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Chitinase and Glucanase Activities of Antagonistic Streptomyces Spp Isolated From Fired Plots under Shifting Cultivation In Northeast India

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Abstract

Antagonistic *Streptomyces* spp (*Streptomyces* sp NEA55 and *Streptomyces cavourensis* NEA5), isolated from fired plots under shifting cultivation in northeast India, are studied for their chitinase and glucanase activities. The species showed strong antagonism against test fungi (*Rhizoctonia solani* and *Cladosporium* sp.) in plate assays. Maximum % inhibition was observed due to the effect of diffusible compounds produced by these species. *Streptomyces* sp. NEA55 showed 54.83 % inhibition against *R. solani* while *S. cavourensis* NEA5 showed up to 50.00 % inhibition against *Cladosporium* sp. The inhibitory effect of volatile compounds by *Streptomyces* sp NEA55 was recorded up to 50.7 % against *R. solani* and 37.50 % against *Cladosporium* sp. While *S. cavourensis* NEA5 showed 49.23 % inhibition against R. solani and 34.37 % inhibition against *Cladosporium* sp. *S. cavourensis* NEA5 and *Streptomyces* sp. NEA55 produced 0.138±0.006 µg/ml and 0.15±0.004 µg/ml chitinase, 0.22±0.001 µg/ml and 0.25±0.002 µg/ml β 1,3 glucanase, respectively. Both the species showed maximum chitinase activity at pH 6 and temperature 50 ºC, while minimum enzyme activity was observed at pH 10 and temperature 20 ºC. Both the species showed glucanase activity maximum at pH 7 and temperature 40 ºC and minimum activity at pH 10 and temperature 20 ºC. Both the species hydrolyzed glycol–chitin as a substrate in denaturing conditions showing variable amount of different isoforms. This study demonstrates that the antagonistic species of *Streptomyces* survive the fire operations under shifting cultivation.

Keywords: *Streptomyces*; Antagonism; Chitinase; Glucanase; Shifting cultivation

Introduction

Actinobacteria are most widely distributed and distinct group of microorganisms in nature. *Actinobacteria, Streptomyces* species in particular, has been a broadly exploited group of microorganisms for the production of important secondary metabolites and enzymes in the field of medicine and agriculture [1, 2]. *Streptomyces* are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi [3]. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes [4, 5]. Many species of *Streptomyces* are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi e.g., *Phytophthora capsici Sclerotinia rolfsii, Fusarium sporotrichiodes, Rhizoctonia solani* and *Sclerotium rolfsii*, *Alternaria alternata* and *Phomopsis archeri* [6-9]. Furthermore, *Streptomyces* produce bioactive compounds such as antimicrobial, antiparasitic and immune-suppressing compounds via secondary metabolism. *Streptomycetes* have been found in beneficial associations with plants where they improve plant growth and protect against pests; this has attracted the attention of researchers worldwide [10].

Chitinase and β-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls [11]. Chitinolytic enzymes have been identified in several *Streptomyces* spp. including *Streptomyces* sp. M-20, *S. venezuelae* P10, and *S. anulatus* CS242 [12-14]. Glucanase has been known to be produced by several microorganisms and playing important role in biocontrol [15]. Several *Streptomyces* have been studied for antifungal properties along with the production of glucanase, some of the examples are *Streptomyces* sp. S27 and *Streptomyces* sp. Mo [16, 17]. Shifting cultivation, refers to 'slash and burn', is a predominant form of agricultural practice in hills of northeast India. The microbiological aspects, basically survival of bacterial, fungal and actinobacterial communities after fire events have been studied in recent times [18-20]. The focus of present study is on the antagonistic potential of two *Streptomyces* species that were isolated after the fire events. These species have been studied with respect to production of diffusible and volatile compounds

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against test fungi along with the chitinase and β-1, 3-glucanase activities. In addition, both the species are also studied with hydrolyzed glycol–chitin as a substrate in denaturing conditions.

Material and Methods

Study site and isolation of actinobacteria

Actinobacteria were isolated from the soil samples that were collected after the completion of fire events in Papumpare District, Itanagar, Arunachal Pradesh under shifting cultivation. The details of the study sites have been described in Pandey et al. [18]. Among the two actinobacterial isolates used in the present study, isolate no. NEA5 showed maximum similarity with *Streptomyces cavourensis* NR_043851 and NEA55 with *Streptomyces* sp. YIM8 AF389344 [19]. The test fungi (*Rhizoctonia solani* and *Cladosporium* sp.) were also originally isolated from shifting cultivation site [20].

Scanning electron microscopy

In order to see the deep morphological pattern (substrate and aerial mycelia) of *Streptomyces* spp. scanning electron microscopy was performed. Glutaraldehyde (2.5 %) was added to the culture and then centrifuged. After centrifugation, washing was given with phosphate buffer saline (PBS) twice and centrifuged. The sample was dehydrated with CPD (Critical dry point), and then the samples were coated with gold by auto fine coater (JFC-1600). After coating, the sample was viewed under scanning electron microscope (JSM-6610LV).

Dual culture technique for determination of the production of diffusible antifungal compounds

To test the ability of the *Streptomyces* spp. to inhibit the phytopathogens, the test fungal culture and *Streptomyces* sp. was spot inoculated off-center on each potato carrot agar (PCA) plate. After 7 days of incubation at 28 ºC, the zone of inhibition was measured. Per cent growth inhibition was calculated using the following formula: (R1-R2)/R1 × 100 (where R1 represents the radius of the test fungus in the direction with no bacterial colony and R2 is the radius of the fungal colony in the direction of the bacterial colony) as described in Trivedi et al. [21].

Sealed double Petri plate technique for determination of the production of volatile antifungal compounds

Each *Streptomyces* sp. was streaked on Petri plate containing PCA and a 6.0 mm disc of 4 days old culture of the test fungus was placed in the middle of another PCA plate. The lid of both the plates were replaced with the plates of same diameter, placed face to face, and sealed by parafilm, preventing any physical contact between the pathogen and the *Streptomyces* sp. Petri plates were incubated at 28 ºC for 7 days and growth of the pathogen was measured and compared to control developed in the absence of the antagonist (mocked inoculation with a 6.0 mm disc of PCA). Results were expressed as means of per cent inhibition of the growth of each fungus in the presence or absence of the antagonistic *Streptomyces* sp. Per cent growth inhibition was calculated by the following formula: (R1-R2)/R1 × 100, where R1 represents the diameter of the test fungus on control plate and R2 is the diameter of the growth on inoculated plate.

Quantitative estimation of production of chitinase and glucanase on different temperature and pH

Quantification of chitinase and glucanase has been done following prescribed procedures [22]. The *Streptomyces* species were cultured at 28 ºC for 5 days on a rotary shaker in 250.0 ml of chitin peptone medium for chitinase production and peptone medium containing laminarin (0.2 %) (from Laminaria digitata, Sigma) for glucanase production [23]. The cultures were centrifuged at 12,000 g for 20 min at 4 ºC and the supernatants were used as enzyme source. The reaction mixture contained 0.25 ml of enzyme solution, 0.3 ml of pH buffer (1.0 M citrate buffer pH 5, phosphate buffer pH 6 to 8, and glycine buffer pH 9). The reaction mixtures were incubated at different temperatures (20, 30, 40, 50 and 60 ºC) for 4 h in a water bath. One unit of chitinase was determined as 1 µmol of N acetyl glucosamine (GlcNAc) released min-1 and β-1, 3-glucanase activity was determined as 1 µmol of glucose released min-1. Protein content was determined as described by Lowry method [24].

SDS-PAGE analysis for chitinase

Streptomyces spp. was grown as shake culture in chitin-peptone medium at 28 ºC for 7 days. The supernatant was centrifuged at 12000 g for 20 min at 4 ºC and filtered through 0.22 µl sterile filter (Millipore) and collected in conical flask. Protein content was analyzed by SDS-PAGE [25]. Polyacrylamide slab gel consisted of 4 % stacking gel and 10 % separating gel containing 0.01 % glycol–chitin. Electrophoresis was carried out at a constant voltage of 65 V; gel was stained with coomassie brilliant blue R-250 and analyzed under gel documentation system (Alpha Imager 2200). Glycol chitin was prepared as described by Trudel and Asselin [26].

Results and Discussion

The scanning electron microscopy of both the species is presented in Figure: 1A and Figure: 1B. Scanning electron microscopy revealed definite structures of mycelium and spores of both the *Streptomyces* spp. Two of the isolates that were used for detailed studies based on their antagonistic properties were subjected to scanning electron microscopy. The scanning electron microscopy has been referred to provide perfect characterization of *streptomycetes* [27]. The effect of diffusible compounds produced by the *Streptomyces* spp., evaluated in terms of reduction in radial growth of two test fungi viz. *Rhizoctonia solani* and *Cladosporium* sp., following 7 days of incubation at 28 ºC, are presented in Figure:2 & Table 1. The results showed that volatile compounds produced by *Streptomyces* spp. were inhibitory to the growth of the test fungi, viz. *R. solani* and *Cladosporium* sp. Inhibition (%) in fungal growth by Streptomyces species is presented in Table 1. Microscopic observations revealed that the diffusible as well as volatile compounds produced by

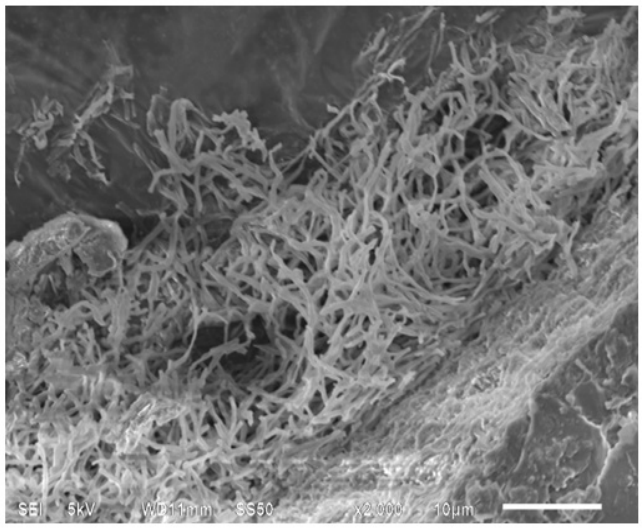

Figure 1A: Scanning electron micrograph of *Streptomyces cavourensis* NEA5

Figure 1B: Scanning electron micrograph of *Streptomyces* sp. NEA55

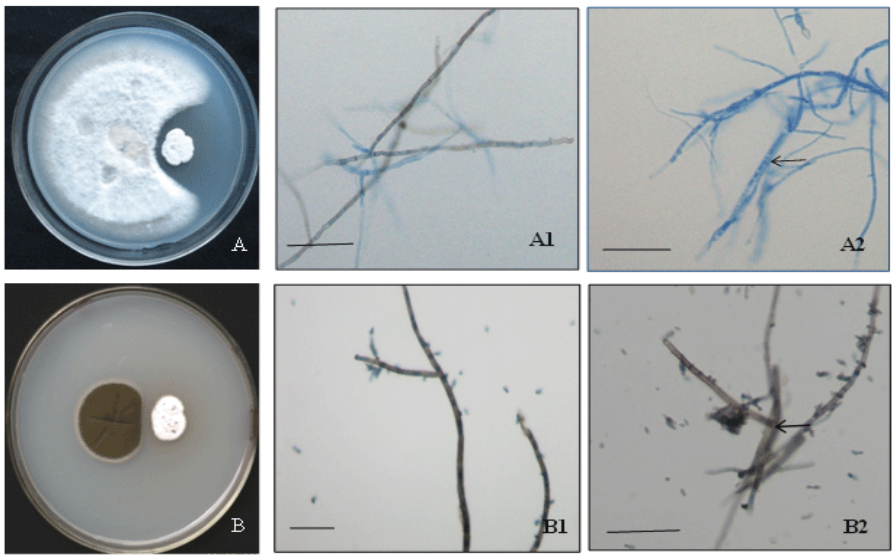

Figure 2: Production of antifungal compounds by *Streptomyces cavourensis* NEA5 & *Streptomyces* sp. NEA55 (A&B) respectively: Inhibition of *R. solani* and *Cladosporium* sp. due to diffusible compounds produced. Morphological deformities: (A1&B1) Normal structures of *R. soloni* and *Cladosporium* sp. (A2&B2) deformed structures of respective fungus. (Bar = 5 µm)

Table 1: In vitro percent *inhibitory* effect of diffusible and volatile metabolites of *Streptomyces* species on growth of test fungi

Pathogenic test fungi	**% Inhibition in fungal growth by *Streptomyces* spp. after 7 days of incubation**			
	***Streptomyces cavourensis* NEA5**		***Streptomyces* sp NEA55**	
Rhizoctonia solani	51.61	49.23	54.83	50.07
Cladosporium sp.	50	34.37	47.7	37.5

Table 2: Comparative account of structures of normal and antagonized pathogen

Test fungi	Structure	Normal	Antagonized
Rhizoctonia	Mycelium	Somatic and fertile hypha well distinguished	Irregular
Solani	Somatic hypha	Septate, dia. 2.5- 3.5 µm	Irregular
			Septate, dia 3.0-6.0
	Fertile hypha	Septate and dark in colour, dia. 3.0-4.5 µm	Irregular septate, dia 3.0-7.5 µm
	Hyphal wall	Present	Lysed
Cladosporium sp.	Mycelium	Somatic hypha septate, dia. 2-3.5 µm	Somatic hypha septate, dia. 2-5 µm
	Conidia	Curved or straight width 2.0-2.5 µm	Irregular width 2-4 µm

Streptomyces species induced morphological abnormalities in the fungal structures (Table 2). Deformation was observed in *mycelial, hyphal* or *conidial* structures. The longitudinal septae completely disappeared and the conidia became thick walled and spherical or irregular in shape. Lysis of fungal hyhae and vacuolization as well as granulation in mycelium was observed in *R. solani.* Size of the somatic and fertile hypha of *R. solani* increased due to the antagonistic effect of actinobacteria. Due to the antifungal activity of *Streptomyces* sp., hyphal wall of *R. solani* was completely lysed. In case of *Cladosporium* sp., size of mycelium and conidia increased with irregular shape of conidia. The inhibition of growth of the test pathogenic fungi continued to increase with increasing incubation time. A clear inhibitory area and dense sporulation ring was observed near the growth of the isolate which antagonized respective test fungi. No physical contact was observed between the *Streptomyces* species and the test fungi that were observed to be antagonized. Moreover, the formation of the inhibitory halo suggested the presence of fungicidal metabolites secreted by *Streptomyces.* Similar observations have been reported by Aghigni et al. [28] from a number of actinobacterial isolates from Iranian soil. These isolates formed inhibition zones in dual culture based assays inhibiting the growth of *Alternaria solani, A. alternata, Fusarium solani, Phytophthora megaperma, Verticillium dahlia* and *Saccharomyces cerevisiae*. The antifungal potential of actinobacteria against *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* was assessed by dual culture technique [10]. Al-Askar et al. [29] reported that *Streptomyces spororaveus* RDS28 produces antifungal compounds against some phytopathogenic fungi, viz., *Rhizoctonia solani, Fusarium solani, F. verticillioides, Alternaria alternata* and *Botrytis cinerea.* There was a change in colour of the test fungi in inoculated plates, indicative of the inhibitory effect of volatile compounds. The test fungi, inhibited by the *Streptomyces,* under present study exhibited morphological abnormalities due to the production of diffusible and volatile antifungal compounds. Abnormal hyphal swelling, degradation and lysis of mycelia were observed by Joo [6] when *Phtophthora capsici* was grown with the high or low molecular fraction of *Streptomyces halstedii* AJ-7.

Quantitative estimation of production of chitinase and β 1, 3 glucanase activities is presented in Figure 3 (A&B) showing maximum activity of chitinase and glucanase. Effect of different pH and temperature on chitinase activity of both the species is presented in Figure 4 (A&B). Both the species showed maximum activity of chitinase at pH 6 and temperature 50 ºC, while minimum enzyme activity was observed at pH 10 and temperature 20 ºC. In case of glucanase activity both the species showed maximum enzyme activity at pH 7 and temperature 40 ºC and minimum activity at pH 10 and temperature 20 ºC (Figure 5A&B). Kim et al. [12] reported maximum activity of chitinase

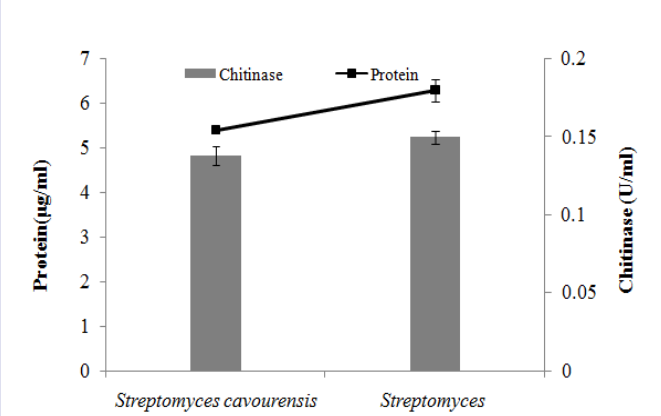

Figure 3A: Quantitative estimation of chitinase activity

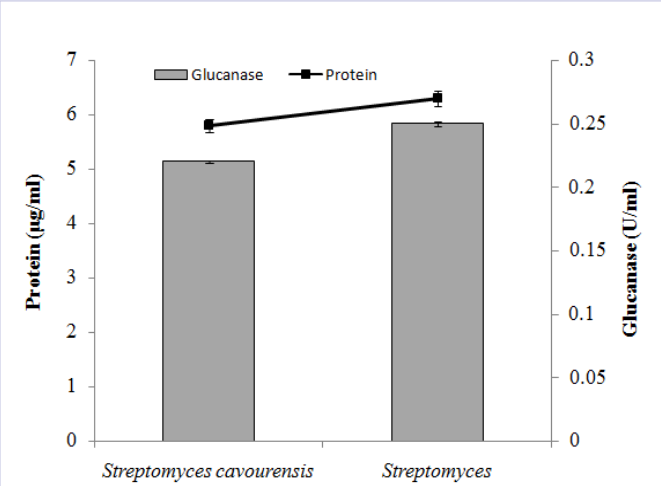

Figure 3B: Quantitative estimation of glucanase activity

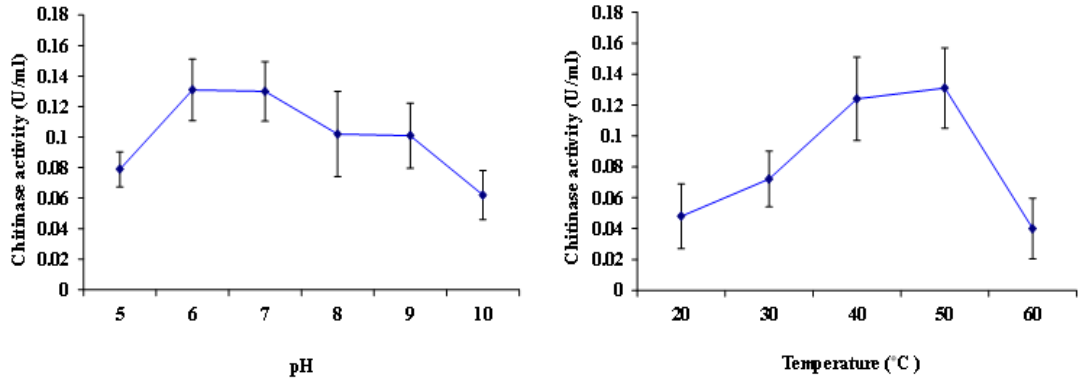

Figure 4A: Chitinase activity of *Streptomyces cavourensis* NEA5 at different pH & temperature

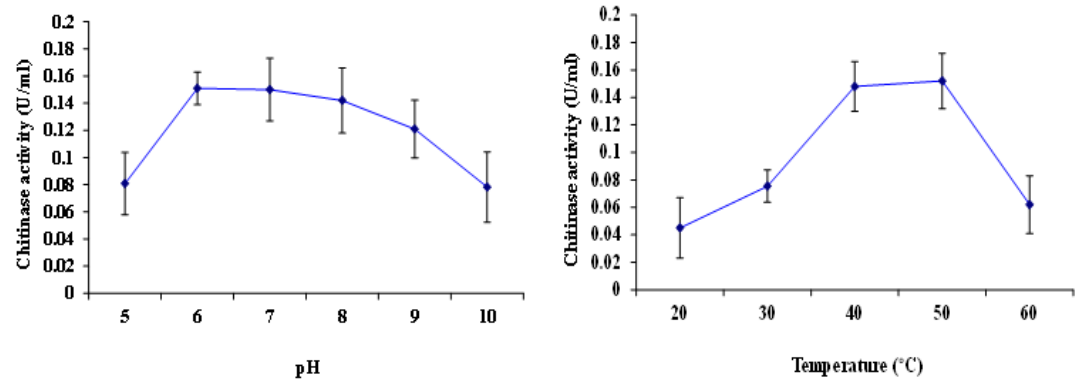

Figure 4B: Chitinase activity of *Streptomyces* sp NEA55 at different pH & temperature

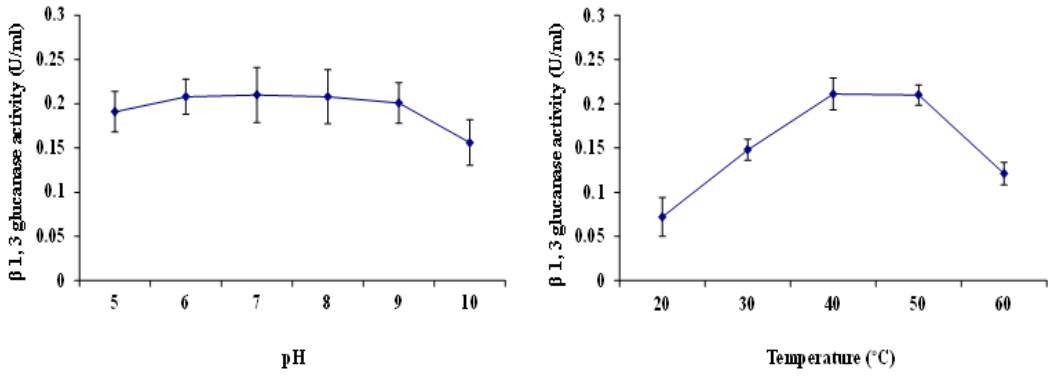

Figure 5A: Glucanase activity of *Streptomyces cavourensis* NEA5 at different pH & temperature

between pH 5.0 to 6.0 and it was relatively stable at pH 4.0 to 8.0 when kept at 4 ºC. Beyond this pH range there was a rapid loss in the activity. Glucanase activity of the two isolates was found maximum at pH 7 and temperature 40 ºC. Highest activity was found between pH 5 to 8 and temperature 30 to 50 ºC, in previous studies. The optimum activity for short term incubation is often seen at temperature in the range of 30 to 50 ºC, while many fungal glucanases appear stable between 50 to 60 ºC [30].

SDS-PAGE exhibited the chitinase activity of the two *Streptomyces* species (NEA5 & NEA55). *Streptomyces* species hydrolyzed glycol-chitin as a substrate in denaturing conditions. Species showing variable amount of different isoforms with molecular weights between 31 to 40 kDa are presented in Figure 6. Majority of bacterial chitinases has been reported to be in the range of 20 to 60 kDa. Chitinases from various *Streptomyces* were found to possess molecular weights as 20 kDa from *Streptomyces*

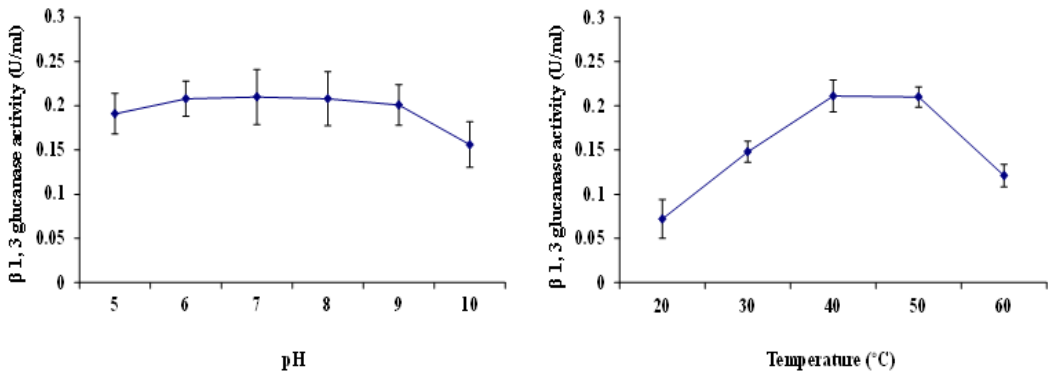

Figure 5B: Glucanase activity of *Streptomyces* sp NEA55 at different pH & temperature

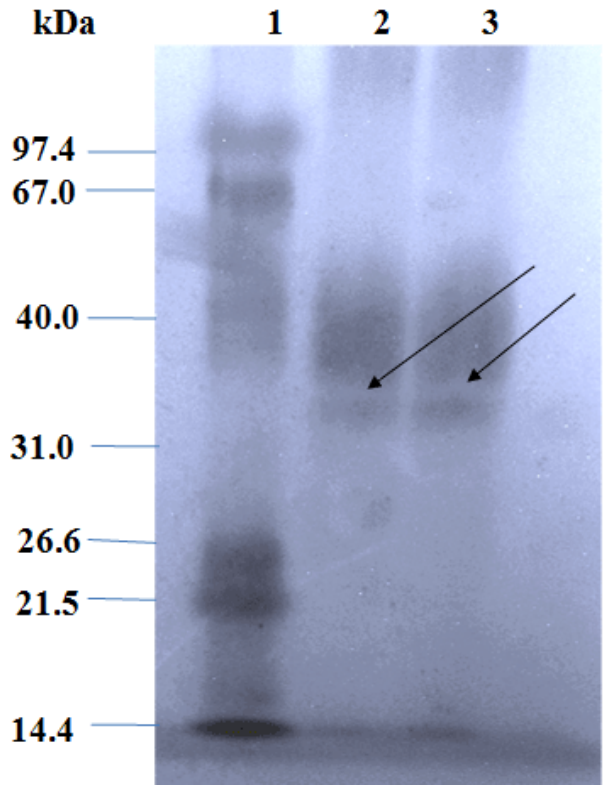

Figure 6: Detection of chitinase activity after SDS-PAGE using glycol chitin as substrate, lane 1 (Molecular weight markers), lane 2 and 3 sample *Streptomyces cavourensis* NEA5 and *Streptomyces* sp NEA55, respectively

sp. M-20 [13] and some strains of *Streptomyces* varying from approximately 25 kDa to 200 kDa [31].

The aim of this work is to prove the potential of *Streptomyces* species to survive the fire operations retaining their potential to reduce or eliminate the plant pathogenic fungi. Such robust isolates may prove to be the potential biocontrol agents for field applications. Further studies are needed in order to determinate the nature of *Streptomyces* species metabolites and their mechanism of action.

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