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PRODUCTION OF CHITINASE FROM BIOCONTROL AGENTS: *Trichoderma* spp.

Hassan, H. M.
Microbial Chemistry Dept., National Research Centre Dokki, Cairo, Egypt

ABSTRACT

Trichoderma spp. play an important role in the destruction of plant pathogenic fungi, and were found to produce chitinase, which hydrolyzes chitin, a major component of many pathogenic fungi cell walls. Out of four *Trichoderma* isolates, *Trichoderma harzianum* T3 and T4 strains showed high chitinolytic activity on chitin – basel agar medium. Factors involved the production of chitinase were investigated. Maximum enzyme levels of (10 to15.9 U / ml) were achieved after six days incubation at 28°C and initial pH of medium 6.5 The enzyme was produced only in the presence of colloidal chitin at a concentration of 1 % (w/v), as a sole carbon source , suggesting the inducible nature of the enzyme. *Trichoderma harzianum* T4 was found to be very efficient to lyse mycelia of *Microphomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Partial purification of chitinase from *Trichoderma harzianum* T4 was achieved by $(NH_4)_2 SO_4$ precipitation followed by gel filtration using Sephadex G – 200. The partially purified enzyme was found to be highly active at pH 6.5 and 50°C, and thermostable at 50°C for 60 min. Mn^{2+} , Ca^{2+} and Fe^{2+} activated the enzyme, whereas Co^{2+} , Cu^{2+} , Zn^{2+} and Hg^{2+} had different inhibitory effects .

Keywords: *Trichoderma*, biological control, chitinase, partial purification, enzyme properties.

INTRODUCTION

Biological control based on application of antagonistic microorganisms is an alternative to chemical control of plant diseases and environmentally more safe. *Trichoderma* spp. are free living fungi that are highly interactive in root , soil and foliar environments and have been extensively used as biological control agents because they attack a large variety of phytopathogenic fungi responsible for major crop diseases(Chet,1987;Küçük and kivanç , 2003 and Harman *et al.*,2004). Several modes of action have been proposed to explain the suppression of plant pathogens by *Trichoderma*. One of these is mycoparasitim which involve production of cell wall degrading enzymes (Haran *et al.*, 1996), such as extracellular chitinase, β-1,3-glucanase and a proteinase which hydrolyze chitin , β-1,3 glucan and protein which are the main structural components of most fungal cell walls (Peberdy , 1990) , hereby destroying plant pathogens (Chet *et al.*, 1979 ; Chet and Baker , 1981 and Lima *et al.*, 1997). Chitinase has received increased attention for biocontrol of plant pathogenic fungi and chitinases producing strains were used directly or indirectly process as purified enzyme in the biocontrol (Ordentlich *et al.*, 1988). The antifungal activity of chitinases produced by bacteria has been known for a long time including *Aeromonas* sp. (Hamed, 1986); *Bacillus* spp. (Watanabe *et al.*, 1990 ; Takayanagi *et al.*,1991 ; Trachuk *et al.*,1996 ; Pleban *et al.*,1997 and Hassan *et al.*,2004);

Pseudomonas fluorescens (Nielsen *et al.*, 1998) ; *Serratia* spp. (Brurberg *et al.*,1996 and Berg *et al.*,1999) and *Streptomyces* spp. (Broadway *et al.*, 1995 ; Mahadevan and Crawford, 1997 and Saad , 2002). A number of *Trichoderma* chitinases have been the subject of basic and applied researches including formation, production, properties, purification and application against some plant pathogenic fungi (Elad *et al.*, 1982 ; Elad and Kapat , 1999 , Saad and Nawar , 2001 and Sid-Ahmed *et al.*, 2003) . Chitinolytic enzymes from *Trichoderma* have a substantial inhibitory effect on the germination of spores and hyphal elongation of several fungal pathogens , including *Botrytis cinerea , Fusarium* spp., *Alternaria* spp., *Ustilago avenae , Uncinula necator* and virtually all chitin containing fungi (Di Pietro *et al* ,1993 and Lorito *et al.*,1993&1994) . *Trichoderma* enzymes were not only able to lyse the soft structure of the hyphal tip but also the hard chitinous wall of mature hyphae, conidia, chlamydospores and sclerotia (Benhamou and Chet, 1993 & 1996; Lorito *et al.*, 1993 and Rousseau *et al.*, 1996).

The present work study the effect of some culture condition factors on chitinase production by *Trichoderma* spp. In addition, general properties, heat stability of the partially purified enzyme and the application of *Trichoderma harzianum* on the lysis of dead mycelium of some plant pathogenic fungi were also studied.

MATERIALS AND METHODS

Microorganisms:

1 – Antagonistic fungi: four isolates of *Trichoderma* spp. were isolated from the Egyptian soil were used in this study. Two isolates were identified as *Trichoderma viride* (T1 & T2), while T3 & T4 are referred to as *Trichoderma harzianum.*

2 – Pathogens: three plant pathogenic fungi were isolated from diseased plants belonging to *Microphomina phaseolina*; *Rhizoctonia solani* and *Sclerotium rolfsii* were used.

All the isolated strains were identified by the Plant Pathology Dept. (Fac. Agric., Alex. Univ.), maintained on potato dextrose agar medium (PDA) and stored at 5 °C.

***In-vitro* preliminary tests of chitinolytic enzymes production and fungal antagonism** : for chitinase production , *Trichoderma* isolates were inoculated on chitin – basel agar medium according to Monreal & Reese (1969) , it contained (g/L): colloidal chitin, 15 ;yeast extract, 0.5 ;($NH_4)_2$ SO_4 1.0; $MgSO_4.7H_2O$, 0.3; KH_2PO_4, 1.36 in distilled water and the pH was adjusted to 6.5. After incubation at 28°C for 7 days the width of clear zones around the growth of fungi were measured. For antagonism against the three plant pathogenic fungi , the *Trichoderma* spp. were tested for their ability to produce zone of inhibition when grown with the pathogenic fungi in PDA medium in the same plate according to the method described by Hamed *et al.* (1996) . Zones of growth inhibition (mm) were recorded after 7 days of incubation at 28°C.

Factors affecting chitinase production : the production of chitinase by *Trichoderma* isolates was followed in shaking flasks (250 mL) containing 50 mL of a synthetic medium developed by Okon *et al.* (1973) which contained (g/L) : $MgSO_4.7H_2O$, 0.2 ; K_2HPO_4, 0.9 ; KCl, 0.2 ; NH_4NO_3, 1.0 ; $FeSO_4.7H_2O$, 0.002 ; $MnSO_4.H_2O$, 0.002 and $ZnSO_4.7H_2O$, 0.002 . This medium (pH 6.3) was supplemented with colloidal chitin, 10g/L as inducer for enzyme production. The medium was inoculated by one disk (4 mm diameter) of the fungal strain and shaken (150 rpm, 28°C ± 2) for 7 days in a rotatory shaker. The time-course of enzyme production was followed from 3 to 7 days, and the effect of initial pH was studied at the pH range 5.5 – 8.5. Also, the effect of different carbon sources at a concentration of 1.0 %(w/v) was studied. The ability of *Trichoderma harzianum* (T4) to lyse the mycelia of *Microphomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* was estimated as the residual mycelium weight (g) after lysis at the end of incubation period (5 and 10 days) when grown in the synthetic medium supplemented with autoclaved pathogenic fungal mycelia as a sole carbon source and the results were expressed as % lysis of mycelium as compared to the original weight.

Enzyme assay: chitinase was assayed be following the release of N – acetylglucosamine according to the method of Jeuniaux (1966). One ml of 1.0 % (w/v) colloidal chitin in 0.05 M citrate-phosphate buffer (pH 6.5) was incubated with 1.0 ml of enzyme at 50°C for 60 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 0.5 µ mole / ml of N-acetylglucosamine per hour.

Determination of protein: protein content was determined by Folin phenol reagent, according to Lowry *et al.* (1951).

Partial purification of chitinase:

A) Ammonium sulphate precipitation: the fungal biomass was removed from the broth by centrifugation at 4000g for 20 min. The remaining liquid was brought to 60 % saturation with ammonium sulphate, and the precipitate after 2 h was collected by centrifugation at 6000g for 30 min at 4°C. The precipitate was dissolved in 5 mL of 0.02 M citrate-phosphate buffer (pH 6.5), and dialyzed against the same buffer overnight in the refrigerator and lyophilized.

B) Gel filtration using Sephadex G – 200: lyophilized enzyme was resuspended in the buffer mentioned above, then applied to Sephadex G – 200 columns that was equilibrated with 0.02 M citrate phosphate buffer, pH 6.5 .Then chitinase was eluted at a flow rate of 30 mL / h and collecting 5 mL fractions.

RESULTS AND DISCUSSION

***In – vitro* preliminary tests of chitinolytic production and fungal antagonism against some plant pathogenic fungi:**

The results in Table 1 showed *in-vitro* preliminary tests of antagonism toward the plant pathogens *Microphomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Inhibition was clearly discerned by

the complete absence of fungal mycelium in the inhibition zone between antagonistic isolates and the plant pathogenic fungi mentioned above. *Trichoderma harzianum* isolates T3 & T4 had a strong activity against the three pathogenic fungi (the inhibition zones between 8.5 – 9.7 mm), while, *Trichoderma viride* isolates T1 & T2 had a moderate activity (the inhibition zones between 4.0 – 6.6 mm). The four *Trichoderma* isolates possessed chitinolytic activities but, the clear zones surrounding *Trichoderma harzianum* isolates were larger than that of *Trichoderma viride* isolates. These results are in accordance with those obtained by Saad and Nawar (2001) who found that four strains of *Trichoderma harzianum* possessed large chitinolytic activities in comparison with *Trichoderma viride* isolates.

Table 1: *In – vitro* preliminary tests of chitinolytic production and fungal antagonism against some plant pathogenic fungi.

Antagonistic isolate	Antagonism toward *			Production of chitinase**
	Microphomina phaseolina	*Rhizoctonia solani*	*Sclerotium rolfsii*	
Trichoderma viride T 1	5.8	5.2	4.5	+3
Trichoderma viride T2	4.0	5.2	6.6	+4
*Trichodermaharzianum*T3	8.5	8.8	9.6	+5
*Trichoderma harzianum*T4	9.5	9.7	8.7	+6

*** The size of the inhibition zone mm (distance between antagonistic fungal mycelium and pathogenic fungal mycelium).**
****Width of clear zone: +5 & +6 width of clear zone more than 10 mm.**
+4 width of clear zone from 6 – 10 mm.
+3 width of clear zone less than 6 mm.

Factors affecting chitinase production :
Enzyme production time-course:

The four isolates of *Trichoderma* grown in the synthetic liquid medium amended with colloidal chitin as a sole carbon source produced extracellular chitinases (Table 2). Maximum levels of chitinase were achieved after 6 days of incubation for all isolates (8.86 – 14.20U / mL), after which (7 days) a slight decrease in enzyme activity was recorded (8.70 – 14.00 U/mL). *Trichoderma harzianum* T4 was the most highly potent and produced the highest active chitinase (14.20 U / mL) followed by *Trichoderma harzianum* T3 , *Trichoderma viride* T2 and *Trichoderma viride* T1.Their values were 12.50 , 12.00 and 8.86 U / mL , respectively .

Table 2: Effect of incubation period on chitinase production by *Trichoderma* isolates.

Fungal strains	Chitinase activity (U / ml) Incubation period (days)				
	3	4	5	6	7
Trichoderma viride T 1	1.98	4.45	8.30	8.86	8.70
Trichoderma viride T2	2.30	6.50	10.30	12.00	11.80
*Trichoderma harzianum*T3	9.90	11.24	11.96	13.50	12.23
*Trichoderma harzianum*T4	10.50	13.01	13.12	14.20	14.00

Incubation temperature 28°C.

Effect of initial pH of medium on chitinase production:

The results in Table 3 showed the effect of initial pH of production medium on the productivity of chitinase by all *Trichoderma* isolates. Generally, the maximal production of enzyme was found at pH 6.5 .At less or more this value (5.5 or 8.5) the enzyme activity still high and the loss did not exceed than 25 %. These results indicated that the *Trichoderma* isolates produced chitinase on broad spectra of initial pH values. T4 isolate produced the highest level of chitinase (15.90 U / mL) while T1 isolate produced the lowest level (9.99U/ml). The enzyme production time-course as well as the initial pH effect on chitinase production varies according to the organisms used and even with the strains of the same species. In this respect, Elad *et al.*, 1982 reported that a high level of chitinase was excreted into the growth medium by *Trichoderma harzianum* when grown for 44 h at pH 5 also, Elad and Kapat 1999 found that the maximum levels of *Trichoderma harzianum* NCIM 1185 chitinase were achieved when grown for 5 days at pH 5 and De Marco *et al.*, 2003 reported that chitinase was produced by three strains of *Trichoderma* including *T. harzianum* when grown on liquid medium at pH 5.5 for 72 h.

Table 3: Effect of the initial pH of medium on chitinase production by *Trichoderma* isolates.

Fungal strains	Chitinase activity (U / ml) pH							
	5.5	6.0	6.3	6.5	7.0	7.5	8.0	8.5
Trichoderma viride T1	7.49	8.46	8.86	9.99	9.80	8.86	8.00	7.89
Trichoderma viride T2	9.50	11.20	12.00	12.30	12.09	12.00	11.99	10.50
*Trichoderma harzianum*T3	12.10	13.00	13.50	14.50	13.60	12.55	12.00	11.76
*Trichoderma harzianum*T4	12.60	13.70	14.20	15.90	14.31	14.21	13.64	12.14

Incubation period and temperature: 6 days and 28°C.

Effect of different carbon sources on chitinase production:

The results (Table 4) indicated that the *Trichoderma* isolates produced chitinase only when grown on medium supplemented with 1 % (w/v)colloidal chitin and the maximum level was reached by *T. harzianum* T4 (15.90 U / ml) . All the other carbon sources tested except colloidal chitin failed to produce any enzyme activity and this pointed out the inducible nature of chitinase produced by all the four *Trichoderma* isolates. The same conclusion had been drawn by other workers (Elad *et al.*, 1982; Elad and Kapat, 1999; Saad and Nawar, 2001 and De Marco *et al.*, 2003). They found that the maximum production of chitinase was achieved at a colloidal chitin concentration range of 0.1- 1.0 %(w/v). Also, chitinase was found to be induced in bacteria by chitin as well as fungi (Monreal and Reese, 1969; Hamed *et al.*, 1998) .Accordingly, *Trichoderma harzianum* T4 as the most potent strain for chitinase production was selected for further studies.

Table 4: The effect of different carbon sources in culture medium on the production of chitinase by *Trichoderma* isolates.

Carbon source 1 % (w/v)	Chitinase activity (U / ml)			
	Trichoderma viride T 1	*Trichoderma viride T2*	*Trichoderma harzianumT3*	*Trichoderma harzianumT4*
Colloidal chitin	9.98	12.31	14.51	15.89
N.A.G.A*	0.0	0.0	0.0	0.0
Glucose	0.0	0.0	0.0	0.0
Galactose	0.0	0.0	0.0	0.0
Lactose	0.0	0.0	0.0	0.0

* N-acetylglucosamine.
Incubation period and temperature: 6 days and 28°C.

Lysis of some plant pathogenic fungi by *Trichoderma harzianum* T4:

The ability of *T. harzianum* T4 to lyse pathogenic fungal mycelia and production of chitinase was studied. The results (Table 5) showed that sharp decrease in enzyme production related to control. Contrary to the enzyme production, very efficient lysis of mycelia was reached, whereas 96.3% of *Microphomina phaseolina* mycelia was hydrolyzed after 10 days of incubation, while 98.6 and 95.2 % lysis was achieved for *Rhizoctonia solani* and *Sclerotium rolfsii,* respectively. The sharp decrease in chitinase production due to the variation in the chemical structure of fungal mycelium and its cell wall as well as the amounts of their components are agreement with the fact that antagonistic organism attacks the pathogen mycelium first by dissolving its cell wall in certain locations by several enzymes including chitinase followed by hyphal penetration . Then the other extracellular enzymes i.e., lipases and proteases act on the other lipids and protein components (Chet *et al.*, 1981 and Harman *et al.*, 2004).

Table 5: Lysis of three plant pathogenic fungi by *Trichoderma harzianum* T4.

Plant pathogenic fungi (3 g dry weight / 50ml)	Lysis (%)		Chitinase activity (U / ml)	
	Incubation period (day)			
	5	10	5	10
Control *	-	-	13.12	-
Microphomina phaseolina	61.3	96.3	0.523	2.80
Rhizoctonia solani	53.6	98.6	0.539	2.92
Sclerotium rolfsii	51.2	95.2	0.430	1.09

Incubation temperature 28°C.
* Control: culture filtrate of *Trichoderma harzianum* T4 grown on colloidal chitin containing medium .

Partial purification of *Trichoderma harzianum* T4 chitinase:

Chitinase was precipitated from culture broth by 60 % saturation ammonium sulphate followed by gel filtration using Sephadex G – 200, the fractions containing chitinase activity were collected. The yield and purity of the enzyme at different purification steps are summarized in (Table 6). The recovery from ammonium sulphate precipitation was 87.92 % of the total activity which affords 1.06 fold purification; also gel filtration recovery was 4.9 % of the total activity and affords 20.55 fold purification.

Table 6: Summary of the purification protocol of the chitinase produced by *Trichoderma harzianum* T4.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U / mg)	Purification (fold)	Yield (%)
Culture filtrate	1590	280.2	5.67	1.00	100
Ammonium sulphate saturation (60 %)	1398	231.8	6.03	1.06	87.92
Gel filtration (Sephadex G-200)	77.84	0.668	116.53	20.55	4.9

All purification steps were performed at 5°C.

Properties of the partially purified *Trichoderma harzianum* T4 chitinase:

Effect of pH:

The optimum pH of the partially purified enzyme was determined in different 0.02M buffers (citrate – phosphate, phosphate and carbonate – bicarbonate) under the standard assay conditions at 50°C. The enzyme was optimally active at pH 6.5 (Fig. 1). These results are similar to that found for *Trichoderma* chitinase (Saad and Nawar, 2001) and differ from those reported for *Trichoderma harzianum* (Elad *et al.*, 1982) and *Aspergillus carneus* (Abdel-Naby *et al.*, 1992).

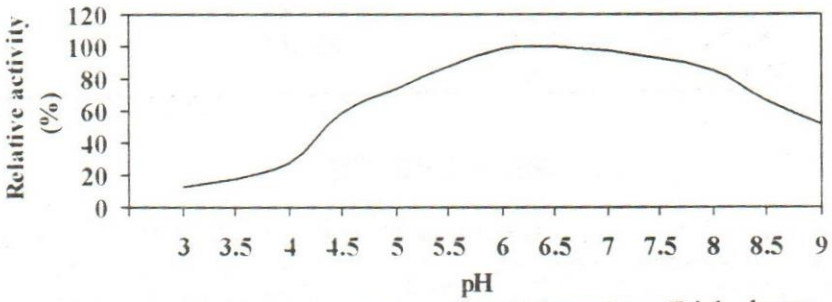

Fig. 1: Effect of pH on the partially purified chitinase from *Trichoderma harzianum* T4.

Effect of temperature:

The optimum temperature was investigated at pH 6.5. As shown in Fig. 2, the enzyme was most active at 50°C. At 70°C the enzyme lost only 19 % of its activity, and this indicated good thermo-stability of the partially purified *T.harzianum* T4 chitinase. This result is in accordance with that found for the other chitinases from *T. harzianum* and *Asp. carneus* (Saad and Nawar, 2001 and Abdel-Naby *et al.*, 1992).

Thermal stability:

Thermal stability of the enzyme was examined after preincubation of the enzyme solution in citrate – phosphate buffer (0.02 M, pH 6.5) at different time intervals at 50°C. The results in Fig. 3 show that *T. harzianum* chitinase was stable for 60 min at 50°C, and lost about 34 % of its activity after 80 min of incubation at the same temperature. The enzyme seems to be more stable than those of *Trichoderma* spp. and *Asp. carneus* chitinases (Saad and Nawar, 2001 and Abdel-Naby *et al.*, 1992).

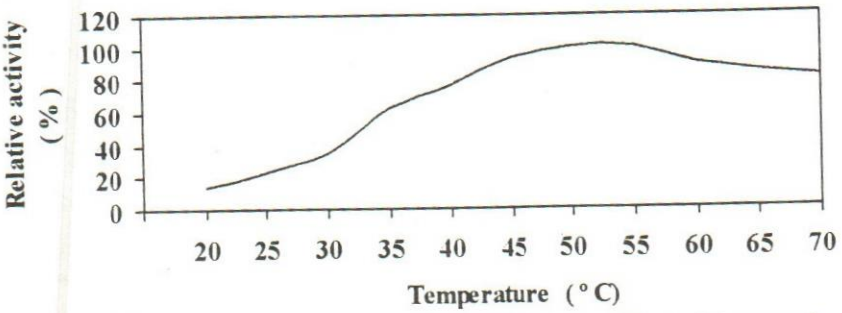

Fig. 2: Effect of temperature on the partially purified chitinase from *Trichoderma harzianum* T4.

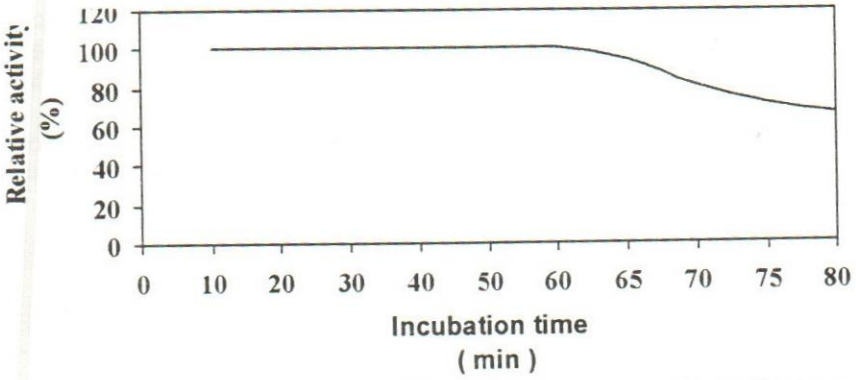

Fig. 3: Thermal stability of partially purified chitinase from *Trichoderma harzianum* T4.

Effect of reaction time:

The progress of enzymatic reaction with time was studied under the optimum conditions (50°C, pH 6.5). Data illustrated in Fig. 4, indicated that the reaction was linear with time up to 60 min. The maximal reaction rate was reached after the first 30 minutes of the chitinase reaction (0.36Umin^{-1}) after which, the rate decreased to the lowest value after 90min (0.17Umin^{-1}).

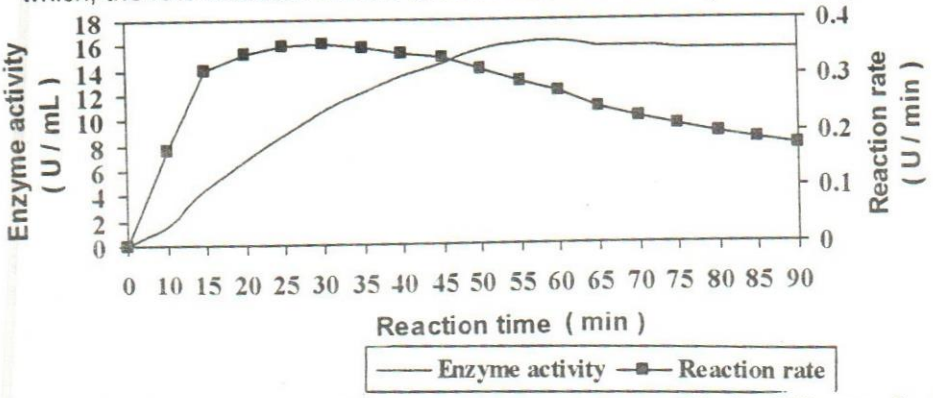

Fig. 4: Effect of reaction time on partially purified chitinase from *Trichoderma harzianum* T4 at pH 6.5 and 50°C.

Effect of metal ions:

The effect of some metal ions on the activity of *Trichoderma harzianum* T4 chitinase is summarized in Table 7. The metal ions under investigation Mn^{2+}, Ca^{2+} and Fe^{2+} activated the enzyme. However , the other metal ions Co^{2+} , Cu^{2+} , Zn^{2+} and Hg^{2+} had different inhibitory effects on the enzyme activity .Generally , the effects of used metal ions on the partially purified *T.harzianum* T4 chitinase very similar to that on the other chitinases from *Trichoderma* isolates (Saad and Nawar , 2001), *Streptomyces venezulae* (Hamed *et al.*, 1998) and *Aspergillus carneus* (Abdel-Naby *et al.*, 1992) .

These results suggest that *Trichoderma harzianum* T4 and its chitinase may provide a possible biological control against some plant pathogenic fungi. Such suggestion requires another further study.

Table 7: Effect of some metal ions on the partially purified chitinase.

Activators		Inhibitors	
Metal ions 10 µmole	Relative activity (%)	Metal ions 10 µmole	Relative activity (%)
None*	100.00	None	100.00
Mn^{2+}	153.00	Co^{2+}	63.20
Ca^{2+}	142.80	Cu^{2+}	43.02
Fe^{2+}	110.00	Zn^{2+}	36.10
-	-	Hg^{2+}	16.20

* No metal as control .

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إنتاج إنزيم الكيتينيز من فطر المقاومة الحيوية الترايكودرما

حلمى محمد حسن

قسم كيمياء الكائنات الدقيقة ، المركز القومى للبحوث – الدقى – القاهرة – مصر

يلعب فطر الترايكودرما دورا هاما فى تدمير الفطريات الممرضة للنبات ، وقد وجد أن الفطر ينتج إنزيم الكيتينيز ، الذىيحلل الكيتين أحد المكونات الرئيسية فى كثير من جدر خلايا الفطريات . من أربعة عزلات من فطر الترايكودرما أظهر فطر الترايكودرما هارزيانم عزلة ت٣ ، ت٤ قدرة كبيرة على تحليل الكيتين فى بيئة الأجار الأساسية المحتوية على الكيتين . وقد درست بعض العوامل المؤثرة على إنتاج الإنزيم. لقد وجد أن أعلى مستوى لإنتاجيةالإنزيم تحقق بعد التحضين لمدة ستة أيام عند درجة حرارة ٢٨ م ودرجة حموضة أولية للبيئة ٦,٥ (من ١٠ إلى ١٥,٩ وحدة / مل) وينتج الإنزيم فقط فى وجود الكيتين كمصدر وحيد للكربون فى البيئة مما يدل على أنه من النوع المستحث . وقد وجد أن فطرة الترايكودرما هارزيانم ت ٤ ذات كفاءة عالية لتحليل الميسيليوم الخاص ببعض الفطريات الممرضة للنبات مثل ميكروفومينا فاصولينا ، ريزوكتونيا سولانى و سكليروشيم رولفسيى. تم إجراء التنقية الجزئية لإنزيم الكيتينيز المنتج من فطرة الترايكودرما هارزيانم ت ٤ باستخدام الترسيب بكبريتات الأمونيوم يتبعه استخدام الترشيح بالجيل بمادة السيفادكس جى – ٢٠٠ . وبدراسة بعض خواص الإنزيم المنقى جزئيا وجد أن أعلى نشاط إنزيمى عند درجة حموضة ٦,٥ ودرجة حرارة ٥٠ م لمدة ستين دقيقة . لقد ثبت أن لأيونات المنجنيز ، الكالسيوم والحديد تأثيرا منشطا للإنزيم بينما أدت أيونات الكوبالت ، النحاس ، الزنك والزئبق إلى تأثيرات تثبيطية مختلفة على نشاط الإنزيم.