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Research Article

# *In vitro* screening for enzymatic activity of Trichoderma species for biocontrol potential

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**Abstract:** A total of seven *Trichoderma* species were isolated from rhizosphere soils of brinjal on potato dextrose agar medium. Based on morphological and cultural characters, the isolates were assigned to different species viz., *Trichoderma viride, T. harzianum, T. virens, T. atroviride, T. koningii, T. pseudokoningii* and *T. reesei*. *Trichoderma* species were screened for the production of extracellular enzymes to identify the strain with high antagonistic potential against fungal pathogens. The screening was done following plate assay method on the respective solid media. These strains were positive for cellulase, amylase, pectinase, protease and chitinase activity. The excretion of extracellular lytic enzymes reveals their usefulness in the application of *Trichoderma* species as biocontrol strains in agricultural soils. The use of simple solid media permits the rapid screening of large populations of fungi for the presence or absence of specific enzymes

**Keywords**: Agar plate assay, lytic enzymes, cellulase, amylase, pectinase, protease, chitinase.

## Introduction

Fungi of the genus *Trichoderma* Pers. are soil borne, green-spored ascomycetes that can be found all over the world. It has gained importance since last few decades due to its bio control ability against several plant pathogens. *Trichoderma* spp. has been found effective against aerial, root and soil pathogens (Weller, 1988; Whipps *et al.,* 1993; Elad *et al.,* 1998 a, b; Van Loon *et al.,* 1998; Elad, 2000; Chaube *et al.,* 2002; Harman *et al.,* 2004). Biocontrol mechanisms are specific for particular antagonists and plant pathogens and several mechanisms could operate in any microbial interaction. Biocontrol agents differ fundamentally from chemical fungicides as they grow and proliferate effectively. Therefore, effective antagonists established in crop ecosystem remain active against targeted pathogens under favorable conditions (Lewis and Papavizas, 1984). *Trichoderma* spp. are able to use a wide range of compounds as carbon and nitrogen sources and secrete a variety of enzymes to break down compound plant polymers into simple sugars for energy and growth. There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis; competition for nutrients; and mycoparasitism whereby *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as cellulase, chitinase, β-1, 3 glucanase and protease (Chet, 1987).

The objective of this study was to isolate *Trichoderma* spp., and screening of lytic enzymes such as cellulase, amylase, pectinase, protease and chitinase, in the presence of corresponding substrates to identify the strain with high antagonistic potential against fungal plant pathogens.

## Materials and Methods

### Isolation of antagonist

The rhizosphere soil samples were collected from the brinjal fields from different areas of Kodad Mandal, Suryapet Dist., Telangana, India. *Trichoderma* spp. were isolated on Potato Dextrose Agar (PDA) medium by soil dilution plate technique (Johnson and Curl, 1972) using $10^{-3}$ to $10^{-5}$ dilutions. The plates were incubated at 26 ± 2°C for 5 days. *Trichoderma* colonies appeared in the plates were noted and sub cultured. They were purified by single spore isolation method and maintained on potato dextrose agar (PDA) slants. Based on culture characters as well as microscopic parameters, *Trichoderma* isolates were identified up to species level (Rifai, 1969; Nagamani *et al.,* 2006). The pure cultures were stored in the refrigerator at 4°C for further studies.

### Qualitative assay of enzymes

Enzyme assay of *Trichderma* isolates was carried out by plate assay on the respective solid media for extracellular enzymes. Assay was based on the formation of clear zones, change of colour and its intensity around the fungal colonies for production of cellulase, amylase, pectinase, protease and chitinase enzymes. The independent experiments were performed for this screening step with three replicates for each strain.

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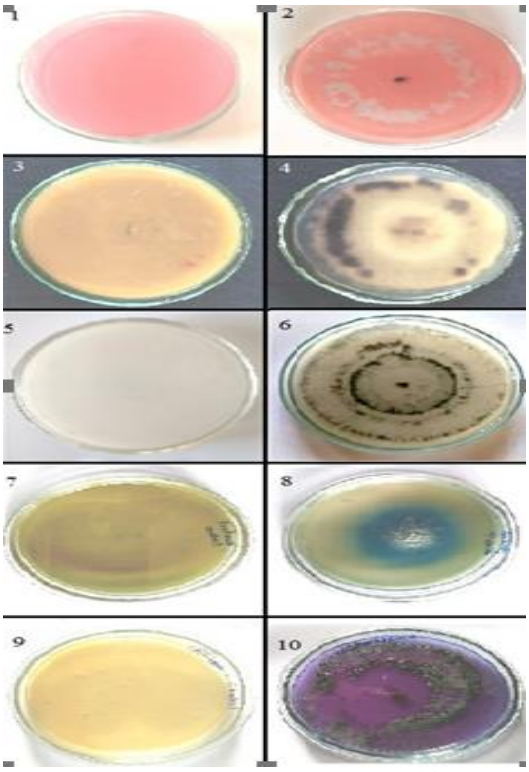
**Cellulase assay:**
For cellulase assay (Hankin and Anagnostakis, 1975), the *Trichoderma* strains were grown on the Czapek-Mineral Salt Agar Medium ($KH_2PO_4$ 1.00 g, $MgSO_4.7H_2O$ 0.50 g, $NaNO_3$ 2.00 g, KCl 0.50 g, Peptone 2.00 g, and Agar 20.00g, Distilled water 1000ml) supplemented with Carboxy Methyl Cellulose (CMC) 5.00g. The medium was aseptically transferred to petri dishes and inoculated with a 6mm agar disc cut from 5-day old fungal culture of each strain separately and incubated at 26 ± 2°C in darkness for 3 to 5 days. The plates were flooded with aqueous Congo red (2% w/v) solution for 15 min. Then, the agar surface was washed with distilled water and the plates were flooded with NaCl (1 M) for 1.5 min. Production of cellulase was observed by the formation of yellow-opaque area around the colonies. Diameters of the colony and the clear zone were measured (Fig. 1, 2).

**Amylase assay:**
Amylase activity (Hankin and Anagnostakis, 1975) was assessed by growing the *Trichoderma* strains on Starch Agar Medium (Starch 20.00g, Beef extract 3.00g, Peptone 5.00g, Agar 16.00g, Distilled water 1000 ml). The medium was aseptically transferred to petri dishes and inoculated with a 6mm agar disc cut from 5-day old fungal culture of each strain separately and incubated at 26 ± 2°C in darkness for 3 to 5 days. The plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered positive for amylase activity (Fig. 3, 4).

**Pectinase assay:**
For pectinase activity (Hankin and Anagnostakis, 1975), the *Trichoderma* strains were grown on Pectinase Agar Medium (Pectin 5.00g, Yeast extract 1.00g Agar 15.00g, Distilled water 1000 ml). The medium was aseptically transferred to petri dishes and inoculated with a 6mm agar disc cut from 5-day old fungal culture of each strain separately and incubated at 26 ± 2°C in darkness for 3 to 5 days and the plates were flooded with 1% (10 g/lt in distilled water) hexa decyl trimethyl ammonium bromide (CTAB). A clear zone formed around colony indicates pectinase activity (Fig. 5, 6).

**Protease assay:**
For protease screening (Vijayaraghavan and Samuel, 2013), the *Trichoderma* strains were grown on Casein Agar Medium (Peptic digest of animal tissue 5.00g, Beef extract 1.50g, Yeast extract 1.50g, Sodium chloride 5.00g, Agar 15.00g, Casein 10.00g, and Distilled water 1000ml). The medium was aseptically transferred to petri dishes and inoculated with a 6mm agar disc cut from 5-day old fungal culture of each strain separately and incubated at 26 ± 2°C in darkness for 3 to 5 days. The plates were flooded with Bromo Cresol Green dye. The clear distinct zone indicates proteolytic activity. A distinct zone surrounded by greenish-blue colour is pH dependent (8.0±0.2). The proteolytic activity appears as a colourless zone, while the rest of the plates as greenish-blue in colour (Fig. 7, 8).

**Chitinase assay:**
The Chitinase Detection Medium (Agrawal and Kotasthane, 2012) consisted of a basal medium comprising (4.5 g of Colloidal chitin, 0.30g of $MgSO_4.7H_2O$, 3.00g of $NH_4SO_4$, 2.00g of $KH_2PO_4$, 1.00 g of Citric acid monohydrate, 15.00g of Agar, 0.15g of Bromo cresol purple and 0.20 ml of Tween-80) per liter, pH was adjusted to 4.7 and then autoclaved at 121°C for 15 min. Colloidal chitin was prepared from commercial chitin (HiMedia) and was amended in the chitinase assay medium as a sole carbon source. Colloidal chitin (Murthy and Bleakley, 2012) was prepared and stored at 4°C until further use. After cooling, the medium was poured in to petri plates and allowed to solidify. The actively growing *Trichoderma* culture plugs of the isolates to be tested for chitinase activity was inoculated into the medium and incubated at 26 ± 2°C for 3-5 days and observed for the colored zone formation. Chitinase activity was identified by the formation purple colored zone. Bromo cresol green reagent sharply increases the colour intensity of the plate, as it binds on unhydrolyzed protein in the plate. Color intensity and diameter of the purple colored zone were taken as the criteria to determine the chitinase activity (Fig. 9, 10).




**Figutre** 1-2. Cellulase plates; 1. Control, 2. Treatement; 3-4 Amylase plates; 3. Control, 4. Treatement; 5-6 Pectinase plates; 5. Control, 6. Treatement; 7-8 Protease plates; 7. Control, 8. Treatement; 9-10 Chitinase plates; 9. Control, 10. Treatement.

## Results and Discussion

A total of seven *Trichoderma* spp. were obtained from rhizosphere soil samples. The isolates found in this study showed variation in culture characters as well as microscopic parameters. *Trichoderma* was initially white and after two days, it turned slightly green. At the moment, the wooly conidia filled up and were compact at the midpoint of a petri dish with mostly dark green colour. Microscopic examinations were conducted on the conidiophores that typically formed paired branches and displayed pyramidals arranged along the length of the primary axis. Variations were seen in the phialides, typically enlarged in the middle; long, bottle shaped, inflated at the base, and some were cylindrical. Phialides were held in the whorls that were commonly flask shaped, densely clustered on a wide main axis. The isolates were identified up to species level. Based on these results the isolates were assigned to different species viz., *Trichoderma viride, T. harzianum, T. virens, T. atroviride, T. koningii, T. pseudokoningii* and *T. reesei.* The species found in this study were commonly reported earlier either from India or elsewhere.

*Trichoderma* spp. were screened for secretion of lytic enzymes such as cellulase, amylase, pectinase, protease and chitinase. The isolates were grouped according to the diameter of the decoloured or/and colour intensity as: - isolates showing no enzyme activity, + isolates showing very low enzyme activity, ++ isolates showing low enzyme activity, +++ isolates showing high enzyme activity, ++++ isolates showing very high enzyme activity (Table 1).

*T. harzianum, T. reesei, T. atroviride* isolates showed very high cellulase activity. *T. koningii and T. pseudokoningii* showed low cellulase activity whereas, *T. viride and T. virens* showed very low cellulase activity. The fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source (Khalid *et al.,* 2006). Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. The appearance of the clear zone around the colony after the addition of congo red solution was strong evidence for the secretion of cellulase enzymes. Cellulase and the -1, 3- glucanase are the two enzymes that play important role in the enzymatic degradation of cell walls of phytopathogenic fungi like *P. ultimum* during mycoparasitic interaction (Kamala and Indira, 2014). Benhamou and Chet (1997) reported that large amounts of cellulytic enzymes are produced and play a key role in breaching the host cell walls at sites of attempted penetration of *Trichoderma* into the host cell walls. Vinit Kumar Mishra, (2010) observed that the *T. viride* exhibited highest cellulase activity of 3.6µ/ml and it was found to suppress the mycelial growth of *Pythium aphanidermatum.*

*T. viride, T. virens, T. koningii and T. pseudokoningii* showed very high amylase activity, *T. harzianum* high amylase activity and *T. reesei* exhibited low enzyme activity. Whereas, *T. atroviride* exhibited very low enzyme activity. Amylase excretion, not known to be associated with phytopathogens was detected in *Trichoderma* species (Maria *et al.,* 2001). Our results are partially in agreement with Maria *et al.,* (2001) that *T. harzianum, T. viride* and *T. koningii* produced extracellular cellulase and pectinases. Amylases are also employed in the starch processing industries for the hydrolysis of polysaccharides.

*T. koningii* showed high pectinase activity, *T. pseudokoningii*, exhibited low pectinase activity, whereas *T. harzianum, T. viride, T. virens* and *T. atroviride* showed very low enzyme activity. *T. reesei* showed no pectinase activity. Fungi produce several extracellular enzymes that result in the decomposition of organic matter and one such enzyme is pectinolytic enzymes. *Trichoderma* produces these enzymes to break down the middle lamella in plants so that it can extract nutrients from the plant tissues and insert fungal hyphae. Our results are in agreement with Claudia *et al.,* (1997) that *Trichoderma* spp. demonstrated aggressive biocontrol ability towards *Aspergillus* and *Fusarium moniliforme* explained by the liberation of extracellular enzymes such as amylase, pectinase, protease and cellulase activity.

*T. harzianum,* showed very high protease activity, *T. koningii* and *T. atroviride* high enzyme activity whereas, *T. reesei, T.viride, T. virens* and *T.pseudokoningii* exhibited low enzyme activity. It has been suggested that this protease is involved in the degradation of pathogen cell walls, membranes and even proteins released by the lysis of the pathogen, thus making nutrients available for the mycoparasite (Goldman *et al.,* 1994). Filamentous fungal cell wall also contains lipids and proteins (Hunsley *et al.,* 1970). Biocontrol of *Botrytis cinerea* by *T. harzianum* has been attributed to the action of proteases produced by the biocontrol agent that inactivate hydrolytic enzymes produced by the pathogen (Howell, 2003). Kapat *et al.,* (1998) suggested that biocontrol of *B. cinerea* by *T. harzianum* might be due to production of proteases by *T. harzianum* that inactivate the hydrolytic enzymes produced by *B. cinerea* on bean leaves. Fungal proteases play a significant role in cell wall lyses by catalyzing the cleavage of peptide bonds in proteins (Mata *et al.,* 2001). Proteases secreted by *T. harzianum* were effective in reducing brown spot disease severity and pathogen sporulation on faba bean leaves inoculated with *Botrytis fabae* (Haggag *et al.,* 2006).

*T.harzianum, T.pseudokoningii* and *T.atroviride* showed very high chitinase activity, *T.viride, T.virens* and *T.koningii* showed high enzyme activity, where as *T.reesei* showed low enzyme activity. Cell wall of the phytopathogen includes chitin and other complex

macro molecules. The final step is penetration of the host mycelium, which is enabled by partial degradation of its cell wall via secretion of mycolytic enzymes, mainly chitinases and glucanases. In order to metabolize these compounds, the antagonistic fungi need to synthesize chitinase to break down chitin and other allied compounds. Hence quantification of this enzyme relates to the extent of mycoparasitism shown against the pathogen. Benitez *et al.,* 2004 demonstrated that *Trichoderma* strains that over produce chitinases have been shown to be effective biocontrol agents against various pathogens. Because the skeleton of pathogenic cell walls contain chitin, glucan and proteins, enzymes that hydrolyze these components have to be present in a successful antagonist in order to play a significant role in cell wall lysis of the pathogen. It is well documented that chitinases and glucanases play key role in mycolytic activity of *Trichoderma* spp. against several fungal pathogens (Elad *et al.,* 1982). The possible role of chitinolytic enzymes in biocontrol is supported by the work of Lorito *et al.,* (1998) who transferred the gene encoding endochitinase from *T. harzianum* into tobacco and potato and demonstrated high level and broad spectrum of resistance against number of plant pathogens.

*Trichoderma* sp. through the mechanisms of mycoparasitism can be able to cleave the hyphae cell wall of the pathogen by secreting enzymes such as glucanases and chitinases (Eduardo *et al.,* 2011). Our results are in agreement with works of Anitha *et al.,* (2012) who reported various extracellular enzymatic activities such as cellulolytic, amylolytic, chitinolytic, pectinolytic and proteolytic in substantial quantity from *T. atroviride*. These lytic enzymes function by breaking down the polysaccharides, chitin, and -glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity of onion roots, infected with *Sclerotium cepivorum* by *T. koningii* (Metcalf and Wilson, 2001).

The antagonistic activities of *Trichoderma* spp. evaluated *in vitro* against *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *melongenae* by dual culture plate technique and production of volatile and non-volatile metabolites showed considerable reduction in the growth of the pathogens (Ramaraju *et al.,* 2016, 2017). The *Trichoderma* restrict the growth of phytopathogens and in most cases, grow over the zone of inhibition, overgrowing the pathogen and sporulating thereafter. The inhibition in radial growth of two interacting organisms in dual culture has been attributed to secretion of extracellular hydrolytic enzymes (Schirmbock *et al.,* 1994) or cell wall degrading enzymes such as chitinases, glucanase, there by destroying cell wall integrity (Elad, 2000).

## Conclusion

In the present study, *Trichoderma* species isolated from brinjal rhizosphere soil samples were of common occurrence. Occurrence of seven species isolated from a single sample reveals the existence of diversity of *Trichoderma* species in the soil samples of brinjal cultivated fields. The results obtained showed that the qualitative methods are valid and important in selection of biocontrol agents. These methods in plates reveal feasibility for an initial selection of strains for screening large number of samples. Most of the *Trichoderma* isolates have exhibited ability in hydrolyzing lignin, cellulose, and starch. These *Trichoderma* isolates can be applied as biocontrol agents in controlling disease and increasing yield and production in the agriculture. The use of simple solid media permits the rapid screening of large populations of fungi for the presence or absence of specific enzymes. However, knowledge of the types, amounts and characteristics of enzymes produced by *Trichoderma* cited above would be studied for selecting organisms best suited for biocontrol in agriculture and industrial requirements. Further research has to be done to quantify the lytic enzymes and *in vivo* experiments to be conducted against phytopathogens.

**Table 1.** Qualitative and quantintitative primary screening of *Trichoderma* spp. for lytic enzymes.

| | Amylase | Cellulase | Protease | Pectinase | Chitinase |
|---|---|---|---|---|---|
| Th | +++ | ++++ | ++++ | + | ++++ |
| Tr | ++ | ++++ | ++ | _ | ++ |
| Tvd | ++++ | + | ++ | + | +++ |
| Tvns | ++++ | + | ++ | + | +++ |
| Tk | ++++ | ++ | +++ | +++ | +++ |
| Tpk | ++++ | ++ | ++ | ++ | ++++ |
| Ta | + | ++++ | +++ | + | ++++ |

Th- *T. harzianum*; Tr- *T. reesei*; Tvd- *T. viride*; Tvns- *T. virens*; Tk- *T. koningii*; Tpk- *T. pseudokoningii*; Ta- *T. atroviride*;

_ Isolates showing no enzyme activity, + Isolates showing very low enzyme activity, ++ Isolates showing low enzyme activity, +++ Isolates showing high enzyme activity, ++++ isolate showing very high enzyme activity.

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