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Activity of Thermostable Proteases of some Actinomycetes and Fungi

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ABSTRACT. The activities of thermostable proteolytic enzymes produced from the most potent. *Streptomyces sp., T. vulgaris, Aspergillus fumigatus* and *Penicillium sp.*, isolated from soils of the Eastern Region of Saudi Arabia were investigated. The enzymes were most active toward gelatin at pH 5-7, 6-7, and 5-5.6, and the optimum temperatures were 50, 50, 40 and 50-55°C, respectively.

The proteases exhibited extreme thermostability. The enzymes from *T. vulgaris* and *Penicillium sp.* retained 100% activity after 30 minutes at 70°C. and those from *Streptomyces sp.* and *A. fumigatus* after up to 20 min. After 2 hr at 70°C they lost 22, 45, 33 and 45% of activities, respectively. The protease of *Streptomyces sp.* was a specific enzyme for gelatin, but the proteases of the other species could hydrolyze casein and albumin also. The enzymes exhibited fair stability at 4°C during 3 months.

The nutritional and environmental factors which affect the production of proteases by *Streptomyces sp., Thermoactinomyces vulgaris, Aspergillus fumigatus* and *Penicillium sp.* were investigated previously (Ali 1990, 1991a) and the optimum conditions for the production of these enzymes were determined. These species the most active producers of proteases among all the actinomyces and fungi isolated previously.

Many works have been done on proteases from actinomycetes (Mizusawa *et al.* 1964 and 1966, Stachowicz 1973 and Ansari *et al.* 1984), from *Aspergillus spp.* (Cohen 1973, Naguib *et al.* 1978, Ansari and Steven 1983) and from *Penicillium spp.* (Kim 1974).

Generally, the production of proteases by actinomycetes and fungi is rare in Saudi Arabia. Therefore, the aim of the present investigation was to characterize the enzyme from the most potent producers.

Materials and Methods

Organisms

Proteolytic actinomycetes (*Streptomyces sp.* and *Thermoactinomyces vulgaris*) and fungi (*Aspergillus fumigatus* and *Penicillium sp.*) were previously isolated from Dammam and Thogbah soils from Saudi Arabia (Ali 1990 and 1991a).

Each strain was grown in liquid media previously found satisfactory for proteases production (Ali 1990 and 1991a).

Modified media for *Streptomyces sp.* and *Thermoactinomyces vulgaris,* consisted of (g/L) : K_2HPO_4, 2.0; Starch, 5.0; Gelatin, 15.0; H_2O, IL. pH of this media was adjusted using citrate - phosphate buffer at 6.0 for *Streptomyces sp.* and 6.6 for *T. vulgaris* (St. G. medium). Modified media for *Aspergillus fumigatus* and *Penicillium sp.* consisted of (g/L): K_2HPO_4, 2.0; sucrose, 5.0; gelatin, 15.0 H_2O, IL, pH, 5.0 for *A. fumigatus* and pH 6.0 for *Penicillium sp.* using the same buffer (S.G. medium).

Cultivation

The four organisms were grown separately in 500 ml Erlenmeyer flasks containing 100 ml suitable production media (St. G. and S.G.) and receiving 1 ml of *Streptomyces sp.* (72 hr culture); 1 ml of *Th. vulgaris* (16 hr culture); 1 ml of *A. fumigatus* (5 days culture) and 1 ml of *Penicillium sp.* (5 days culture) inocula respectively. The flasks of *Streptomyces sp., T. vulgaris; A. fumigatus* and *Penicillium sp.* were incubated at 30, 55; 30 and 25°C for 60 hr for actinomycetes and 5 days for fungi respectively.

Crude enzymes

The cell-free sterile culture filtrate from each organism was separately dialyzed against sucrose (Elwan *et al.* 1977 and 1978a), to obtain crude enzymes (2 × dialysate), used as a stock crude enzyme solution. This was stored at 4°C.

Proteases assay

The clear zone cup agar plate method (GCZ) was used (Ali 1990, 1991 and 1991a).

Properties of proteases

Using the GCZ method, three cups were made per plate and in each cup 0.1 ml of crude enzyme solution was transferred. Triplicate plates were set for each enzyme of each organism and the mean diameters of the clear zones were measured to the nearest 0.5 mm.

(a) To determine the effect of different temperatures on enzyme activity, the plates were incubated for 6 hr at controlled temperatures between 20 and 65°C.

(b) The effect of different pH was examined in phosphate buffers covering the range 5.0-8.0 at suitable temperature for each enzyme.

(c) The influence of different concentrations of gelatin, between 0.5 and 3.5% (w/v) was explored.

(d) The thermostability of the enzymes was examined by heating 5 ml samples in closed culture tubes at 70°C, with aliquots taken at 10 min. intervals for up to 2 hr for assay by GCZ technique.

(e) The effect of storage was examined weekly by assaying the proteolytic activity of the crude enzyme of each organism, during storage at 4°C, for three months.

(f) The effect of different substrates on the enzyme activity was examined with gelatin, casein and albumin in the assay medium at a concentration of 1.5% (w/v).

Results

Factors affecting protease activity

The crude enzymes produced under the optimal conditions from *Streptomyces sp., Th. vulgaris, A. fumigatus* and *Penicillium sp.* had activities equivalent to 31, 348, 5565 and 31 μg/ml respectively using the standard curve previously described (Ali 1990 and 1991a).

(a) Fig. (1) illustrates the effect of temperatures ranging from 20 to 60°C upon proteases activity of each organism. The optimal temperatures were observed to be 50, 50, 40 and 50-55°C for the enzymes of *Streptomyces sp., Th. vulgaris; As. fumigatus* and *Penicillium sp.* respectively. All enzymes were approximately stable between 40-50°C, but a significant reduction in their activities occurred over 65°C.

(b) Fig. (2) shows the optimum pH of enzymes activity of *Streptomyces sp., Th. vulgaris; As. fumigatus* and *Penicillium sp.* had broad optima ranging

from pH 5.0-7.0, 6.0-7.0, 5.0-5.6 and 5.0-5.6 respectively.

(c) The activity increased with increasing the concentration of gelatin substrate. Optimum activity was at 7.5% for enzymes of all organisms as shown in Fig. (3).

(d) Studies were conducted to determine the thermostability of the crude enzyme proteases. Fig. (4) shows that the enzymes were impressively thermostable at 70°C. The enzymes from *Th. vulgaris* and *Penicillium sp.* were found to be completely thermostable at 70°C for up to 30 min. while the enzymes from *Streptomyces sp.* and *As. fumigatus* retained 71 and 95% of their activities. The enzymes from *Th. vulgaris, Streptomyces sp.; Penicillium sp.* and *As. fumigatus* lost only 22, 33, 45 and 45.5% of their activity heating at 70°C for as long as 160 min.

(e) The enzymes from *As. fumigatus, Th. vulgaris, Streptomyces sp.* and *Penicillium sp.* were found fairly stable at 4°C after 3 months of storage, with losses of 27, 28, 30 and 33% respectively. After 1.5, 2 and 2.5 months of storage respectively the losses were 6.7, 8, 6.7 and 10%; 6.7, 20, 16.7 and 16.7%; and 23.3, 24, 23.3, 26.7% Fig. (5).

(f) With regard to the specificity of the enzymes, gelatin, casein and albumin as substrates were tested; the maximum activity of all enzymes was obtained with gelatin. The enzyme from *Streptomyces sp.* could not hydrolyze either casein or albumin. The enzyme of *Penicillium sp.* hydrolyzed all substrates at the same rate. The results are shown in Fig. (6).

Discussion

Only a few investigations have been made of the activity of proteases of *Aspergillus spp.* (Sugiura *et al.* 1972, Cohen 1973, Danno 1973, Kolodzeeiskaya *et al.* 1973, Lyublinskaya *et al.* 1973, Sekine 1973, Tulaganov and Zakirov 1973 and Klocking and Markwardt 1975). There is no information about proteases of *A. fumigatus.* Very little is known about proteases of *Penicillium spp.* (Ghosh and Thangamani 1973 and Kim 1974).

Likewise there is little information on the proteases from *Streptomyces spp.* and thermophilic actinomycetes (Orcharov 1961, Mizusawa *et al.* 1964 and 1966, Petrova and Dolidze 1972, Dolidze and Petrova 1973, Stachowicz 1973, Tsyperovych *et al.* 1973 and Nakanishi and Yamamoto 1974). Also information on proteases of *Th. vulgaris* is lacking. In this report we were able to obtain thermostable proteases from *Streptomyces sp., Th. vulgaris, A. fumigatus* and *Penicillium sp.* with a high yield from each organism (31, 348, 5565 and 31 μg/ml respectively) as crude enzymes.

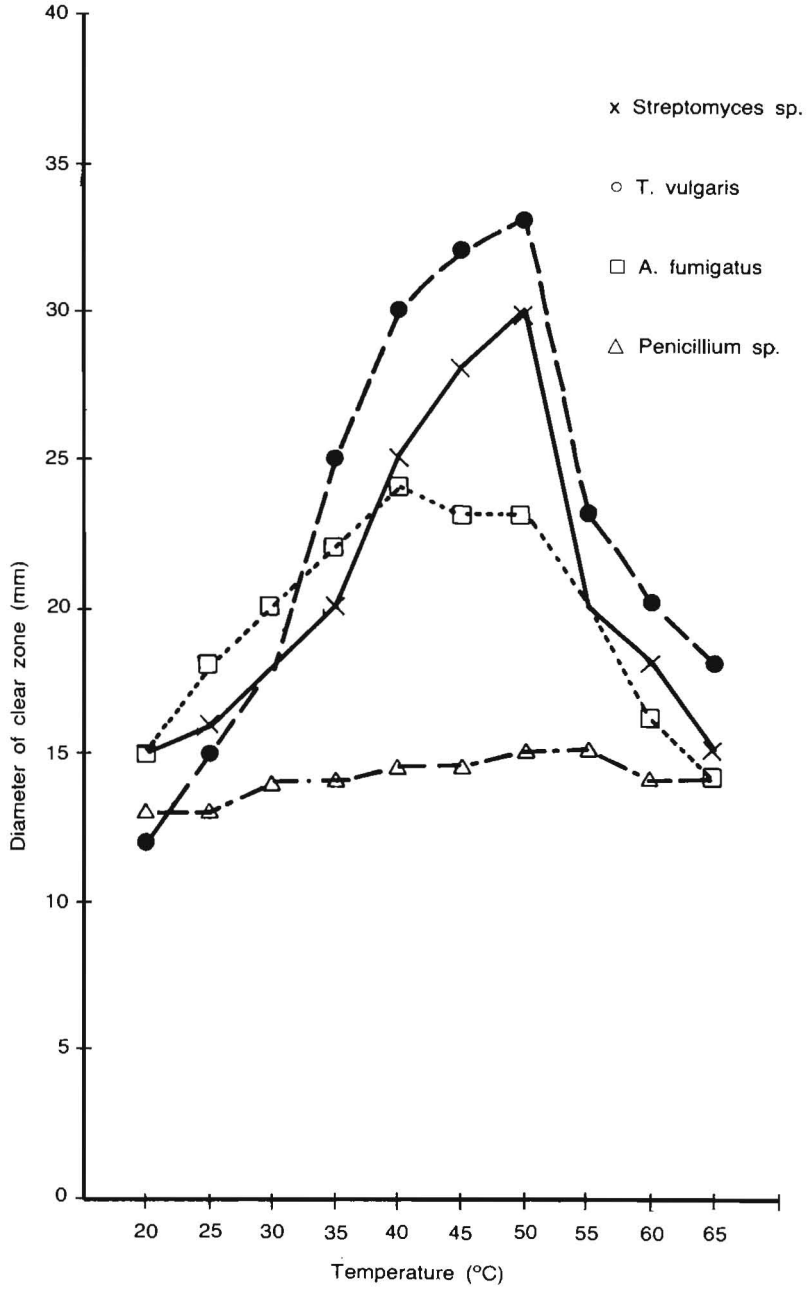

Fig. 1. Effect of different temperatures over 6 hr in the GCZ assay on the activity of the crude proteases from selected actinomycetes and fungi.

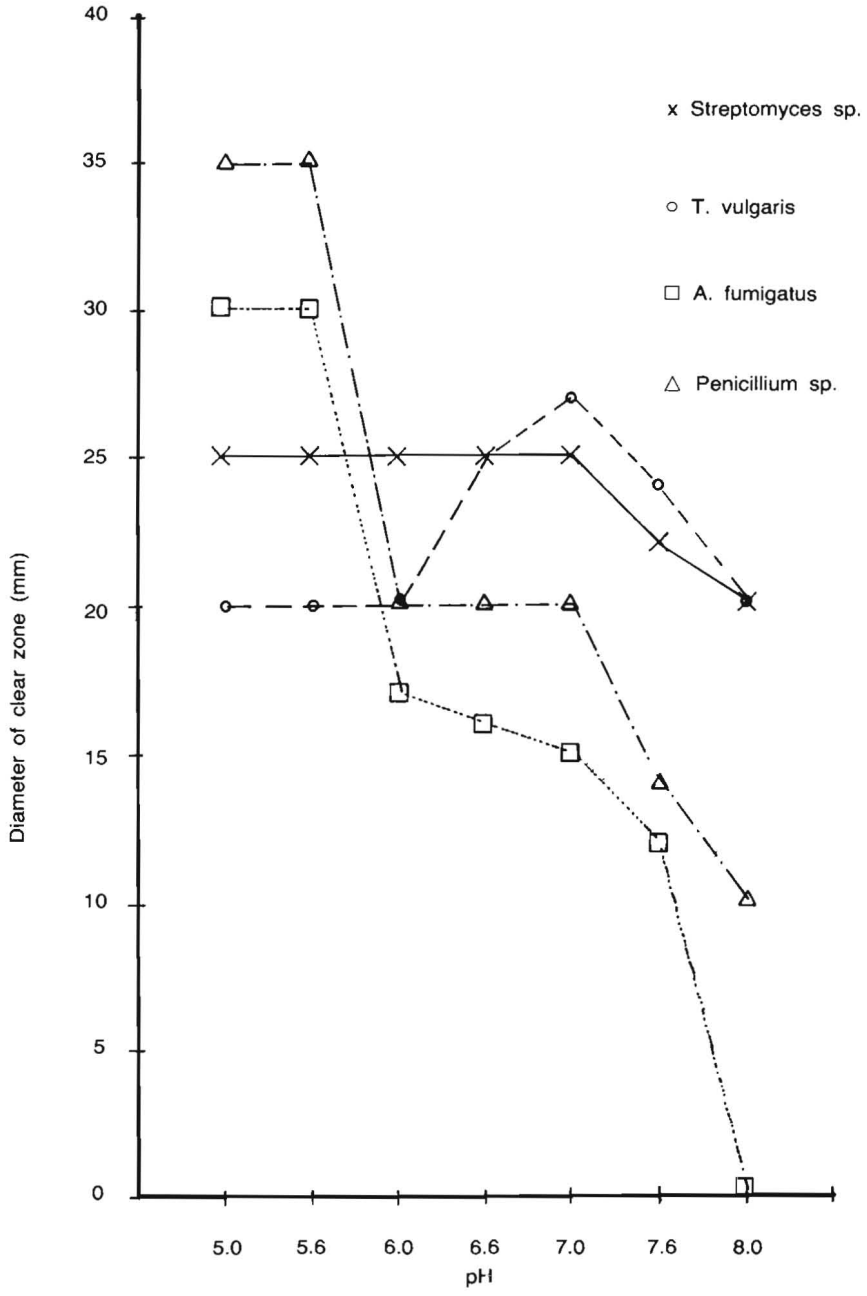

Fig. 2. Effect of different pH of phosphate buffer on the protease activity from selected actinomyces and fungi at suitable temperatures at 6 hr as shown by the GCZ assay.

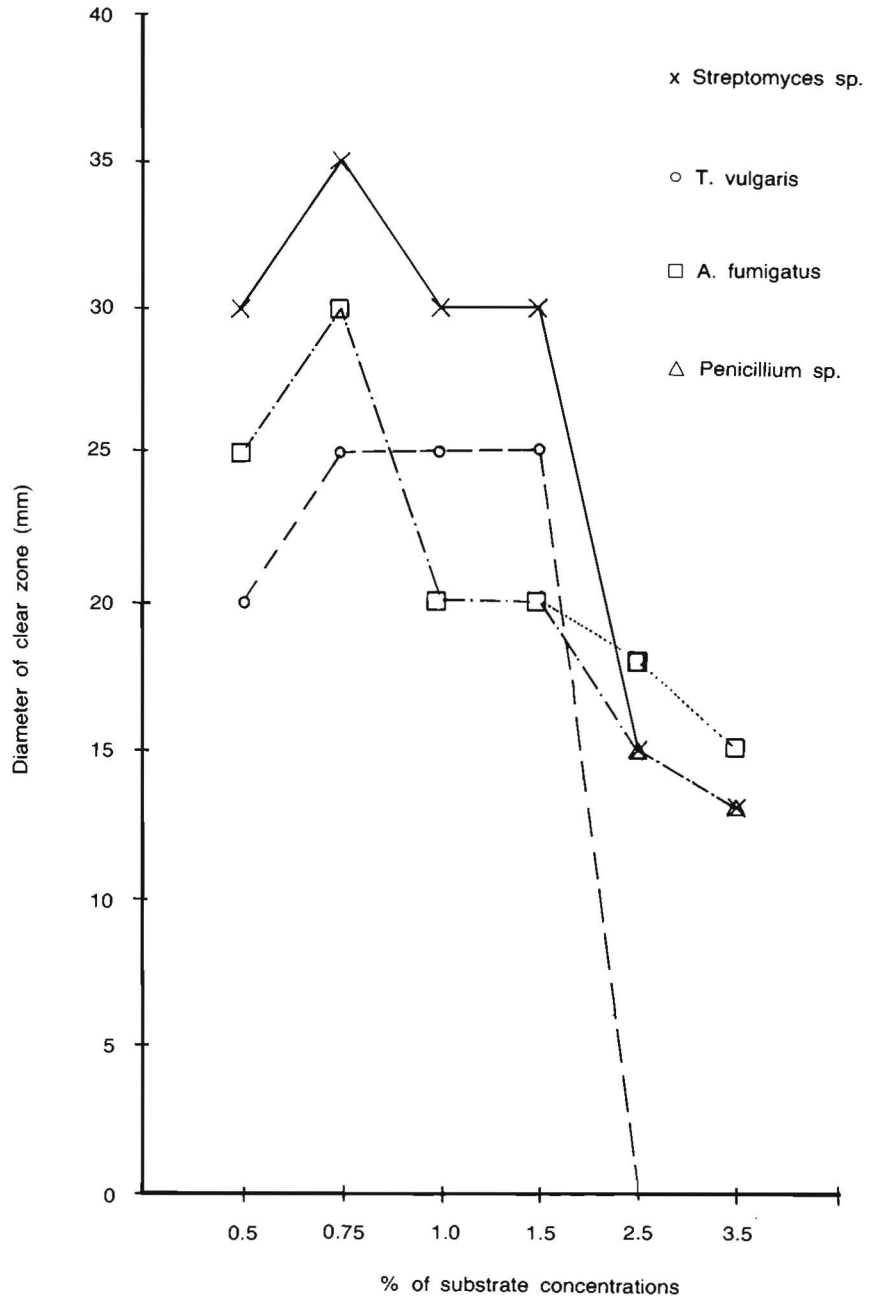

Fig. 3. Effect of substrate concentrations on protease activity from selected actinomyces and fungi as shown by the GCZ assay for 6 hr at suitable temperatures.

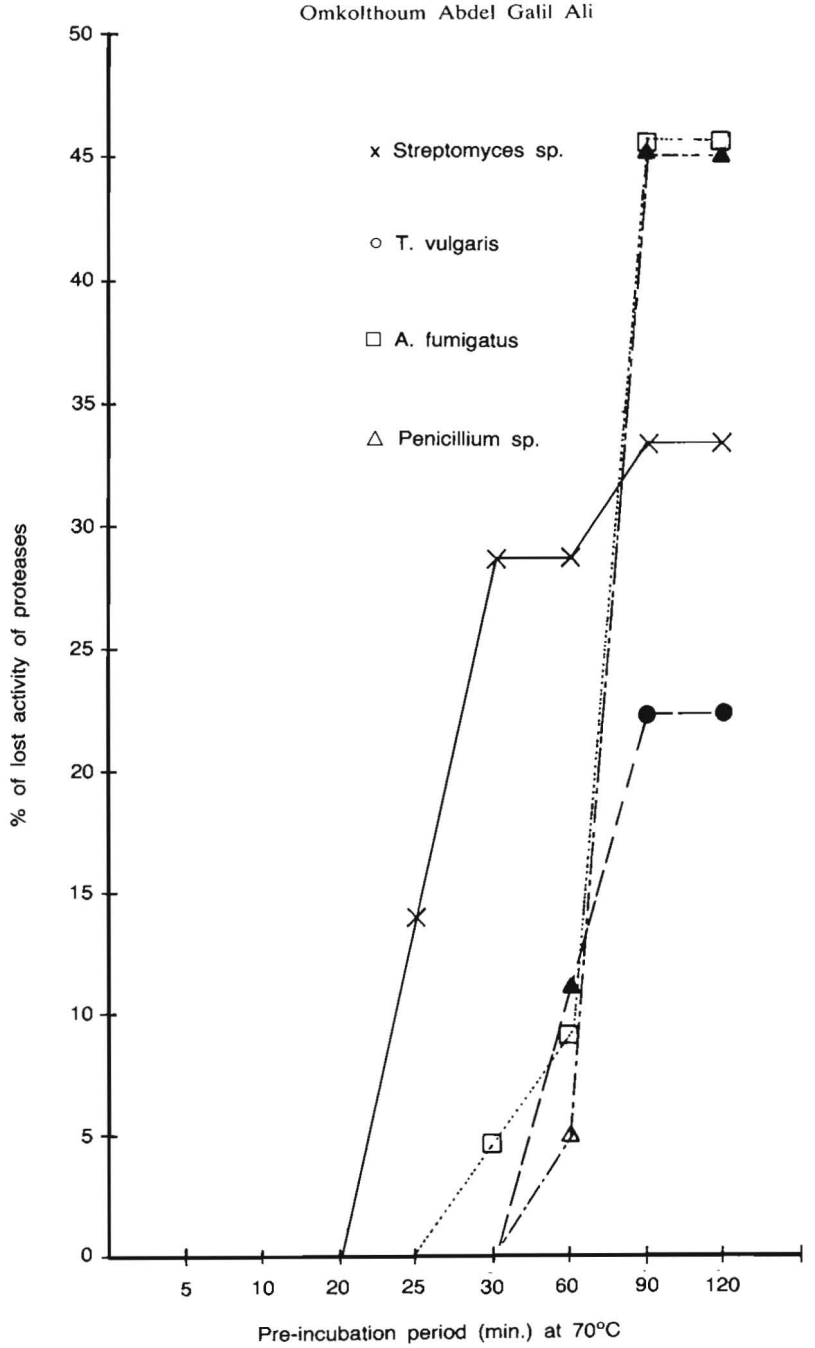

Fig. 4. Thermostability of proteases after heating at 70°C and subsequently tested by the GCZ assay for 6 hr at suitable temperatures.

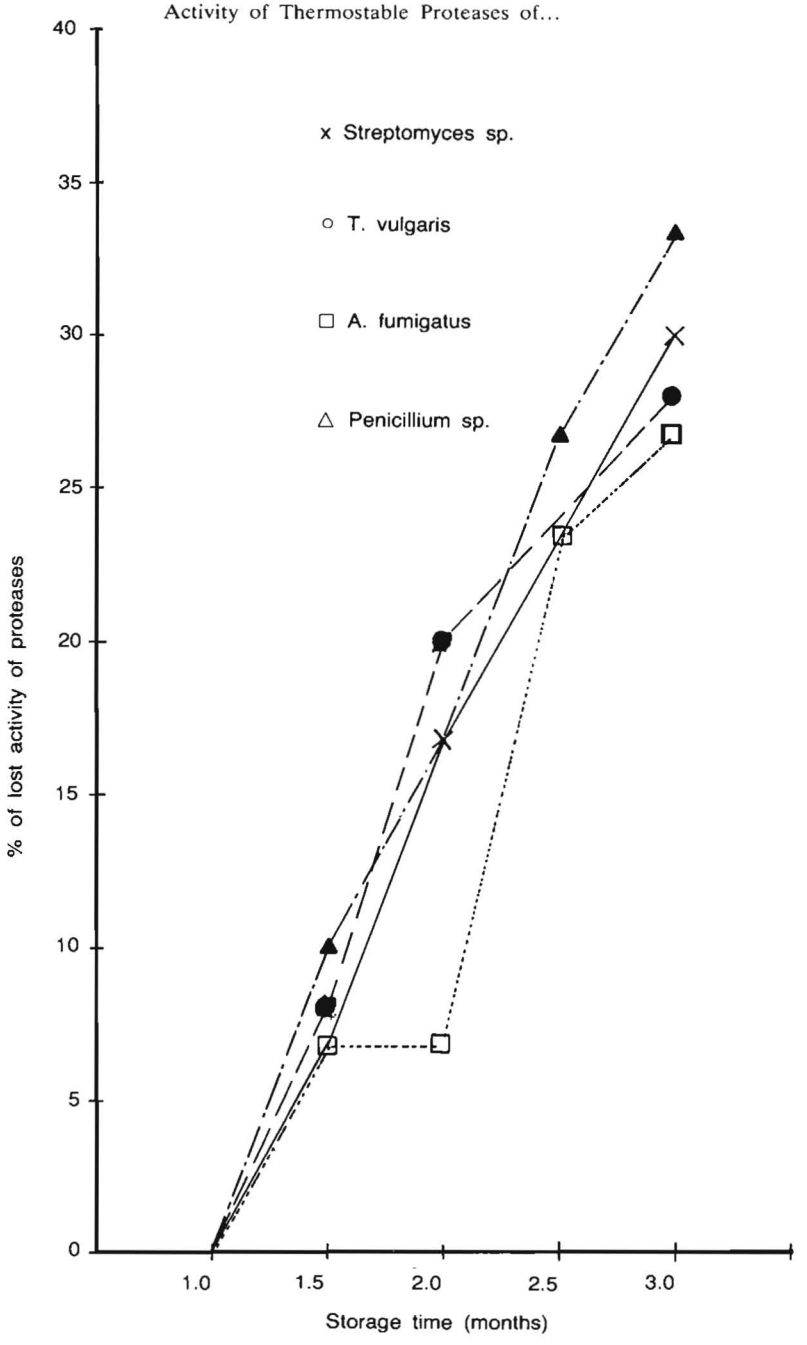

Fig. 5. Effect of three months storage at 4°C on protease activity, as measured by the GCZ assay for 6 hr at suitable temperatures.

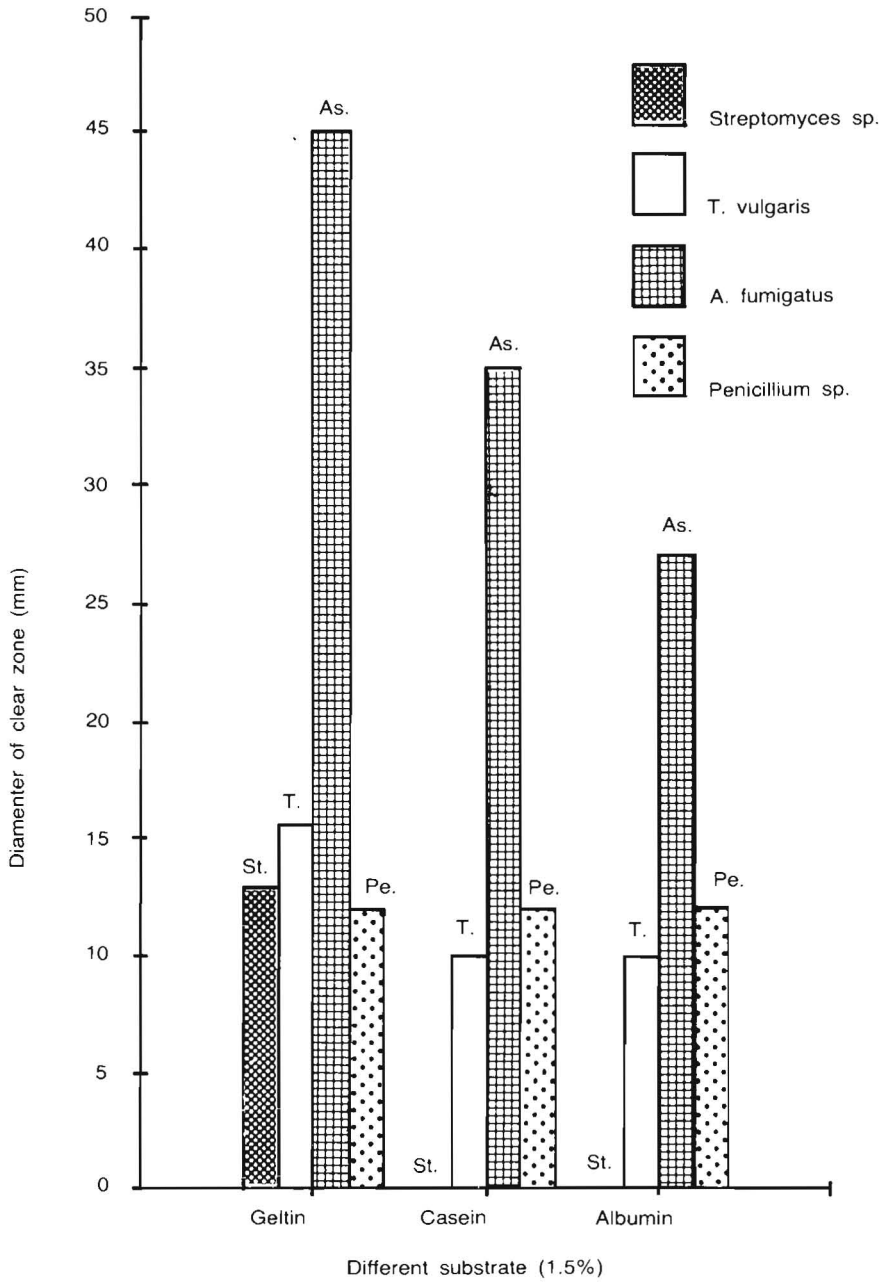

Fig. 6. Effect of different substrates at concentration of 1.5% in the assay medium. Incubation was for 6 hr at suitable temperatures.

The clear zone cup agar plate technique used is considered to be semiquantitative. Many investigators have used this technique. Doery *et al.* 1965, Lawrence *et al.* 1967, Elwan *et al.* 1977 and 1978a used it for assay of lipase activity. And Naguib *et al.* (1978) and Ali (1990, 1991 and 1991a) used it for proteases activity.

Proteases activity of *Streptomyces sp.* and *T. vulgaris* was optimum at 50°C and pH 5.0-7.0 and 6.0-7.0 respectively. Stachwicz 1973 reported that the crude proteolytic enzyme of *Streptomyces erythraeus* possesses maximum activity at pH 8.4 and 60-80°C. The optima temperature and pH of thermostable protease of *Bacillus spp.* have been recorded pH 5.6 at 40°C, pH 9 and 45°C, pH 12-13 at 60°C by (Yoshio and Tsujisaka 1975, Manachini *et al.* 1988 and Takami *et al.* 1989) respectively. The results suggest that the enzymes of *Streptomyces sp.* are acidic and neutral proteases, while the enzyme of *T. vulgaris* is a neutral protease. Cohen 1973 found that the neutral and alkaline proteases of *A. nidulans* were separated to B_1 and B_2 by electrophoresis. In the case of protease activity of *A. fumigatus* and *Penicillium sp.* we showed optima at pH 5.0-5.6 and 40, 50-55°C respectively. Thus also can suggest that the enzyme of *A. fumigatus* and *Penicillium* sp. are acidic proteases.

Proteases activity from *Penicillium sp.*, *Aphanomyces* and *Choenephora cucurbitarum* and *Phascolomyces articulosus* had pH and temperatures optima at pH 8-11 and 50°C, pH 7.0 and 45-50°C and pH 7.0 and 37°C (Kim 1974, Kenneth and Vnestam 1975 and Balasubramania and Manocha 1987). These findings, approximately agreed with our results.

The presence of more than one enzyme in the crude preparation as judged by the broad activities at pH 5.0-9.0 and at temperatures from 20-65°C was suggested. Such results have been observed with proteases of *Bacillus spp.* (Ali 1991). Similarly (Kundu *et al.* 1968), suggested that the enzyme system of *A. oryzae* proteinase(s) is either mixture of two enzymes or that it possesses two different kinds of functional groups that act differently. Also Naguib *et al.* 1978 stated that they found that the fungi under test have the ability to produce more than one proteolytic enzyme.

The protease from *T. vulgaris* and *Penicillium sp.* were extremely thermostable. They remained active after 30 min. heating at 70°C, whereas the thermostabilities of proteases from *Streptomyces sp.* and *A. fumigatus* were some what less: during 20 min. at 70°C, they retained all their activity, but after 30 min. they had 71 and 95% activity, respectively. The protease from *Th. vulgaris*, *Streptomyces sp.*, *Penicillium sp.* and *A. fumigatus* lost 22, 33, 45 and 45% of the activities at 70°C during 2 hr. Thus these enzymes were extremely thermostable.

Such results have been observed with the crude enzyme of proteases from the proteolytic *Bacillus spp.* isolated from Eastern Region of Saudi Arabia (Ali 1991). They retained 100% activity at 70°C for 30 min. This finding also shows the similar trend of thermostable proteases from thermophilic bacteria (Hatton and Regoeczi 1973, Chopra and Mathur 1983 and 1984 and Manachini *et al.* 1988). These findings support the fact that thermostability of protease producing strains could be due to their genetic adaptability to carry out their biological activity at high temperature (Gaur *et al.* 1989). Whereas the proteases from *B. polymyxa* and *Bacillus sp.* were inactivated rapidly at higher temp. and were destroyed after 10 min. at 60°C (Patrick and Fogarty 1973 and Fujiwara and Yamamoto 1987). Informations about thermostabilities of proteases of actinomycetes and fungi are lacking.

The broad activity towards substrate concentrations from 0.5 to 3.5% and the relative stability of the activities towards the concentration from 0.5 to 1.5%, also suggest the presence of more than one proteases in the crude enzymes.

Tsyperovych *et al.* (1973) reported that *Streptomyces griseus* produced a proteolytic complex possessed 14 different exoproteases which differed in their proteolytic activity.

The protease of *Streptomyces sp.* was a specific enzyme, but proteases of the other organisms could hydrolyze casein and albumin beside gelatin.

Proteases of *Penicillium chrysogenum* (Bezborodova 1960) showed their maximum activity on gelatin. Smirnov *et al.* (1972) found that proteases of *A. awarnari, A. niger* and *A. oryzae* hydrolyzed albumin, casein and gelatin. Casein and albumin hydrolyzed by proteases of *Streptomyces fradiae* (Petrova and Dliolze 1972). Casein was the preferred substrate for the proteases of *Bacillus spp.* and *Pseudomonas fluorescence* (Yan *et al.* 1985).

The results have established that proteases from all organisms were fairly stable at 4°C during 3 months storage. Cohen (1973) reported that proteases from *A. nidulans* converted from B_1 and B_2 to B during storage of mycelial extracts at 4°C, but did not occur in stored culture filtrates.

Many investigations have been done on the enzyme properties of the supernatunt fluids or of the crude enzymes (Elwan *et al.* 1971, 1977 and 1978a, Yamamoto *et al.* 1972, Day 1978, Gaur *et al.* 1989, El-Rahman 1990 and Ali 1991).

We hope to make investigations about these enzymes after purifications.

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نشاط انزيم البروتياز الصامدة للحرارة من بعض الفطريات والاكتينوميسيتات

أم كلثوم عبدالجليل علي

كلية العلوم للبنات ـ الدمام ـ ص. ب: ٢٨٣٨ ـ الدمام ٣١١١٣ ـ المملكة العربية السعودية

أنتجت الانزيمات المحللة للبروتينات من أفضل السلالات الفطرية والاكتينوميسيتية المنتجة لهذا الانزيم والتي عزلت من تربة المنطقة الشرقية بالمملكة العربية السعودية وهي سلالة من الاستربتوميسيس وثيرمواكتينوميسيس فلجاريس واسبرجلس فيوميجاتس وسلالة من البنسيليوم.

درست العوامل التي تؤثر على نشاطات هذه الانزيمات، وأظهرت النتائج أن أفضل درجة تركيز أيون الهيدروجين هو (٥ ـ ٧ ، ٦ ـ ٧) لنشاط الاستربتوميسيس وثيرمواكتينوميسيس فلجاريس و(٥ ـ ٦ ، ٥) لنشاط الانزيمات المنتجة من الاسبرجلس فيوميجاتس وسلالة البنسيليوم. وكانت درجة الحرارة المثلى لنشاط هذه الأنزيمات (٥٠، ٥٠، ٤٠، ٥٠ ـ ٥٥°م) على التوالي. وأظهرت النتائج أيضاً أن هذه الأنزيمات صامدة بشدة لدرجات الحرارة العالية حيث أن أنزيمات كيرمواكتينوميسيس فلجاريس وسلالة البنسيليوم لا تفقدان أي شيء من نشاطهما عند تسخينها لدرجة ٧٠°م لمدة ٣٠ دقيقة ويفقدان ٢,٢٢ ، ٤٥ ٪ من نشاطهما بعد ساعتين، أما انزيمات سلالة الاستربتوميسيس واسبرجلس فيوميجاتس فلا تفقدان أي شيء من نشاطهما عند نفس الدرجة لمدة ٢٠ دقيقة وتفقدان ٣٣,٣، ٤٥,٥ ٪ من نشاطهما بعد ساعتين عند نفس الدرجة. وقد

وجد ان الانزيمات تستطيع أن تحلل الكازين والالبومين أيضاً، ولكن انزيم الاستربتوميسيس أظهر التخصص في نشاطه أي أنه يحلل الجيلاتين فقط. وأظهرت النتائج أيضاً أن هذه الانزيمات لا تفقد أي شيء من نشاطها عند التخزين لمدة ثلاثة شهور عند درجة ٤°م.