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Chitinase Production by *Bacillus thuringiensis* and *Bacillus licheniformis*: Their Potential in Antifungal Biocontrol

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Thirty bacterial strains were isolated from the rhizosphere of plants collected from Egypt and screened for production of chitinase enzymes. *Bacillus thuringiensis* NM101-19 and *Bacillus licheniformis* NM120-17 had the highest chitinolytic activities amongst those investigated. The production of chitinase by *B. thuringiensis* and *B. licheniformis* was optimized using colloidal chitin medium amended with 1.5% colloidal chitin, with casein as a nitrogen source, at 30°C after five days of incubation. An enhancement of chitinase production by the two species was observed by addition of sugar substances and dried fungal mats to the colloidal chitin media. The optimal conditions for chitinase activity by *B. thuringiensis* and *B. licheniformis* were at 40°C, pH 7.0 and pH 8.0, respectively. Na^+, Mg^{2+}, Cu^{2+}, and Ca^{2+} caused enhancement of enzyme activities whereas they were markedly inhibited by Zn^{2+}, Hg^{2+}, and Ag^+. *In vitro*, *B. thuringiensis* and *B. licheniformis* chitinases had potential for cell wall lysis of many phytopathogenic fungi tested. The addition of *B. thuringiensis* chitinase was more effective than that of *B. licheniformis* in increasing the germination of soybean seeds infected with various phytopathogenic fungi.

Keywords: Chitinase, *Bacillus thuringiensis*, *Bacillus licheniformis*, optimization, characterization, biological control

Introduction

Chitin is an insoluble polysaccharide composed of linear chains of β-1, 4-*N*-acetylglucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds. Chitin is one of the most abundant biopolymers on earth, next to cellulose. Chitin is widely distributed in nature as it is found in the outer skeleton of insects, fungi, algae, crabs, shrimp, lobsters and in the internal structures of other invertebrates (Bhattachrya *et al*., 2007). Each year, a vast amount of chitin waste material is released into the environment, which creates a serious environmental problem. The recycling of chitin is extremely important for retaining the carbon-nitrogen balance in the ecosystem (Hayes *et al*., 2008).

The catabolism of chitin typically occurs in two steps, involving the initial cleavage of the chitin polymer by chitinase into chitin oligosaccharides, and then further cleavage to *N*-acetylglucosamine monomers by chitobiases (Suginta *et al*., 2000). These derivatives have a broad range of agricultural, industrial and medical applications such as antitumor activity and elicitor action and even in cosmetics (Bansode and Bajekal, 2006).

Chitinases are produced by various microorganisms such as bacteria (Ajit *et al*., 2006), actinomycetes (Akagi *et al*., 2006), fungi (Viterbo *et al*., 2001) and also by higher plants (Matsushima *et al*., 2006). During the last decade, chitinases have received increased attention worldwide because of their wide range of applications. Chitinases are used as potential biocontrol agents for many fungal pathogens through its chitin degradation activity, and hence find use as food and seed preservative agents (de la Vega *et al*., 2006; Chang *et al*., 2007), as candidates for the bioremediation and bioconversion of chitin wastes (Wang and Huang, 2001), and for preparation of oligosaccharides and *N*-acetyl D-glucosamine (Wang *et al*., 2006). Moreover, potential roles of chitinase in biocontrol of insects and mosquitoes and in production of single cell protein (SCP) have also been suggested (Hayes *et al*., 2008). Chitinase has also been employed in human health care, such as making ophthalmic preparations, and in many other biotechnological areas (Dahiya *et al*., 2006).

Several bacterial species *viz*. *Pseudomonas*, *Serratia*, and *Bacillus* are known to produce chitinase for the utilization of chitin as a source of carbon and nitrogen, and chitinase production is further increased when the medium is supplemented with a chitin source or fungal cell walls (Ajit *et al*., 2006).

In the present study, chitin degrading bacteria were isolated from soil samples collected from agricultural fields in Egypt. Optimization and characterization of chitinases, which were produced by the most potent species, *Bacillus thuringiensis* and *Bacillus licheniformis*, were studied. These studies also included an examination of the antagonistic activity of the produced chitinases against some fungal pathogens and their effectiveness in increasing the germination of soybean seeds infected with various phytopathogenic fungi.

Materials and Methods

Isolation of chitin degrading bacteria

A total of 17 different rhizospheric soil samples of maize, wheat and rice were collected from agricultural fields of Cairo, Kaluobia, Giza, Helwan, and Dakahlia governorates.

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Chitin degraders were isolated by serial dilutions of soil samples and plated on colloidal chitin agar (CCA) medium, a minimal salt medium containing colloidal chitin as sole carbon and energy source, as described by Kamil *et al.* (2007). The isolates showing distinct zones of clearance were selected as potent chitinase producers.

Colloidal chitin preparation

Colloidal chitin was prepared from chitin flakes (Sigma Chemicals Co.) by the method of Mathivanan *et al.* (1997). The chitin flakes were ground to powder, added slowly to 10 N HCl and kept overnight at 4°C with vigorous stirring. The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze dried to powder and stored at 4°C until further use.

Strain identification

16S rRNA sequencing and analysis: Bacterial isolates were tested for species identity using the 16S rRNA sequencing method according to the methods of Rochelle *et al.* (1995). The gene sequencing was done at Macrogen (South Korea). DNA sequences were aligned using Gene Mapper v4.1 & Data Collection v3.1 Communication Patch1. To extract the genomic DNA, bacterial colonies are picked with a sterilized toothpick, and suspended in 0.5 µl of sterilized saline, then centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 µl of Insta Gene Matrix (Bio-Rad, USA), incubated at 56°C for 30 min and then heated to 100°C for 10 min. After heating, the supernatant can be used for the PCR reaction.

Bacterial 16S rRNAs were amplified by using the following universal bacterial 16S rRNA primers: forward primer 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1792 R (5′-TACGGYTACCTTGTTACGACTT-3′). Polymerase chain reaction was performed using kits with Ampli Taq DNA polymerase (FSenzyme) (Applied Biosystems). 1 µl of template DNA was added to 20 µl of PCR reaction solution. Amplification was performed using 35 cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. The PCR amplicon was purified using a Montage PCR clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers 518 F (5′-CCA GCA GCC GCG GTA ATA Cg-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′). Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems). Sequence analysis was performed with sequences in the National Center for Biotechnology Information (NCBI), USA database using Basic Local Alignment Search Tool for Nucleotides (BLASTN) (Altschul *et al.*, 1997).

Chitinase production and partial purification: Colloidal chitin broth (50 ml) in 250 ml capacity Erlenmeyer flasks was inoculated with a 1.0 ml spore suspension (adjusted to 1.0 OD_{600}) of each species and incubated at 30°C in a rotary incubator (150 rpm) for 72 h. The culture fluids were centrifuged at 10,000×g for 20 min and the cell free supernatants were saturated with ammonium sulphate to 60–70% levels, kept at 4°C overnight, and then collected by centrifugation at 10,000×g for 20 min. The precipitates were dissolved in 50 mM phosphate buffer (pH 7) and extensively dialyzed against the same buffer. The resultant dialysates were regarded as partially purified chitinases (PPC) and were used for further studies (Bansode and Bajekal, 2006).

Chitinase assay: Chitinase activity was measured using colloidal chitin as a substrate following the method of Sun *et al.* (2006). The amount of reduced sugar released was determined by recording the absorbance at 575 nm. One unit (U) of chitinase activity was defined as the amount of enzyme that released 1 µ mol *N*-acetylglucosamine (GlcNAc) from colloidal chitin per min under the same conditions as used for the GlcNAc (Sigma) standard.

Optimization of chitinase production: The effect of incubation period on chitinase production was studied by growing *B. licheniformis* and *B. thuringiensis* in colloidal chitin broth medium up to 7 days. The impact of pH and temperature on chitinase production was investigated by cultivating the strains in the colloidal chitin medium at various pH (5.0–9.0) and temperature (20–40°C) settings. The optimum chitin concentration for chitinase production was determined by amending different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%) of colloidal chitin into the medium. Carbon sources such as glucose, sucrose, galactose, arabinose, rafinose, mannose, maltose, fructose, lactose, xylose, starch, and cellulose at a concentration of 0.5% and nitrogen sources such as ammonium chloride, ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate, yeast extract, casein, peptone, tryptone and soybean meal were supplemented to the colloidal chitin medium to study their influence on chitinase production.

Utilization of fungal cell wall: *Rhizoctonia* sp., *Trichoderma harzianum*, *Fusarium oxysporum*, and *Penicillum chrysogenum* were grown in Czapek-Dox broth. After 7 days of incubation, the fungal mat was harvested and autoclaved at 121°C, for 20 min. The autoclaved fungal mats were washed twice with sterile distilled water and dried in an oven at 80°C to constant weight. The dried fungal mat was powdered and used as chitin source at a concentration of 2 g/L for the production of chitinases. Flasks containing colloidal chitin medium without any fungal mat were used as control (Goel *et al.*, 2004).

Enzyme characterization

Effect of pH and temperature on chitinase activity: The optimal pH for chitinase activity was determined by measurements at different pH values using colloidal chitin as the substrate under standard assay conditions. The buffers used were as follows (50 mM): citrate-phosphate buffer (pH 5.0–6.0), phosphate buffer (pH 7.0–8.0) and glycine-NaOH buffer (pH 9.0). The optimal temperature was determined by incubating the reaction mixtures at different temperatures from 20 to 50°C and measuring the enzyme's activity under standard assay conditions using colloidal chitin as a substrate.

Effect of metal ions and inhibitors on chitinase activity: Effects of several metal ions and inhibitors on the activity

of the enzymes were investigated by incubating the produced chitinases in colloidal chitin solution plus the metal ions under test (K^+, Na^+, Ag^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, and Zn^{2+}) for 30 min and inhibitors such as ethylenediamine tetraacetic acid (EDTA), and L-cystein at a final concentration of 5 and 10 mM. The relative activity was calculated with respect to the control where the reaction was carried out in the absence of any additive under the optimum assay conditions.

***In vitro* antifungal activity:** The chitinase enzymes obtained from *B. licheniformis* and *B. thuringiensis* were tested for their potential to act as biocontrol agents against important fungal phytopathogens. The phytopathogenic fungi were obtained from Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Center, Al-Azhar University, Cairo, Egypt. Fungal mycelium (2.5 mg) was transferred to 250 ml Erlenmeyer flasks containing 50 ml 1% potato-dextrose medium and partially purified chitinase of each species (2%). Cultures were incubated at 30°C, 250 rpm for 120 h in triplicate. Fungal growth was determined by dry weight. Cultures without chitinase were used as a control.

Effect of chitinase on seed germination: To investigate the effect of chitinase on the germination of soybean seeds infested with each fungus, plastic trays, measuring 52 × 25 × 4 cm, with 200 wells each, were used. Soybean seeds were mixed with the dried mycelium of each fungus (5 to 6 mg/kg). One seed was planted in each well. Two milliliter of each chitinase was applied separately to each well. The number of germinated seeds was recorded and the percentage (%) of germination was calculated. Soybean seeds infested with each fungus without chitinase were used as controls.

Statistical analysis

Results are presented as mean value ± standard deviation (SD). The Microsoft Excel 2003 and SAS 9.1.3 statistical program were used for data analysis.

Results and Discussion

Isolation and screening of chitinolytic bacteria

Thirty bacterial strains were isolated from agricultural fields of different localities in Egypt. Screening of chitinolytic bacterial isolates was carried out by spreading inocula of each colony onto plates containing a minimal salt medium with colloidal chitin as a sole carbon and energy source. The chitin degrading bacteria formed colonies of 1–2 mm in diameter, surrounded by clear zones indicating chitinase activity. Only 5% of isolates exhibited different clear zone sizes. Of these, two isolates designated S1and S2 gave the widest clear zones (1.1 and 0.9 cm, respectively) and therefore, they were selected for further study.

The presence of a greater number of chitin degrading bacteria in agricultural fields has already been demonstrated (Nawani and Kapadnis, 2003). Detection of chitin-degrading bacteria from natural sources such as rhizospheric soils is useful in the isolation of bacteria that produce antifungal or other novel compounds. The presence of chitinolytic bacteria in the crop rhizosphere soils is highly beneficial as they can suppress plant pathogenic fungi near the root zone, by attacking the chitin-rich fungal cell wall, and thus provide sustainable plant protection against root diseases.

Identification of strains

To confirm the identification of isolates S1and S2, a 16S rRNA gene sequence analysis was performed. The sequence alignment using BLASTN software for the comparison of up to 1,500 bp gave high homology (98%) with *Bacillus thuringiensis* NM101-19 and *B. licheniformis* NM120-17, respectively. *B. thuringiensis* subsp. *kurstaki* (Driss *et al.*, 2005), *B. thuringiensis* sub sp. *aizawai* (de la Vega *et al.*, 2006), and *B. licheniformis* (Waldeck *et al.*, 2006) were previously reported to produce chitinase.

Factors affecting chitinase production

Analysis of the results revealed that the production of different metabolites in the microbial cultures could be increased by adopting suitable medium components and culture techniques.

Incubation period

The time course of chitinase production by the two species is shown in Fig. 1. For *B. thuringiensis* and *B. licheniformis* the highest level of chitinase production reached 1.28 and 1.14 U/ml, respectively on the 5th day of the incubation period. The time lag probably results from the fact that chitin is a high molecular weight compound and organisms need a longer time to decompose it completely. Similarly, Nawani and Kapadnis (2004) reported that the production of chitinase by *Streptomyces* sp. NK1057 was high after 5 days of incubation. Chitinase synthesis was found to decline as the incubation period was further extended. The decrease in chitinase production after 5 days of incubation may be related to the increase of pH due to the release of carbonate

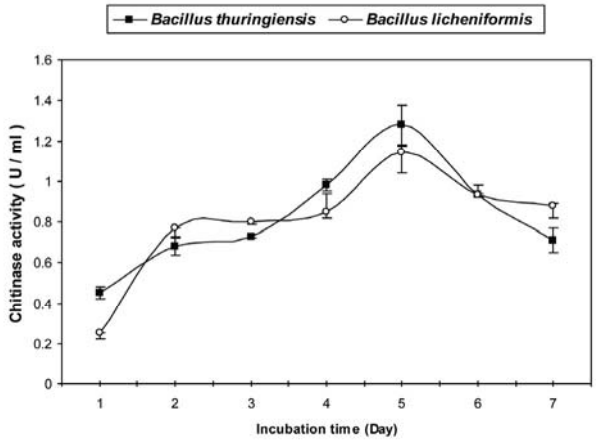

Fig. 1. Time course of chitinase production by *B. thuringiensis* NM101–19 and *B. licheniformis* NM120–17 grown in a medium supplemented with colloidal chitin as a sole carbon and energy source. Bars represent standard deviations.

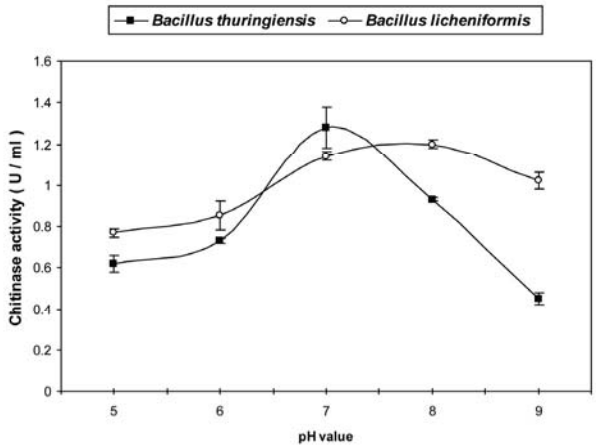

Fig. 2. Effect of initial pH of media on the chitinases production by *B. thuringiensis* and *B. licheniformis*. Bars represent standard deviations.

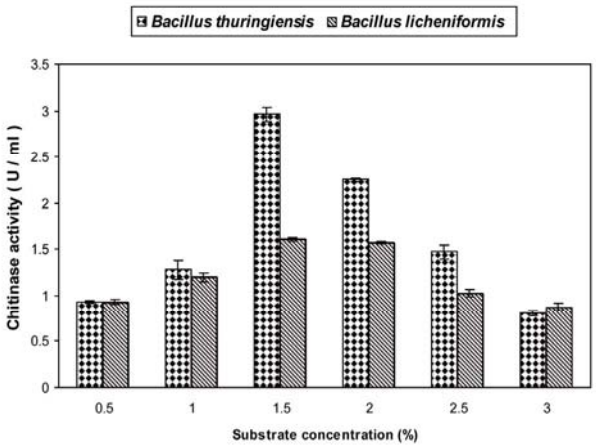

Fig. 4. Production of chitinase by *B. thuringiensis* and *B. licheniformis* in different concentrations of colloidal chitin after incubation at 30°C for 5 days. Bars represent standard deviations.

salts and protein into the fermentation medium, the presence of proteolytic enzymes, or the accumulation of *N*-acetyl-glucosamine resulting from chitin decomposition.

pH and temperature

It is well known that pH of the culture medium affects the availability of certain metabolic ions and permeability of bacterial cell membranes, which in turn supports cell growth and enzyme production. A majority of bacteria was reported to produce the maximum level of chitinase at neutral or slightly acidic pH, whereas fungi mostly secret it in acidic conditions (Sharaf, 2005). However, results illustrated in Fig. 2 demonstrated that pH 7.0 and 8.0 favored chitinase production by *B. thuringiensis* and *B. licheniformis* at the maximum of 1.28 and 1.20 U/ml, respectively. Similarly, Nawani *et al.* (2002) reported that the optimum pH for chitinase production by *Microbispora* sp. V2 was pH 7. Moreover, a similar optimum pH of 8.0 for chitinase production was reported for *B. pabuli* K1 and *B. laterosporus* MML2270 (Shanmugaiah *et al.*, 2008).

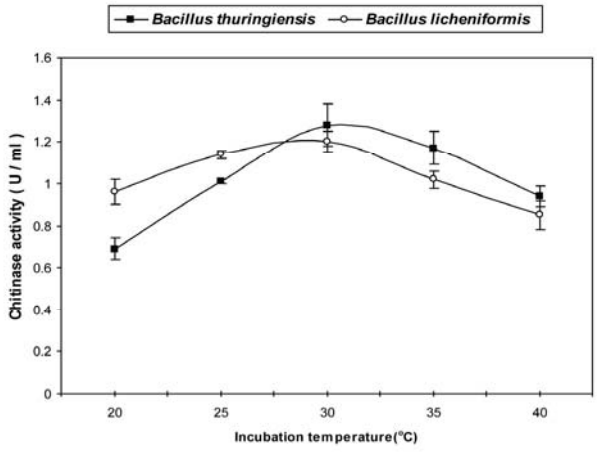

Fig. 3. Impact of incubation temperature on the chitinases production by *B. thuringiensis* and *B. licheniformis*. Bars represent standard deviations.

Growth temperature is another critical parameter that needs to be controlled. For the temperatures tested, *B. thuringiensis* and *B. licheniformis* produced maximum chitinase activity at 30°C (Fig. 3). It has been observed that in both lower and higher temperatures, the chitinase activities were sharply decreased. Taechowisan *et al.* (2003) and Shanmugaiah *et al.* (2008) reported that the production of chitinase by *Streptomyces aureofaciens* and *B. laterosporus* MML2270, respectively were optimal at temperatures of 30–40°C.

Substrate concentration

Taechowisan *et al.* (2003) reported that chitinase produced by bacteria hydrolyzed colloidal chitin more rapidly than crude chitin or chitin from fungal cell walls. The concentration of colloidal chitin is an important factor as it is reported to induce chitinase production in several microorganisms (Soiuza *et al.*, 2005). Results illustrated in Fig. 4 showed that chitinase produced by *B. thuringiensis* and *B. licheniformis* increased along with increasing colloidal chitin concentration up to 1.5% and reached 2.96 and 1.61 U/ml, respectively, only to drop soon after. This is in accordance with the fact that most of the chitinolytic systems reported in the literature are inducible (Ulhoa and Peberdy, 1991). Beyond 1.5%, the substrate concentrations decreased the enzyme activities. This conforms with the finding of Gupta *et al.* (1995) who reported that the production of chitinase from *Streptomyces aureofaciens* was optimal with 1.5% of chitin amended medium.

It is worth mentioning that the addition of chitin to soil leads to an increase in the population of chitinolytic microbes, which in turn, reduces the plant diseases caused by fungal pathogens (Lafontaine and Benhamou, 1996). Hence it is worth considering the utilization of chitinolytic bacteria in the control of plant pathogens.

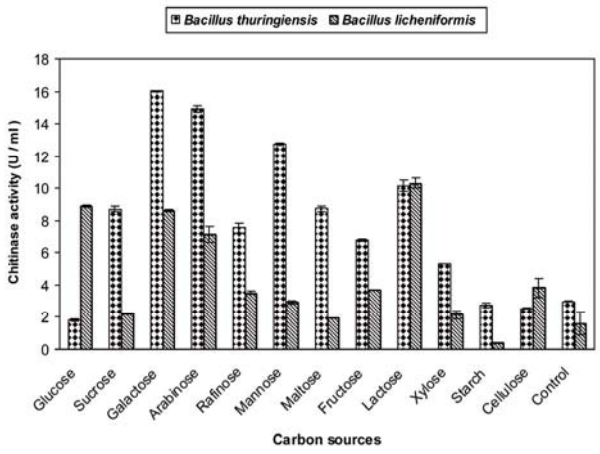

Fig. 5. Influence of additional carbon sources (0.5%) on chitinase production by *B. thuringiensis* and *B. licheniformis* cultured in colloidal chitin medium containing 1.5% colloidal chitin. Control: without additional carbon source. Bars represent standard deviations.

Addition of different carbon sources

The influence of additional carbon sources on chitinase production was studied by adding various carbon sources to the culture medium. Enhanced chitinase production by *B. thuringiensis* was found in colloidal chitin medium amended with galactose (16.02 U/ml) On the other hand, in case of chitinase production by *B. licheniformis,* lactose caused enhancement of chitinase production (10.33 U/ml) (Fig. 5). Different results were reported by Taechowisan *et al.* (2003) who found that amendment of pectin, starch and carboxymethyl cellulose to the colloidal chitin medium increased chitinase production by *Streptomyces aureofaciens.* Moreover, Joo (2005) reported that glucose (0.4%) along with chitin induced high levels of chitinase by *Streptomyces halstedii.*

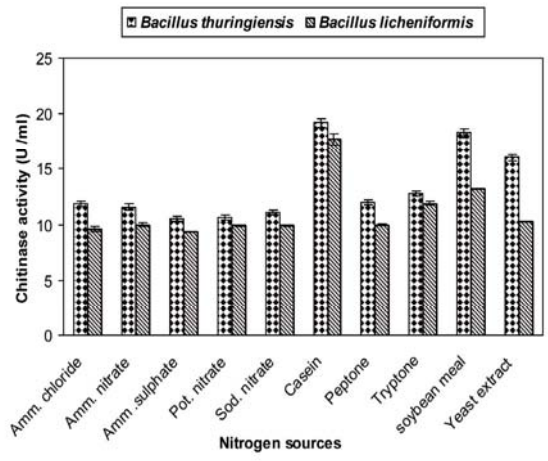

Fig. 6. Effect of various nitrogen sources on chitinase production by *B. thuringiensis* and *B. licheniformis*. Bars represent standard deviations.

Nitrogen source

The nitrogen source has a profound influence on enzyme production as it is the ultimate precursor for protein biosynthesis. The nitrogen source can also affect the pH of the medium, which in turn, may influence the activity and stability of the enzyme (Nizamudeen and Bajaj, 2009). Inorganic nitrogen sources proved less favorable for enzyme secretion (Fig. 6). This is expected as organic nitrogen sources are rich in amino acids and short peptides that support enzyme production. Casein yielded the highest chitinase production by *B. thuringiensis* and *B. licheniformis*, reaching 19.21 and 17.75 U/ml, respectively. Casein contains some essential amino acids as well as some carbohydrates and the inorganic elements calcium and phosphorus. These results were in agreement with Vaidya *et al.* (2001) who reported that organic nitrogen sources significantly increased chitinase production by *Alcaligenes xylosoxydans.*

Role of fungal biomass on chitinase production

As the fungal cell wall contains chitin as the major component, chitinase is well known to lyse the cell wall of both live and dead fungi (Ueno *et al.*, 1990). An enhancement of chitinase production, over that of the control, by the two species was observed in colloidal chitin medium amended with dried fungal mats (Table 1). In the same regard, an increase in chitinase production was observed from *Streptomyces aureofaciens* and *Streptomyces halstedii* when cultured in a medium containing colloidal chitin supplemented with fungal cell wall preparations (Taechowisan *et al.*, 2003; Joo, 2005).

From the previous results, it could be concluded that colloidal chitin (1.5%), addition of galactose for *B. thuringiensis* and lactose for *B. licheniformis* (0.5%), casein as a nitrogen source, and addition of dried fungal mats were the most suitable ingredients for securing higher growth and chitinase production by the two species. High chitinase production could also be obtained by providing the following conditions.

1) Initial pH of 7.0 for *B. thuringiensis* and 8.0 for *B. licheniformis*.
2) Incubation temperature at 30°C.
3) Incubation period for 5 days using a rotary shaker (150 rpm).

It is note worthy to state that by optimizing the above medium components and culture conditions, the production of chitinases has been increased by about 20 fold from 1.28 U/ml and 1.14 u/ml for the initial medium composition to

Table 1. Production of chitinase by *B. thuringiensis* and *B. licheniformis* on mixed substrate containing colloidal chitin and chitin derived from different fungal cell walls

Substrate	*B. thuringiensis*	*B. licheniformis*
Control	19.21±0.01	17.75±0.05
Rhizoctonia sp.	21±0.03	22.79±1.16
Trichoderma harzianum	25.36±0.00	23±0.03
Fusarium oxysporum	22.42±0.04	19.64±0.04
Penicillum chrysogenum	21.24±0.11	18.34±0.01

Control : Colloidal chitin only
Data represent the mean of 3 different readings±standard deviation

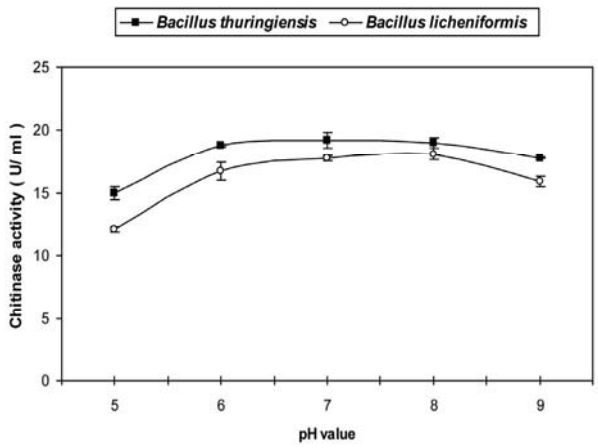

Fig. 7. Impact of pH upon activity of chitinases from *B. thuringiensis* and *B. licheniformis*. Bars represent standard deviations.

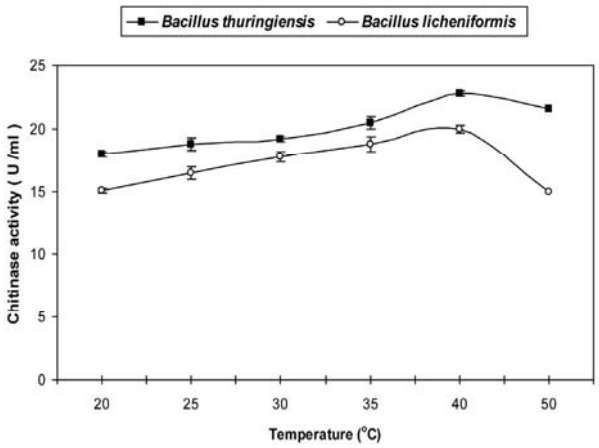

Fig. 8. Impact of temperature on activity of chitinases from *B. thuringiensis* and *B. licheniformis*. Bars represent standard deviations.

25.36 U / ml and 23 U/ml under the final conditions for *B. thuringiensis* and *B. licheniformis*, respectively.

Enzyme properties

Effects of pH and temperature on chitinase activity: These experiments examined the effect of pH on the activity of chitinases produced by *B. thuringiensis* and *B. licheniformis*. The two species could produce the enzymes over a broad pH range (5.0–9.0) (Fig. 7). Under standard conditions, the pH profile was normal (bell shaped) with a maximum value at pH 7.0 and pH 8.0 for *B. thuringiensis* and *B. licheniformis*, respectively. Similarly, Wang and Chang (1997) previously stated that activity of several bacterial chitinases showed pH optima under neutral or slightly alkaline conditions.

Chitinases with alkaline pH optima and stability are considered to have a major potential in biological control of insect pests. The peritrophic gut lining of insects is chitinous and has an alkaline pH. Chitinases with a better stability and activity in these conditions can be used in synergism with other biocontrol agents. Moreover, alkaline chitinases are also considered useful in management of chitinous wastes, such as those generated by seafood manufacturing industries (Nawani and Kapadnis, 2003).

With regard to temperature, *B. thuringiensis* and *B. licheniformis* showed maximum chitinase activity at 40°C under the standard assay conditions. Thereafter, the activity sharply declined (Fig. 8). Similarly, Mane and Deshmukh (2009) reported that *Streptomyces canus, Streptomyces pseudogriseolus*, and *Micromonospora brevicatiana* showed maximum chitinase activity at 40–60°C.

Effects of metal ions and inhibitors on chitinase activity: The effects of metal ions and inhibitors on enzyme activities are presented in Table (2). There were increases in enzyme activities in the presence of Na^{+}, Mg^{2+}, Cu^{2+}, and Ca^{2+}, whereas the activities were markedly inhibited by Zn^{2+}, Hg^{2+}, and Ag^{+}. Metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate, combining with both, and thus holding the substrate at the active site of the enzyme (Mahmoud *et al.*, 1968). The effect

Table 2. Effect of metal ions and inhibitors on activity of chitinases produced by *B. thuringiensis* and *B. licheniformis*

Metal ions or inhibitors	Relative activity (%)			
	B. thuringiensis		*B. licheniformis*	
	5 mM	10 mM	5 mM	10 mM
Control (no addition)	100	100	100	100
NaCl	112.14±0.02	98.22±0.61	117.02±0.00	102.83±0.11
KCl	98.51±0.13	83.33±1.05	97.85±0.53	87.82±1.61
$AgNO_3$	76.74±0.01	58.51±0.00	54.43±0.02	39.64±0.00
$MgCl_2$	117.88±0.00	112.92±0.00	102.87±0.00	97.39±0.01
$CaCl_2$	160±0.05	148.88±0.07	167.73±0.07	160.73±0.13
$CuSO_4$	103.33±0.04	94.44±0.03	98.93±0.03	85.36±0.01
$ZnSO_4$	77.74±0.06	61.85±2.19	70.88±0.02	50.89±0.02
$FeSO_4$	97.51±0.03	89.25±0.03	84.73±0.01	77.12±0.00
HgI_2	30±0.13	25.55±0.00	60.42±0.09	40.56±0.03
EDTA	94.81±0.13	81.48±0.11	88.25±0.01	77.56±1.05
L-Cystein	37.40±3.03	34.81±0.03	70.92±0.00	65.30±0.02

Data represent the mean of 3 different readings±standard deviation

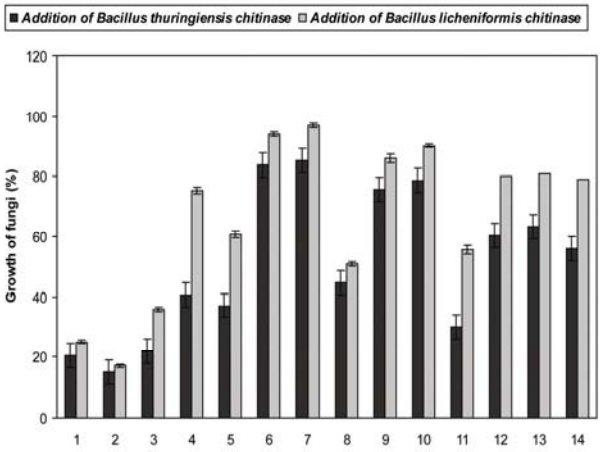

Fig. 9. Growth of phytopathogenic fungi in the presence of *B. thuringiensis* and *B. licheniformis* chitinases. 1, *Aspergillus flavus*; 2, *Aspergillus niger*; 3, *Aspergillus terreus*; 4, *Fusarium oxysporum*; 5, *Fusarium* sp.; 6, *Penicillum chrysogenum*; 7, *Pythium* sp.; 8, *Ralstonia solanacearum*; 9, *Rhizoctonia solani*; 10, *Rhizoctonia* sp.; 11, *Rhizopus* sp.; 12, *Trichoderma harzianum*; 13, *Trichoderma viride*; and 14, *Verticillium* sp. Bars represent standard deviations.

of metal ions on enzyme activity may be due to a change in electrostatic bonding, which would change the tertiary structure of enzymes (Roy *et al.*, 1990). This may also due to the possible participation of sulfhydryl groups in the active site of the enzyme. Moreover, Ca^{2+} is a known stimulator of extracellular enzymes, especially helping them to withstand high temperatures (Tsujibo *et al.*, 1993). The inhibition of chitinases by Zn^{2+} and Hg^{2+} could be related to the residues of aspartic and glutamic acid in chitinases. It has been shown that these amino acids in the active sites of chitinases bind to certain divalent cations, thereby possibly inhibiting chitinases (Milewski *et al.*, 1992). In contrast, EDTA did not affect activity appreciably indicating that divalent cations are not required for the enzyme activities.

In vitro antifungal activity

Biological control with bacteria offers an effective method of managing plant pathogens. These bacteria inhibit the fungal pathogens by producing antibiotics, lytic enzymes and by inducing resistance systemically in the plant by activating defensive genes such as chitinase, β-1, 3-glucanase, peroxidase, and phenylalanine ammonia lyase (Chang *et al.*, 2007). Chitinolytic enzymes have been considered important in the biological control of soil-borne pathogens because of their ability to degrade fungal cell walls whose major component is chitin (Ulhoa and Peberdy, 1991). Different morphological effects on the fungal cell wall have been reported in studies using bacterial chitinases. These effects were mainly spore germination inhibition, bursting of spores and hyphal tips, and germ tube elongation (Taechowisan *et al.*, 2003).

In this study, the ability of the selected chitinolytic strains in suppression of mycelial growth of phytopathogenic fungi was conducted and the results illustrated in Fig. 9 demonstrated that chitinase produced by *B. thuringiensis* was more effective against phytopathogenic fungi than that produced by *B. licheniformis*. In the presence of *B. thuringiensis* chitinase, growth ranged from 15.11% to 44.66% for *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Fusarium oxysporum*, *Fusarium* sp., *Ralstonia solanacearum*, and *Rhizopus* sp. While *B. licheniformis* chitinase was most effective against only *Aspergillus flavus*, *A. niger*, and *A. terreus*, growth ranged from 17.06% to 35.79% and for *Pythium* sp., the least affected, it was 97%.

Bacillus strains have been intensively investigated as biological control agents. For example, Driss *et al.* (2005) observed that the culture supernatant of *B. thuringiensis* strain BUPM255 had high antifungal activity against *A. niger*. Also, de la Vega *et al.* (2006) reported that a purified chitinase from *Bacillus thuringiensis* subsp. *aizawai* exhibited lytic activity against the cell walls of six phytopathogenic fungi and inhibited the mycelial growth of *Fusarium* sp. and *Sclerotium rolfsii*.

Table 3. Germination of soybean seeds infested with different phytopathogenic fungi, in the absence and presence of *B. thuringiensis* and *B. licheniformis* chitinases

	Germination of soybean seeds (%)		
Fungi	Without chitinase	With *B. thuringiensis* chitinase	With *B. licheniformis* chitinase
Aspergillus flavus	25±0.01	50±0.03	27.27±0.02
Aspergillus niger	15.50±0.02	80.80±0.02	70.75±0.01
Aspergillus terreus	6.66±±0.01	46.66±0.08	38.46±0.60
Fusarium oxysporum	20.20±0.02	46.66±0.00	30.30±1.21
Fusarium sp.	6.66±0.01	43.75±0.04	12.14±3.03
Penicillum chrysogenum	50±0.60	66.66±0.03	54.54±0.00
Pythium sp.	40±0.02	65.11±0.09	50±0.03
Ralstonia solanacearum	10.45±0.00	33.33±0.01	28.57±0.01
Rhizoctonia solani	25.76±0.04	36.33±1.21	30.76±0.02
Rhizoctonia sp.	25±1.21	35.29±0.01	28.57±0.00
Rhizopus sp.	15.38±0.00	46.15±0.00	28.57±0.04
Trichoderma harzianum	18.18±0.01	46.15±0.03	35.35±0.00
Trichoderma viride	21.42±0.01	38.46±2.19	30.76±0.01
Verticillium sp.	25.25±0.00	45.45±0.05	37.50±0.04

Data represent the mean of 3 different readings±standard deviation

Effect of chitinase on the germination of soybean seeds

Soybeans are a particularly important crop because of their high protein (40%) and oil (20%) content. A limiting factor for their production is the disease caused by phytopathogenic fungi, affecting seeds from germination and throughout development. Consequently, yield reductions of up to 30% have been reported (Reyes-Ramirez *et al.*, 2004). Fungicides have commonly been used; however, pathogen resistance has developed, as well as damages for man and the environment. Recently, the use of biological control has increased. Chitinolytic microorganisms have been suggested for the control of some fungi.

The effect of chitinase produced by the selected chitinolytic isolates on the germination of soybean seeds infested with phytopathogenic fungi was tested and the results are presented in Table 3. All fungi tested reduced the germination of infested seeds. *Aspergillus terreus* and *Fusarium* sp. were the most aggressive, reducing germination to 6.66%. Seeds protected with chitinase of *B. thuringiensis* showed a very good response for germination.

This ranged from 33.33% for seeds infested with *Ralstonia solanacearum* to 80.80% for seeds infested with *A. niger*. On other hand, the germination of seeds protected with *B. licheniformis* ranged from 12.14% for seeds infested with *Fusarium* sp. to 70.75% for seeds infested with *A. niger*.

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