

## CHITINASE PRODUCTION BY *STREPTOMYCES* SP. ANU 6277

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### ABSTRACT

Chitinase production by a terrestrial *Streptomyces* sp. ANU 6277 was studied under sub-merged fermentation. Chitinase production started after 24 h of incubation and reached maximum levels after 60 h of cultivation. A high level of chitinase activity was observed in the culture medium with pH 6 at 35°C. Culture medium amended with 1% chitin was found to be suitable for maximum production of chitinase. An optimum concentration of colloidal chitin for chitinase production was determined. Studies on the influence of additional carbon and nitrogen sources on chitinase production revealed that starch and yeast extract served as good carbon and nitrogen sources to enhance chitinase yield. Chitinase was purified from crude enzyme extract by single step gel filtration by Sephadex G-100. Purified chitinase of the strain exhibited a distinct protein band near 45 kDa by means of SDS-PAGE.

**Key words:** Chitinase, *Streptomyces* sp. ANU 6277, Optimization, Characterization

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### INTRODUCTION

Actinomycetes, particularly *Streptomyces* spp. have been a widely exploited group of microorganisms in the production of secondary metabolites and enzymes of commercial importance in medical and agricultural applications (11). Several chitinolytic enzymes have been identified in several *Streptomyces* spp. including *S. antibioticus*, *S. griseus*, *S. plicatus*, *S. lividans*, *S. aureofaciens* and *S. halstedii* (21, 9). Chitinase has received attention due to its use as a biocontrol agent (14, 25) and also for developing transgenic plants (3). They have been used in a biological research for the generation of fungal protoplasts to degrade the fungal cell wall and also for being employed in human health care such as making ophthalmic preparations with

chitinases (4). An actinomycete strain isolated from laterite soils of Acharya Nagarjuna University campus was identified by 16S rRNA analysis and designated as *Streptomyces* sp. ANU 6277. The taxonomic study of the strain which was reported previously (15) showed that it was closely related to the *Streptomyces albidoflavus* cluster. In the present study, an attempt was made to optimize the culture conditions for the production of chitinase by the strain and its characterization.

### MATERIALS AND METHODS

A pure culture of the strain, *Streptomyces* sp. ANU 6277 was maintained on a yeast extract- malt extract- dextrose agar (ISP-2) medium. Colloidal chitin was prepared according to the method described by Sandhya *et al.* (20).

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A culture suspension of the strain was inoculated in a chitin-yeast extract-salts (CYS) medium (g.L<sup>-1</sup>): chitin, 5.0; yeast extract, 0.5; K<sub>2</sub>HPO<sub>4</sub>, 2.0; MgSO<sub>4</sub>.7H<sub>2</sub>O, 1.0; and FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.1 and final pH of the medium adjusted to 7. For every 12 h intervals up to 84 h, a culture filtrate was collected and used as an enzyme source. The harvested cells attained through the filtration were used for biomass estimation which was determined by the cell-dry-weight.

### **Chitinase assay**

Extracellular chitinase activity was determined by incubating 1 ml of crude enzyme with 1 ml of 1% colloidal chitin in a 0.05M phosphate buffer, pH 7.0 at 35°C for 1 h. After centrifugation of reaction mixture, the amount of N-acetyl-D-glucosamine released in the supernatant was determined by the method of Reissig *et al.*, (19) using N-acetyl-D-glucosamine (NAG) as a standard. NAG present in 0.5 mL of aliquot of supernatant was determined by adding 0.1 ml of K<sub>2</sub>B<sub>4</sub>O<sub>7</sub> and then boiled for 3 min in a water bath. The tubes were cooled and 3 ml of p-dimethylaminobenzaldehyde was added. Absorbance was read within 10 min at 585 nm against the blank prepared with distilled water without the enzyme presence. One unit (U) of chitinase is defined as the amount of enzyme which releases 1 μM N-acetyl-D-glucosamine per hour under the conditions of the study.

### **Optimization of chitinase production**

An optimum level of chitin concentration on chitinase production was determined by amending different concentrations of chitin in a CYS medium. The impact of pH and temperature on chitinase production was investigated by cultivating strain in the CYS medium at various pH (4-9) and temperature (15-45°C) ranges for 60 h. The carbon sources as starch, glucose, maltose, cellulose and arabinose and nitrogen sources such as ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrate, L-asparagine, L-glutamine, soybean meal, peptone and yeast extract were supplemented with the CYS medium to study their influence on chitinase

production. There were three sets of flasks were maintained. One set of flasks contained the CYS (1% chitin) medium supplemented with respective carbon and nitrogen sources whereas another set, besides the amended CYS medium and respective carbon and nitrogen sources was not supplemented with chitin. The third set, as control flasks, had its CYS medium kept constant (24).

### **Utilization of fungal biomass as a source of chitin for chitinase production**

*Fusarium oxysporum* and *Penicillium citrinum* were grown in Czapek-Dox broth. After 15 days of incubation, the fungal mat was harvested and autoclaved at 121 °C, 15 lbs for 20 min. The autoclaved fungal mat was washed twice with sterile distilled water and dried in an oven at 80°C till constant weight (6). The dried fungal mat was powdered and used as chitin source (2 g.L<sup>-1</sup>) for the production of chitinases. Utilization of fungal mat by the strain for chitinase production was determined by using three sets of flasks – 1) CYS medium in which colloidal chitin was replaced by fungal mat (2 g/l), 2) CYS medium supplemented with fungal mat (2 g/l) and 3) Control only with CYS medium without any fungal mat.

### **Purification of chitinase**

A single-step purification of chitinases was performed according to the method suggested by Nawani and Kapadnis (16). The culture filtrate (500 mL) of 60-h old culture broth was subjected to precipitation with ammonium sulphate to 80% saturation and kept at 4°C for 24 h. The precipitate thus obtained was collected by centrifugation at 10,000 g for 20 min. The pellet was dissolved in a 0.01M citrate phosphate buffer, pH 6 and extensively dialyzed against the same buffer. The protein concentrate was loaded on Sephadex G-100 (Sigma, USA) column (2x40 cm) pre-equilibrated with a 0.01M citrate phosphate buffer, pH 6 and eluted with the same buffer. Fractions thus collected were tested for chitinolytic activity. Chitinolytic active fractions were recovered and concentrated.

Enzyme concentrate thus obtained through a gel filtration was checked for purity by SDS-PAGE (12) and its molecular weight was determined by comparing with known standard proteins. SDS-PAGE was carried out in a 2 mm slab gel of 10% acrylamide in a Tris-HCl buffer (pH 8.0) containing 0.1% SDS. Enzyme samples of 20  $\mu$ L were loaded into the wells. After electrophoresis, the gel was stained with 0.025% Coomassie brilliant blue R-250. The gel was de-stained with a solution (10% v/v glacial acetic acid and 30% v/v methanol in distilled water) till a clear background of the gel was obtained. The bands present on the gels were observed and compared with standard proteins. SDS-PAGE broad-molecular weight range proteins were used as standard proteins (Bio-Rad, USA).

#### Assay of chitinolytic activity of partially purified chitinase

Chitinolytic activity of the partially purified enzyme was examined by agar well diffusion method (21). Enzyme (50  $\mu$ L) was added to the wells of CYS agar medium plates. Control was maintained with 50  $\mu$ L of heat inactivated enzyme. Chitinolytic zones around the wells were observed after 12-24 h of incubation.

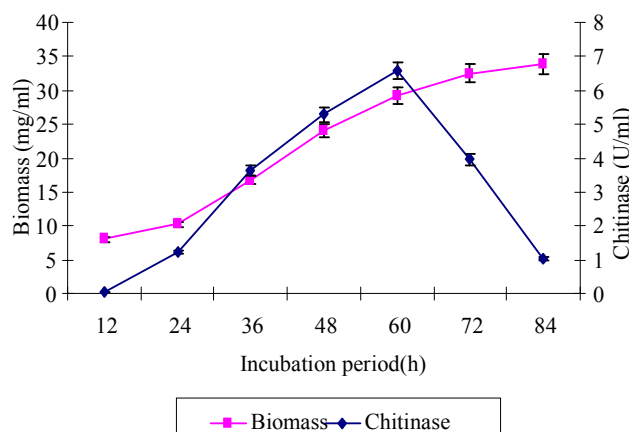
#### Inhibition of fungal growth by partially purified chitinase from the strain

*Fusarium udum* MTCC 2204 the causative agent of wilt of *Cajanus cajan* L. was cultured on a Czapek-Dox agar (CDA) medium (15). Spore suspension was spread onto the plates of CDA. Antifungal activity of purified chitinase against *F. udum* was studied by agar well diffusion method (21). Chitinase (50  $\mu$ L) of the strain was placed in wells of the CDA plate. Control was maintained with heat inactivated 50  $\mu$ L of purified enzyme. Inhibition of fungal growth was observed after 3-5 days.

## RESULTS AND DISCUSSION

#### Effect of incubation period on chitinase production

The effect of the course of time on chitinase production by the strain is presented in Figure 1. Chitinase production was initially found after 24 h of incubation and reached maximum levels after 60 h of cultivation. Chitinase synthesis was found to decline as the incubation period further extended. Nawani *et al.*, (18) reported the maximum production of extracellular chitinase by *Microbispora* sp. V2 after 48 h of incubation which declined subsequently. Nawani and Kapadnis (17) reported that the production of chitinase by *Streptomyces* sp. NK1057 was high after 5 days of incubation while Joo (9) found that maximum yield of chitinase by *Streptomyces halstedii* after 72 h of incubation.

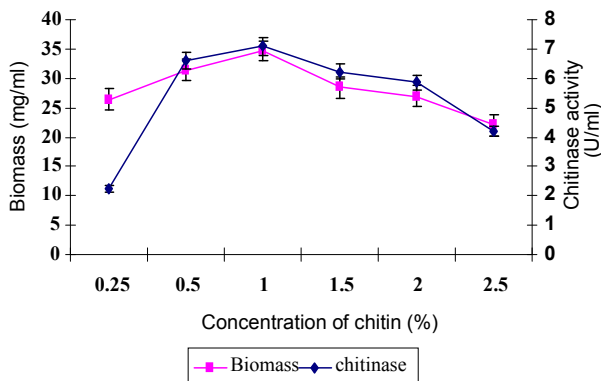


**Figure 1.** Effect of course of time on chitinase and biomass production (values are means of three replicates  $\pm$  SD)

#### Effect of chitin concentration on chitinase production

Optimal concentration of chitin for maximum chitinase production was studied (Fig. 2). Culture medium amended with 1% chitin exhibited maximum enzyme activity. It was in conformity with the finding of Taechowisan *et al.* (21), who reported that the production of the chitinase from *S. aureofaciens* CMU Ac 130 was optimal with 1% colloidal chitin concentration. According to Gupta *et al.* (8), *S. viridificans* produced maximum levels of chitinase in 1.5% of

chitin amended medium after six days of incubation at 30°C. The strain ANU 6277 produced maximum chitinase in CYS medium amended with 1% chitin after 60 h of incubation.



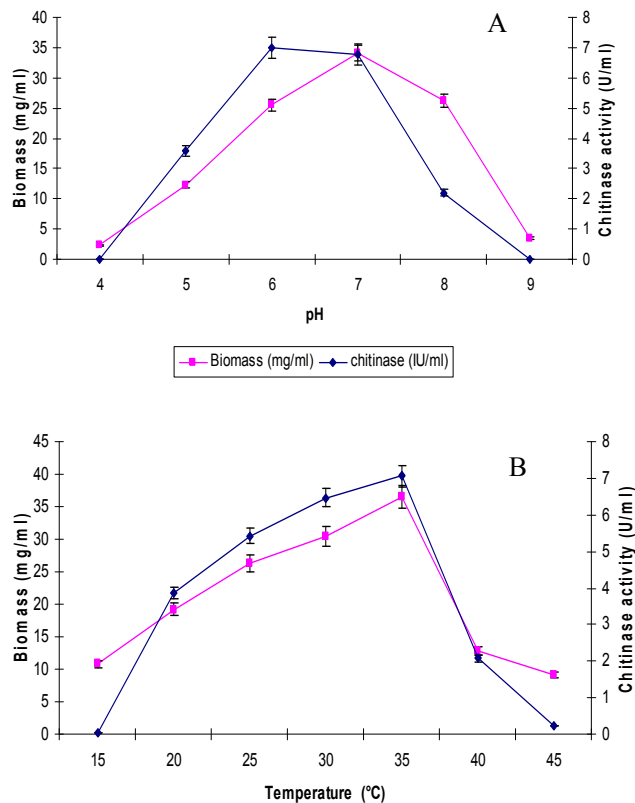
**Figure 2.** Role of chitin concentration on chitinase and biomass production (values are means of three replicates ± SD)

**Impact of pH and temperature on chitinase production**

The influence of pH and temperature on chitinase production by the strain is depicted in Figure 3. Chitinase production varied as medium pH changed between 4 and 9. A high level of chitinase activity was observed in the culture medium with pH 6 and optimum temperature for chitinase production was at 35°C. In *Microbispora* sp. V2, the optimum pH and temperature for chitinase production were reported to be 7 and 40°C (18). Taechowisan *et al.*, (21) reported that the production of chitinase by *S. aureofaciens* was optimal at pH 6.5-7 and temperature 30-40°C. Maximum chitinase production by the strain ANU 6277 was observed in 1% chitin amended CYS medium incubated at pH 6 and temperature 35°C for 60 h.

**Influence of different carbon and nitrogen sources on chitinase production**

The influence of additional carbon and nitrogen sources



**Figure 3.** Impact of A) pH and B) temperature on chitinase and biomass production (values are means of three replicates ± SD)

on chitinase production was studied by supplementing different carbon and nitrogen compounds to CYS broth. Data on the effect of several carbon sources on chitinase production by the strain are presented in Table 1. Enhanced chitinase production was found in CYS medium amended with 0.2 % starch, while enzyme production was suppressed in the CYS medium with glucose and arabinose. Cellulose and maltose did not influence chitinase production as compared to control. In *S. viridificans*, chitin medium amended with arabinose with chitin, enhanced the production of chitinase (8). Joo (9) reported that glucose (0.4 %) along with chitin induced high levels of chitinase by *S. halstedii*. Taechowisan *et al.* (21) found that amendment of pectin,

starch and carboxymethyl cellulose to the colloidal chitin medium increased chitinase production by *S. aureofaciens*.

**Table 1.** Influence of additional carbon sources on chitinase production by the strain ANU 6277 cultured in CYS medium

Carbon sources	Chitinase activity	
	(U.mL <sup>-1</sup> )	± SD
*Control	6.98	± 0.17
Starch (0.2%)	7.22	± 0.08
Starch (0.4%)	6.14	± 0.30
Glucose (0.2%)	4.16	± 0.21
Glucose (0.4%)	1.45	± 0.07
Cellulose (0.2%)	6.5	± 0.14
Cellulose (0.4%)	6.12	± 0.05
Maltose (0.2%)	6.19	± 0.51
Maltose (0.4%)	6.05	± 0.26
Arabinose (0.2%)	3.06	± 0.19

\* CYS medium with 1% chitin; Mean ± SD from three experiments

Among the different inorganic nitrogen sources supplemented to CYS medium, none could enhance chitinase production as compared to CYS medium (Table 2). However, there was a significant increase in chitinase production in CYS medium amended with organic nitrogen sources as yeast extract (0.4%) and soybean meal (0.6%). Vaidya *et al.*, (24) reported that organic nitrogen sources as yeast extract and peptone significantly increased the chitinase production by *Alcaligenes xylosoxydans*. In the present study, CYS medium amended with starch (chitin, 1%; starch, 0.2%; yeast extract, 0.4%; K<sub>2</sub>HPO<sub>4</sub>, 0.2%; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.1% and FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.01%) with pH 6 was found to be suitable for maximum production of chitinase by the strain cultured at 35°C for 60 h.

#### Role of fungal biomass on chitinase production

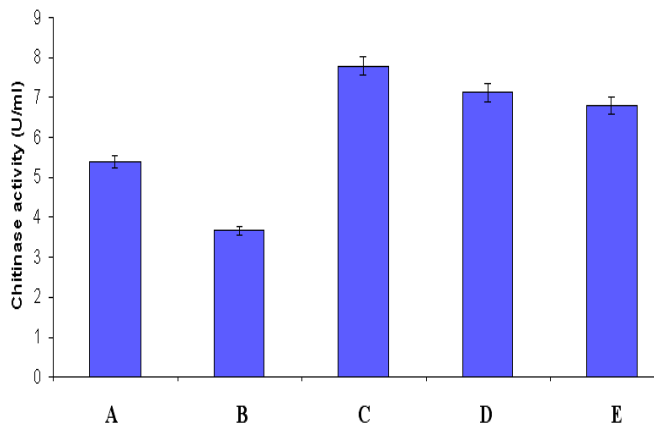
As the fungal cell wall contains chitin as the major

component, chitinase are well known to lyse the cell wall of both live and dead fungi (23). Utilization of dead mass of *F. oxysporum* and *P. citrinum* by the strain for the chitinase production was studied (Fig. 4). Little enhancement of chitinase production was observed in CYS medium amended with dried fungal mats over CYS medium. The enzyme production was found to decline when the strain was cultured in CYS medium in which colloidal chitin was replaced by dried fungal mats as chitin source. Beyer and Diekmann (1) reported cell wall degradation of *Penicillium chrysogenum* by chitinase system of *Streptomyces* sp. ATCC 11238. An increase in chitinase production was observed from *S. aureofaciens* and *S. halstedii* when cultured in a medium containing colloidal chitin supplemented with fungal cell wall preparations (9, 21).

**Table 2.** Influence of additional nitrogen sources on chitinase production by the strain ANU 6277 cultured in CYS medium

Nitrogen source (%)	Chitinase activity	
	(U.mL <sup>-1</sup> )	± SD
*Control	6.98	± 0.17
NH <sub>4</sub> Cl (0.2%)	3.68	± 0.16
NH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub> (0.2%)	5.68	± 0.19
NaNO <sub>3</sub> (0.2%)	6.84	± 0.09
KNO <sub>3</sub> (0.2%)	6.65	± 0.23
L-glutamine (0.2%)	6.83	± 0.57
L-asparagine (0.2%)	7.12	± 0.39
Soybean meal (0.2%)	7.19	± 0.07
Soybean meal (0.4%)	7.26	± 0.30
Soybean meal (0.6%)	8.05	± 0.24
Soybean meal (0.8%)	7.24	± 0.76
Peptone (0.2%)	6.95	± 0.69
Yeast extract (0.2%)	7.16	± 0.50
Yeast extract (0.4%)	8.89	± 0.34
Yeast extract (0.6%)	7.2	± 0.65

\* Control- CYS medium with 1% Chitin; Mean ± SD from three experiments



**Figure 4.** Utilization of dried fungal mat for chitinase production by *Streptomyces* sp. ANU 6277 (values are means of three replicates  $\pm$  SD)

- A – CYS medium in which colloidal chitin was replaced by dried fungal mat of *Fusarium oxysporum*  
 B – CYS medium in which colloidal chitin was replaced by dried fungal mat of *Penicillium citrinum*  
 C – CYS medium supplemented with dried fungal mat of *Fusarium oxysporum*  
 D – CYS medium supplemented with dried fungal mat of *Penicillium citrinum*  
 E – Control only with CYS medium without any fungal mat

#### Purification of chitinase

The crude extract of enzyme was partially purified by a single-step procedure using Sephadex G-100 gel filtration. Proteins present in culture filtrate were extracted by ammonium sulphate (80%). Dialyzed protein precipitate was subjected to gel filtration for purification. In the gel filtration, four protein peaks were observed but maximum chitinase activity was detected in the fractions of third peak (Fig.5). The purification steps are summarized in Table 3. Following Sephadex G-100 gel filtration, the purification of the chitinase was increased to 5 fold with over all yields of 46.2 %. Chitinase of *Microbispora* sp.V2 was purified up to 6-fold with 4.6% recovery using Sephadex G-100 filtration (18). Purification of crude chitinase from *Streptomyces* sp.

M-20 was increased to 6-fold using DEAE-cellulose and Sephadex G-100 filtration (10).

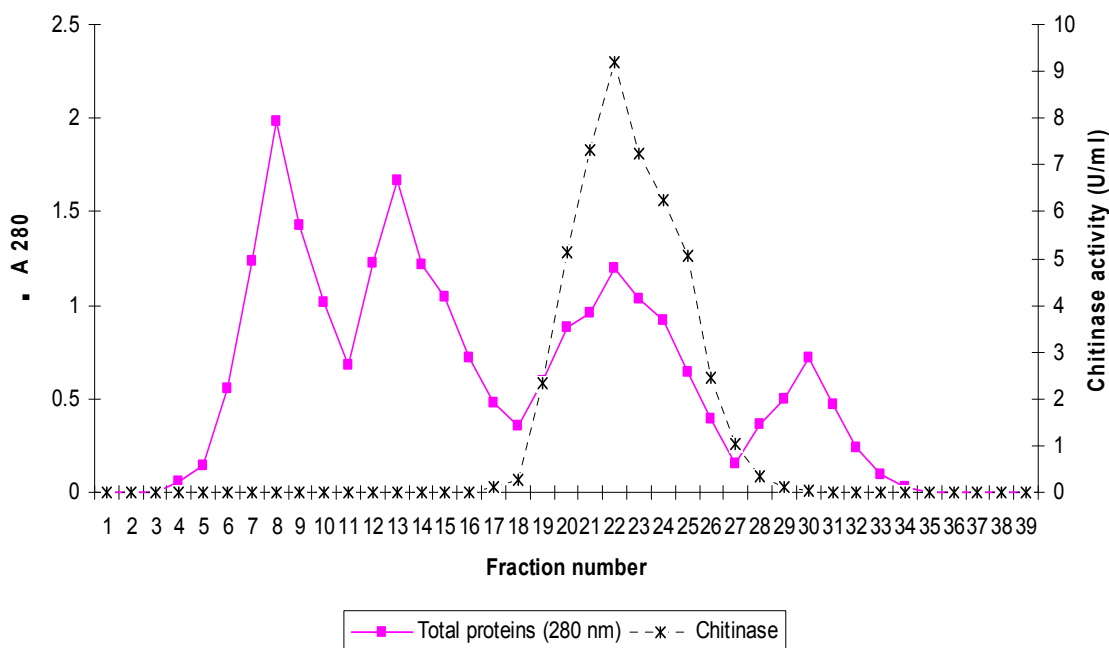
Chitinolytic active fractions thus collected during gel filtration were concentrated and analyzed by SDS-PAGE for the determination of molecular weight of chitinase. Partially purified chitinase of the strain ANU 6277 revealed a distinct protein band near 45 kDa (Fig. 6). Majority of bacterial chitinases were reported to be in the range of  $\sim$  20-60 kDa (2). Chitinases from various *Streptomyces* were shown to possess molecular weights as 20 kDa from *Streptomyces* sp. M-20 (10), 28 kDa, 35 kDa and 45 kDa from *Streptomyces* sp. NK 1057 (17), 43 kDa and 45 kDa from *S. albovinaceus* S-22(5), and 49 kDa from *S. griseus* HUT 6037 (22) by SDS-PAGE analysis.

The chitinase obtained by Sephadex G-100 gel filtration was tested for chitinolytic property on CYS agar medium. Partially purified enzyme was tested for chitinolytic property by cup-plate method. Controls were maintained with heat inactivated enzyme. Clear chitinolytic zones were observed around the wells loaded with 50  $\mu$ L of partially purified enzyme in CYS medium but heat inactivated enzyme failed to hydrolyze the colloidal chitin present in the CYS medium (Fig. 7A). Chitinases produced by *Streptomyces* spp. such as *S. viridificans* (8), *S. lydicus* WYEC 108 (13) and *S. aureofaciens* CMU Ac 130 (21) could hydrolyze colloidal chitin more rapidly than crude chitin or chitin from fungal cell walls.

Partially purified chitinase from strain was tested for antifungal activity against *Fusarium udum*. Controls were maintained with heat inactivated enzyme. Chitinase from the strain exhibited inhibitory activity against the growth of *F. udum* but heat inactivated enzyme taken as control did not show any antifungal activity (Fig. 7B). Kim *et al.*, (10) determined the antifungal activity of purified chitinase from *Streptomyces* sp. M-20 against *Botrytis cinerea* by using agar diffusion test. Chitinases from *Streptomyces* were reported to exhibit several antifungal activities like inhibition of spore germination, germ tube elongation, bursting of spores and

hyphal tips (21, 7). In the present study, cultural conditions were optimized for chitinase production by *Streptomyces* sp. ANU 6277 and the partially purified enzyme exhibited a protein band near 45 kDa. As the enzyme

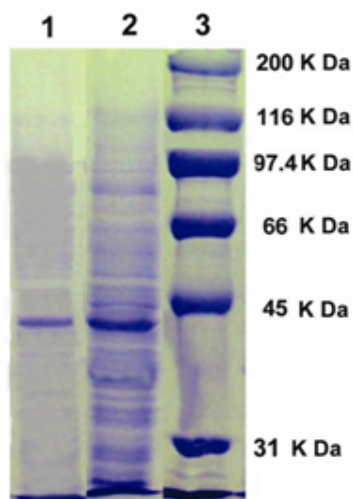
exhibited antifungal activity against a phytopathogenic mold *F. udum*, it may be used as a biocontrol agent against wilt disease.



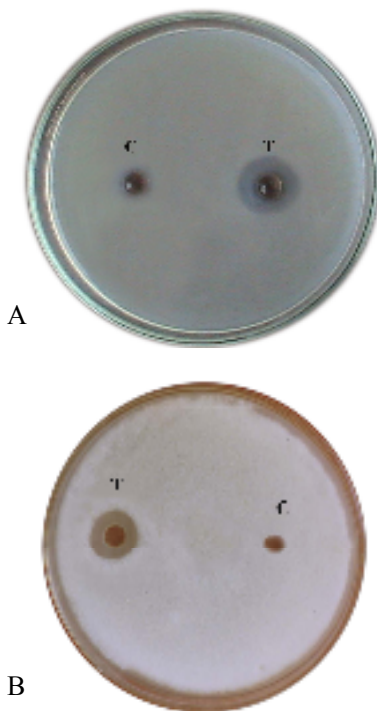
**Figure 5.** Chromatogram of the chitinase from *Streptomyces* sp. ANU 6277 on a Sephadex G-100 column. The column was eluted with 0.01M phosphate buffer, pH 7.0

**Table 3.** Purification steps of extracellular chitinase from the strain ANU 6277

Purification step	Total proteins (mg)	Total activity(U)	Specific activity (U.mG <sup>-1</sup> )	Purification (fold)(%)	Yield (%)
Culture filtrate	302	3570	11.8	0	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitation	118	3120	26.4	2.2	87.4
Sephadex G-100	27.5	1649	59.9	5	46.2



**Figure 6.** SDS-PAGE of chitinase from strain ANU 6277. Lane 1, Sephadex G – 100 gel filtration fraction; Lane 2, Ammonium sulphate precipitate; Lane 3, Standard proteins.



**Figure 7.** Chitinolytic activity (A) and Antifungal activity (B) of purified enzyme (50  $\mu$ L) T- Purified enzyme; C - heat inactivated enzyme (5-min boiled).

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