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Partial purification and antifungal activity of crude chitinase from *Neisseria* species

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Abstract
Aim: To partial purify and determine antifungal activity of *Neisseria* chitinases.
Materials and Methods: Characterized and optimized chitinase were obtained from *Neisseria* species and partially purified by ammonium sulphate precipitation, dialysis and SDS-PAGE for molecular weight determination. Further antifungal activities were determined against *Trichoderma* sp, *Aspergillus* sp and *candida* sp by agar well diffusion method at 25°c.
Results: The partially purified chitinase showed 43kDa molecular weight. Half-life of the chitinase enzyme was three and half hours at 28°c and showed effective antifungal activity against *Candida* sp, *Aspergillus* sp and *Tricoderma* sp.

Keywords: crude chitinase, partially purification, antifungal activity

Introduction
In many plant species, local invasion of the pathogen induces production of PR- proteins like chitinases, b-1,3-glucanases, proteinases, inhibitors, etc. (Aziz *et al*., 2006). As pathogenic fungi and insects contain chitin in their protective covers, induction of chitinases in plants is the main defense response. Most of these chitinases are induced in vegetative plant organs by infection but some are also present in seeds demonstrated that extracts of the pea endocarp contain chitinase and chitobiase activity. In fact, there is no other better proof for the contribution of plant chitinases in self defense than the formation of chitosan in the cell wall of a bean rust fungus, *Uromyces viciae-fabae* to combat with the chitinase activity (Bailey *et al*., 2002; Adesina *et al*., 2007; Aziz *et al*., 2008) [6, 1, 4].

Most of the chitinases preferentially cleave highly acetylated substrates and the activities decrease with decrease in the degree of acetylation. Therefore, increase in the deacetylation level on the surface of hyphae may be useful for the fungus to resist plant chitinases. And the presence of chitin deacetylase activity during the formation of infection structures supported this hypothesis. Numbers of soil bacteria produces chitinase by Roberts and Selitrennikoff. studied plant and bacterial Chitinases for anti-fungal activity and enzyme specificity(Bailey *et al*., 2002) [6] before or simultaneously along with the pathogenic fungus, itself was successfully tried (Agullo *et al*., 2003). The insect-pathogenic fungi, *Beauveria bassiana, B. brongniartii*, and *Verticillium lecanii* produced cuticle degrading enzymes when grown on chitin containing medium. The pretreatment of insects with the enzyme solution was reported to be useful. In this paper we descried novel *Neisseria* chitinases for partial purification and their antifungal activity.

Methods and materials
Previously reported chitinases from *Neisseria* species were optimized by solid state fermentation and further used for partial purification and antifungal activity.

Ammonium sulphate precipitation
25 ml of culture filtrate solution was treated with 70% of saturated ammonium sulphate solution. The crude enzyme was continuously stirred using magnetic stirrer and kept at 4˚C for overnight, the enzyme was centrifuged at 5000 rpm for 20 minutes, then supernatant was discarded and the pellets were collected using 0.05M phosphate buffer (pH 6.0).

Dialysis
Dialysis membrane was treated with 0.05 phosphate buffer in water bath at 50°C for 20 minutes. Then washed thoroughly with distilled water. A knot was made at one end the membrane and is used for filling the sample. To this dialysis bag, the sample was added and it was dialyzed against 0.05 M phosphate buffer of pH 6.0 for overnight at 4˚C by placing on a magnetic stirrer. The buffer used for dialysis should be removed twice for the complete removal of salts.

Half Life of Chitinases enzyme
0.5 ml of chitinase enzyme was incubated in 0.5 ml of 0.05M Phosphate buffer at 28^{0}C for a 5 hrs. And chitinase enzyme activity was determined by DNS method after every 30 min. time intervals.

Sodium Dodecyl Sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE was performed to carry out the purification of chitinase enzyme. SDS-PAGE was performed according to the method of Weber and Osborn (Weber and Osborn-1969).

Determination of antifungal activity of chitinases
Isolation of fungi: *Trichoderma* sp, *Aspergillus* sp and *candida* sp were isolated from the fish market soil sample on sterile PDA at 28ºC for (7-10) days and identified on the basis of morphological characterization.

Inoculums preparation: Fungal inoculum was prepared by inoculating a 1cm agar blocks of test organisms in 5 ml of

PDB and incubated at room temperature for 3 days.

Antifungal Activity

The antifungal activity was assayed *in vitro* by inhibiting the growth of fungus on sterile PDA medium. PDA medium were prepared and sterilized and poured into the Petri plates. After solidification, the fungal colonies were spread along the plates and Wells were prepared. 0.1 ml Chitinase enzyme were placed in the well and incubated at room temperature. After five days of incubation, the diameter of the zone of inhibition was measured and expressed in mm.

Results and discussion

In previous papers we documented and discussed about isolation, characterization and production of acidic chitinase from *Neisseria* species under controlled conditions [1, 5]. In the present study, partial purification and antifungal activity of crude chitinase from *Neisseria* species was planned. Partial purification of acidic chitinase was obtained by ammonium sulphate precipitation and dialysis under controlled conditions [3, 6]. The results are shown in table I & Figure I.

Crude acidic chitinase was 25 ml and partially purified up to 25 ml by using standard protocols. Crude enzyme showed 29.80 enzyme activity (µM/ml/min.) and 1.19 Specific Activity (uM/mg) with 1 fold purification while Partially purifird Crude acidic chitinase showed 18.79 enzyme activity (µM/ml/min.) and 3.75 Specific Activity (uM/mg) with 3.15 fold purification. Further, protein content was determined by folein-Lowery method. 3.15 folded acidic chitinase was processed for SDS-PAGE for molecular weight determination under controlled conditions where it showed 43kDa molecular weight as compared to standard values of proteins.

Table 1: Acidic chitinases concentration at different purification steps.

Sr. No	Protein purification stage	Protein Volume (ml)	Enzyme activity (µM/ml/min.)	Specific Activity (uM/mg)	Fold Purification
1	Crude enzyme (A)	25	29.80	1.19	1
2	Partilly purified enzyme (B)	15	27.69	1.84	1.54
3	Purified chitinase (C)	5	18.79	3.75	3.15

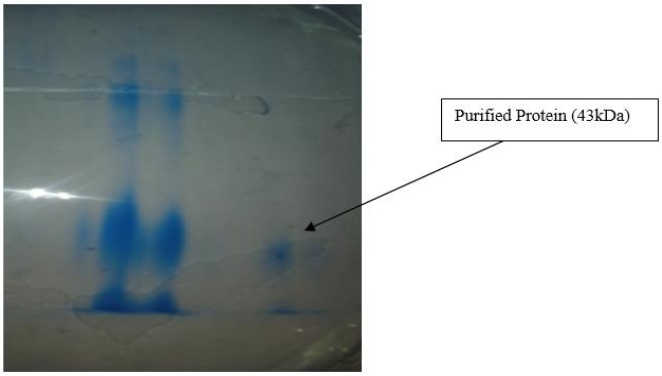

Fig 1: SDS-PAGE for purification and molecular weight determination of partially purified chitinases, 43kDa molecular weight of purified chitinase

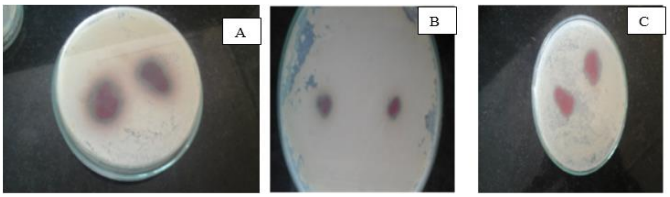

Fig 1: Antifungal activity of *Neisseria* chitinases against (A) *Trichoderma* sp. (B) *Aspergillus* sp.(C) *Candida* sp. by agar diffusion method at 25^{O}C with 0.1ml of chitinases.

The molecular weight of partially purified chitinase was determined by SDS-PAGE (43kDa) chitinases reported by Adesina *et al*., 2007 [1] were in the similar molecular weight range. Isolated chitinases from *Neisseria* species is effective against different fungi such as *candida* sp, *Tricoderma* sp. and *Aspergillus* sp. These results were performed somewhat similarities with Bansode *et al*., 2006; Adesina *et al*., 2007 [1]; Aziz *et al*., 2008 [4].

Conclusion

This study concludes that present chitinases would be effective against phytopathogens such as C*andida* sp, *Tricoderma* sp. and *Aspergillus* sp.

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