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CHARACTERIZATION OF EFFICIENT CHITINOLYTIC ENZYME PRODUCING *TRICHODERMA* SPECIES: A TOOL FOR BETTER ANTAGONISTIC APPROACH

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Abstract: *Trichoderma* is opportunistic, avirulent plant symbiont and pathogenic to other fungi. Chitinolytic enzyme producing *Trichoderma* species have long been recognized as an agent for controlling plant diseases caused by various phytopathogenic fungi. Chitin is the main structural component of fungi. Chitinase is an enzyme responsible to metabolize the chitin. Primary screening of chitinase producing fungus is essential to obtain an efficient and novel bio control agent. In this study, three *Trichoderma* species were isolated (*Trichoderma harzianum, Trichoderma flavofuscum, Trichoderma viride*) and their macro- and microscopic observation was done. Furthermore, they were screened for its chitinase production, using Chitinase Detection Medium. The basic findings will be helpful to a mycologist for study and screening of chitinase overproducing fungi at the primary level.
Key words: Biocontrol mechanisms, *Trichoderma*, Chitinase, Phytopathogen, Chitin.

Introduction

Imperfect *Trichoderma* fungi has application as an antagonist of phytopathogenic fungi to control plant diseases (Monte, 2001), few strains have the ability to kill the plant pathogen under variety of environmental conditions. Fungi showing biocontrol activity under the genus *Trichoderma* has developed surprising ability to interact parasitically and symbiotically (Harman and Kubick, 1998).

The most commonly used BCAs of genus *Trichoderma* is *T. harzianum, T. flavofuscum and T. viride.* There are several antagonistic mechanisms used by *Trichoderma,* mainly antibiosis and mycoparasitism where by bio control agent directly attack the plant pathogen by secreting lytic enzymes such as chitinase, β-1, 3-glucabase, cellulase and proteases (Haran et al*.,* 1996a; Balasubramanian, 2003). These enzymes hydrolyse the pathogen's cell wall components such as chitin, glucan, cellulose and proteins successfully limiting the growth of fungal pathogens (Lorito et al., 1994; Carsolio et al*.,* 1999). As the skeleton of the fungal cell wall mainly contains chitin, glucan and proteins, mycoparasitism and enzymes that hydrolyze these components are one of the main mechanisms accounting for showing antagonistic

activity against plant pathogenic fungi. Chitinase, β-1, 3-glucanase and cellulase are important in the hyper-parasitic mechanism. The distribution of chitinases in nature is very common. It has been established that the chitinase producing *Trichoderma* spp. can be effective biocontrol agents against fungal pathogens (Tokimoto, 1982; Sivan and Chet, 1989; Kubicek et al., 2001). Several distinct chitinolytic enzymes have been reported in *T. harzianum* (De la Cruz et al., 1992), which are secreted by in liquid culture supplemented with only chitin as a carbon source.

Material and Methods

Isolation of efficient *Trichoderma* species

Triplicate soil sample were randomly collected and the isolations of antagonistic fungal species were performed by using serial dilution plate technique (pour plate method) on Rose Bengal Medium and Potato Dextrose Agar. Emerging colony of fungi were isolated, stained with lacto-phenol cotton blue, based on the microscopic observation and colony characteristics, colonies of were selected. Purification was done by successively striking and then transferred to new PDA plates **(Figure. 2)** and stored at 4^{o}C for further experiments.

Genus and species-level identification of *Trichoderma* were carried by studying the microscopic features such as conidiophores, branching, phialides shape and position, spore size and shape (Gams and Bisset, 1998). The confirmations of species-level identification of *Trichoderma* species were carried with the support of ITCC, Indian Agriculture Research Institute, New Delhi (India).

Morphological, cultural and microscopic characteristics of isolated *Trichoderma* species

Taxonomic identification of fungi (based on purely morphologically macro- and microscopic characteristics) was carried out according to International Scientific Mycological References. The major and remarkable macroscopic features in species identification were the colony features, including diameter after 7 days, color of conidia, mycelial color, colony reverse, colony texture and shape.

For microscopic study, Lacto-phenol cotton blue staining procedure was used. For the proper visualization of characteristic features, slides were prepared from older colonies, because they were covered with too many spores. However, for examination of characters of spores, the slides were prepared from two-week old culture. Microscopic characteristics for the identification were conidial head; conidia shape, roughness and vesicle serration was taken into consideration.

Study of cultural characteristics of *Trichoderma harzianum*, *T. flavofuscum and T. viride* was done. Isolated *Trichoderma* species were grown on different medium, i.e. PDA, (Potato Dextrose Agar), RBA (Rose Bengal Agar), CzA (Czapeks Dox Agar) and TSM (*Trichoderma* selective media). Morphological features like growth rate, colony color, colony texture, rate of sporulation and color of colony reverse were studied. Depending upon the rate of sporulation and growth rate, the proper sporulating medium was selected for further study.

T. harzianum grows very fast, having smooth surfaced at submerged conidiation with watery white color of mycelia. In case of *T. flavofuscum* conidiation appears late. Mycelia appear in white color with submerged condition. *T. viride* grows with fewer yellow pale green coloration with very high and early sporulation, mycelia with submerged growth, on the reverse side appeared colorless.

Screening of *Trichoderma* species for its chitinolytic enzyme overproducing activity

The strains of *Trichoderma* were screened for overproducing chitinolytic activity, by using the method of Kotasthane and Agrawal, (2009), which is the basis of selection, performance and *in vivo* bioefficacy. Here to study the chitinase activity chitinase detection medium was used. The final chitinase detection medium [(all amounts are per liter) 4.5 g of colloidal chitin, 0.3 gm of MgSO4. 7H2O, 3.0 g of (NH_4)SO_4,2.0 2.0 2.0 g KH_2po_4, 1 g of citric acid monohydrate, 15 g agar, 0.15 g bromocresol purple and 200 µl of tween-80)], pH was adjusted to 4.7 and was autoclaved at 121° C for 15 min. The medium was poured into the 90mm Petri's plates and allow it to solidify. The fresh culture plugs of the *Trichoderma* to be tested for chitinase activity was inoculated and incubated at 25^o C for 3-4 days. Formation of the purple colored zone was observed and recorded.

Result and Discussion

Trichoderma species were isolated from soil samples collected from various sites of agricultural field, by using serial dilution's method. Genus and species- level identifications were done based on colony morphology and microscopic observation was done by using lacto-phenol cotton blue stain. The confirmation of species-level identification of *Trichoderma* was carried out with the support of ITCC, Indian Agriculture Research Institute, New Delhi (India).

The genus *Trichoderma* was identified when grown on Rose Bengal Agar **(Figure 1)** Colony had key characteristics that can be used to identify them as *Trichoderma,* including growth

pattern, growth speed, odor and color **(Table 1)** (Gams and Bissett, 1998). *Trichoderma* species was isolated by dilution plate method (Weinhold and Bowman, 1968). The cultures were purified, and species-level identification of *Trichoderma* species was based on the growth of the colony on culture media, conidiation; exudates formed, branching of conidiophore, septatation of mycelium, color, structure and size of conidia **(Table 2).** Microscopic examination (Figure. 3) was carried out according to Bissett (1984, 1991a, b, c) classification method.

Cultures were found to be fast growing between the ranges of temperature 25-30°C. A characteristic sweet or 'coconut' odor is produced by some species. Conidiophores were highly branched and difficult to define, and loosely or sometime compactly tufted, often observed in distinct concentric rings. Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and sometimes phialides arising directly from the first axis to the tip. All primary and secondary branches arise at or near 90° C with respect to, the main axis. The diameter of the purple colored zone was taken as main criteria to determine the chitinolytic activity after 3-4 days of incubation.

Three species of *Trichoderma* were screened and selected for their chitinolytic enzyme production based on the diameter of purple color zone surrounding the colony on chitinase detection media in a shorter time **(Figure. 4).** *Trichoderma harzianum*, shows the larger purplish color zone as compared to *T. viride* and *T. flavofusssscum*. *Trichoderma* exhibiting higher chitinase activity on chitinase detection media were selected maintained on PDA (Potato Dextrose Agar) and RBA (Rose Bengal agar) medium and were used for further studies. Among all the three *Trichoderma* species, *T. harzianum* shows the higher diameter of the purple color zone. In case of *T. viride* and *T. flavofuscum* measured purple colored zone was nearly equal in diameter.

Purple color zone was due to bromocresol purple that was supplemented with media as a pH indicator. As the medium containing colloidal chitin, *Trichoderma* breaks down the chitin with chitinolytic enzymes, changing chitin to N-acetylglucosamine, which is fundamental in nature. Thus, change in the pH from acidic to basic, color of media also changes from yellow to purple color (Kotasthane and Agrawal, 2009). Chitin agar plate has been used earlier for isolating chitinolytic microorganisms by observing clear zone around the colony of microorganism (Wirth and Wolf, 1990). In fungi, chitinases play important biological and physiological roles, containing autolytic, nutritional, morphogenetic, and parasitic roles.

Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism (Haran et al., 1996)

Conclusion

Based on the results of this study it can be concluded that, the selection of suitable biological agents and optimization of its accepted cultural characteristics for their better antagonistic effect is important in designing an effective and safe biocontrol strategies. However, the medium used here could be very effectively used as an economical source for basic screening and categorization of large fungal populations for its chitinase activity. This medium is suitable for rapid and user friendly sensitive plate assays. Formation of the purple color zone was found to be the easier alternative method for the selection of chitinolytic strains of *Trichoderma* species.

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Tables-

Table 1. Morphological studies of wild *Trichoderma harzianum, T. flavofuscum and T. viride.*

SN.	*Trichoderma* Species	Diameter	Conidiation	Color of mycelia	Spore color	Sporulation
1	WH	56mm	Smooth surface submerged	Watery white	No sporulation	No sporulation
2	WK	40mm	No conidiation	White submerg-ed dull	No sporulation	No sporulation
3	WV	35mm	Less, yellow-pale green, submerged, reverse-colorless	White	Yellow to pale green	Sporulation appears

Table 2. Comparative accounts of macroscopic characters of *Trichoderma* species

SN.	Characteristics	*T. viride*	*T. harzianum*	*T.flavofuscum*
1.	Growth rate (cm), colony color	8-9.0cm after 4 days, watery white-bluish green.	9.5cm in 4 days, whitish green-bright green.	7-9.5cm in 4 days, yellowish-olive green.
2.	Colony mycelia	Floccose, whitish	Floccose, compact, whitish	Effuse to wooly, white or grayish.
3.	Colony reverse	Yellow to amber, old colonies emits coconut odor.	Colorless to drab color.	Colorless
4.	Conidiophore branching	Irregularly branched, compact forming conifer like branching.	Much branched, form loose tufts which arise in ring like zone.	Complexly branched, basal part is unbranched.
5.	Conidiation, conidial color	Abundant, loose tuft, green	Moderate, compact, yellow- pale green	Abundant, effused, cushion shape near margin, pale green.
6.	Conidia shape	Rough, globose to obviod	Smooth, subglobose.	Smooth subglobose.
7.	Exudates/Pigmentation	No exudates or pigmentation.	Colorless to pale amber, yellowish in some species.	Colorless to pale amber in small droplets.

Photoplates:

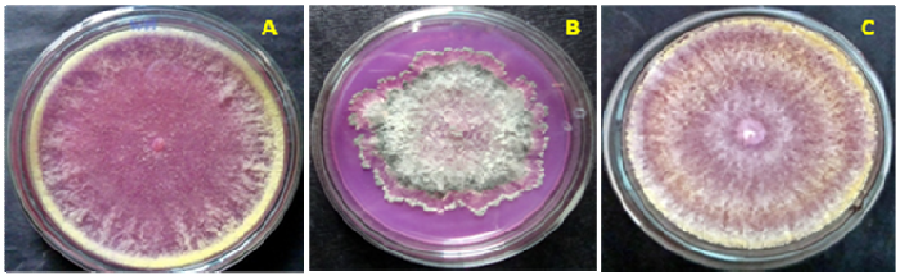

Fig1. **A-** *T. harzianum*, **B -** *T. viride*, **C-** *T. Flavofuscum* on Rose Bengal Agar media.

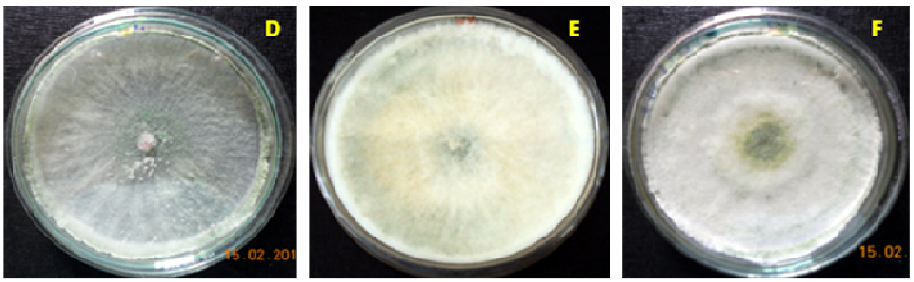

Fig.2. **D-** *T. harzianum*, **E-** *T. Viride*, **F-** *T. flavofuscum* on Potato Dextrose Agar media.

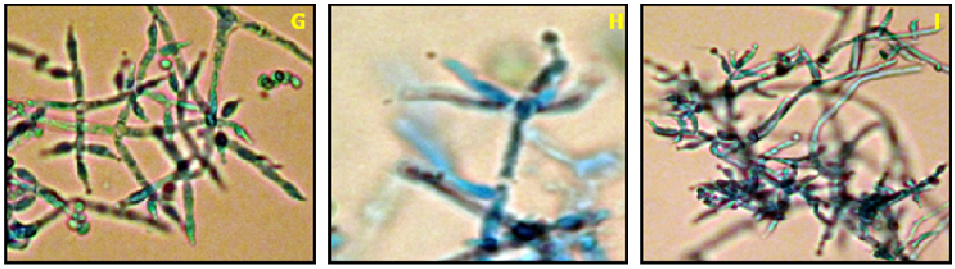

Fig 3. **Microscopic photographs** of **G** -*T. harzianum*, **H**-*T. viride*, **I**-*T. flavofuscum*

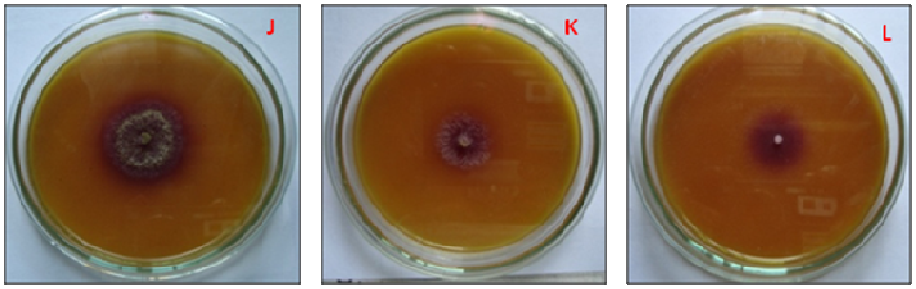

Fig 4. Screening of chitinase overproducing isolates of *Trichoderma* species on Chitinase detection medium. J - *T. harzianum,* K - *T. viride,* L - *T. flavofuscum*

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