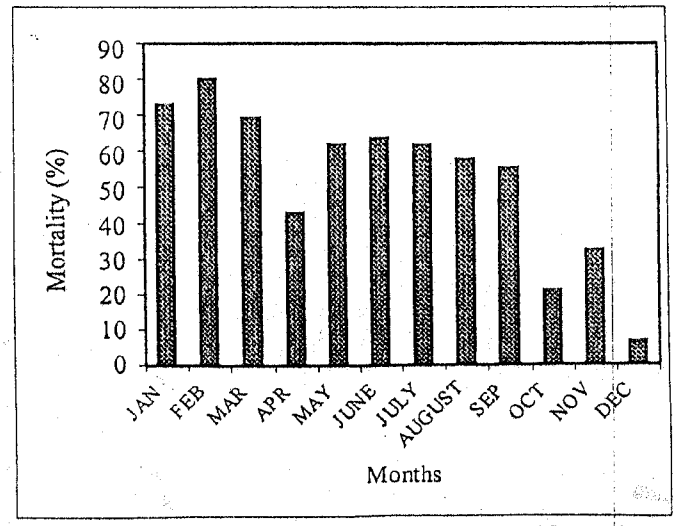

Fig. 2. Percentage mortality of *B. suppressaria* population collected from terai tea plantation in different months of the year

Table 1. Toxicity of *Bacillus* sp. against *Buzura suppressaria* within 2-9 days

Crude spore concentration of *Bacillus* sp. (µg/ml)	Log. Conc. (X)	No. of tested larvae	Actual mortality	Percentage mortality	Corrected mortality (%)
500	2.69	60	25	41.66	16.65
750	2.87	60	39	65	35.71
1000	3	60	48	80	71.42
Control	Nil	60	18	30	Nil

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)= 2415.92$	Y=8124.45-2836.75X	727.8	724.9 (Lower limit) 730.6 (Upper limit)

Table 2. Effect of different concentrations of spores (*Bacillus* sp.) on LT_{50} against second instar larvae of *B. suppressaria*

Dosage (µg/ml)	LT_{50} (days)	% Mortality
1000	5.93	80
750	8.05	65
500	43.36	41.66

extent its distinct nature as a *Bacillus* which is different from *Bacillus thuringiensis* kurstaki and its potential as a microbial bioagent.

Acknowledgement

The authors wish to thank Dr. R. Chakraborty, Head, Department of Biotechnology, University of North Bengal for extending the laboratory facility and Prof. P. K. Sarkar, Microbiology laboratory, Department of Botany, University of North Bengal for giving valuable suggestions. The authors are also thankful to Dr. Bishwajit Paul, Scientist (Senior scale), Entomology Division, IARI, New Delhi for his suggestion on the character of the bacterium and to an unknown reviewer.

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SHORT COMMUNICATION

A novel bacterial pathogen (*Enterobacter* sp.) isolated from the leaf roller, *Caloptilia theivora* of tea of Darjeeling foothills

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Received: 5 January 2008 / Accepted: 17 April 2008 / Published online: 1 May 2008

Abstract *Enterobacter* sp. was isolated from the diseased and dead caterpillars of the tea leaf roller (*Caloptilia theivora*) from the Darjeeling foothill region. When the vegetative form of the bacterium was applied via food, mortality of *C. theivora* showed an LC_{50} value at 363.1 μg/ml (bacterial wt./vol. of water) with fiducial limits 363.25 and 362.94 μg/ml respectively. The LT_{50} values for *C. theivora* were 6 days for 100 μg/ml, 5.96 days for 300 μg/ml, 5.81 days for 500 μg/ml, 4.96 days for 750 μg/ml and 4.61 days for 1,000 μg/ml concentrations. The finding would enable one to contemplate development of a microbial pesticide using this novel *Enterobacter* sp. DD01 for control of the leaf rolling pest.

Keywords *Caloptilia theivora* · *Camellia sinensis* · *Enterobacter* sp. · Tea · Darjeeling

Introduction

The tea leaf-roller, *Caloptilia theivora* (Lepidoptera: Gracillariidae), attacks and consumes young leaves of tea bushes, *Camellia sinensis*, causing substantial loss in tea production and deterioration of its quality. Synthetic pesticide sprays have their limitation to readily reach caterpillars and pupae of the species that remain covered in the leaf rolls. Moreover, residue-free organic tea has a great demand in domestic and international (export) market. In view of this, biological control of this tea pest has become a necessity, which could possibly be achieved through development of microbial pesticides.

The present study was aimed to isolate and characterize the naturally killing bacterial pathogen of *C. theivora* followed by bioassay to determine lethal concentration (LC_{50}) and lethal time (LT_{50}) of *Enterobacter* sp. DD01 in order to find its potential.

Materials and methods

DD01 was isolated from larvae of *C. theivora*. For isolation of bacterium of *C. theivora*, the larvae dying of disease were taken for surface sterilization with 70% alcohol and then washed three times with sterilized double distilled water. Then they were kept in sterilized Eppendorf tube with sterilized double distilled water. The Eppendorf was wrapped with Parafilm and stored at −20°C (Lacey and Brooks 1997). The stored dead larvae were taken and thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3,000 rev/min for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was taken for pure culture isolation by the 'dilution streak method' in nutrient agar medium. Two different types of colonies appeared on the nutrient agar medium after inoculation. One was the 'white glossy circular' colony and another was the 'yellow pigmented colony'.

From the white glossy circular colonies innocula were taken and dilution-streaked on four culture plates. The same was done in case of yellow pigmented colonies.

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From each of the four white glossy circular and yellow pigmented colonies, single colonies were purified on nutrient agar media. The purified strains having white glossy colony phenotype were named as WGC01, WGC02, WGC03 and WGC04 and similar yellow pigment-producing strains were named as YP01, YP02, YP03 and YP04. The purified cultures of all WGC and YP strains were individually fed to healthy *C. theivora* larvae. The result showed that only one white glossy circular-colony-producing strain (WGC03) created the disease syndrome in healthy larvae. On the other hand all four yellow pigmented culture failed to produce the disease syndrome. From the diseased larvae again plating on nutrient agar was repeated. The predominance of white glossy colonies was found on the culture plate. From WGC colonies single colony was purified which was named, DD01. From that DD01 stock, bacterial innoculum was taken to reinfect the healthy larvae for bioassay.

Several biochemical tests like indole, Voges-Proskauer, methyl red, citrate utilization, esculin hydrolysis lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using Enterobacteriaceae Identification Kit (KB003 Hi25) (Himedia).

Aqueous formulation of the DD01 (vegetative body) (100, 300, 500, 750 and 1,000 μg/ml) of *C. theivora* was used for bioassay after the method of Jackson et al. 1992). Leaf dipped in different concentrations of the above bacterial solution were offered as food. Second instar larvae ($n = 100$) were used for each treatment (concentration). Treatment with sterile distilled water was used in the controls. The mortality was observed at an interval of 24 h from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula. Data were subjected to probit analysis (Finney 1954) and the median lethal concentration (LC_{50}) value was calculated from the regression equation. The median lethal time (LT_{50}) value was also determined simultaneously following the method of Biever and Hostetter (1971).

Results and discussions

Vegetative cells of the bacterial strain DD01 were aerobic, Gram-negative, motile and rod shaped. The strain was positive in Voges-Proskauer, β-galactosidase, lysine decarboxylase, ornithin decarboxylase, nitrate reduction, Simmons citrate, esculin hydrolysis tests, but negative in phenylalanine deaminase, H_2S production, methyl red, indole, oxidase, urea hydrolysis and gelatin liquefaction tests. It utilized malonate, L-arabinose, D-xylose, D-adonitol, L-rhamnose, cellobiose, mellibiose, saccharose, raffinose, trehalose, D-glucose, lactose and D-sorbitol. The bacterial strain, DD01 was therefore tentatively assigned to the genus *Enterobacter*. This *Enterobacter* sp. has thus been isolated for the first time from diseased caterpillars of *Caloptilia theivora* of tea grown in the Darjeeling foothill region. When the bacterium was applied via food, mortality of *C. theivora* showed the LC_{50} value at 363.1 μg/ml with fiducial limits 363.25 and 362.94 μg/ml, respectively. The LT_{50} values for *C. theivora* were 6 days for 100 μg/ml, 5.96 days for 300 μg/ml, 5.81 days for 500 μg/ml, 4.96 days for 750 μg/ml and 4.61 days for 1,000 μg/ml concentrations (Table 1).

The use of the Gram-negative non-spore forming bacterium *Serratia entomophila* is well established as microbial bioagents for controlling of grass grub larvae in New Zealand (Jackson et al. 1992). There are approximately 100 species of bacteria pathogenic to insects but

Table 1 LC_{50} and LT_{50} values of *Enterobacter* sp. DD01 for the pest *C. theivora* during first seven days of inoculation

Enterobacter sp. (μg/ml)	Log. Conc. (*X*)	No. of tested larvae (*n*)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
100	2	100	64	64	6.0
300	2.47	100	74	74	5.96
500	2.69	100	76	76	5.81
750	2.87	100	79	79	4.96
1000	3	100	86	86	4.61
Control	0.0	100	20	20	–

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi2(5) = 39.73$ for 100 μg/ml	$Y = 18.72 + 0.69X$	363.1 μg/ml	362.94 (Lower limit)
$\chi2(5) = 58.53$ for 300 μg/ml			363.25 (Upper limit)
$\chi2(5) = 62.82$ for 500 μg/ml			
$\chi2(5) = 69.62$ for 750 μg/ml			
$\chi2(5) = 87.43$ for 1,000 μg/ml			

hardly one percent of these are used as biocontrol agents. Nevertheless, the gut-colonizing bacterium *Enterobacter cloacae* has been reported to be used in pest control (Watanabe et al. 2000).

So, the present investigation reasonably substantiates the potentiality of the *Enterobacter* sp. DD01 bacterium as a microbial bioagent effective against the pest, *C. theivora*.

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CORRIGENDUM

Clarification of the points raised by the examiner of the Ph.D thesis of Smt. Damayanti De, Department of Zoology, NBU

(No: Ph.D./Zoo/910/R.11 dt: 20.5.2011)

Clarifications

Point no 1:

The standard style of presenting LC_{50} & LT_{50} tables has not been followed. Graphs showing dose response curve are lacking. The heterogeneity as shown in LC_{50} & LT_{50} tables should be checked.

LC_{50} & LT_{50} value have been furnished in tabular form where the column of mortality (response) has been presented against the dose/concentration of the entomopathogenic bacterial isolate. The results of 'dose-response' are further being presented graphically (Ref: Öztürk *et al.*, 2008; Nun˜ez-Valdez *et al.*, 2008) below as requested by the reviewer. The heterogeneity of LC_{50} is also checked as proposed.

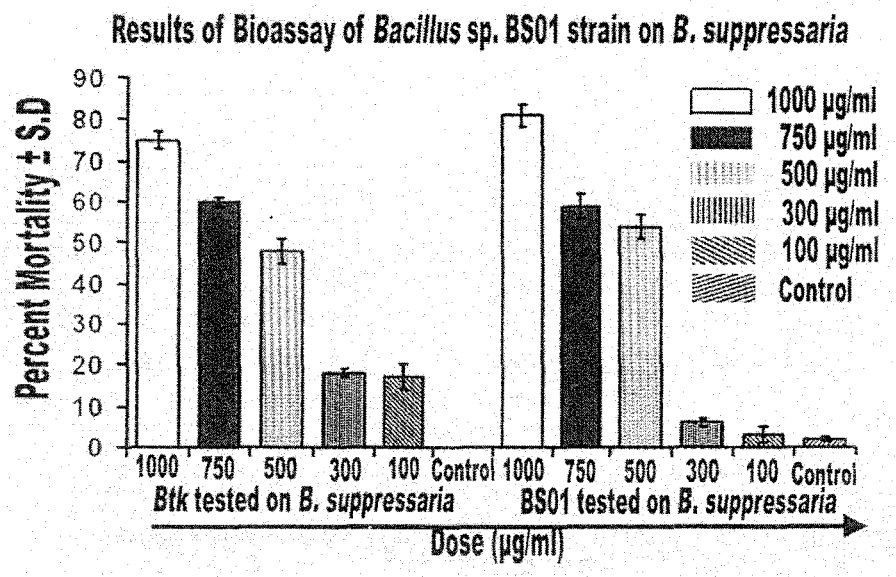
References:

Öztürk F., Açık L., Ayvaz A., Bozdoğan B and Suludere Z. 2008. Isolation and Characterization of Native *Bacillus thuringiensis* Strains from Soil and Testing the Bioactivity of Isolates Against *Ephestia Kuehniella* Zeller (Lepidoptera: Pyralidae) Larvae. *Turk J Biochem* 33(4):202-208.

Eugenia Nun˜ez-Valdez M., Calderón M A., Aranda E., Herna´ndez L., Ramı´rez-Gama R M., Lina L., Rodrı´guez-Segura Z., Gutie´rrez M C. and F J. 2008. Identification of a Putative Mexican Strain of *Serratia entomophila* Pathogenic against Root-Damaging Larvae of Scarabaeidae (Coleoptera). *Applied and Environmental Microbiology*. 74(3): 802-810.

Dose response curves are graphically presented below:

Graphical representation of table presented in page no. 51



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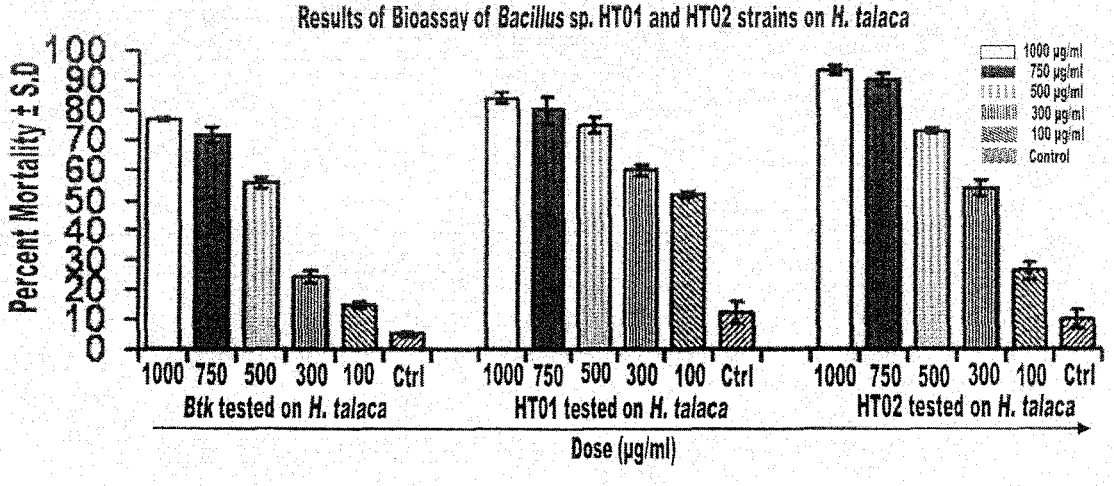

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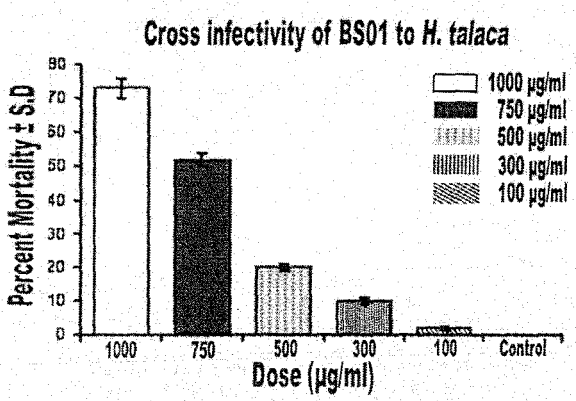
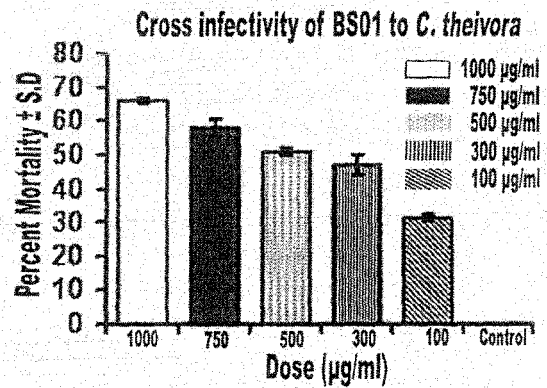
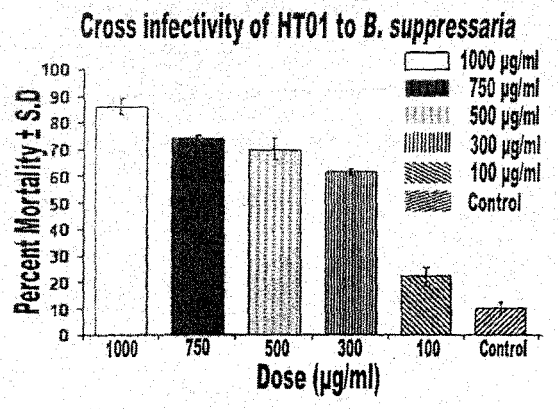


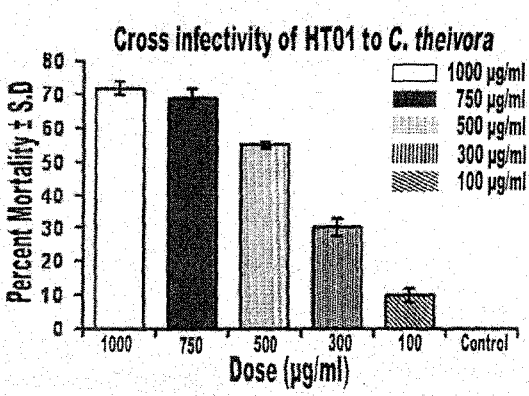
Graphical representation of table presented in page no. 56



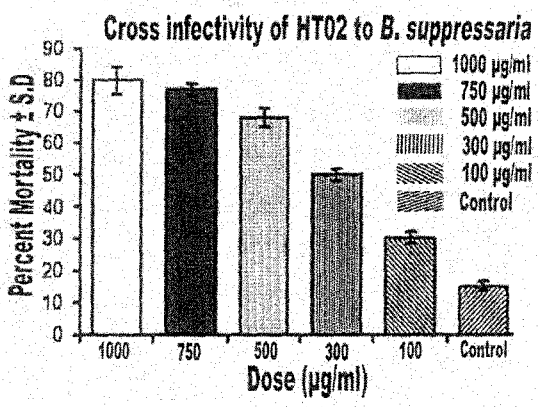
Graphical representation of table presented in page no. 58



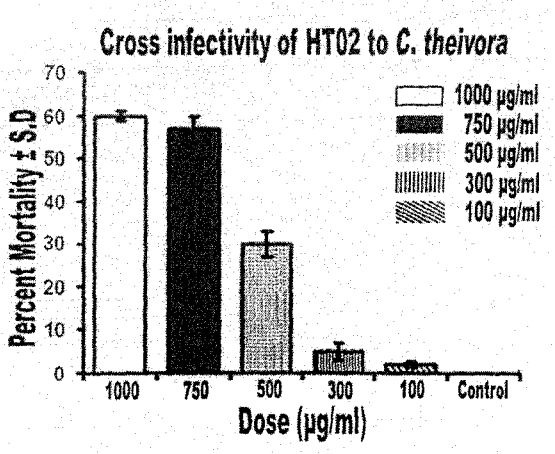
Graphical representation of table presented in page no. 59



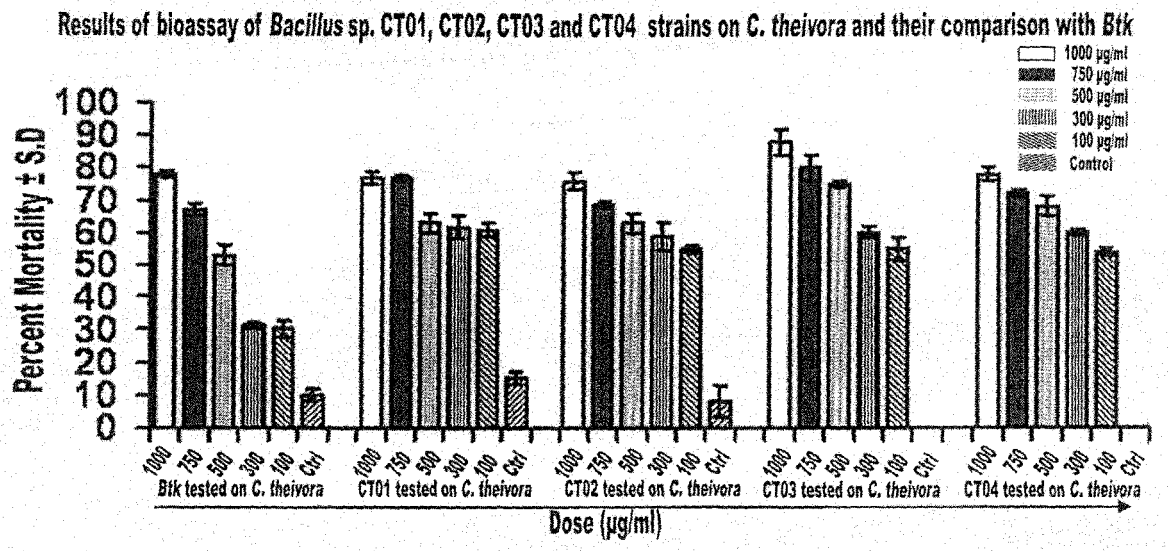
Graphical representation of table presented in page no. 61



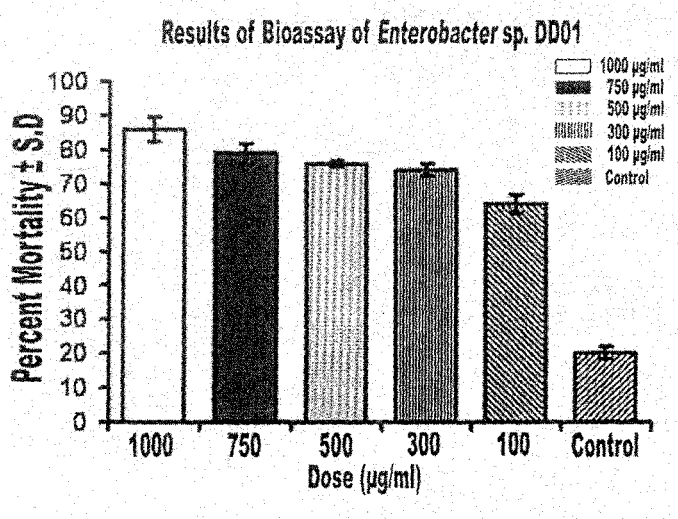
Graphical representation of table presented in page no. 62



Graphical representation of table presented in page no. 80



Graphical representation of table presented in page no. 81



Refernces:

Guerrero C, Pereira D and Net L (2007) Evaluation of the *Bacillus thuringiensis* based insecticide in the control of lepidopterous caterpillars on golf courses. *International Journal of Energy and Environment* 1(2): 106-108.

Ravi M, Santharam G and Sathiah N (2008) Ecofriendly management of tomato fruit borer, *Helicoverpa armigera* (Hubner). *Journal of Biopesticides* 1(2): 134-137.

Point no 3:

The observed selectivity of the bacteria, especially *Bacillus* to silkworm larvae needs clarification, as *Bacillus* is not species specific.

The bacterial strains selected to test cross infectivity to the lepidopteran tea pests of the same habitat and beneficial insect, such as silkworm is based on the most virulent strains (say of *Bacillus*) found to cause high mortality to the concerned tea pest from where it has been isolated. As the silk worm industry is running side by side with the tea industry, so before going into field study of the *Bacillus* strains it is necessary to know the cross-infectivity of the selected strains in silk worm population. So, the *Bacillus* strains were taken to cross-infect the silk worm population.

(Signature)

Damayanti De 1.6.2011

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