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Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Metarhizium anisopliae* isolates

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Abstract - *Metarhizium anisopliae,* an entomopathogenic hyphomycete, is being used effectively in Integrated Pest Management (IPM) system. Foliar application of these fungi is quite satisfactory as it invades its host by adhering to insect cuticles and formation of penetration structures called appresoria, which produces various extracellular enzymes, including chitinase that causes the insect cuticle breaching. The induction and repression mechanism of chitinase activity is not entirely understood and activity of this enzyme is different in response to different carbon and nitrogen sources. This report illustrates the effect of two carbon sources viz. colloidal chitin and dextrose and a nitrogen source, yeast extract on the chitinase production of fourteen *M. anisopliae* isolates. The chitinase activity varied among the isolates and the different media used. A high enzymatic activity was observed in the medium containing an extra nitrogen source (yeast extract) followed by the medium containing colloidal chitin as a sole source of carbon and nitrogen. The exochitinase activity and the chitinase activity gel were also studied for the isolates showing high chitinase enzyme production. An array of chitinase isozymes were observed on chitinase activity gel with a common 14.3 kDa enzyme for all the isolates.

Key words: chitinase activity induction; chitinase activity repression; colloidal chitin; exochitinase.

INTRODUCTION

Entomopathogenic fungi are being recognized as alternatives to the chemical counterparts for the effective pest management. *Metarhizium anisopliae*, a natural soil borne entomopathogenic fungus, is currently being explored for its wide host range as it is active against corn borer, root weevil, cockroaches and many other insect pests as well (Inglis *et al.,* 2001). Chitin, the insoluble polymer of N-acetyl glucosamine, is the second most abundant polymer in nature, generally found in insect cuticle. It is quite difficult to chemically modify chitin but it has been found that several micro organisms and also the plants and invertebrates have developed enzymatic systems that could metabolize this insoluble polymer (Muzzarelli, 1999). Cuticle degrading enzymes from entomopathogenic fungi can be attributed for the comparison of isolates differing in pathogenicity. Insect cuticle is the foremost obstacle in the pathogenesis. The composite nature of cuticle comprises a lipid-protein rich epicuticle, procuticle and chtinatious exo and endo cuticle (Anderson, 1979). Invasion occurs through the initiation of a secretion array of cuticle hydrolyzing enzymes. The degree of pathogenicity is related to the production of the chitinolytic enzymes in entomopathogenic fungi *Nomuraea rileyi* (El-Sayed *et al.,* 1989). Overproduction of an endochitinase radically increases the pathogenicity of entomopathogenic fungus *B.bassiana* (Fang *et al.,* 2005).The effect of various carbon sources on the chitinolytic activity was described by Campos *et al.* (2005). The medium containing insect cuticle and crystalline chitin produced highest chitinase activity in contrast to media containing glucose and high levels of GlcNAc repressed the enzyme activity (De Moraes *et al.,* 2003). Nitrogen source also has a profound effect on chitinase activity. Peptone and Yeast extract as a nitrogen source has proved to be the most affirmative regulator of chitinase enzyme (Lopes *et al.,* 2008). Fungal growth on chitin induces both endo and exochitinase activity. Among different exochitinases, N-acetyl glucosaminidase is the prominent one, releasing acetylglucosamine from non reducing ends of chitin chains. Kang *et al.* (1999) purified a novel chitinase from *M. anisopliae* which showed high chitinolytic activity against colloidal chitin as well as synthetic substrates of exochitinase enzymes indicating both endo and exo chitinase activity. A 110 kDa N-acetylglucosaminidase activity was observed in *M. anisopliae* (Charnley and St. Leger, 1991). Almost ten chitinase isozyme activities were observed on polyacrylamide gel using glycol chitin as a substrate for chitinase from *M. anisopliae* (St. Leger *et al.,* 1993).

In order to evaluate the induction and regulation mechanism of chitinase activity fourteen *M. anisopliae* isolates were grown on four different media containing various carbon and nitrogen sources. Chitinase activity was observed for a time period of ten days so as to understand the influence of different carbon and nitrogen sources and also the appropriate incubation time to

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TABLE 1 - Source of *Metarhizium anisopliae* isolates

Isolates	Code No./Accession No. ARSEF	Host Insect	Geographical location
UM1	1745	*Nilaparvata lugens*	India
UM2	2735	*Spodoptera sp.*	Philippines
UM3	2153	*Nephotettix virescens*	Indonesia
UM4	2424	*Lepidpotera larva*	Indonesia
UM5	3210	*Coleoptera*	India
UM6	2596	*Pyrausta machaeralis*	India
UM7	1080	*Helicoverpa zea.*	U.S.A
UM8	1724	*Nilaparvata lugens*	India
UM9	1727	*Nilaparvata lugens*	India
UM10	3295	*Anticarsia gemmatalis*	Mexico
UM11	1729	*Nilaparvata lugens*	India
UM12	1744	*Nilaparvata lugens*	India
UM13	1823	*Nilaparvata lugens*	India
AR1	Local	Unknown	India

optimize the chitinase activity. N-acetylglucosaminidase activity and chitinase activity gel was also determined for the selected isolates which demonstrated high exochitinase activity.

MATERIALS AND METHODS

Fungal isolates. A total of thirteen isolates were obtained from ARSEF (USDA-ARS Plant Protection Unit, Ithaca, NY) and one isolate was from India (Table 1). The isolates were routinely subcultured on Sabourauds dextrose agar (SDA) slants at 28 °C and maintained at 4 °C.

Preparation of colloidal chitin. Colloidal chitin was prepared with a diminutive modification of the Simahara and Takiguchi (1988) method. Ten grams of practical grade crab shell chitin (Sigma Chemicals) were mixed with 150 ml 12 N HCl with a continuous stirring for 2 h at 4 °C. The suspension was repeatedly mixed with 1 l water and filtered through a course filter paper. This step was followed four to five times and the pH of the suspension was adjusted to 7.0 by addition of 5N NaOH and the colloidal suspension was washed several times with ddH_2O for desalting. After desalting the suspension was centrifuged at 8000 rpm for 10 min and the precipitate was collected for further use as colloidal chitin.

Different media and culture conditions. Seven day old SDA slants were used for the preparation of conidial suspension (1 x 10^6 conidia/ml) to inoculate SDY broth (4% dextrose, 1% peptone, and 1% yeast extract) and incubated at 28 °C and 180 rpm for three days. The harvested mycelium was washed twice with sterilized distilled water and inoculated in to different media at 20% (v/v) based on the final volume (50 ml) of the culture. Four different media were used with various carbon and nitrogen sources. Colloidal chitin (2%) was constant for all the four media used. Medium I was constituted by basal salts: KH_2PO_4 (1 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $FeSO_4{\cdot}7H_2O$ (0.2 mg/l), $ZnSO_4{\cdot}7H_2O$ (1 mg/l), $NaMoO_4{\cdot}2H_2O$ (0.02 mg/l), $CuSO_4{\cdot}5H_2O$ (0.02 mg/l), $MnCl_2{\cdot}4H_2O$ (0.02 mg/l) (Cooper and Wood, 1975). Medium II was supplemented with 2% dextrose and Medium III with 1% yeast extract, respectively. The components in Medium IV contained both 2% dextrose and 1% yeast extract. The pH of the culture media was adjusted to 5.6 and the cultures were incubated at 28 °C and 180 rpm for 10 d. The cultures were centrifuged and the culture filtrate was taken in order to study the enzyme assays on alternate days of growth till tenth day of culture.

Protein assay. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays.

Chitinase assay. Chitinase assay was determined by the method of Yanai *et al*., (1992). The culture supernatant (500 µl) was incubated with 300 µl of 10% (w/v) colloidal chitin and 300 µl of 0.2 M acetate buffer (pH 4.0) at 37 °C for 2 h. The reaction product N-acetyl glucosamine was determined by the method of Reissig *et al.* (1955) by using para-dimethyl-amino benzaldehyde reagent (DMAB) and was prepared as described by Kang *et al.* (1999). Absorbance at 585 nm (A_{585}) was taken against water as blank. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µM of N-acetyl glucosamine per min under the above conditions. N-Acetyl glucosamine was taken as standard for all the enzyme assay calculations.

Exochitinase assay. Basal salt medium supplemented with colloidal chitin (2%) was used to suspend 10^6 conidia/ml and incubated at 28 °C and 180 rpm for 72 h. N-Acetylglucosaminidase activity was determined by the method of Coudron *et al.* (1984) using synthetic substrate, para-nitro phenyl-N-acetylglucopyranoside (PNP-NADG). A 200 µl of PNP-NADG (1 mg/ml) and 200 µl of 0.2 M citrate-phosphate buffer (pH 5.6) was added to 200 µl of culture supernatant. The reaction mixture was incubated for 1 h at 37 °C and reaction was terminated using 1 ml of 0.01 M NaOH. Absorbance at 400 nm (A_{400}) was observed and concentration of para-nitro phenol was determined. One unit of exochitinase activity was defined as the amount of enzyme that produced 1 µM of para-nitro phenol per min under the above conditions.

Chitinase activity gel. Chitinase activity on gel was observed using glycol chitin as substrate. Glycol chitin was prepared by the

method of Trudel and Asselin (1989). Samples were boiled for 3 min in sample buffer excluding β-mercaptoethanol and electrophoresed on 12.5% polyacrylamide gel containing 0.01% (w/v) glycol chitin. Gels were incubated in 100 mM sodium acetate buffer (pH 5.4) containing 1.5% (v/v) TritonX-100 at 30 °C for 20 h with gentle shaking. After incubation, gels were stained for 10 min with 100 ml freshly prepared Fluorescent Brightener 28 (Sigma Chemicals) in 500 mM Tris-HCL buffer (pH 8.9) and repeatedly washed with ddH_2O for 1 h. Dark lytic zones were visualized against fluorescent background by UV illumination.

Statistical analysis. Statistical analyses were performed by SPSS software. Test of significance were carried out using Tukey's test.

RESULTS

Significant difference in the enzyme activity on second day of culture was observed for Medium II in comparison to the other three media. Medium III showed highest chitinase activity for most of the isolates, with highest enzyme activities of 15.20, 15.74 and 15.20 U/ml for isolates UM10, UM12 and UM13 respectively (Table 2). A moderate chitinase activity was observed for Medium I while Medium II showed low chitinase activity for most of the isolates. High specific activities were observed in Medium III (0.3 mU/mg-1.88 U/mg); although a very high specific activity of 5.90 U/mg was observed in Medium II for isolate UM11. Highest enzyme activity was observed in Medium I and Medium III for isolate UM6 (4.30 U/ml) and UM7 (11.40 U/ml) respectively. No significant difference in the chitinase activity in any of the medium was observed for isolate UM8. Isolate AR1 showed trivial change in the chitinase activity in the four media investigated.

Medium III showed highest chitinase activity on fourth day of culture for most of the isolates except UM1 and UM13 with highest chitinase activities in Medium IV (6.50 U/ml) and medium II (12.54 U/ml) respectively (Table 3). Medium III showed highest chitinase activity of 17.92 U/ml for isolate UM10. High specific activity was observed for both Medium II and Medium III on fourth day of culture with highest specific activity of 4.56 U/mg for isolate UM11. The comparative study of enzyme activity as well as specific activity among the four media for isolates UM3, UM8 and AR1 showed no significant difference.

On the sixth day of culture, all the four media showed moderate to high chitinase activity for the different isolates. High chitinase activity was observed in Medium IV for isolate UM1 (10.31 U/ml) and UM9 (12.19 U/ml) (Table 4). Highest enzyme activity was observed in Medium III for isolate UM7 (14.86 U/ml), while the highest specific activity was observed in Medium II for isolate UM13 (23.92 U/mg). Most of the isolates showed high specific activities in Medium II.

Exceptionally high chitinase activity was observed for Medium IV with the highest chitinase activity of 15.74 U/ml for isolate UM1 although an extremely low enzyme activity of 0.54 U/ml was observed in Medium II on eighth day of culture (Table 5). Almost all the isolates showed high chitinase activities in Medium IV and isolate UM10 showed high chitinase activity of 14.12 U/ml in Medium II. High specific activities were observed for Medium II in isolate UM11 (2.66 U/mg) and for Medium IV in isolate and UM7 (2.28 U/mg) respectively.

A similar trend in the enzyme activity profile was observed for different isolates on tenth day of culture. Although the chitinase activity decreased with increasing age of incubation time, medium IV still showed high enzyme activities for most of the isolates (Table 6). Specific activity also decreased compared to preceding culture days. High specific activity was observed in Medium II and Medium IV but highest specific activity was observed in Medium I for isolate UM4, (4.70 U/mg). No significant difference in chitinase activity was observed for the isolates UM5 and UM8 in the four media studied.

Seven out of fourteen isolates of *M. anisopliae* were screened out based on the chitinase activity in Medium III at 6th day of incubation as it showed good enzyme production when compared to other three media. The protease activity profile of these selected isolates was also studied and found to be high when compared to other isolates (data unpublished).

Exochitinase activity for the selected strains

Exochitinase activity was studied with seven isolates showing high chitinase activity. Isolate UM13 showed highest exochitinase activity of 7.19 U/ml and highest specific activity of 0.98 U/mg. isolates UM7, UM11 and UM12 showed an enzyme activity of 5.81, 5.84 and 5.95 U/ml respectively although specific activities were low for these isolates. Isolates UM4, UM6 and UM10 showed low exochitinase activity and correspondingly low specific activity. (Table 7).

Chitinase activity gel

A 14.3 kDa chitinase was observed for all the seven isolates investigated (Fig. 1). A different zymogram was observed for isolate UM6 with three additional bands of 24, 66 and 80 kDa along with the 14.3 kDa band. An additional 23.8 kDa band was also observed for isolate UM7 along with the 14.3 kDa band and an additional chitinase isozyme of 20.1 kDa was observed for isolate UM11 whereas isolate UM13 showed a chitinase isozyme of 17.2 kDa.

DISCUSSION

The paradigm of infection mechanism is more complex based on physiological and morphological factors. Different extracellular enzymes from entomopathogenic fungi are involved in the cuticle hydrolysis, viz chitinase, protease, esterase (Gabriel, 1968; Bajan *et al.,* 1979). The amount of chitinase release is directly related to the degree of pathogenicity in entomopathogenic fungi as well as other pathogenic fungi (Yanagita, 1980; El-Sayed *et al.,* 1989). *Metarhizium anisopliae* was reported to secrete a range of extracellular chitinolytic enzymes (St. Leger *et al.,* 1993). The late appearance of chitinase during the *Manduca sexta* infection suggests that this enzyme is induced by the chitin present in the host cuticle which is revealed eventually from the protein matrix by the action of fungal protease (St.Leger *et al.,* 1996), although this induction mechanism is not fully understood till date.

Extracellular enzymes like chitinases facilitate to breach the chitinatious insect cuticle and invade the host. Extracellular enzyme production is also subjective to factors such as the carbon and nitrogen sources and the media pH (St Leger *et al.,* 1998; Barreto *et al.*, 2004). Cruz *et al.* (1993) reported a raise in the chitinase production from *Trichoderma harzianum* with the increase in the chitin concentration in the media and then a quick decrease with the addition of glucose. Colloidal chitin seemed to contain a minute amount of GIcNAc that helps to induce the enzyme initially but it is reported that GIcNAc causes catabolite repression when present in high concentration in the medium (Campos *et al.,* 2005). In the present study, it was observed that a very minimal amount of chitinase was produced especially during the first few days of incubation in Medium II containing two carbon sources (colloidal chitin and dextrose). The presence of easily available carbon source (dextrose), suppresses the chi-

TABLE 2 - Enzyme and specific activity of *Metarhizium anisopliae* isolates in the four different media on day 2

Media	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
Enzyme activity (U/ml)														
MI	2.71^{bF}	7.05^{aA}	4.80^{aD}	4.89^{bD}	4.30^{bE}	4.30^{aE}	5.83^{bC}	2.31^{aF}	1.86^{bF}	3.38^{bF}	4.54^{bE}	6.64^{bA}	5.29^{cC}	5.97^{aC}
MII	0.54^{cI}	0.54^{bI}	1.09^{cF}	3.26^{bC}	1.09^{cF}	1.63^{bE}	5.43^{bB}	1.98^{aD}	1.90^{bD}	1.63^{bE}	1.36^{bE}	7.98^{bA}	9.23^{bA}	2.40^{bC}
MIII	2.17^{bI}	7.60^{aF}	2.17^{bI}	9.7^{aE}	6.50^{aG}	1.30^{bJ}	11.40^{aC}	1.65^{aJ}	13.03^{aB}	15.20^{aA}	10.32^{aD}	15.74^{aA}	15.20^{aA}	4.30^{aH}
MIV	5.90^{aAB}	6.50^{aA}	2.17^{bDE}	3.8^{bBC}	3.80^{bBC}	2.70^{bD}	3.58^{cBC}	2.59^{aD}	3.64^{bCD}	2.23^{bDE}	2.71^{bD}	4.51^{cBC}	0.81^{dE}	3.40^{abCD}
Specific activity (U/mg)														
MI	0.34^{aB}	0.11^{aC}	0.09^{aD}	0.12^{bC}	0.36^{aB}	0.67^{aA}	0.10^{dC}	0.07^{cD}	0.04^{dD}	0.11^{cC}	0.10^{cC}	0.20^{cB}	0.18^{bB}	0.11^{aC}
MII	0.13^{bH}	0.02^{bI}	0.06^{aI}	1.05^{aC}	0.39^{aG}	0.42^{aG}	0.93^{bD}	0.59^{aF}	0.73^{bE}	0.32^{bG}	5.90^{aA}	1.67^{aB}	1.53^{aB}	0.13^{aH}
MIII	0.07^{cG}	0.11^{aF}	0.05^{aG}	0.18^{bE}	0.10^{bF}	0.03^{cG}	1.58^{aC}	0.13^{bE}	1.81^{aA}	1.29^{aD}	1.71^{bB}	1.88^{aA}	1.59^{aC}	0.15^{aE}
MIV	0.18^{bDE}	0.10^{aF}	0.06^{aF}	0.13^{bF}	0.13^{bF}	0.17^{bF}	0.45^{cB}	0.43^{aB}	0.23^{cC}	0.15^{cE}	0.07^{cF}	0.65^{bA}	0.18^{bDE}	0.09^{aF}

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey's test.

TABLE 3 - Enzyme and specific activity of *Metarhizium anisopliae* isolates in the four different media on day 4

Media	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
Enzyme activity (U/ml)														
MI	3.6^{bF}	5.97^{bC}	3.26^{aG}	5.43^{bD}	3.80^{bF}	2.71^{aH}	6.64^{bB}	4.57^{aE}	4.32^{bE}	7.48^{bA}	5.54^{bCD}	8.92^{aA}	6.59^{bB}	5.43^{aD}
MII	1.63^{cH}	1.09^{cI}	1.63^{aH}	3.80^{cF}	2.70^{bG}	3.26^{aFG}	6.79^{bC}	4.84^{aE}	3.04^{bG}	5.86^{bD}	6.57^{bCD}	10.65^{aB}	12.54^{aA}	3.26^{aFG}
MIII	4.34^{bH}	8.14^{aD}	3.26^{aI}	8.14^{aD}	6.51^{aF}	1.63^{aJ}	13.03^{aB}	4.34^{aH}	13.57^{aB}	17.92^{aA}	14.60^{aB}	10.04^{aC}	7.33^{bE}	5.43^{aG}
MIV	6.50^{aC}	7.60^{bB}	2.71^{aI}	3.40^{cH}	3.26^{bH}	2.17^{aI}	6.41^{bD}	3.64^{aGH}	12.43^{aA}	6.19^{bD}	7.00^{bB}	5.21^{bE}	3.80^{cG}	4.34^{aF}
Specific activity (U/mg)														
MI	0.33^{aA}	0.10^{aC}	0.07^{aDE}	0.11^{bC}	0.15^{aB}	0.07^{aDE}	0.09^{cD}	0.07^{dD}	0.04^{cE}	0.13^{cC}	0.09^{dD}	0.12^{cC}	0.12^{dC}	0.08^{bDE}
MII	0.09^{cG}	0.02^{bG}	0.06^{aG}	0.43^{aF}	0.07^{bG}	0.08^{aG}	0.69^{bE}	0.85^{aD}	2.39^{aB}	0.75^{bED}	4.56^{aA}	1.19^{aC}	1.53^{aC}	0.09^{bG}
MIII	0.13^{bFG}	0.14^{aFG}	0.08^{aH}	0.14^{bFG}	0.08^{bH}	0.02^{aI}	1.62^{aB}	0.25^{cF}	1.81^{bA}	1.48^{aC}	1.75^{bA}	1.16^{aD}	0.54^{bE}	0.10^{bAH}
MIV	0.19^{bD}	0.12^{aEF}	0.05^{aF}	0.10^{bEF}	0.07^{bEF}	0.05^{aF}	1.15^{aA}	0.43^{bC}	1.25^{bA}	0.59^{bB}	0.39^{cC}	0.71^{bB}	0.31^{cC}	0.19^{bD}

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey's test.

TABLE 4 - Enzyme and specific activity of *Metarhizium anisopliae* isolates in the four different media on day 6

Media	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
Enzyme activity (U/ml)														
MI	3.26^{bC}	5.43^{bB}	2.71^{aC}	5.97^{aB}	3.80^{aC}	2.44^{bC}	7.59^{bA}	5.55^{aB}	5.59^{cB}	7.87^{bA}	7.76^{bA}	5.44^{cB}	7.48^{bA}	4.89^{bcB}
MII	1.09^{cF}	1.09^{cF}	2.70^{aBE}	4.34^{aD}	3.80^{aD}	6.50^{aC}	10.26^{aA}	5.37^{aC}	8.47^{bB}	5.10^{cC}	7.00^{bB}	8.88^{aB}	10.29^{aA}	5.90^{bC}
MIII	4.89^{bC}	5.97^{bC}	3.80^{aD}	5.43^{aC}	3.26^{aD}	2.17^{bD}	14.86^{aA}	6.75^{aB}	10.97^{aA}	13.06^{aA}	12.22^{aA}	7.53^{bB}	7.89^{bB}	7.06^{aB}
MIV	10.31^{aA}	8.69^{aB}	3.80^{aD}	4.80^{aD}	2.70^{aD}	2.17^{bD}	8.31^{bB}	6.47^{aC}	12.19^{aA}	8.98^{bB}	8.34^{bB}	5.95^{cC}	7.45^{bC}	4.34^{cD}
Specific activity (U/mg)														
MI	0.22^{aA}	0.10^{aB}	0.05^{bCD}	0.11^{bB}	0.09^{bB}	0.04^{bD}	0.07^{cCD}	0.06^{cCD}	0.03^{cD}	0.13^{cB}	0.11^{dB}	0.06^{cCD}	0.12^{cB}	0.06^{cD}
MII	0.29^{aH}	0.29^{aH}	1.34^{aE}	1.17^{aF}	1.06^{aF}	0.90^{aD}	2.58^{aC}	1.38^{aE}	3.71^{aB}	1.32^{aE}	0.84^{bG}	1.93^{aD}	23.93^{aA}	0.29^{aH}
MIII	0.14^{aF}	0.13^{aF}	0.09^{bG}	0.09^{bG}	0.04^{bG}	0.03^{bG}	1.06^{bC}	0.54^{bE}	1.36^{bB}	1.03^{aC}	1.77^{aA}	0.84^{bD}	1.01^{bC}	0.15^{bF}
MIV	0.25^{aG}	0.11^{aH}	0.06^{bI}	0.11^{bH}	0.05^{bI}	0.04^{bI}	1.66^{bA}	0.59^{bE}	1.08^{bB}	0.45^{bF}	0.47^{cF}	0.95^{bC}	0.79^{bD}	0.06^{cI}

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey's test.

tinase production at first but in the later phases of growth there is little enhancement in chitinase production when the dextrose is fully or partially being used up. Sandhya *et al.* (2004) documented the same pattern of chitinase production with the use of different carbon and nitrogen source for the fungus *Trichoderma harzianum*. Maximum chitinase activity was observed in Medium III constituting colloidal chitin as carbon source and Yeast extract as a nitrogen source, compared to the other three media. The exact mechanism of induction of this enzyme by extra nitrogen source is not known but it is assumed that either Yeast extract supports the mycelial growth and hence enhancement in the initial growth leads to more chitinase production or it could be due to the presence of oligomers of GlcNAc which can directly induce the chitinase gene (De Moraes *et al.,* 2003). Nawani

TABLE 5 - Enzyme and specific activity of *Metarhizium anisopliae* isolates in the four different media on day 8

Media	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
							Enzyme activity (U/ml)							
MI	3.26^{bF}	5.43^{bC}	2.71^{bH}	4.89^{bD}	2.40^{bH}	1.63^{bH}	6.34^{cB}	2.99^{bG}	2.22^{dH}	8.02^{cA}	4.47^{bE}	5.03^{cCD}	4.44^{bE}	4.89^{bD}
MII	0.54^{cH}	4.34^{bE}	1.09^{cG}	1.92^{cF}	4.34^{aE}	1.63^{bG}	9.99^{bB}	5.99^{aD}	9.34^{bB}	14.12^{aA}	4.45^{bE}	7.93^{aC}	9.18^{aB}	2.18^{cF}
MIII	4.30^{bF}	5.97^{bE}	4.89^{aF}	4.89^{bF}	3.26^{aG}	2.71^{aG}	7.60^{cD}	2.79^{bG}	7.06^{cD}	10.31^{bA}	8.14^{aC}	6.52^{bD}	9.23^{aB}	8.80^{aC}
MIV	15.74^{aA}	8.69^{aE}	4.80^{aH}	6.51^{aF}	2.17^{bI}	2.40^{aI}	11.08^{aB}	5.45^{aG}	11.67^{aB}	11.08^{bB}	9.18^{aD}	7.87^{aB}	10.42^{aC}	8.14^{aE}
							Specific activity (U/mg)							
MI	0.21^{aA}	0.07^{bB}	0.03^{aC}	0.05^{aBC}	0.03^{aC}	0.01^{aC}	0.08^{dB}	0.04^{cC}	0.02^{dC}	0.20^{dA}	0.04^{dC}	0.05^{cBC}	0.07^{cB}	0.03^{bC}
MII	0.01^{bD}	0.09^{bD}	0.03^{aD}	0.07^{aD}	0.09^{aD}	0.04^{aD}	1.11^{bBC}	1.68^{aAB}	1.06^{aBC}	1.73^{aAB}	2.66^{aA}	1.02^{aC}	0.57^{bCD}	0.01^{bD}
MIII	0.09^{bFG}	0.15^{aF}	0.08^{aG}	0.05^{aG}	0.03^{aG}	0.02^{aG}	0.64^{cB}	0.22^{bE}	0.33^{cD}	0.72^{bA}	0.32^{cD}	0.38^{bD}	0.46^{bC}	0.14^{aF}
MIV	0.24^{aE}	0.15^{aF}	0.08^{aG}	0.09^{aG}	0.04^{aH}	0.03^{aH}	2.28^{aA}	0.38^{bD}	0.69^{bC}	0.46^{cD}	0.65^{bC}	1.14^{aB}	1.09^{aB}	0.08^{bG}

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey's test.

TABLE 6 - Enzyme and specific activity of *Metarhizium anisopliae* isolates in the four different media on day 10

Media	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
							Enzyme activity (U/ml)							
MI	2.40^{cE}	4.89^{bB}	2.71^{bDE}	4.89^{aB}	2.40^{aE}	1.63^{bF}	4.44^{cC}	2.12^{aE}	1.02^{dG}	6.54^{bA}	3.32^{cD}	4.28^{bC}	3.26^{cD}	4.89^{bB}
MII	0.54^{dH}	1.09^{cG}	0.54^{cH}	1.09^{bG}	1.63^{aF}	0.54^{cH}	3.26^{cE}	4.34^{aD}	3.20^{cE}	5.27^{cC}	3.09^{cE}	7.18^{aB}	9.77^{aA}	1.63^{cF}
MIII	4.30^{bE}	5.43^{bD}	6.51^{aC}	4.34^{aE}	2.17^{aG}	6.51^{aC}	7.52^{bB}	2.66^{aG}	6.40^{bC}	7.42^{bB}	5.92^{bD}	3.03^{bF}	3.83^{cF}	8.69^{aA}
MIV	12.49^{aA}	8.69^{aE}	5.97^{aG}	4.34^{aH}	1.63^{aJ}	1.09^{bJ}	10.42^{aC}	3.34^{aI}	11.29^{aB}	9.93^{aD}	9.28^{aD}	7.71^{aF}	6.62^{bG}	7.60^{aF}
							Specific activity (U/mg)							
MI	0.16^{aB}	0.20^{aB}	0.09^{bC}	4.70^{aA}	0.09^{aC}	0.04^{aE}	0.07^{cC}	0.04^{cE}	0.01^{cE}	0.19^{cB}	0.04^{cE}	0.08^{cC}	0.07^{cC}	0.09^{bC}
MII	0.06^{bF}	0.02^{bF}	0.02^{bF}	0.02^{bF}	0.03^{aF}	0.01^{aF}	0.77^{bC}	1.95^{aA}	0.41^{bE}	0.35^{bE}	1.33^{aB}	0.73^{aC}	0.59^{aD}	0.06^{bF}
MIII	0.09^{bDE}	0.15^{aD}	0.14^{aD}	0.04^{bE}	0.02^{aE}	0.08^{aDE}	0.77^{bA}	0.26^{bC}	0.44^{bB}	0.65^{aA}	0.28^{bC}	0.49^{bB}	0.25^{bC}	0.19^{aC}
MIV	0.18^{aE}	0.12^{aF}	0.08^{bF}	0.05^{bF}	0.02^{aF}	0.01^{aF}	1.62^{aA}	0.24^{bE}	0.61^{aB}	0.40^{bD}	0.46^{bD}	0.57^{bBC}	0.49^{aC}	0.06^{bF}

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey's test.

and Kapadnis (2004) reported almost the same effect of organic and inorganic nitrogen sources on the chitinase production from *Streptomyces* sp. Yeast extract alone or in combination with other nitrogen sources could significantly improve the enzyme secretion (Lopes *et al.,* 2008). Medium IV with two carbon source and one nitrogen source also showed high chitinase activity in most of the isolates with isolate UM1 showing 15.74 U/ml on 8th day of culture being high compared to others. St Leger *et al.* (1986) reported the repression of chitinolytic enzymes by the addition of extra carbon and nitrogen sources and catabolite repression by GlcNAc as well, although a low level of chitinase activity could be found in the medium containing restricted amount of non inducing sugars. Among the isolates screened out for studying the exochitinase activity, isolate UM13 showed high enzyme and specific activity. In a study, chitinase from *Verticillium alboatrum* released N-acetyl glucosamine as a major end product along with di and tri saccharides (Pegg and Young, 1982). The effective concentration of GlcNAc for induction of exochitinase activity for biocontrol agent *Trichoderma harzianum* was found to be in the range of 0.001-0.002 mM (Omero *et al.,* 2001). Kang *et al.* (1999) purified a 60 kDa chitinase enzyme from *M. anisopliae* comprising both endo and exo chitinase activities. In this study, three separate chitinase isozymes were observed on gel for the seven *M. anisopliae* isolates. An additional 80 kDa band was observed for isolate UM6 differing from a 48 kDa band from *M. anisopliae* as described by St. Leger *et al.* (1993).

Repression of enzymes by glucose has been extensively studied for many bacteria and fungi (Deutscher 2008; Tamayo *et al.,* 2008). In case of fungi, glucose repressed genes are divided into three categories. First, those genes which are involved in the glycolysis and gluconeogenesis. Secondly genes of those enzymes that are involved in the Krebs cycle and the third group comprises those genes that encode enzymes for the uptake and metabolization of

TABLE 7 - Exochitinase activity of *Metarhizium anisopliae* isolates.

Isolates	Enzyme activity (U/ml)	Specific activity (U/mg)
UM4	4.55^{c}	0.29^{bc}
UM6	4.00^{c}	0.24^{cd}
UM7	5.81^{b}	0.36^{b}
UM10	3.81^{c}	0.34^{b}
UM11	5.84^{b}	0.17^{d}
UM12	5.95^{b}	0.26^{c}
UM13	7.19^{a}	0.98^{a}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test.

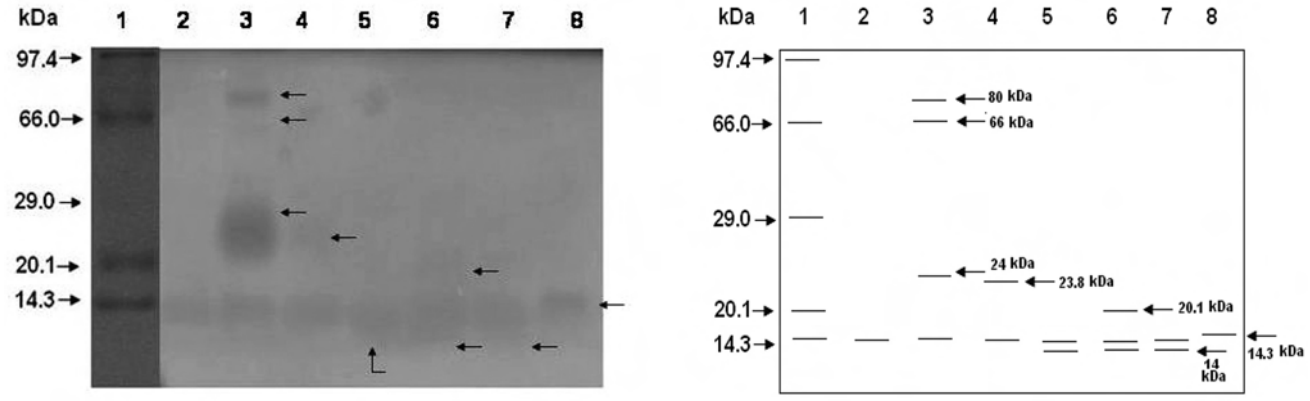

FIG. 1 - A: Chitinase activity of *Metarhizium anisopliae* isolates in polyacrylamide gel containing 0.01% (w/v) glycol chitin. Lane 1 corresponds to Molecular Weight marker after staining of the gel. Lanes 2-8 corresponds to isolates UM4, UM6, UM7, UM10, UM11, UM12 and UM13. B: Schematic representation of Fig 1A with the corresponding MW for each isozyme.

carbon sources other than glucose (Ronne, 1995). Different regulatory signals were identified in mycoparasitic fungus *Trichoderma atroviride* which include physiological stress along with chitinase induction by chito-oligomers. Further experiments carried out at the molecular level can lead to the identification of regulatory elements of chitinolytic enzymes from *M. anisopliae.*

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