Lytic Activity of Bacterial Chitinases in *Aspergillus flavus* Link. ex Fries and *A. parasiticus* Speare

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Abstract

The mycolytic activity of the crude chitinases obtained from two strains of **Bacillus circulans** *(5Bc and 7Bc), two strains of* **Serratia marcescens** *(11Sm and 14Sm),* **Arthrobacter luteus** *(6Al) and* **Streptomyces griseus** *(9Sg) was evaluated against two species of aflatoxigenic fungi,* **Aspergillus flavus** *and* **A. parasiticus.**

Lysis was confirmed based on the presence of N-acetylglucosamine (GleNAc), a product of chitin degradation, in a reaction mixture containing hyphae as substrate for chitinase activity. Microscopic examination of the mycelia treated with crude chitinase enzymes showed restricted mycelial growth. All the undiluted crude enzymes caused significant reduction in the mean diameter of fungal colonies in PDA 24 hours after treatment.

Introduction

One of the most common mechanisms of interaction between populations in natural environments is enzyme-induced lysis, that is, one species eliminating another by digesting the cells or hyphae of the second species (Skujins et al., 1965). This type of microbial antagonism is involved in the suppression of fungal pathogens. Mycolytic bacteria that abound in the natural environment release/excrete hydrolytic enzymes that lyze the fungal cell walls. One group of hydrolytic enzymes is chitinases.

Chitinases are a complex and diverse group of enzymes (Gooday, 1991) found in a wide variety of organisms (Flach et al., 1992) that cleave a bond between C1 and C4 of two consecutive N-acetylglucosamines of chitin.

Some early investigations on mycolysis as a means to control certain pathogenic fungi involved addition of chitin and other organic substrates to the soil (soil amendment). This proved effective in the control of *Fusarium oxysporum f. cubense* from artificially infested soil (Mitchell and Alexander, 1961; 1962). The practice of soil amendment resulted in the selective stimulation of chitinoclastic/mycolytic population of microorganisms (Mitchell, 1963). Mitchell and Alexander (1962) reported marked stimulation of chitinase production

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and activity in actinomycetes following soil amendment. This suggested that mycolytic activity and toxin production may be implicated in the selective influence of chitin in suppressing soil fungi.

Working with one chitinoclastic strain of *Bacillus cereus*, Mitchell and Alexander (1963), however, reported that chitinase along with laminarinase appeared to be associated with lysis of *Fusarium oxysporum*. Incubation of the mycelium with chitinase alone did not result in lysis. Likewise, Skujins et al. (1965) showed that extracellular b-(1—3) glucanase and chitinase were instrumental in the dissolution of hyphal walls of *Aspergillus oryzae* and *Fusarium solani*. Earlier, Horikoshi and Iida (1958; 1959) reported enhancement of lysis of *Aspergillus oryzae* by *Bacillus circulans* through the addition of chitinase preparation.

This study demonstrates the lytic activity of some bacterial chitinases in *Aspergillus flavus* and *A. parasiticus in vivo*.

Materials and Methods

Six accessions of bacteria obtained from the Microbial Culture Collection and Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH) were previously selected. Based on their ability to show moderate to heavy growth and form moderate to large zones of clearing around colonies in chitin-containing agar, the bacteria selected include 2 strains each of *Bacillus circulans* and *Serratia marcescens* and a strain each of *Arthrobacter luteus* and *Streptomyces griseus*. Production of crude chitinase enzymes from each bacterium followed the procedure of Ueda and Arai (1992). Chitinase activity in each culture filtrate was assayed using turbidimetric method (Imoto and Yagashita, 1971) and cylinder cup assay method (modified from Roberts and Selitennikoff, 1988).

Aspergillus flavus* and *A. parasiticus used in this study were likewise obtained from BIOTECH . The molds were grown in 0.5% peptone- 0.5% glucose broth medium and incubated with shaking at room temperature for 18-20 hours. The mycelial balls were washed free of medium by centrifugation with 0.85% saline solution prior to use.

The mycolytic activity of the six crude chitinases was assayed based on: (1) optical density changes (turbidimetric method by Imoto and Yagashita, 1971) using wet suspension of mycelial balls instead of colloidal chitin in the reaction mixture, (2) microscopic examination of the mycelial balls immersed in the crude enzyme extract for 24 hours, and (3) growth of the treated mycelial balls on potato dextrose agar (PDA) 24 hours after plating.

Results and Discussion

Aspergillus flavus* Link ex Fries and *A. parasiticus Speare (Figs. 1a and 1b) are two species of molds widely known to produce aflatoxin as secondary metabolites in agricultural commodities before and after harvest. As aflatoxin are highly toxic, consumption of these compounds in contaminated foods has been linked to acute and chronic diseases in humans and animals (Bhat, 1991). As such, control of growth of these toxigenic molds in agricultural produce such as corn, peanuts, copra and the like has become the object of extensive research.

The ability of the crude chitinase enzymes to lyze the cell walls of *Aspergillus flavus* and *A. parasiticus* was determined through turbidimetry. Table 1 summarizes the changes in the optical density and the estimated amount of GlcNAc released in the different reaction mixtures containing the crude enzyme and wet suspension of fungal mycelia. There was no significant difference on the mean change in optical density among the different crude enzymes indicating relatively similar enzymatic activity on the cell wall of *Aspergillus flavus*. On the other hand, much higher amount of GlcNAc was released in the reaction mixtures containing *Arthrobacter luteus* (6Al) and *Bacillus circulans* (7Bc) crude enzymes compared to those containing *B. circulans* (5Bc), *Streptomyces griseus* (9Sg) and *Serratia marcescens* (11Sm) using *A. parasiticus* cell walls as substrate. All the observed changes in the optical density in all the crude enzymes differed significantly from the control for both *A. flavus* and *A. parasiticus*.

Fungal cell wall is a fabric of interwoven microfibrils embedded in or cemented by amorphous matrix substances. Chitin, a polymer of N-acetylglucosamine, is one of the major components of the microfibrillar component of the wall. Blumenthal and Roseman (1957) estimated quantitatively the chitin content of some fungi which included several strains of *Aspergillus flavus* and *A. parasiticus*. Among the aspergilli that were studied, *A. parasiticus* QM 884 was reported to contain the highest chitin (26.2%) after 8 days of growth. In similar experiments, Mitchell and Alexander (1963) and Skunjins et al. (1965) showed that *Pythium debaryanum*, a fungus not known to contain a chitin component in the cell wall was not suppressed by microorganisms degrading chitin.

In the present study, undiluted crude enzymes lysed the mycelia of both *Aspergillus flavus* and *A. parasiticus*. Generally, higher amounts of GlcNAc was released from the mycelia *of A. parasiticus* than from *A. flavus*. Probably, the strain *of A. parasiticus* that was used contained more chitin in their walls compared to *A. flavus* strain, thereby becoming more prone to the attack of chitinases (Mitchell and Alexander, 1963).

It was also observed that a higher amount of GlcNAc was released by the crude enzymes from colloidal chitin than from live mycelia. In fungal cell wall, chitin is normally present in a rigid crystalline state; hence, the chitin is more resistant to attack by the chitinases compared to the colloidal chitin. Gooday (1991) mentioned that nascent chitin (e.g. as newly formed in fungal apices) and colloidal chitin are readily hydrolyzed by chitinases. In addition, bacterial chitinases are exochitinases, so they are restricted to locating non-reducing termini of chitin as substrates which may be difficult to locate in intact fungal cell wall (Roberts and Selitennikoff, 1988). Nevertheless, lysis of fungal cell walls by bacterial chitinases has been reported in *Aspergillus oryzae* (Horikoshi and Sakaguchi, 1958; Horikoshi and Iida, 1959; and Skunjins et al., 1965), *A. candidus, A. oryzae* Sakae and *A. flavus* Link (Tsujisaka et al., 1973), *A. soyae* (Hirokoshi and Sakaguchi, 1958); various species of *Fusarium* (Mitchell and Alexander, 1962; Lloyd et al., 1965; Skunjins et al., 1965; and Morrissey et al, 1976), *Sclerotium rolfsii* (Ordentlich et al., 1988); *Rhizopus* (Tominaga and Tsujisaka, 1976) and *Rhizoctonia solani, Aspergillus* sp., *Fusarium oxysporum, Curvularia lunata* and *Sclerotinia oryzae* (Gupta et al., 1995).

Microscopic observations on the mycelial balls of *A. flavus* and *A. parasiticus* treated with pure chitinase (commercial chitinase) and with the different crude chitinases were done. Distilled water and pure chitinase enzyme served as negative and positive controls, respectively. As shown in Figure 2, some changes in the hyphae of *A. flavus* treated with pure chitinase enzyme were noted including restricted or inhibited mycelial elongation and

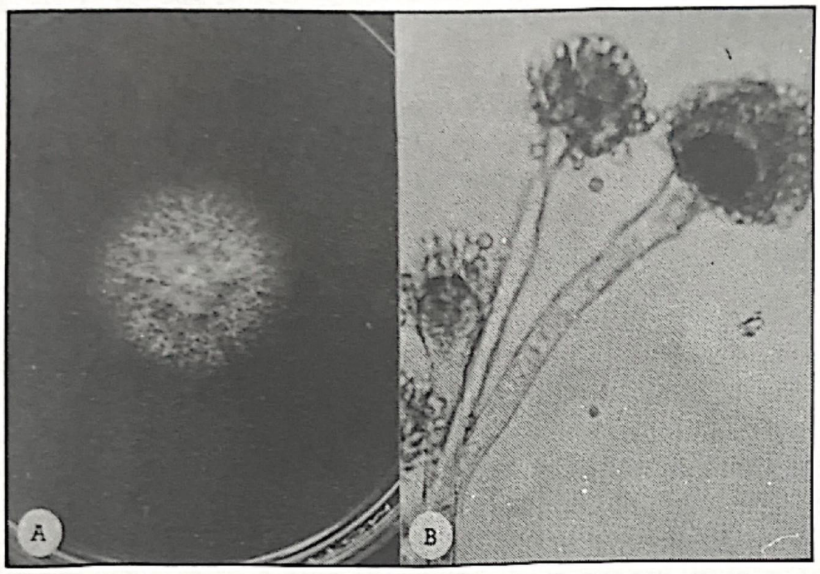

Figure 1a. *Aspergillus flavus* Link ex Fries. BIOTECH 3092 A. Colony, B. Fruiting bodies

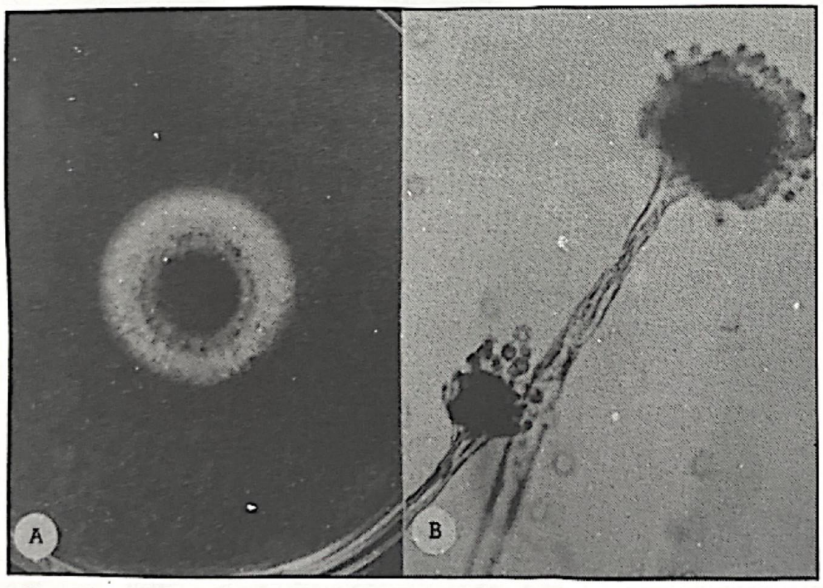

Figure 1b. *Aspergillus parasiticus* Speare BIOTECH 3055 A. Colony, B. Fruiting bodies

Table 1. Activity of the crude chitinase extracts on the mycelia of *Aspergillus flavus* and *A. parasiticus* (turbidimetric method).*

EXTRACT	CULTURES			
	Aspergillus flavus		*A. parasiticus*	
	Δ in O.D. (Mean +/- S.E.)	mg GlcNAc	Δ in O.D. (Mean +/- S.E.)	mg GlcNAc
Control (-)	0a 0	0	0a 0	0
5Bc	0.309b 0.117	0.1087	0.322b 0.076	0.1147
6Al	0.339b 0.117	0.1227	0.330bc 0.055	0.1185
7Bc	0.319b 0.117	0.1134	0.352c 0.072	0.1287
9Sg	0.303b 0.120	0.1059	0.321b 0.072	0.1143
11Sm	0.297b 0.120	0.1031	0.314b 0.065	0.1110
14Sm	0.327b 0.119	0.1166	0.343b 0.077	0.1245

Mean values with dissimilar letters show significant differences based on DMRT.
*Average of three trials with two replications per trial

Legend:

Control - distilled water
5Bc - *Bacillus circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

hyphal swelling which were not observed in the mycelia treated with distilled water. When mycelia of *A. flavus* were treated with different dilutions (undiluted, 4:1; 1:1 and 1:5) of the six crude chitenase enzymes, only those treated with undiluted and 4:1 consistently caused inhibited mycelial elongation. In general, the 4:1 dilution caused only a lesser extent of inhibition on the elongation of mycelia compared to the undiluted. Hyphal swelling was not observed even if the treatment was extended beyond 24 hours. No other changes on the mycelia were noted.

Similarly, treating the mycelial balls of *A. parasiticus* with pure chitinase enzyme inhibited mycelial elongation and caused hyphal swelling (Fig. 3). Distilled water did not cause any observable change. With various dilutions (undiluted, 4:1, 1:1 and 4:1) of the crude enzymes, only the undiluted and 4:1 dilutions inhibited mycelial elongation. No hyphal swelling was observed. Some mycelial balls of *A. flavus* and *A. parasiticus*, which were immersed in the crude chitinase enzymes for 24 hours were grown in potato dextrose agar (PDA). After incubation for 24 hours, the colony diameter was measured. Tables 2 shows significant reduction in mean diameter of the colonies of *A. flavus* treated with the two *B. circulans* and two *S. marcescens* undiluted enzymes 24 hours after transferring the mycelial balls on PDA compared with the negative control. Further, these undiluted enzymes inhibited *A. flavus* growth as effectively as pure chitinase. All the diluted samples did not hinder *A. flavus* proliferation.

All the undiluted crude enzymes caused significant reduction in the mean diameter of the colonies *of A. parasiticus* 24 hours after growing the mycelial balls on PDA (Table 3). At 4:1 dilution, only *Bacillus circulans* (5Bc) and *B. circulans* (7Bc) enzymes inhibited colonial development.

The pronounced swelling of hyphae of *A. flavus* and *A. parasiticus* in the chitinase-treated mycelia must have been due to chitin breakdown on some sites thus weakening them. Actively growing sites in the tips, septa and branches of fungal hyphae are sensitive to chitinases and as mentioned by Gooday (1991), chitin that is newly-formed in fungal apices is readily hydrolyzed owing to their still amorphous form (Cabib, 1988). Frandberg and Schnurer (1994) stated that in hyphal apex, chitin is plastic and sensitive to chitinases. The activity of the enzyme, however, is insufficient to weaken the wall to its bursting point, but enough to temporarily increase the 'plastic' area in the growing tips thereby giving rise to swollen hyphae. Benhamou et al. (1993) demonstrated morphological changes in the hyphal tips of *Rhizoctonia solani* about one hour after treatment with bean endochitinase. Hyphal tip swelling was the main feature of the reaction of most fungal cells. Observations at higher magnification revealed that the walls appeared shredded, swollen and less electron opaque. With prolonged exposure to chitinase, nearly all fungal cells were severely damaged and showed disintegration. These observations could have resulted from disruption of chitin molecule causing wall breakdown and protoplasm alteration. Similarly, *Serratia marcescens* culture filtrate caused swift swelling and bursting of hyphal tips of *Sclerotium rolfsii*. Futhermore, Inbar and Chet (1991) provided evidence that chitinase of *Aeromonas caviae* can disintegrate and cause collapse of mycelia of *S. rolfsii* and *F. oxysporum* f. sp. *vasinfectum*.

Similar microscopic observations were reported by Barrows-Broaddus and Kerr (1981) wherein *Arthrobacter* caused inhibition of *Fusarium moniforme* var. *subglutinans*. Hyphae of the fungus growing near the isolate *Arthrobacter* were enlarged producing vesicular-like structures. In addition, Lopez-Romero et al. (1982) reported that a chitinase from *Mucor rouxii* was an inhibitor of chitin synthethase, the enzyme that catalyzes the formation of

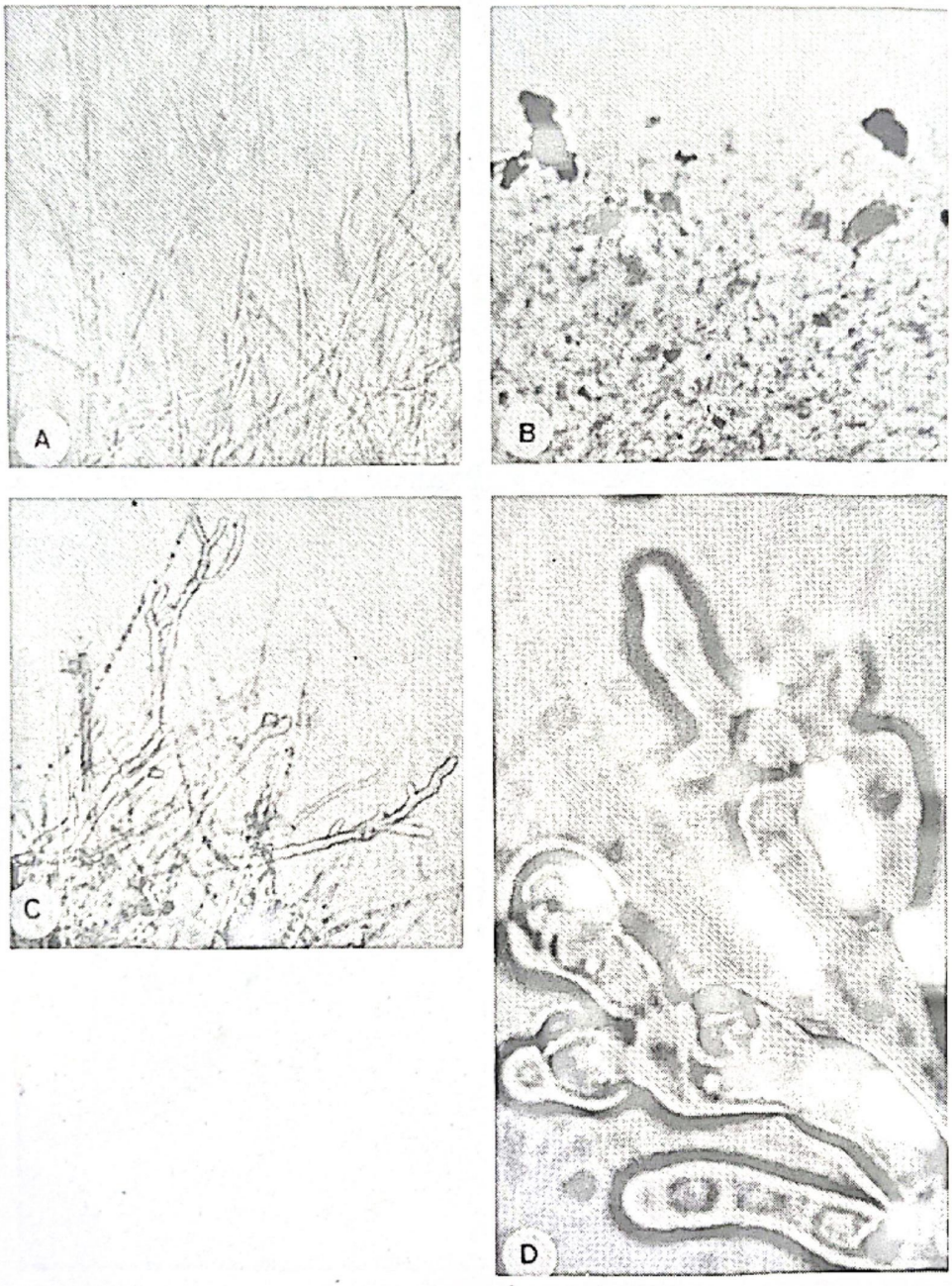

Figure 2. Mycelia of *Aspergillus flavus* treated with distilled water and pure chitinase. Restricted growth and hyphal swelling on mycelia treated with pure chitinase (B,C,D), but not in distilled water treated mycelia (A). A,B,C-100X,D-1000X.

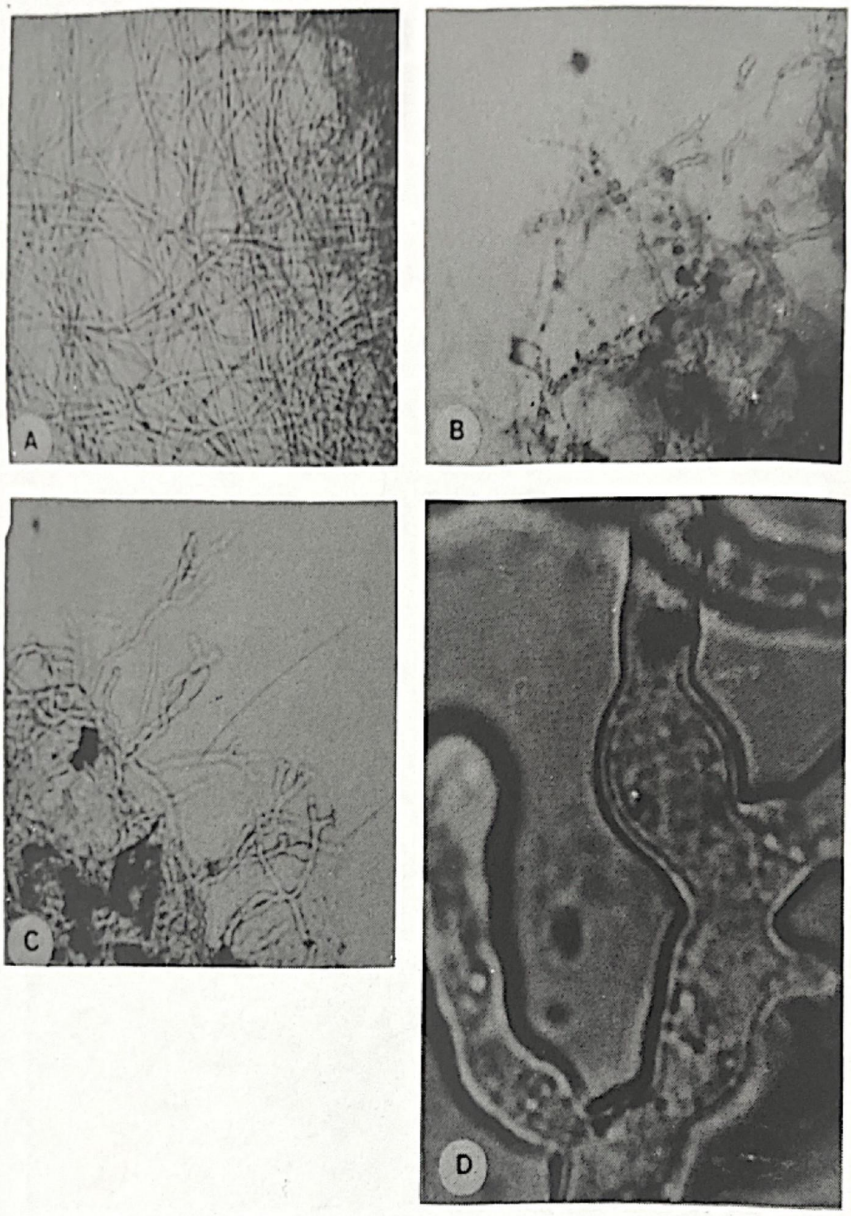

Figure 3. Mycelia of *Aspergillus parasiticus* treated with distilled water and pure chitinase. Restricted growth and hyphal swelling on mycelia treated with pure chitinase (B,C,D), but not in distilled water treated mycelia (A). A,B,C-100X, D-1000X.

Table 2. Growth of crude chitinase extract-treated *Aspergillus flavus* on potato dextrose agar (PDA) after 24 hours.

EXTRACT	DIAMETER OF COLONIES (cm)*			
	Undiluted (Mean+/- S.E.)	4:1 (Mean+/- S.E.)	1:1 (Mean+/- S.E.)	1:5 (Mean+/-S.E.)
Control (-) (distilled water)	1.20a 0.14			
Control (+) (pure chitinase)	0.30b 0.16			
5Bc	0.40b/y 0.20	1.48a/z 0.11	1.78a/z 0.26	1.53a/z 0.28
6Al	0.78ab/y 0.36	1.65a/z 0.27	1.63a/z 0.19	1.48a/z 0.11
7Bc	0.40b/y 0.14	1.48a/z 0.24	1.80a/z 0.27	1.50a/z 0.14
9Sg	0.90a/y 0.21	1.63a/z 0.15	1.60a/z 0.21	1.40a/z 0.17
11Sm	0.20b/y 0.14	1.50a/z 0.14	1.73a/z 0.26	1.48a/z 0.18
14Sm	0.20b/y 0.14	1.28a/y 0.23	1.98a/z 0.11	1.83a/z 0.24

Mean values with dissimilar letters in columns (extract) and in rows (dilution) show significant differences based on DMRT.
*Average of three trials with two replications per trial.

Legend:

Control (-) - distilled water
Control (+) - pure chitinase
5Bc - *Bacillus circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm- *Serratia marcescens*
14Sm- *S. marcescens*

Table 3. Growth of crude chitinase extract-treated *Aspergillus parasiticus* on potato dextrose agar (PDA) after 24 hours.

EXTRACT	DIAMETER OF COLONIES (cm)*			
	Undiluted Mean +/-S.E.	4:1 (Mean +/-S.E.)	1:1 (Mean+/-S.E.)	1:5 (Mean+/-S.E.)
Control (-) (distilled water)	1.35a 0.18			
Control (+) (pure chitinase)	0.28b 0.11			
5Bc	0.30b/y 0.14	0.49b/y 0.16	1.53a/z 0.22	1.83a/z 0.43
6Al	0.40b/y 0.21	1.50a/z 0.14	1.60a/z 0.19	1.98a/z 0.41
7Bc	0.10b/y 0	0.48b/y 0.18	1.55a/z 0.25	1.80a/z 0.45
9Sg	0.30b/y 0.20	1.55a/z 0.12	1.65a/z 0.24	1.73a/z 0.42
11Sm	0.50b/y 0.14	0.88a/z 0.24	1.55a/z 0.21	1.95a/z 0.42
14Sm	0.20b/y 0.14	0.93a/z 0.25	1.58a/z 0.23	1.93a/z 0.18

Mean values with dissimilar letters in columns (extract) and in rows (dilution) show significant differences based onDMRT.
*Average of three trials with two replications per trial.

Legend:

Control (-) - distilled water
Control (+) - pure chitinase
5Bc - *Bacillus circulans*
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7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm- *Serratia marcescens*
14Sm- *S. marcescens*

chitin from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) located mainly in the cell wall fraction. With this information, it can also be speculated that the swelling of hyphal tips is due to incomplete synthesis of the cell wall. Since metabolism still continues, additional cell constituents could have caused enlargement of the hyphal tips.

The restricted elongation of mycelia of *A. flavus* and *A. parasiticus* treated with the crude chitinases is also attributed to chitin breakdown. Barrows-Broadduss and Kerr (1991) observed that hyphae of *Fusarium moniliforme* var. *subglutinans* growing near the chitinolytic *Arthrobacter* were distorted and wrinkled in comparison with the normal hyphae. The absence of hyphal swelling in these treated mycelia is probably due to low enzyme activity. Diluting the crude enzymes further lowered the enzyme activity thus no effects were observed in the enzymes diluted above 4:1.

Significant reduction in the colony diameter of *A. flavus* treated with the two *B. circulans* and two *S. marcescens* extracts 24 hours after transferring the mycelial balls on PDA was noted when compared to the negative control. The reduction in the diameter of the colonies grown from balls treated with crude enzymes might have been a consequence of inhibition of mycelial elongation.

References Cited

Barrows-Broaddus, J., and T.K. Kerr. 1981. Inhibition of *Fusarium moniliforme* var. *subglutinans*, the causal agent of pine pitch canker, by the soil bacterium *Arthrobacter* sp. Can. J. Microbiol. 27:20-27.

Benhamou, N., K. Broglie, R. Broglie and I. Chet. 1993. Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultrastructural changes and cytochemical aspects of chitin breakdown. Can. J. Microbiol. 39:318-328.

Bhat, R. V. 1991. Aflatoxins: Successes and failures of three decades of research, In: B.R.Champ, E. Highly, A.D. Hocking and J.I. Pitt (eds.). Fungi and mycotoxins in stored products. Proceedings of an international conference held at Bangkok, Thailand, 23-26 April 1991.

Blumenthal, J. H. and S. Roseman. 1957. Quantitative estimation of chitin in fungi. J. Bact. 74:222-224.

Cabib, E. 1988. Chitinase from *Serratia marcescens*. In: W.A. Wood and S.T. Kellogg. (eds.). Methods in Enzymology. Vol. CLXI. California: Academic Press, 574 pp.

Flach, J., P.E. Pilet and P. Jolles. 1992. What's new in chitinase research? Experentia (Basel) 48:701-716.

Frandberg, E. and J. Schnurer. 1994. Evaluation of a chromogenic chito-oligosaccharide analogue, p-nitrophenyl-β-D-N,N'- diacetylchitobiose, for the measurement of the chitinolytic activity of bacteria. J. Appl. Bact. 76:259-263.

Gooday, G. W. 1991. Chitinases. In: G.F. Leatham, and M.E. Himmel (eds.) Enzymes in Biomass Conversion. Washington D.C.: American Chemical Society, 520pp.

Gupta, R. R.K. Saxena, P. Chaturvedi and J. S. Virdi. 1995. Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. J. Appl. Bact. 78:378-383.

Horikoshi, K. and S. Iida. 1958. Lysis of fungal mycelia by bacterial enzymes. Nature 181:917-918.

Horikoshi, K. and K. Sakaguchi. 1958. Studies on autolysis of *Aspergillus oryzae*. The lytic phenomenon of *Aspergillus oryzae* caused by *Bacillus circulans*. J. Gen. Appl. Microbiol. 4:1-11.

Horikoshi, K. and S. Iida. 1959. Effect of lytic enzyme from *Bacillus circulans* and chitinase from *Streptomyces sp.* on *Aspergillus oryzae*. Nature 183:186-187.

Imoto, T. and K. Yagishita. 1971. A simple activity measurement of lysozyme. Agr. Biol. Chem. 35(7):1154-1156.

Inbar, J. and I. Chet. 1991. Evidence that chitinase poduced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. Soil Biol. Biochem. 23:973-978.

Lloyd, A. B., R. L. Noveroske, and J. L. Lockwood. 1965. Lysis of fungal mycelium by *Streptomyces* spp. and their chitinase system. Phytopathology. 55:871-875.

Lopez-Romero, J. R. Herrera and S. Bartnicki-Garcia. 1982. The inhibitory protein of chitin synthetase from *Mucor rouxii* is a chitinase. 702:233-236.

Mitchell, R. 1963. Addition of fungal cell wall compounds to soil for biological disease control. Phytopathology 53:1068-1071.

Mitchell, R. and M. Alexander. 1961. The mycolytic phenomenon and biological control of *Fusarium* in soil. Nature 190: 109-110.

Mitchell, R. and M. Alexander. 1962. Microbiological processes associated with the use of chitin for biological control. Proc. Soil Science Soc. 26:556-558.

Mitchell, R. and M. Alexander. 1963. Lysis of soil fungi by bacteria. Can. J. Microbiol. 9:169-177.

Morrissey, R. F. E. P. Dugan and J.S. Koths. 1976. Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. Soil Biol. Biochem. 8:23-38.

Ordentlich, A., Y. Elad and I. Chet. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84-88.

Roberts, W.K. and C. P. Selitennikoff. 1988. Plant and bacterial chitinases differ in antifungal activity. J. of Gen. Microbiol. 134:169-176.

Skujins, J.J., H.J. Potgieter and M. Alexander. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1—3) glucanase. Arch. Biochem. Biophys. 111:358-364.

Tominaga, Y. and Y. Tsujisaka. 1976. Purification and some properties of two chitinases from *Streptomyces orientalis* which lyse *Rhizophus* cell wall. Agr. Biol. Chem. 40:2325-2333.

Tsujisaka, Y., Y. Tominaga, and M. Iwai. 1973. Taxonomic characters and culture conditions of a bacterium which produces a lytic enzyme on *Rhizopus* cell wall. Agr. Biol. Chem. 37(11):2517-2525.

Ueda, M. and M. Arai. 1992. Purification and some properties of chitinases from *Aeromonas* sp. No. 10S-24. Biosci. Biotech. Biochem. 56:460-464.