

Full Length Research Paper

# Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus*

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Accepted 22 January, 2010

*In vitro* tests of interactions between *Streptomyces griseus* strains and some soil-borne plant pathogens (*Fusarium oxysporum*, *Alternaria alternata*, *Rhizoctonia solani* and *Fusarium solani*) and 2 isolates of *Aspergillus flavus* were studied on PDA medium. Strains tested produced a metabolite that inhibited growth of plant pathogenic fungi on PDA medium (dual culture test). When grown in liquid medium having fungal cell walls as sole carbon source, *S. griseus* produced chitinase enzyme in the medium. Higher levels of this enzyme were induced by cell wall of *Aspergillus flavus* and the crude chitinase enzyme extracted showed zone of inhibition on all pathogens inoculated PDA plates at all tested concentrations. When lytic enzyme produced by *S. griseus* was incubated with hyphal wall of the test fungi treated with 2 M NaOH and chloropharm: Methanol, the release of glucose and N acetyl glucosamine significantly increased relative to the untreated one. This result suggests that proteins in the cell walls of pathogens may make these walls more resistant to degradation by the extracellular lytic enzymes. Ionic strength of NaOH on lytic activity was tested, where as the enzymes lysed fungal cell wall best at ionic concentration of 2 M treatment. Pretreatment with alkali or proteolytic enzyme increases their susceptibility for lysis. *In vitro* lytic activity provides an appropriate condition and the effect of biocontrol organism in field level treatment.

**Key words:** Chitinase enzyme, dual culture test, plant pathogenic fungi, NaOH treatment and Ionic strength.

## INTRODUCTION

Plants in their environment exposed to deleterious organisms such as fungi, bacteria, viruses, nematodes, etc and they are responsible for plant diseases, which affects the crop production worldwide and cause chronic threat to agricultural food production. The agricultural production in over the past few decades, producers dependent on use of chemical inputs causes several negative effects, that is, development of pathogen resistance to the applied agents and their environmental impact. Thus there is need to control pathogens. Crop rotation, breeding for resistant plant varieties and applying pesticides are inefficient to control the root diseases of vegetative crops (Compant et al., 2005). The use of microorganisms to control plant pathogen is a suitable and eco friendly alternative way of reducing the use

of chemicals in agriculture.

Actinomycetes, particularly *Streptomyces* sp. are gram positive filamentous bacteria that produce and secrete a wide array of biologically active compounds including antibiotics, hydrolytic enzymes and enzyme inhibitors. They are resistant to desiccation and nutrient stress, by their ability to produce spores (Compant et al., 2005; Fravel et al., 2005; Shantikumar Singh et al., 2006). Though the fungal cell wall is made up of mainly of glucan and chitin, the  $\beta$ -1, 3-glucanase and chitinase are key enzymes responsible for fungal cell wall lysis and degradation. (Kucuk and Kivanc, 2004) *Streptomyces* chitinase have been implicated against a variety of plant pathogenit fungi (Gupta et al., 1995; Gomes et al., 2000; Taechowisan et al., 2003). Many biological control agents in the last few years are being tested and are not commercially available. However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their biocontrol effectiveness.

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Our main objective was to increase our understanding of the relationship between chitinolytic and anti-fungal properties of actinomycetes that occur naturally against the biological control of various pathogens.

## MATERIALS AND METHODS

### Organism, source of plant pathogenic fungi

Chitinase enzyme producing *Streptomyces griseus* (MTCC - \*4734) strain purchased from Microbial Type Culture Collection, Chandigarh, India was used for the current enzyme extraction, purification and antagonistic activity studies.

The following plant pathogenic fungi: AFC<sub>1</sub> - *Fusarium oxysporum*; AFC<sub>2</sub> - *Alternaria alternata*; AFC<sub>3</sub> - *Rhizoctonia solani*; AFC<sub>4</sub> - *Fusarium solani*; AFC<sub>5</sub> - *Aspergillus flavus* and AFC<sub>6</sub> - *A. flavus* were kindly obtained from Central Institute for Cotton Research, Coimbatore. The spores were maintained in PDA slants and stored at 4°C until use.

### Dual culture test of *S. griseus*

Antagonistic activity was observed directly on plates of YMA medium (containing the following: 10.0 g glucose; 5.0 g peptone; 3.0 g yeast extract; 3.0 g malt extract; 20.0 g agar; 1000 ml distilled water and pH 6.2) using modification of the hyphal extension-inhibition assay (Fenice et al., 1998), where actively growing *S. griseus* were streaked on the one edge and the phytopathogenic fungal strains on the opposite side of a Petri dish containing YMA medium. After the desired incubation time at 28°C, an inhibitory effect of growing *S. griseus* against growth of all the test pathogenic fungi was measured and tabulated. Percent inhibition (zone of inhibition) of test pathogen by antagonistic strain was calculated.

### Preparation of hyphal wall

All the isolated plant pathogenic fungal cultures were inoculated into 50 ml of YM broth and incubated at 30°C for 5 days. After incubation the mycelia was collected by filtration. The mycelia were thoroughly washed with autoclaved distilled water and homogenized on ice, with a homogenizer for 5 min. The mycelial suspension was centrifuged at 15,000 rpm for 20 min at 4°C (Remi - C 24). The pellet was resuspended in distilled water and sonicated on ice 4 times for 5 min using heat systems - ultrasonics sonicator at full amplitude. The suspension was centrifuged at 8000 rpm for 10 min at 4°C (Remi - C 24) to precipitate the coarse particle. Then precipitate was washed, air dried and stored at 4°C (Sivan and Chet, 1989).

### Qualitative determination of lytic enzyme activity

*S. griseus* which showed antagonistic behavior to one or more fungi were tested to find whether they also produced the lytic enzyme chitinase, on chitin agar plates. In addition enzyme assays were made under conditions that were similar to those existing in the *in vitro* antagonism test, namely in MS medium that contained the following (g/l); 1 g each fungal cell wall, 5 g peptone, 5 g Yeast, 0.7 g KH<sub>2</sub>PO<sub>4</sub>, 0.3 g K<sub>2</sub>HPO<sub>4</sub>, 4 g NaCl, 5 g MgSO<sub>4</sub> 7H<sub>2</sub>O, 1 mg FeSO<sub>4</sub> 7H<sub>2</sub>O, 0.1 mg ZnSO<sub>4</sub> 7H<sub>2</sub>O, 0.1 mg MnSO<sub>4</sub> 7H<sub>2</sub>O, and 1 g NH<sub>3</sub>SO<sub>4</sub>. 1 ml of spore suspension was inoculated and incubated for seven days at 30°C at 125 shakes/min (Remi - R 8C). At the end of incubation, the culture was centrifuged at 15,000 rpm for 10 min at 4°C. (Remi - C 24). The collected supernatant was tested directly

for chitinase enzyme activity (Kim et al., 2003).

### Chitinase assay

Colloidal chitin (Sigma Chemicals Co., USA) was used as a substrate with reference to Wen et al. (2005). 0.3 ml of 1% colloidal chitin in acetate buffer (50 mM, pH 6.0) was taken to that 1 ml of enzyme was added and incubated at 30°C for 30 min. The hydrolysis reaction was terminated by adding 0.6 ml of dinitrosalicylic acid (DNS) reagent. The mixture was kept in a boiling water bath for 15 min, chilled and centrifuged to remove the insoluble chitin. The resulting adduct was measured in UV double beam spectrophotometry (Systronics - 2101) at 540 nm (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of N-acetyl D-glucosamine per ml in minutes.

### Inhibition of fungal growth by crude enzyme extract

The possible involvement of actinomycetes chitinase in the antagonism towards other pathogenic fungi was determined indirectly in YMA medium uniformly spread with plant pathogenic fungi. Filter paper discs were laid on the swabbed plates, in to each disk 50 µl of the purified lyophilized enzyme at the concentration of 5, 50 and 100 U extracted from *S. griseus* was added. For control 50 µl of distilled water was added. Fungal growth was observed over 4 days of incubation at 30°C. The radial diameter of the zone formation was measured (Gomes et al., 2001).

### Modification of hyphal cell walls

For modification of hyphal walls; a total of 4 mg of cell walls of isolated fungal cultures were suspended and kept in a rotatory shaker for 1 h at 50 rpm (Remi - R 8 C) in 6 ml of (a) 2 M NaOH at 25°C, and (b) chloroform/methanol (2:1, v/v) at 37°C. After 1 h treatment, the hyphal walls were collected by centrifugation at 10000 rpm for 10 min at 4°C (Remi - C 24). The pellets were washed thoroughly with distilled water. Finally the pellet was suspended in acetate buffer (50 mM, pH 5.0) (Sivan and Chet, 1989).

### Hydrolytic activity of *S. griseus* culture filtrate

For assessing the hydrolytic activity, the reaction mixture (1 ml) containing 1 mg/ml of cell wall with 0.3 ml of crude enzyme was incubated at 38°C for 24 h. Each test tube was amended with 10 µl of toluene to prevent contamination. The released total reducing sugars (Miller, 1959); glucose (Nelson, 1944); and the N-acetyl D-glucosamine (GlcNAc) was measured (Reissig et al., 1959) in treated and untreated fungal cell walls.

### Effect of ionic strength of NaOH on lytic activity

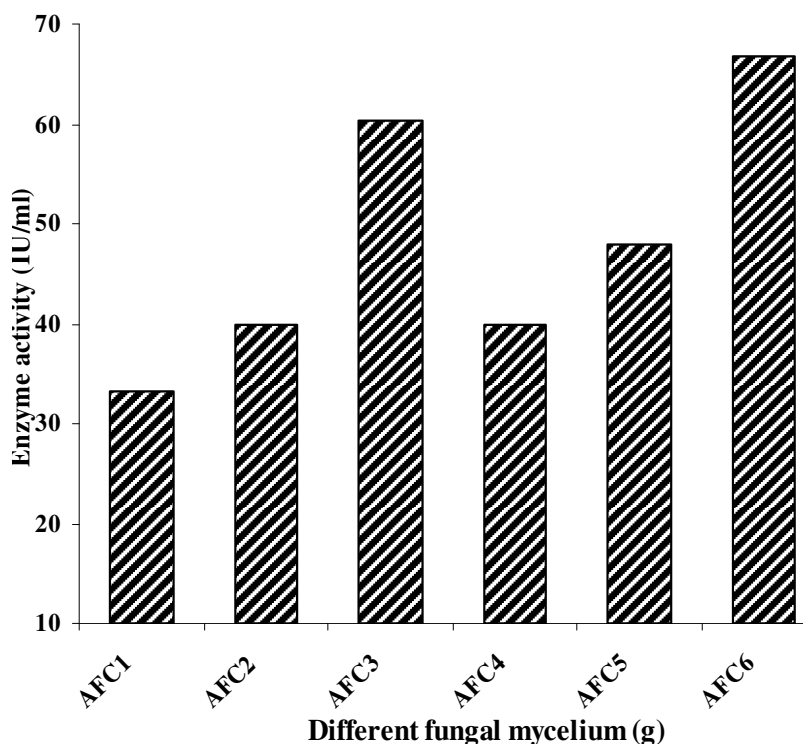
The effect of ionic strength on lytic activity of *S. griseus* producing chitinase enzyme against cell walls of fungal pathogens was determined. For this experiment fungal cell walls were suspended in NaOH concentration ranges from 0.5 - 3.0 M. The tubes were then incubated at 25°C for 1 h at 50 rpm in a shaking incubator. At the end of incubation the tubes were taken and the released N-acetyl D-glucosamine (NAG) was assayed.

Reproducibility: In all the experiments, three replicates of each treatment were used. The results were presented as mean values

**Table 1.** Anti-fungal activity screening of *S. griseus* against fungal pathogens - Dual culture test.

Pathogens studied	Percent Inhibition
AFC <sub>1</sub> - <i>Fusarium oxysporum</i>	55.6 ± 0.12
AFC <sub>2</sub> - <i>Alternaria alternate</i>	66.6 ± 0.12
AFC <sub>3</sub> - <i>Rhizoctonia solani</i>	50.0 ± 0.11
AFC <sub>4</sub> - <i>Fusarium solani</i>	80.0 ± 0.09
AFC <sub>5</sub> - <i>Aspergillus flavus</i>	68.8 ± 0.57
AFC <sub>6</sub> - <i>Aspergillus flavus</i>	72.2 ± 0.17

Values are mean ± standard error of triplicates.



**Figure 1.** Production of endochitinase of *S. griseus* against cell wall of isolated fungal pathogens as a sole carbon source (AFC<sub>1</sub> – *F. oxysporum*; AFC<sub>2</sub> – *A. alternate*; AFC<sub>3</sub> – *R. solani*; AFC<sub>4</sub> – *F. solani*; AFC<sub>5</sub> – *A. flavus*; AFC<sub>6</sub> – *A. flavus*).

of triplicates ± Standard errors.

## RESULTS

In the dual culture, an inhibitory effect of growing *S. griseus* against growth of pathogenic fungal strains was observed. The diameter of zone of clearance was measured and percent inhibition was recorded in Table 1. It showed zone of inhibition of 3.0 cm and percent inhibition of 80.0 ± 0.09 against *F. solani*.

The lytic enzyme production by the strain *S. griseus* were tested using cell walls of six pathogens as the sole

carbon source. Maximum chitinase enzyme activity of 66.67 ± 0.0 IU/ml was observed against *A. flavus* fungal cell wall and minimum enzyme activity of 33.3 ± 0.13 IU/ml was observed in *F. oxysporum* cell wall (Figure 1).

*In vitro* antifungal activity of the crude chitinase of *S. griseus* was confirmed against all phytopathogenic fungi. The diameter of zone of clearance was recorded in Table 2. Zone of clearance of 1.0 ± 0.00 cm at 100 U concentration of chitinase enzyme against *F. oxysporum*, 3.0 ± 0.03 cm against of *A. alternate* and 1.9 ± 0.02 cm against *R. solani* was observed.

The possible interference of fungal cell wall moiety in

**Table 2.** Anti fungal activity of *S. griseus* enzyme chitinase against fungal pathogens.

Pathogens studied	Zone of inhibition in diameter (cm)
AFC <sub>1</sub> - <i>Fusarium oxysporum</i>	1.0 ± 0.00
AFC <sub>2</sub> - <i>Alternaria alternata</i>	3.0 ± 0.03
AFC <sub>3</sub> - <i>Rhizoctonia solani</i>	1.5 ± 0.03
AFC <sub>4</sub> - <i>Fusarium solani</i>	1.9 ± 0.02
AFC <sub>5</sub> - <i>Aspergillus flavus</i>	1.0 ± 0.02
AFC <sub>6</sub> - <i>Aspergillus flavus</i>	1.0 ± 0.03

Values are mean ± standard error of triplicates.

the mycoparasitic activity of *S. griseus* strains was tested by treating them with alkali or organic solvent, prior to incubation with the lytic enzymes. As per Table 3, chitinase enzyme of *S. griseus* incubated with NaOH and treated *F. oxysporum* fungal cell wall released  $49 \pm 0.0$  µg/ml of glucose and  $68.7 \pm 0.0$  µg/ml of NAG compared to  $12 \pm 0.1$  µg/ml of glucose and  $18.6 \pm 0.0$  µg/ml of NAG of untreated cell wall (Table 3). In contrast, chloroform: methanol treatment had only a slight effect on sugar releasing.

Lytic activity of *S. griseus* producing chitinase enzyme against cell walls of fungal pathogens treated with different NaOH concentration was determined. Maximum lytic activity of  $80.95 \pm 0.0$  µg/ml of NAG was released from the *F. oxysporum* fungal cell wall treated with 2.0 M NaOH concentration (Figure 2).

## DISCUSSION

Antifungal activity was also observed directly on plates of YMA medium using spread inoculation instead of punctuate inocula. An inhibitory effect of growing *S. griseus* against growth of all pathogenic fungal strains was observed. The *S. griseus* producing higher inhibition probably due to the presence of some inhibitory substance, antibiotics and other enzymes such as glucanases, protease essential for complete cell-wall lysis. Similarly, an inhibitory effect of growing *Streptomyces* RC1071; *Streptomyces lydicus* WYEC108 against growth of wider range of pathogenic fungal strains was reported previously (Yuan and Crawford, 1995; Gomes et al., 2000, 2001).

The lytic enzyme production by the test strain *S. griseus* induced by the presence of all phytopathogen cell walls as the carbon source, suggested that these substrates can also act as inducers of lytic enzyme synthesis. Higher chitinase enzyme production from *Streptomyces aureofaciens* CMUAc130 using fungal cell wall of different pathogens was reported by Taechowisan et al. (2003). This study was also supported by Sivan and Chet (1986) and Elad et al. (1982).

Antifungal activity of the crude chitinase of *S. griseus* showed zone of clearance of  $1.0 \pm 0.00$  cm at 100 U con-

centration of chitinase enzyme of *S. griseus* against *F. oxysporum* (Table 2). This result suggested that the inhibition factor was thermosensitive and that commercial chitinase alone was not sufficient for complete growth inhibition. Similarly antifungal activity of purified chitinase enzyme against fungal pathogens was proved previously (El-Tarabil et al., 2000; Gomes et al., 2000, 2001; Taechowisan et al., 2003; Von der Weid et al., 2003).

In the present study, *S. griseus* was shown to produce a high level of chitinase enzyme when grown in the presence of all fungal cell wall. The chitinase produced on this substrate was active against all other fungi as measured by the release of sugars from their cell walls (Gomes et al., 2001; Lima et al., 1999; Mahadevan and Crawford, 1997 and Gupta et al., 1995).

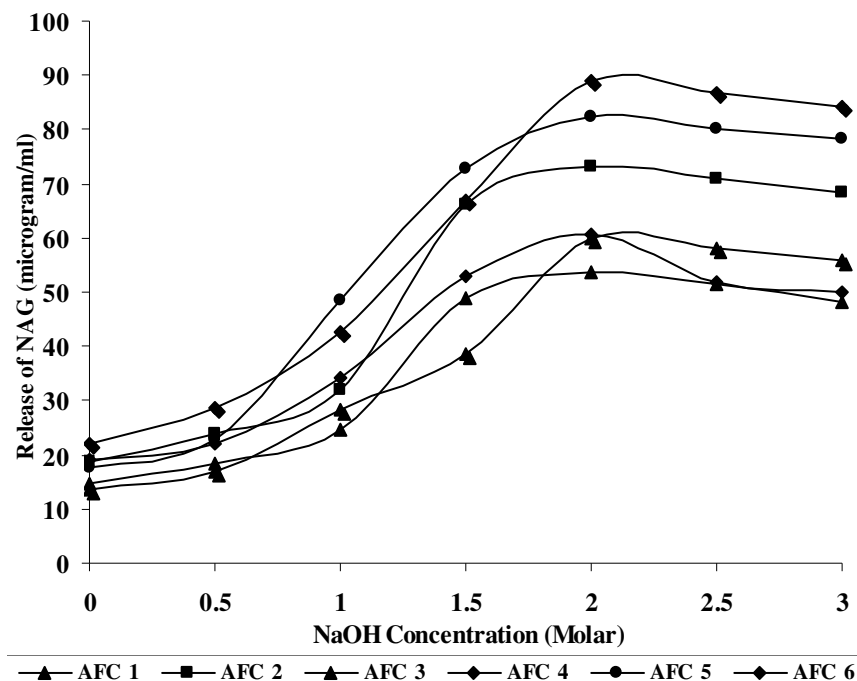
The role of chitinase activity against the fungal cell wall is evident (Lim et al., 1991; Thara and Guanamanickam, 1994). In the present study, we found that enzyme content degraded the hyphal wall of *R. solani* whereas *Fusarium* sp. cell walls were more resistant. Sivan and Chet (1986, 1989) have argued that *Fusarium* species cell walls contain more protein than do walls of other fungi. Our data seem to confirm this hypothesis. Chitinase enzyme incubated with NaOH treated fungal cell walls of pathogens released more glucose compared with untreated one. This result suggested that alkali weaken the forces present between the substances in the cell wall and increases their susceptibility for lysis. From this, the study concludes that NaOH renders the cell wall structures more accessible for the chitinases. It implies that *in vitro* studies of field level, the usage of *S. griseus*, along with NaOH will provide better biocontrol efficiency. Also that, to reduce the implication of cell wall proteins, proteolytic digests should be performed after NaOH treatment. This study was supported by Elad et al. (1982) and Kudu and Kivanc (2004).

Antagonistic activity of several *Streptomyces* sp. against a number of fungal pathogenic species has been known for a long time (Crawford et al., 1993). Also, in greenhouse experiments, *Streptomyces* sp. has conferred various degrees of protection on different plant species against soil-borne pathogenic fungi (Yuan and Crawford, 1995). Very high protein content of clamydospores of *Fusarium* sp. (7 - 28%) may be responsible for their ability to

**Table 3.** Effect of NaOH and Chloroform/Methanol treatment on the release of monomers from plant pathogenic fungal cell wall by chitinase of *S. griseus*.

Pathogens studied*	Release of monomers ( $\mu\text{g/ml}$ ) after treatment								
	Control			2 M - NaOH			Chloroform/ Methanol		
	Total sugars	Glucose	NAG	Total sugars	Glucose	NAG	Total sugars	Glucose	NAG
AFC <sub>1</sub> - <i>Fusarium oxysporum</i>	31 $\pm$ 0.0	12 $\pm$ 0.1	18.6 $\pm$ 0.0	113.7 $\pm$ 0.0	45 $\pm$ 0.0	68.7 $\pm$ 0.0	53.2 $\pm$ 0.0	24 $\pm$ 0.05	31 $\pm$ 0.0
AFC <sub>2</sub> - <i>Alternaria alternate</i>	33.1 $\pm$ 0.0	8.5 $\pm$ 0.0	18.6 $\pm$ 0.0	130.8 $\pm$ 0.0	55.3 $\pm$ 0.0	82.5 $\pm$ 0.0	58.7 $\pm$ 0.0	19.3 $\pm$ 0.0	30.7 $\pm$ 0.0
AFC <sub>3</sub> - <i>Rhizoctonia solani</i>	31.2 $\pm$ 0.1	8.6 $\pm$ 0.0	16.6 $\pm$ 0.0	111.2 $\pm$ 0.15	51 $\pm$ 0.02	60.2 $\pm$ 0.05	58.2 $\pm$ 0.0	26.5 $\pm$ 0.0	31 $\pm$ 0.0
AFC <sub>4</sub> - <i>Fusarium solani</i>	32.2 $\pm$ 0.0	6.2 $\pm$ 0.05	19 $\pm$ 0.25	118.2 $\pm$ 0.0	58 $\pm$ 0.0	60.2 $\pm$ 0.0	51.5 $\pm$ 0.1	29.4 $\pm$ 0.0	38 $\pm$ 0.1
AFC <sub>5</sub> - <i>Aspergillus flavus</i>	32.4 $\pm$ 0.1	8.8 $\pm$ 0.0	17.6 $\pm$ 0.0	124.3 $\pm$ 0.0	52 $\pm$ 0.0	72.3 $\pm$ 0.0	44.6 $\pm$ 0.0	17.3 $\pm$ 0.1	32 $\pm$ 0.0
AFC <sub>6</sub> - <i>A. flavus</i>	35.5 $\pm$ 0.0	12.5 $\pm$ 0.0	22 $\pm$ 0.0	136.9 $\pm$ 0.0	58 $\pm$ 0.0	80.9 $\pm$ 0.0	64.8 $\pm$ 0.0	20.6 $\pm$ 0.0	40 $\pm$ 0.0

Values are mean  $\pm$  standard error of triplicates.



**Figure 2.** Ionic strength optimum for lytic activity of fungal cell wall by enzyme endochitinase of *S. griseus* (AFC<sub>1</sub> - *Fusarium oxysporum*; AFC<sub>2</sub> - *Alternaria alternate*; AFC<sub>3</sub> - *Rhizoctonia solani*; AFC<sub>4</sub> - *Fusarium solani*; AFC<sub>5</sub> - *Aspergillus flavus*; AFC<sub>6</sub> - *Aspergillus flavus*).

resist lysis in soil. Up to this, many works was published in lytic activity of *Trichoderma harzianum* enzyme on NaOH treated and untreated fungal cell wall. (Sivan and Chet, 1986; 1989) In the present study we assed the lytic activity of *S. griseus* chitinase enzyme on treated and untreated fungal cell wall of pathogenic fungi. Our present study suggests that *S. griseus* having interaction with NaOH treated *F. oxysporum*. The significant biological control of *F. oxysporum* can be obtained by *S. griseus* along with NaOH. Further experiments are being carried out to identify those enzymes important in *S. griseus* biological control and to study their individual regulation.

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