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Proteolytic Activity of Some Local Isolates of Yeasts as Affected by Cultural Conditions

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Abstract: The ability of soil yeasts, (*Candida tropicalis*, *Geotrichum candidum*, *G. capitatum*, *Rhodotorula minuta* and *R. rubra)* to produce extra cellular protease *in vitro* was investigated. It appeared that the soil yeasts were proteolytic, with the exception of the yeast *C. tropicalis*. Production of extra cellular protease led to a marked reduction in pH of the medium.
G. capitatum followed by *G. candidum* were excellent proteolytic producers, and they were the most active yeast in this process, both *R. minuta* and *R. rubra* were average producers. *C. tropicalis* failed to produce protease enzyme with exception of trace amount towards the end of incubation temperature 30°C. The optimum incubation temperature for maximum proteolytic activity was 30 °C. The maximal proteolytic activity was attained in culture medium supplemented with maltose as a sole carbon source. Also peptone was found to be the most suitable nitrogen source for protease production by soil yeasts selected.

Key words: soil, yeasts, protease, yeast biomass, environments

Introduction

Fungi have traditionally been regarded as decomposing organisms whose primary role is the degradation of carbon and nitrogen-rich residues such as leaf litter and wood. The production of various extra cellular enzymes by fungi has a great influence on human life, owing to its important role in the pharmaceutical, food, paper, textile and petroleum industries (Pestana and Castillo, 1985; Bokhary and Parvez 1992). The bio degradation of waste materials is another example of the beneficial effects of fungi, as the waste of garbage will become a great problem for our future generations (Hudson, 1980).
Members of several microbial strains have long been known to posess strong proteolytic activities. At present time, microbiological synthesis is one of the most effective methods of obtaining an enzyme (Allam *et al.*, 1977; Porkorni *et al.*, 1979; Gesheva *et al.*, 1982 and Gabr *et al.*, 1987).
Most of the cited literature dealt with the microbial production of protease by different strains including bacteria, actinomyces, streptomyces and fungi (Keay and Bernards, 1970; Egorov *et al.*, 1976; Cohen, 1980; Abdel-Fattah *et al.*, 1985; Lim *et al.*, 1985; Mansour, 1985; Gabr *et al.*, 1987; Haque *et al.*, 1990; Ismail *et al.*, 1990; Saad 1995;). Also a few studies on protease production by the yeast *Saccharomyces cerevisiae* has been published because it is very common in domestic and industrial use (Hindazlotnk *et al.*, 1983; Abou Hamed, 1995; Al-Falih, 1997).
The soil microbiological yeasts in particular, have been neglected, although they are known to appear in most soils. As a result, little is known about their ecology and the role that they play in mineral cycling (Al-Falih and Wainwright, 1996). A recent study showed that some soil yeasts are capable of producing extra cellular enzymes (Al-Falih 1997). It is suggested that soil yeasts might be used as inoculants to stimulate the beneficial processes of mineral cycling in soils.
This study was carried out to investigate the possibilities of producing protease using soil yeasts, isolated from cultivated soil in Saudi Arabia. Besides, the effect of various carbon and nitrogen sources on synthesis of protease, also the enzyme activity was determined at different incubation temperatures.

Materials and Methods

Isolation of soil yeasts: Soil yeasts were isolated from a sandy soil (total C, 0.3 %; total N, 0.1 %; pH, 7.2, obtained from Riyadh, central region, Saudi Arabia). The yeast strains included, *Candida tropicalis* (Cast.) Berkhout, *Geotrichum candidum* Link, *Geotrichum capitatum* (Diddens & Lodder) V. Arx, *Rhodotorula minuta* (Saito) Harrison var. texensis and *Rhodotorula rubra* (Demme), which were isolated and identified according to the method described by Van der Walt (1970).

Media: The basal medium used was Czapek-Dox liquid for the cultivation of soil yeasts. Suspensions (1 ml) containing 1.4 x 10^5 yeast cells were used to inoculate liquid Czapek Dox medium (100 ml in 250 ml capacity Erlenmeyer flasks), adjusted to pH 6.0 with 2N NaOH.
The flasks were incubated with shaking (100 rpm) at 30 °C for 8 days. After 2 days interval, three flasks were removed and the contents filtered through pre-dried and pre-weighed Whatman No.1 filter papers. Yeast biomass was determined in the medium after filtration. The weight of yeast cells retained by the filter papers was then determined (after drying to a constant weight at 80 °C for 24 h) as a measure of yeast cell biomass. The pH of the medium was determined with a glass electrode.

Enzyme production: Enzyme production in liquid Czapek Dox medium inoculated with soil yeasts was estimated during 8 days of incubation at 30 °C. After 2 days interval the flasks were removed and the cells were harvested by cetrifugation at 12000 xg and the clear supernatant was used as enzyme preparation.

Enzyme assay: It was carried out according to the modified method of Saad (1995); the reaction medium contains 40 mg of soluble casein, 100 micromoles of phosphate buffer pH 7.0 and enzyme source (from culture supernatant), in a total volume of 2.5 ml. The reaction mixture was incubated at 40 °C for 30 min after which the reaction was stoped by addition of 2 ml of 15 % trichloroacetic acid. The reaction mixture was then centrifuged at 3000 rpm for 20 min and the precipitated proteins were discarded. From the resulting supernatant 0.5 ml was withdrawn and pipetted in a test tube containing 1.5 ml of 0.33 M sodium hydroxide. The tyrosin was determined as described by Greenberg (1955). Control sample was the mixture with reaction stopped at zero time.
One unit of enzyme was defined as the amount of enzyme

which yield a colour equivalent to m Mole of tyrosine per minute in 1 ml of reaction mixture under the standard conditions of pH and temperature.

Enzyme optimization: Various sources of carbon (at 4% w/v) and nitrogen (at 0.07% as N base) were selected for determining the optimal nutritional requirements of protease production by soil yeasts. Also the enzyme activity was determined at different incubation temperatures (15, 25, 30 and 40 °C).

Results and Discussion

Data on protease activity produced by soil yeasts as affected by incubation temperature are given in Figs 1a - 5a. These yeasts exhibited wide variabilities of their protease activities. The yeast of *G. capitatum* exhibited the highest protease activity followed by *G. candidum*. A moderate protease was produced by *R. minuta* and *R. rubra*. A slight protease was produced by *C. tropicalis* (8 units/mg protein). In the case of *C. tropicalis* yeast, protease activity was not detected in the medium until day 4, after which the activity of the enzyme increased as yeast biomass increased (Fig 1b). The interesting observation here was that the proteolytic activity of *G. capitatum* was stable after certain time, so the enzyme activities of this yeast was reaching the maximal level after 4 days of incubation.

The yeast cultures were incubated at different temperature (from 15 to 40 °C) to determined the optimum temperature for protease enzyme production by these yeasts. It is obvious that the protease activity was minimum when the incubation temperature was 15 °C, then it increased by increasing the incubation temperature till it reached its maximal value at 30 °C after which the enzyme activity decreased by increasing the incubation temperature over 30 °C. Figs 1a - 5a illustrate that 30 °C was the optimum incubation temperature. This result agree with that obtained by Hindazlotnk *et al.*, (1983); Abou Hamed (1995) and Al-Falih (1997) who found that 30°C is the optimum temperature for protease production by the yeast *S. cerevisiae*. However Saad (1995) reported that 28 °C is the optimum incubation temperature for protease production by *Streptomyces venezulae*.

Results showed that all the soil yeasts were able to produce protease in Czapek-Dox medium, a process which was associated with a reduction in pH of the medium (Figs 1b - 5b). Production of extra cellular protease by soil yeasts led to a marked reduction in the pH of the medium, especially in case of *G. capitatum* that produced the highest percentage of protease activity (Fig. 3b). The decline observed in the pH values following the proteolytic activities of soil yeasts could be due to the formation of amino acids.

The largest amount of biomass (0.42 g) was produced by the yeast of *G. capitatum* (Fig. 3b). However the smallest amount of biomass, 0.10 g was produced by *C. tropicalis* (Fig. 1b). The soil yeasts *R. minuta* and *R. rubra* were average producers of biomass forming 0.18 g and 0.26 g respectively at the end of incubation period. Table 1 shows the effect of carbon sources on protease activity produced by soil yeasts. The culture of *G. capitatum* recorded the maximum protease production in a medium amended with maltose as a sole carbon source. It is clearly shown that maltose followed by sucrose were the best inducers, for protease production. Low protease activity resulted in presence of glycerol. This result is consistent with the findings of previous studies (Egorov *et al.*, 1976; Gesheva *et al.*, 1982; Lim *et al.*, 1985 and Haque *et al.*, 1990).

Enzyme production is decreased by the addition of starch. This result is in agreement with that obtained by Abou Hamed (1995) and Al-Falih (1997) and disagreed with that obtained by Abdel-Fattah *et al.* (1985); Ismail *et al.* (1990) and Saad (1995). This may be due to the disability of yeasts to utilize this sugar. Ethanol and cellulose seemed to be unsuitable for protease production by soil yeasts as inducive carbon sources. Such that no amount of protease was detected in a medium amended with ethanol or cellulose.

The basal Czapek-Dox medium was supplemented by different organic and inorganic nitrogen sources. The addition of an appropriate organic nitrogen source supported activity of protease than that induced by the inorganic ones (Table 2). Again difference in the levels of enzyme activities of the soil yeasts was observed. For example, *G. capitatum* has been found to be the best

Table 1: Effect of carbon sources on protease production by soil yeasts (Values given are means of triplicates ± standard deviation).

Carbon sources*	*C. tropicalis*	*G. candidum*	*G. capitatum*	*R. minuta*	*R. rubra*
Sucrose	7.5 ± 0.2	65.2 ± 7	70.1 ± 5	34.8 ± 2	32.1 ± 1
Glucose	-	64.0 ± 3	69.3 ± 4	8.1 ± 0.4	-
Maltose	8.9 ± 0.1	66.1 ± 6	75.9 ± 7	27.4 ± 5	33.0 ± 9
Starch	2.1 ± 0.4	28.3 ± 9	26.1 ± 1	3.3 ± 7	6.4 ± 3
Glycerol	-	40.4 ± 2	45.2 ± 8	15.6 ± 3	11.9 ± 2
Ethanol	-	-	-	-	-
Cellulose	-	-	-	-	-

*: Carbon sources were added at 4% w/v.

Table 2: Effect of nitrogen sources on protease production by soil yeasts (Values given are means of triplicates ± standard deviation).

Nitrogen sources*	*C. tropicalis*	*G. candidum*	*G. capitatum*	*R. minuta*	*R. rubra*
$NaNO_3$	6.1 ± 0.3	65.2 ± 1	62.0 ± 3	23.1 ± 6	25.7 ± 7
KNO_3	3.4 ± 0.1	38.1 ± 3	43.2 ± 4	27.0 ± 8	15.6 ± 3
NH_4Cl	-	40.7 ± 5	43.0 ± 8	-	16.4 ± 4
$(NH_4)_2SO_4$	4.2 ± 3	47.2 ± 7	44.3 ± 9	13.2 ± 1	28.9 ± 5
Casein	8.4 ± 0.9	70.0 ± 8	85.1 ± 2	44.8 ± 3	30.2 ± 6
Peptone	9.1 ± 1.7	79.2 ± 1	88.6 ± 7	42.7 ± 2	35.4 ± 8
Yeast extract	-	77.8 ± 4	83.1 ± 12	9 ± 0.3	-
Urea	-	5.6 ± 2	10.5 ± 0.9	-	-

*: Nitrogen sources were added at 0.07% as N base.

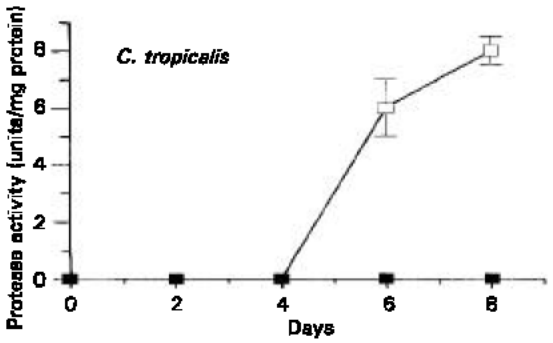

Fig. 1a: The activity of protease enzyme (units/mg protein produced by *C. tropicalis* as affected by incubation temperature. (All values are means of triplicates ± S.D.)

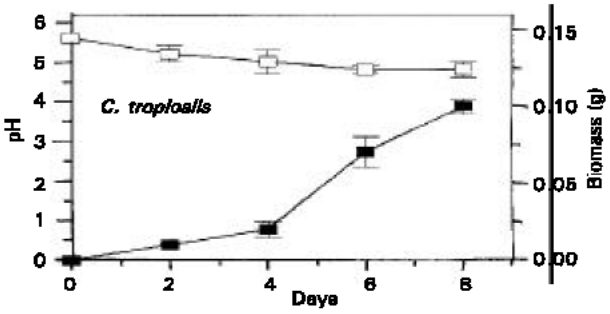

Fig. 1b: Changes in biomass (g) and pH of medium inoculated with *C. tropicalis*. (All values are mean of triplicates ± S.D.)

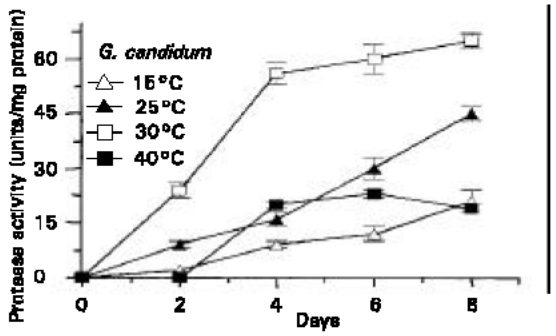

Fig. 2a: The activity of protease enzymes (units/mg protein) produced by *G. candidum* as affected by incubation temperature. (All values are means of triplicates ± S.D).

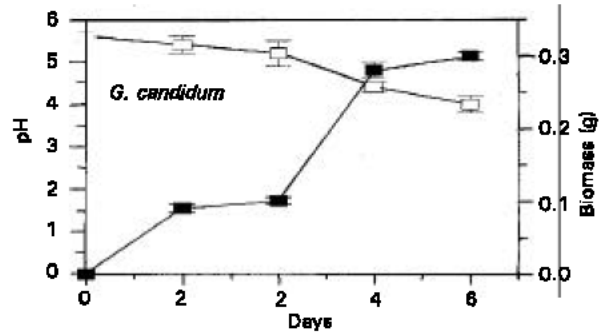

Fig. 2b: Changes in biomass (g) and pH of medium inoculated with *G. candidum*. (All values are means of triplicates ± S.D).

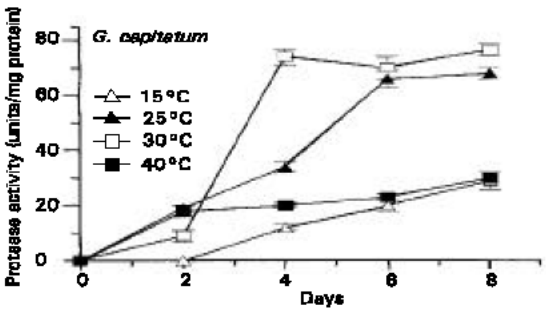

Fig. 3a: The activity of protease enzyme (units/ mg protein) produced by *G. capitatum* as affected by incubation temperature. (All values are means of triplicates ± S.D).

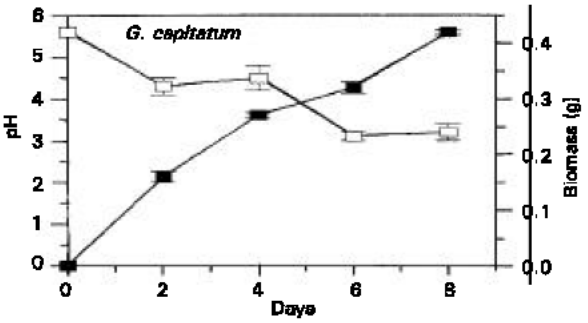

Fig. 3b: Changes in biomass (g) and pH o medium inoculated with *G.capitatum*. (All values are means of triplicates ± S.D).

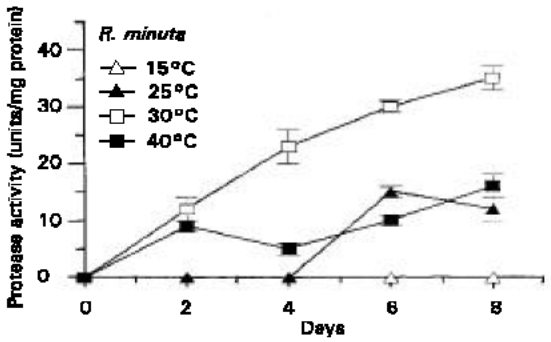

Fig. 4a: The activity of protease enzymes (units/mg protein produced by *R. minuta* as affected by incubation temperature. (All values are means of triplicates ± S.D).

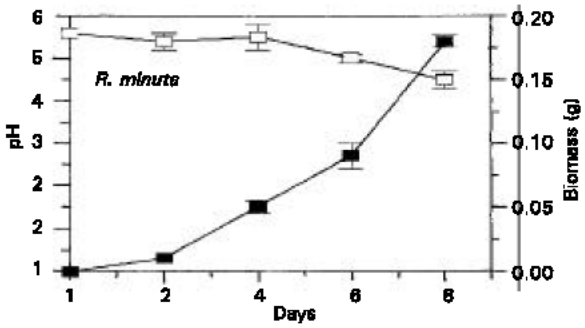

Fig. 4b: Changes in biomass (g) and pH of medium inoculated with *R. minuta*. (All values are means of triplicates ± S.D).

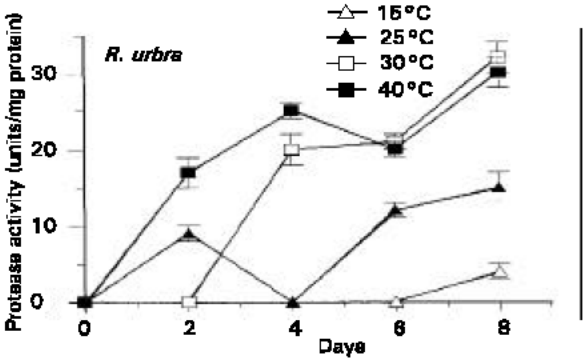

Fig. 5a: The activity of protease enzyme (units/mg protein) produced by *R. rubra* as affected by incubation temperature. (All values are means of triplicates ± S.D).

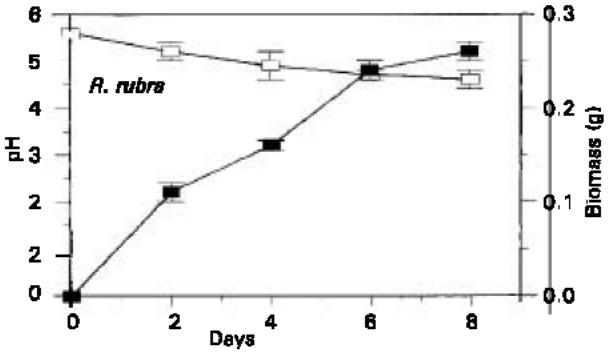

Fig. 5b: Changes in biomass (g) and pH of medium inoculated with *R. rubra*. (All values are means of triplicates ± S.D).

proteolytic enzyme producer with 88.6 units/mg protein in a medium supplemented with peptone. Overall, the highest proteolytic activity of soil yeasts occurred in presence of peptone followed by casein. On the other hand, inorganic nitrogen compounds caused lower proteolytic activities than those produced by the complex organic nitrogen compounds. Result (Table 2) showed that the lowest proteolytic activity was recorded in the presence of urea followed by ammonium chloride and potassium nitrate. The peptone was found to be the best substrate for stimulating protease production for all yeasts tested. These results are in complete accordance with thoseof Abdel-Fattah *et al.* (1985), Lim *et al.* (1985), Mansour 1985; Gabr *et al.* (1987) and Abou Hamed 1995 who found that the protease secretion by certain microorganisms being lowest in media containing inorganic nitrogen sources and highest in media containing complex peptides.

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