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Nutritional controls of proteolytic and keratinolytic activities by a keratinophilic *Chrysosporium* from soil in Kuwait

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ABSTRACT

Proteolytic activity of *Chrysosporium zonatum*, which is a keratinophilic species from soil, has been expressed in the exponential growth phase in Sabouraude dextrose broth (SDB). The four-week-old stationary grown mats were transferred into different minimal media that had been depleted from either carbon, nitrogen, sulfur or phosphorus. These mats expressed higher proteolytic activities at day seven. Addition of keratin as the sole source of carbon and nitrogen further enhanced proteolytic and keratinolytic activity.

Keywords: *C. zonatum*; catabolite repression; dermatophytes; keratinase; protease.

INTRODUCTION

Keratin is an important natural material of animal origin that remains in the environment as a recalcitrant solid waste. It is a very important biodegradable material because of its biotechnological implications in the wool and feather industries (Onifade *et al.* 1998), as well as in medical fields (Grappel & Blank 1972). Fungi and bacteria are the major biodegraders of keratins in the environment (Al-Musallam 1988, 1990, Abdel-Hafez & Sharouny 1990, Malviya *et al.* 1993, Fasasi 1997). Because of their pathological importance, keratinolytic proteases from dermatophyte fungi have been well studied (Asahi *et al.* 1985, Apodaca & McKerrow 1989, Porro *et al.* 1997). The non-dermatophyte species, however, are of wider occurrence in the environment and are potentially viable for keratinolysis in biotechnological applications. The majority of *Chrysosporium* spp. are soil-born and keratinophilic (van Oorschot 1980, Al-Musallam 1988, 1990). They digest keratinous substrates in a manner apparently similar to the dermatophytes (Yu *et al.* 1968, Wawrzkiewicz *et al.* 1991, Dozie *et al.* 1994). However, the control of keratinase expression and activity has not been properly addressed for non-dermatophytic fungi. *Chrysosporium zonatum* was originally isolated as a co-colonizer of horse hair with the well known dermatophyte *Microsporum gypseum* (Al-Musallam & Tan 1989). In the present paper, *C. zonatum*

was selected to investigate the nutritional conditions that control the expression and secretion of the extracellular protease(s) that can potentially digest keratinous substrates.

MATERIALS AND METHODS

Biomass and proteolytic activity of stationary grown cultures

Aliquots (50 μl) of the spore suspension from an active culture *Chrysosporium zonatum* were inoculated into flasks containing 1000 μl Sabouraude dextrose broth (SDB). Flasks were incubated in a still position at 28°C for 32 days. At intervals of 7 days up to 32 days, mycelial mats were removed by filtration through Whatman filter paper and were dried in an oven at 60°C to a constant weight. Un-inoculated controls were harvested at each time interval. Proteolytic activity of the culture filtrates was measured at the same time intervals using the chromogenic substrate azocoll (Sigma) as follows: Five milligrams of azocoll were suspended into 900 μl of 1 mM Tris–HCl/$CaCl_2$ buffer at pH 8.0 in Eppendorf tubes and 100 μl of the culture filtrate was added. The final volume of the reaction mixture was 1 ml. The reactants were incubated at least 4 h in a shaking water bath at 25°C. Samples were then centrifuged for 5 min in a Sorvall microfuge, and the absorbance of the released dye in the supernatant was determined spectrophotometrically at 520 nm. One protease unit (Pu) was defined as the change of 0.1 A_{520} ml/h as described by Apodaca and McKerrow (1989).

Proteolytic and keratinolytic activity in response to carbon, nitrogen, phosphorus and sulfur depletion

Twenty-eight-day-old mycelial mats initially grown on SDB were washed three times with 0. 1M phosphate-buffered saline (pH 7.2), and the mats were then transferred to new flasks containing 100 ml of a minimal medium or a minimal medium depleted from carbon, nitrogen, phophorus or sulfur as described by Apodaca and McKerrow (1989). Cultures were incubated for 7 days in a shaking incubator at 25°C. Samples from the complete minimal medium or from the individually depleted media were separated from mats by filtration using membrane filters (Nalgene). The sterile membrane-filtered media were then assayed for proteolytic and keratinolytic activities using azocoll and keratinazure (Sigma) as protein and keratin substrates, respectively. The complete minimal medium contained 0.25% (w/v) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$, 3.4 mM KH_2PO_4, and 2 mM $MgSO_4 \cdot 7H_2O$ as nitrogen, phosphorus and sulfur sources, respectively. Carbon, nitrogen, phosphorus and sulfur depleted media were made by deleting the appropriate compound from the minimal medium. In media without phosphorus, KH_2PO_4 was omitted and $(NH_4)_2PO_4$ was substituted with 50 mM NH_4HCO_3.

Azocoll assay: In Eppendorf tubes, 5 mg of azocoll was suspended in 900 μl of 1 mM Tris–HCl–1 mM $CaCl_2$ buffer (pH 8.0) according to Apodaca and McKerrow (1989). After addition of 100 μl of the respective membrane filtered medium, the final volume of the reaction was 1 ml. The assay mixtures were incubated for 4, 20, and 24 h in a shaking water bath at 37°C, and then centrifuged for 5 min. The absorbance of the released dye in the supernatant was determined spectro-

photometrically at 520 nm against a control that contained 1 ml buffer and azocoll. One proteolytic unit (PU) was defined as a change of 0.1 A_{520}/ml of the enzyme preparation/h of assay time.

Keratinase assay: In Eppendorf tubes, 5 mg of the dye impregnated wool (keratinazure) was suspended in 900 μl of 100 mM glycine/NaOH/1 mM $CaCl_2$ buffer at pH 9.0, and 100 μl of the membrane filtered medium from each treatment was added to the buffer/substrate mixture. The final volume of the reaction mixture was 1 ml. The assay mixtures were then incubated in a shaking water bath at 37°C for 48, 66 and 72 h, and then centrifuged for 5 min. The absorbance of the released dye in the supernatant was determined spectrophotometrically at 595 nm against a control that contained 1 ml buffer and keratinazure. One keratinase unit (KU) was defined as the change of 0.01 A_{595}/ml of the enzyme preparation/h of assay time.

Proteolytic and keratinolytic activities in response to natural keratinous substrate

Twenty-eight-day-old mycelial mats of *C. zonatum* that were initially grown in SDB were washed with 0.1 M phosphate buffered saline (pH 7.2). The washed mats were then transferred to new flasks containing 100 ml of either minimal medium supplemented with keratin (animal wool, Scholl Ltd. UK), or keratin salt medium (KS) with the following composition per liter: 5 g powdered animal wool, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4 and 100 μg Thiamin–HCl. Mats were incubated for one week and the induction medium was then adjusted to pH 8.0 using 1 mM Tris–HCl–1 mM $CaCl_2$ buffer, and pH 9.0 using 100 mM glycine–NaOH–1 mM $CaCl_2$, and protease and keratinase activities were measured for 4 and 48 h, respectively. The keratinase and protease activities in the minimal medium were used as the control reading. The change in the dry weight of the mat and added wool was measured after drying in the oven at 60°C overnight.

Proteolytic and keratinolytic activities in keratin salt medium at varying pH values

After washing with buffered saline at pH 7.2, the four week old mats were transferred into 1 mM KS medium enriched with 100 μg/L Thiamin–HCl. The control set did not contain Thiamin. After 7 days incubation in a shaking incubator at 25°C, the protease and keratinase activities were assayed at adjusted pH values from 6 to 9 using 1 mM Tris–HCl–1 mM $CaCl_2$ and 100 mM glycine–NaOH–1 mM $CaCl_2$ buffers as described above.

RESULTS

Figure 1 shows the growth and proteolytic activity of *Chrysosporium zonatum* on Sabouraude dextrose broth. The mycelial biomass increased exponentially to the termination of the experiment when the recorded dry weight of the floating mycelial mat was 620 mg. The pH of the medium increased from 6.5 before inoculation to 8.3 at the end of the experiment. The maximal protease units of 0.46 PU were

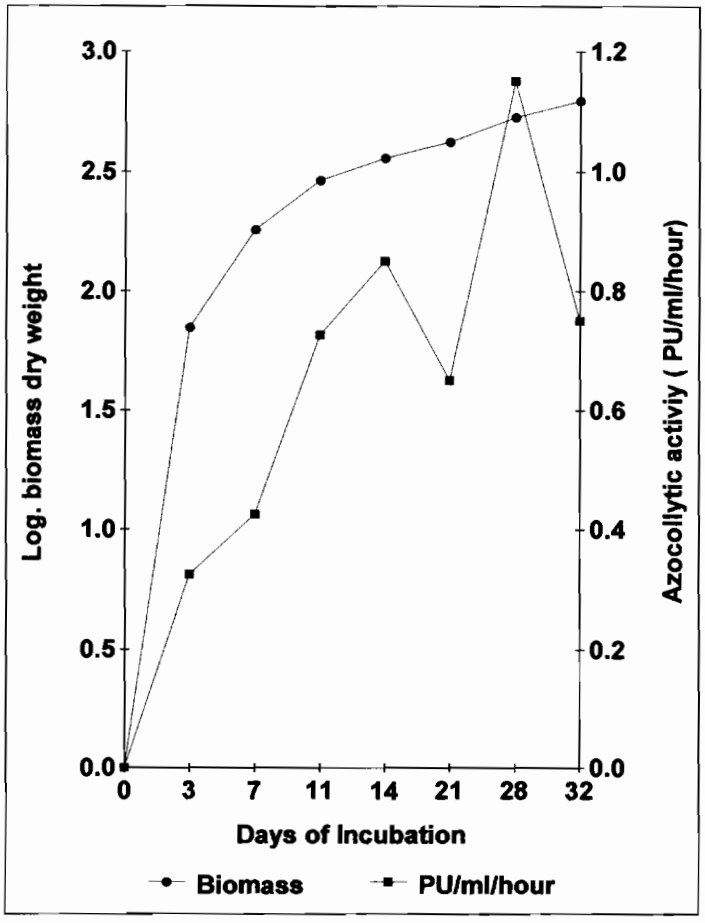

Fig. 1. Daily increase in biomass dry weight of *C. zonatum* on SDB and the corresponding proteolytic activity in culture filtrates measured after 4 h of incubation with the chromogenic substrate *Azocoll* at pH 8.0 and 25°C.

recorded in the culture filtrate of the 28-day-old cultures. The expression of protease activities in the 28-day-old culture filtrate, measured at 30 min intervals, showed a maximum activity of 0.56 PU/ml after 180 min of incubation with the substrate at pH 8.0 (Fig. 2).

Table 1 shows the proteolytic activities of *C. zonatum* cultures, expressed as PU/ml induction medium/h, in relation to the depletion of essential nutrients from the minimal medium. Depletion of carbon or nitrogen significantly enhanced the proteolytic activity. Depletion of carbon caused the highest proteolytic activities after 4 and 24 h assay time (0.24 and 0.143 PU/ml/h, respectively). Nitrogen depletion induced the highest activity after 20 h (0.15 PU/ml/h). Sulfur and phosphorus deficiencies induced slightly higher activities after 4 h and 20 h assay, which gradually declined after 24 h assay to a level that was not significantly different from the control.

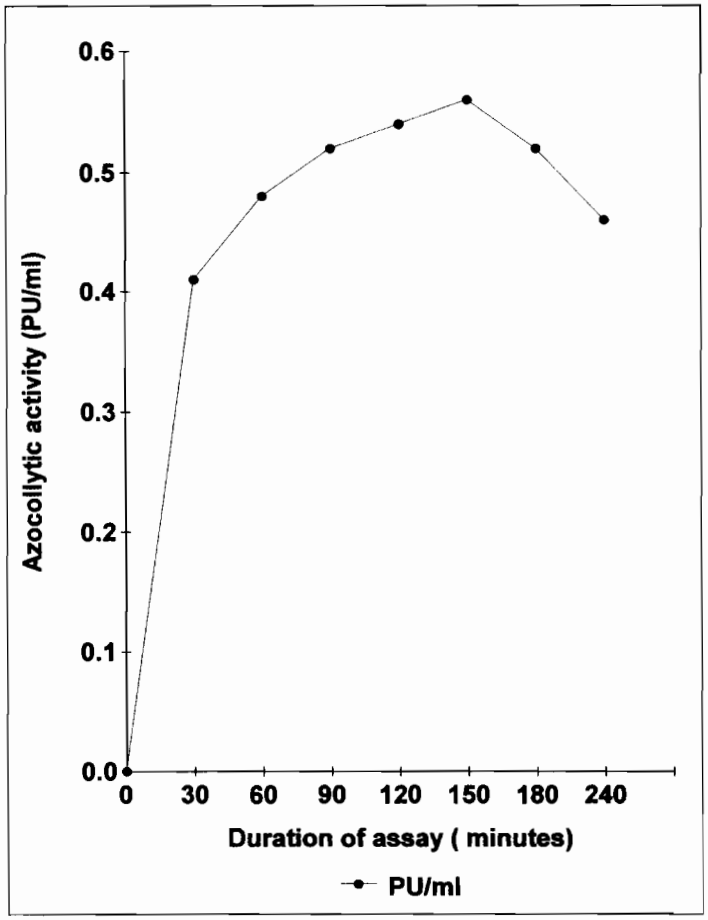

Fig. 2. Azocollytic activity expressed in the culture filtrate of the 28-day-old stationary mat of *C. zonatum* at pH 8 and 25°C measured at 30 min time intervals from 0–240 min.

Table 2 shows the keratinolytic activities in relation to depletion of essential nutrients. Depletion of carbon and nitrogen induced the highest keratinase activities after 48 h assay (0.078 and 0.081 KU/ml/h, respectively). Depletion of phosphorus induced activity significantly higher than the minimal medium only after 66 h assay. Sulfur depletion, on the other hand, caused a minimal increase in keratinase activities after 72 h assay, although none of the treatments appeared to be significant after 72 h assay.

Table 3 shows the enhancement of keratinolytic activity in cultures grown in the minimal medium supplemented with keratin (animal wool) and in the keratin salt medium in which keratin powder is the only source of carbon and nitrogen. The addition of keratin to the minimal medium enhanced the keratinolytic activity from 0.050 to 0.065 KU/ml/h, while the addition of keratin as the sole source of carbon and nitrogen increased the activity to 0.104 KU/ml/h, indicating a two-fold increase in keratinolytic activity. The azocollytic activities in the presence of keratin in either

Table 1. Effects of carbon, nitrogen, phosphorus and sulfur depletion on proteases secreted by the four-week-old mycelial mats of *Chrysosporium zonatum*, expressed after 7 days in the minimal medium and the corresponding depletion treatment. The assay temperature was 37°C, the pH of the assay medium was 8.0. Zero change in absorbance has been adjusted by uninoculated medium with buffer only.

	Azocollytic activity at time intervals (PU*)		
Type of medium	4 h	20 h	24 h
minimal	0.120 ± 0.005a**	0.030 ± 0.003a	0.030 ± 0.001a
carbon depleted	0.240 ± 0.088b	0.103 ± 0.01bc	0.143 ± 0.003b
nitrogen depleted	0.130 ± 0.013a	0.147 ± 0.004b	0.141 ± 0.003b
phosphorus depleted	0.190 ± 0.01ab	0.089 ± 0.001c	0.062 ± 0.003a
sulfur depleted	0.140 ± 0.008a	0.090 ± 0.007c	0.072 ± 0.009a

*1 PU = change of 0.1 absorbance at 520 nm/ml of the medium/h assay time.
**values followed by the same letter in each column are not significantly different, i.e. $P \leq 0.05$ by the least significant difference.

Table 2. Effects of carbon, nitrogen, phosphorus and sulfur depletion on the activity of keratinase enzymes secreted by the four-week-old mycelial mats of *Chrysosporium zonatum*, expressed after 7 days in the minimal medium and the corresponding depletion treatment. The assay temperature was 37°C, the pH of the assay medium was 9.0. Zero change in absorbance has been adjusted by uninoculated medium with buffer only.

	Keratinolytic activity at time intervals (KU*)		
Type of medium	48 h	66 h	72 h
minimal	0.050 ± 0.002a**	0.032 ± 0.001a	0.024 ± 0.001a
carbon depleted	0.078 ± 0.002b	0.036 ± 0.002a	0.021 ± 0.001a
nitrogen depleted	0.081 ± 0.011b	0.046 ± 0.007b	0.035 ± 0.007a
phosphorus depleted	0.051 ± 0.003a	0.058 ± 0.006b	0.000 ± 0.000a
sulfur depleted	0.029 ± 0.003c	0.029 ± 0.004a	0.039 ± 0.008a

*1 KU = change of 0.01 absorbance at 595 nm/ml of the medium/h assay time.
**values followed by the same letter in each column are not significantly different, i.e. $P \leq 0.05$ by the least significant difference.

Table 3. Effects of keratinous substrates in the presence of carbon and nitrogen on the proteolytic and keratinolytic activities expressed by the four-week-old cultures of *Chrysosporium zonatum*, after 7 days in the minimal medium and the keratin salt medium. The pH of the assay media are 8 and 9, the assay times was 4 and 48 h, respectively. Absorbance in the minimal medium has been used as the control reading.

Medium	Azocollytic activity [(PU)* at pH 8.0]	Keratinolytic activity [(KU)* at pH 9.0]
minimal medium	0.122 ± 0.005a**	0.050 ± 0.002a
minimal + keratin	0.241 ± 0.002b	0.065 ± 0.004b
keratin salt medium†	0.243 ± 0.008b	0.104 ± 0.007c

*1 PU = 0.1 change in absorbance at 520 nm/ml medium/h assay time, 1 KU = 0.01 change in absorbance at 595 nm/ml medium/h assay time.
**values followed by the same letter in each column are not significantly different. i.e. $P \leq 0.05$ by the least significant difference.
†see text for the composition of keratin salt medium.

the minimal medium or in the KS medium were significantly higher in relation to the control minimal medium, but there was a minimal difference between the treatments (0.241 and 0.243, respectively) indicating the absence of catabolite repression of carbon and/or nitrogen.

Table 4 shows the effect of assay pH on the protease and keratinase activities derived from the keratin salt medium in the presence or absence of thiamin–HCl. The highest level of the keratinolytic activity was expressed at pH 9.0 in both the presence and absence of the vitamin (0.10 and 0.8 KU/ml/h, respectively). In contrast, in the presence of thiamin, the proteolytic activity showed a significant increase from pH 7.5 up to pH 9.0 with the highest activity at pH 8.5 (0.5 PU/ml/h). In the absence of thiamin–HCl the highest proteolytic activities were observed at pH 7.5 and pH 9.0 (0.485 and 0.488, respectively).

Table 4. Effect of hydrogen ion concentration on the proteolytic and keratinolytic activities expressed by the 4-week-old cultures of *C. zonatum* after 7 days in KS medium in the presence or absence of Thiamin. Uninoculated controls used for zero absorbance at each pH value.

pH of the assay medium	Azocollytic activity (PU)		Keratinolytic activity (KU)	
	+Thiamin	−Thiamin	+Thiamin	−Thiamin
6.0	0.14 ± 0.015c†	0.17 ± 0.01c	ND	ND
6.5	0.14 ± 0.002c	0.12 ± 0.01c	ND	ND
7.0	0.20 ± 0.017c	0.34 ± 0.005b	ND	ND
7.5	0.37 ± 0.005b	0.45 ± 0.007a	0.04 ± 0.003b	0.05 ± 0.013b
8.0	0.44 ± 0.01ab	0.42 ± 0.032ab	0.03 ± 0.013b	0.01 ± 0.005c
8.5	0.50 ± 0.017a	0.38 ± 0.01b	0.07 ± 0.007ab	0.07 ± 0.005ab
9.0	0.44 ± 0.01ab	0.49 ± 0.037a	0.10 ± 0.012a	0.08 ± 0.007a

*Duration of proteolytic activity assay was 4 h, pH was adjusted with Tris–HCl/$CaCl_2$ buffer.
**Duration of keratinolytic assay was 48 h, pH was adjusted with glycine–NaOH/$CaCl_2$ buffer.
†values followed by the same letter in every column are not significantly different. i.e. $P \leq 0.05$ by the least significant difference.

DISCUSSION

Complex polymeric compounds in the environment of actively growing fungi provide alternative sources of carbon, nitrogen or sulfur. The utilization of such compounds demonstrates the presence of extracellular or cell-bound digestive enzymes within the fungus body, whose expression and secretion would usually respond to one or more regulatory mechanisms (Lasure 1980, Apodaca & McKerrow 1989). El-Naghy *et al.* (1998) reported that fungal species belonging to the genus *Chrysosporium* were the most effective in the biodegradation of feathers, despite the fact that feathers are not the ideal substrate to support good growth. In their study, El-Naghy *et al.* (1998) used a minimal medium either supplemented with feathers as the sole source of carbon and nitrogen, or with added glucose and ammonium chloride separately or in combination. They reported an increase in keratinase activity with 1% glucose and a decline in the activity with

ammonium chloride. In contrast, Apodaca and McKerrow (1989) reported that the regulation of keratinolysis by the dermatophyte species *Trichophyton rubrum* was carbon and nitrogen dependent, and that keratinolytic activity was suppressed in the presence of either carbon, nitrogen or sulfur. The late exponential phase of our *Chrysosporium* species responded in a fashion similar to *T. rubrum* in regard to carbon and nitrogen deficiencies, and to a lesser extent to sulfur or phophorus deficiency (Tables 1 & 2). In the absence of carbon, the proteolytic activity increased more than two-fold, and the increase in the keratinolytic activity was nearly 20%. In the absence of nitrogen the increase in the keratinolytic activity was nearly 30%. These results indicate that readily available carbon and nitrogen compounds generate a repressive mechanism similar to catabolite repression for similar extracellular proteases in culture filtrates (Griffin 1994). The depletion of phosphorus enhanced proteolytic activity but did not affect keratinolytic activity. Sulfur depletion, in contrast, produced a negative effect on the keratinolytic activity, suggesting a sulfur dependent extracellular keratinolytic protease only.

The addition of keratin to the minimal medium caused doubling of the proteolytic activity (Table 3). In the keratin salt medium (KS), the proteolytic activity exhibited a slight increase in activity when compared to the keratin-supplemented medium (0.243 and 0.241 PU/ml/h, respectively). Keratinolytic activity, on the other hand, increased nearly 40% in the KS medium compared to the keratin-supplemented medium, and by more than 50% when compared to the minimal medium without keratin.

The present results indicate that the keratinolytic activity of *C. zonatum* is induced by the presence of a keratinous substrate, and is negatively suppressed by a catabolic repressive mechanism caused by the presence of either carbon or nitrogen in the growth environment (Tables 2 & 3). Such regulatory mechanisms had been widely reported in several filamentous fungi (Drucker 1972, 1975, Lasure 1980). However, Malviya *et al.* (1993) and El-Naghy *et al.* (1998) reported that keratinolytic activities of *Chrysosporium queenslandicum* and *C. georgiae* were not repressed in keratin-containing medium supplemented with carbon, nitrogen or sulfur, indicating an inducible nature of their enzymes that was not affected by a catabolite repression. Their conclusions are closer to our results for proteolytic activity. Apodaca and McKerrow (1989) reported that the addition of keratin or elastin to the minimal medium did not induce azocollytic or elastinolytic activities by *T. rubrum*, but they did not use keratinazure for keratinolytic activity. Our results may, therefore, indicate the expression of a specific extracellular keratinase from *C. zonatum* that expresses activity against keratinazure.

Similar to several dermatophytes, the optimal proteolytic activity of *C. zonatum* was expressed in the alkaline pH range, with the optimal azocollytic activity being expressed at pH 8.5 in the presence of thiamin-HCl, and at pH 7.5–9.0 in the absence of thiamin–HCl. In contrast, the keratinolytic activities were optimally expressed at pH 9.0 irrespective of the presence or absence of thiamin–HCl (Table 4). Alkaline keratinolytic enzymes have also recently been reported from *C. keratinophilum* when cultured in the presence of animal (cow) hair (Dozie *et al.* 1994).

C. zonatum was originally described from soil as a colonizer of horse hairs together with the dermatophytic species, *Microsporum gypseum* (Al-Musallam & Tan 1989) suggesting that both species probably occupy closely related ecological niches. Due to their different requirements for the expression of their proteolytic

and/or keratinolytic activities, *C. zonatum* appeared to have a stronger survival potential on the keratinous substrate that supported its keratinolytic activity for a prolonged duration, and thus, it repressed and eventually terminated the growth of *M. gypseum* on the same substrate.

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تأثير عناصر التغذية على تحلل البروتين والكيراتين بواسطة فطر كرايزوسبوريم (*Chrysosporium*) المعزول من التربة في الكويت

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الخلاصة

تم قياس قدرة تحلل البروتين بواسطة فطر كرايزسبوريم زوناتم *Chrysosporium zonatum* الذي يعيش على تحلل الكيراتين في التربة في مزرعة على وسط سابرود (SDB) الغذائي. وقد تسجل أعلى نشاط تحللي في مراحل النمو اللوغارتمي. وعند نقل المزرعة المتكونه بعد أربعة أسابيع إلى أوساط غذائية متعددة أزيل منها عنصرا واحدا من العناصر الغذائية الأساسية (كربون، نيتروجين، فوسفور، كبريت) ترتفع درجة النشاط التحللي للبروتين والكيراتين الذي يبلغ أقصاه في اليوم السابع. كما أن إضافة الكيراتين كبديل لعنصري الكربون والنيتروجين يحفز النشاط التحليلي للكيراتين لدي الفطر.