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International Journal of Current Research in Life Sciences
Vol. 11, No. 01, pp.3436-3440, January, 2022

ISSN: 2319-9490

RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF TRICHODERMA SPECIES FROM SOIL SAMPLES FOR ESTIMATION OF CHITINASE ENZYME

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Received 29th November, 2021; Accepted 20th December, 2021; Published 30th January, 2022

ABSTRACT

One of the most important strategies towards the biological control of several phytopathogenic fungi is the application of *Trichoderma* species for as a possible eco-friendly pesticide. The secretion of chitinase breakdown the chitin cell wall of most of the fungal pathogens. Chitinase hydrolyses the glycosidic bonds of the fungal cell wall and weakens the pathogen. The present study shows that the *Trichoderma* spp. can be easily isolated from the soil and have different chitinase activities against fungal pathogens. In this study, three Trichoderma species were isolated (*harzianum*, *Polysporum and viride*) and their Chityinase assay were done to check their Chitinase wall degradation potential accordingly. The maximum amount of protein (486 mg) in the incubated broth was revealed for the isolate SIT2 i.e. for *Trichoderma polysporum* and least (265mg) for the isolate SIT5 i.e. for *Trichoderma viride.* The total chitinase activity were found to be highest (62.56 µmolmin^{-1}) for *T. harzianum* isolate (SIT1) and least (21.36 µmolmin^{-1}) for the T. polysporum isolate (SIT2). Furthermore, it was revealed that the specific activity of Trichoderma harzianum (SIT1) was maximum (0.190 µmolmin^{-1}mg^{-1}) and Trichoderma polysporum (SIT2) as minimum (0.0 43µmolmin^{-1}mg^{-1}).

Key words: *Phytopathogenic, Pathogen, spp., Chitinase, Glycosidic bonds.*

Citation: Niraj Kumar Singh, Choudhary, D.P. and Prasad Gajendra. 2022. "Isolation and characterization of trichoderma species from soil samples for estimation of chitinase enzyme" *International Journal of Current Research in Life Sciences*, 11, (01), 3436-3440

INTRODUCTION

Trichoderma is a well known genus of fungi belonging to the family Hypocreaceae and present in almost all kind of soils (Harman *et al*. 2004). *Trichoderma* species shows variable behaviour, most of them are plant symbionts (Baiyee *et al*. 2019a). *Trichoderma* is generously used as bio-pesticide against number of fungal phytopathogens (Hamid *et al*. 2013). *Trichoderma* species are known to excrete enzymes such as chitinases, β-glucanases and proteases during the interaction with cell wall of phytopathogenic fungi (Wonglom *et al*. 2019; Baiyee *et al*. 2019b). Apart from chitinse, other enzymes may also be involved in the complete degradation of cell walls of phytopathogenic fungi, butthe enzyme chitinase is considered an important enzyme since its substrate, chitin, is the most abundant component in cell walls of many fungus species (Baek *et al*. 1999). Chitinases (E.C 3.2.2.14) are glycosyl hydrolases and are present in a wide range of organisms such as bacteria, fungi, yeasts, plants, actinomycetes, and arthropods (Hamid *et al*. 2013). There are approx 89 species of the genus is recorded. The genus is easily culturable at the room temperature within basal media such as CDA (Commercial Dextrose Agar) or PDA (Potato Dextrose Agar).

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Many species in this genus can be characterized as opportunistic avirulent plant symbionts. Use of morphological characteristics is one of the conventional methods to identify Trichoderma species and it remains as a potential method to identify Trichoderma up to genus level (Samuels *et al*., 2002). The most commonly used biocontrol agents 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, Beta-1, 3-glucanase, cellulase and proteases. These enzymes hydrolyse the pathogen's cell wall components such as chitin, glucan, cellulose and proteins successfully limiting the growth of fungal pathogens. 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. Studies on characterisations and activity of extracellular chitinase from *Trichoderma* species had been started long time ago such as *T. harzianum* (Ulhoa and Peberdy 1991; El-Katatny *et al*. 2000, 2001; Sandhya *et al*. 2004), *Trichoderma* sp. (Lima *et al*. 1999), *T. virens* (Baek *et al*. 1999). However, studies in *T. asperellum* have only recently begun and only focused on chitinase coding genes, its characterisation and activity (Loc *et al*. 2011, 2013; Kumar *et al*. 2012; Nadarajah *et al*. 2014; Asad *et al*. 2015; Wu *et al*. 2017; Pandian *et al*. 2018; Liu *et al*. 2019, etc.). In the present piece of work trichoderma species were isolated for the quantification of their chitinase activity, so they could be used for a bio-pesticide against number of phyto-pathogens.

MATERIALS AND METHODS

Sample Collection: Soil samples were collected from five different sites of the campus of Department of Botany, C. M. Science College, L. N. Mithila University, Darbhanga, Bihar. Soil samples were collected at the depth of 10 centimetres below the top soil and packed immediately within the pre-sterilized zipper poly-bags and immediately moved to the laboratory for further isolation of fugal colonies. The pH and temperature of the soil collection sites were recorded as per standard methods.

Isolation of Colony Forming Units (CFU): Soil samples were serially diluted within normal saline up to Dilution Factor 10^{-7}. The fungal isolation media Potato dextrose agar was used for the isolation of fungal colonies from working samples having the Dilution Factors (DF) 10^{-5}, 10^{-6} and 10^{-7} by using pour plate isolation technique.

Culture Characterization and Identification of Trichoderma Spp: By the methods of Domsch *et al*., 1980, based on Cultural and Microscopic characteristics of *Trichoderma* isolates and were verified through Gilman and Barnnet Manuals for soil fungi

Total Protein Estimation: The amount of total protein present in broth filtrate was estimated by Lowry's method (Lowry, 1953) in order to determine the total and specific activity of chitinase enzyme produced by the individual isolates.

Chitinase activity assay: Spectrophotometric method was used to detect the Chitinase activity according to Tsujibo *et al*. (1998) using p-Nitrophenyl-β-N-acetyl glucosaminide (pNp-GlcNAc) as a substrate. Enzyme samples were partially purified by column chromatography. Seventy µl of partially purified enzyme was added to 140 µL of 2.5 mM pNp-GlcNAc that dissolved in 50 mM acetate buffer, pH 6, and incubated for 10 minutes at 50°C. The ongoing hydrolysis process was then stopped using 1.4 mL of 0.2 M sodium carbonate. The sample was then meseared for optical absorbance at a wavelength of 420 nm. Chitinase activity was defined as the amount of enzyme released 1 µmol *p*-nitrophenol from pNp-GlcNAc during 1 min. To determine the chitinase activity regression equation equation of $y = 0.673x$ ($R^2 = 0.988$), where y is *p*-nitrophenol content and x is absorbance at 420 nm.

RESULTS AND DISCUSSION

Eight different Trichoderma isolates out of 124 isolated CFU on 25 PDA plate were purifies on Dextrose Agar slants. Individual pure strains were cultured on PDA pate to study the culture characteristics as depicted in Table 1.0,2.0 and fig 1.0 and 2.0 respectively. The isolates that had culture characteristics of Trichoderma were given isolate codes SIT (Soil IsolateTrichoderma) such as SIT1, SIT2. SIT3, SIT4, SIT5, SIT6, SIT7 and SIT8. Morphology of the spores and sporulating structures of the isolates were more or less similar, but significantly different in length and width.

Table 1.0 Culture characteristics of different purified isolates on PDA plates

S.N	IsolateCode	Culture Colour	Morphology	Size of Spores (µm)	Size of Phialide (µm)	Mycelial Width (µm)
1	SIT1	Whitish Green	Circular	Width- 2.65±0.23 Length-2.61±0.33	Width- 2.13±0.33 Length-7.95±0.54	4.85±0.31
2	SIT2	Blackish Green	Diffused	Width - 2.23±0.19 Length -2.21±0.22	Width- 3.43±0.51 Length-8.97±0.09	5.12±0.18
3	SIT3	Dark Green	Circular, 2 rings	Width - 2.51±0.41 Length -2.42±0.31	Width- 2.75±0.11 Length-8.26±0.32	5.02±0.24
4	SIT4	Whitish Grey	Circular	Width - 3.47±0.82 Length -3.40±0.64	Width-3.52±0.21 Length-7.12±0.22	4.54±0.17
5	SIT5	Light Green	Circuar, 3-4 rings	Width - 2.65±0.42 Length -2.55±0.61	Width-3.11±0.42 Length-8.80±0.37	4.92±0.31
6	SIT6	Bluish Green	Circular	Width - 3.88±0.19 Length -3.82±0.14	Width-3.57±0.17 Length-7.54±0.24	4.61±0.26
7	SIT7	Whitish Blue	Diffused	Width - 2.15±0.22 Length -2.08±0.31	Width- 3.32±0.33 Length- 9.18±0.34	5.22±0.54
8	SIT8	Dark Green	Circular, 2 rings	Width - 2.73±0.41 Length -2.71±0.23	Width- 2.23±0.44 Length- 7.88±0.24	4.04±0.25

{SIT= Soil Isolate Trichoderma, n=7, sevsn experimental sets}

Table 2.0: Identified Isolates of Trichoderma

S.N.	Isolate Code	Trichoderma Species Identified
1	SIT1	*harzianum*
2	SIT2	*polysporum*
3	SIT3	*viride*
4	SIT4	*viride*
5	SIT5	*viride*
6	SIT6	*harzianum*
7	SIT7	*harzianum*
8	SIT8	*viride*

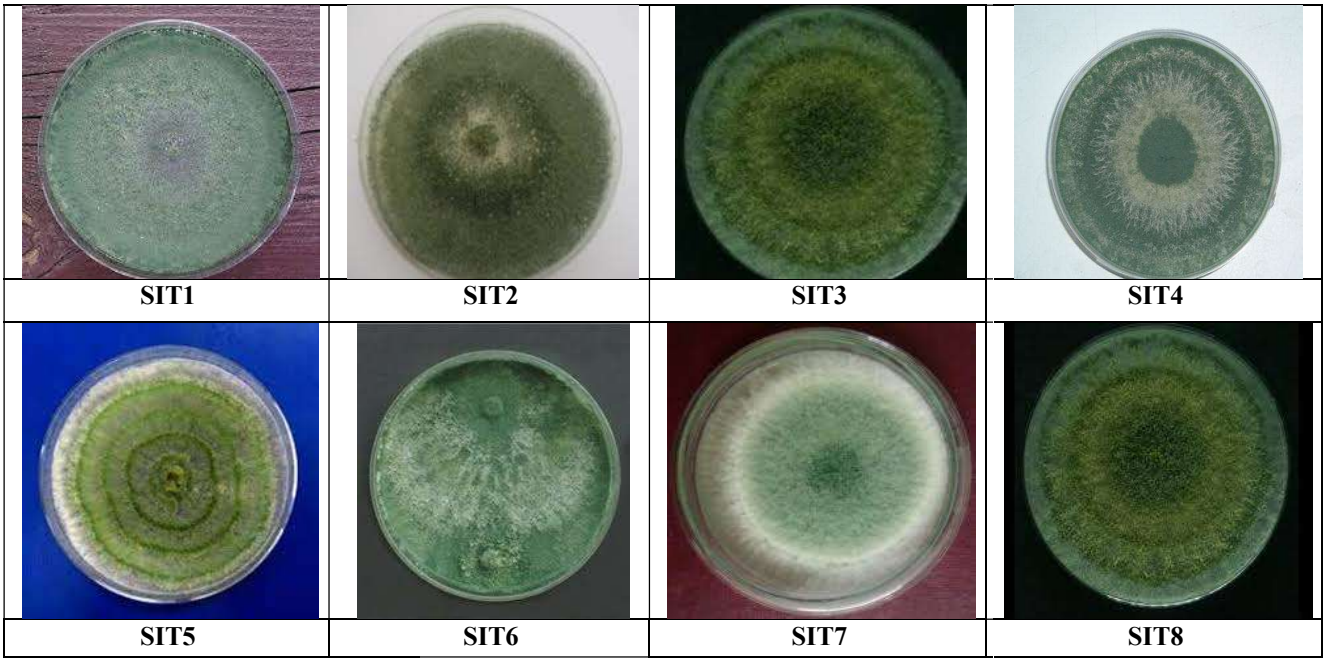


Fig 1.0: Culture of Different Trichoderma Isolates on PDA

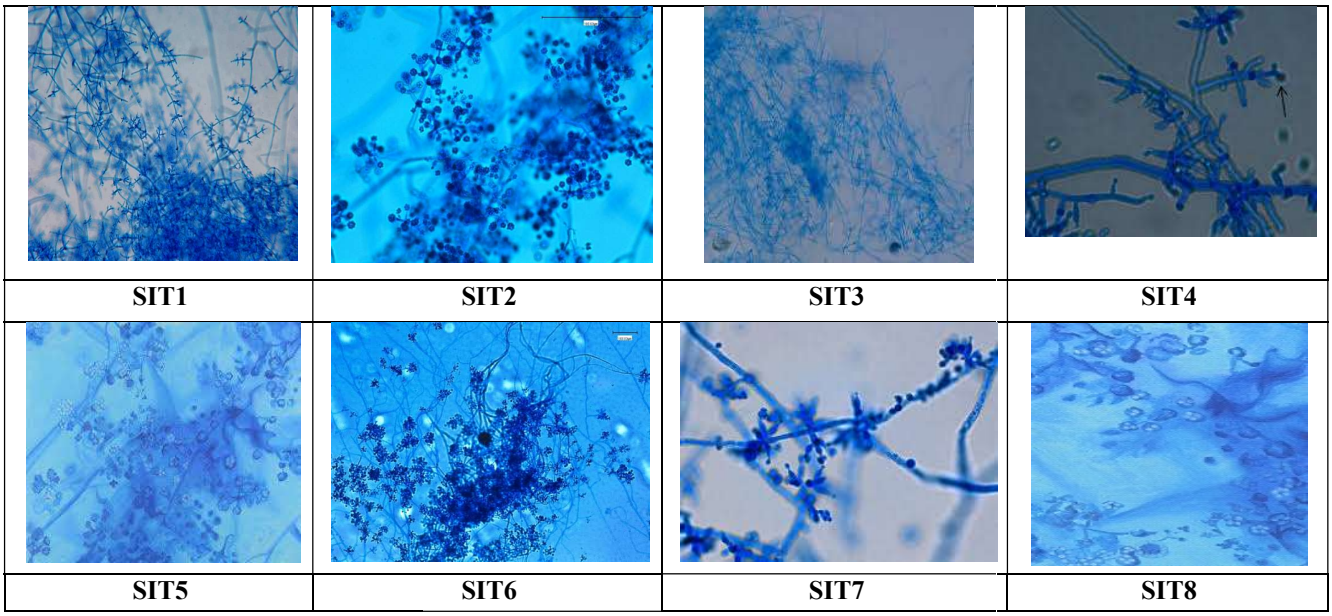
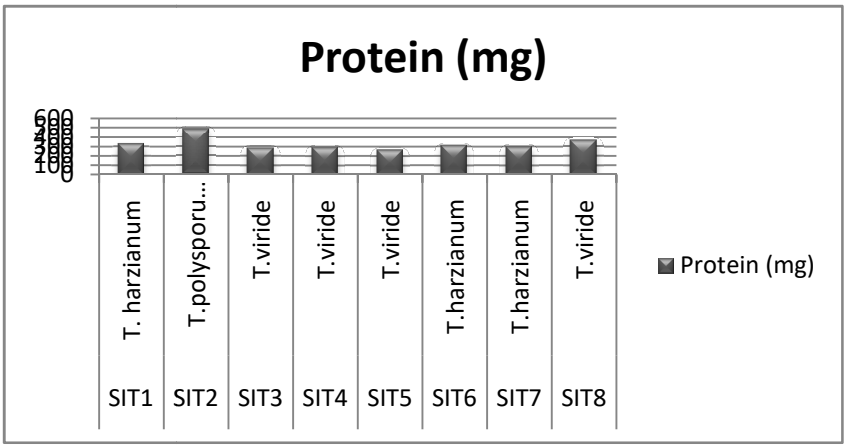


Fig.2.0. Micrographs of different Trichoderma isolates



Graph 1.0: Amount of protein estimated for100 ml of purofied borth of Trichoderma isolates.

Table 3.0: Purification Folds and Units of Chitinase Activity

S.N	Code	Trichoderma Spp.	Protein (mg)	Total Activity ($\mu molmin^{-1}$)	Specific Activity ($\mu molmin^{-1}mg^{-1}$)
1	SIT1	*T. harzianum*	328	62.56	0.190
2	SIT2	*T.polysporum*	486	21.36	0.043
3	SIT3	*T.viride*	282	32.15	0.114
4	SIT4	*T.viride*	293	29.42	0.100
5	SIT5	*T.viride*	265	27.61	0.104
6	SIT6	*T.harzianum*	311	41.23	0.132
7	SIT7	*T.harzianum*	296	55.25	0.186
8	SIT8	*T.viride*	364	30.41	0.083

Trichoderma Isolates showed varied pattern of colony development on Potato Dextrose Agar Media (Fig. 1.0). SIT3, SIT5 and SIT8 showed circular rings of dark green colour while SIT1, 2,4, 6 and 7 showed diffesed growth pattern without any distinct circular rings. The micrographs of Trichoderma isolates were depicted in fig 2.0. the micrographs shows somewhat similar structure of spores and phialids with different sizes along with their mycelium. From the culture and micrograph characteristics, three different species were identified according to the Keyes provided by Gilman and Barnnet Manual for Soil fungi (J. C. Gilman, 2001; *Barnett* and Hunter; 2006) followed by the online identification key provided by Samuels and his coworkers (http://nt.arsgrin.gov/taxadescriptions/keys/ FrameKey.cfm? gen=*Trichoderma*) and the Compendium of soil fungi (Domsch *et al*., 1980). Use of morphological characteristics is one of the conventional methods to identify species and it remains as a potential method to identify *Trichoderma* up to genus level (Samuels *et al*., 2002). Table 1.0 indicates the colony morphology and microscopic characteristics such as length and width (µm) of spore, phialides and mycelia of eight *Trichoderma* isolates. It was reviled that the eight Trichoderma isolates belongs to three different species i.e. *T. harzianum, T. officinale and T. viride* (Table 2.0). Isolates were identified on the basis of culture and morphological keys as per the standard methods and verified accordingly. The amount of total protein was estimated by Lowry's method and the amount of protein present in broth after the incubation of 120 hrs (5 days) is shown in graph 3.0 for all the eight *Trichoderma* isolates. The highest amount of protein (486 mg) was found in the SIT2 Isolate i.e. in the Culture broth of *polysporum* followed by the SIT8 (*T. viride*) isolate (364 mg). The least amount of protein (265 mg) secreted within broth was found to be of the isolate SIT5 (*T. viride*). The activity of chitinase enzyme of different trichodrma isolates were summarised in table 3.0. It was revealed that the chitinase activity of *T. harzianum* isolates were higher among the isolated, SIT1 was found to have highest total activity (62.56 $\mu molmin^{-1}$) followed by SIT7 (55.25 $\mu molmin^{-1}$) and SIT6 (41.23 $\mu molmin^{-1}$) isolates. *T. viride* isolates showed considerable total chitinase activity for the isolates SIT3 (32.15 $\mu molmin^{-1}$), SIT4 (29.42 $\mu molmin^{-1}$), SIT5 (27.61 $\mu molmin^{-1}$) and SIT8 (30.41 $\mu molmin^{-1}$) respectively. The isolate SIT2, confirmed as *T. polysporum* showed least amount of chitinase activity (21.36 $\mu molmin^{-1}$) among all the Trichoderma isolates. The specific activities of the chitinase enzyme produced by different isolates shows the similar pattern as total activity. Highest specific chitinase activity (0.190 $\mu molmin^{-1}mg^{-1}$) was shown by SIT1 (*T. harzianum*) and the least (0.043 $\mu molmin^{-1}mg^{-1}$) specific activity was shown by SIT2 (*T. polysporum*) isolate.

Among *T. viride* isolates, SIT3, SIT$, SIT5 and SIT8 showed a specific activity of 32.15, 29.42, 27.61 and 30.41 $\mu molmin^{-1}mg^{-1}$ respectively. The present work is in accordance with the works of Sandhya *et al*. (2004) , Asad *et al*. (2015) and of Urbina-Salazar *et al*. (2018) for the production of extracellular chitinse within broth culture and for the estimation of theit total and specific activity..

Disclosure statement: There is no conflict of interest was reported by the authors.

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