African Journal of Biotechnology Vol. 9(47), pp. 8092-8099, 22 November, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1161
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Beauveria bassiana* isolates

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Accepted 15 December, 2009

Beauveria bassiana **a natural soil borne insect pathogen is being used effectively these days in integrated pest management system. Foliar application of these fungi is quite satisfactory as it invades its host by adhering to insect cuticles through the formation of penetration structures called appresoria, which produces various extracellular enzymes, including chitinase that causes the insect cuticle breaching. Although many investigations have been done in this regard, only a little is known about the induction and repression mechanism of this hydrolytic enzyme. This report illustrates the effect of two carbon sources; colloidal chitin and dextrose and a nitrogen source, yeast extract on the chitinase production of seventeen *B. bassiana* isolates. The chitinase activity varied among the isolates and the different media studied. A high enzymatic activity was observed in the medium with colloidal chitin as a sole source of carbon followed by the medium containing an extra nitrogen source, yeast extract. Exochitinase activity and the chitinase activity gel were also determined for the isolates showing high chitinase enzyme activity. An array of chitinase isozymes were observed on chitinase activity gel with a common 70 kDa enzyme for all the isolates.**

Keywords: *Beauveria bassiana*, induction, colloidal chitin, chitinase, exochitinase.

INTRODUCTION

Chitin, the insoluble polymer of N-acetyl glucosamine (GlcNAc), is the second most abundant polymer in nature, generally found in insect cuticle. It is quite difficult to chemically modify chitin (Muzzarelli, 1999) but it has been found that several microorganisms and also plants and invertebrates have developed enzymatic systems that could metabolize this insoluble polymer. Microorganisms utilize chitin as a source of carbon. Plants employ this enzymatic system in defensive purposes and in the case of invertebrates, it is used during ecdysis period. Microorganisms, specifically several insect pathogenic hyphomycete fungi produce hydrolytic enzymes that penetrate insect's cuticle. The fungal conidia adhere to the cuticle surface through non specific hydrophobic interaction and produce penetration structure called appresoria which produces many cuticle hydrolytic enzymes Like chitinases, proteases, esterases and lipases which together help to breach the insect cuticle and initiate the infection process (Inglis et al., 2001). This property of insect pathogenesis of these natural soil borne fungi is currently being exploited as a better alternative to the chemical control of insect pests.

Hyphomycete *Beauveria bassiana* is one of the most studied entomopathogenic fungi with a wide host range. Cuticle degrading enzymes from entomopathogenic fungi can be attributed to the comparison of isolates differing in pathogenicity. The degree of pathogenicity is related to the production of the chitinolytic enzymes in entomopathogenic fungi *Nomuraea rileyi* (EL-Sayed et al., 1989). *B. bassiana* also has a well developed chitinolytic system which can be induced by a number of chitooligosaccharides (Smith and Grula, 1983). Although the *B. bassiana* chitinolytic system has been extensively studied, very little is known about the induction and repression of this enzyme. The effect of various carbon sources on the chitinolytic activity was described by Campos et al. (2005). The medium containing insect

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Table 1. Source of *B. bassiana* isolates.

Isolates	Code No./ Accession No.ARSEF	Host insect	Geographic location
UB1	1788	*Helicoverpa virescens*	Spain
UB2	2041	*Cnaphalocrocis medinalis*	Philippines
UB3	5278	*Bemicia tabaci*	U.S.A
UB4	2417	*Hyblaea puer*	India
UB5	2597	*Spodoptera litura*	India
UB6	6646	*Coccinella septumpunctata*	India
UB7	4027	*Coccinella septempunetata*	Denmark
UB8	1166	*Helicoverpa armigera*	Spain
UB9	2033	*Coccinella* sp.	U.S.A
UB10	2034	*Coccinella* sp.	U.S.A
UB11	4018	*Coccinella septempunetata*	Denmark
UB12	1886	*Chilo infuscatellus*	India
UB13	2412	*Xyloryctes jamaicensis*	India
UB14	8250	*Basilepta fulvicornis*	India
UB15	6650	*Spodoptera litura*	India
UB16	2660	*Adult Coleoptera*	India
AB1	Local	Unknown	India

cuticle and crystalline chitin produced highest chitinase activity in contrast to media containing glucose and high levels of GlcNAc which repressed the enzyme activity (Campos et al., 2005). Nitrogen source also has profound effect on chitinase activity. Peptone and yeast extract as a nitrogen source has proved to be the most affirmative regulator of chitinase enzyme (Vaidya et al., 2001). Fungal growth on chitin induces both endo and exo-chitinase activity. Among different exochitinases, N-acetyl glucosaminidase is the prominent one, releasing acetyl-glucosamine from non reducing ends of chitin chains. Kang et al. (1999) purified a novel chitinase from *Metarhizium anisopliae* which showed high chitinolytic activity against colloidal chitin as well as synthetic substrates of exochitinase enzymes indicating both endo and exo chitinase activity (Kang et al., 1999). A 110 kDa N-acetylglucosaminidase activity was observed for *M. anisopliae* (Charnley and St. Leger, 1991). In order to evaluate the induction and repression mechanism of chitinase activity, seventeen *B. bassiana* isolates were grown on four different media containing various carbon and nitrogen sources in the present study. 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 effect of cultivation time on chitinase activity. N-acetylglucosaminidase activity was also determined for the selected isolates which demonstrated high endochitinase activity.

MATERIALS AND METHODS

Fungal Isolates

A total of sixteen 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 SDA (Sabouraud Dextrose Agar) 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 continuous stirring for 2 h at 4°C. The suspension was repeatedly mixed with 1 litre 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 5 N NaOH and the colloidal suspension was washed several times with distilled H_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 ($1x10^6$conidia/ml) to inoculate SDY broth (dextrose (4%), peptone (1%) and yeast extract (1%)) and incubated at 28°C and 180 rpm for three days. The harvested mycelium was washed twice with sterilized distilled water and inoculated into 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 (basal salts) constituted KH_2PO_4 (1 gm/l), $MgSO_4.7H_2O$ (0.5 gm/l), $FeSO_4.7H_2O$ (0.2 mg/l), $ZnSO_4.7H_2O$ (1 mg/l), $NaMoO_4.2H_2O$ (0.02 mg/l), $CuSO_4.5H_2O$ (0.02 mg/l), $MnCl_2.4H_2O$ (0.02 mg/l) (Cooper and Wood, 1975). Medium II was supplemented with dextrose (2%) and Medium III with yeast extract (1%), respectively. The components in Medium IV contained both dextrose (2%) and yeast extract (1%). The pH of the culture media was adjusted to 5.6 and the cultures were incubated at 28°C and 180 rpm for 10 days. The cultures were centrifuged and the culture filtrate was taken in order to study the enzyme assays on alternate

Table 2. Enzyme and specific activity of *B. bassiana* isolates in the four different media on day 2. 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.

Media	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
Enzyme activity (U/ml)																	
MI	33.55^{aE}	47.83^{Aa}	9.72^{Ah}	4.09^{I}	20.9^{aG}	7.55^{bI}	4.35^{cL}	6.52^{cJ}	26.76^{aF}	5.43^{cK}	6.52^{cJ}	38.49^{aD}	45.11^{aB}	5.97^{cK}	7.60^{bI}	7.05^{aI}	39.19^{aC}
MII	1.30^{cGH}	1.62^{dF}	3.22^{cB}	1.20^{cGH}	1.23^{dGH}	1.29^{dGH}	2.39^{dD}	1.79^{dF}	3.34^{cB}	0.65^{dH}	2.99^{dC}	2.48^{dD}	2.94^{cC}	2.12^{dE}	4.89^{cA}	3.96^{bB}	1.13^{dGH}
MIII	6.89^{bG}	6.14^{cIJ}	6.57^{bH}	2.30^{bL}	2.93^{cL}	5.92^{cJ}	6.24^{bI}	10.04^{aE}	5.70^{cJ}	15.74^{aD}	19.27^{aA}	5.86^{cJ}	10.91^{bE}	17.92^{aC}	18.46^{aB}	8.14^{aF}	5.38^{cK}
MIV	7.44^{bH}	12.65^{bB}	7.28^{bH}	3.53^{aI}	8.52^{bF}	13.03^{aA}	10.26^{aD}	9.07^{bE}	10.26^{bD}	7.33^{bH}	8.09^{bG}	13.40^{bA}	9.23^{bE}	8.47^{bE}	7.55^{bFG}	9.28^{aE}	11.20^{bC}
Specific activity (U/mg)																	
MI	0.52^{aIH}	0.29^{bJ}	0.51^{bIH}	0.22^{bJ}	0.36^{bIJ}	0.12^{bK}	0.6^{bH}	1.62^{bF}	2.91^{aD}	3.14^{aC}	0.89^{bG}	2.02^{aE}	2.19^{aE}	1.5^{bF}	4.39^{aA}	4.08^{aB}	0.15^{Bk}
MII	0.52^{aG}	0.59^{aF}	1.66^{aA}	1.21^{aB}	0.69^{aE}	1.04^{aC}	1.24^{aD}	0.59^{cF}	0.51^{bG}	0.09^{cJ}	1.06^{aC}	0.78^{bD}	0.44^{bH}	0.48^{cH}	1.66^{bA}	0.29^{dI}	0.78^{aD}
MIII	0.06^{bH}	0.08^{cGH}	0.35^{bF}	0.14^{bG}	0.20^{bG}	0.06^{bH}	1.66^{aC}	2.18^{aB}	0.17^{cG}	1.43^{b}	1.31^{aD}	0.06^{cH}	0.31^{bF}	2.7^{aA}	1.56^{bD}	0.76^{cE}	0.06^{Bh}
MIV	0.08^{bE}	0.22^{bB}	0.1^{cDE}	0.09^{cE}	0.22^{bB}	0.10^{bDE}	1.88^{aA}	1.89^{bA}	0.13^{cCD}	1.88^{bA}	1.89^{aA}	0.08^{cE}	0.16^{bC}	1.89^{bA}	1.88^{bA}	1.88^{bA}	0.16^{bC}

days of growth till the tenth day of culture.

Protein and enzyme assays

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

Chitinase assay

Chitinase assay was determined by the method of Yanai et al. (1992). 500 µl of culture supernatant 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 10^6conidia/ml and incubated for 72 h at 28°C and 180 rpm. N-acetylglucosaminidase activity was determined by the method of Courdon 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 paranitro phenol was determined. One unit of exo-chitinase 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) containing1.5% (v/v) TritonX-100 for 20 h at 30°C 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 distilled H_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

Chitinase assay

Isolates behaved differently for the four different media and different pattern of enzyme profile was observed for the seventeen *B. bassiana* isolates. Enzyme activity was investigated for a period of ten days and highest chitinase activity was observed between four to six days of culture. The specific activity profile of the isolates differed indiscriminately from the enzymatic activity. Activity of chitinase enzyme decreased with the aging culture, probably due to nutrient limitations.

Medium to high chitinase activity was observed for medium I, III and IV on the second day of culture (Table 2). Highest chitinase activity of

Table 3. Enzyme and specific activity of *B. bassiana* isolates in the four different media on day 4. 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.

Media	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
Enzyme activity (U/ml)																	
MI	33.72^{aB}	52.99^{aA}	28.52^{aD}	6.84^{aL}	33.03^{aB}	29.88^{aD}	7.6^{bL}	8.15^{bK}	31.32^{aC}	14.38^{bJ}	16.02^{aI}	27.76^{aE}	20.75^{bG}	16.55^{aI}	19^{aH}	22.27^{aF}	16.03^{aI}
MII	1.73^{cGH}	2.34^{dFG}	2.89^{dF}	1.45^{cH}	2.01^{dGH}	1.67^{dGH}	9.99^{aA}	7.17^{cB}	4.46^{cD}	4.94^{dCD}	7.55^{cB}	5.49^{bC}	3.46^{dEF}	4.89^{cCD}	3.64^{dE}	5.7^{dC}	2.64^{cF}
MIII	16.95^{bD}	10.98^{cF}	10.09^{bF}	2.43^{bK}	5.08^{cJ}	4.77^{cJ}	10.65^{aF}	9.53^{bG}	9.64^{bG}	17.29^{aC}	15.88^{aE}	6.69^{bI}	36.98^{aA}	18.74^{aB}	15.66^{bE}	9.07^{cH}	5.05^{bJ}
MIV	16.29^{bD}	17.27^{bC}	7.41^{cJ}	5.59^{aK}	8.60^{bI}	18.14^{bA}	9.65^{aG}	10.91^{aF}	9.14^{bH}	7.65^{cJ}	9.77^{bG}	17.87^{aB}	17.6^{cB}	9.01^{bH}	8.58^{cI}	11.51^{bE}	16.4^{aD}
Specific activity (U/mg)																	
MI	0.39^{aG}	0.08^{cH}	0.09^{cH}	0.05^{bH}	0.38^{aG}	0.09^{bH}	0.82^{cE}	0.58^{cF}	1.29^{aD}	1.85^{aB}	1.29^{aD}	3.33^{aA}	1.76^{aB}	1.85^{bB}	1.61^{aC}	1.76^{aB}	0.03^{bH}
MII	0.34^{aDE}	0.62^{aC}	0.23^{aFG}	0.16^{aG}	0.09^{bG}	0.25^{aF}	4.19^{aA}	0.54^{cC}	0.39^{bD}	0.48^{bC}	0.27^{dF}	0.3^{bF}	0.34^{bDE}	0.74^{cBC}	0.79^{bB}	0.39^{cD}	0.15^{aG}
MIII	0.12^{bGH}	0.07^{cG}	0.13^{bGH}	0.08^{bH}	0.07^{bH}	0.08^{bH}	2.46^{bB}	1.65^{aD}	0.17^{cG}	1.88^{aC}	0.72^{bF}	0.05^{bH}	0.21^{bG}	2.7^{aA}	1.02^{abE}	0.69^{bF}	0.05^{bH}
MIV	0.11^{bEF}	0.29^{bD}	0.03^{dF}	0.09^{bF}	0.03^{bF}	0.08^{bF}	0.50^{dC}	0.78^{bA}	0.06^{dF}	0.61^{bB}	0.58^{cC}	0.10^{bEF}	0.17^{bE}	0.51^{cC}	0.81^{bA}	0.75^{bA}	0.24^{aD}

Table 4. Enzyme and specific activity of *B. bassiana* isolates in the four different media on day 6. 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.

Media	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
Enzyme activity (U/ml)																	
MI	13.89^{bE}	29.05^{aB}	18.19^{aD}	16.93^{aD}	23.07^{aCD}	26.71^{aC}	6.66^{bF}	7.9^{aF}	35.08^{aA}	12.23^{aE}	14.62^{aE}	19.49^{aD}	16.72^{bD}	14.89^{aDE}	17.57^{aD}	16.49^{aD}	13.41^{aE}
MII	4.05^{cF}	2.77^{dH}	1.57^{dI}	1.34^{dI}	3.33^{cG}	2.81^{cG}	8.74^{aB}	8.58^{aB}	7.09^{dE}	6.95^{cE}	7.93^{cC}	7.84^{cC}	3.67^{dG}	7.44^{bD}	7.6^{cD}	9.77^{cA}	2.93^{bH}
MIII	34.96^{aB}	6.95^{cI}	15.65^{bC}	3.03^{cL}	4.39^{cJ}	3.94^{cK}	7.97^{bH}	9.17^{aJ}	13.74^{bE}	10.38^{bF}	10.32^{bF}	10.69^{bF}	89.96^{aA}	12.23^{aE}	14.05^{bD}	12.34^{bE}	4.34^{bJ}
MIV	12.49^{bC}	11.83^{bD}	8.79^{cG}	6.77^{bI}	17.48^{bA}	10.59^{bE}	8.63^{aG}	8.52^{aG}	9.01^{cF}	6.51^{cI}	5.21^{dJ}	11.99^{bD}	11.83^{cD}	4.29^{cK}	4.72^{dK}	7.49^{dH}	13.84^{aB}
Specific activity (U/mg)																	
MI	0.56^{aH}	0.13^{aJ}	0.34^{aI}	0.06^{aJ}	0.45^{aH}	0.21^{aIJ}	3.17^{aC}	0.83^{aG}	1.98^{aD}	1.54^{aE}	1.56^{aE}	5.78^{aA}	1.3^{aF}	5.75^{aA}	1.85^{aD}	5.2^{aB}	0.08^{aJ}
MII	0.19^{bG}	0.15^{aGH}	0.08^{bI}	0.09^{aI}	0.11^{bI}	0.29^{aF}	1.26^{bB}	0.29^{bF}	1.37^{aB}	0.69^{bD}	0.37^{bDE}	0.32^{bDEF}	0.36^{bDE}	1.17^{bC}	1.89^{aA}	0.67^{bD}	0.13^{aHI}
MIII	0.17^{bH}	0.04^{bI}	0.07^{bI}	0.07^{aI}	0.03^{cI}	0.09^{bI}	1.84^{bA}	1.49^{aB}	0.08^{bI}	1.05^{aC}	0.73^{bF}	0.07^{cI}	0.45^{bG}	1.58^{bB}	0.86^{bE}	0.97^{bD}	0.04^{aI}
MIV	0.11^{bED}	0.17^{aD}	0.09^{bE}	0.08^{aE}	0.06^{cE}	0.05^{bE}	0.45^{cA}	0.39^{bB}	0.08^{bE}	0.53^{bA}	0.32^{bBC}	0.05^{cE}	0.05^{cCE}	0.25^{cC}	0.24^{cC}	0.42^{bA}	0.14^{aD}

47.83 U/ml was observed in medium I for isolate UB2 whereas highest chitinase activity of 19.27 U/ml for medium III and 13.40 U/ml for medium IV for isolate UB11 and UB12, respectively. Specific activity was observed to be highest in medium I and medium IV.Highest specific activity of 4.39 U/mg was observed in medium I for isolate UB15.

A very high chitinase activity was observed in medium I on the fourth day of culture. Among the four media investigated, highest chitinase activity was observed in medium I for isolate UB2 (52.99 U/ml) followed by medium III (36.98 U/ml) for isolate UB13 (Table 3). Chitinase activity in medium II was reasonably low for all the isolates. Highest specific activity of 4.19 U/mg was observed for medium II in case of isolate UB7 followed by 3.33 U/ml for medium I with the isolate UB12.

An exceptionally high chitinase activity of 89.96 U/ml was observed in medium III for isolate UB13 on the sixth day of culture (Table 4). Moderate to high enzyme activity was in both medium I and IV although chitinase activity was typically low in medium II. A very high specific activity was observed in medium I of 5.78, 5.75, 5.2 U/mg for

Table 5. Enzyme and specific activity of *B. bassiana* isolates in the four different media on day 8. 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.

Media	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
Enzyme activity (U/ml)																	
MI	5.21^{bJ}	7.82^{bE}	4.07^{bK}	5.83^{aI}	5.54^{bI}	5.59^{bI}	5.43^{bI}	7.87^{aE}	7.82^{bE}	11.08^{aC}	10.59^{aC}	7.11^{aF}	8.14^{aD}	14.66^{aA}	12.49^{aB}	12.76^{aB}	5.16^{bJ}
MII	6.43^{aD}	7.56^{bC}	1.44^{cIJ}	1.32^{cJ}	4.86^{bF}	6.35^{bD}	4.51^{bF}	1.25^{bK}	3.36^{cG}	2.49^{cH}	1.25^{cK}	8.04^{aB}	6.09^{bE}	1.54^{cI}	1.54^{bI}	13.19^{aA}	4.74^{bF}
MIII	5.59^{bG}	5.38^{cG}	35.72^{aA}	4.7^{abI}	4.04^{bI}	3.32^{cJ}	7.6^{aF}	8.14^{aE}	21.12^{aB}	7.06^{bF}	8.69^{bE}	3.64^{cJ}	5.1^{bH}	10.32^{bD}	12.86^{aC}	13.03^{aC}	2.82^{cK}
MIV	7.05^{aE}	9.59^{aB}	5.01^{bG}	4.49^{bH}	12.26^{aA}	9.68^{aB}	8.25^{aD}	7.11^{aE}	8.32^{bD}	3.93^{cI}	2.71^{cJ}	5.27^{bG}	8.91^{aC}	2.28^{cJ}	1.85^{bK}	6.24^{bF}	9.30^{aB}
Specific activity (U/mg)																	
MI	0.07^{bH}	0.12^{bH}	0.82^{aD}	0.08^{bH}	1.09^{aC}	0.37^{aG}	0.64^{bF}	0.49^{bG}	1.57^{aB}	1.01^{aCD}	0.63^{aF}	2.45^{aA}	0.84^{aD}	1.03^{aC}	0.69^{aDEF}	0.7^{bDEF}	0.09^{bH}
MII	0.28^{aEH}	0.37^{aCD}	0.08^{cG}	0.24^{aE}	0.27^{bEH}	0.18^{bF}	0.41^{bC}	0.06^{cG}	0.59^{bB}	0.18^{dF}	0.05^{cG}	0.34^{bDH}	0.44^{bC}	0.12^{bF}	0.15^{bF}	0.91^{aA}	0.16^{aF}
MIII	0.03^{bG}	0.04^{cG}	0.47^{bE}	0.07^{bG}	0.03^{cG}	0.23^{abF}	1.72^{aA}	1.12^{aB}	0.25^{bF}	0.77^{bC}	0.65^{aD}	0.03^{cG}	0.02^{cG}	1.71^{aA}	0.66^{aD}	1.02^{aB}	0.06^{bG}
MIV	0.08^{bE}	0.12^{bCD}	0.06^{cE}	0.08^{bE}	0.05^{cE}	0.05^{cE}	0.34^{bA}	0.31^{bAB}	0.08^{cE}	0.32^{cAB}	0.16^{bC}	0.03^{cE}	0.05^{cE}	0.1^{cDE}	0.07^{cE}	0.27^{cB}	0.08^{bE}

Table 6. Enzyme and specific activity of *B. bassiana* isolates in the four different media on day 10. 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.

Media	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
Enzyme activity (U/ml)																	
MI	3.53^{bG}	2.71^{bI}	4.89^{bF}	2.47^{bI}	2.28^{cJ}	3.64^{bG}	1.63^{bJ}	5.7^{aE}	4.99^{bF}	8.69^{aC}	10.32^{aB}	6.03^{bDE}	2.99^{dH}	11.94^{aA}	4.61^{bF}	2.99^{cH}	6.24^{Ad}
MII	5.55^{bE}	2.23^{bH}	1.39^{cI}	1.11^{cJK}	6.05^{bD}	6.58^{aC}	4.34^{aF}	1.10^{cJK}	2.45^{cG}	2.42^{bG}	1.02^{cK}	8.84^{aB}	6.59^{aC}	1.32^{cI}	1.22^{cJ}	21.12^{aA}	4.88^{bF}
MIII	3.80^{bH}	3.58^{bI}	11.02^{aB}	4.2^{aJ}	1.89^{cK}	2.10^{cJ}	4.89^{aF}	3.80^{bH}	6.14^{bDE}	8.69^{aC}	5.97^{bE}	2.23^{dJ}	3.96^{cH}	6.52^{bD}	11.94^{aA}	8.14^{bC}	1.09^{cL}
MIV	6.84^{aB}	6.83^{aB}	3.38^{bFG}	3.33^{bFG}	8.58^{aA}	5.62^{aC}	4.29^{aE}	4.34^{bE}	8.47^{aA}	3.42^{bF}	2.33^{cH}	4.64^{cD}	5.09^{bC}	1.09^{cI}	1.09^{cI}	3.37^{cFG}	6.56^{aB}
Specific activity (U/mg)																	
MI	0.16^{bG}	0.13^{bG}	0.3^{bF}	0.5^{bF}	0.96^{aD}	0.12^{aG}	0.15^{bG}	0.37^{aF}	1.49^{aB}	0.59^{bE}	0.75^{aDE}	2.14^{aA}	0.31^{aF}	1.22^{aC}	0.39^{bF}	0.31^{bF}	0.22^{aG}
MII	0.56^{aB}	0.36^{aCD}	0.1^{aG}	0.75^{aA}	0.46^{bC}	0.18^{aF}	0.16^{bF}	0.07^{cG}	0.46^{bC}	0.29^{cD}	0.06^{cG}	0.66^{bAB}	0.10^{bG}	0.04^{bG}	0.03^{cG}	0.19^{cEF}	0.23^{aE}
MIII	0.04^{cG}	0.05^{cG}	0.18^{aF}	0.08^{cG}	0.03^{cG}	0.04^{bG}	0.94^{aB}	0.28^{aE}	0.09^{dFG}	0.97^{aB}	1.18^{aA}	0.02^{cG}	0.02^{bG}	1.26^{aA}	0.55^{aD}	0.69^{aC}	0.09^{bFG}
MIV	0.09^{cC}	0.07^{cC}	0.05^{bC}	0.07^{cC}	0.04^{cC}	0.03^{bC}	0.09^{cC}	0.15^{bB}	0.15^{cB}	0.28^{cA}	0.14^{bB}	0.03^{cC}	0.04^{bC}	0.05^{bC}	0.03^{cC}	0.13^{cB}	0.09^{bC}

isolates UB12, UB14 and UB16. Specific activity was lowest n medium IV on sixth day. An interesting feature in the enzyme activity profile was observed on the eighth day of culture (Table 5). Chitinase activity decreased for media I, III and IV while it slightly increased for medium II. Highest enzyme activity was observed in medium III (21.12 U/ml) for isolate UB9. Highest specific activity was observed in medium I (2.45 U/mg) for isolate UB12 followed by 1.72 and 1.71 U/mg in medium III for isolate UB7 and UB14, respectively.

Highest chitinase activity was observed in medium II (21.12 U/ml) for isolate UB16 followed by medium I and medium III,11.94 U/ml for isolate UB14 and UB15, respectively, on the tenth day of culture (Table 6). Specific activity was low for the all four medium investigated on the tenth day with highest specific activity in medium I, 1.49 U/mg for isolate UB9.

Seven out of the seventeen isolates of *B. bassiana* were screened out based on the chitinase activity in medium I on the fourth day of incubation as it showed good enzyme production when compared to other three media for further

Table 7. Exochitinase activity by *B. bassiana* isolates.

Isolates	Enzyme activity (U/ml)	Specific activity (U/mg)
UB1	7.72[c]	0.77[b]
UB2	8.63[b]	0.88[a]
UB3	9.47[a]	0.37[d]
UB5	7.14[d]	0.49[c]
UB9	7.31[d]	0.74[b]
UB10	4.56[e]	0.24[e]
UB13	5.50[f]	0.26[e]

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

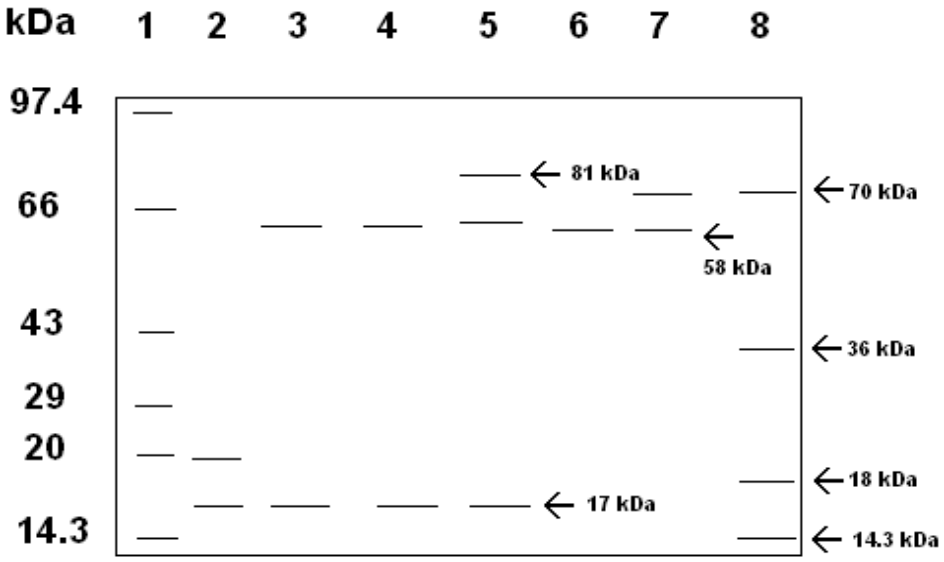

Figure 1b. Schematic representation of Figure 1a with the corresponding MW for each isozyme.

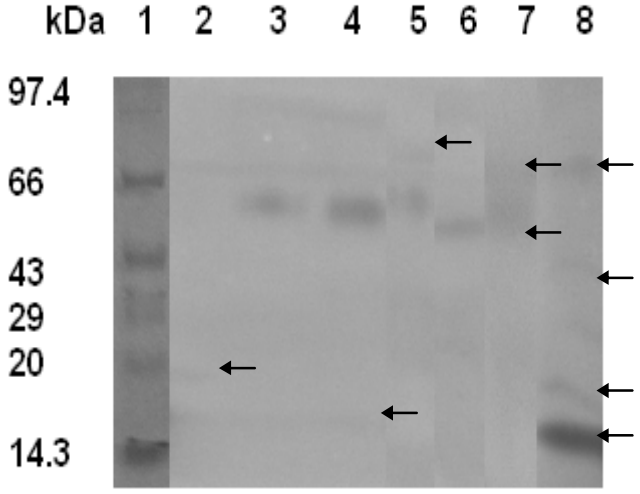

Figure1a. Chitinase activity of *B. bassiana* isolates in polyacrylamide gel containing 0.01% (w/v) glycol chitin. Lane 1 = Molecular Weight marker after staining of the gel; Lanes 2 - 8 = isolates UB1, UB2, UB3, UB5, UB9, UB10 and UB13.

studies. The protease activity profile of these selected isolates was also studied and found to be high compared to other isolates (data not shown).

Exochitinase activity

Exochitinase activity was studied with seven isolates showing high chitinase activity. Among the isolates studied, isolate UB3 showed highest exochitinase activity of 9.47 U/ml followed by isolate UB2 (8.63 U/ml) which also showed highest specific activity of 0.88 U/mg (Table 7). The rest of the isolates showed lower exochitinase enzyme and specific activity. Isolate UB1 showed a specific activity of 0.77 U/mg though its enzyme activity was lower at 7.72 U/ml when compared to isolates UB2 and UB3. Isolate UB10 showed the lowest enzyme

activity at 4.50 U/ml and specific activity was also low at 0.24 U/mg.

Chitinase activity gel

A 70 kDa chitinase isozyme was observed for almost all the isolates (Figure 1). A different zymogram was observed for isolate UB1 with bands of 17 and 20 kDa. Isolate UB2 and UB3 showed two chitinase bands of 17 and 58 kDa. An additional 81 kDa band was also observed for isolate UB5 along with the 70 kDa band. Isolate UB9 and UB10 showed a 58 kDa chitinase isozyme whereas a 70 kDa band was observed for isolate UB10. A 14.3 kDa chitinase was observed for the isolate UB13 along with an 18, 36 and 70 kDa chitinase isozyme.

DISCUSSION

The role of entomopathogenic fungi in the integrated pest management is inevitable due to its broad host range and host specificity. Each isolate of *B. bassiana* is specific towards different hosts like, Colorado potato beetle, European corn borer, white flies and grasshoppers and locusts (Inglis et al., 2001). Extracellular enzymes like chitinase help to breach the chitinatious insect cuticle and invade its host. The paradigm of infection mechanism is more complex based on physiological and morphological factors. Different factors including temperature, UV radiation and humidity influence the insect pathogenicity of this fungus (Inglis et al., 2001). Extracellular enzyme production is also subjective on factors such as the carbon and nitrogen sources of the media and also the pH (Vaidya et al., 2001; St Leger et al., 1999). Maximum amount of chitinase activity was observed in medium I constituting colloidal chitin as a sole carbon source compared to the other three media. De la Cruz et al. (1993) reported a rise in the chitinase production from *Trichoderrna harzianum* with the increase in the chitin concentration in the media and a quick decrease in the enzyme production with the addition of glucose (De la Cruz et al., 1993). Colloidal chitin seemed to contain a minute amount of GlcNAc that helps to induce the enzyme initially but it has being reported that GlcNAc causes catabolite repression when present in high concentration in the medium (Campos et al., 2005). Low amount of chitinase activity was observed for all the isolates especially the first few days of the culture in medium II containing colloidal chitin and dextrose (that is, two carbon sources). The presence of easily available carbon source, dextrose, suppress the chitinase production at first but in the later phase of growth there was little enhancement of chitinase production when the dextrose is fully or partially being used up.

Addition of an extra nitrogen source, yeast extract, in medium III showed a trivial change in the enzyme production with an exception of isolate UB12 which showed highest enzyme production of 10.69 U/ml on the 6^{th} day of culture. The exact mechanism of induction of this enzyme by extra nitrogen source is not known but it is assumed that it either supports the mycelium growth and hence enhancement in the initial growth leads to more chitinase production or it could be the presence of oligomers of GlcNAc which can directly induce the chitinase gene. Nawani and Kapadnis (2005) reported almost the same effect of organic and inorganic nitrogen source on the chitinase production from *Streptomyces* sp. Yeast extract alone or in combination with other nitrogen sources can significantly improve the enzyme secretion (Lopes et al., 2008). Medium IV with two carbon source and one nitrogen source did not have any significant effect on the chitinase secretion. Chitinase production was suppressed for the availability of the easily available carbon source dextrose. Sandhya et al. (2004) documented the same pattern of chitinase production with the use of different carbon and nitrogen source for the fungus *T. harzianum*. 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. The effective concentration of GlcNAc for induction of exochitinase activity for biocontrol agent *T. harzianum* was found to be in the range of 0.001-0.002 mM (Omero et al., 2001). Investigation of the chitinolytic activity in *N. rileyi* isolates showed almost 10-17 times more endochitinase activity and 15-18 times more exochitinase activity in highly virulent isolates (EL-Sayed et al., 1989). A trend of high exochitinase activity for isolates with high chitinolytic activity was observed in the present study. *B. bassiana* grown in a liquid medium containing N-acetyl-D-glucosamine and colloidal chitin produced two N-acetyl-D-glucosaminidases, NAGase 1 (97 kDa) and NAGase of 2 consisting two subunits of molecular weights 64 and 66 kDa (Bidochka et al., 1993). Inducible chitinolytic system is frequently observed in other filamentous fungi also. *Aspergillus fumigatus,* a ubiquitous saprophyte produces an extracellular chitinolytic enzyme which is regulated by a negative feedback mechanism (Escott et al., 1998).

Repression of enzymes by glucose has been extensively studied for many bacteria and fungi (Deutscher, 2008; Tamayo et al., 2008). In the case of fungi, glucose repressed genes are divided into three categories. First in the list are 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 comprise those genes that encode enzymes for the uptake and metabolism of carbon sources other than glucose (Ronne, 1995). Further experiments should be carried out at the molecular level to identify regulatory elements of chitinolytic enzymes

from *B. bassiana*

ACKNOWLEDGEMENT:

We acknowledge the financial support provided by Department Science and Technology (Project No. SR/FT/L-144) and Ministry of Human Resource and Development (Project No. F.26-11/2004 TS V). We also thank Dr. Richard Humber (USDA-ARS) for providing fungal cultures.

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