



## **Induction and Expression of Chitinases from Four Sub Species of *Bacillus thuringiensis***

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### **Author's contribution**

*The sole author designed, analyzed and interpreted and prepared the manuscript.*

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### **ABSTRACT**

Chitin is a naturally occurring linear polymer of *N*-acetylglucosamine and the major structural component of fungal cell walls and exoskeletons of insects and arthropods. Chitinases are the enzymes that breakdown chitin to economically important derivatives and found in a range of organisms including bacteria, insects, crustaceans, invertebrates, some vertebrates and higher plants. In the present study, four *Bacillus thuringiensis* (*Bt*) isolates were grown in media supplemented with 0.1% (w/v) regenerated chitin and chitinase inducing medium and screened for chitinolytic activity. All isolates showed notable extracellular chitinase activity with very low levels in cell bound fractions. When the isolates were screened with fluorogenic substrates, *Bt* HD133, *Bt* T7002 and *Bt* IPS78 showed a similar pattern of distribution with the highest production from *Bt* IPS 78. The lowest activity was detected from the isolate *Bt* 36-3. Similar to the results obtained with fluorogenic substrates, *Bt* 36-3 and *Bt* IPS 78 expressed the lowest and the highest activities with the substrate Carboxymethyl-Chitin Remazol Brilliant Violet 5R (CM-chitin RBV 5R) respectively. When the culture supernatants of isolates grown in nutrient broth supplemented with regenerated chitin were electrophoresed on activity gels, one chitinase band of approximate molecular mass of 36 kDa was obtained from all four sub species. Future experiments can be carried out to find out whether these chitinases have any insecticidal activity aiming, to develop environmental-friendly biopesticides, and *in vivo* antifungal activities to test against plant pathogenic fungi in growth chambers and under glasshouse condition.

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## 1. INTRODUCTION

Chitin is a long unbranched polymer of an amino sugar *N*-acetyl-  $\beta$  -D-glucosamine residues (GlcNAc) linked together by  $\beta$ -1,4-glycosidic linkages [1]. This naturally occurring polymer is the second most frequent biopolymer in the environment after cellulose and present in insects, crustaceans and most fungi, but not in plants, vertebrates and prokaryotes. Chitin is hydrolyzed by two types of enzymes, namely chitinase (E.C. 3.2.1.14) and *N*-acetyl glucosaminidase (E.C. 3.2.1.52).

According to the nomenclature suggested by [2], the chitinolytic enzymes are divided into three principal types: endochitinase, exochitinase and *N*-acetylglucosaminidase. Endochitinases and exochitinases are the two major categories of chitinases. Chitinases catalyse the conversion of chitin to its monomeric components. They are produced by a large variety of chitin-degrading organisms including several bacteria [3,4,5] actinomycetes [6,7], fungi [8] and also by higher plants [9,10,11,12].

*Bacillus thuringiensis* (*Bt*) is an insecticidal, Gram-positive rod-shaped, motile, spore forming bacterium widely used as a biocontrol agent against pests. *Bt* produces parasporal crystalline inclusion bodies containing highly specific insecticidal proteins called Cry proteins or  $\delta$ -endotoxins. These toxins are mainly active against lepidopteran species and some also shows toxicity against dipteran and coleopteran species and other organisms [13]. Under suitable conditions, *Bt* is capable of producing many biological active molecules, for examples bacteriocins and hydrolytic enzymes such as chitinases. Chitinases of *B. thuringiensis* are worth of consideration due to their potential role as biological control agents for insects and plant-pathogenic fungi [14,15].

In this present study, chitinases produced by four subsp. of *Bacillus thuringiensis* were assayed using 4-methylumbelliferyl fluorogenic substrates and a dye substrate and their antifungal activity was investigated against *Botrytis cinerea*, the causative organism of grey mold disease.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Isolates and Culture Conditions

*Bacillus thuringiensis* subsp. *israelensis* IPS 78 and subsp. *aizawai* HD 133 were kindly provided by the University of Cambridge, United Kingdom. *B. thuringiensis* subsp. *aizawai* T7002 and subsp. *israelensis* 36-3 (isolated in Brazil) were obtained from the Heriot-Watt University, Edinburgh, Scotland. All bacteria were grown in liquid media; nutrient broth (NB) without regenerated chitin, nutrient broth supplemented with 0.1% (w/v) regenerated chitin and Chitinase inducing medium (CIM) [16].

### 2.2 Preparation of Bacterial Cultures for Chitinases Assay

Cultures were incubated with shaking (Varishaker-Incubator, Dynatech) for 96 hours. Following incubation, cultures were centrifuged at 11,600 g for 15 min. The supernatant and the pellet of each culture were collected separately to individual sterile containers and stored at – 20°C until required.

### 2.3 Synthesis of Glycol Chitin

Glycol chitosan (Sigma, UK) was prepared according to the method of [17], an adaptation of the method by [18]. The final concentration of the stock suspension of glycol chitin was made as a 1% (w/v) and stored at 4°C until use.

### 2.4 Preparation of Regenerated Chitin

Regenerated chitin was prepared according to the method by [18] using chitosan (Sigma, UK). The concentration of prepared chitin was determined by lyophilisation and weighing of a small sample. The suspension was stored at 4°C until use.

### 2.5 Assay for Chitinases with 4-methylumbelliferyl Fluorogenic Substrates

The fluorometric assay developed by [19] was used. In this assay, four 4-methylumbelliferyl fluorogenic substrates 4- MU-(GlcNAc)<sub>1-4</sub> (S1-S4) were used. Working solutions of monomer

[4-MU-(GlcNAc)<sub>1</sub>], dimer [4-MU-(GlcNAc)<sub>2</sub>] and trimer [4-MU-(GlcNAc)<sub>3</sub>] (Sigma Chemical Co.) were prepared at a concentration of 0.8 mM in sterile distilled water. The tetramer [4-MU-(GlcNAc)<sub>4</sub>] (Janssen Biochemica) was prepared at the same concentration in 50% (v/v) ethanol. All substrate solutions were kept at -20°C. The use of these substrates allows discrimination between  $\beta$ -N-acetylglucosaminidase [hydrolysing 4-MU-GlcNAc<sub>1</sub>], exochitinase [hydrolysing 4-MU-(GlcNAc)<sub>2</sub>] and endochitinase [hydrolysing 4-MU-(GlcNAc)<sub>3</sub> and 4-MU-(GlcNAc)<sub>4</sub>] [19].

The reaction mixture in each well contained 20  $\mu$ l enzyme preparation, 80  $\mu$ l McIlvaine's buffer (0.1 M citric acid and 0.2 M dibasic sodium phosphate, pH = 6) and 5  $\mu$ l of each 0.8 mM 4-MU substrate. First, the enzyme extract was incubated with the buffer at 37°C for 5 min in a shaking incubator (Varishaker-Incubator, Dynatech). Then the substrates were added to the wells and the plates were incubated at 37°C for 60 min. After incubation, the plates were read with the Fluoroskan II fluorometer (Titertek Instruments). Then 120  $\mu$ l of stop buffer (1 M NaOH/glycine pH 10.6) was added to each well and the plates were read again to get the end-point fluorescence after 5 min incubation with stop buffer at 37°C. Control wells contained the reaction mixture without the substrates and the reaction mixture with boiled enzyme extract. The control wells without substrate contained 5  $\mu$ l more buffer to keep the final volume constant. In calculating the activity, the mean fluorescence of control wells was subtracted from the test samples. All assays were performed in triplicate. Enzyme activity was expressed as katal. One katal is the amount of enzyme catalysing the conversion of one mole of substrate to product, in one second.

## 2.6 Assay for Chitinases with Carboxymethyl-chitin Remazol Brilliant Violet 5R

Assay for Chitinases with Carboxymethyl-Chitin Remazol Brilliant Violet 5R (CM-chitin-RBV 5R) was carried out as described by [20]. Reaction mixtures contained the enzyme preparation, 0.1M sodium citrate buffer (pH = 5) and CM-chitin-RBV 5R solution. The enzyme activity was expressed as the change in absorbance at 550 nm h<sup>-1</sup>. The assays were carried out in triplicates for each sample and the activity was expressed as a mean of these three replicates.

## 2.7 Chitinase Isozyme Patterns in SDS-PAGE Activity Gels

SDS-PAGE was carried out as described by [17] using glycol chitin impregnated separating gels, with the buffer system of [21]. For activity gels, 12% separating gels containing 0.1% (w/v) glycol chitin and 4% stacking gels were used. Lytic zones in gels, where chitin had been digested, were detected by inspection of gels in a UV illuminator, where they appeared as dark bands against the fluorescent background of intact glycol chitin [17].

## 2.8 Screening of *B. thuringiensis* for Antagonism *in vitro* against *Botrytis cinerea*

All four subsp. of *B. thuringiensis* were screened against *B. cinerea* using a dual culture technique [22]. Further, supernatant fractions of bacterial cultures used for chitinase assays were also screened. Central wells (10 mm diameter cork borer) were cut in PDA plates seeded with conidia of *B. cinerea*. The wells were filled to capacity (200  $\mu$ l) with filter-sterilised (0.2  $\mu$ m Acrodisc) culture supernatants of *Bt* isolates grown in nutrient broth supplemented with regenerated chitin. Plates were incubated at 21°C and observed for zones of inhibition. Proteins in wells were concentrated by adding up to 200  $\mu$ l of appropriate culture supernatant daily at the first 3 days of incubation.

## 3. RESULTS AND DISCUSSION

Various species of *Bacillus* have been shown to secrete chitinases, including *Bacillus amyloliquefaciens* [23], *Bacillus cereus* [24], *Bacillus circulans* [25], *Bacillus licheniformis* [26], *Bacillus megaterium* [23], *Bacillus subtilis* [27], *Bacillus thuringiensis* sub sp. *aizawai* [15] and *B. thuringiensis* sub sp. *kurstaki* [14]. Production of chitinases by different strains of *B. thuringiensis* and the involvement of these chitinases during pathogenesis of insects has been demonstrated [16].

Most of the microbial chitinolytic systems are inducible [28], but the production of chitinolytic enzymes in bacteria has been reported to be both constitutive [29] and inducible [30,31]. Hence in this study, *Bt* isolates were grown in appropriate liquid media with and without the supplement of regenerated chitin in order to assay both constitutive and inducible chitinase activities. All four sub species of *B. thuringiensis*

screened in this study produced chitinases when grown in liquid media containing chitin. But [32] have purified a 36 kDa chitinase by ion exchange and gel filtration chromatography from the culture supernatant of *Bacillus thuringiensis* HD-1 and this chitinase production was independent of the presence of chitin in the growth medium and was produced even in the presence of glucose.

The fluorometric assay was used as the principal assay to determine the chitinolytic activities. Although this assay is highly sensitive and reliable, it is expensive. Four of the 4-methylumbelliferyl fluorogenic substrates detect different enzymes in the chitinolytic system (*N*-acetylglucosaminidase, exochitinase and endochitinase). Therefore the results can be used to get an idea about the overall chitinase production of each isolate as well as the level of production of individual enzyme in the chitinolytic system. Both extracellular (culture supernatant) and cell-bound (cellular) activities were detected, but the latter with very low levels. According to the results obtained, [30] suggested that the occurrence of chitinase activity in cellular fraction of highly chitinolytic bacteria could actually be due to an intracellular enzyme, but could also result from an incomplete release of the enzyme to the growth medium.

Negligible levels of chitinolytic activities were detected in both assays, upon growth of *B. thuringiensis* in nutrient broth in the absence of chitin (results not shown). This indicated that chitinases were induced in the presence of

regenerated chitin in the growth medium. Significant levels of chitinolytic activities were detected in the supernatant fractions of all four *B. thuringiensis* sub species when grown in NB supplemented with 0.1% (w/v) regenerated chitin and chitinases inducing medium (CIM) (Fig. 1) in contrast to the very low activities of cellular (cell-bound) fractions (Table 1). Except for *Bt* 36-3, other three isolates showed higher chitinase activities with 4-methylumbelliferyl fluorogenic substrates when grown in NB supplemented with regenerated chitin. This result indicates that all isolates of *B. thuringiensis* showed notable extracellular exochitinase [hydrolysing 4-MU-(GlcNAc)<sub>2</sub>] and endochitinase [hydrolysing 4-MU-(GlcNAc)<sub>3</sub> and 4-MU-(GlcNAc)<sub>4</sub>] activities.

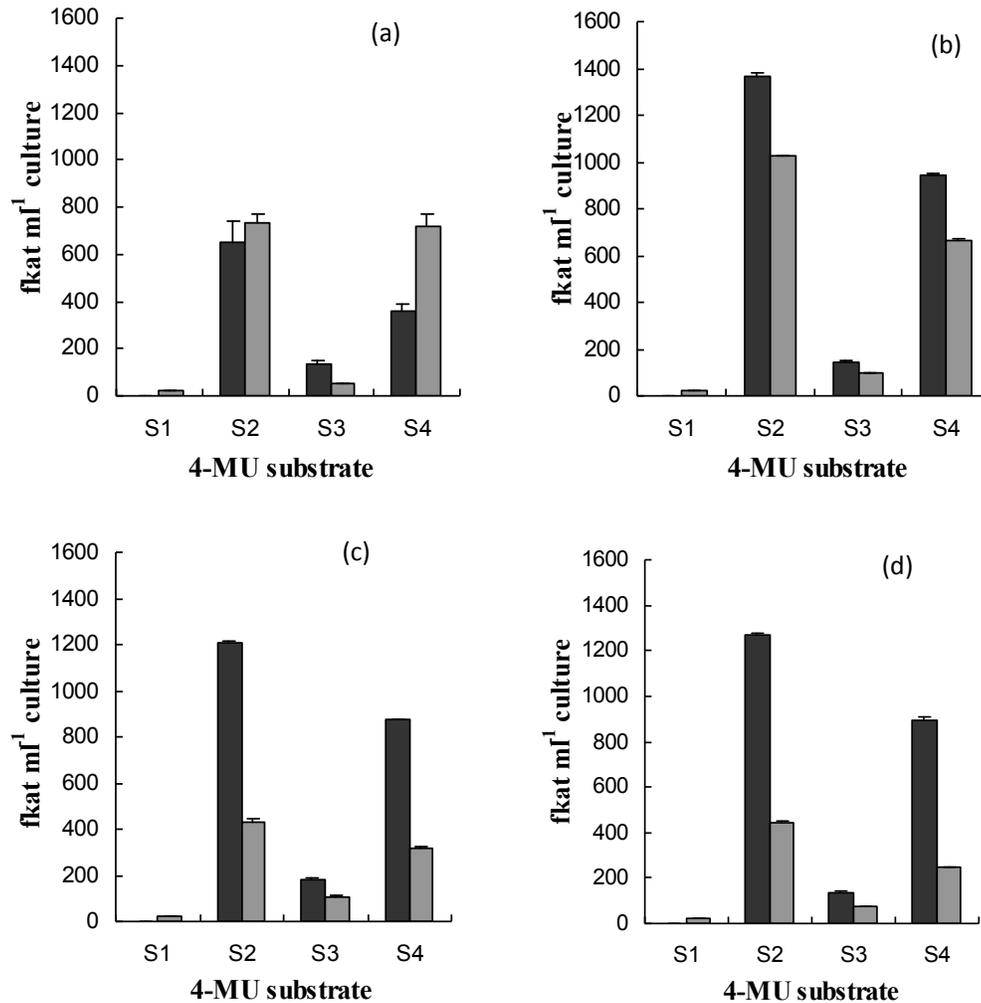
There were no detectable *N*-acetylglucosaminidase activities in cell-bound or supernatant fractions when the cells were grown in NB medium either with or without regenerated chitin. In contrast, very low levels of *N*-acetylglucosaminidase activities were detected only in culture supernatants of chitinase inducing medium (Fig. 1).

When four subsp. of *B. thuringiensis* were screened for chitinolytic enzymes with fluorogenic substrates, *Bt* HD133, *Bt* T7002 and *Bt* IPS78 showed a similar pattern of distribution (Fig. 1) with the highest production from *Bt* IPS78. For all isolates, the highest was the exochitinase activity with 4-MU-(GlcNAc)<sub>2</sub> and the lowest was the *N*-acetylglucosaminidase activity with 4-MU-(GlcNAc)<sub>1</sub>. When [16]

**Table 1. Chitinase activities (fkat ml<sup>-1</sup> culture) of culture supernatant (S) and cell-bound (C) fractions of four subsp. of *B. thuringiensis* against 4-MU substrates. Mean activities of triplicate wells ± SE are given**

Sub species	S/C	NB + regenerated chitin				CIM	
		4-MU-(GlcNAc) <sub>2</sub>	4-MU-(GlcNAc) <sub>3</sub>	4-MU-(GlcNAc) <sub>4</sub>	4-MU-(GlcNAc) <sub>2</sub>	4-MU-(GlcNAc) <sub>3</sub>	4-MU-(GlcNAc) <sub>4</sub>
<i>Bt</i> 36-3	S	650 ± 90	133 ± 14	358 ± 28	733 ± 38	50 ± 0	716 ± 52
	C	102 ± 4	28 ± 0	71 ± 4	118 ± 5	19 ± 1	69 ± 1
<i>Bt</i> IPS78	S	1366 ± 16	142 ± 8	942 ± 8	1025 ± 8	100 ± 8	667 ± 8
	C	126 ± 10	26 ± 0	78 ± 3	123 ± 4	25 ± 1	97 ± 7
<i>Bt</i> HD133	S	1208 ± 8	183 ± 8	875 ± 0	433 ± 8	108 ± 0	317 ± 0
	C	144 ± 6	29 ± 3	81 ± 1	110 ± 7	17 ± 1	35 ± 3
<i>Bt</i> T7002	S	1267 ± 8	133 ± 8	892 ± 12	442 ± 0	75 ± 0	250 ± 8
	C	132 ± 4	32 ± 2	82 ± 2	102 ± 1	15 ± 1	33 ± 2

Key: S: Supernatant, C: Cell-bound, NB: Nutrient broth, CIM: Chitinase inducing medium



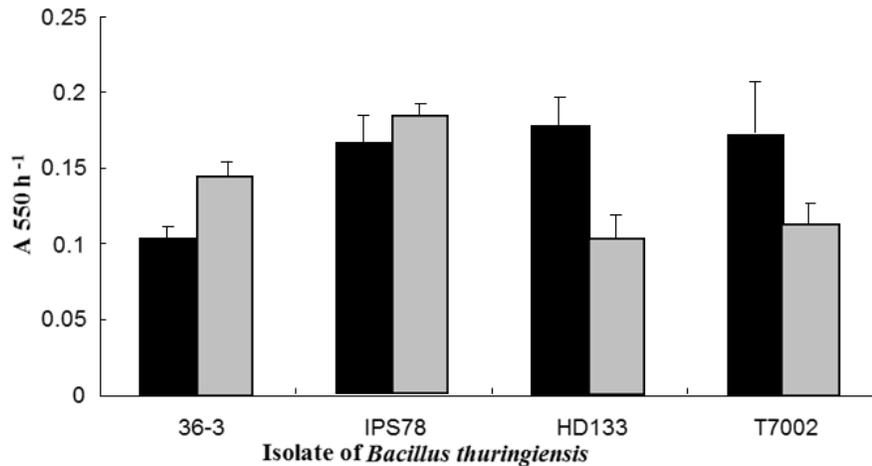
**Fig. 1. Chitinolytic activities of supernatant fractions of *B. thuringiensis***

(a) *B. thuringiensis* 36-3, (b) *B. thuringiensis* IPS78, (c) *B. thuringiensis* HD133 and (d) *B. thuringiensis* T7002. Cells were grown in nutrient broth supplemented with 0.1% (w/v) regenerated chitin (■) and chitinase inducing medium (□). The mean activities of three replicate wells  $\pm$  SE are given

assayed several isolates of *B. thuringiensis* including *Bt* HD133 and *Bt* IPS78 in fluorometric assay, high activities were obtained with 4-MU-(GlcNAc)<sub>2</sub> (exochitinase) followed by 4-MU-(GlcNAc)<sub>3</sub> (endochitinase) and lower activities with 4-MU-(GlcNAc)<sub>1</sub> (*N*-acetylglucosaminidase) and 4-MU-(GlcNAc)<sub>4</sub> (endochitinase). But in this present study, higher endochitinase activity with 4-MU-(GlcNAc)<sub>4</sub> was obtained for all four sub species than that of with 4-MU-(GlcNAc)<sub>3</sub> (both these substrates detect the activity of endochitinase). This may be due to some different growth conditions applied in two studies such as the concentration of regenerated chitin in the growth medium.

The chitinase activities of the culture supernatants of *B. thuringiensis* against the substrate CM-chitin RBV are shown in Fig. 2. Chitinase activities of the supernatant fractions of four sub species of *Bt* detected in two assays used in this study showed a same pattern of distribution. Similar to the results obtained with 4-methylumbelliferyl fluorogenic substrates, *Bt* 36-3 and *Bt* IPS 78 expressed the lowest and the highest activities with the substrate CM-chitin RBV respectively.

When the culture supernatants of four *B. thuringiensis* sub species grown in nutrient broth supplemented with regenerated chitin were

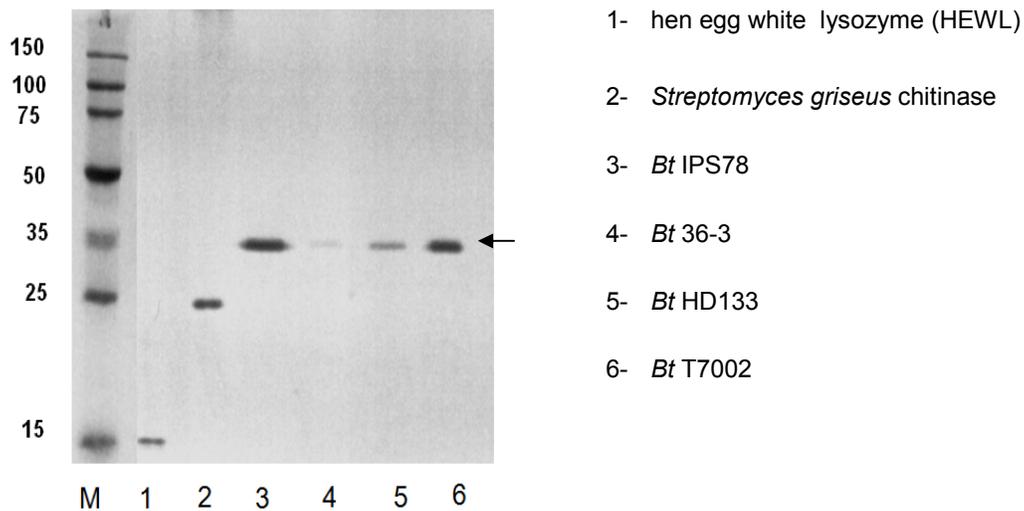


**Fig. 2. Chitinase activities of the supernatant fractions of four subsp. of *B. thuringiensis* with CM-chitin RBV**

Cells were grown in Nutrient broth supplemented with regenerated chitin (■) and Chitinase inducing medium (▒). The mean absorbances of triplicate assays ± SE are given

electrophoresed on activity gels, one chitinase band of approximate molecular mass of 36 kDa was obtained from all four sub species (Fig. 3). *B. thuringiensis* 36-3 which showed the lowest activity in assays gave a faint band in the activity gel. Hen egg white lysozyme (HEWL) and purified chitinase from *Streptomyces griseus* were used as positive controls.

A chitinase of approximate molecular mass of 39 kDa (Chi39) had been detected by [33] from *B. thuringiensis* serovar *konkukian* S4 and purified Chi39 has shown antifungal activity against the phytopathogenic fungi such as *Fusarium oxysporum* and *Aspergillus niger*. A chitinase Chi255 from *B. thuringiensis* subsp. *kurstaki* showed *in vitro* antifungal activity against



**Fig. 3. Detection of chitinase activity of four subsp. of *B. thuringiensis* in SDS-PAGE activity gels containing glycol chitin.** The supernatants of the cultures grown in nutrient broth supplemented with regenerated chitin were used. The standard molecular markers (Novagen perfect protein markers) are shown in the left. The position of 36 kDa chitinase is indicated with an arrow

*A. niger* that causes black blight of onion and peanut [14]. Further, when several *Bt* isolates were screened for the antifungal activity by [34], a complete inhibition of the growth of *Fusarium oxysporum* f. sp. *cubense* was noticed with *Bt* isolates 50E and 48F and *Beauveria bassiana* with 50E, 48F, 47C, 24B, 13.2, A12.3, 50B and 30, whereas some of the *Bt* isolates were found to inhibit *Sclerotium rolfisii* to an extent of 75%. Among these, some of them have also inhibited *Aspergillus flavus* to an extent of almost 50%. In this present study, when the crude culture supernatants of four subsp. of *Bt* were screened against *Botrytis*, no growth inhibition was obtained *in vitro*. A possible explanation was given by [35] for the limitation of antifungal activity of many bacterial chitinases when tested in growth inhibition experiments. One reason may be that the bacterial exochitinases, digest chitin chains starting from the non-reducing end of the chain, which may be difficult in intact fungal cell walls. In contrast, most plant chitinases (endochitinases) can cleave any portion of the chitin polymer with which they come in contact. But in this study, this may be not the only reason and there could be some other factors such as the crude condition of the enzyme, concentration of enzyme extract applied in each well and the incubation conditions used for *Botrytis* (eg. 21°C) might be not the optimum for the activity of chitinases.

#### 4. CONCLUSION

In conclusion, the present work shows the chitinolytic activities of four subsp. of *Bacillus thuringiensis* under *in vitro* conditions. But more work is needed towards further characterization of chitinases in these isolates. Future experiments can be carried out to reveal whether these enzymes have any insecticidal activity and furthermore, to develop environmental-friendly biopesticides, *in vivo* antifungal activities of these chitinases can be examined against plant pathogenic fungi in growth chambers and under glasshouse condition.

#### COMPETING INTERESTS

Author has declared that no competing interests exist.

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