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# Production and regulation of cell wall degrading hydrolytic enzymes in mycoparasitic *Trichoderma* spp.

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Ten isolates of *Trichoderma* spp., collected from dfferent agro-ecological habitat of West Bengal, were evaluated for their hydrolytic enzymes activity and subsequently the best one was used for the further study. Highest chitinase and β-1,3 glucanase activities were detected in $T_{10}$ isolate while $T_2$ exhibited highest activity of cellulase (23.43 U) followed by $T_5$ (17.53 U) and $T_{10}$ (16.63 U), which were statistically significant. When the effects of time course and carbon sources on the activity of three hydrolytic enzymes were studied it was found that the activity of chitinase level increased with time and reached a maximum of 85 IU at 7th day in MSM supplemented with chitin. Enzyme activity was not detected both in media supplemented with cellulose and media with no sugar with few exceptions. Highest activity of β-1,3 glucanase was detected and reached peak of 20.7 U at 3rd day of incubation and then declined to 6.4 U at 8th day in MSM supplemented with laminarin. There was a considerable amount of β-1, 3 glucanase activities in media without any sugar and which reached the peak of 2.3 U at 4th day. Similarly, activity of cellulase increased with incubation period and reached it's peak at 5th day of incubation when cellulose was used in the medium as the sole carbon sources. The acidic range of pH (5-5.5) was the best for the production of these cell wall degrading enzymes. It was revealed that 1.0% substrate concentration for β-1, 3 glucanase and 0.75% for chitinase and cellulase induced the highest activity of respective enzymes.

**Key words** : Production, regulation, β-1,3 glucanase, chitinase, cellulase, *Trichoderma* spp.

## INTRODUCTION

A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cell-wall-degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium (Jijakli and Lepoivre, 1998; Giuliano *et al.,* 2001). Since the report of Weindling (1934) the *Trichoderma* spp. produce antifungal substances several investigators have suggested that this fungus may serve as a biocontrol agent against soil borne plant pathogenic fungi (Jash and Pan, 2004a, b; Jash *et al.,* 2005; Jash, 2006; Jash *et al.,* 2006). Physical contact followed by enzymatic degradation of host cell wall and disorganizaion of the cell components have been proved as the principal mechanisms of antagonism (Tu, 1980). The ability of *T. harzianum* to produce extracellular β-1,3-glucanases and chitinase into the medium when grown in presence of laminarin and chitin (Elad *et al.,* 1982) has led several authors to postulate that the released enzymes are actively involved in microbiological control (Benhamou *et at.,* 1990; Cherif and Benhamou, 1990; Lorito *et al.,* 1994, 1996; Harman *et al.,* 1993; Di Pietro *et al.,* 1993; El-Katatny *et al.,* 2000; 2004). However, conclusive evidence on the role played by these hydrolases could not be reached in the absence of data concerning precise *in situ* localization of sites of lytic activity. In an attempt to address this question, Elad *et al.* (1983) have studied the interaction between *T. harzianum* and some pathogenic fungi using SEM and fluorescence techniques. Although this study has provided strong support for the assumptions that *Trichoderma*-hydrolases are active determinats in the antagonistic process, it does not allow an accurate demonstration of a lytic activity at the molecular level.

Expression of these extracellular cell wall degrading enzymes frequently has been reported to be induced by fungal cell wall components and repressed by carbon catabolite repressors, such as glucose (Tronsmo and Harman, 1992; De La Cruz *et al.,* 1995; Carsolio *et al.,* 1994; Peterbauer *et al.,* 1996; Donzelli *et al.,* 2001; Giuliano *et al.,* 2001). in some cases, starvation conditions alone could trigger cell wall enzyme production (Ramot *et al.,* 2000), while in others, cell walls or cell wall components are needed (Elad *et al.,* 1982). Giuliano *et al.,* (2001) have indicated that the expression and secretion of cell wall-degrading enzymes is nitrogen repressed, that effects of carbon and nitrogen nutrition are interactive, and that especially for chitinolytic enzymes, the inductive effect of chitin is altered by the level of ammonium or glucose in the medium. This paper reports production and regulation of these cell wall degrading enzymes under different conditions to exploit the efficacy of antagonistic strain of *Trichodema* spp.

## MATERIALS AND METHODS

### *Fungal isolates and culture conditions*

Ten isolates of *Trichoderma* were obtained from the rhizosphere soil of different ecological habitats of West Bengal with diverse cropping history by dilution plate technique (Harris and Sommers, 1968) using *Trichoderma*-specific medium (TSM) (Elad and Chet, 1983) modified by Saha and Pan (1997). The isolates were identified into different species (Table 1) based on available literature and monograph of Rifai (1969) and Bissett (1984). All the identified species of *Trichoderma* were maintained on potato dextrose agar (PDA) slant at 4°C for further use.

The different strains of *Trichoderma* spp. were grown on a minimal synthetic medium (MES, El-Katatny *et al.,* 2000) supplemented with dried mycelium of *Macrophomina phaseolina* @ 5 g/l as sole carbon source. The medium was adjusted to pH 6.0 and sterilized by autoclaving at 121°C in Erlenmeyer flasks. The Erlenmeyer flask of 250 ml containing 50 ml medium was inoculated with 1 ml spore suspension at 2 × $10^7$/ml and placed on a rotary shaker at 150 rpm for 15 hrs. in each day at 25°C. After 5 days of incubation the culture medium containing the enzymes of interest was separated from biomass by centrifugation at 4°C for 10 min at 5000 rpm and clear supernatants were either immediately tested for enzyme activity or stored at 20°C until assayed.

**Table 1**: Isolates of *Trichoderma* investigated and their sources

| Code | Taxa | Crop associated | Location |
|---|---|---|---|
| $T_1$ | *T. viride* | Potato field | Falakata, Jalpaiguri |
| $T_2$ | *T. harzianum* | Chilli field | Kakdwip, South 24 Pgs |
| $T_3$ | *T. viride* | Mustard field | Bishnupur, Bankura |
| $T_4$ | *T. harzianum* | Brinjal field | Kalyani, Nadia |
| $T_5$ | *T. harzianum* | Sunflower field | Namkhana, South 24 Pgs |
| $T_6$ | *T. virens* | Gladiolus field | Kalimpong, Darjeeling |
| $T_7$ | *T. virens* | Jute field | Arambagh, Hooghly |
| $T_8$ | *T. virens* | Cabbage field | Ranaghat, Nadia |
| $T_9$ | *T. roseum* | Potato field | Alipurduar, Jalpaiguri |
| $T_{10}$ | *T. roseum* | Rice field | Sehara Bazar, Bardhaman |

### *Preparation of dry mycelium*

For obtaining the mycelial powder, 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB) was inculated with 5 mm diameter disc of actively growing mycelium of *M. phaseolina* and *Sclerotium rolfsii.* The inoculated flasks were incubated at 28±1°C for 7 days. The mycelium was then collected by filtration through Whatman No. 1 filter paper, washed with distilled water and homogenized in distilled water. The suspension was centrifuged. three times at 6000 rpm for 10 min. The mycelium was stored in lyophilized state and used as carbon source.

### *Effect different pH and temperatures on enzyme activity*

Enzyme activity was determined in medium with pH values of 4 to 8 using 50 mM of acetate buffer (4 to 5.5 pH) and phosphate buffer (pH 6-8.5). To study the effect of temperature, the fungus was grown on MSM, pH 6.0 for chitinase and pH 5.5 for β-1,3 glucanase and cellulase at 20°, 25°, 30°, 35° and 40°C.

### *Effect of time course and carbon sources on enzyme activity*

To test the effect of time course and carbon source on the enzyme activity, the highly effective strain of *Trichoderma* was used and grown in MSM amended with different carbon sources i.e. laminarin @ 1 g/l (from *Laminaria digitata*), colloidalchitin @ 2 g/l

purified as the method described by Vessey and Pegg (1973), cellulose @ 5 g/l, mycelial powder of *M. phaseolina* and *S. rolfsii* @ 5 g/l each and with no sugar. The pH of the medium was adjusted to 6.0.

Six levels of carbon concentration (0.25, 0.50, 0.75, 1.0, 1.25 and 1.50 %) by amending laminarin, colloidal chitin and cellulose in MSM were evaluated for determination of β-1,3 glucanase, chitinase and cellulase activities of highly efficient strains, respectively.

### *Enzyme assay*

#### *β-1,3 glucanase (E.C. 3.2.1.58)*

The reaction mixture containing 0.5 ml laminarin (concentration 3.2 mg/ml distilled water), 1.0 ml of 0.05 M citrate buffer, pH 4.8 and 0.5 ml enzyme solution (culture filtrate) was incubated at 40°C for 60 min and the reaction was stopped by boiling. The amount of reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). In this method equal volume of dinitrosalicylic acid reagent was added to the reaction mixture and warmed in boiling water bath for 15 min. The absorbance of the reaction mixture was measured at 575 nm in a spectrophometer and compared with the standard graph drawn by following the same procedure but using different concentration of glucose instead of culture filtrate. The quantity of reducing sugar was calculated from the glucose standards included in the assay and activity of β-1,3 glucanase was expressed in nkat/ml. One nkatml corresponds to the release of 1 nmol glucose-equivalent per second under the condition given above.

#### *Chitinase (E.C. 3.2.1.14)*

A mixture of 0.5 ml enzyme solution (culture filtrate), 0.5 ml suspension of 0.5% colloidal chitin and 1.0 ml of McIlvaines buffer pH 4.0, was incubated at 37°C for 2 hrs in a water bath with constant shaking. The reaction was stopped by boiling for 3 min in heated water bath. In the reaction mixture 3 ml potassium ferricyanide reagent was added and was heated in boiling water bath for 15 min. The amount of N-acetyl glucosamine (NAG) released was estimated by the method of Reissing *et al.* (1955) from the absorbance of the reaction mixture at 420 nm comparing with the standard graph drawn by performing the same procedure but using different concentration of NAG suspension instead of culture filtrate. The amount of reducing sugars released was calculated from standard curves for NAG and the activity of chitinase was expressed in pkat (pmol/s) per milliliter.

#### *Cellulase (E.C. 3.2.1.4)*

The reaction mixture containing 1 ml of 1% cellulose, 2 ml of 0.05 M citrate buffer, pH 4.8 and 1 ml of culture filtrate was incubated for 30 min at 55°C in a water bath with periodical shaking. The reaction was stopped by boiling. The amount of glucose released in the reaction was estimated by dinitrosalicylic acid method and enzyme activity was expressed as release of 1 μmol glucose/ml/min for one unit.

## RESULTS

In this study experiments were conducted to find out the ability of different species of *Trichederma* to produce different kind of hydrolytic enzymes e.g. chitinase, β-1,3 glucanase and cellulase in various sugar supplemented minimal media (Table 2). Among the 10 isolates highest chitinase and β-1,3 glucanase activities were detected in $T_{10}$ isolate while $T_2$ exhibited highest activity of cellulase (23.43 U). $T_1$ (32.96 U) and $T_5$ (27.50 U) were the next highest in their chitinase activity while $T_9$ (6.43 U) and $T_1$ (5.83 U) isolates followed the $T_{10}$ for β-1,3

**Table 2** : Activity of extracellular hydrolytic enzymes produced by different isolates of *Trichoderma* grown on minimal synthetic medium

| Isolate | Chitinase (pkat/ml) | β-1,3 glucanase (nkat/ml) | Cellulase (μg of glucose /ml/min) |
|---|---|---|---|
| $T_1$ | 32.96 | 5.83 | 10.96 |
| $T_2$ | 16.00 | 2.26 | 23.43 |
| $T_3$ | 21.60 | 4.80 | 4.73 |
| $T_4$ | 14.86 | 2.63 | 9.86 |
| $T_5$ | 27.50 | 1.90 | 17.53 |
| $T_6$ | 13.40 | 3.83 | 15.60 |
| $T_7$ | 18.60 | 1.96 | 12.36 |
| $T_8$ | 24.76 | 3.06 | 6.96 |
| $T_9$ | 24.83 | 6.43 | 14.23 |
| $T_{10}$ | 38.26 | 7.76 | 16.63 |
| S Ed(±) | 1.04 | 0.24 | 0.24 |
| CD (P=0.01) | 2.96 | 0.69 | 0.68 |

glucanase activity. Very low activity of both chitinase and β-1,3 glucanase was noticed in case of $T_6$ and $T_5$ respectively. Activity of cellulase was highest in $T_2$ isolate followed by $T_5$ (17.53 U) and $T_{10}$ (16.63 U), which were statistically significant. Very low activity of cellulase was detected in $T_3$ isolate (4.73 U). Based on these results $T_{10}$ isolate for chitinase and β-1,3 glucanase and $T_2$ for cellulase were selected for all the further experiment.

Effects of time course and carbon sources on the activity of three hydrolytic enzymes were also studied. Activity of chitinase was tested in media supplemented with five types of sugar i.e. laminarin, mycelial powder of *M. phaseolina* and *S. rolfsil*, cellulose and also in media without sugar (Fig. 1). Activity of chitinase was detected in all the sugar amended MSM except in those amended with cellulose and with no sugar. Again, activity of

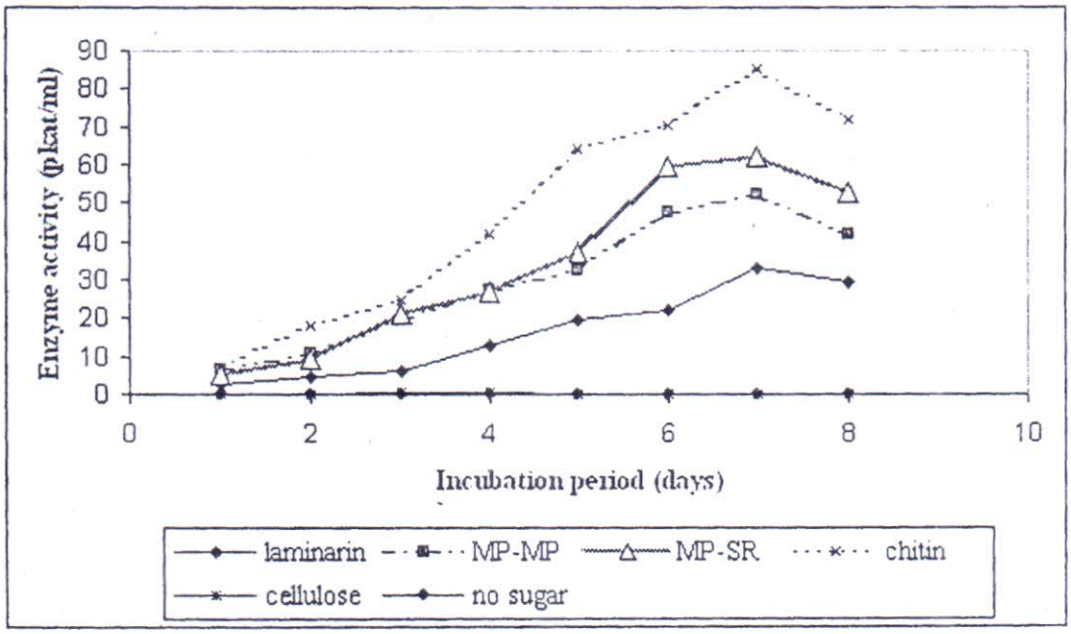


**Fig 1.** Effect of time course and carbon sources on the chitinase activity

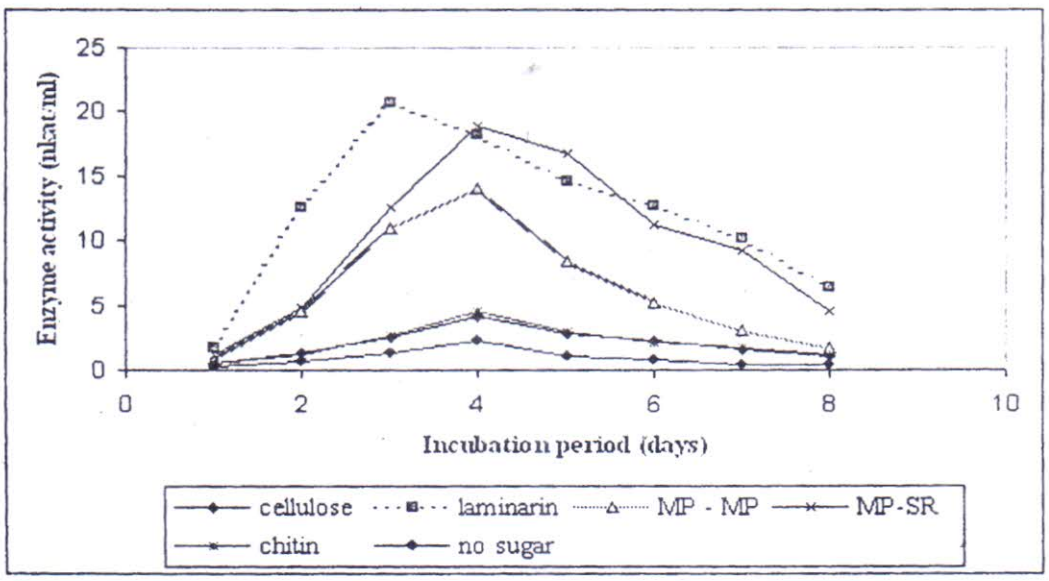


**Fig 2.** Effect of time course and carbon sources on β-1,3 clucanase activity

enzyme increased with incubation period and reached it peak at 7th day of incubation and then declined. When $T_{10}$ isolate was grown in MSM supplemented with chitin, the activity of chitinase level increased with time and reached a maximum of 85.1 U at 7th day. Enzyme activity was not detected both in media supplemented with cellulose and media with no sugar with few exceptions. Chitinase activity was also higher in media supplemented with mycelial mat of *S. rolfsii* and *M. phaseolina* as compared to laminarin.

When $T_{10}$ isolate was grown in MSM supplemented with laminarin, highest activity of β-1,3 glucanase was detected which reached its peak of 20.7 U at 3rd day of incubation and then declined to 6.4 U at 8th day (Fig. 2). In the other sugar supplemented media highest peak was recorded at 4th day of incubation and declined upto 8th day. It was interesting to note that there was a considerable amount of β-1,3 glucanase activities in media without any sugar and reached the peak of 2.3 U at 4th day. A significance activity of β-1,3 glucanase was produced by $T_{10}$ isolate in culture media amended with dried mycelium of pathogen. Levels of enzyme production started to decrease after 4th day of incubation when dry mycelium of phytopathogenic fungi *M. phaseolina* and *S. rolfsil* was used as carbon source. Activity was greater when mycelium of *S. rolfsil* was used as compared to those of *M. phaseolina.*

Similarly, activity of cellulase increased with incubation period and reached it peak at 5th day of incubation when cellulose was used in the medium as the sole carbon sources (Fig. 3). 56.15 U activity was found at 5th day and then declined to 31.7 U at 8th day of incubation. When chitin was supplemented in the medium the activity was very low as compared to media with no sugar. Mycelial mat of both these pathogens induced the cellulase activity much more than in laminarin supplemented media. the cellulase activity of 6.4 U was found at 4th days of incubation in media with no sugar and declined to zero at 8th day.

The effect of pH on the activity of extracellular hydrolytic enzymes was studied by incubating the $T_{10}$ strain in media with variable pH between 4.0 and 8.0. The β-1,3 glucanase was active from pH 4.0 to 8.0 with an optimum at pH 5.5 (Fig. 4). Activity was lowest at pH 4.0 (2.99 U) as compared to pH 8.0 (3.02 U). The optimum pH for chitinase activity was pH 5.0 (Fig. 5). These enzymes preferred acidic condition for their activity than alkaline. Very low chitinase activity was detected at pH 8.0. Highest cellulase production was measured when the initial pH was set to 5.0 as compared to others (Fig. 6).

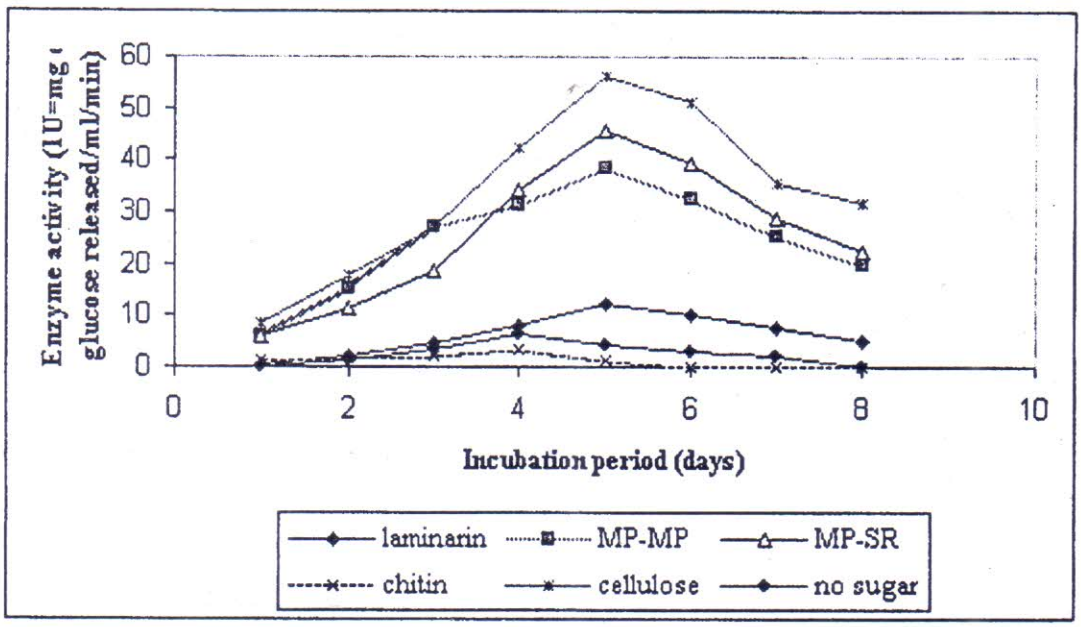


**Fig 3.** Effect of time course and carbon sources on cellulase activity

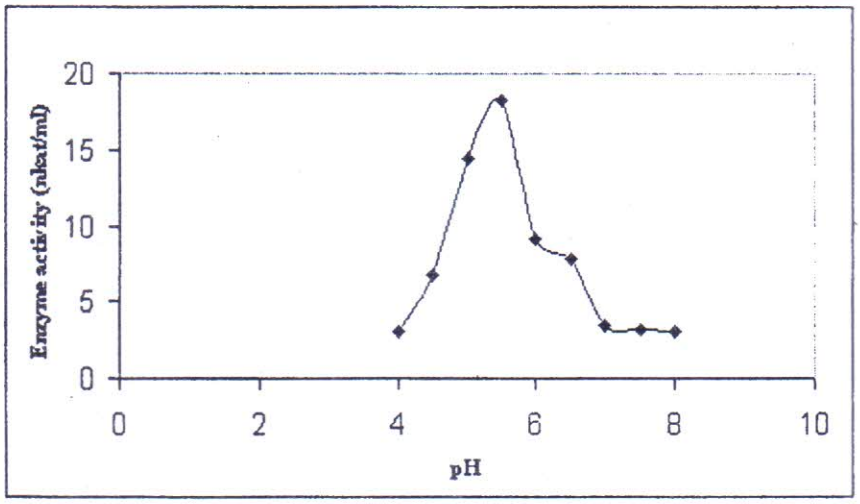


**Fig 4.** Effect of pH on the activity of β-1,3 glucanase

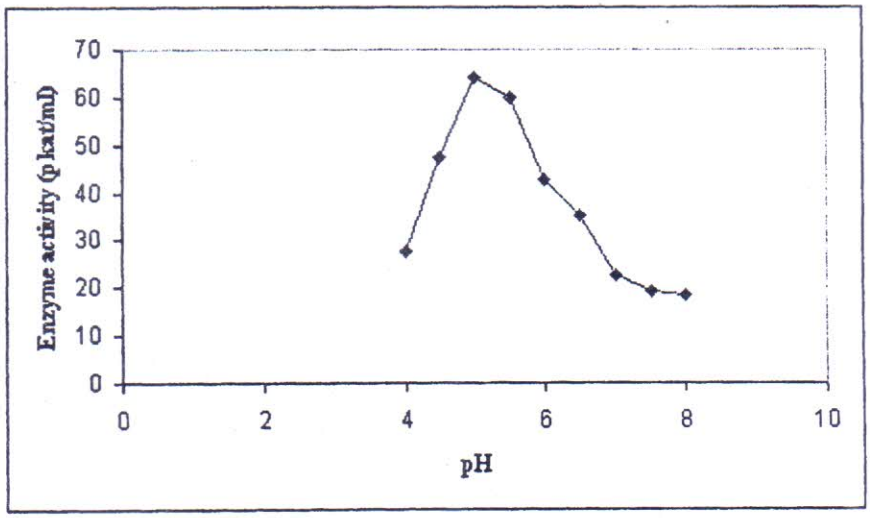


**Fig 5.** Effect of pH on the activity of chitinase

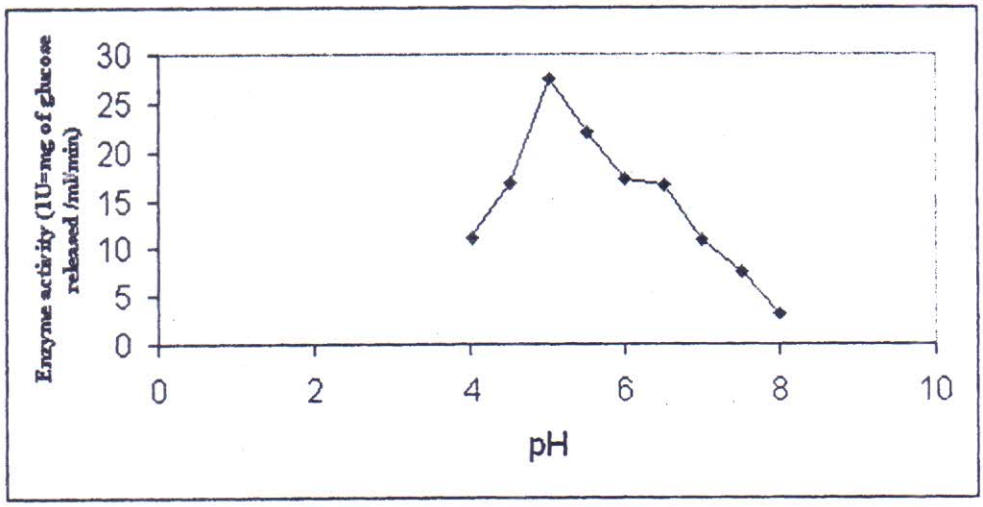


**Fig 6.** Effect of pH on the activity of cellulase

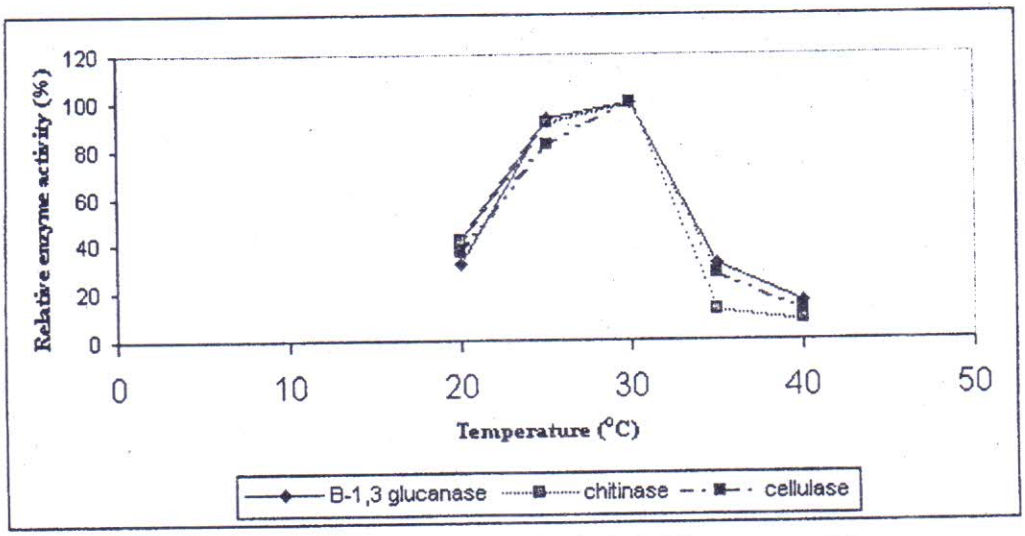


**Fig 7.** Determination of optimum temperature for the hydrolytic enzymes activity

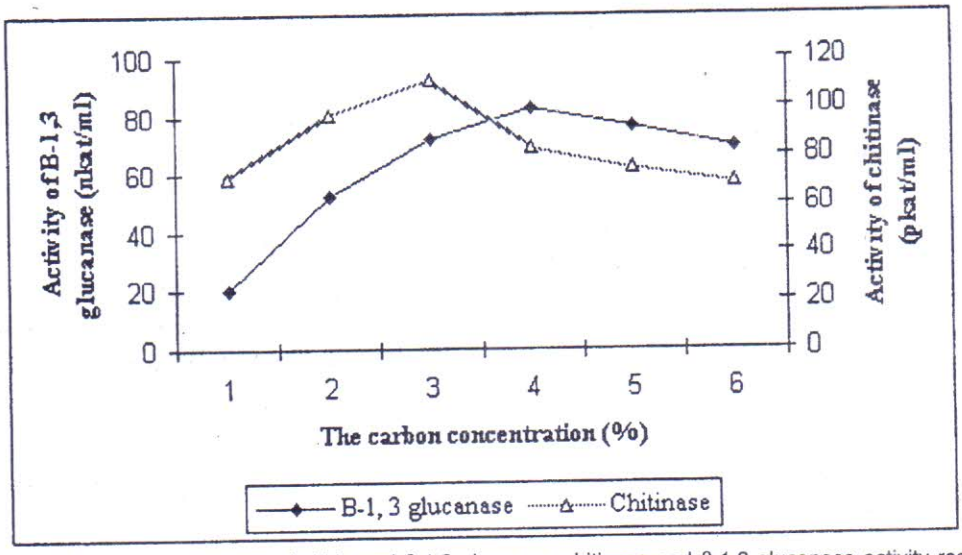


**Fig 8.** Effect of different concentrations of chitin and β-1,3 glucan on chitinase and β-1,3 glucanase activity respectively

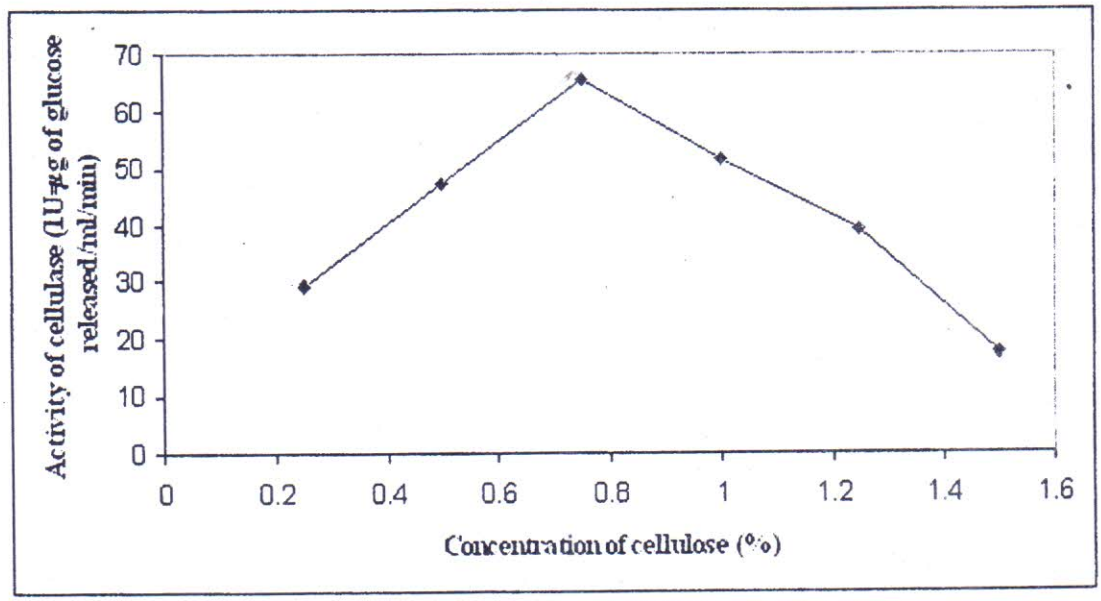


**Fig 9.** Effect of different concentrations of cellulose on cellulase activity

Very low activity was found at pH 8.0. The five levels of temperature i.e. 20°, 25°, 30°, 35°, and 40°C were studied for the enzyme activity and results showd that all three enzymes were active at 30°C than others (Fig. 7).

Effect of chitin, laminarin and cellulose concentration was tested for the activity of chitinase, β-1,3 glucanase (Fig. 8) and cellulase (Fig. 9), respectively. Activity of β-1,3 glucanase increased with concentration of its sugar substrate laminarin and reached a peak at 1.00% concentration (82.3 U) and then declined to 69.2 U when the substrate was 1.5%. As chitinase production was substrate depended, its highest activity was detected at 0.75% chitin concentration and then decreased with increase in concentration of chitin. Similarly, activity of cellulase was increased up to 0.75% of substrate concentration and above 0.75% of cellulose there was no further increase of cellulase activity and declined to 17.6 U at 1.5% substrate concentration.

## DISCUSSION

In the present investigation all the test isolates produces the cell wall degrading hydrolytic enzymes i.e. β-1,3 glucanase, chitinase and cellulase. However, these was a considerable variation in the production of the enzymes or secretion in the culture filtrate among the isolates. For successful biological control against a wide spectrum of fungi the strain should produced all cell degrading enzyme in a significant amount. Cherif and Benhamou (1990) have revealed that local alteration of chitin macromolecules in mycelium of *F. oxysporum* f.sp. *radicis-lycopersici* has an early event preceding visible wall disruption and leakage of cytoplasm. The observation that, as early as 48 hrs after inoculation, N-acetyl glucosamine residues are released in the growing medium, correlates well with the idea that wall-bound chitin may be locallly hydrolyzed by extracellular chitinase produced by *Trichoderma*. If one considers that most fungal cell walls are mainly composed of chitin and β-1, 3-glucans embedded in a matrix of amorphous materials (Farkas, 1979), it is to be expected that successful wall degradation may result from the activity of more than one enzyme, In most cases chitin is buried in β-glucans, thus not readily accessible to chitinase. It is, thus, likely that chitinase activity is preceded by or coincides with the hydrolytic activity of other enzymes, especially β-1,3 glucanase (Cherif and Benhamou, 1990). Hydrolytic enzymes such as glucanase, chitinase, cellulase, xylanase, acid and alkaline phosphatase, esterase, lipase, leucine arylaminidase, α- and β-galactosidase, α- and β-glucosidase, N-acetyl glucosaminidase and protease are known to be produced by *Trichoderma* upon induction (Aziz *et al.,* 1993). Production of extracellular β-1.3 glucanase, chitinase, cellulase and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Carsolio *et al.,* 1994; De La Cruz *et al.,* 1995; Kumar and Gupta, 1999; Roy *et al.,* 2005). These observation, together with fact that, chitin, β-1.3 glucan and protein are the main structural components of most fungal cell wall (Peberdy, 1990), are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens. Thus, new strains, which are more effective in biocontrol, could have considerable industrial potential. The physiological age of the mycelium used for inoculation of shake flasks seems to be an important factor of enzyme production. Ulhoa and Peberdy (1991) have found in their study on chitinase production using washed mycelium of another. *T. harzianum* strain that both mycelium harvested at 18 hrs (exponential phase) and at 24 hrs (early stationary phase) show similar production of extracellular chitinase.

Most of the chitinolytic enzyme systems reported in the literature is inducible and products of chitin degradation also regulate chitinase synthesis in *T. harzianum* (Ulhoa and Peberdy, 1991). In agreement with these findings the result of present investigation show high chitinase activity only in culture supplied with chitin and mycelial mat of pathogen but not with other polymers such as cellulose, which is further indicative of induction. Kumar and Gupta (1999) have reported that cell walls of *M. phaseolina* and *S. rolfsii* are known to have glucan and chitin that should have resulted in the induction of glucanase and chitinase in mycelial mat amended media. These findings support the result of the present investigation. High β-1.3 glucanase and chitinase activities are detected in dual culture when *T. harzianum* parasitize *R. solani* and *S. rolfsii* compared with low levels in absence of pathogen (Elad *et al.,* 1983). However, Tronsmo and Harman

(1992) have claimed that *T. harzianum* show low enzyme activity in simple mineral salt solution plus single carbon sources including chitin while addition of any of several complex materials substantially increase enzyme yield.

A low level of β-1.3 glucanase and cellulase and no chitinase have been measured when the antagonistic strain has been deprived of a carbon source and its growth is low in this present experiment. Although the exact reasons for this remain to be established, it seems probable that in condition of carbon starvation and poor growth the fungus actively secrets some levels of hydrolytic enzymes. Alternatively, the production of β-1.3 glucanase in carbon deprived media could be related to metabolism, such as mobilization of wall glucans and to some morphogenetic functions and changes. No production or low level of chitinase activity in deprived carbon source confirms that chitinase enzyme is produced inducibly not constitutively. Expression of these cell wall degrading enzymes frequently has been reported to be induced by fungal cell wall components and repressed by carbon catabolite repressor such as glucose (Peterhauer *et al.,* 1996; Donzelli *et al.,* 2001; Giuliano *et al.,* 2001).

Chitinase, β-1, 3 glucanase and cellulase production has been favoured by acidic pH (5.0, 5..5 and 5.0 respectively). Acidic pH has been reported to be an important growth parameter in the production of chitinase and β-1, 3 glucanase in mycoparasite *T. harzianum* (Elad *et al.,* 1982). Ulhoa and Pederby (1991) have found that the production of chitinase has been markedly affected by pH with the optimum at pH 6.0. The pH and temperature optima of these three enzymes support the work of Jijakli and Lepoivre (1998) and Harman *et al.* (1993). Prasad and Devaraj (1997) have reported that the optimum pH of the activity of endo-N-acetyl-galactos-aminidase, an enzyme which cleaves O-glycosidic linkage between α-N-acety-D-galactosamine and serine/threonine residue of the asialofeltin an O-linked glycoprotein, has been found to be pH 6.

It has been found that chitinase activity increase with increasing chitin concentration up to 0.75%. Ulhoa and Peberdy (1991) have suggested that chitinase production is substrate concentration dependent; above 0.5% (w/v) chitin there is no further promotion of synthesis. Elad *el al.* (1982) have reported that chitinase secretion into the growth medium by *T. harzianum* increase up to concentration of 1%. In the present experiment β-1, 3 glucanase enzyme production increases up to 1.0% concentration of laminarin but decreases at higher concentration. This may be due to fact that at higher concentration of sugar activity of enzyme is inhibited. At high levels of glucose and ammonium, expression and secretion of all cell wall degrading enzymes measured are completely repressed. When both nutrients are at low levels all enzymes are strongly expressed (Giuliano *et al.,* 2001).

The control of enzyme expression is of ecological importance. Soil condition i.e. presence of readily available simple sugars and organic acid and available nitrogen may also affect the enzyme expression. Thus, the regulation of cell wall degrading enzyme expression in *Trichoderma* is complex and is regulated by multiple interactive factors.

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