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Chitinolytic assay for *Trichoderma species* isolated from different geographical locations of Uttar Pradesh

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Chitin is the most commonly available polymer on the earth. Cell walls of most of the fungi are made up of chitin. As we all know that *Trichoderma* produces a wide variety of cell wall degrading enzymes (CWDEs) such as chitinase, xylanase, glucanase and cellulase. Out of these CWDEs chitinase is of prime importance as it is the building block of fungal cell walls. For the detection of chitinase activity we used a simple and sensitive method. We supplemented the chitinase detection media with coloidal chitin as a carbon source and bromocresol purple as pH indicator dye. This method is easy, sensitive, reproducible and economical. Colloidal chitin derived from sea-shells and commercial chitin is supplemented as carbon source in chitinase broth and solid media for the detection of chitinolytic and exochitinase activity. The chitinolytic activities were ranged from 6.2 to 3.9 and 4.8 to 1.8 mg/ml and exochitinase activities ranged from 0.0133 × 10^{-3} to 0.0076 × 10^{-3} and 0.00609 to 0.0055 × 10^{-3} U/ml), respectively, with colloidal chitin derived from commercial chitin and sea-shells.

Key words: Bromocresol purple, chitin, N-acetyl-β-D-glucosamine, p-nitrophenol, *trichoderma*, volume activity.

INTRODUCTION

Chitin is an unbranched polymer of 1,4-β- linked N-acetyl-D-glucosamine (NAGA). Chitin is the building block of fungal cell walls. Chitinase are enzyme that degrade the chitin by breaking the β-1,4 linkages. Chitinases occur in a wide variety of microorganisms including bacteria, fungi, insects etc. In fungi, chitinases are believed to have autolytic, nutritional and morphogenetic roles. In mycoparasitic fungi, chitinases are associated with the degradation of cell walls. *Trichoderma* spp. is the most commonly used biocontrol agents against several soil-borne fungal plant pathogens such as *Sclerotium rolfsii, Rhizoctonia solani and Pythium* spp. Etc Bhattachrya et al., 2007). Members of the fungal genus *Trichoderma* spp. produce cell wall degrading enzymes such as glucanase, chitinase xylanase etc., that are involved in the mycoparasitic action. Chitinase enzymes are of great importance as compared to other CWEDEs, as fungal cell wall is made up of chitin that is why chitinolytic enzyme degrade phytopathogenic fungi easily (González et al., 2012). *Trichoderma* spp. employ different strategies of defense against phytopathogens such

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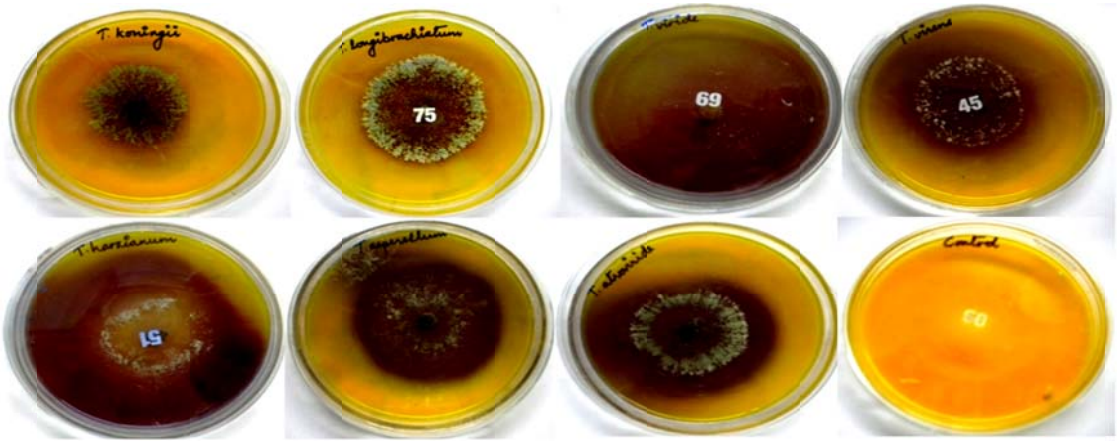

Figure 1. Chitinase activity observed after 3 days of inoculation in chitinase detection media supplemented with colloidal chitin (set-1).

as: competition for space and nutrients, secretion of cell wall degrading enzymes, induction of resistance etc. (Rifat et al 2013). *Trichoderma* inhibit the hyphal growth of phytopathogens by coiling, it uses hooks to penetrate the fungal cell walls with the help of cell wall degrading enzymes such as xylanase, chitinase, cellulase etc., among the different mechanisms used by *Trichoderma* spp. parasitism, competition and antibiosos are the main mechanisms which are involved in mycoparasitic action. Cell wall degrading enzymes are the key factors which involved in the cell wall destruction of pathogen (Kowsari et al., 2014).

MATERIALS AND METHODS

Isolation and maintenance of fungal isolates

Trichoderma strains were isolated from soil samples collected from the different geographical locations of U.P. and were maintained on potato dextrose agar (PDA) plates. Colloidal chitin used as a carbon source was derived from sea-shell and commercial chitin (CDH). For colloidal chitin preparation, acid hydrolysis was done by conc. HCl during acid hydrolysis of which the flasks were kept in constant stirring using magnetic stirrer for 24 h. After 24 h, this chitin and acid mixture was kept at 4°C and left for overnight. After incubation period the acid mixture is treated with 2000 ml of ice cold 95% ethanol and kept at 26°C for overnight. After incubation period, it was centrifuged at 3000 rpm for 20 min at 4°C. After centrifugation the supernatant was discarded while pellet is washed with distilled water by centrifugation at 3000 rpm for 5 min, till the smell of alcohol is removed (Saraswathi et al. 2013). The colloidal chitin thus obtained has a soft and white consistency with 90 to 95% moisture and stored at 4°C till use (Roberts and Selitrennikoff, 1988).

Agar medium for detection of chitinase-positive microorganisms

Chitinase detection medium supplemented with colloidal chitin as carbon source was used for the chitinase plate assay (Shahidi et al., 2005). Lukewarm media was poured into the Petri plates and allowed to solidify; after solidification, fresh culture plugs of the *Trichoderma* spp. tested for chitinase activity were placed in the middle of the plate. Plates were incubated at 25±2°C and were observed for purple colour zone formation. Chitinase activity exhibited by seven *Trichodrema* spp. was determined by measuring the diameter of purple color zone after three to seven days of incubation (Agrawal and Kotasthane, 2012) (Figures 1 to 4).

Total chitinolytic activity

Total chitinolytic activity was calculated by measuring the release of reducing saccharised from carbon source (colloidal chitin). A reaction mixture was prepared containing 1 ml culture supernatant and 0.3 ml of sodium acetate buffer (pH 4.6); to this mixture, 0.2 ml of colloidal chitin was added and incubated for 20 h at 40°C. After incubation, the contents of the tube were centrifuged at 13000 rpm for 5 min at 5°C.

After centrifugation 0.75 ml of supernatant was taken and 0.25 ml of 1% salicylic acid was added to the mixture 1 ml of 0.7 M NaOH and 10 M NaOH were added and heated at 100°C for 5 min. OD of the reaction mixture was taken at 582 nm. Reference curve was made with N-acetyl-β–D-glucosamine (NAGA). Chitinolytic activity was expressed in terms of mg/ml (Muzzarelli et al 1997).

Exochitinase activity

Exochitinase activity was measured by the release of p-nitrophenol (pNP) from p-nitrophenyl-Nacetyl- β-Dglucosaminide (pNPg). A reaction mixture was prepared containing 25 ul of culture filtrate and 0.2 ml of p- nitrophenol solution, to this reaction mixture 1 ml of 0.1 M sodium acetate buffer was added. This reaction mixture was incubated at 40°C for 20 h. After incubation period, contents of the tube were centrifuged at 13000 rpm. After centrifugation, 0.125 M sodium tertaborate- sodium hydroxide buffer was added to the 0.6 ml of supernatant. OD was taken at 400 nm.

RESULTS AND DISCUSSION

When *Trichoderma* strains were inoculated on chitinase media containing colloidal chitin (carbon source) and bromocresol purple (pH indicator dye) breakdown of

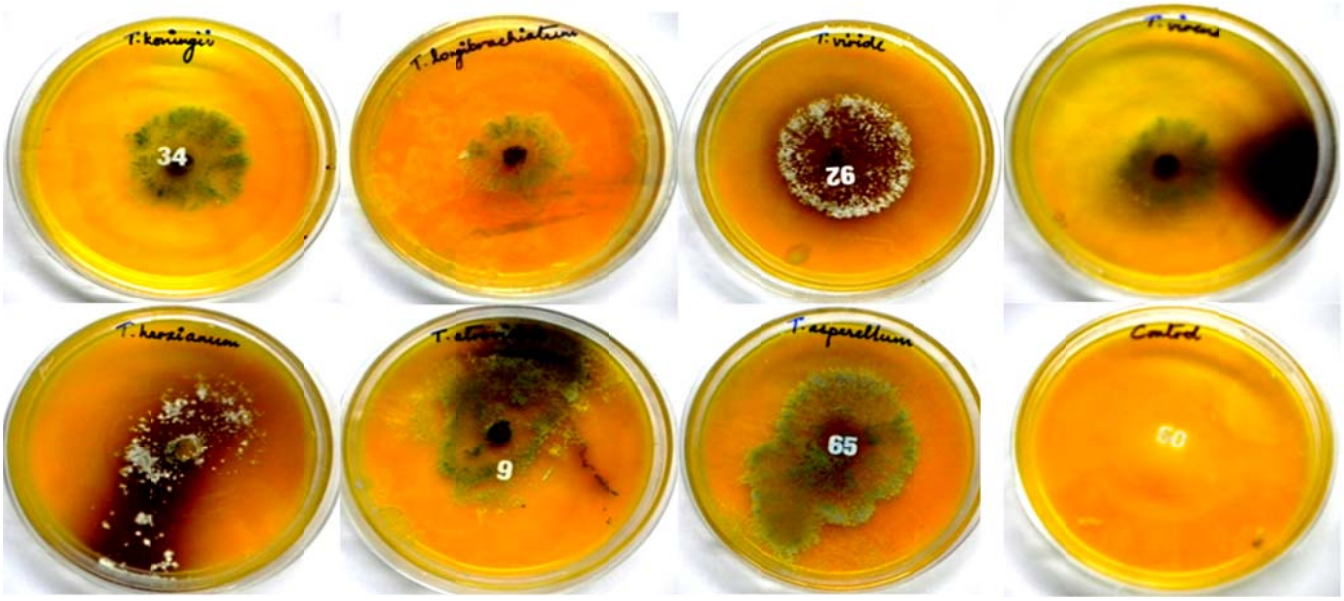

Figure 2. Chitinase activity observed after 3 days of inoculation in chitinase detection media supplemented with sea- shell chitin (set-1).

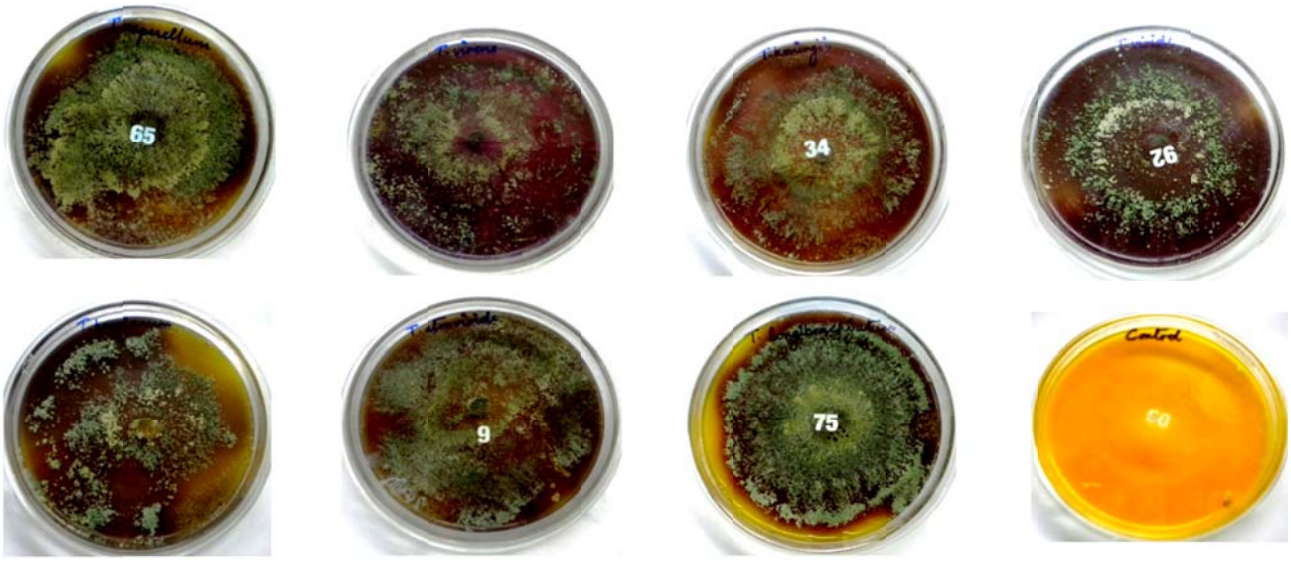

Figure 3. Chitinase activity observed after 7 days of inoculation in chitinase detection media supplemented with seashell chitin (Set-2).

chitin occurs into N-acetyl glucosamine which causes a change in pH (acedic to alkaline). This change in pH is indicated by the change in colour of media from yellow to purple zone surrounding the inoculated culture plug area.

Chitinase activity exhibited by the seven strains of *Trichoderma* was evaluated through the formation of purple coloured zone after three and seven days of incubation. No complicated protocols were adopted for the evaluation of chitinase activity (Gomez et al., 2004). Total chitinolytic activity was assayed by measuring the release of reducing saccharides from colloidal chitin (Table 1).

Standard curve generated by the use of NAGA is used to evaluate the reducing saccharide conc. The observations were in close resemblance with those De la Cruz et al. (1992) and Lorito et al. (1994). Production of hydrolytic enzymes is greatly affected by the cultural conditions. For exochitinase activity, release of p-nitrophenol (pNP) from pnitrophenyl-N-acetyl-β-D-glucosaminide (pNPg) was measured. The volume activity

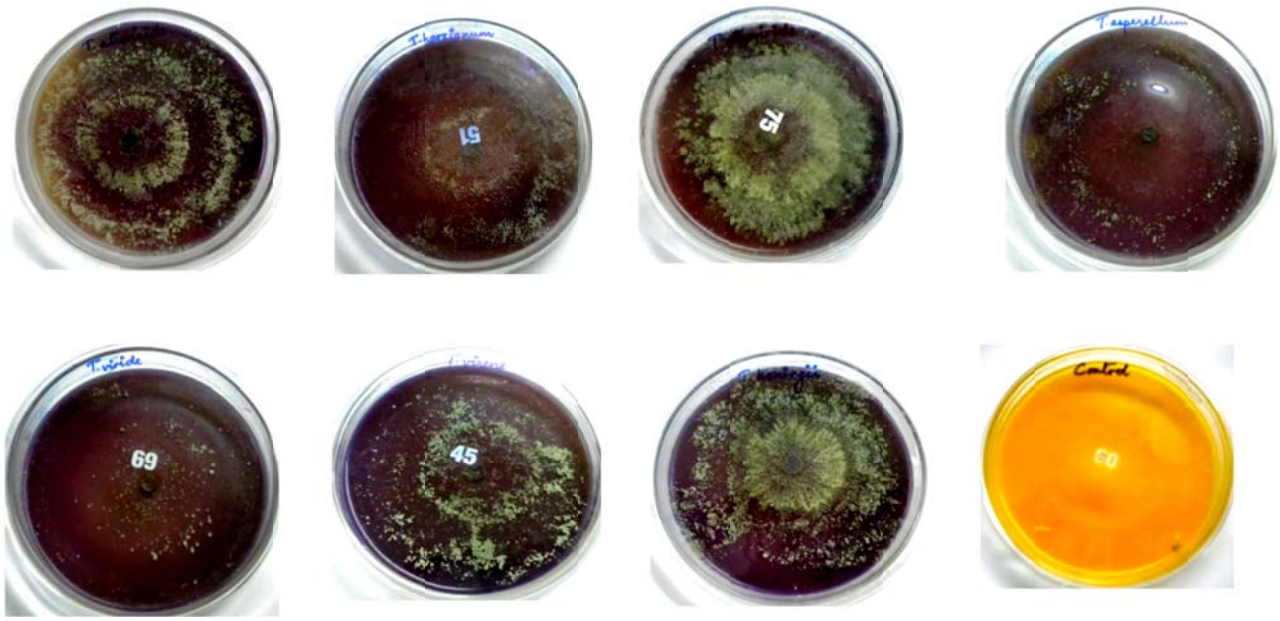

Figure 4. Chitinase activity observed after 7 days of inoculation in chitinase detection media supplemented with colloidal chitin (Set-1).

Table 1. Chitinolytic and exochitinase activity of *Trichoderma* species.

Isolate name	Total chitinolytic activity (mg/ml)		Exochitinase activity (U/ml X 10^{-3})	
	Colloidal chitin	Seashell chitin	Colloidal chitin	Seashell chitin
T. viride	6.0	4.3	0.0125	0.00609
T. harzianum	6.2	4.8	0.0133	0.00607
T. asperellum	5.4	3.3	0.0110	0.00604
T. koningii	5.5	2	0.0097	0.0060
T. atroviride	3.9	3.3	0.0116	0.0061
T. longibrachiatum	5.6	3.1	0.0084	0.0055
T. virens	5.0	1.8	0.0076	0.0056

of pNP ranged from 0.0125 to 0.0076 × 10^{-3} U/ml and 0.0069 to 0.0055 × 10^{-3} U/ml in commercial chitin and sea shell derived colloidal chitins, respectively.

Conclusion

Based on the results of above observations, it is clear that for choosing an effective biocontrol agent, it is essential to provide the optimum cultural conditions. The medium used here for chitinase assay was very effective and economical. The medium used here is very friendly and sensitive. Formation of the purple color zone was found to be the easier alternative method for the selection of chitinolytic strains of *Trichoderma* species.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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