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Some properties of chitinase produced by a potent *Aspergillus carneus* strain

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Summary. Seven fungal isolates characterized by high chitinolytic activity were isolated from soil and identified. *Aspergillus carneus* in a 7-day-old shaken culture was the most promising chitinase producer. The use of chitin as a carbon source favoured production of extracellular chitinase enzymes. Maximum chitinase activity was reached at 10 g chitin/l. An initial pH value of the culture medium of 5.0 gave the highest chitinolytic activity. Some properties of the crude enzyme produced by *A. carneus* were studied. Maximal enzyme activity was reached at pH 4.5 and 40° C after 30 min. Thermal treatments at 70° C and pH 4.5 had the most adverse effect on enzyme activity.

Introduction

Enzymes for the breakdown of chitin are found in a variety of living organisms.

Several microorganisms have been studied with respect to the liberation of chitinolytic activity (▮▮▮). The decomposition of chitin by bacteria has been a subject of sporadic interest and its breakdown by fungi has so far attracted even less attention.

The present work was undertaken to investigate certain factors affecting the production of chitinase by the fungal isolate *Aspergillus carneus*. The general properties of the enzyme preparation were also studied.

Materials and methods

Isolation of chitin-degrading fungi. Enrichment cultures were set up using a mineral salt medium containing (g/l distilled water): KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4$, 0.001. The pH was adjusted to 5.0, then fine powdered chitin (0.5%) was added as the sole C and N source. The flasks containing media were inoculated with diluted soil samples obtained from a mixture of different Egyptian solis and incubated at 28° C for 2 weeks.

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To isolate chitin-decomposing fungi, 1-ml portions of the previous flasks were appropriately diluted and plated out, using mineral salts agar medium containing colloidal chitin to render it cloudy. The fungi showing clear zones were isolated and transferred to ▮▮▮ (PDA) slants.

Identification of isolated fungi. Seven fungi were obtained and identified locally. Some isolates were confirmed by the International Commonwealth Mycological Institute, Kew, Surrey, London. These fungal cultures were: *Aspergillus carneus, A. candidus, A. niger, Bipolaris specifera* (IMI332266), *Penicillium lilicinium, Sporotrichum pruinosum* (IMI332268) and *Trichoderma koningii.*

Growth studies. The basal growth medium for enzyme production had the following composition (g/l): colloidal chitin (Sigma, St. Louis, Mo., USA), 4.0; yeast extract, 3.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5. The pH was adjusted to 4.5. For preparation of inoculum, 1 ml spore suspension of a 7-day-old culture was transferred to 50 ml basal medium in 250-ml erlenmeyer flasks and incubated on a rotary shaker (180 rpm) for 5 days. Cultivation was in 250-ml erlenmeyer flasks each containing 50 ml sterile medium. One millilitre of inoculum was transferred to the growth medium. The flasks were incubated at 28° C on a rotary shaker (180 rpm) for 7 days. The culture broth from each flask was filtered off and the clear culture filtrate was taken for enzyme assays.

Chitinase activity. One millilitre of appropriately diluted enzyme sample was incubated with 1.0 ml of 1.0% (w/v) pure colloidal chitin (Sigma) in 0.05 M acetate buffer, pH 5.0. The reaction mixture was incubated at 37° C for 1 h. The reducing sugar was measured by the method of Reissing et al. (1955) using a standard curve of *N*-acetyl glucosamine. One unit of enzyme activity is defined as the amount of enzyme required to produce 0.5 µmol *N*-acetylglucosamine under the assay conditions.

Chitosanase activity. Chitosanase was assayed by a modification of the method reported by Balasubramanian and Manocha (1986). One ml of 1.0% (w/v) chitosan (Sigma) in 0.05 M acetate buffer, pH 5.0, was incubated with 1 ml enzyme sample at 37° C for 30 min. The reaction was stopped by the addition of 0.1 ml of 1 M KOH to precipitate chitosan. The reaction mixture was centrifuged. The supernatant was assayed for glucosamine by the method of Rondle and Morgan (1955). One unit of enzyme activity was defined as the amount of enzyme which produces 0.5 µmol glucosamine.

Estimation of protein. The protein content of the enzyme preparation was determined according to Lowry et al. (1951).

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Results and discussion

Examination of fungal isolates for the production of extracellular chitinase

Fungal isolates showing chitinolytic activity were examined for the production of extracellular chitinase in shake flasks. The production of chitinase and chitosanase in the culture filtrates is shown in Table 1. Of the fungal cultures investigated, *A. carneus* was the most potent for chitinase production (30.82 units/ml), followed by *A. candidus* and *P. licinium* (28.8 and 26.1 units/ml, respectively). The least chitinase activity was showen by *B. specifera* (0.01 unit/ml). Therefore *A. carneus* was selected for further and more detailed studies.

Table 1. Examination of chitinase and chitosanase activities of fungal isolates

Fungus	Protein content (mg/ml)	Enzyme activity (units/ml)	
		Chitinase	Chitosanase
Aspergillus carneus	1.03	30.82	0.20
A. candidus	1.12	28.80	0.08
Penicillium lilicinium	0.95	26.10	0.70
Sporotrichum pruinosum	1.22	4.90	0.02
Trichoderma koningii	0.85	4.35	0.00
A. niger	1.36	0.06	0.08
Bipolaris specifera	0.84	0.01	0.00

Culture conditions for maximization of chitinase production by A. carneus

The effect of initial pH of the culture medium on the production of chitinase was investigated over the pH range 3.0–8.0. As shown in Fig. 1a, maximal chitinase and chitosanase production were attained at an initial pH of 5.0. These results are in agreement with those reported for chitinase production from *A. fumigatus* (Monreal and Reese 1969) and *T. harzianum* (Elad et al. 1982).

Several carbon sources were investigated for the production of extracellular chitinase by *A. carneus*. Chitin served as a superior carbon source for chitinase production. Excretion of chitinase into the culture medium was enhanced by the concentration of chitin. Maximum enzyme production was achieved at 1.0% (w/v) chitin concentration. A little chitinase activity (0.92 and 1.8 units/ml) was detected with 1.0% chitosan and cellulose respectively. On the other hand no enzyme was detected with cellobiose (0.5%), glucose (1.0%), glucosamine (0.2%), lactose (1.0%), starch (1.0%), and sucrose (1.0%). These results coincide with those reported by other investigators for chitinase production (Monreal and Reese 1969; Ohtakara et al. 1979; Elad et al. 1982; Robert and Cabib 1982; Young et al. 1985).

On an equivalent nitrogen basis, the yeast extract in basal medium was replaced by peptone, meat extract, corn steep, soybean, $(NH)_2SO_4$, urea and $NaNO_3$. Of the nitrogen sources investigated yeast extract was the best for chitinase production (44.3 units/ml) followed by peptone (38.47 units/ml) and meat extract (30.0 units/ml). Urea was comparatively the least favourable (1.33 units/ml). In this connection, yeast extract was found to enhance chitinase production from *Serratia marcescens* (Monreal and Reese 1969).

The effect of phosphate level in the culture medium using 0.125–2.5% KH_2PO_4 was also investigated. The highest chitinase activity (47.3 units/ml) was obtained

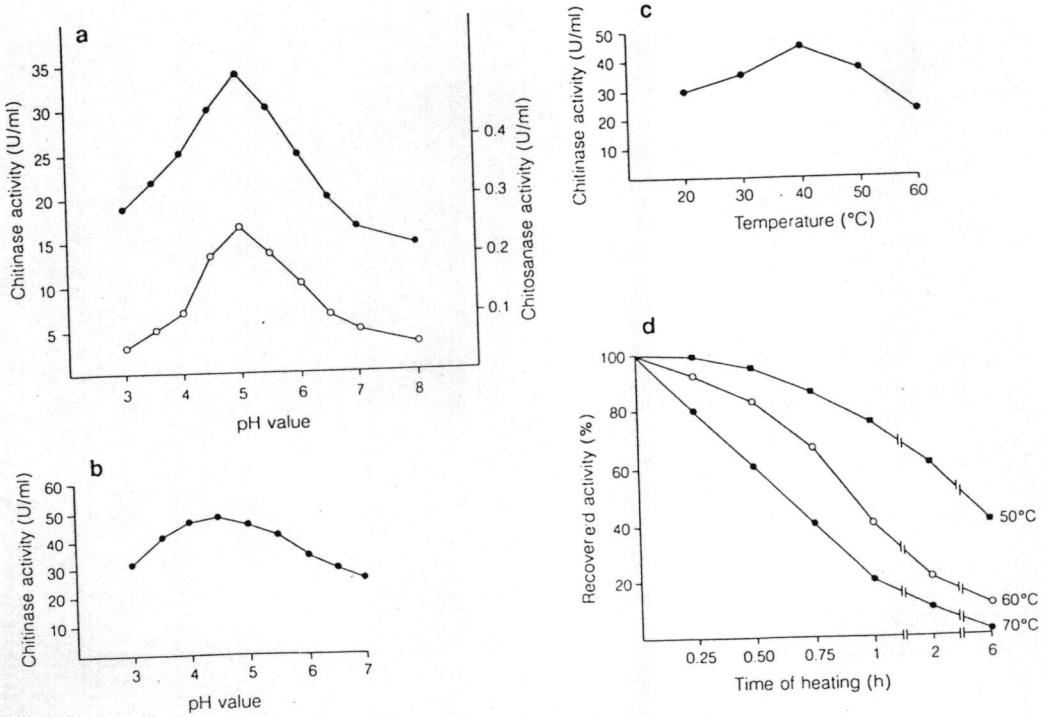

Fig. 1a–d. Production of chitinolytic enzyme activity by *Aspergillus carneus*. **a** Effect of initial culture pH on production of chitinase and chitosanase. **b** Effect of reaction pH on chitinase activity. **c** Effect of reaction temperature on chitinase activity. **d** Thermal stability of the chitinase

with 0.15% KH_2PO_4. The chitosanase activity appeared to be only slightly affected (0.32–0.38 unit/ml) over the KH_2PO_4 range used.

Surfactants have been shown to increase cell permeability and hence promote enzyme release (Reese et al. 1969). However, no significant increase in enzyme yield was observed by the addition of 1.0% (v/v) of Tween 80 and Tween 20 to the culture medium of *A. carneus*. On the other hand, the production of chitinase by *S. marcescens* was repressed by the addition of Tween 80 to the culture medium (Monreal and Reese 1969).

Chitinase production in optimum medium

From the above results and optimal medium was formulated consisting of (g/l): colloidal chitin, 10.0; yeast extract, 3.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; and KCl, 0.5. The pH was adjusted to 5.0. In the optimum culture medium *A. carneus* yielded 47.3 units/ml of chitinase and 0.38 unit/ml of chitosanase, a 43.73% increase in chitinase and 90% increase in chitosanase compared to the original basal medium (Table 1).

Properties of A. carneus crude enzyme preparation

Chitinase showed its maximal activity at pH 4.5 (Fig. 1b). These results partially agree with those of Roberts and Cabib (1982), who reported a broad pH optimum between 4.0 and 7.0 for *S. marcescens* chitinase. On the other hand, the results obtained in the present work are lower than those obtained by Ohtakara et al. (1979), who recorded an optimal pH between 6.0 and 8.0 for chitinase from *Vibrio* sp. These differing pH optima may reflect variations in the properties of enzymes from different strains.

The enzyme showed maximal activity at 40° C (Fig. 1c). This is higher than that reported earlier by Reisert (1972) who recorded a temperature optimum of 25° C for *Chytriomyces hyalins* chitinase. On the other hand an optimum of 50° C was obtained for chitinase activity from *S. marcescens* (Monreal and Reese 1969).

The thermal stability of *A. carneus* chitinase was investigated by preincubation of the enzyme solutions in acetate buffer (0.05 M, pH 4.5) for different periods with the temperature range 50–70° C (Fig. 1d). The results showed that the enzyme lost about 25% of its original activity on heating in the absence of substrate at 50° C for 1 h. At higher temperatures the enzyme activity rapidly decreased. Only 3.5% of the original activity was retained after heating the enzyme solution for 6 h at 70° C.

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