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In vitro Evaluation of Enzymatic activities of *Trichoderma* spp. Isolated from Copper Mining Areas of Uttarakhand

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ABSTRACT: A total of 29 *Trichoderma* stains were isolated and purified from rhizospheric soil samples collected from different copper mining area (Nainital, Bageshwar and Tehri Garwal districts) of Uttarakhand hills. These *Trichoderma* isolates were assessed for their enzymatic potential under *in vitro* conditions using colorimetric method. Among the isolates, TCMS-36 showed maximum (116.5EU) chitosanolytic activity followed by TCMS-65 andTh-14 (53.5 EU), TCMS-5 (51.0 EU) and TCMS-2(47.0 EU). TCMS-2 showed maximum β-1, 3 endoglucanase activity of 85.56 EU followed by TCMS-43 (84.89 EU), TCMS-34 (82.0 EU), TCMS-14b (80.67 EU) and TCMS-4(80.22 EU). While TCMS-36 showed maximum chitinase activity of 15.5 EU. However, TCMS-16 was found to have maximum (93.77 EU) cellobiase activity which was followed by TCMS-24 (19.33 EU) and Th-32 (14.44 EU). Among different enzymes assayed, the activity of chitosanase was found to be maximum followed by β-1,3 endoglucanase activity and cellobiose degrading enzymes activity. However, chitinase activity was least (≤15.56 EU) in all the tested isolates. The enzymatic activity is one of the important attributes of a potential bio-control agent to dissolve the cell wall of the pathogenic fungi. Hence, the enzymatic activity can be directly correlated with degree of parasitism. The selected *Trichoderma* isolates with maximum enzymatic potential could be further screened against various plant pathogenic fungi and can be used for effective, economical, ecofriendly and sustainable management of fungal diseases of agricultural and horticultural crops.

Keywords: Biocontrol, Trichoderma, Enzymes, Antagonism, Sustainability, plant disease.

INTRODUCTION

Trichoderma is present in nearly all kind of soil and can survive in diverse habitats. In relation to other soil fungi, they are found to be the most prevalent and belonging to the genus under Deuteromycotina (Sub Division), Hyphomycetes (Class), Moniliales (Order), and Moniliaceae (Family). It comprises large number of species like *T. viride*, *T. harzianum*, *T. virens*, *T. asperellum*, *T. atroviride*, *T. hamatum*, and *T. koningii* they are commonly used for biological control of plant pathogens. In addition, it was also increasing plant growth and development (Hjeljord and Tronsmo 1998; Singh *et al*., 2006). *Trichoderma* spp. are compatible with various chemical fungicides which are used for to control of several diseases (Samuels, 1996).

The teleomorphic stage of Trichoderma belonging to the Hypocreales order of the Ascomycotina Sub-division. Some *Trichoderma* species are very good cellulase producers (Reczey *et al.*, 1996).

Trichoderma strains exert bio-control activity against fungal phytopathogens either directly by mechanism of mycoparasitism or indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis. Mycoparasitic *Trichoderma* strains are able to recognize the host hyphae, to coil around them, develop haustoria, penetrate the cell wall of the host with cell-wall degrading enzymes like chitinases, glucanases and proteases, and utilize the contents of the host hyphae as nutrient source. *Trichoderma* strains with effective antagonistic abilities are potential candidates for the biological control of plant diseases. Keeping the above factors in view, a study was carried out on "*In vitro* screening of *Trichoderma* spp for various enzymatic activities". Enzymatic activities have been found in plant pathogenic and mycoparasitic fungi (Nogawa *et al.* 1998). Chet (1987) suggested that chitinase producing fungi, such as *Trichoderma* sp., can be effective as biocontrol of soil borne plant pathogenic fungi.

Trichoderma spp. are known to produce chitinolytic enzymes that can able to degrade the cell wall of various fungi. *Trichoderma harzianum* is a mycoparasite and it secretes multiple cell wall degrading enzymes viz., endochitinase, exochitinase, and exo-β-D-*N*-acetylhexosaminidase (Peterbauer *et al.*,

1996). The chitinases of this mycoparasitic species are also involved in the antagonistic ability of these fungi against plant pathogens and in biocontrol (Chet *et al.*, 1998). Haran *et al.* (1995) reported that the chitinolytic system of *T. harzianum* consists of five to seven distinct enzymes, depending on the strain. In the best characterized *Trichoderma* isolate (isolate TM), the system is apparently composed of two β-(1,4)-*N*acetyl-glucosaminidases and four endochitinases. Diffusion of this enzyme catalyses the release of cell-wall oligomers from target fungi, and this in turn induces the expression of fungi toxic endochitinases (Brunner *et al.*, 2003), which also diffuse and begin the attack on the target fungus before contact in actually made (Zeilinger *et al.*, 1999; Viterbo *et al.*, 2002). The combined activities of these compounds result in parasitism of the target fungus and dissolution of the cell walls (Mendoza *et al*., 2003; Pozo *et al*., 2003). Different components of the chitinolytic system of *T. harzianum* probably involve complementary modes of action of the component enzymes.

Trichoderma is well-known for cellulase hyper production whose members have been attracted continued interest as sources of various enzymes. It secretes at least two cellobiohydrolases (exo type; EC 3.1.2.91), four endoglucanases (EC 3.1.2.4), and two β-glucosidases (EC 3.1.2.20) (Nevalainen and Penttila 1995). Janice Lisboa De Marco *et al*. (2003) evaluated *Trichoderma* spp. for potential to produce hydrolases. The tested *Trichoderma* spp. secreted substantial amounts of chitinolytic and glucanolytic enzymes. Apart from producing extracellular β-(1,3)-glucanases and chitinases, *Trichoderma* spp. also produce lipases and proteases when they are grown on cell walls of pathogenic fungi (Haran *et al.*, 1996). The chitosanase activity was of 27.3 U/mg of protein against completely deacetylated chitosan (Nogawa *et al*., 1998). Xiao *et al.* (2005) reported that chitosanlytic activity from an *Aspergillus* mutant CJ22-326 was 3.61 u/ml of culture fluid.

MATERIAL AND METHODS

A. Isolation, purification and naming of Trichoderma spp.

Extensive collections of soil samples were carried out in different farming situations from the copper mining areas of Uttarakhand such as Nainital, Bageshwar and Tehri Garwal districts. *Trichoderma* selective medium (TSM) was used for isolation and purification of *Trichoderma*. The isolates were purified by single spore culture. Colony arising from single spore was picked up and inoculated on a fresh plate. This culture was finally maintained and used for further studies. The fungus was identified on the basis of cultural and morphological characteristics. 49 isolates of *Trichoderma* were obtained and were coded on the bases of location and serial number of the soil sample as *Trichoderma* from copper mining site (TCMS 1 to 116). Abiotic stress tolerant (six) isolates of *Trichoderma* collected from the Biological Control laboratory, Department of Plant Pathology, were named as Th-Series.

B. Evaluation of Trichoderma spp. for their enzymatic activity

Thirty-three *Trichoderma* isolates (20-TCMS series, 6-Th series and 7- SBIT series) were selected for evaluation of Chitosanase, Chitinase, β, 1, 3 Endoglucanase and cellobiose degrading activity.

Trichoderma spp. were cultured in 100 ml of basal mineral medium (g/L): 12 NaH_2PO_4, 2 KH_2PO_4, 0.330 $CaCl_2.2H_2O$, 0.030 $ZnSO_4.7H_2O$, 0.030 $MgSO_4.7H_2O$, 0.005 $FeSO_4.7H_2O$), in a 250 ml conical flask shaken at 150 rpm at 30°C for 7 days. In order to remove fungus cells and spores, the culture was first filtered through filter paper (Whatman No.1) and then centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant (crude enzyme solution) was then collected to perform different enzymatic assays. The crude extract was stored at -20 °C until used.

C. Preparation of glucose standard curve

Glucose stock solution (1000 µmol=0.1802g/l) was prepared and different concentrations from stock solutions were made by mixing with citrate buffer in separate test tubes and incubated at 37°C for 1 hr. The concentration of reducing sugars was measured by the modified Schales"s method as described by Imoto and Yagishita (1971) with d-glucose as a reference compound. One unit of chitosanase activity was defined as the amount of enzyme that produced one µmol of D-glucose equivalent in 1 min. Then 3 ml DNS reagent was added and the mixture was placed in boiling water for 5 minutes. Then it was cooled to room temperature. The final volume was made upto 10 ml by adding double distilled water (DDW). Optical Density (OD) value was recorded at A_{540} nm with water blank for 100% transmittance. Subsequently, standards on semilog paper (log % Transmission versus concentration) were prepared.

D. Chitosanolytic activity

100µl of crude enzyme solution with 400µl of substrate (chitosan) solution and 500µl of Na-acetate buffer in a test tube of volume 25 ml were mixed well and incubated at 37°C for 1 hr. 3.0 ml DNS reagent was added to it after incubation and boiled for 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks and the spectro zero were boiled together and transferred immediately to a cold-water bath. The final volume was made to 10 ml by adding DDW and was mixed thoroughly. The colour thus formed was measured against the spectro zero at 540 nm. Translated the absorbance of the sample to glucose production during the reaction with the help of glucose standard curve. The enzyme activity was expressed in Enzyme Unit (EU-µmol/min/ml).

E. Chitinase activity

500µl of crude enzyme solution with 500µl of substrate (chitin) solution in a test tube of volume 25 ml were mixed well and incubated at 37°C for 30 min. 3.0 ml DNS reagent was added to it after incubation. Boiled for 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks and the spectro zero were boiled together. After boiling

it was transferred immediately to a cold water bath. The final volume was made to 10 ml by adding DDW and was mixed thoroughly. Measured the colour formed against the spectro zero at 540 nm. Translated the absorbance of the sample to glucose production during the reaction using a glucose standard curve. The enzyme activity was expressed in Enzyme Unit (EU; µmol/min/ml)

F. Carboxymethyl Cellulase assay for endo-ß-1, 4-glucanase

Crude enzyme solution (0.5 ml) with 0.5 ml substrate solution in a test tube of volume 25 ml were mixed well and incubated at 50°C for 30 min. 3.0 ml DNS reagent was added to it after incubation. Boiled for 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks and the spectro zero were boiled together. After boiling it was transferred immediately to a cold-water bath. The final volume was made to 10 ml by adding DDW and was mixed thoroughly. Measured the colour formed against the spectro zero at 540 nm. Translated the absorbance of the sample to glucose production during the reaction using a glucose standard curve. The enzyme activity was expressed in Enzyme Unit (EU- µmol/min/ml)

G. Cellobiose degrading activity

One ml crude enzyme solution with 1 ml ml substrate solution in a test tube of volume 25 ml were mixed well and incubated at 50°C for 30 min. To this 3.0 ml DNS reagent was added and boiled for five min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks and the spectro zero were boiled together. After boiling it was transferred immediately to a cold-water bath. The final volume was made to 10 ml by adding DDW and mixed thoroughly. Measured the colour formed against the spectro zero at 540 nm. Translated the absorbance of the sample to glucose production during the reaction using a glucose standard curve. The enzyme activity was expressed in Enzyme Unit (EU; µmol/min/ml).

RESULTS AND DISCUSSION

A. Evaluation of Trichoderma isolates for the production of different enzymes

Twenty-six *Trichoderma* isolates were analyzed for different enzymatic *viz.*, chitosanase, β-1, 3 endoglucanase, chitinase and cellobiose degrading assays. The glucose standard curve was plotted by analyzing different concentrations of glucose for optical density (O.D.) values. Results were analyzed using the Y and R values obtained the glucose standard curve (Fig. 1).

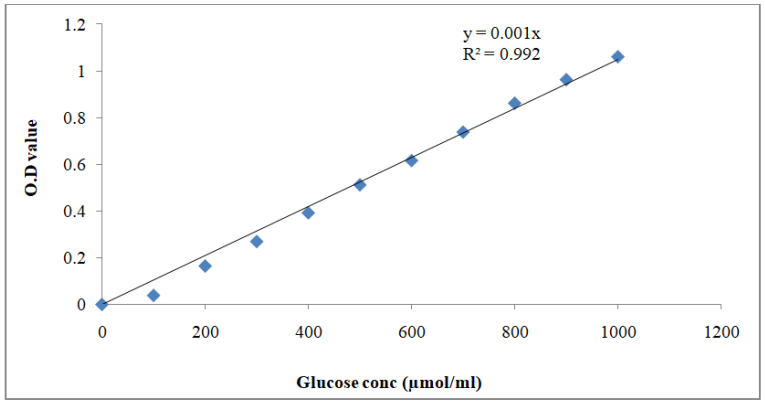

Fig. 1. Glucose standard curve (optical density v/s glucose concentrations).

Enzymatic activity in different *Trichoderma* isolates

Different enzymatic activity in *Trichoderma* isolates is depicted in Table 1 and Fig. 2.

Chitosanase activity. All the isolates were found producing chitosanolytic enzymes. The isolate TCMS-36 showed maximum chitosanolytic activity (116.5 EU) followed by TCMS-65, Th-14, TCMS-5 and TCMS-2 with 53.5, 53.5, 51 and 47 EU of enzyme activity, respectively. However, TCMS-93 was found to be having least (25 EU) chitosanolytic activity followed by TCMS-24 and TCMS 14a. Remaining isolates recorded having chitosanase enzyme activity in the range of 26.5 - 47 EU.

All the isolates tested showed significant β-1, 3 endoglucanase activity. TCMS-2 showed maximum activity of 85.56 EU followed by TCMS-43, TCMS-34, TCMS-14b and TCMS-4 with 84.89, 82, 80.67 and 80.22 EU, respectively with a significant difference among each other. However, minimum enzyme activity was observed in TCMS-5 (6EU) followed by TCMS-12 (7.33EU).

Chitinase activity of the assayed isolates was lesser than the other two assayed enzyme activity. Isolate TCMS-36 showed maximum activity (15.5 EU) followed by TCMS-65 (6 EU), TCMS-24 (5.33 EU), TCMS-93 (4.44 EU) and Th-1 (3.83 EU). However, isolate Th-32 exhibited least chitinolytic activity (0.39 EU) which was followed by the isolates Th-14, Th-13, Th-19, Th-3, TCMS-72, TCMS-85 and TCMS-14b with 0.44, 0.5, 0.67, 0.78, 0.89, 0.89 and 1.11 EU, respectively.

TCMS-16 was found to have maximum (93.77 EU) cellobiase activity which was followed by TCMS-24 (19.33 EU) and Th-32 (14.44 EU). However, TCMS-32 was the least (0.67 EU) producer of cellobiose degrading enzymes followed by TCMS-69 (0.89 EU), which was at par with TCMS-32.

Among different enzymes assayed, the activity of chitosanase was found maximum (up to 116.5 EU) in all the isolates followed by β-1,3 endoglucanase

activity (up to 85.56 EU) and cellobiose degrading enzymes activity (up to 15.56 EU). However, chitinase activity was least (≤15.56 EU) in all the tested isolates.

Similar kind of enzymatic activity of *Trichoderma* was reported earlier. Nogawa *et al.* (1998) reported chitinolytic and chitosanolytic activities in mycoparasitic fungi. *Trichoderma* isolates are able to excrete hydrolytic enzymes such as chitinases, proteases and β-glucanases into the medium when grown in the presence of laminarin, chitin or cell walls of phytopathogenic fungi. Chitin together with β-1, 3-glucan is the major constituent of the cell walls of asco- and basidiomycetes (Peberdy, 1990).

Similarly, Peterbauer *et al.* (1996) reported that *T. harzianum* secretes multiple chitin-degrading enzymes, including endochitinase, exochitinase, and exo-β-D-*N*-acetyl hexosaminidase. Nevalainen and Penttila (1995) also observed that different components of the chitinolytic system of *T. harzianum* probably involve complementary modes of action of the component enzymes. *T. reesei* secretes at least two cellobiohydrolases, four endoglucanases and two β-glucosidases.

Janice Lisboa De Marco *et al*. (2003) evaluated two isolates of *Trichoderma* for their potential to produce chitinolytic and glucanolytic enzymes. The highest total cellulase and endoglucanase activities were produced byisolate1051. However, although the cellobiase activity produced by the isolate 1051. Besides other modes of action of *Trichoderma*, cell wall-degrading enzymes such as chitinases (De Marco *et al*., 2000; Janice, 2003) and β-1, 3 glucanases have been associated with the ability of *Trichoderma* spp. tocontrol plant pathogens (Noronha and Ulhoa 1996). The chitosanase activity was 27.3 U/mg of protein against completely deacetylated chitosan (Masahiro Nogawa *et al*., 1998). *Trichoderma* sp. secretes endochitinases (Brunner *et al.,* 2003), which are induced by the oligomers present in cell wall of many fungi (Zeilinger *et al.,* 1999; Viterbo *et al.,* 2002). The combined action of these enzymes released by the *Trichoderma* sp. helps to dissolve the cell wall of pathogenic fungi leading to suppression plant diseases (Mendoza *et al*., 2003; Pozo *et al*., 2003). Such potential Trichoderma isolates with higher level of enzymatic activities can be further screened and utilized for the management of soil-borne plant diseases under integrated disease management systems and organic farming systems. Some *Trichoderma* sp, can even be utilized for commercial production of enzymes by the industries. The genes responsible for the enzyme secretion may be further identified and incorporated into the plants for the induction of diseases resistance.

Table 1: Enzymatic activity of *Trichoderma* isolates.

Sr. No.	Isolate	Chitosanase		β, 1-3-Endoglucanase		Chitinase		Cellobiase	
		O.D. value	EU	O.D. value	EU	O.D. value	EU	O.D. value	EU
1.	TCMS2	0.03	47.00	1.28	85.56	0.03	2.00	0.02	1.33
2.	TCMS4	0.02	32.50	1.20	80.22	0.03	2.00	0.04	2.67
3.	TCMS5	0.04	51.00	0.09	6.00	0.02	1.56	0.04	2.67
4.	TCMS 12	0.03	27.00	0.11	7.33	0.04	2.67	0.03	2.00
5.	TCMS 14a	0.03	25.00	0.94	62.67	0.02	1.33	0.03	2.22
6.	TCMS14b	0.02	26.50	1.21	80.67	0.02	1.11	0.03	2.00
7.	TCMS15	0.02	36.50	1.13	75.11	0.03	2.00	0.05	3.11
8.	TCMS16	0.02	42.00	1.10	73.33	0.03	2.22	1.41	93.78
9.	TCMS24	0.03	25.00	0.05	3.33	0.08	5.33	0.29	19.33
10.	TCMS32	0.03	116.50	0.97	64.44	0.05	3.33	0.01	0.67
11.	TCMS34	0.02	33.00	1.23	82.00	0.05	3.33	0.05	3.33
12.	TCMS36	0.08	53.00	1.12	74.44	0.23	15.56	0.08	5.33
13.	TCMS 43	0.02	31.00	1.27	84.89	0.03	1.78	0.06	3.78
14.	TCMS60	0.02	28.50	0.99	66.22	0.03	2.00	0.05	3.56
15.	TCMS62	0.04	29.00	1.11	74.22	0.02	1.33	0.02	1.56
16.	TCMS 64	0.03	39.00	0.98	65.56	0.03	1.78	0.01	0.89
17.	TCMS 65	0.02	53.50	1.14	76.00	0.09	6.00	0.04	2.67
18.	TCMS 72	0.04	28.00	1.02	68.00	0.01	0.89	0.10	6.67
19.	TCMS 85	0.04	28.00	1.10	73.33	0.01	0.89	0.02	1.33
20.	TCMS 93	0.02	15.00	1.01	67.33	0.07	4.44	0.02	1.56
21.	Th 1	0.02	0.02	0.91	30.44	0.23	3.83	0.07	2.33
22.	Th 3	0.02	0.02	1.14	38.11	0.05	0.78	0.12	3.89
23.	Th 13	0.01	0.01	0.70	23.33	0.03	0.50	0.04	1.33
24.	Th 14	0.04	0.04	0.92	30.56	0.03	0.44	0.03	1.00
25.	Th 19	0.02	0.02	0.87	29.00	0.04	0.67	0.07	2.44
26.	Th 32	0.02	0.02	0.50	16.56	0.02	0.39	0.43	14.44
SE (m) ±		-	0.33	-	0.18	-	0.22	-	0.30
CD (1%)		-	1.31	-	0.82	-	0.88	-	1.42

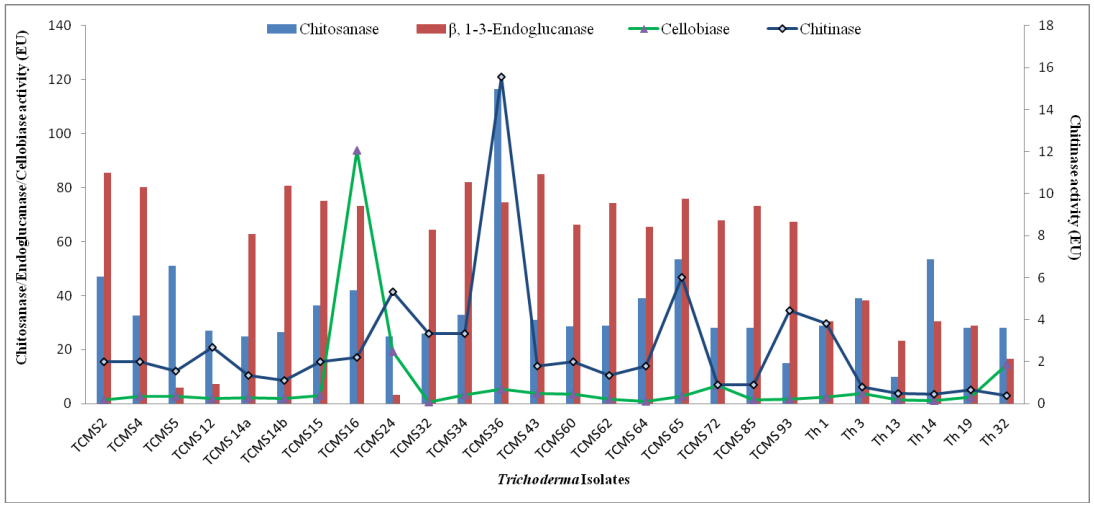

Fig. 2. Enzymatic activity in different *Trichoderma* isolates.

CONCLUSIONS

Trichoderma species are highly efficient producers of many extracellular enzymes and it is a unique feature of *Trichoderma* species that is the main reason of their antagonistic nature. The extracellular hydrolytic enzyme activities were exhibited at the different rates by different *Trichoderma* species. The enzymatic activity is directly related to the antagonistic potential of the *Trichoderma* spp. The enzymes produced by *Trichoderma* spp. affect the chemistry of cell wall which is accompanied by cell wall degradation. The extracellular hydrolytic enzymes played important role in mycoparasitism and antibiosis which are essential aspect for the bio-control of pathogens. The *Trichoderma* strains with high enzymatic potential could be screened further for their antagonistic activity and can be used for the sustainable crop disease management and in organic agriculture systems.

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Conflicts of Interest. None.

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