Selection and characterization of *Bacillus thuringiensis* strains from northwestern Himalayas toxic against *Helicoverpa armigera*

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Abstract
In this study, we present the selection and the characterization of *Bacillus thuringiensis* (Bt) strains with respect to their *cry/cyt* gene content and toxicity evaluation toward one of the most important polyphagous lepidopteran pest, *Helicoverpa armigera*. Fifty-six Bt isolates were obtained from 10 different regions of northwestern Himalayas, recording a total *B. thuringiensis* index of 0.62. Scanning electron microscopy revealed presence of bipyramidal, spherical, flat and irregular crystal shapes; SDS-PAGE analysis of spore-crystal mixtures showed the prominence of 130, 70, and 100 kDa protein bands in majority of the isolates; PCR analysis with primers for eight *cry* and *cyt* gene families and 13 *cry* gene subfamilies resulted in isolates showing different combinations of insecticidal genes. Strains containing *cry1* were the most abundant (57.1%) followed by *cry12* (46.42%), *cry11* (37.5%), *cry2* (28.57%), *cry4* (21.42%), *cry1* (19.64%), *cry3* (8.9%), and *cry7, 8* (7.14%). A total of 30.35% of the strains did not amplify with any of the primers used in this study. Median lethal concentration 50 (LC₅₀) estimates of spore-crystal mixtures of Bt-JK12, 17, 22, 48, and 72 against second instar larvae of *H. armigera* was observed to be 184.62, 275.39, 256.29, 259.93 μg ml⁻¹, respectively.

*B. thuringiensis* presents great diversity with respect to the presence of crystal protein encoding genes and insecticidal activity. Four putative toxic isolates identified in this study have potential application in insect pest control. *B. thuringiensis* isolate JK12 exhibited higher toxicity against *H. armigera* than that of *B. thuringiensis* HD1, hence can be commercially exploited to control insect pest for sustainable crop production. The results of this study confirm the significance of continuous exploration of new Bt stains from different ecological regions of the world.

**KEYWORDS**
*Bacillus thuringiensis*, Bioassay, Cry proteins, *Helicoverpa armigera*, LC₅₀, PCR

1 | INTRODUCTION

The escalating public concern, stringent environmental regulations, and build-up of resistant insect populations to synthetic pesticides have led to an increased interest in alternative eco-friendly pest control strategies. One of the most successful substitutes to the manmade pesticides is the use of entomopathogenic bacterium, *Bacillus thuringiensis* (Bt). The entomopathogenic potential of Bt is primarily due to its
ability to synthesize during sporulation, the crystalline proteins, Cry and Cyt, encoded by cry and cyt genes, respectively (Schneef et al., 1998). Up to March 2017, 74 classes of Cry proteins (Cry1 to Cry74) and three classes of Cyt proteins (Cyt1-Cyt3) have been designated based on their amino acid sequence homology (Crickmore et al., 2017). These toxins are highly specific in action, harmless to humans and other vertebrates and are biodegradable. The reasons for increased acceptability of Bt over synthetic insecticides are due to the nonselective deleterious effects of chemicals (Eriksson & Wiktelius, 2011) and the emergence of resistance in insect pests against the synthetic insecticides (Ahmad, Sayyed, & Saleem, 2008). For the said reasons, continuous efforts are being made to isolate novel B. thuringiensis strains with distinctive host range or higher toxicity potential.

The presence of Bt has been extensively studied in different ecological habitats such as soil, stored product dust, dead insects, food grains, phyllosphere, and aquatic environments (Ben-Dov et al., 1997; Bravo et al., 1998; Martin & Travers, 1989). Diversity of B. thuringiensis and their cry genes from different regions of India have been studied earlier (Kaur & Singh, 2000; Patil, Purani, & Ingle, 2013). However, distribution and diversity of B. thuringiensis and their cry and cyt genes in B. thuringiensis isolates from northwestern Himalayan region has not yet been approached in detail (Yadav, Sachan, Verma, & Saxena, 2016). The variations in topographical features along longitude, latitude, and altitude of the region create climatic variations resulting in unique and rich biodiversity (Ray, Doshi, Alag, & Sreedhar, 2011), thereby making the northwestern Himalayan region a critical biodiversity hotspot of the world. These distinctive features and diversity of insects in the region provide an opportunity for prospecting novel B. thuringiensis strains with novel combinations of crystalline protein coding genes having wide insecticidal spectrum. Various methods such as polymerase chain reaction (PCR), Southern blotting, protein profiling, serotyping, and bioassay have been employed for the characterization of B. thuringiensis strains and their crystalline protein-encoding genes; among all, PCR-based methods have proven to be the most efficient in terms of rapidity in large-scale screening programs (Porcar & Juarez-Perez, 2003). In this study, a combination of three methods viz. PCR, protein profiling, and insect bioassay was used to characterize the B. thuringiensis strain collection.

The aim of this study was to isolate potentially toxic B. thuringiensis strains from northwestern Himalayas, which represents one of the hotspots of biodiversity. The strains were characterized based on the presence of protein crystals, SDS-PAGE analysis, and PCR analysis to identify different cry and cyt gene combinations and toxicity screening against Helicoverpa armigera. The type of cry/cyt genes present in a particular strain defines their host range, so the strains harboring several types of cry/cyt can be predicted to have a wider spectrum or additive activity. Such studies are useful in understanding the distribution of cry and cyt genes and may lead to identification of broad-spectrum and effective isolates for the control of various insect pests.

### 2 | MATERIALS AND METHODS

#### 2.1 | Sample collection, isolation of B. thuringiensis, and crystal characterization

The 89 strains analyzed in this study were isolated from 207 soil samples collected from three zones consisting of 10 locations of northwestern Himalayas. Sites with no history of use of Bt or its products were selected (Table 1). Isolation of Bt was done by enrichment using acetate selection as described by Travers, Martin, and Reichelderfer (1987). Following acetate selection, isolates that tested positive for growth on Luria medium amended with penicillin at a concentration of 10 μg ml⁻¹ were examined for the presence of parasporal crystals (Ohba & Aizaw, 1986; Yang, Wu, Wang, & Xie, 2000). Crystals were

| TABLE 1 | Distribution of sample collection sites and recovery of Bacillus thuringiensis |
| --- | --- | --- | --- | --- |
| Geographical areas (northwestern Himalayas) | No. of samples examined | No. of colonies obtained | No. of Bacillus like isolates | Bt index* |
| Agricultural land | | | | |
| Lolab | 23 | 132 | 14 | 9 | 0.64 |
| Aru | 25 | 118 | 13 | 11 | 0.84 |
| Saripara | 15 | 126 | 12 | 8 | 0.66 |
| Urusa | 23 | 118 | 9 | 3 | 0.33 |
| Venkra | 17 | 115 | 8 | 4 | 0.50 |
| Forests | | | | |
| Brarin | 28 | 136 | 8 | 8 | 1.00 |
| Afferwat | 22 | 122 | 7 | 5 | 0.71 |
| Sonnarg | 27 | 114 | 4 | 3 | 0.75 |
| Zanskar | 22 | 128 | 6 | 3 | 0.50 |
| Lake sediments | | | | |
| Pangkong | 5 | 92 | 8 | 2 | 0.25 |
| Total | 207 | 1201 | 89 | 56 | 0.62 |

*Ratio between number of B. thuringiensis colonies and total number of sporulating colonies examined.
characterized using phase contrast microscopy and the shape of crystals was confirmed by scanning electron microscopy (Lone, Yadav, Malik, & Padaria, 2016). Bt index was calculated as described by Baig, Bukhari, and Shakoori (2010).

2.2 | Standard bacterial strains

Four standard B. thuringiensis (Bt) strains; Bt. subsp. kurstaki (BGSC 4D1), Bt. subsp. aizawai (BGSC 4J3), Bt. subsp. israelensis (BGSC 4Q1), and Bt. subsp. morrisoni biovar. tenebrionis (BGSC 4AA1) were kindly provided by Dr. Daniel R. Zeigler from the Bacillus Genetic Stock Center (Ohio, USA). These strains were used as reference wherever required.

2.3 | PCR analysis of crystalline protein genes

DNA template was prepared by lysis precipitation (Rampersad & Ammons, 2005). All primer pairs used for detection of cry and cyt gene families (cry1, cry2, cry3, cry4, cry7, cry8, cry11, cyt1, cry2) have already been documented (Juarez-Perez, Ferrandis, and Frutos, 1997; Ben-Dov et al., 1997; Ibarra et al., 2003). Table S1 shows sequences of all primers used in the study, their source, expected amplicon sizes, optimal annealing temperatures and respective positive control DNA used. For detection of crystalline protein gene combinations, all the native B. thuringiensis isolates were subjected to PCR analysis. PCR was performed in an Eppendorf Mastercycler and amplification conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at variable temperature depending on the primer pair (Table S1) for 60 s, and extension at 72°C for 60 to 90 s (depending on amplicon expected size), and a final extension step at 72°C for 10 min. Isolates showing the presence of lepidopteran-specific cry1 and cry2 genes were further screened using cry1 (cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D, cry1E, cry1F, cry1G) and cry2 (cry2Aa, cry2Ab, cry2Ac) subfamily primers, respectively.

2.4 | Protein profiling of B. thuringiensis isolates

The purpose of protein analysis was to select isolates exhibiting distinct protein profiles and to characterize each isolate of B. thuringiensis collection. All the native B. thuringiensis isolates and reference strain (Bt HD1) were grown in T3 broth for 72 hr at 30°C, the cultures were pelleted by centrifugation at 12,000g at 4°C for 5 min and the pellets were washed once with sterile distilled water followed by washing with 1 mML⁻¹ NaCl containing 5 mML⁻¹ EDTA. Protein composition of spore-crystal mixtures was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (Laemmli, 1970).

2.5 | Helicoverpa culture

Helicoverpa armigera larvae were initially maintained on natural diet consisting of chick pea leaves and pods up to second generation and the subsequent generations were reared on artificial diet consisting of gl⁻¹/mL⁻¹ pollen (15), soy flour (8), wheat germ (60), yeast extract (50), ascorbic acid (3), nipagin (3), sorbic acid (1), sunflower oil (5), and agar (12) in water. During oviposition, H. armigera adults were provided with 5% (w/v) honey solution. Larvae were maintained up to 10 generations before proceeding with bioassay experiments. Both rearing and bioassays were carried out at 25°C, 50 ± 10% relative humidity, with 14 hr photoperiod (Teakle & Jensen, 1985).

2.6 | Bioassay

Mass screening of B. thuringiensis collection for toxicity against H. armigera was performed using relatively higher concentration (1 mg mL⁻¹) of spore-crystal mixtures. All the B. thuringiensis strains were cultured in 50 ml CCY medium (Stewart, Johnstone, Hagelberg, & Ellar, 1981) for 72–96 hr at 30°C, optimal crystal formation was checked by phase contrast microscopy. The cells were harvested by centrifugation at 9,000g for 10 min, the pellet obtained was washed twice with cold 1mol/L NaCl and twice with cold autoclaved distilled water. The wet and dry weight of final pellet (spore-crystal, mixture) was determined and stored at −20°C until bioassay (Baig et al., 2010). The density of bacterial doses was optimized on cry1Ac harboring positive control strain of Bt HD-1. All steps were carried out at 4°C to limit proteolysis. Second instar larvae of H. armigera were used for bioassay by diet incorporation method (Song et al., 2003). The conditions for bioassay were as mentioned above, 30 larvae per treatment were used. In the control group, the culture was substituted with sterile distilled water. Mortality was recorded after 7 days, larvae were scored as dead if they failed to respond to gentle probing, Bt HD1 was used as positive control for bioassays. Isolates showing 96%–100% mortality in the preliminary bioassay screening were subjected to more precise bioassays using six concentrations (50 μg ml⁻¹ to 800 μg ml⁻¹) of spore-crystal mixtures. Each dose was thoroughly mixed in 3 ml of artificial diet prior to solidification and poured into wells. Twenty-four second instar larvae per treatment were used, and the experiment was set in triplicate, making total number of larvae tested per treatment to 72. The mortality observed was corrected to control mortality by Abbott’s formula (Abbott, 1925). The median lethal concentration 50 (LC₅₀) was determined by probit analysis using the software SPSS version 19.0. for windows. In order to rule out toxicity as a function of β-exotoxin production, the supernatant from the toxic isolates was collected and heated at 95°C for 20 min, and subsequently assessed for its toxicity against H. armigera (Lone et al., 2016).

3 | RESULTS

3.1 | Bacillus thuringiensis phenotypic characterization

Eighty-nine Bacillus strains were isolated by applying acetate-penicillin selection methodology to 207 soil samples collected from 10 different regions representing three different environments of northwestern Himalayas (Table 1). Fifty-six isolates were found to synthesize
crystal protein during sporulation by phase contrast microscopy, the parasporal crystals were differentiated from the endospores by appearing as dark particles in contrast to endospores which appeared bright. Parasporal crystals were classified into four morphological classes based on SEM observations (Fig. 1); bipyramidal, spherical, cuboidal, and irregular types. *B. thuringiensis* was successfully isolated from all types of samples, recovery was the highest from forest soils (0.76) followed by plain agricultural soils (0.62) and the least from lake sediments (0.25) (Table 1). The average Bt index of the study was 0.62 (Table 1), (Bt index: ratio between number of *B. thuringiensis* colonies and total number of sporulating colonies examined).

### 3.2 Molecular characterization

The collection of *B. thuringiensis* isolates was characterized by assessing the number and type of cry and or cyt genes (PCR analysis) and the number and size of Cry and/or Cyt protein bands (SDS-PAGE analysis).

#### 3.2.1 cry/cyt gene content

PCR screening using eight pairs of specific primers was performed to detect seven cry and two cyt gene families in our collection (Table S1). Primers for the detection of cry1, cry2, cry3, cry4, cry7, 8, cry11, cyt1, cyt2 showed successful amplification as indicated by their specific product sizes of 1500, 701, 589, 439, 420, 445, 477, and 355 bp, respectively, on agarose gel electrophoresis (data not shown). Thirty-nine isolates of the collection were found to be positive for the presence of cry/cyt genes tested, harboring either single or combination of these genes. Overall in our collection, isolates containing cry1 were the most abundant (57.1%) followed by cyt2 (46.42%), cry11 (37.5%), cry2 (28.57%), cry4 (21.42%), cyt1 (19.64%), cry3 (8.9%), cry7, 8 (7.14%), and 30.35% of the isolates did not react with any of the primers tested (Figure 2a). Four (10.24%) of the isolates carried single cry/cyt gene, whereas 7 (17.94%) isolates had combination of two, 9 (23.07%) isolates had combination of three, 13 (33.33%) isolates had combination of four and 6 (15.38%) isolates harbored combination of five cry/cyt genes (Figure 2b). The isolates harboring single gene showed equal dominance of cry1 and cyt2 (5.12% each), all the remaining genes were found in combination with other cry/cyt genes. Isolates harboring two genes showed two different combinations consisting of cry1 + cyt2 (15.38%) and cry3 + cry7, 8 (2.56%) (Figure 2b, Table 2). Isolates harboring three genes showed eight different combinations consisting of cry1 + cry2 + cry11 (2.56%), cry4 + cry11 + cyt2 (5.12%), cry1 + cry4 + cyt1 (2.56%), cry4 + cry11 + cyt1 (2.56%), cry2 + cry11 + cyt1 (2.56%), cry1 + cry3 + cry7, 8 (2.56%), cry1 + cry2 + cyt2 (2.56%), cry1 + cry2 + cry7, 8 (2.56%); within the group, cry1 is common in five of the eight combinations, while as, cry2 and cry11 is common in four combinations (Figure 2b, Table 2). Isolates harboring four genes showed seven different combinations consisting of cry1 + cry3 + cry7, 8 + cyt2 (2.56%), cry1 + cry11 + cyt1 + cyt2 (2.56%), cry1 + cry2 + cry3 + cry4 (2.56%), cry1 + cry4 + cry11 + cyt2 (10.25%), cry1 + cry2 + cry11 + cyt1 (7.69%), cry1 + cry2 + cyt1 + cyt2 (5.12%), cry1 + cry2 + cry3 + cyt2 (2.56%); within the group cry1 was found in all the combinations, cyt2 was found in five of the seven combinations and cry2, cry11 in four combinations (Fig. 2b, Table 2). Isolates harboring five genes showed four different combinations consisting of cry1 + cry2 + cry11 + cyt1 + cyt2 (7.69%), cry1 + cry2 + cry4 + cry11 + cyt2 (5.12%), cry1 + cry4 + cry11 + cyt1 + cyt2 (5.12%), cry1 + cry2 + cry4 + cry11 + cyt2 (5.12%); within the group cry1, cry11, and cyt2 were found in all the combinations, cry2 and cry4 in three of the four combinations (Fig. 2b; Table 2).

Isolates harboring cry1 and cry2 genes were further characterized by detection of the secondary rank of these genes using several sets of primers (Table S1). Both cry1 and cry2 harboring isolates showed the presence of different subtype genes. Two isolates showed the combination of three cry1, 10 isolates had combination of two cry1 and five isolates showed the presence of only one cry1-subtype gene. cry1Ac was the most abundant constituting 34.3%, followed
by cry1Aa (31.25%), cry1Ab (15.62%), cry1C (6.25%), cry1D (3.12%), and cry1E (3.12%) of the cry1-positive isolates (Figure 2c). Among the cry2-subtypes genes tested, cry2Aa was the most predominant, observed in 43.75%, followed by cry2Ab observed in 31.25% of the cry2-positive isolates (Figure 2c). Four cry1 subtype genes, cry1Ad, cry1B, cry1F, cry1G, and one cry2 subtype, cry2Ac were not amplified in any of the Bt isolates with the sets of primers used in this study (Figure 2c).

3.2.2 | Protein profiling

SDS-PAGE analysis revealed great diversity with respect to the number and size of protein bands observed (Figure 3), even strains from the same sample showed diverse protein profiles (Table 2). Several strains in the collection showed identical protein profiles, such strains were considered as duplicate and only one strain per profile and sample was selected. The protein profiles of the isolated strains were compared to that of B. thuringiensis 4D1, the protein profiles are summarized in Table 2. The most common protein profile exhibited several polypeptides ranging in molecular mass from 25 kDa to 130 kDa (Figure 3). Most of the strains (42.8%) showed a protein composition resembling that of Lepidoptera-toxic B. thuringiensis subsp. kurstaki consisting of two major protein bands of 130 kDa and 65 kDa, approximately (Figure 3 Lanes 6, 7, 11). A total of 28.5% of the isolates showed the presence of major polypeptide of 65–70 kDa resembling Diptera toxic B. thuringiensis subsp. israelensis (Figure 3 Lane 10), whereas 14.28% isolates represented a unique profile consisting of major polypeptides
b, Sc, Tc, Ic, Tc indicate bipyramidal, spherical, irregular, and tiny crystal shapes (too small to record their shape), respectively.

70 kDa (Figure 3) and 35–55 kDa. Two polypeptides between 25 and 35 kDa were consistently observed in all the B. thuringiensis isolates including the reference strain.

| Isolates | Crystal shape | cry gene profiles | Major protein bands (kDa) |
|----------|---------------|-------------------|---------------------------|
| JK3      | Bc, Sc, Ic, Tc | cry1C, cry3, cry7,8, cyt2 | 55, 65, 130 |
| JK7      | Bc, Sc, Ic, Tc | cry1Aa, cry1Ac, cry2Aa, cry2Ab cry11, cyt1, cyt2 | 55, 65, 70, 130 |
| JK9      | Bc, Sc, Tc    | cry1Aa, cry1Ab, cry2Aa, cry2Ab cry11, cyt1, cyt2 | 55, 65, 70, 130 |
| JK10     | Tc            | cyt2              | 25, 28, 35 |
| JK11     | Tc            | cyt2              | 25, 28, 35 |
| JK12     | Bc, Sc, Tc    | cry1Aa, cry1Ac, cry4, cry11, cyt2 | 55, 65, 130 |
| JK14     | Bc            | cry1              | 65, 130 |
| JK16     | Sc, Tc        | cry1, cry2, cry11, cyt1, cyt2 | 65, 70, 130 |
| JK18     | Sc, Ic, Tc    | cry1Ab, cry4, cry11, cyt1, cyt2 | 65, 130 |
| JK19     | Bc, Sc        | cry1Aa, cry1Ac, cry2Aa, cry11, | 65, 70, 130 |
| JK20     | Sc            | cry4, cry11, cyt2 | NA |
| JK21     | Sc            | cry1, cry4, cyt1 | 65, 130 |
| JK22     | Bc            | cry1Ac, cyt2     | 65, 130 |
| JK26     | Bc            | cry1Ab, cyt2     | 65, 130 |
| JK32     | Sc            | cry4, cry11, cyt1 | NA |
| JK33     | Bc, Sc        | cry1Aa, cry1Ac, cry1C, cry4, cry11, cyt2 | 65, 130 |
| JK34     | Bc            | cry1Ab, cry1E    | 65, 130 |
| JK36     | Sc            | cry2, cry11, cyt1 | 70, 65 |
| JK37     | Bc            | cry1Aa, cry1Ac, cry1D, cyt2 | 65, 130 |
| JK38     | Bc            | cry1, cyt2       | 65, 130 |
| JK40     | Bp, Tc        | cry1, cry11, cyt1, cyt2 | 65, 130 |
| JK48     | Bp, Sc, Ic    | cry1Aa, cry1Ac, cry2Ab, cry3, cry4 | 65, 70, 130 |
| JK53     | Sc, Tc        | cry1Ac, cry4, cry11, cyt2 | 65, 130 |
| JK55     | Bc, Sc        | cry1, cry2, cry11, cyt1 | 65, 70, 130 |
| JK56     | Bc            | cry1, cyt2       | 65, 130 |
| JK58     | Sc            | cry2Aa, cry2Ab, cry11, cyt1 | 65, 70 |
| JK60     | Ic, Tc        | cry3, cry7,8     | 70 |
| JK61     | Ic, Tc        | cry3, cry4, cry11, cyt2 | 70 |
| JK63     | Bc            | cry1, cyt2       | 65, 70 |
| JK64     | Sc            | cry1Ab, cry2, cry11, cyt2 | 65, 70, 130 |
| JK65     | Bc, Sc        | cry1Aa, cry1Ac, cry2, cry3, cyt2 | 65, 70, 130 |
| JK66     | Sc            | cry2Aa, cry11, cyt1 | 70 |
| JK67     | Sc            | cry1, cry4, cry11, cyt2 | 65, 130 |
| JK72     | Bc            | cry1Aa, cry1Ac, cry2Aa, cry11, cyt2 | 65, 70, 130 |
| JK73     | Bc, Ic        | cry1, cry3, cry7,8 | 65, 70, 130 |
| JK78     | Sc            | cry1, cry4, cry11, cyt2 | 65, 130 |
| JK79     | Sc            | cry1, cry2, cyt2 | 65, 70, 130 |
| JK82     | Sc            | cry4, cry11, cyt2 | NA |
| JK86     | Bc, Ic        | cry1, cry2Aa, cry2Ab, cry7,8 | 65, 70, 130 |

aBc, Sc, Ic, Tc indicate bipyramidal, spherical, irregular, and tiny crystal shapes (too small to record their shape), respectively.

bNA indicates that no major band was observed.

of 110 kDa and 30 kDa (Fig. 3 Lanes 2-4). The majority of the isolates showed the presence of a number of polypeptides of approximately 70 kDa (Figure 3) and 35–55 kDa. Two polypeptides between 25 and 35 kDa were consistently observed in all the B. thuringiensis isolates including the reference strain.

3.3 | Insect bioassay

Toxicity screening against H. armigera was conducted in parallel to the PCR analysis, therefore, all the 56 B. thuringiensis strains of our collection were subjected to bioassays. Concentrated spore-crystal
suspensions (1,000 μg ml⁻¹) were used for initial toxicity screening. Barring four isolates (JK12, JK22, JK48, JK72), which killed 96%–100% of Helicoverpa larvae within 7 days, preliminary screening revealed different levels of toxicity among the isolates with mortality ranging from 0% to 100% (Figure 4). These potentially toxic isolates were subjected to additional bioassays to determine the LC₅₀ concentrations, using B. thuringiensis kurstaki (HD1) as positive control. The LC₅₀ values of native isolates ranged from 184.62 to 259.93 μg ml⁻¹, whereas that of HD1 was 195.32 μg ml⁻¹. The negative control assays did not show any mortality. Although LC₅₀ of all the native isolates was comparable to that of HD1, JK12 was found to be the most potent, showing the least LC₅₀ (184.62 μg ml⁻¹) compared to all the native isolates and to the positive control (HD1) as well (Table 3). All four toxic isolates and the Bt HD1 strain showed a drastic (~100%) reduction in mortality following heat treatment, which indicates that the toxic component is heat-labile high molecular mass fraction and not the heat-resistant β-exotoxin.

### DISCUSSION

In the Bacillus cereus group, the presence of a parasporal inclusion body is the single most discriminatory criterion for the identification of isolates in the species B. thuringiensis (Soufiane, Baizet, & Cote, 2013). Based on the presence of parasporal crystals, 56 out of 89 Bacilli strains were classified as B. thuringiensis. The results revealed that B. thuringiensis was ubiquitously distributed in the soils of northwestern Himalayas tested, with no history of Bt application. However, the Bt index (the estimation of the success of Bt isolation)

### FIGURE 3
SDS-PAGE of spore-crystal mixture of B. thuringiensis strains showing diverse protein profiles. Lanes: M, prestained molecular mass marker (Thermo Scientific, USA); 1, 5, 8, no major proteins; 2, 3, 4, major protein of 110 kDa; 6, 7, 9, major proteins of 130 kDa and 65 kDa; 10, major protein of 70 kDa; 11, Bt kurstaki (4D1) showing major protein of 130 kDa

### FIGURE 4
Mortality of H. armigera larvae fed with diets containing highly concentrated spore-crystal mixtures (1,000 μg ml⁻¹) of different B. thuringiensis isolates

### TABLE 3
Toxicity of spore-crystal mixtures of Bt isolates toward H. armigera

| Strain tested | Slope ± SE | LC 50 (μg ml⁻¹) | 95% Fiducial limits (μg ml⁻¹) | Toxicity index* |
|---------------|------------|-----------------|-----------------------------|-----------------|
| JK12          | 1.72 ± 1.66| 184.62          | 151.99–220.65               | 1.05            |
| JK22          | 1.68 ± 0.54| 275.39          | 179.49–434.47               | 0.70            |
| JK48          | 1.62 ± 0.72| 256.29          | 158.02–419.69               | 0.76            |
| JK72          | 1.72 ± 0.92| 259.93          | 217.68–310.68               | 0.75            |
| HD1           | 1.33 ± 1.64| 195.32          | 158.80–236.49               | 1.00            |

*Toxicity index indicates the relative toxicity of native to reference strain. It is the ratio of LC₅₀ of HD1 to that of the tested strain; the larger the ratio, the higher the toxicity.
varied in samples collected from different regions. The average Bt index recorded in the study was 0.62, which means that 62% of the sporulating colonies examined showed the presence of crystals. The average is higher compared to some previous isolation programs (less than 0.1 to 0.52) (Bravo et al., 1998; Chak, Tsen, & Yamamoto, 1994; Vidal-Quist, Castañera, & González-Cabrera, 2009), lower compared to that observed (0.85) by Martin and Travers (1989) and similar to that observed (0.6 to 0.7) by Obeidat, Hassawi, and Ghabeish (2004). Lake sediments showed the least Bt index (0.25), whereas forest soils showed the highest Bt index (0.76) (Table 1). It is difficult to assess the likely reason for the differences in Bt index values, but the sampling size, difference in geomorphology of the region, complex interaction between the bacterium and its insect host could influence their numbers in a particular environment. The predominance of bipyramidal and round crystal shapes observed in this study corroborates with the previous reports (Jara, Maduell, & Orduz, 2006; Vidal-Quist et al., 2009).

PCR-based identification of cry genes was introduced by Carozzi, Kramer, Warren, Evola, and Koziel (1991) to predict insecticidal activity of B. thuringiensis strains and has become popular tool for the large-scale screening of B. thuringiensis collections for the presence of insecticidal genes. In this study, different cry and cyt gene profiles have been detected, the prevalence of cry1 was found to be the most abundant followed by cry2, cry11, cry2, cry4, cry1, cry3, and cry7, 8. The frequency of genes observed was in accordance with the frequency reported by Porcar and Juarez-Perez (2003); Bravo et al. (1998); Baig and Mehnaz (2010), all of whom reported the predominance of cry1 genes, and differed from that observed by Ben-Dov et al. (1997); Pinto and Fiuza (2003); Baig et al. (2010); and Mendoza, Portillo, Arias, Ribas, and Olmos (2012), who reported the predominance of cry9, cry2, cry4, and cry4 genes, respectively. These data indicate the difference in the diversity of different crystalline protein coding genes in B. thuringiensis of different geographic regions. Only four isolates showed the presence of single gene, two each harboring cry1 and cry2, all remaining positive strains showed presence of cry/ cyt genes in combinations (Figure 2b). The most frequent combinations observed consisted of lepidopteran-specific cry1 with dipteran-specific cry2, cry4, cry11, and cyt2 genes, less frequent combinations included lepidopteran-specific cry1 with dipteran-specific cry2, cry4, and coleopteran-specific cry3 genes (Figure 2b). Strains containing presence of intergroup crystalline protein-coding genes are ideal candidates for the development of broad-spectrum biocontrol agents. Other researchers have reported different combinations of cry genes in the same Bt strains, cry3 and cry7 or cry9 (aronson, 1993; Ben-Dov et al., 1997), cry1, cry3A, cry3B, and cry7A (Bravo et al., 1998). The presence of more than one cry and or cyt genes in these isolates suggests that they have high frequency of genetic information exchange. Among the genes identified in this study, cry1, cry2, encode lepidopteran-specific toxins; cry2, cry4, cry11, cry1, cry2, encode dipteran-specific toxins, and cry3, cry7, 8 encode coleopteran-specific toxins. As a result, 33, 26, and 6 strains of our collection are at least theoretically toxic to lepidopteran, dipteran, and coleopteran pest, respectively.

Among the cry1 gene subtypes tested in this study, cry1Ac was the most dominant, present in (34.37%) by cry1Ab (15.62%) of the cry1-harboring isolates. cry1Ad, cry1B, cry1F, cry1G genes were not observed in any of the cry1-harboring isolates and 46.87% of the cry1 gene-harboring isolates did not show amplification of any cry1 subtypes tested suggesting that these isolates may contain other cry1 subtype genes not tested in this study. Various reports have shown a high frequency of one or the other subtype of cry1 genes, like, Chak et al. (1994) reported abundance of cry1A genes, whereas Arrieta, Hernandez, and Espinoza (2004) reported abundance of cry1D, and Baig et al. (2010) reported high frequency of cry1K subtypes. The cry1 gene-harboring isolates showed the presence of different combinations of cry1 subtype genes with cry1Aa-cry1Ac combination constituting 28.15% of the cry1 gene-harboring isolates (Figure 2c). Two of the isolates JK33 and JK37 had unique combinations of cry1Aa, cry1Ac, cry1C, and cry1Aa, cry1Ac, cry1E, respectively (Table 2).

Among the cry2 subtypes tested, the combination of cry2Aa-cry2Ab was the most frequent followed by cry2Aa and cry2Ab alone, whereas cry2Ac was not observed in any of the cry2-positive isolates (Figure 2c), the similar pattern of distribution was observed in soils of Argentina (Sauka, Cozzi, & Benintende, 2005) and China (Liang et al., 2011). However, Ben-Dov et al. (1997) observed highest frequency of cry2Ab alone followed by cry2Aa-cry2Ab and cry2Ab-cry2Ac in Bt isolates in samples from Israel, Kazakhstan, and Uzbekistan. The prevalence of cry2Aa-cry2Ab gene combination, as observed in this study may be commonly found in nature but its biological significance is yet to be studied. Of the 17 isolates that did not react with any of the primers tested, many showed presence of crystals on microscopy. Although such isolates did not show appreciable toxicity against H. armigera, the presence of crystals in these isolates suggests that they may harbor insecticidal proteins specific to some other target insect. Previous studies have reported varying number of isolates producing no PCR product (ranging from 14% to 40%) from different geographical regions of the world (Bravo et al., 1998; Uribe, Martinez, & Ceron, 2003; Vilas-Bôas & Lemos, 2004). All these results indicate the presence of numerous isolates harboring novel and unidentified crystalline protein genes throughout the world. The results presented here demonstrate that PCR-based approach can be used for systematic, large-scale screening of B. thuringiensis isolates to identify known cry/ cyt genes and to characterize their toxicity.

SDS-PAGE profiles of the sporulated Bt strains revealed great diversity in our collection (Figure 3, Table 2), the diverse profiles observed are in accordance with the earlier reports (Chak et al., 1994; Jara et al., 2006) and lower compared to others (Iriarte, Porcar, Lecadet, & Caballero, 2000; Vidal-Quist et al., 2009). Correspondence of SDS-PAGE profiles to the cry/cyt gene profiles detected by PCR, however, has not been straightforward in all the strains. The most likely reasons for this difference are: the strains tested could produce endotoxins whose genes are not tested in this study, the protein bands observed could be either protoxins, activated toxins, or proteolysis intermediates, the same trend has been observed earlier (de Barros Moreira & Silva-Filha, 2007; Mohan & Gujar, 2003). The most
prominent molecular mass polypeptides (130 kDa, 65 kDa) observed correspond to the Cry1 and Cry2 proteins, respectively (Armengol, Escobar, Maldonado, & Orduz, 2007; Seifinejad, Salehi Jouzani, Hosseinizadeh, & Abdimashani, 2008). The lower molecular mass polypeptides (35–55 kDa) observed match with the Cry proteins or digestion products of many Cry proteins (Gough, Kemp, Akhurst, Pearson, & Kongsuwan, 2005).

Various bioassay methods like diet incorporation (Dulmage, Dulmage, Boening, Rehnborg, & Hansen, 1971), surface contamination (Ignoffo, 1965), and droplet feeding (Hughes & Wood, 1981) have been used to obtain lethal concentration (LC) estimates. The first two methods are suitable for post-first instar larvae as they are able to fully consume small blocks of food, whereas the third method is suitable for neonates as their transparent body makes it easy to track the colored feeding solution in their gut. In this study, diet incorporation method was used against second instar larvae of *H. armigera*, the earlier instar larvae were used because the older larvae tend to be more tolerant to *Bt*-based pesticides (Sanahuja, Banakar, Twyman, Capell, & Christou, 2011). The preliminary toxicity screening of *Bt* isolates using higher doses of spore-crystal suspensions (1,000 μg ml⁻¹) revealed that these strains exhibit a wide range of toxicity (causing 0%–100% mortality) toward second instar larvae of *H. armigera* (Figure 4). Twenty-seven percent of the isolates causing mortality of 60% or above were considered as toxic, among them, four isolates causing (96%–100%) mortality were considered to be highly toxic and were subjected to detailed toxicity analysis to determine their LC₅₀ values. The LC₅₀ values of native isolates observed were in the range of 184.62 to 259.93 μg ml⁻¹ in comparison to HD1 which showed LC₅₀ of 195.32 μg ml⁻¹ (Table 3).

As the strain JK12 was actually most toxic to *H. armigera* than any other strain tested in this study, there is a possibility of synergism between the two different lepidopteran-specific (cry1Aa and cry1Ac) Cry proteins observed in this strain by PCR analysis. It must, however, be noted that mere presence of more than one cry genes does not account for additive effect in toxicity, as all genes may not be expressed simultaneously or even if expressed, they may act antagonistically by competing for the same binding site in insect gut as reported earlier (Liao, Heckel, & Akhurst, 2002; Morse, Yamamoto, & Stroud, 2001). Previous studies have reported pronounced differences in the toxicity response of *H. armigera* to *B. thuringiensis* insecticidal proteins (Avilla, Vargas-Osuna, González-Cabrera, Ferré, & González-Zamora, 2005; Bird & Akhurst, 2007; Liao et al., 2002). These differences could be a result of variation in insecticidal genes present, differences in insect strain used and difference in the diet composition or bioassay method employed (Avilla et al., 2005; Bird & Akhurst, 2007). Therefore, comparison of the toxicity data in different studies must be based on the relative toxicity rather than the absolute values of toxicity estimates. Moreover, in order to have more realistic estimates of toxicity of a particular strain, many independent populations of susceptible insect should be tested, preferably with the same protocols.

*B. thuringiensis* presents great diversity with respect to their crystalline protein content and insecticidal activity even in the isolates from same soil sample. The diversity and activity might have relationship with the geographical location of the samples. An important characteristic observed in this study was the presence of more than one cry/cyt genes in many of the isolates, with differences, at least in theory, in terms of their host range. Moreover, the presence of cry/cyt genes from different groups were also observed in the same strain, suggesting that some of these strains (particularly *Bt* JK12) after proper evaluation against different insect species can be developed into broad-spectrum biopesticides. Seventeen of the *Bt* isolates in the collection did not interact with the eight pairs of primers tested, and were also not toxic to *H. armigera*. However, presence of crystals in these isolates suggests that they may harbor novel cry/cyt genes not tested in this study, hence, their toxicity toward other insect species needs to be evaluated. The results of this study confirm the significance of continuous exploration of new *Bt* strains from different ecological regions of the world.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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