

## Production and Regulation of Extracellular Chitinase by *Aschersonia aleyrodis* (Cordycipitaceae: Hypocreales)

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**Abstract.-** Chitinases are virulence determination factors of entomopathogenic fungi which perform critical functions during degradation of insect cuticle. Production of extracellular chitinases by *Aschersonia aleyrodis* (Cordycipitaceae; Hypocreales) isolate A005 was investigated using different combinations of basal medium components. The effects of different carbon sources on chitinase activity and biomass production by *A. aleyrodis* were tested in media supplemented with simple or complex carbon sources individually or in combination. Maximum chitinase activities ( $221.59 \pm 1.20$  mU/ml) as well as maximum biomass production ( $16.37 \pm 1.59$  mg/ml) were found in the supernatants from 1.5% *N*-acetylglucosamine (GlcNAc) plus 0.8% chitin. The effects of the divalent metal ions (iron and magnesium) on lipase activity were also studied. The divalent metal salts,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  and  $\text{ZnSO}_4$ , inhibit chitinase activity at 10 and 100mM concentration whereas inhibition of chitinase activity by KCl,  $\text{FeSO}_4$  and EDTA was observed at higher concentrations only. The optimum pH for chitinase production was 5.7, and the optimum temperature 25°C.

**Key Words** *Aschersonia aleyrodis*, chitinase, carbon sources

### INTRODUCTION

Insect cuticles are composite structural materials with mechanical properties that are optimal for their biological functions. The cuticle consists of a thin outer epicuticle containing lipids and proteins and a thick procuticle mainly consisting of chitin and proteins (Andersen *et al.*, 1995; Samson *et al.*, 1988). Entomopathogenic fungi enter their hosts by direct penetration of the cuticle, which acts as a barrier against penetration of microbes. Consequently, fungal pathogens have a potential as a biological means of controlling sap-sucking insects that have not been easily controlled with chemical pesticides (Charnely, 1997). During the fungal penetration through the host cuticle, hydrolytic enzymes such as proteases, chitinases, and lipases are produced and secreted and are important for the initiation of the infection process (Schagger and von Jagow, 1987; Yang and Yeh, 2005). Chitinases catalyze the hydrolysis of chitin, which is a  $\beta$ -(1,4)-linked polymer of *N*-acetyl-D-glucosamine and one of the important structural

components of insect cuticle (Tsigos and Bouritos, 1995). Chitinases are produced by a large number of organisms including plants, fungi, and bacteria. Chitinases play an important role in the defense mechanism of plants against pathogens and in the mycoparasitic process of fungi. They also play an important role in nutrition, development, and morphogenesis of fungi. However, the role of the chitinases in the host infection process is not yet fully understood.

*Aschersonia aleyrodis* (Clavicipitaceae: Hypocreales) is a promising insect pathogenic fungal species being used as a biological control agent. Its successful use in Florida citrus groves dates from nearly 1900s, when citrus branches with *A. aleyrodis* were introduced into citrus groves to seed epizootics in whitefly populations (Berger, 1921). *A. aleyrodis* has been successfully used against greenhouse whitefly in Bulgaria, China, Japan and USSR (Evans and Hywel-Jones, 1990). Recent studies on spore production, germination and pathogenicity have provided a better understanding of *A. aleyrodis* biology yet very little information is available about the chitinolytic activity as well as characterization of chitinases produced by *A. aleyrodis*. The regulation of cuticle-degrading enzyme is probably complex and may involve a combination of carbon/nitrogen induction and/or

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repression (Ali *et al.*, 2009). This paper deals with the effects of different carbon sources, growth temperature, pH of the growth medium, on chitinolytic activity of *A. aleyrodis* isolates which have shown considerable pathogenic ability during the previous studies (Musa, 2006). The effect of different metal ions on the apparent enzymatic activity was also determined.

## MATERIALS AND METHODS

### *Fungal strains*

For all assays, *A. aleyrodis* isolate (A005) deposited at the Engineering Research Center of Biological Control, South China Agricultural University, was cultured on potato dextrose agar (PDA) and incubated at  $26\pm 2^\circ\text{C}$  for 10 days. Conidia were harvested with deionized water containing 0.03% Tween 80 and sieved through filter paper into sterile vials. Conidia were counted in a compound microscope using a hemocytometer ( $0.0625\text{m}^2$ ; Fuchs-Rosenthal Merck Eurolab) to calibrate a suspension of  $1\times 10^6$  conidia/mL.

### *Effect of different carbon sources on chitinase activity and biomass production*

Effect of carbon source on chitinolytic activity and biomass production by *I. fumosoroseus* was studied by growing the fungal isolates on basal liquid Sabouraud dextrose medium consisted of glucose 10%, peptone 10% and yeast extract 3%. As carbon sources, glucose (0.8% or 1.5%) or *N*-acetylglucosamine, GlcNac (0.5% or 1%) were added. Complex substrates such as chitin (0.8%); combinations of chitin plus different concentrations of glucose (0.8% or 1.5%) or chitin 0.8% plus GlcNac (0.5% or 1%) were also used. All media were heat sterilized ( $121^\circ\text{C}$  for 15 min). The flasks were inoculated with one mL of  $1 \times 10^6$  spores/mL and incubated at 180 rpm and  $28^\circ\text{C}$  for 5 days. For biomass determinations the culture supernatants were separated from the mycelium by filtration through Whatman filter no. 1 and dried at  $80^\circ\text{C}$  until a constant weight.

### *Effect of different salts on chitinolytic activity*

To evaluate the effects of different metal ions

such as  $\text{K}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Zn}^{++}$  and EDTA on the apparent enzymatic activity by growing the fungal isolates in liquid medium having 0.8% chitin as a carbon source. All the salts were used at concentration of 100mM in 50 mL of basal medium in 250 mL Erlenmeyer flasks. The flasks were inoculated with one mL of  $1 \times 10^6$  spores/mL and incubated at 180 rpm and  $28^\circ\text{C}$  for 5 days.

### *Evaluation of best pH and temperature for chitinase production*

The effect of pH on enzymatic activity was determined by varying the pH of the 50 mL of basal media using with 0.1 M sodium phosphate in the following pH: 5.7, 6.3, 7.0 and 8.0 whereas non buffered basal medium was used as control. 50 mL of sterile growth medium inoculated with one mL of  $10^6$  spores/mL was incubated for 5 days in an orbital shaker operating at 180 rpm and  $28^\circ\text{C}$ .

The range of optimum temperature for chitinolytic activity was studied by incubating 50 mL of basal medium inoculated with one mL of  $10^6$  spores/mL was incubated for 5 days at different temperature (20, 25, 30, 35 and  $40^\circ\text{C}$ ) in an orbital shaker operating at 180 rpm.

### *Analytical determinations*

The chitinase activity in the culture supernatant was estimated as described earlier by Nahar (2004) using acid swollen chitin as the substrate. To prepare acid swollen chitin, the chitin (10g, were suspended in chilled *O*-phosphoric acid (88%, w/v) and left at  $0^\circ\text{C}$  for 1h with stirring. The acid swollen chitin was repeatedly washed with chilled distilled water, followed with a 1% (w/v)  $\text{NaHCO}_3$  were further dialyzed against cold distilled water. After homogenization in Waring blender (1 min), 50 mM acetate buffer, pH 5.0, were added to the suspension so that one mL of suspension contained 7 mg of chitin. The reaction mixture for chitinase assay will contain 1mL 0.7% acid swollen chitin, 1mL 50 mM acetate buffer, pH 5.0 and one milliliter enzyme solution that were incubated at  $50^\circ\text{C}$  for 1h. The GlcNac produced were estimated colorimetrically with *p*-dimethyl amino benzaldehyde (DMAB) (Reissig *et al.*, 1955). One international unit was defined as the activity that produced  $1\mu\text{mol}$  of GlcNac per min.

*Chemicals and reagents*

*p*-dimethyl amino benzaldehyde (DMAB) and *N*-acetylglucosamine, (GlcNac) was purchased from Sigma (St Louis, MO, USA). All the other chemicals were obtained from Guangzhou Jinhua chemical reagent company, Guangzhou, China. Chitin obtained from crab shell was purchased from Sinopharm chemical reagent company, Shanghai, China.

*Experimental design and statistical analysis*

Each study was conducted three times with freshly prepared fungal suspension. Chitinase activity as well as biomass production under different conditions was analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's studentized range test for mean comparisons at 5% level of significance. All statistical analysis was performed using SAS 8.01(SAS, 2000).

**RESULTS***Effect of different carbon sources*

The effects of different carbon sources on chitinase production by *A. aleyrodis* were tested in medium supplemented with simple or complex carbon sources individually or in combination. As shown in Tables I, *A. aleyrodis* produced chitinases in all media tested; however, the amount of secreted enzymes varied significantly among different treatments and control ( $F= 10.51$ ;  $df = 9$ ;  $P<0.0001$ ). The highest levels of chitinase activities ( $221.59 \pm 1.20$  mU/ml) were observed for the cultures grown on 1.5% *N*-acetylglucosamine (GlcNac) plus 0.8% chitin. When chitin was added to the media, without GlcNac, chitinase activities were detected at comparatively lower levels, suggesting an induction of chitinolytic enzymes by GlcNac. When different concentrations of glucose were used as a sole carbon sources, very low levels of chitinase activity was also detected even when the fungus was grown in high concentration of this compound but higher rates of chitinase activity were observed when glucose was used in combination with 0.8% chitin (Table I).

The biomass production *A. aleyrodis* in the basal medium supplemented with simple or complex carbon sources individually or in combination after

5 days of inoculation was significantly different when compared to the control ( $F= 20.73$ ;  $df = 9$ ;  $P<0.0001$ ). The lowest biomass production was observed in control having a mean biomass production of  $6.14 \pm 0.90$  mg/ml whereas the highest biomass ( $16.37 \pm 1.59$  mg/ml) was obtained from the basal medium having a combination of 1.5% *N*-acetylglucosamine (GlcNac) and 0.8% chitin as a carbon sources. Biomass produced in the media having 0.8% and 1.5% glucose as a carbon source were significantly similar to control whereas biomass production at different concentrations of glucose differed significantly from control when it was used in combination with 0.8% chitin (Table I).

**Table I-** Effect of carbon sources on chitinase activity and biomass production by *Aschersonia aleyrodis*.

Carbon sources	Chitinase activity (mU/mL)	Biomass (mg/mL)
Glucose 0.8%	66.92±2.02 c	6.39±0.65 e
Glucose 1.5%	87.98±3.69 c	7.36±0.72 de
GlcNac 0.8%	176.23±2.58 b	8.56±0.56 de
GlcNac 1.5%	183.67±2.84 b	13.98±1.50 b
Chitin 0.8%	129.39±1.43 bc	9.66±0.80 d
Glucose 0.8% + Chitin 0.8%	191.11±1.64 ab	7.73±1.18 de
Glucose 1.5% + Chitin 0.8%	199.28±2.44 ab	11.27±1.31 c
GlcNac 0.8% + Chitin 0.8%	213.41±2.08 ab	12.62±1.05 bc
GlcNac 1.5% + Chitin 0.8%	221.59±1.20 a	16.37±1.59 a
Control	52.05±2.88 c	6.14±0.90 e
F, df, P	10.51; 9; <0.0001	20.73; 9; <0.0001

Means in the same column with different letters are significantly different from each other (Tukey's,  $P<0.05$ )  
±: Standard error (Based on three independent replicates)

*Effect of different salts*

The effects of different metal compounds used at different concentrations (10mM and 100mM) on the enzyme activity are reported in Table 2. At 10 mM, chitinase activities observed from cultures having KCl, FeSO<sub>4</sub> and EDTA was significantly similar to the control whereas significantly different rates of chitinase activity were observed for CaCl<sub>2</sub> and MgCl<sub>2</sub> (Table II). At 100 mM, all the above mentioned compounds significantly affected chitinase activity of *A. aleyrodis* when compared to control ( $F= 46.79$ ;  $df =6$ ;  $P <0.001$ ). The divalent

metal salt,  $MgCl_2$  caused an activity reduction of 72% when compared to the control. Chitinase activity observed for the cultures having  $FeSO_4$  and  $CaCl_2$  were significantly similar to each other having mean values of  $114.47 \pm 3.12$  and  $109.41 \pm 2.16$  mU/ml (Table II).

**Table II.- Effect of metal compounds at different concentrations on chitinase activity of *Aschersonia aleyrodis*.**

Compounds	Chitinase activity (mU/ml) at different concentrations	
	10mM	100mM
KCl	168.22±1.91 a	152.13±2.03 b
CaCl <sub>2</sub>	147.40±2.04 b	109.41±2.16 c
FeSO <sub>4</sub>	163.86±1.77 a	114.47±3.12 c
MgCl <sub>2</sub>	123.12±2.54 c	49.73±1.80 f
ZnSO <sub>4</sub>	141.33±1.26 b	78.16±2.06 e
EDTA	164.44±2.01 a	97.54±1.27 d
Control	174.87±2.61 a	174.87±2.61 a
F, d.f, P	32.49; 6; <0.001	23.67; 6; <0.001

Means in the same column with different letters are significantly different from each other (Tukey's,  $P < 0.05$ )  
 $\pm$ : Standard error (based on three independent replicates)

#### Effect of pH of basal medium

Chitinase activity of *A. aleyrodis* differed significantly at different pH levels ( $F = 23.41$  df = 4;  $P < 0.001$ ). The best chitinase production was obtained when media was buffered with pH 5.7 having mean chitinase activity of  $177.18 \pm 9.41$  mU/ml. Chitinase activity decreased as the pH value of media was increased (Fig. 1).

#### Effect of temperature

Examination of the relationship between growth temperature and chitinase production indicated that growth temperature had a significant effect on chitinase production ( $F = 12.31$  df = 3;  $P = 0.021$ ). Substantially less chitinase activity ( $133.05 \pm 4.46$  mU/ml) was obtained at a growth temperature of 20°C. The best temperature range for chitinase production in the basal medium was 25–30°C (Fig. 2).

## DISCUSSION

The existence of external hydrolytic enzymes in micro-organisms might be attributed to the

impermeability of the cell membrane to their corresponding substrate. Some of these enzymes are formed only in the presence of their specific substrates. This situation may pertain to chitinase formation in entomopathogenic fungi, where the enzyme appeared at high levels in response to the presence of chitin, its usual substrate (Barreto *et al.*, 2004). One of the major roles of chitinases found in

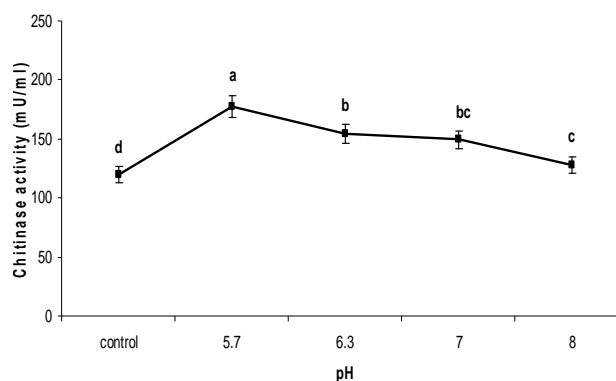


Fig. 1. Effect of different pH on chitinase activity of *Aschersonia aleyrodis*. Legends having different letters are significantly different from each other (Tukey's,  $P < 0.05$ ). Bars represent standard error of means (based on three independent replicates).

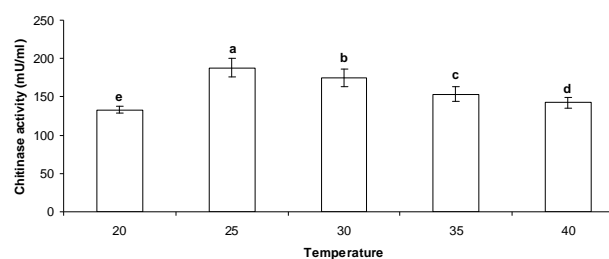


Fig. 2. Chitinase activity of *Aschersonia aleyrodis* at different temperatures. Legends with different letters are significantly different from each other (Tukey's,  $P < 0.05$ ). Bars represent standard error of means (based on three independent replicates).

fungi is to modify the organism's structural constituent chitin, present in their cell walls (Gooday, 1990; Sahai and Manocha, 1993), but the extracellular chitinolytic enzymes produced by entomopathogenic fungi have also been suggested to be pathogenicity determinants involved in host invasion (Clarkson and Charnely, 1996). The effects

of different carbon sources on chitinase activity and biomass production by entomopathogenic fungi were tested in medium supplemented with simple or complex carbon sources individually or in combination. Extracellular chitinase activity as well as biomass production increased progressively when glucose was added in the culture medium with 0.8% chitin. When compared to the cultures having chitin as sole carbon source. In addition, the chitinase activity and biomass production were drastically reduced when glucose was used a sole carbon source in the same culture system which may be due to the repression of glucose for protein utilized in the carbohydrate degradation pathways. GlcNAc induced the production and secretion of the chitinases and these effects were also observed when GlcNAc was added to media containing chitin (Table I). In this respect, the same regulation of chitinase activity for *A. aleyrodis* was shown by Morases *et al.* (2003) who observed highest levels of chitinase activities in the supernatants from 0.5% N-acetylglucosamine (GlcNAc) alone and in combination with 0.8% chitin. When chitin or was added to the media, without GlcNAc, chitinase activities were detected at lower levels, suggesting an induction of chitinolytic enzymes by GlcNAc. According to St Leger *et al.* (1986) GlcNAc might cause catabolic repression of chitinases when in excess of the immediate growth requirements of the organisms, and N-acetyl- $\beta$ -D-glucosaminidase production could be related to cellular growth.

The divalent metal salts, CaCl<sub>2</sub>, MgCl<sub>2</sub> and ZnSO<sub>4</sub>, inhibit chitinase activity at 100mM concentration whereas higher rates of chitinase inhibition were observed for KCl, FeSO<sub>4</sub> and EDTA. This agrees with data presented by Fencie *et al.* (1998) on inhibition of *Verticillium lecanii* chitinases by these salts. They showed that at 10 mM, K<sup>+</sup>, Fe<sup>++</sup> and EDTA did not produce any inhibitory effect, while the other substances caused 30% inhibition. At 100 mM, the inhibitory effects were more evident: Zn<sup>++</sup> and EDTA caused an activity reduction of about 50% and with Mg<sup>++</sup> the residual activity was about 30%. A possible reason of this reduction in enzyme activity can be the presence of some binding forces between the salts and the enzyme which can ultimately lead to the inactivation of enzyme (Iwai *et al.*, 1970).

Chitinase production by *A. aleyrodis* was also affected by pH and temperature changes. Maximum chitinase activity was observed at a pH of 5.7 which is almost similar to the findings of St Leger *et al.* (1998) who reported maximum chitinase production at a pH of 5.0 and a reduction in enzyme activity was observed by increase in pH. During these studies a temperature of 25°C proved most optimum for chitinase production. Similar results were also obtained by Fencie *et al.* (1998) who found maximum chitinase activity at a temperature of 25°C when *Verticillium lecanii* was grown at different temperatures.

The study of the regulation of the virulence factors in entomopathogenic fungi is of particular importance because pathogenic specialization may operate by way of regulatory controls that allow their expression. The results presented in this study increase the knowledge about chitinase production in *A. aleyrodis* opening new avenues for the study of the role of this enzyme in virulence against different insect pests during the infection process.

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