Physiological and biochemical analysis of *Trichoderma* species isolated from different location U. P.

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Seven different species of *Trichoderma* isolated from the different locations of Uttar Pradesh were used in this study. A comparative analysis was done among all the seven species for the determination of glucanase enzyme production and nitrogen content determination the results showed that among all the seven tested species, *Trichoderma harzianum* shows the highest glucanase enzyme production as well as the highest nitrogen content. Optimal physical parameters such as pH, temperature, and effect of agitation were also tested for the biomass production on all the eight species. The results revealed that the optimal pH temp and agitation speed for *Trichoderma* biomass production were 6.0 and 6.5, 25 and 30°C and 150 rpm. In present study we also evaluated the effect of two different carbon sources on the glucanase enzyme induction. Carboxymethyl cellulose (CMC) was found to be the best carbon source for glucanase enzyme induction.

**Key words:** *Trichoderma*, nitrogen content, biological control.

INTRODUCTION

Soil borne pathogens have a broad host range and persist for longer periods in soil by resistant resting structures. Chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Therefore, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists against plant pathogenic fungi (Margolles-Clark et al., 1996; Harman et al., 2004; Chet, 1987). In which, *Trichoderma harzianum* has been accepted as one of the most potent biocontrol agents against plant diseases and used as an antagonist against many soil borne phytopathogenic fungi over the past few years (Samuels et al., 1998). Various strategies of biocontrol have been proposed. They include the creation of competition for nutrients or space; the production of antibiotics and lytic enzymes; the inactivation of the enzymes of phytopathogenic fungi; and parasitism (Viterbo et al., 2002). The cell wall-degrading enzymes (CWDEs), mostly chitinases, glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents (Bisset, 1991b). CWDEs attack the cell wall of phytopathogenic fungi to cause cell lysis and

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subsequent death. Although the mechanism of mycoparasitism is not completely understood, this process has been assumed to involve the expression of extracellular CWDEs. Recently, homologues of proteins encoded by avirulence genes have been identified in Trichoderma strains. These proteins can induce hypersensitive responses and other defense-related reactions in plant cultivars that contain the corresponding resistance genes (Tseng et al., 2008).

The knowledge of nutritional requirements is the main need in the cultivation of microorganisms using any cultural technique. The carbohydrates, proteins, lipids, nucleic acids are made up of macro elements like carbon, hydrogen, nitrogen, sulphur, phosphorus and these are involved in mechanisms like host pathogen interaction and self defense mechanisms. Nitrogen is a major element found in many of the simple compounds and nearly all of the complex macromolecules of living cells. Proteins and nucleic acids are especially rich in nitrogen. Thus, it should not be surprising that a substantial cellular investment is made in the metabolic machinery comprising nitrogen catabolic pathways to ensure a constant nitrogen supply for growth. Extensive studies of nitrogen metabolism and its control have been carried out in three fungi, Neurospora crassa, Aspergillus nidulans and Saccharomyces cerevisiae. The ability of some filamentous fungal species to produce gram quantities of protein per liter of culture medium has been exploited by enzyme industry e.g. Trichoderma reesei (Kendrick and Ratledge, 2006).

MATERIALS AND METHODS
Purification and morphological characterization of Trichoderma species

Trichoderma isolates collected from soil samples of different lactation of Uttar Pradesh, isolated with the help of serial dilution plate technique, (Johnson and Curl, 1972) were grown on PDA medium for proper identification. These potential isolates of Trichoderma species were identified by light microscope for morphological characters such as the branching pattern of conidiophores, the conidiophores apex elongation and shape (coiled, straight or undulate), the phialides shape, structure, size and the conidial shape (Table 1). The cultures were identified using the available literature (Bisset, 1991b; Griffin, 1994) and monographic contribution provided by Bisset (1991a, b) also reconfirmed by ITCC, Division of Plant Pathology IARI, New Delhi.

Physical parameters

pH

The influence of initial medium pH on fungal growth was investigated at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. A 10% (v/v) standard inoculum was inoculated in a 500ml Erlenmeyer flask containing 100 ml broth of PDA media and incubated at 28°C in an orbital shaker at 150 rpm for 7 days. The pH that promoted the highest biomass production was used for subsequent steps of the investigation.

Temperature

The effects of temperature on fungal growth were studied at 25, 30, 35 and 40°C in Trichoderma specific medium at the determined optimum pH and incubated in BOD incubator for 7 days. The temperature that promoted the highest biomass production was used for the subsequent steps of the investigation.

Speed of agitation

The effects of agitation during incubation on growth were carried out in Trichoderma specific medium at optimum pH using an orbital shaker at 100, 150, 200 and 250 rpm. Incubation was conducted at the determined optimum pH and temperature. The agitation speed that promoted the highest biomass production was used for the subsequent steps of the investigation.

Induction of β-1,3 Glucanase enzyme taken from Trichoderma sp. on different carbon sources

Two different carbon sources were selected for the induction of glucanase enzyme viz- CMC and wood dust. These carbon sources were added in Czapek Dox medium at the rate of 1%. Cultures were incubated for 10 days at 28°C on orbital shaker at 150rpm. At the end of the incubation time, wood dust residues were removed and filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was considered as a source of crude enzyme. This obtained supernatant was used for measuring enzyme activity. Glucanase enzyme activity was assayed using 1% (w/v) CMC as a substrate. Enzyme activity is expressed as U/mg.

1 ml enzyme solution was added to 1ml of 1% carboxymethyl cellulose dissolved in 50 mM sodium acetate buffer, pH 5.0. After incubation at 50°C for 60 min, the reaction was stopped by the addition of 3 ml DNS reagent. After incubating for 10 min in a boiling water bath enzymatic activity was determined at 540 nm. One unit of CMCase activity was expressed as the amount of enzyme that liberate reducing sugar equivalent to glucose per minute under assay conditions.

Determination of protein concentration

Protein content of the crude enzyme preparation was assayed by Lowry (1951) method, using BSA, as standard. 0.5 mg/ml of BSA standard was used. We made different dilutions of this standard. To each tube 2 ml of complex forming reagent was added and keep for 10 min at room temperature. After this 0.2 ml of solution Folin-Ciocalteu reagent was added and the sample was incubated for 20 to 30 min. The same steps used for the test sample, absorbance was taken at 660 nm. Calibration curve was constructed by plotting absorbance reading on Y axis against standard protein concentration (mg/ml). Sample concentration was calculated through this standard graph (Figure 2).

Kjeldahl method for nitrogen estimation

Total Nitrogen content of different strains of Trichoderma sp. was estimated by Kjelplus nitrogen analyzer. About 1 g dried and well powdered sample of Trichoderma was accurately weighted on a piece of filter paper and transferred along with the filter paper to 30 ml Micro Kjeldahl digestion tube. Then 10 ml of concentrated H2SO4 and 500 mg digestion mixture (CuSO4· K2SO4· 1: 5) were added to digestion tube. Sample was digested on an electric heater at 400°C for 1 h. After cooling, the digested sample was transferred to micro Kjeldahl distillation apparatus using successive small quantities of
Table 1. Identification of potential strains of Trichoderma species.

| Strain No. | Name of bioagent | ITCC Acc. No. | GenBank accession no. | Strain code | Source | GPS Location |
|------------|------------------|---------------|-----------------------|-------------|--------|--------------|
| T1         | T. harzianum     | 6796          | KC800922              | Th azad     | CSA Kanpur Nagar | Latitude: 25° 8’ 34.821”  
|            |                  |               |                       |             |        | Longitude: 81° 59’ 2.979” |
| T2         | T. viride        | 8315          | JX119211              | 01PP        | Hardoi  | Latitude: 27° 23’ 40.729”  
|            |                  |               |                       |             |        | Longitude: 80° 7’ 47.751”  |
| T3         | T. asperellum    | 8940          | KC800921              | Tasp/CSAU   | CSA Kanpur Nagar | Latitude: 25° 8’ 34.821”  
|            |                  |               |                       |             |        | Longitude: 81° 59’ 2.979”  |
| T4         | T. koningii      | 5201          | KC800923              | Tk (CSAU)   | CSA Kanpur Nagar | Latitude: 26° 29’ 33.384”  
|            |                  |               |                       |             |        | Longitude: 80° 18’ 6.518”  |
| T5         | T. atroviride    | 7445          | KC 008065             | 71 L        | Hardoi  | Latitude: 26° 29’ 28.323”  
|            |                  |               |                       |             |        | Longitude: 80° 18’ 26.361”  |
| T6         | T. longibrachiatum | 7437     | JX978542              | 21 PP       | Kaushambi  | Latitude: 26° 34’ 27.61”  
|            |                  |               |                       |             |        | Longitude: 79° 18’ 24.623”  |
| T7         | T. virens        | 4177          | KC800924              | Tvi (CSAU)  | CSA Kanpur Nagar | Latitude: 25° 21’ 39.794”  
|            |                  |               |                       |             |        | Longitude: 81° 24’ 11.414”  |

Soil samples collected from different places of Uttar Pradesh, India, were identified as T. harzianum (Th Azad) which is isolated from soil sample of chickpea crop of Kanpur district. T. viride (01pp) isolated from soil sample of pigeon pea crop of Hardoi district. T. atroviride (Tk/CSAU) and T. koningii (Tk/CSAU) were isolated from rhizospheric soil sample of Nawabganj farm, Kanpur. T. longibrachiatum (21pp) isolated from soil sample of Neveda block of Kaushambi and T. virens (Tvi/CSAU) isolated from soil sample of chickpea field of Student farm, CSAU Campus, Kanpur. They were morphologically identified by slide mounting and re-confirmation by ITCC, IARI, New Delhi and allotted with unique accession number, as well as molecular identified by ITS marker, sequences deposited to NCBI GenBank. Finally, potential and effective bioagent were submitted to microbial data bank at NBAIM, Mau, India (Table 2).

Effect of pH, temperature and agitation on the biomass of Trichoderma sp

The mycelial growth was observed among all isolates of Trichoderma species described in Table 3 at all tested pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 each of every 0.5 interval of pH range. Maximum number of isolates showed high biomass production at pH 6.0 and 6.5 followed by 5.5 and 7.0 and minimum at pH 4.5 and 7.5. The biomass production of T. harzianum (Th Azad), T. viride (01pp) and T. asperellum (Tasp/CSAU) were significantly higher than any other species at all pH levels whereas, T. longibrachiatum (21pp) and T. atroviride (71L) showed moderate biomass production and minimum was observed with T. koningii (Tk/CSAU).
### Table 2. Morphological and cultural observation of *Trichoderma* species.

| Isolates/characters | *T. harzianum* (*Th* azad) | *T. viride* (01PP) | *T. asperellum* (*tasp*/CSAU) | *T. longibrachiatum* (71 L) | *T. atroviride* (21 PP) | *T. koningii* *T*<sub>K</sub> (CSAU) | *T. virens* *T*<sub>vi</sub> (CSAU) |
|---------------------|-----------------------------|-------------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------------|---------------------------------|
| Colony growth rate  | Colony colour                | Reverse colony colour | Colony edge | Culture smell | Mycelial form | Mycelial colour | Conidiation | Conidiophores branching | Phialide shape | Conidial shape | Conidial colour | Clamydospore |
| (cm/day)            | (cm/day)                     | Colourless         | Smooth          | Coconut like | Floccose to arachnoids | Cottony white | Circular | Flexuous regularly highly branched | Flask shape, swollen in middle, narrow at the tip | Subglobule to ovoid | Light green | Observed |
| 7-8 in 5 days      | Light green to dark green     | Whits ring like zones wavy | Smooth          | Coconut like | Floccose to arachnoids | Whitish white | Circular | Repeated, highly branched | Flask shape swollen in middle, narrow at the base | Globose to ovoid | Light green | Not observed |
| 7-8 in 5 days      | Bright green to dark green    | Colourless         | Smooth          | Coconut like | Floccose to arachnoids | Watery white | Circular | Irregularly branched | Long, swollen in middle, horn shaped | Globose to subglobule | Yellowish green | Not observed |
| 6-7 in 5 days      | dark green                   | Colourless         | Smooth          | Coconut like | Floccose to arachnoids | Watery white | Circular | Often paired | Lageniform, slightly swollen near the middle | Ellipsoidal to obvoid | Yellowish green | Not observed |
| 7-8 in 5 days      | Yellowish green              | Colourless         | Smooth          | Coconut like | Floccose to arachnoids | Watery white | Circular | Irregularly, crowed smooth, branched | Cylindrical, slightly flask shape, swollen in middle | Subglobule | Light green | Not observed |
| 5-6 in 5 days      | Dark Green                   | Colourless         | Smooth          | Coconut like | Floccose to arachnoids | Watery white | Circular | Simple, pyramidal, smooth, | Nine-pin bowling shape | Oblong, obvoid, ellipsoid | Light green | Not observed |
| 6-7 in 5 days      | Dull green                   | Light yellow       | Smooth          | Malt           | Floccose to arachnoids | Watery white | Circular | Simple, rebranched, few lateral branches | Lageniform to ampuliform swollen at the middle | Ellipsoidal to obvoid | Yellowish green | Not observed |

### Table 3. Effect of pH on the biomass of *Trichoderma* species.

| Stains name | pH 4.5 | pH 5.0 | pH 5.5 | pH 6.0 | pH 6.5 | pH 7.0 | pH 7.5 |
|-------------|--------|--------|--------|--------|--------|--------|--------|
| *T. harzianum* (T. Azad) | 0.107  | 0.152  | 0.313  | 0.607  | 0.709  | 0.301  | 0.223  |
| *T. viride* (01PP) | 0.098  | 0.119  | 0.210  | 0.564  | 0.702  | 0.321  | 0.122  |
| *T. asperellum* (*tasp*/CSAU) | 0.105  | 0.117  | 0.398  | 0.667  | 0.732  | 0.438  | 0.235  |
| *T. longibrachiatum* (21PP) | 0.098  | 0.138  | 0.295  | 0.655  | 0.803  | 0.465  | 0.463  |
| *T. atroviride* (71 L) | 0.089  | 0.122  | 0.284  | 0.583  | 0.789  | 0.378  | 0.148  |
| *T. koningii* (T.k/CSAU) | 0.086  | 0.109  | 0.376  | 0.540  | 0.653  | 0.366  | 0.073  |
| *T. virens* (T.vi/CSAU) | 0.078  | 0.102  | 0.270  | 0.398  | 0.537  | 0.302  | 0.980  |
Table 4. Effect of Temperature on the biomass of Trichoderma species.

| Stains name       | 15°C | 20°C | 25°C | 30°C | 35°C | 40°C |
|-------------------|------|------|------|------|------|------|
| T. harzianum (T. Azad) | 2.1  | 3.2  | 5.9  | 6.7  | 6.5  | 4.9  |
| T. viride (01PP)   | 1.9  | 3.1  | 5.7  | 6.6  | 6.8  | 4.6  |
| T. asperellum (T-asp)/CSAU | 1.9  | 2.7  | 5.5  | 6.7  | 6.7  | 4.9  |
| T. longibrachiatum (21PP) | 2.0  | 2.8  | 5.7  | 6.5  | 6.4  | 4.0  |
| T. atroviride (71 L) | 1.8  | 2.2  | 5.4  | 6.3  | 6.2  | 3.8  |
| T. koningii (T.k/CSAU) | 1.6  | 2.0  | 5.5  | 6.4  | 6.0  | 3.6  |
| T.virens (T.vi /CSAU) | 1.4  | 1.9  | 4.2  | 5.5  | 5.9  | 3.2  |

Table 5. Glucanase enzyme production by Trichoderma spp. grown on different carbon sources.

| Carbon source (1%) | T. viride | T. harzianum | T. asperellum | T. koningii | T. atroviride | T. longibrachiatum | T. virens |
|--------------------|-----------|--------------|---------------|-------------|--------------|--------------------|----------|
| CMC                | 1.66      | 2.01         | 1.42          | 1.39        | 1.35         | 1.20               | 0.82     |
| Wood dust          | 0.5       | 0.41         | 0.4           | 0.39        | 0.35         | 0.33               | 0.3      |

and T. virens (Tvi/CSAU) after 7 days (Table 4). Along with pH all the species of Trichoderma produces good biomass at different temperatures. In which maximum biomass produced by T. harzianum, T. viride, T. asperellum followed by T. longibrachiatum, T. atroviride, T. koningii and T. virens when incubated at 25, 30 and 35°C compared to incubation at 15, 20 and 40°C. There was no significant difference between 25 and 30°C with p-value at 0.041 (Table 4). As for the effects of aeration, Trichoderma species showed an increase biomass as the rate of agitation increased up to 150 rpm and reduced when the speed of agitation increased up to 250 rpm. Statistical analysis showed no significant difference between speed of agitation of 150 and 200 rpm with p-value at 0.059, although species of Trichoderma produced higher biomass at 150 rpm than at 250 rpm.

**Calculation of results**

The general equation for the protein content is:

\[
\text{Percent protein} = \frac{\text{Adolescent} \times \left(\frac{V_b - V_s}{W}\right) \times f}{(1.4007) \times \text{normality of acid} \times \text{weight of the sample (mg)}}
\]

\[V_b = \text{ml titrant for the blank} \quad V_s = \text{ml titrant for the individual samples} \quad N = \text{Normality of the acid titrant (nominally 0.1)} \quad 1.4007 = \text{A single factor that takes into account the molecular weight of nitrogen} \quad \text{The conversion of the mille – equivalent result of} \quad V \times N \quad \text{and the conversion to} \quad 1; W = \text{the weight of the sample in grams} \quad \text{The error is sufficiently small that, for Sample weighted to} \quad 1.000 \text{ g + / - 0.0005 g} \quad \text{This can be assumed to be} \quad 1; F = \text{the factor for converting the percent nitrogen in sample to percent protein.}

N content of the sample was calculated on the basis of the following formula:

\[N\% = \frac{(\text{ml } H_2SO_4 \text{ in sample}) - (\text{ml } H_2SO_4 \text{ in blank}) \times \text{normality of acid}}{\text{Weight of the sample (mg)}}
\]

Total protein content was determined by, multiplying a factor with the observed nitrogen values.

To achieve maximum production two different carbon sources were added in the culture media for maximum glucanase enzyme production (Figure 1). CMC was found to be the best glucanase inducer as compare to wood dust (Table 5). T. harzianum and T. viride produced the highest amount of glucanase enzyme. So these strains can be commercialized on large scale for the control of phytopathogens.

The chemical factors which in turn influenced the occurrence of Trichoderma sp. Trichoderma species that were high in nitrogen favored the occurrence of this fungus. From the data presented in Table 6, it was revealed that the maximum Nitrogen was recorded in T. asperellum (10.2%) followed by T. longibrachiatum (7.1%), T. harzianum and T. koningii (6.8%) but in T. viride (4.1%). Nitrogen content was found to be too low.

Mineral nutrition is essential for growth, sporulation and stimulation of fungal secondary metabolism (Griffin, 1994). High total N availability increased sporulation, production of antifungal anthroquinone pigments, hyphal growth rate (Fargasona, 1992), and the antagonistic activity of Trichoderma sp. against wood rot fungus Serpula lacrymans (Score and Palfreyman, 1994). Soil nitrate levels were positively correlated with cellulose production (Widden and Breil, 1988) and may favor competitiveness of the biocontrol agent with the pathogen. Nitrogen is a major element found in most simple compounds and nearly all of the complex microorganisms of living cells. Proteins and nucleic acids are especially rich in nitrogen.

The method was developed by Johan Kjeldahl, and is used extensively in the determination of protein, since
Table 6. Glucanase enzyme protein content of seven fungal strains.

| Fungal strain      | Glucanase enzyme protein concentration (mg/ml) |
|--------------------|-----------------------------------------------|
| T. harzianum       | 0.23                                          |
| T. viride          | 0.21                                          |
| T. koningii        | 0.19                                          |
| T. asperellum      | 0.17                                          |
| T. atroviride      | 0.15                                          |
| T. longibrachiatum | 0.12                                          |
| T. virens          | 0.09                                          |

Table 7. Evaluation of N% of Trichoderma species.

| Trichoderma species | Titration value | Nitrogen percentage | Protein percentage |
|---------------------|-----------------|---------------------|--------------------|
| T. harzianum        | 0.79            | 1.10                | 6.8                |
| T. viride           | 0.48            | 0.67                | 4.1                |
| T. koningii         | 0.80            | 1.1                 | 6.8                |
| T. asperellum       | 1.17            | 1.63                | 10.2               |
| T. atroviride       | 0.54            | 0.75                | 4.6                |
| T. longibrachiatum  | 0.82            | 1.14                | 7.1                |
| T. virens           | 0.49            | 0.68                | 4.2                |

Figure 1. Effect of carbon sources on glucanase enzyme induction.

Protein is a macromolecule and made up of nitrogen containing amino linkage together. For the given sample of Trichoderma sp., the percent nitrogen measured is converted to the equivalent protein content by the use of an appropriate numerical factor. For this sample, the factor is 6.25 since fungal protein is approximately 16% of nitrogen. Data revealed (Table 7) that highest protein is found in T. asperellum followed by T. longibrachiatum.

Conflict of Interests

The authors have not declared any conflict of interests.
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