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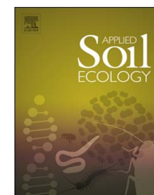

Diversity and functional annotation of chitinolytic *Bacillus* and associated chitinases from north western Indian Himalayas

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ABSTRACT

Chitinolytic *Bacillus* and associated chitinases have prospective implication in both industry and biological control of agricultural pests. A total of 83 chitinolytic *Bacillus* strains were isolated from native soils of Uttarakhand state, north western Indian Himalayas. A multiphasic characterization of the collection showed a vast diversity in bacterial kinetics (growth and enzyme production), pH stability in ten isolates, thermal-stability in 23 isolates and psychrotolerance in three isolates. The collection was characterized by chief existence of 148 kDa (65 isolates) and 152 kDa (7 isolates) chitinases, the high molecular weight enzymes. Partial sequencing of 16S rRNA and *chi* genes showed *Paenibacillus* species are the major chitinase producers of the region. In addition, *licheniformis* and *circulans* group chitinases were also found to be associated with the collection. The molecular variation in sequences revealed existence of different *Paenibacillus* species and especially a special evolutionary status of chitinases in phylogeny which is also evidenced by associated high molecular weight chitinases. The bioactivity of isolates against insect pests, *Pieris brassicae* and *Helicoverpa armigera* showed that the isolates were not lethal except at very high concentrations (10^8 cfu/ml) that to in only 9 isolates. However, they are primarily involved in growth reduction which led to identification of three prominent isolates (UKCH27, UKCH29 and UKCH77) that caused a significant larval weight reduction at lowest concentration tested (10^2 cfu/ml). Further testing of synergism between Cry toxins of *B. thuringiensis* strain HD 1 and these isolates showed near cent percent mortality of test insects at LC_{30} concentrations.

1. Introduction

The agricultural importance of chitin comes from the fact that it is the major structural component of cell walls of plant pathogenic fungi and the exoskeletons of arthropod pests, the two major limiting factors of crop production. The native rigidity of this polysaccharide offers structural integrity (Park et al., 1997) and protection against invading pathogenic microbes (Kramer and Koga, 1986; Cohen, 1987) being which can be an ideal target for pest management programs. Besides, chitin is the second most abundant polysaccharide in nature whose degradation is one of the key steps in carbon and nitrogen cycling (Gooday, 1990). Chitinases (EC 3.2.1.14) are fairly versatile group of hydrolytic enzymes that cleaves β 1–4 glycosidic bonds of N-acetyl glucosamine residues of chitin. Association of these enzymes is irrespective to the evolutionary hierarchy that span from prokaryotic organisms (bacteria, yeast etc) to eukaryotes (fungi, insects, crustaceans etc including higher plants). The key role of chitinases in both chitinogenic and non-chitinogenic organisms is either nutritive (Hamid et al., 2013 Langner and Göhre, 2016), protective (Kubicek and Druzhinina, 2007; González-Teuber et al., 2010; Grover, 2012; Sharma, 2013), or pathogenic (Sampson and Gooday, 1998; Busby et al., 2012; Frederiksen et al., 2013).

Based on amino acid sequence similarity in the catalytic domain, chitinases comes under glycoside hydrolase and are classified into family 18 and 19 (Henrissat and Bairoch, 1993). Family 18 chitinases are mainly from bacteria (Suzuki et al., 1999), whereas, majority of family 19 chitinases are from plants (Henrissat, 1999). In bacteria, the primary role of chitinases is thought to be catabolism and utilization of chitin as a carbon and energy source (Cohen-Kupiec and Chet, 1998). In bacteria, they are often associated with the outer membrane or secreted as extracellular enzymes (Terahara et al., 2009). The vast diversity of bacteria has made family 18 chitinases the most frequently detected in different environments (Ramaiah et al., 2000; Cottrell et al., 2000; Metcalfe et al., 2002; Terahara et al., 2009; Yasir et al., 2009). Several

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chitinolytic strains of Actinobacteria and Streptomyces are thought to have direct toxicity to plant pathogenic fungi (Kawase et al., 2006) and nematodes (Woo-Jin et al., 2002; Abbasi et al., 2014) by degrading the chitinous cell wall. In addition, synergistic potential of bacterial chitinases with insect pathogens against a variety of phytophagous insect pests is also well documented (Sampson and Gooday, 1998; Ding et al., 2008; Singh et al., 2014)

The members of genus, *Bacillus* are well-known producers of extracellular enzymes, including cellulase, glucanase, amylase, proteinase, chitinase and other important secondary metabolites. Different species and strains of this bacterial genus were extremely utilized whose implications ranges from plant growth promotion to protection against biotic stresses (Santoyo et al., 2012; Bouizgarne, 2013). The possible applications, huge diversity and ubiquitous nature made the bacterial genus one of the most explored and commercially utilized (Bravo et al., 2011; Santoyo et al., 2012). To date, only a few studies reported chitinase diversity within terrestrial ecosystems such as upland pastures (Metcalfe et al., 2002), maize fields (Ikeda et al., 2007), lake sediments of Antarctica (Xiao et al., 2005) apart from vermicompost and (Yasir et al., 2009) aquatic ecosystems (Cottrell et al., 2000; Ramaiah et al., 2000). Although, these studies revealed high diversity of whole bacterial chitinases and associated microbiota, mostly by culture independent methods, identification of major chitinase producers in a given ecosystem is lacking. Present study reports the major chitinase producers and associated diversity of enzymes in Uttarakhand Himalayan region by culture dependent methods. This is also a first report on extensive isolation and identification of high chitinase producing *Bacillus* and related species from the region.

2. Materials and methods

2.1. Sample collection and handling

A total of 987 soil samples from different ecological niches of Uttarakhand state, north western Indian Himalayas (30°′N and 79°30′ E) were collected from all the 13 districts covering the whole state. The collection sites distributed widely between 1250 and 2600 m amsl and were irrespective to standing crop covering agricultural fields, grazing lands, forest areas *etc* and also barren lands. At each collection site, soil sample of approximately 10 g was collected from a depth of 10–15 cm using a sterile spatula in to individual self sealing plastic bags and stored under laboratory conditions, until analysed.

2.2. Preparation of colloidal chitin

Commercial chitin flakes (Himedia) was made into colloidal chitin according to Berger and Reynolds (1988). Chitin flakes (10 g) were powdered in a mortar and pestle and mixed with 400 mL of concentrated hydrochloric acid under continuous stirring for 4 h at 4 °C. The resulting solution was incubated at 37 °C for 2 h and was passed through four layer muslin cloth to eliminate the impurities. To the filtrate, four liters of ice cold distilled water was slowly added and allowed to stand overnight at 4 °C for precipitation of colloidal chitin. The precipitate was collected by centrifugation and was washed thoroughly with distilled water until neutral pH was achieved. The weighed final precipitate was made in to 20% stock, autoclaved and stored at 4 °C, until used.

2.3. Isolation of chitinolytic bacillus

The collected soil samples were recovered from plastic bags and suspensions were made by mixing 1 g of sample (Fresh weight) in 10 mL of sterile distilled water. An aliquot of 100 μL was subjected to heat shock at 80 °C for 5 min and was evenly spread on to chitinase detection (CHD) agar plates (Kamil et al., 2007) and incubated at 30 °C. After 48 h, well separated colonies with clear halo zones around the bacterial growth were picked and spot inoculated on to separate CHD agar plates. Plating and colony selection was done at appropriate dilutions (10^{-3} to 10^{-7}) in samples with more number of colonies or with overlapping colonies and halos. The selected bacterial colonies were measured for the diameter of the halo after 7 days of incubation at 30 °C and colonies with halo radius greater than or equal to 1 cm were selected for further study. A total of 83 bacterial colonies were selected and further tested for their enzyme characteristics, biocontrol potential and diversity analysis. All the isolates were maintained at 4 °C in nutrient agar slants for customary use and stored as 25% glycerol stocks at −20 °C.

2.4. Isolation of partially purified chitinases

The extracellular chitinases from each isolate were obtained by culturing them in half strength nutrient broth infused with 1% colloidal chitin. Each isolate was inoculated to the autoclaved medium and incubated at 30 °C at 250 rpm. After 3 days, the fermented broth supernatants were obtained by centrifugation at 10,000 rpm for 20 min. Four volumes of ice cold acetone was added to the supernatants and allowed to stand overnight at −20 °C. The precipitated proteins were collected by centrifugation at 10,000 rpm for 10 min and dissolved in appropriate quantity of 15 mM Tris-HCl buffer (pH 6.8) after 80% ethanol wash. The protein content of sample was measured by standard Bradford dye binding method and designated as partially purified chitinases (PPC).

2.5. Chitinase activity assay

The enzyme activity of chitinases was estimated by using natural substrate, colloidal chitin at pH 5 and 7 using 50 mM acetate and phosphate buffers, respectively. The reaction mixture consists of equal volumes of appropriately diluted enzyme and buffer containing 1% colloidal chitin. The mixture was incubated at 50 °C for 30 min followed by terminating the reaction by boiling in a water bath for 10 min. The remaining colloidal chitin was precipitated by centrifugation at 10,000 rpm for 5 min and supernatant was estimated for released reducing sugars by modified Schales reagent (Imoto and Yagishita, 1971). The absorbance was measured at 420 nm and the reducing sugar was estimated from a standard curve of *N*-Acetylglucosamine (NAG). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute.

2.6. Screening for thermal stability and psychrotolerance

Partially purified chitinases from each isolates was incubated in a microcentrifuge tube at 50 °C for 1 h in a thermostatic chamber. The residual enzyme activity was estimated as described above and expressed as percent activity to the initial enzyme activity. All the estimates were done in autonomous triplicates and corrected with blank values of supernatant.

2.7. Kinetics of bacteria

The bacterial growth of each isolate (obtained as above) was observed under fluorescent microscope in 100 x magnification to identify the vegetative cells and spores. The bacterial growth was measured indirectly by estimating OD_{660} using spectrophotometer (Biorad). Protein content of supernatant was also measured by standard Bradford dye binding method following estimating the optical density at 595 nm.

2.8. Identification of chitinase active proteins

The supernatant proteins from each isolate were separated under native conditions using polyacrylamide gels to retain enzyme activity and further identification. Ten micrograms of protein was subjected to

electrophoresis using 10% separating gel and 5% stacking gel in a Bio-Rad mini-protein II cell assembly at 4 °C. The protein samples were mixed with sample buffer devoid of mercaptoethanol and loaded on to gels. Electrophoresis was performed in duplicate at a constant voltage of 100 V. After protein separation, the gel was cut to separate the duplicates and one replica was used for protein bands visualization by Coomassie brilliant blue R-250 dye binding method. The other replica gel was washed thoroughly with 50 mM acetate buffer (pH 5) and laid on to a substrate gel made of 2% agarose and 0.1% colloidal chitin. The assembly was incubated in a thermostatic chamber at 50 °C for 4 h to allow enzymatic activity. At the end of incubation, the agarose gel was recovered and stained for 15 min in chitin binding fluorescent dye solution (0.01% Calcofluor white M2R in 50 mM Tris HCl pH 8) followed by destaining in distilled water for 2 h. The lytic zones were visualized on UV illuminator and compared with Coomassie stained replica for identification of protein band.

2.9. 16S rRNA and chitinase gene libraries

Genomic DNA of the each isolate was isolated from the overnight grown cultures by CTAB method of cell disruption followed by PCI purification. The purified DNA was used as template for PCR amplification of 16S rRNA and chitinase gene fragments using *Bacillus* specific and family 18 chitinase specific primers, respectively (Table 1). The PCR reaction mix (50 μL) contained 100 ng of total DNA, 0.5 μM primers, 1.5 mM $MgCl_2$, 200 nM dNTPs, and 2.5 U of *Taq* DNA polymerase. The reactions were performed in a Bio-Rad thermal cycler with an initial 5 min denaturation step at 94 °C followed by 30 cycles of amplification consisting of a 1 min denaturation at 94 °C, 45 s of annealing (Table 1), 2 min of extension at 72 °C, with an extra extension step of 10 min at 72 °C. The amplifications were confirmed by investigating 10 μL of PCR product by electrophoresis in a 1% agarose gel.

The obtained PCR products of 16S rRNA and chitinase gene fragments were purified using gel elution columns (Sigma) and sequenced from Scigenome labs, Kochi, India. The obtained nucleotide sequences were aligned with the Clustal Omega (1.2.1) multiple sequence alignment (McWilliam et al., 2013) and molecular evolutionary analyses were performed using the software MEGA4 (Molecular Evolutionary Genetic Analysis version 4) (Tamura et al., 2007). A phylogenetic tree was constructed along with other sequences of different *Bacillus* strains by the neighbor-joining method using the distance matrix from alignment. The obtained sequences were submitted to National Center for Biotechnology Information (NCBI) Gene Bank nucleotide sequence databases and the accession numbers were presented in Tables 3 and 4.

2.10. Screening for psychrotolerance

The low temperature tolerance and chitinase production of the *Bacillus* isolates was tested by growing them in agar medium containing nutrient broth at half strength and 1% colloidal chitin at 5 °C. Each isolate was spot inoculated in triplicate per plate and allowed to grow at the desired temperature for 7 days. At the end of the incubation period, halo diameter around the bacterial colony was measured as an estimate of psychrotolerance.

2.11. Insect bioassay

The bioefficacy of chitinolytic *Bacillus* isolates were estimated in a two tier evaluation where 3rd instar *Plutella xylostella* was used to establish the bioactivity followed by estimation of growth reduction and synergism of potent isolates against 2nd instar larvae of *H. armigera*. The bacterial cultures were obtained by growing them in half strength nutrient broth supplemented with 1% colloidal chitin for 4 days (at 250 rpm and 30 °C) followed by adjusting the cell strength to 10^8 cfu/ml using sterile 0.1% Triton X-100. Leaf spread bioassay (Lacey, 2012) was carried out to study the toxicity and growth reduction of chitinolytic *Bacillus* against *P. xylostella.* Fully opened matured cabbage leaves were cut into 5 cm diameter leaf discs and spread with 300 μL of test concentration (10^8 cfu/ml) using camel hair brush. After air drying, ten freshly molted second instar larvae were allowed to feed on treated leaves. Larval mortality and weight of larvae was recorded after 48 and 96 h after treatment.

Chickpea based artificial diet (Singh and Rembold, 1992) was used for bioassays against *H. armigera*. The molten diet mix was poured in to 24 well tissue culture plates and after solidification, 200 μL of bacteria preparation was evenly spread on to the surface. After air drying, freshly molted second instar larvae was carefully transferred in to each well and allowed to feed. A total of 30 larvae were tested in each experiment at 30 ± 2 °C, 60% humidity and 14L:10D photoperiod. As preliminary assays revealed no substantial mortality at low concentrations, weight of the larvae was estimated to study the percent growth reduction at concentrations, 10^6, 10^4 and 10^2 cfu/ml. The synergistic potential was estimated from the bioassays where LC_{30} concentration (estimated from bioasssays at six different concentrations as described above) of spore crystal mixture of *B. thuringiensis* strain HD-1 was mixed with two different concentrations (10^2 and 10^3 cfu/ml) of identified chitinolytic *Bacillus* species.

3. Results

3.1. Isolation and multiphase characterization of chitinolytic bacillus

The environmental samples collected in the study comprises of soil samples from different ecosystems including agricultural fields, horticultural lands, forest areas, barren lands. Intensive screening of these collected 987 soil samples yielded a total of 83 isolates producing halo size of greater than 1 cm around the bacterial colony after 7 days of incubation. All the isolates were designated as UKCH prefixed to a number. In addition to these selected isolates, each soil sample contained an average of 4–10 chitinolytic heat resistant bacteria colonies with varying levels of chitinase production as evidenced by the diameter of halo around the bacteria colony (Annexure 1). The selected 83 isolates were initially studied for cell strength, supernatant proteins content, enzyme activity, thermal stability and cold tolerance (Annexure 2). An indirect estimate of bacterial growth by OD660 showed high bacteria count in UKCH17 (0.842) followed by UKCH55 (0.190). The protein content of culture supernatants was at its peak in UKCH73 and UKCH80 with 0.94 and 0.9 mg/ml, respectively. Most of the isolates showed less enzyme activity at pH 7 compared to pH 5. Peak enzyme activity of 217 U/ml was observed in UKCH23 and UKCH62 at pH 5. However, at pH 7 highest activity was observed in

Table 1
Details of primers used in the study.

Gene	Primer	Sequence ('5–3′)	Amplicon size	Annealing temperature	Reference
16S rRNA	16S rRNA(F)	'5-CAGGCCTAACACATGCAAGTC-3′	1.3 kb	45 °C	Yoon et al., 2001
	16S rRNA (R)	'5-GGGCGGTGTGTACAAGGC-3′			
Chitinase	GA1F	5′-CGTCGACATCGACTGGGARTDBCC-3′	440 bp	62 °C	Williamson et al., 2000
	GA1R	5′-ACGCCGGTCCAGCCNCKNCCRTA-3′			

UKCH38 (247 U/ml). About 10 isolates viz., UKCH19, UKCH20, UKCH31, UKCH32, UKCH34, UKCH39, UKCH51, UKCH73, UKCH74 and UKCH78 showed comparable activity at both the pH (Annexure 2).

3.2. Thermal stability and psychrotolerance of chitinases

The thermal stability study of enzymes after a temperature stress at 50 °C for 1 h showed 23 isolates (UKCH6, UKCH11, UKCH14, UKCH15, UKCH16, UKCH22, UKCH23, UKCH27, UKCH28, UKCH33, UKCH34, UKCH51, UKCH52, UKCH53, UKCH55, UKCH59, UKCH60, UKCH70, UKCH72, UKCH75, UKCH78, UKCH82 and UKCH83) were thermally stable by retaining their cent percent activity. However, psychrotolerance was observed in nominal isolates despite of existing annual low temperatures. Significant low-temperature tolerance was observed in UKCH6, UKCH32 and UKCH47, which was manifested by 4 mm halo after 7 days of incubation at 5 °C (Annexure 2).

3.3. Identification of chitinase active proteins

Chitinase active protein bands were identified by electrophoresis of culture supernatant proteins followed by outgel enzyme active assay (Annexure 3). The analysis showed that the collection was characterized by high molecular weight chitinases. Out of 83, a total of 78 isolates showed more than 100 kDa chitinase active proteins among which chief occurrence of 148 kDa was observed in 64 isolates. The second most abundant chitinases were 152 kDa in 7 isolates. The remaining 12 isolates showed 10 different chitinase active proteins among which five isolates showed low molecular weight chitinases viz., 83, 70, 66 and 45 kDa (Table 2).

3.4. Diversity of chitinase genes

A total of 15 isolates were studied for chitinase gene characteristics by PCR amplification followed by direct sequencing of amplicon. Sequence homology study using entire GenBank database by BLASTN search revealed 10 isolates were 78 to 80% identical to chiA gene from *Paenibacillus* sp. FPU7 (Accession no. AB683959). Two isolates (UKCH17 and UKCH77) showed 99% identity to a variety of *B. licheniformis* chitinases and with variable identity (88 to 95%) to other *Bacillus* species (*B. paralicheniformis, B. circulans, B. pumilus, B. subtilis* etc) chitinases. Interestingly, three isolates (UKCH19, UKCH20 and UKCH44) showed 89 to 90% similarity with *B. circulans* chitinase (Table 3). Multiple sequence alignment of obtained nucleotide sequences showed 40 and 16 single nucleotide polymorphisms (SNPs) in *paenibacillus* (UKCH19, UKCH20 and UKCH44; Annexure 4) and *circulans* (Annexure 5) grouped chitinases, respectively. The *licheniformis* sequences (UKCH17 and UKCH77) were identical (Annexure 6). Multiple sequence alignment of deduced primary structure (approximately 120 amino acids) showed 8 conserved, 19 semiconserved, 22 non conserved amino acids substitutions (Fig. 1) between all 15 sequences covering the three groups. In particular, the *circulans* group reported only one semi-conserved amino acid substitution (A to S) despite of observed 16 SNPs, whereas, the *Paenibacillus* group reported 2 conserved (especially in UKCH68), 8 semi conserved and one non conserved substitution.

Table 3
Details of chitinase gene fragments sequenced from chitinolytic *Bacillus* of Uttarakhand Himalayas.

S. No.	Isolate	NCBI accession number	BLAST similarity		Group
			Identity (%)	Accession No	
1.	UKCH5	KX235872	79	AB683959	*Paenibacillus*
2.	UKCH17	KX235873	99	CP014781	*Licheniformis*
3.	UKCH19	KX446921	90	M57601	*Circulans*
4.	UKCH20	KX446922	89	M57601	*Circulans*
5.	UKCH21	KX446923	79	AB683959	*Paenibacillus*
6.	UKCH39	KX446924	79	AB683959	*Paenibacillus*
7.	UKCH42	KX446925	79	AB683959	*Paenibacillus*
8.	UKCH44	KX446926	90	M57601	*Circulans*
9.	UKCH47	KX446927	80	AB683959	*Paenibacillus*
10.	UKCH56	KX446928	80	AB683959	*Paenibacillus*
11.	UKCH59	KX446929	79	AB683959	*Paenibacillus*
12.	UKCH60	KX446930	79	AB683959	*Paenibacillus*
13.	UKCH66	KX446931	78	AB683959	*Paenibacillus*
14.	UKCH68	KX446932	80	AB683959	*Paenibacillus*
15.	UKCH77	KX446933	99	CP014781	*Licheniformis*

The molecular variations clearly differentiated the three groups (*Paenibacillus*, *circulans* and *licheniformis*) in bootstrap consensus clustering (phylogeny) of the sequences along with selected sequences from NCBI database (Fig. 2). Clear clustering of annotated groups with high bootstrap values also confirms phylogenetic discrimination between isolates. Interestingly, *Paenibacillus* group showed separate and distinct clustering among the isolates indicating a unique and diverse status of these chitinases amongst other published *Paenibacillus* spp. (data not shown).

3.5. 16S rRNA gene analysis

16S rRNA sequences were used to analyze the phylogenetic position and molecular identification of selected strains. Approximately, 850 bp

Table 2
Summary on molecular weights of chitinase active proteins of native chitinolytic *Bacillus* from Uttarakhand Himalayas.

S. No.	Chitinase active band (~kDa)	Number of isolates	Isolates
1	152	7	UKCH18, UKCH36, UKCH42, UKCH43, UKCH59, UKCH71 and UKCH80
2	148	64	UKCH1, UKCH2, UKCH3, UKCH4, UKCH5, UKCH6, UKCH10, UKCH7, UKCH8, UKCH9 UKCH11, UKCH12, UKCH13, UKCH14, UKCH15, UKCH16, UKCH19, UKCH21, UKCH23, UKCH26, UKCH27, UKCH28, UKCH29, UKCH30, UKCH32, UKCH33, UKCH34, UKCH35, UKCH37, UKCH38, UKCH39, UKCH40, UKCH41, UKCH46, UKCH47, UKCH48, UKCH49, UKCH50, UKCH51, UKCH52, UKCH53, UKCH54, UKCH55, UKCH57, UKCH58, UKCH60, UKCH61, UKCH62, UKCH63, UKCH64, UKCH65, UKCH67, UKCH68, UKCH69, UKCH70, UKCH72, UKCH73, UKCH74, UKCH75, UKCH76, UKCH78, UKCH81, UKCH82 and UKCH83
3	136	1	UKCH44
4	130	1	UKCH31
5	129	2	UKCH24 and UKCH25
6	128	1	UKCH66
7	122	1	UKCH22
8	117	1	UKCH45
9	83	1	UKCH56
10	70	2	UKCH17 and UKCH77
11	66	1	UKCH20
12	45	1	UKCH79

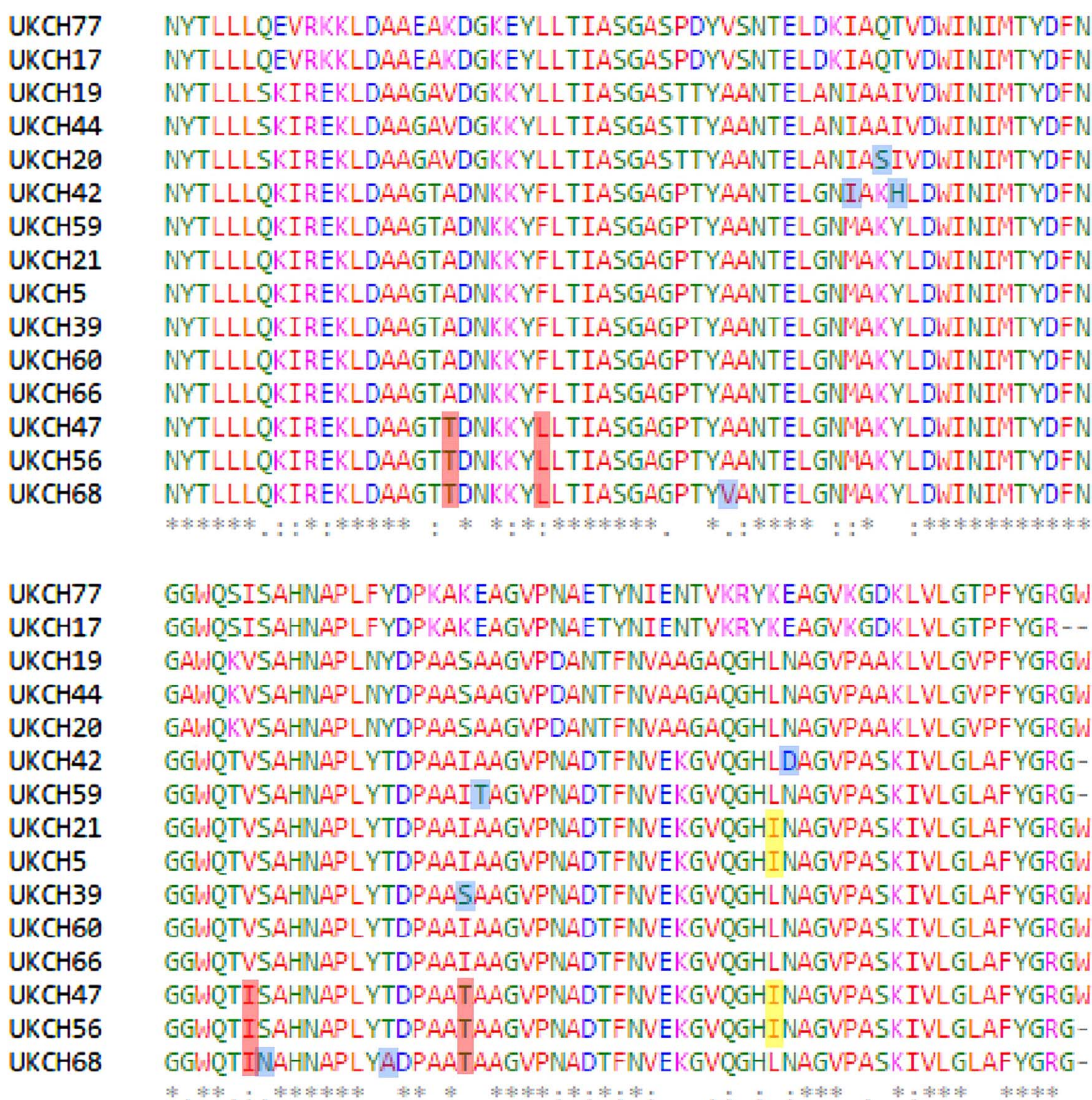

Fig. 1. Multiple sequence alignment of deduced amino acid sequences of partial *chi* gene from chitinolytic *Bacillus* of Uttarakhand Himalayas.
UKCH77 and UKCH17 were *licheniformis* group; UKCH19, UKCH20, UKCH44 were circulans group; remaining sequences were *Paenibacillus* group. The amino acid substitutions in each group were highlighted.
*,: and. indicates conserved, semi conserved and unconserved amino acid residues, respectively.

region of targeted gene was sequenced and the similarity search using BLASTN of GenBank revealed high similarity of isolates with 16S rRNA sequences of various *Bacillus* and *Paenibacillus* species (Table 4). A 16S rRNA sequence based phylogenetic tree clearly differentiated both the *Bacillus* and *Paenibacillus* species in to two major clusters (Fig. 3). In addition, grouping amongst the observed clusters clearly inferred phylogenetic proximity and species identity. Out of the tested isolates, UKCH17 and UKCH77 were found to be *B. licheniformis* strain and the remaining were found to be the species of *Paenibacillus*. The highest boot strap values also support this species identity.

3.6. Bioactivity of chitinolytic Bacillus against P. xylostella

A preliminary bioassay screening of all the isolates against *P. xylostella* (Table 5 and Annexure 7) led to identification of eight bioactive isolates based on mortality and growth reduction of the test insect. Further bioefficacy testing of these identified isolates against *H. armigera* revealed poor mortality (data not shown) except at higher concentrations (Table 6). Despite of this poor mortality, significant growth reduction was observed when evaluated at three different concentrations. Among the eight studied isolates, only three isolates (UKCH27, UKCH29 and UKCH77) showed a sizeable reduction in weight of larvae even at the lowest concentration (10^2 cfu/ml) tested (Table 6). However, the effect was not much dependent on dosage although a nominal variation was observed.

The three potent isolates were selected to test the synergistic potential with Cry toxins of *B. thuringiensis* strain HD-1. An initial bioefficacy of HD1 Cry toxins against *H. armigera* and *P. brassicae* revealed LC_{30} of 0.15 and 0.6 μg/ml, respectively (Table 7a). These LC_{30} values were tested in combination with the selected isolates at two different concentrations. The results showed a soaring increase in mortality of both the test insects. Especially, *P. xylostella* was found to be highly susceptible to the mixtures of chitinolytic bacteria and Cry toxis with cent percent mortality even at the lowest concentration combinations. *Helicoverpa armigera* was also found to be highly susceptible to the mixture as indicated by elevated mortality range of 85–97% (Table 7b). Besides nominal differences in mortality, the selected three isolates were found to be potent in increasing the larval mortality of test insect pests.

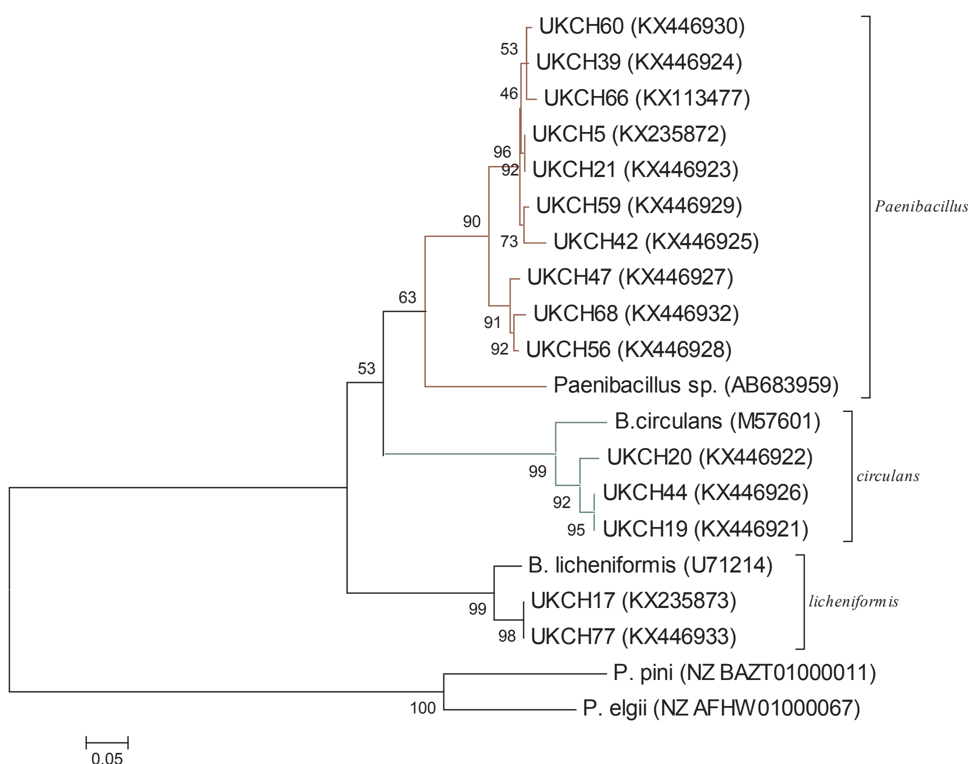

Fig. 2. Phylogenetic tree showing clustering of bacterial chitinases constructed by the neighbor-joining method. The scale represents 0.1 substitutions per site. The tree was constructed with known chitinase sequences taken from GenBank. The accession numbers were given in parenthesis. Numbering at each branch indicates bootstrap supports.

Table 4
Details of 16S rRNA gene sequences from chitinolytic *Bacillus* of Uttarakhand Himalayas.

S. No.	Isolate	NCBI accession number	Species identified
1.	UKCH5	KX113470	*Paenbacillus chitinolyticus*
2.	UKCH9	KX113471	*P. chitinolyticus*
3.	UKCH15	KX113472	*P. amylolyticus*
4.	UKCH17	KX113464	*Bacillus licheniformis*
5.	UKCH20	KX113473	*P. amylolyticus*
6.	UKCH21	KX113474	*P. Illinois*
7.	UKCH23	KX113468	*P. lautus*
8.	UKCH25	KX113475	*P. lautus*
9.	UKCH26	KX113469	*P. gansuens*
10.	UKCH44	KX113476	*P. amylolyticus*
11.	UKCH66	KX113477	*P. chitinolyticus*
12.	UKCH77	KX113463	*B. licheniformis*
13.	UKCH79	KX113478	*P. chitinolyitcus*
14.	UKCH83	KX113479	*P. elgii*

4. Discussion

The vast range of geo-environmental conditions and undisturbed soils of Indian Himalayas offer an enormous diversity of micro-biota and has been little explored in the past (Pandey et al., 2006). Soil is a dynamic natural resource of chitin degrading bacteria whose exploration and identification broadens the activity spectrum of bacterial biocontrol agents (Lorito et al., 1994 Pardo-lópez et al., 2009). Bacterial chitinases are the predominant degrading factors of Chitin (LeCleir et al., 2004). *Bacillus* sps are well known chitinase producers with high levels of chitinolytic activity (Cody et al., 1990). The present study reports isolation of 83 chitinolytic *Bacillus* which indicates their natural occurrence in soils of Uttarakhand hills. Besides, an average count of 4–10 chitinolytic bacteria in every sample with variable levels of enzyme production also signifies high prevalence of chitin in Uttarakhand soils.

The enzyme activity estimation at pH 5 and 7 showed most isolates have high enzyme activity at either of them or any other pH specific to bacterial species or enzyme. So, the apparent activity of given bacterial chitinase is a function of exiting pH and estimation of optimum pH is a prerequisite for maximized expression of enzyme activity. However, comparable activity observed in 10 isolates also indicates some chitinases have the ability to resist pH fluctuations (Bansode and Bajekal, 2006). Additionally, a comparable high activity at pH7 in most of the isolates in a sample of 83 isolates also establishes that the bacterial chitinases shows optimal activity at neutral pH (Wang and Chang, 1997). However, it should also be noted that naturally occurring family 18 chitinases display different pH optima (Synstad et al., 2004).

The geographic distribution of psychrotolerance and thermostability was found to depend significantly on the prevailing annual average temperature which forms temperature-dependent selection regimes. Predominance of thermostability (23 out of 83 isolates) up to 50 °C, a prominent temperature reported by most of the studies (Karthik et al., 2015), may be a function of high seasonal fluctuations in temperature in Uttarakhand hills. Studies also reported specific featured chitinases with respect to pH (Loni et al., 2014; Fu et al., 2016) and thermal stability (Takayanagi et al., 1991; Toharisman et al., 2005; Liu et al., 2015; Ueda and Kurosawa, 2015) having potential in industrial applications.

In particular, the bacterial chitinases were mostly around 60,000 to 110,000 Da (Annamalai et al., 2010). However, preponderance of high molecular weight chitinases in the collection (152 and 148 kDa) was found to be a unique characteristic feature. The novelty was also clearly evident in the chitinase sequence based phylogenetic tree, which showed a special grouping. Besides, high occurrence of *Paenibacillus* chitinases (10 out of 15) and sequence variation also reveals existence of different species in collection which was also confirmed in 16S rRNA phylogeny (12 out of 14 isolates). Chitinase diversity reports from different ecosystems (Metcalfe et al., 2002; Hobel et al., 2005; Das et al., 2010; Nampally et al., 2015) and other bacteria diversity studies from soils (Someya et al., 2011; Cihan et al., 2012; Yadav et al., 2015) also reported occurrence of these species. The study also suggests that the paenibacillus group was the major chitinolytic bacteria community in the soils of Uttarakhand Himalayas. Another characteristic features associated with *Paenibacillus* chitinases of the collection was high molecular weight chitinases. Itoh et al. (2013, 2014) also reported high

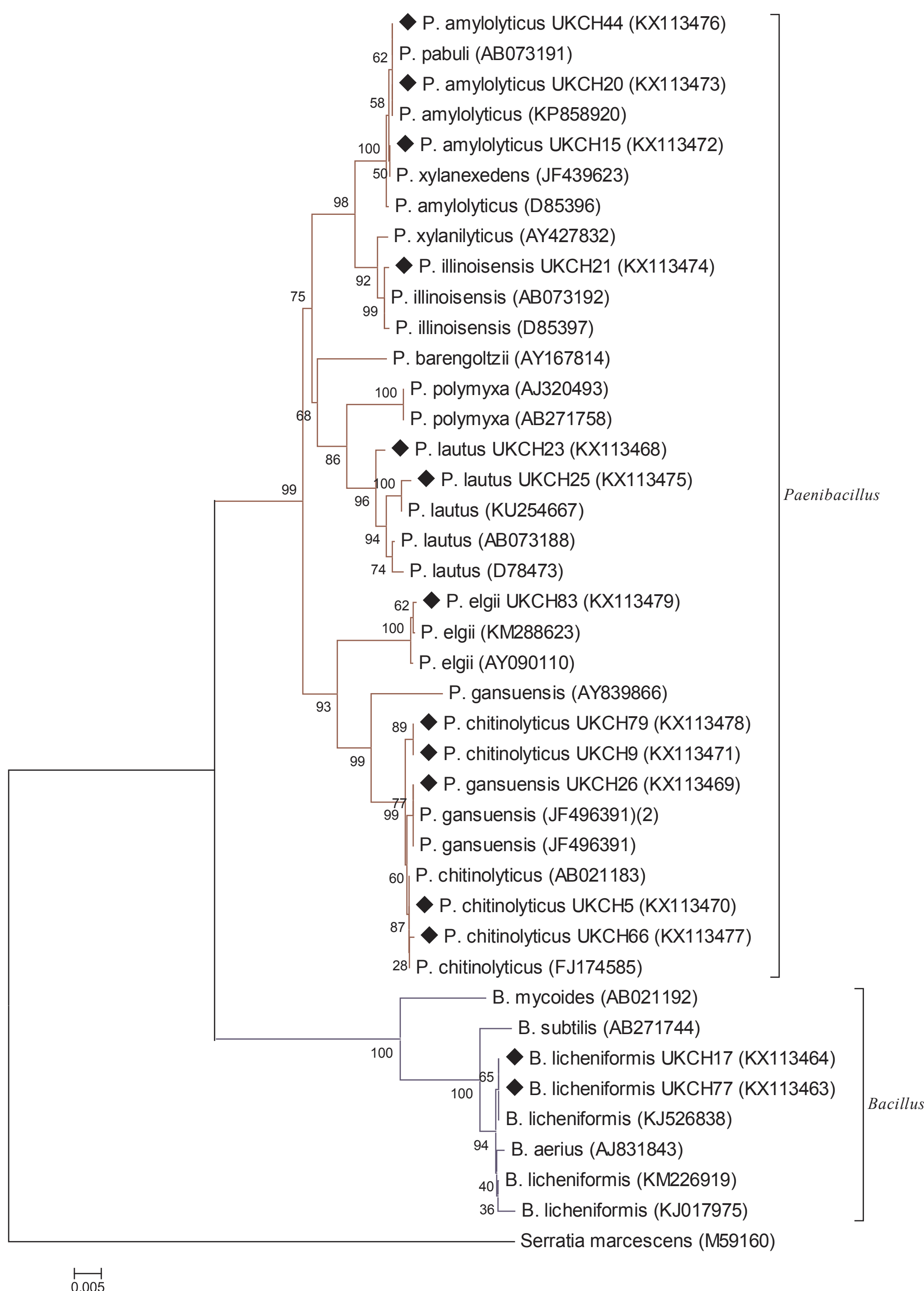

Fig. 3. Phylogenetic tree showing clustering of bacterial 16S rRNA gene sequences constructed by the neighbor-joining method. The scale represents 0.1 substitutions per site. The tree was constructed with known chitinase sequences retrieved from GenBank. The accession numbers are given in parenthesis. Numbers at each node indicate percentage of confidence levels generated from 1000 bootstrap trees.

molecular weight chitinases in different *Paenibacillus* species but with isozymes. In contrast, majority of studies reported occurrence of low molecular weight chitinases as well as multiple forms and or isozymes in *Paenibacillus* sp. For example, *P. illinoisensis* KJA-424 (three chitinase isozymes viz., 63, 54, and 38 kDa, Jung et al., 2003), *Paenibacillus* sp. D1 (56 kDa, Singh and Chhatpar, 2011), *P. pasadenensis* (35 kDa, Loni et al., 2014), *P. barengoltzii* (70 kDa, Yang et al., 2016; 67 kDa, Fu et al., 2016), *P. thermoaerophilus* strain TC22-2b (48 kDa, Ueda and Kurosawa, 2015) etc.

The *licheniformis* group isolates (UKCH17 and UKCH77) showed 70 kDa chitinases which is in agreement with previous reports on *B. licheniformis* strains (Roberts and Selitrennikoff, 1988; Nguyen et al., 2012; Laribi-Habchi et al., 2015) with a variation of 2–5 kDa among them. This minor difference may be explained by association of signal peptides as observed in sister species, *B. thuringiensis* (Barboza-Corona et al., 2003), which needs to be studied. Amongst, the *circulans* group isolates, UKCH44 showed existence of 66 kDa which was in close agreement with presence of 66 kDa (Siwayaprahm et al., 2006) and 45 kDa (Wiwat et al., 1999) and 52 kDa (Watanabe et al., 1992) in different strains of *B. circulans*. However, 16S rRNA gene sequence identified the isolate as *P. amylolyticus*. Similarly, UKCH20 was also identified as *P. amylolyticus* but with high molecular weight chitinases (136 kDa). These signify high genomic DNA exchange and or inter species existence of high molecular weight chitinases.

The *in vitro* bioassay of chitinolytic *Bacillus* species of Uttarakhand Himalayas revealed only a nominal isolates are primarily toxic. This lack of toxicity can be explained by primarily *exo*- instead of *endo*-cleaving enzymes, the former being substantially less effective than the

Table 5
Bioefficacy of chitinolytic *Bacillus* species from Uttarakhand Himalayas against *P. xylostellaa.*

Mortality		
Percent	Isolates	No.
< 50	UKCH1, UKCH2, UKCH4, UKCH5, UKCH6, UKCH7, UKCH8, UKCH10, UKCH12, UKCH13, UKCH15, UKCH17, UKCH18, UKCH19, UKCH20, UKCH21, UKCH22, UKCH23, UKCH24, UKCH25, UKCH26, UKCH28, UKCH30, UKCH31, UKCH32, UKCH33, UKCH34, UKCH35, UKCH36, UKCH37, UKCH38, UKCH39, UKCH40, UKCH41, UKCH42, UKCH43, UKCH44, UKCH45, UKCH46, UKCH47, UKCH48, UKCH49, UKCH50, UKCH51, UKCH52, UKCH53, UKCH54, UKCH55, UKCH56, UKCH57, UKCH59, UKCH60, UKCH61, UKCH62, UKCH63, UKCH64, UKCH65, UKCH66, UKCH67, UKCH68, UKCH69, UKCH70, UKCH71, UKCH73, UKCH74, UKCH75, UKCH76, UKCH78, UKCH79, UKCH81, UKCH82, UKCH9	72
50–80	**UKCH3,** UKCH14, UKCH16, **UKCH58,** UKCH72, **UKCH83**	6
> 80	**UKCH11, UKCH27, UKCH29, UKCH77, UKCH80**	5
Growth reduction		
Percent	**Isolates**	**No.**
< 50	UKCH1, UKCH2, UKCH4, UKCH5, UKCH6, UKCH7, UKCH8, UKCH10, UKCH12, UKCH15, UKCH17, UKCH18, UKCH19, UKCH20, UKCH21, UKCH22, UKCH23, UKCH25, UKCH26, UKCH28, UKCH31, UKCH32, UKCH33, UKCH34, UKCH35, UKCH36, UKCH37, UKCH38, UKCH39, UKCH40, UKCH41, UKCH42, UKCH43, UKCH44, UKCH45, UKCH46, UKCH47, UKCH48, UKCH49, UKCH50, UKCH51, UKCH52, UKCH53, UKCH54, UKCH55, UKCH56, UKCH57, UKCH59, UKCH61, UKCH62, UKCH63, UKCH65, UKCH66, UKCH68, UKCH69, UKCH70, UKCH71, UKCH72, UKCH73, UKCH74, UKCH75, UKCH76, UKCH78, UKCH79, UKCH82, UKCH9	66
50–80	UKCH13, UKCH14, UKCH16, UKCH24, UKCH30, UKCH60, UKCH64, UKCH67, **UKCH80,** UKCH81	10
> 80	**UKCH11, UKCH27, UKCH29, UKCH3, UKCH58, UKCH77, UKCH83**	7

Table 6
Mortality and growth reduction of *H. armigera* by selected chitinolytic *Bacillus* of Uttarakhand Himalayas.

Isolate	Mortality (%)	Percent growth reduction at different dilution (cfu/ml)		
	10^8	10^6	10^4	10^2
UKCH3	71.3	82.5^{bc}	62.5^{c}	34.6^{bc}
UKCH11	82.3	72.3^{d}	58.6^{cd}	33.1^{c}
UKCH27	**92.5**	$\mathbf{84.6^{b}}$	$\mathbf{69.2^{a}}$	$\mathbf{40.7^{a}}$
UKCH29	**91.2**	$\mathbf{90.2^{a}}$	$\mathbf{68.5^{ab}}$	$\mathbf{41.3^{a}}$
UKCH58	61.3	78.7^{cd}	52.6^{d}	22.4^{d}
UKCH77	**88.2**	$\mathbf{82.9^{bc}}$	$\mathbf{52.8^{d}}$	$\mathbf{38.7^{ab}}$
UKCH80	75.5	63.3^{e}	42.8^{e}	19.6^{d}
UKCH83	45.3	72.6^{d}	62.8^{bc}	30.9^{c}
CD (P = 0.05)	ND	4.53	3.53	2.82
CV	ND	4.18	4.07	4.7
SEd	ND	2.14	1.66	1.33

Means followed by a common letter(s) are not significantly different by LSD ($P = 0.05$) with "a" representing a better group.

latter in degrading chitin (Kramer and Muthukrishanan, 1997). Additionally studies also reported change in feeding profiles of insect pests to counteract the toxic effects by way of reduced food intake (Alchanatis et al., 2000) which ultimately results in reduced damage to crop plants. Moreover, a prominent growth reduction observed in test insects either increases larval period or weakens the pest that ultimately result in increased accessibility to natural enemies. With an exception of UKCH77, the identified nine bioactive isolates were found to have high molecular weight chitinases (148 and 152 kDa) which represents *Paenibacillus* species. The growth reduction observed may be a result of direct degradation of chitin rich midgut peritrophic membrane leading to cessation of feeding (Bahar et al., 2012). Furthermore, irregularities in protective layer increase accessibility of pathogenic microorganisms to midgut receptors, a key mechanism of synergistic interaction between Cry toxins and chitinases (Regev et al., 1996; Wiwat et al., 1996; Sampson and Gooday, 1998). The high degree of synergistic interaction (an increase in mortality from 30% to 100%) observed between HD-1 Cry toxins and chitinolytic isolates in the present study also supports this hypothesis. Thus, chitinases are the most important enzymes and unique biological control material by means of these versatile effects.

In conclusion, present study established a collection of chitinolytic *Bacillus* isolates native to north western Indian Himalayas with vast diversity of associated chitinases. The study was earliest of its type in the region and recognized unique status of chitinases with an evolutionary class of high molecular weight enzymes. It also proposes *Paenibacillus* species were the major chitinase producers and chitin degrading factors in the hill soils. Besides, possible growth inhibition and synergistic interaction reported also supports their possible utilization in resistance management programs and biotechnological applications. Moreover, chitinases and associated bacteria were also found to be the major biocontrol agents of plant pathogenic fungi and nematodes. Keeping this in view, further studies are being planned to study the pathogenicity of established collection against these promising constraint of agriculture.

Table 7
Synergism of potent native chitinolytic *Bacillus* isolates with Cry toxins of *B. thuringiensis* strain HD-1.

a Bioefficacy of *B. thuringiensis* strain HD-1.

Pest	Lethal concentrations (μg/ml)			FL limits for LC_{30}	Equation	X^{2*}
	LC_{30}	LC_{50}	LC_{90}			
H. armigera	0.15	0.27	3.21	0.02 − 0.230	Y = 4.304 + 0.72×	0.285
P. xylostella	0.6	1.4	4.23	0.031 − 0.812	Y = 4.23 + 1.14×	0.952

b Synergistic potential of potent chitinolytic *Bacillus* with *B. thuringiensis* strain HD-1.

Combination	Mortality (%)					
	H. armigera			*P. xylostella*		
	UKCH27	UKCH29	UKCH77	UKCH27	UKCH29	UKCH77
$LC_{30} + 10^2$	85.4	88.8	87.6	100	100	100
$LC_{30} + 10^3$	92	97	95	100	100	100

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.05.024.

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