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Isolation and Insecticidal Potential of Native *Bacillus thuringiensis* against *Helicoverpa armigera* and *Spodoptera litura*

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A B S T R A C T

The present investigation was carried out with an aim to isolate and characterize native *Bacillus thuringiensis* from Anand district of Middle Gujarat. Total 67 probable *B. thuringiensis* were isolated from 17 diverse soil samples, out of which 9 isolates were selected based on presence of parasporal crystal body and primary toxicity test. Selected nine native isolates were characterized based on their morpho-cultural and biochemical characteristics. Among the selected nine isolates, Abt10 isolated from soil of Navli village, Anand found the most potent. Systemic laboratory bioassay of isolate ABt-10 shown LD_{50} value of 2.15 $X10^7$ spores/ml and 3.7 X 10^6 spores/ml with the highest mortality (69.53 %) on *Helicoverpa armigera and Spodoptera litura*, respectively.

Introduction

Biological control is a crucial tool of Integrated Pest Management. It involves the use of bio-agents like parasitoids, predators and insect pathogens e.g. fungi, bacteria, virus, nematodes etc. to manage the pests. Among these, bacterial bio-agents have gained significant attention since inception of insect pathology (Morales-Ramos *et al*., 2014). There are several families of entomopathogenic bacteria such as *Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae* and *Micrococaceae* which are used widely for insect pest management throughout world (Tanada and Kaya, 1993). Bacteria belonging to family *Bacillaceae* possess wide range of insecticidal activity; from which, *B. thuringiensis* (*Bt*) is the most widely used and successful microbial pesticide (Jurat-Fuentes and Jackson, 2012). *B. thuringiensis* was first discovered by Japanese biologist Ishiwata Shigetane in 1901. The sales of *B. thuringiensis* since its registration, account for more than 90% share amongst all microbial pesticides sold worldwide through its products represent about 1% of the total

'agrochemical' market of fungicides, herbicides and insecticides across the world.

B. thuringiensis comprises of gram-positive, big rod shaped, spore-forming bacterium. They possesses unusual property of producing a parasporal protein crystal, popularly known as 'Cry' protein (δ-endotoxin), which is toxic to many insect pests (Crickmore *et al.*, 2014). Efforts have been made in many countries to isolate new strains with increased potency against target pest insects having a wider host range. These toxins have not only shown activity against *Lepidoptera, Diptera, Hymenoptera, Isoptera, Orthoptera* and *Coleoptera* but also against nematodes, mites, lice, aphids and ants also (van Frankenhuyzen, 2009).

Thus, long established approach of isolating naturally existing strains of *B. thuringiensis* with novel toxicities is an attractive alternative. *B. thuringiensis* have been differentiated by various methods including presence of parasporal bodies, biochemical tests, serotyping, 16S *rRNA* gene sequencing, cell membrane fatty acid analysis, esterase production, antibiotic production, enzymes, phages and lectin grouping (El-kersh *et al*., 2012). Several biotechnological tools are available for rapid identification and differentiation of organisms (Sanahuja *et al*., 2011).

In various regions of the world, studies of *B. thuringiensis* have required new strains with different potentialities from those that are at present known for new *cry* genes with different insecticidal effects (Liang *et al*., 2011). Gujarat, India harbors diverse environments that are suitable for microorganism development, comprising natural sources for the isolation of new bacteria. In view of the biodiversity of the Middle Gujarat environment, isolation of *B. thuringiensis* isolates from this region may be helpful in finding new insect pathogen with combinations of *cry* genes, which are different from those currently known. Accordingly, this study aimed to isolate *B. thuringiensis* from soils of Anand district of middle Gujarat, for use in the microbial control of *Helicoverpa armigera* and *Spodoptera litura*.

Materials and Methods

Isolation of *B. thuringiensis* from soil samples

Native *B. thuringiensis* were isolated following the method described by Patel *et al.* (2013). One gram soil was added to 10 ml of buffered T3 medium in a 100-ml flask and incubated at 100 rpm at 30 ^{0}C for 36 to 48 h. From this, 1 ml broth was re-inoculated in 10 ml of buffered Luria Broth and incubated at 30 ^{0}C for 4-6 h on environmental shaker. One ml of broth was heat treated at 80 ^{0}C for 3 min in a water bath and allowed to settle at room temperature (28±1 ^{0}C). The supernatant was transferred to sterile test tube, vortexed and serially diluted with sterile distilled water (10^{-1}, 10^{-2}, and 10^{-3}). From each dilutions, 500 µl aliquot was spread on Mannitol-Egg Yolk-Polymyxin (MYP) agar plates and incubated for 36-48 h at 30 ^{0}C. Colonies with fried egg appearance (Off white, with irregular margin which produce red color and precipitation on MYP agar) were transferred on T3 agar plates and incubated at 30 ^{0}C for 72 h. Isolated colonies obtained on T3 agar plates were examined microscopically for presence of spores and crystals under phase-contrast microscope as well as stained with 0.13 % Coomassie brilliant blue stain. The cultures were then transferred on Nutrient agar slants and stored at 4 ^{0}C for further studies.

Establishment of Koch's postulates and preliminary toxicity test of native *B. thuringiensis* isolates

It is necessary to prove Koch's postulates for an organism to consider it as pathogen. So,

accordingly, insect pathogens need to be screened for its pathogenicity against preferred host and it will give preliminary idea regarding its potency also (Poinar and Thomas, 1984).

Diet contamination technique (Navon *et al.*, 1990) was employed to establish Koch's postulates. Test insects belonging to order Lepidopteran *viz. H. armigera* and *S. litura* were selected to establish Koch's postulates and preliminary toxicity test. Artificial diet for each test insect was prepared separately and mixed thoroughly with respective bacterial isolates approximately 10^9CFU/ml and fed to the respective test insects. Periodic mortality was recorded up to 120 h. Haemolymph of the dead larvae was observed for the presence of spores and parasporal body of *B. thuringiensis* by preparing wet mounts, under 400X in Phase-Contrast Microscope.

Identification and characterization of native *B. thuringiensis* isolates

All isolates were identified based on their morphological, cultural and biochemical characteristics using 9th edition of Bergey's Manual of Determinative Bacteriology and standard literature (Halt *et al.*, 1994).

Based on the results of preliminary toxicity test, partial 16S *rRNA* gene sequencing was carried out for promising isolate and was performed using the ABI PRISM® BigDye™ Terminator cycle sequencing kit on the ABI PRISM 3100 genetic analyzer (Chromous biotech). This sequencing has emerged as a powerful technique for phylogenetic placement, identification and diversity analysis of bacteria and was carried out using the set of primers suggested by Perez *et al*., 2007. The 16S *rRNA* gene sequences were assembled using MEGA 4 software, compared with other strains using NCBI BLAST analysis for identification purpose and comparison of homologies of isolated strains with previously characterized strains.

Bioassays of Native *B. thuringiensis* isolate ABt 10 on Lepidopteran Pest

Laboratory bioassay of selected isolate ABt 10 was carried out by diet contamination technique (Navon *et al.*, 1990) against *H. armigera* and *S. litura* along with standard strain of *B. thuringiensis var. kurstaki* HD-73.

A series of concentrations of isolate ABt 10 and *Bt*k HD-73 *viz*., 10^7, 10^8 and 10^9CFU/ml were prepared in sterile distilled water. The number of *B. thuringiensis* spores and crystals in the suspension was pre-determined microscopically using an improved Neubauer's haemocytometer. Each treatment was replicated four times having 10 larvae per each treatment. The observations on larval mortality were recorded at 24 h interval up to 168 h. Dead larvae were recovered from the trays and presence of *B. thuringiensis* in the haemolymph was confirmed by observing the bacterial spores and crystals under 400X in a phase contrast microscope. Data were analyzed using Completely Randomized Block Design (Gomez and Gomez, 1984). Periodic observations were analyzed following the Analysis of Variance (ANOVA) method (Steel and Torries, 1980). Data were transformed using Arc sin transformation and subjected to statistical analysis. Insecticidal activities (LD50) of *B. thuringiensis* isolate Abt 10against the test insect was assessed following Probit analysis by Finney's method (Finney, 1971).

Results and Discussion

Isolation of native *B. thuringiensis* from soil

In present investigation, we approached to study the natural distribution of

B. thuringiensis in the soils from diverse locations of Anand district. The type of soil from which the samples were collected was Sandy loam. Preliminarily 67 *B. thuringiensis* like colonies were selected for further screening from 109 isolated colonies on MYP agar plates. Fried egg like *Bt* and non-*Bt* distinguished from each other as *Bt* colonies gave pink coloration and precipitation of the medium around it, with an average of 3.94% per samples. Selected 67 *B. thuringiensis* like isolates obtained after primary screening were further screened for presence of parasporal crystal inclusion body, popularly known as '*Cry* protein' which is responsible for *Bt*'s insecticidal activity using Trinocular Phase-Contrast research microscope (400X) (Patel *et al*., 2013). Total 9 isolates were screened out having presence of parasporal crystal body and designated as *B. thuringiensis* isolates ABt 2, 10, 17, 21, 33, 49, 54, 61 and 63, respectively. Isolate ABt10 obtained from Navli village shown larger size of inclusion body as compared to other isolates.

Establishment of Koch's postulates for native *B. thuringiensis*

Selected nine isolates shown presence of parasporal crystal inclusion body, which were subjected to establishment of Koch's postulates. According to Torres-Quintero *et al*. (2015) Koch's postulates is the basic step for establishing the microorganism as the causal agent of a disease to particular insect host. All nine isolates successfully proved Koch's postulates in the laboratory against *H. armigera* and *S. litura* as target insect with varying level of larvicidal activity after 120 h. Typical symptoms recorded were cessation of feeding, paralysis, diarrhea, and vomiting. After passing through the gut epithelial barrier, pathogens proliferate in the hemocoel, producing bacteremia or septicemia as a result of the action of bacterial toxins and pathogenic factors; this typically results in color and tissue changes in the host. Insects killed by tested *B. thuringiensis* turned dark in color, soft and flaccid. Among the tested isolates, ABt 10 gave maximum mortality 80% and above (Table 1) against both tested insects. Isolate ABt 10 possessed the largest inclusion body, which can be directly correlated with results of primary toxicity test where highest mortality was recorded (Whiteley and Schnepf, 1986).

Characterization and identification of native *B. thuringiensis* isolates

All the nine isolates were found gram positive, straight, thick, sporulating rods, occurring in long chains. The spores were ellipsoidal and sub terminal with round edges. Parasporal crystalline bodies were bipyramidal and smaller than spores (Fig. 1 A and B). On Nutrient agar the colony was round, medium sized, elevated with irregular margins and the color was creamish white, which later on showed dark center (Fig. 2 A and B). Similar results were found by Martin and Travers (1989) and Ashokan and Puttaswamy (2007) during their studies.

All the test isolates were confirmed *B. thuringiensis* by biochemical characters. In the present study, all the isolates were found positive for citrate utilization, production of catalase and arginine dehydrogenase enzyme. While malonate, Voges Proskauer's and ONPG tests were negative in this study.

Similar findings have also been reported by Lacey and Goettel (1995), Patel (2006) and Kaur *et al*. (2006). All the test isolates successfully fermented sucrose, glucose and trehalose, while for mannitol and arabinose fermentation the test isolates were found negative. The findings are comparable with the findings of Lacey and Goettel (1995), Patel (2006).

After preliminary characterization and toxicity testing, isolate ABt 10 acquired from Navli village soil (Anand) was identified at molecular level using 16S *rRNA* gene sequencing along with phylogenetic analysis and proceeded for further systemic insect bioassay against Lepidopteran insect pest *H. armigera* and *S. litura*.

Table.1 Efficacy of isolates against *H. armigera* and *S. litura*

Sr. No.	Isolate No.	Mortality	
		H. armigera	*S. litura*
1	ABt 2	++	++
2	ABt 10	++++	++++
3	ABt 17	++	++
4	ABt 21	+	+
5	ABt 33	+++	+++
6	ABt 49	+	+
7	ABt 54	++	++
8	ABt 61	++	++
9	ABt 63	+	+

Note: + = Poor effect, ++ = Moderate effect, +++ = Good effect, ++++ = Excellent effect.

Table.2 Bioassay of Native *B. thuringiensis* isolate ABt-10 against *H. armigera*

Treatments (spore-crystal mixture/cm^3)	**% mortality after h**						
	24	**48**	**72**	**96**	**120**	**144**	**168**
T1- ABt 10(**1 x 10^7**)	5.29* (0.85)**	11.70 (4.11)	30.28 (25.42)	44.36 (48.87)	44.36 (48.87)	44.36 (48.87)	44.36 (48.87)
T2- ABt 10(**1 x 10^8**)	9.67 (2.82)	20.73 (12.53)	31.78 (27.74)	44.20 (48.61)	44.20 (48.61)	44.20 (48.61)	44.20 (48.61)
T3- ABt 10(**1 x 10^9**)	20.47 (12.23)	33.05 (29.75)	50.83 (60.11)	69.53 (87.77)	69.53 (87.77)	69.53 (87.77)	69.53 (87.77)
T4 **-Btk HD-73(1 x 10^9)**	14.05 (5.90)	28.23 (22.37)	45.00 (50.00)	63.81 (80.52)	63.81 (80.52)	63.81 (80.52)	63.81 (80.52)
T5**Control (Untreated)**	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)
S. Em.	**3.69**	**3.31**	**2.12**	**2.14**	**2.14**	**2.14**	**2.14**
CD at 5%	**11.13**	**9.99**	**6.39**	**6.45**	**6.45**	**6.45**	**6.45**
C.V. %	**73.27**	**35.03**	**13.35**	**9.61**	**9.61**	**9.61**	**9.61**

* Figures in table are Arcsine transformed values.
** Figures in parentheses are the retransformed values

Table.3 Bioassay of Native *B. thuringiensis* isolate ABt-10 against *S. litura*

Treatments (spore-crystal mixture/cm^3)	% mortality after h						
	24	**48**	**72**	**96**	**120**	**144**	**168**
T1- ABt 10(**1 x 10^7**)	5.29* (0.85)**	18.12 (9.67)	33.29 (30.12)	48.88 (56.74)	50.32 (59.23)	50.32 (59.23)	50.32 (59.23)
T2- ABt 10(**1 x 10^8**)	9.67 (2.82)	26.58 (20.03)	42.76 (46.10)	56.43 (69.43)	56.43 (69.43)	56.43 (69.43)	56.43 (69.43)
T3- ABt 10(**1 x 10^9**)	20.47 (12.23)	39.17 (39.89)	56.95 (70.25)	69.53 (87.77)	69.53 (87.77)	69.53 (87.77)	69.53 (87.77)
T4 **-Btk HD-73(1 x 10^9)**	14.05 (5.90)	36.22(34.92)	49.39 (57.63)	63.81 (80.52)	63.81 (80.52)	63.81 (80.52)	63.81 (80.52)
T5**Control(Untreated)**	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)	0.91 (0.03)
S. Em.	**3.69**	**3.31**	**2.61**	**2.28**	**2.20**	**2.20**	**2.20**
CD at 5%	**11.13**	**9.98**	**6.86**	**6.87**	**6.64**	**6.64**	**6.64**
C.V. %	**73.27**	**27.36**	**14.23**	**9.51**	**9.15**	**9.15**	**915**

* Figures in table are Arcsine transformed values.
** Figures in parentheses are the retransformed values.

Table.4 LD_{50} of Native *B. thuringiensis* isolate ABt 10 and Btk HD-73 at various time intervals against *H. armigera* and *S. litura*

Treatment	**Time (h)**	**Chi^2**	**Slope**	**LD_{50} Value**	**Regression Equation**
H. armigera	48	0.165	0.335	8.497 $X10^{10}$	y= -3.662 + 0.335x
	72	0.548	0.393	6.364$X10^8$	y= -3.463 + 0.393x
	96	1.286	0.518	2.150$X10^7$	y= -3.801 + 0.518x
S. litura	48	0.076	0.370	7.812$X10^9$	y= -3.661 + 0.370x
	72	0.338	0.404	1.363$X10^8$	y= -3.286 + 0.404x
	96	0.219	0.401	3.700$X10^6$	y= -2.635+ 0.401x

Figure.1 Morphological characters of *B. thuringiensis* ABt 10 (A) Phase contrast Micrograph (400 X) (B) Bright field micrograph of Spores

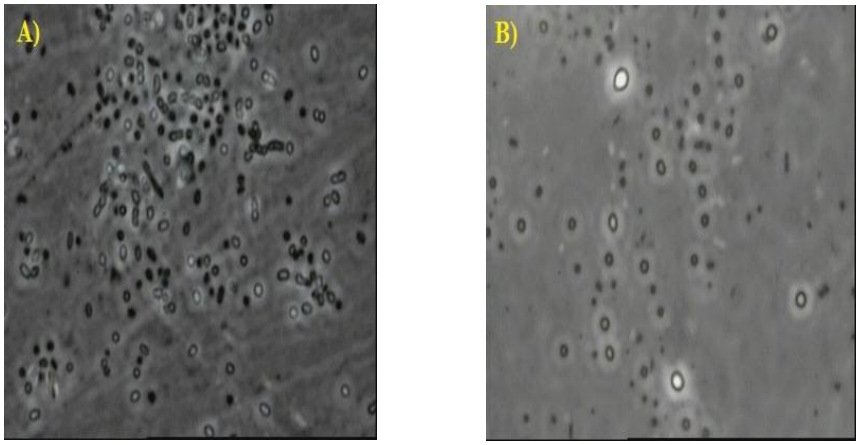

Figure.2 Cultural characters of *B. thuringiensis* ABt 10 (A) Colony on MYP Agar (B) Gram's staining

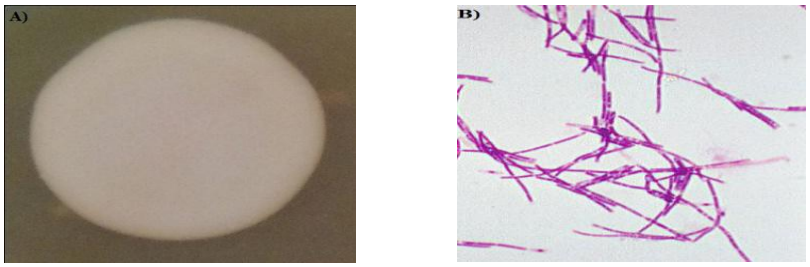

Figure.3 Symptoms of *B. thuringiensis* isolate ABt10 on *H. armigera* and *S. litura* A) Healthy larvae of *H. armigera*, B) Dead larvae of *H. armigera*, C) Healthy larvae of *S. litura* and D) Dead larvae of *S. litura*

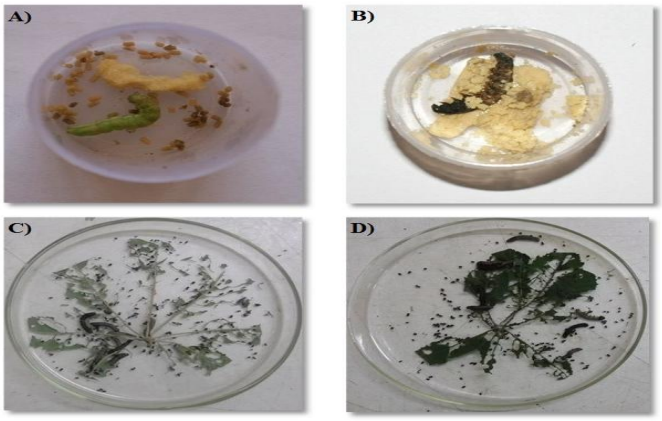

Figure.4 Phylogenetic tree based on 16S *rRNA* sequence of *B. thuringiensis* isolate ABt 10

For 16S *rRNA* gene sequencing based identification, DNA was extracted from native *B. thuringiensis* isolate ABt 10 as described by Wilson (1987). PCR amplification of 16S *rRNA* gene from isolate ABt 10 was carried out using universal primers (U27f and U1492r). 16S *rRNA* partial gene sequence of ~ 1500 bp was carried out and the output data were stored in FASTA. The output sequences were subjected for BLAST (Basic Local Alignment Search Tool) analysis to identify the cultures and to find out the nearest match of the cultures. Isolate ABt 10 was identified as *B. thuringiensis* sp. with 99 % similarity and 100 % query coverage to *B. thuringiensis* sp. IAM 12077 and designated as *B. thuringiensis* ABt 10 (Gene Bank Accession No. KF 279356). Additionally, the phylogenetic position of the isolate was also worked out within the available database of NCBI (presented as phylogenetic tree in Figure 4). The phylogenetic tree indicated, ABt 10 was placed in cluster one with more than 99 % similarity with known *B. thuringiensis* sp. indicating phylogenetic closeness.

Laboratory bioassays of native *B. thuringiensis* isolate ABt 10 on lepidopteran pest

Native *B. thuringiensis* isolate ABt 10 was systematically tested through bioassay by Diet contamination technique given by Navon *et al.,* (1990) against second instar larvae of *H. armigera* and *S. litura* to quantify the optimum bacterial dose for its effect for the suppression of the pest.

Spore-crystal mixture of *B. thuringiensis* isolate ABt 10 was tested at 10^7, 10^8 and 10^9 concentrations with that of *Btk* HD-73 (standard check). The larval mortality was recorded from 24 h, after the treatment, at an interval of 24 h up to 168 h. Amongst the different concentrations of *B. thuringiensis* isolate ABt 10 tested; mortality started after 24 h in T_3 (*B. thuringiensis* isolate ABt 10@ 10^9 spore crystal mixture/ cm^3 diet) and was significantly superior over control. Larval % mortality was observed to increase with a consequent increase in dose as well as time up to 120 h; however, the quantum of increase lowered after 96 h of the treatment. All the treatments were found effective from 24 h and T_3 was the best among all the treatments of test, standard strain as well as control.

All the treatments exhibited significant differences in per cent mortality of the test insect *H. armigera* and *S. litura* amongst different treatments as well as control ranging from 0.91 to 69.53 %. T_3 was found to be at par with *B. thuringiensis* HD-73 @ 10^9 spore crystal mixture/ cm^3 diet at 24, 48, 72 and 96 h periods after treatment. No larval mortality was noticed in untreated control (Table 2 and 3) (Fig. 3).

The dose mortality response of *H. armigera* at different concentrations of *B. thuringiensis* isolate ABt 10 was subjected to probit analysis and the LD_{50} value were calculated (Table 4). The LD_{50} value for *B. thuringiensis* isolate ABt 10 against *H. armigera* was found to be $2.15X10^7$ spores/ml at 96 h with regression equation y= -3.801 + 0.518x, Chi^2 value 1.286 and slope of 0.518 and *S. litura* was found to be 3.7 X 10^6 spores/ml at 96 h with regression equation y= -2.635+ 0.401x, Chi^2 value 0.219 and slope of 0.401.

Laboratory evaluation of different *B. thuringiensis* subspecies showed that 10^{10} spore/ml concentration caused 100 % mortality of larvae of the *H. armigera* during leaf bioassay (Rehman *et al.,* 2002). According to Chandrashekar *et al.,* (2005) *B. thuringiensis* var. *kurstaki* HD-1 based formulations are mostly used for lepidopteran control, but HD-73 showed higher mortality than HD-1 by 3.2-fold. HD-73 showed relatively higher mortality than HD-1 to neonates of *H. armigera*.

In this study, isolate ABt 10 shown the best performance during primary toxicity test and in bioassay in laboratory conditions. This isolate can be tested in field for its potency and can be developed commercially as a formulation. The research work presented here opens the door for future research on developing a novel *B. thuringiensis* of Middle Gujarat farmers for biological pest control.

Based on the results obtained from present investigations, it is concluded that modified method applied for isolation of *B. thuringiensis* from soil revealed good results and can be very useful in future. According to cultural, morphological, biochemical characters, 16S *rRNA* based molecular phylogenetic analysis and primary toxicity tests, *B. thuringiensis* ABt 10Accn No. KF 279356 found to be the most promising as biocontrol agent against *H. armigera* and *S. litura*.

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