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Invitro Study of Antagonistic Activity, Extraction and Optimization of Amylase Enzyme from *Trichoderma Harzianum* Against Selected Soil- Borne Fungal Isolates

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Abstract—*Trichoderma harzianum* has been well known as a potent biocontrol agent against several plant pathogenic fungi. But information with regard to dual role of *Trichoderma* as biocontrol and plant growth promoting agent is less reported. The extracellular hydrolytic enzymes are known for their application as biocontrol agents in agricultural fields. Henceforth, the present study was conducted to determine the myriad level of antagonistic activity against various soil borne fungal isolates. Collected soil samples from four distinct Groundnut Agricultural fields, undergone serial dilution followed by inoculation in Potato Dextrose Agar (PDA) supplemented with Streptomycin. Based on morphological and cultural characteristics, 23 isolates were identified, out of which 12 fungi belong to Ascomycota & one of Zygomycota phylum. The isolates were tentatively identified as *Aspergillus terreus, A. niger, A. ochraceous, A. tamarii, A. parasiticus, A. nidulans, A. flavus, A. nidulans, Trichoderma harzianum, Penicillium sp., Rhizopus sp., Curvularia sp.,* and *Fusarium oxysporum*. Antagonistic screening activity had revealed that isolates of *Trichoderma* had shown the highest antagonism against 19 isolated fungal strains. It had also revealed that, *Trichoderma harzianum* had shown the minimum percentage of growth inhibition in a range of 11.11% to maximum of 94.11% among all selected soil borne fungal isolates. *Trichoderma* had also represented the highest antagonistic activity against *Curvularia* sp. Further, extracellular enzyme viz. cellulase, amylase, pectinase, protease and chitinase activity had been determined qualitatively by zone of clearance, as representation of enzyme potency. Quantitative broth culture assay had revealed, maximum amylase activity 49.60U/ml. Further, optimum amylase activity was recorded 87.69 U/ml at optimum pH 7.0, incubation period of 5 days and temperature 31˚C respectively.

Keywords: *Trichoderma harzianum*. Soil Borne Fungal Isolates, Antagonistic activity, Amylase activity, Submerged fermentation.

INTRODUCTION

Nowadays plant diseases can be controlled by using various biocontrol agents that inhibit the growth of plant pathogens by several mechanisms. Among them, antagonism refers to the action of an organism that suppresses or interferes with normal growth and their pathogenic activity. Several strains of *Trichoderma* utilize biocontrol agents and provide beneficial effects in plant growth promotion. *Trichoderma harzianum* is generally

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found in normal rhizospheric soil and wood. It has the ability to control seed borne, foliar and soil borne disease (Charan et al., 2016). *Trichoderma* plays a significant role in the bioremediation and plant growth promotion as well as serving as a biocontrol agent too. *Trichoderma sp.* has various modes of actions to prevent the plant pathogens such as mycoparasitism, antibiosis, competition, and lysis. It can bind with the plant root and inhibit the growth of other fungal parasites onto the root of the plants (Cook and Baker 1983). *T. harzianum* have the capability to produce the extracellular hydrolytic enzymes which have significant effect to control the mycoparasitism (Bhale et al., 2012). *Trichoderma sp*. has the facility that consumes the majority of carbon and nitrogen containing compounds, for their rapid growth and development than other fungi. It's able to secret some enzymes that break down the complex plant polymers into simplest sugars and these sugars will be taken up by the *T. harzianum* for their growth and energy (Ramaraju et al., 2017). The aim of the experiment was to conduct an *in vitro* study of antagonistic activity of *Trichoderma harzianum* against soil borne fungal isolates, screening, extraction and optimization of amylase enzyme production by *T. harzianum.*

MATERIALS AND METHODS

Collection of Soil Samples

The soil samples were collected from the four different agricultural fields (groundnut seed crop) at four various places of Railnagar, Bedi, Ratanpar and Gauridad areas in district Rajkot, Gujarat. The samples were collected from the month of January 2021. Therefore, soil samples were collected from 15-20 cm depth with the help of a sterilized scalpel by maintaining V-shaped depth into the ground. The collected soil samples were kept into the sterile polyethylene bags with the appropriately labeled such as collection site, date, time and place of collected samples for further isolation purpose. Nomenclature and collected sample location were described in Table-1.

Isolation of Fungi from the Soil Samples

The soil dilution (Waksman,1922) was utilized for the isolation of fungal culture in the Potato Dextrose Agar medium. Soil dilutions were prepared by taking 1.0gm of soil sample in a test tube by adding 10.0ml of sterile distilled water for every sample. 10^{-3}, 10^{-4} and 10^{-5} dilutions were employed to isolate fungi culture from soil samples in order to avoid overcrowding of the fungal colonies in plates. 1.0ml serially diluted soil sample was added in 1% streptomycin containing potato dextrose agar medium petri plates and to maintain the triplicates. Then plates were incubated at 28˚C for 7 days. At higher dilutions fungal colonies are easily isolated because they format well dispersed surface hyphal colonies (Ratna et al., 2015).

Identification of the Soil Fungi

For the identification of fungal colonies morphological characterization and microscopic examination were employed. For the morphological identification presence or absence of aerial mycelium, length and width of the colony, colour, pigment production and wrinkled furrows characteristics are to be evaluated (Gilman 2001).

Microscopic Identification of Fungal Isolates

For the microscopic identification of fungal strains Lactophenol Cotton Blue staining method has been performed with the usages of inoculating needle and Bunsen burner. With the help of an inoculating needle, a small portion of grown culture was transferred into the lacto-phenol cotton blue containing glass slide with placing to the cover-slip. Then slightly squashed the culture to avoid overcrowding of the mycelium. Then stained fungal species were observed under the light microscope (40X) for the morphological identification (Aneja 2002).

Invitro Antagonistic Activity of *Trichoderma Harzianum* Against Soil Borne Fungal Isolates

Dual culture method has been used for the determination of antagonistic activity of Trichoderma harzianum against various soil borne fungal isolates on Potato Dextrose Agar medium. Approximately 3.0 to 4.0 mm in diameter mycelial disc was employed from *Trichoderma harzianum* and test fungal cultures were placed on PDA medium in the same petri dish, which is basically 4.0cm away from each other individually. All the culture inoculated petri plates were incubated at 28±2°C for six days. After the completion of incubation period plates were observed for antagonistic activity of *T. harzianum* against soil borne fungal isolates. Index of antagonism percentage of growth inhibition of soil borne fungal isolates, was determined by method of Watanabe (1984).

$$\% \text{ of Growth Inhibition} = \frac{C-T}{C} \times 100$$

Where,

C = Colony diameter of control pathogen

T = Colony diameter of pathogen in inhibition on plate

Qualitative Screening of Various Enzyme Assay

Enzyme assay was performed by using respective solid media for the screening of extracellular enzymes. Enzyme assay was assessed based on the zone of clearance, colour changes and its intensity around the fungal colonies for production of amylase enzymes.

Amylase Assay

The selected *T. harzianum* was screened for amylolytic activity by starch hydrolysis test, utilizing Starch Agar Medium containing Peptone 5.0gm, Beef extract 3.0gm, Starch 20.0gm and Agar-Agar 18.0gm were used in the 1000ml of distilled water then medium was aseptically transferred to petri plates and line inoculated of 5-day old fungal culture and incubated at 28ºC for 5 days. 1% iodine solution was flooded into the starch culture plate. The clear zone of hydrolysis around growth indicates positive results for amylase producers (Sujeeta et al., 2017; Shah et al., 2014; Shalini et al., 2014).

Cellulase Assay

Cellulase activity (Hankin and Anagnostakis, 1975) was performed by growing *T. harzianum* isolated strain on the Czapek-Mineral Salt Agar Medium and was amplified with Carboxymethyl Cellulose 5.00 g. The medium is then poured into sterile petri-plates and line inoculation of *T. harzianum* has been carried out. The plates were allowed to incubate at 28ºC for 5 days. The plates were flooded with 2% w/v aqueous Congo red solution for 15 min. Then rinse the agar surface with distilled water. Further the plates were flooded with 1 M NaCl for 1.5 min. The yellow-opaque zone has been observed around the colony which was considered as a positive result for cellulose assay (Ramaraju et al., 2017).

Pectinase Assay

Pectinase activity (Hankin and Anagnostakis, 1975) was performed by growing *T. harzianum* isolated strain on Pectinase Agar Medium. The medium is then poured into sterile petri-plates and line inoculation of *T. harzianum* has been carried out. The plates were allowed to incubate at 28ºC for 5 days. After the completion of the incubation period the plates were flooded with 1% CTAB (Hexadecyl trimethyl ammonium bromide) was dissolved in 10 g/lit. in distilled water. The zone of clearance has been observed around the colony which was considered as a positive result for pectinase assay (Geetha et al., 2012).

Protease Assay

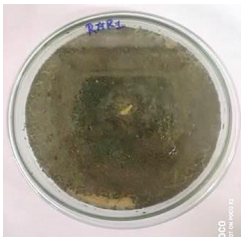
Protease activity (Vijayaraghavan and Samuel, 2013) was performed by using *T. harzianum* isolated strain on Casein Agar Medium. Line inoculation of *T. harzianum* has been carried out. The plates were allowed to incubate at 28ºC for 5 days. The plates were flooded with Bromo-Cresol Green dye. The zone of clearance around the colony which indicates the positive result for protease assay and indicates proteolytic activity (Ramaraju et al., 2017).

Extraction of Crude Amylase Enzyme from *T. Harzianum*

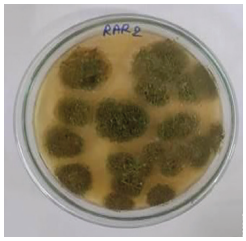
For the extraction of crude amylase enzyme from *T. harzianum* has been carried out by submerged fermentation by using three different production media such as Starch media containing NH_4NO_3 10.00g, $FeSO_4.7H_2O$ 0.01g, KH_2PO_4 1.4g, KCl 0.50g, $MgSO_4.7H_2O$ 0.10g, Starch 20.00g, Distilled water 1000 ml, Potato Dextrose Broth containing 0.1g yeast extract and rice water all these production medium were prepared individually and sterilized at 121°C for 20 mins. Then one loopful culture of *T. harzianum* was inoculated into the medium and incubated on a rotary shaker incubator at 100rpm at 28°C for 5-7 days. Fungal mycelial mat was filtered with the help of Whatman filter paper no.-1 and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was collected and used as the crude amylase enzyme (Shah et al., 2014).

Quantitative Screening of Amylase Assay

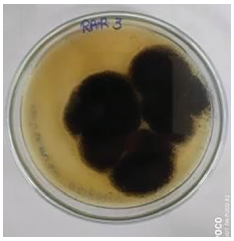
An amylase activity was determined as described by Jones and Varner (1991). Amylase enzyme activity determined by using Dinitro salicylic acid (DNSA) method. To measure the amount of reducing sugar i.e. glucose 1% soluble starch dissolved in phosphate buffer of 0.1 M and pH adjusted to 7. Then 1 ml of enzyme aliquot and 5 ml of starch solution has been taken and incubated at 55°C for 15 minutes. 1 ml of 3,5 Dinitro salicylic acid was added as a stopping reagent and allowed to incubate at 55°C for 10 minutes. Distilled water was added to make the final volume upto 12 ml. The amount of reducing sugar was measured by



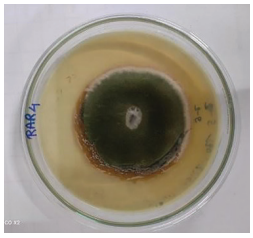
Trichoderma harzianum (RAR1)



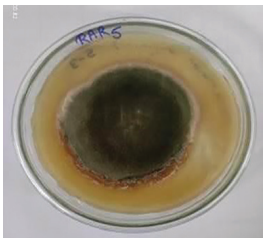
Aspergillus parasiticus (RAR 2)



Aspergillus niger (RAR 3)



Penicillium sp. (RAR 4)



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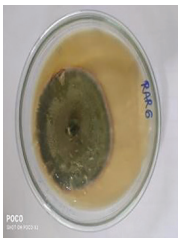
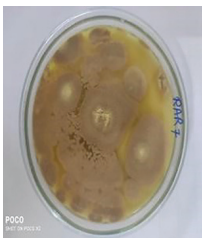
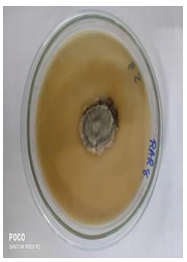
(RAR 5



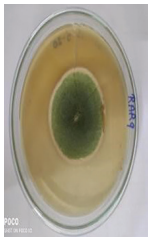
(RAR 6)



Aspergillus ochraceous (RAR 7)

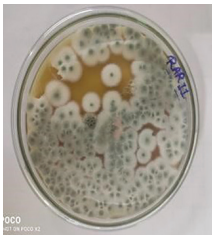


(RAR 8)
Curvularia sp



Aspergillus fumigatus (RAR 9)

Rhizopus sp. (RAR 10)



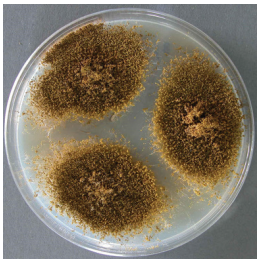
Penicillium sp. (RAR 11)

(RAR 12)

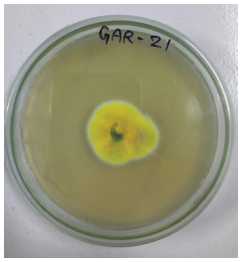
Aspergillus nidulans (RAR 13)

Fusarium oxysporum (BAR14)

Aspergillus flavus (BAR15)



Aspergillus tamarii (GAR19)



(GAR21

Fig. 1: Representative Pure Cultures of Isolated Fungal Strains from Four Different Agricultural (Groundnut) Soils of Railnagar (RAR1 to RAR13), Bedi (BAR14 & BAR15), and Gauridad (GAR19 & GAR21)

taking Optical Density (O.D.) of reaction mixture at 540 nm of spectrophotometer (UV 1900 Shimadzu). Concentration of reducing sugar was determined by following formula Suganyadevi et al., (2012).

$$\text{Concentration of Reducing Sugar } (\mu g/ml) = \frac{Optical\ Density \times 1}{Slop \times Aliquot}$$

Enzyme Activity was Determined by Following Formula

$$\text{Amylase Activity } (U/ml) = \frac{\mu g\ of\ Glucose\ Produced}{Vol.\ of\ enzyme \times Solution \times Time}$$

Optimization of Crude Amylase Production of *T. Harzianum*

Effect of Temperature

For the measurement of optimum temperature of amylase production by the fungal strain bioprocess fermentation was carried out at various temperatures such as 21, 23, 25, 27, 29, 31, 33, 35 and 37°C on at 150 rpm rotation in shaker incubator and the values has been observed by one-factor at-a-time approach (Chitra et al., 2019).

Effect of Incubation Time

To optimum incubation time of amylase production and growth of *T. harzianum* the fungal culture was cultured into the potato dextrose production medium and incubated for various incubation time 3, 5, 7, 9 and 11 days at 31°C on shaker incubator at 150 rpm rotation speed and the data has been observed by one-factor at-a-time approach (Nehad et al., 2020).

Effect of pH

For the investigation of pH value for the cultivation of *T. harzianum* into the production medium. Media were adjusted to varying from the pH values 5.0, 6.0, 7.0, 8.0 and 9.0 with the adjustment of NaOH (0.1 N) or HCl (0.1 N) in 150 rpm at 31°C for 5 days observe the data by one-factor at-a-time approach (Chitra et al., 2019).

Statistical Analysis

All the optimization studies were conducted in triplicate and the data were analyzed using mean standard deviation (± SD) of the value.

RESULTS

Isolation of Soil Borne Fungi

Soil microflora plays an important role in the plant community, their productivity as well as a causative agent for plant diseases. Environmental factors such as moisture present in soil, pH and temperature are the essential features in the distribution of mycoflora. These are the main factors affecting the biodiversity of fungi. The soil mycoflora in four different agricultural (groundnut crop) fields viz., Railnagar, Bedi, Gauridad and Ratanpar from Rajkot region, Gujarat, India were observed. In the present investigation, soil dilution plate was employed for the isolation of fungi. During the month of January 2021, the total number of twenty-three (23) fungal colonies were isolated on Petri plates containing PDA medium from Railnagar area total 13 fungal colonies i.e. (RAR1 to RAR13), Bedi (BAR14 to BAR16), Ratanpar (R_nAR17, R_nAR18) and Gauridad (GAR19 to GAR23) have been isolated as soil borne fungi. Purification was done by culturing of the hyphal tips and was transferred by using the point inoculation method to the fresh PDA medium plates (Fig. 1).

MORPHOLOGICAL AND MICROSCOPIC IDENTIFICATION OF FUNGAL ISOLATES

Generally saprophytic fungi significantly contribute to the reprocessing of nutrients from nature; they play a major role as decomposers of dead and decay organic matter. Although four soil samples from four different locations of Rajkot region were collected for the examination of fungal biodiversity. Results had revealed from the study the presence of 13 fungal species out of 23 isolates belonging to the 4 different genera, identified and characterized on the PDA plates (Table-2). The maximum number of fungal species belonging to the Ascomycota (12 colonies) and Zygomycota (01 colony) and 09 colonies were left unknown is to be identified on the PDA medium. In our current studies among all fungal isolates the genera *Aspergillus, Penicillium, Fusarium* and *Rhizopus* were the most dominant species. The most common species among them are *A. niger, A. ochraceous, A. tamarii, A. parasiticus, A. nidulans, A. flavus, A. fumigatus, Curvularia sp., Trichoderma harzianum, Penicillium sp., Rhizopus sp.,* and *Fusarium oxysporum* were isolated and characterized from the different soil mycoflora. They are isolated from four groundnut crop fields and further evaluated by morphological i.e. colour, shape, size and pigmentation features. While, in microscopic identification the conidia design, hyphal arrangement colour were identified by using lacto-phenol cotton blue staining technique and observation was done at 40X microscope & the details are reflected in the Table-2.

Table 1: Agricultural Soil Samples Collected from Different Places in Rajkot Region

Sample Number	Agricultural Field	Place and Nomenclature
1	Groundnut	Railnagar (RAR*)
2	Groundnut	Bedi (BAR*)
3	Groundnut	Ratanpar (R_nAR*)
4	Groundnut	Gauridad (GAR*)

***Note**: In naming of the places **AR** is denoted of Agricultural Region

Table 2: Morphological and Microscopic Identification of Fungal Isolates from Different Agricultural Field of Soil Samples

Sample	Colony Colour	Front View Arial Mycelia Vegetative/ Arise	Area (cm)		Pigmentation	Wrinkle Furrow	Microscopic Observation	Identified Fungal Isolates
			Height	Width				
RAR 1	Dark green or pustules fringed by sterile mycelium	Vegetative	9	9	Dull yelloweye	NO	Conidiophore- Frequently branching, Ampulliform Phialide, conidia shape- Sub globose to obovoid	*Trichoderma harzianum*
RAR 2	Dark Green	Arise	9	9	NO	NO	Subglobose, Filamentous extension rough conidiophore septate hyphae	*Aspergillus parasiticus*
RAR 3	Black	Arise	9	9	NO	NO	Smooth conidia septate hyphae phialides- rough	*Aspergillus niger*
RAR 4	White border to Dark green center	Vegetative	7.5	4.5	NO	NO	Conidia- round, Phialides- Flask shaped brush like clusters	*Penicillium sp.*
RAR 5	White to yellowish border with greenish center	Border: Vegetative Center: Arise	5	4	Reverse mycelium pigmentation (yellow)	NO	–	Not-Identified
RAR 6	Gray	Vegetative	1	1	NO	Wrinkled	–	Not-Identified
RAR 7	Cream to brownish color with light yellow center	Vegetative	9	9	NO	NO	Vesicle- elongated conidia- rough & spherical	*Aspergillus ochraceous*
RAR 8	Velvety-Black grey	Arise	4.5	6	Reverse mycelium pigmentation (black)	Wrinkled	Septate geniculate conidia	*Curvularia sp.*
RAR 9	Border: Off white & dark green Center: Light green	Vegetative	9	9	NO	NO	Septate Hyphae, single series of Phialides, round and rough conidia	*Aspergillus fumigatus*
RAR 10	Gray to White color	Arise	9	9	NO	NO	Non-septate, root like hyphae sporangiophore- unicellular shape- ovoid	*Rhizopus sp.*

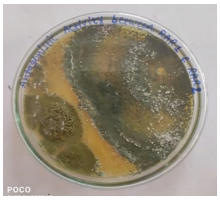
Table 2 contd...

Table 2 contd...

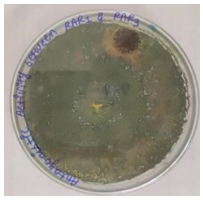
RAR 11	White to dark greenish	Vegetative	9	9	NO	NO	Conidia- round, Phialides- Flask shaped brush like clusters	*Penicillium sp.*
RAR 12	Border: Light green Internal: Dark green & brown	Border: Vegetative Center: Arise	5.5	6	NO	NO	–	Not-Identified
RAR 13	Off White; Border Internal: Dark green	Arise	6.5	6	NO	NO	Septate hyphae, conidial head heads-columnar, conidiophores-brown and short	*Aspergillus nidulans*
BAR 14	White	Arise	7.7	6	Pink reverse mycellium pigmentation	NO	Conidiophore- short, unbranched septate macrospores sickle shaped	*Fusarium oxysporum*
BAR 15	Yellowish-Green	Center: Arise Border: Vegetative	7.5	7.5	NO	NO	Septate hyphae phialides- arranged in one arrow	*Aspergillus flavus*
BAR 16	White border with black center	Vegetative	9	6	NO	NO	–	Not-Identified
R_nAR 17	White	Vegetative	2	2	NO	NO	–	Not-Identified
R_nAR 18	White	Arise	6	6	NO	NO	–	Not-Identified
GAR 19	Browhish	Vegetative and arise	7.5	6.5	NO	NO	Non-septate phialides- arrange in one arrow	*Aspergillus tamarii*
GAR 20							–	Not-Identified
GAR 21	Yellow with center dark green	Vegetative	3	2.5	NO	NO	–	Not-Identified
GAR 22	Light Yellow-Green	Vegetative	2	2	NO	NO	–	Not-Identified
GAR 23	White	Vegetative and arise	7	6	NO	NO	Conidiophore- short, unbranched septate sickle shaped	*Fusarium sp.*

Antagonistic Activity of *Trichoderma Harzianum* (RAR 1) Against all the Soil Borne Fungal Isolates

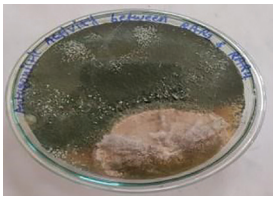
Invitro antagonistic activity of *Trichoderma harzianum* (RAR 1) was screened against various isolated soil borne fungal cultures by dual culture plate method on PDA media with an incubation time of 7 days at 28°C. The inoculum size of *Trichoderma* was 237.5 × 10^5 spores/mm^3. The results had revealed *Trichoderma harzianum* was shown inhibition of minimum 11.11% to maximum 94.11% growth inhibition of all selected soil borne fungal isolates. Here, *Trichoderma* shows the highest growth inhibition percentage is 94.11% against *Curvularia sp*. (RAR 8), while 71% to 80% were observed from RAR 3 (*A. niger*), RAR 7 (*A. ochraceous*), GAR 20, GAR 21, then 50% to 70% growth inhibition was from GAR 22, RnAR 17, BAR 14 (*Fusarium oxysporum*), RAR 5, RAR 4 (*A. terreus*), RAR 2 (*A. parasiticus*), less than 50% antagonism was obtained from RAR 9 (*A. fumigatus*), RAR 10 (*Rhizopus sp.*), RAR 11 (*Penicillium sp.*), RAR 12, RAR 13 (*A. nidulans*), BAR 16 and GAR 23. On other hand 3 soil borne fungal strains i.e. BAR 15 (*A. flavus*), RnAR 18 and GAR 19 (*A. tamarii*) has shown the negative impact to inhibit the growth of *Trichoderma harzianum* (RAR 1). Where Growth in millimeters of *Trichoderma harzianum* and selected soil borne fungal isolates were given in the Table-3 respectively. With all the soil borne fungal isolates *T. harzianum* shows antagonistic activity against all the fungal strains that inhibits the growth of all fungi.



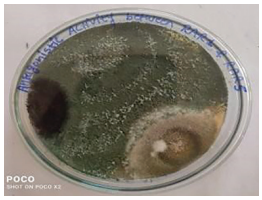
T. harzianum (RAR 1) against RAR 2



T. harzianum (RAR 1) against RAR 3



T. harzianum (RAR 1) against RAR 4



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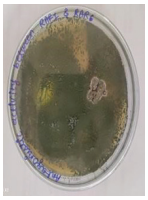

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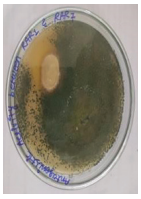

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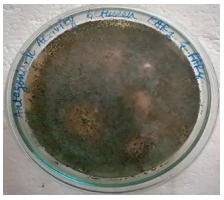
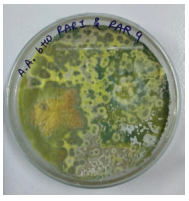
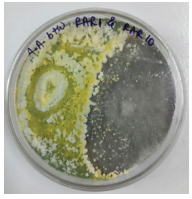


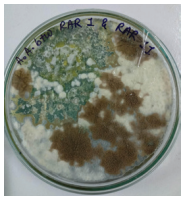
T. harzianum (RAR 1) against RAR 8



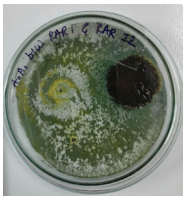
T. harzianum (RAR 1) against RAR 9



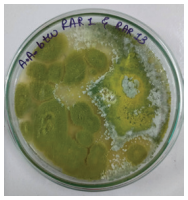
T. harzianum (RAR 1) against RAR 10



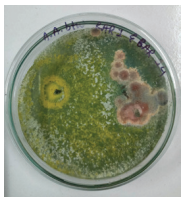
T. harzianum (RAR 1) against RAR 11



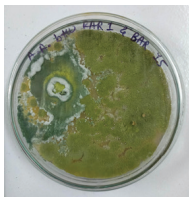
T. harzianum (RAR 1) against RAR 12



T. harzianum (RAR 1) against RAR 13

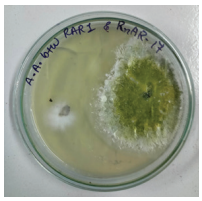


T. harzianum (RAR 1) against BAR 14



T. harzianum (RAR 1) against BAR 15

T. harzianum (RAR 1) against BAR 16



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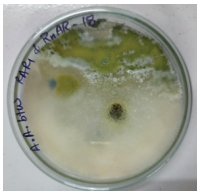

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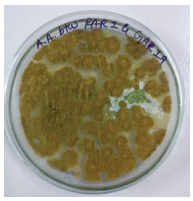
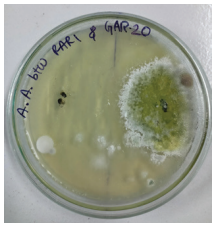
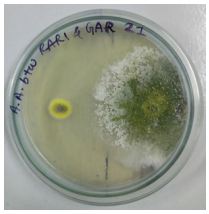


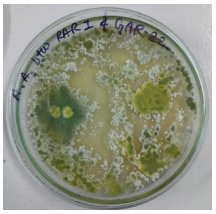
T. harzianum (RAR 1) against GAR 19



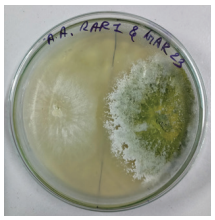
T. harzianum (RAR 1) against GAR 20



T. harzianum (RAR 1) against GAR 21



T. harzianum (RAR 1) against GAR 22



T. harzianum (RAR 1) against GAR 23

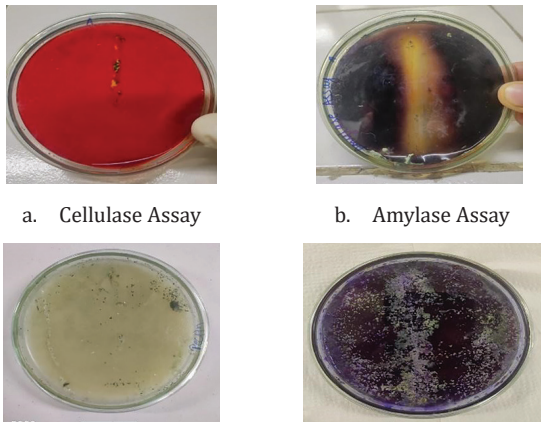
Fig. 2: Antagonistic Activity of *Trichoderma Harzianum* Against Various Soil Borne Fungal Isolates

Table 3: Antagonistic Activity of *T. Harzianum (RAR 1)* Against Remaining 22 Soil Borne Fungal Isolates by Employing Dual Culture Method

Sr. No.	Tested Fungal Isolate Against *T. Harzianum (RAR 1)*	Colony Diameter of Control Pathogen (*T. Harzianum-RAR 1)* (mm)	Colony Diameter of Pathogen in Inhibition on Plate (mm)	Percentage (%) Growth Inhibition
1.	RAR 2	60	30	50.00
2.	RAR 3	75	15	80.00
3.	RAR 4	69	21	69.56
4.	RAR 5	53	22	58.49
5.	RAR 6	73	17	76.71
6.	RAR 7	74	16	78.37
7.	RAR 8	85	05	94.11
8.	RAR 9	52	40	23.07
9.	RAR 10	45	40	11.11
10.	RAR 11	45	39	13.33
11.	RAR 12	55	30	45.45
12.	RAR 13	45	40	11.11
13.	BAR 14	65	30	53.84
14.	BAR 15	42	45	-07.14
15.	BAR 16	50	35	30.00
16.	R_nAR 17	60	25	58.33
17.	R_nAR 18	30	55	-83.33
18.	GAR 19	23	55	-139.13
19.	GAR 20	35	05	85.71
20.	GAR 21	50	12	78.00
21.	GAR 22	55	20	63.63
22.	GAR 23	50	37	26.00

QUALITATIVE SCREENING OF VARIOUS ENZYME ASSAY

The qualitative screening of enzymes such as amylase, cellulase, protease and pectinase produced by *T. harzianum* were performed by plate assay method. Amylase assay was done by using Starch Agar Medium, for cellulose assay Mineral Salt Agar Medium, pectinase assay: Pectinase Agar Medium and Protease assay was done by Casein Agar Medium. Absence of hydrolytic zone was observed on cellulase, pectinase and protease assay while the starch agar plate method resulting in clear zone of hydrolysis after iodine treatment which implied the presence of extracellular amylase enzyme (Fig. 3). *T. harzianum* exhibited the highest amylase activity around the starch assay medium. Amylase enzyme was selected for the extraction and optimization by submerged fermentation. So, *T. harzianum* was considered as a potent amylase producer and can be utilized in industrial applications.



a. Cellulase Assay b. Amylase Assay

c. Pectinase Assay d. Protease Assay

Fig. 3: Qualitative Screening of Enzyme Assay by Plate Assay Method: (a) Cellulase (b) Amylase (c) Pectinase and (d) Protease Assay

QUANTITATIVE SCREENING OF AMYLASE ENZYME ASSAY

Amylase activity was estimated by Dinitrosalicylic acid assay method (Table-4). Amount of sugar concentration has been estimated. Three different production media namely, Potato Dextrose Broth, Starch Broth and Rice Water were utilized for amylase enzyme extraction. In the present investigation, highest crude amylase activity i.e. 49.60U/ml found in Potato Dextrose Broth, was gradually decreased in Starch broth (19.68U/ml). The lowest enzyme production was obtained in Rice water (0.48U/ml).

Table 4: Amylase Activity of *T. Harzianum* by Employing Three Different Media

Sr. No.	Medium used for Enzyme Extraction	Optical Density (O.D.) at 540 nm	Amount of Reducing Sugar (µg/ml)	Enzyme Activity (U/ml)
1.	Potato Dextrose Broth	0.620	1240	49.60
2.	Starch Broth	0.246	492	19.68
3.	Rice water	0.006	12	0.48

OPTIMIZATION OF CRUDE AMYLASE PRODUCTION OF *T. HARZIANUM*

Effect of Temperature

In the present investigation data clearly indicates that amylase activity by *T. harzianum* varied significantly with an increase towards the optimum temperature. Maximum (91.05±2.20 U/ml) amylase activity was obtained at 31˚C under submerged fermentation condition (SmF).

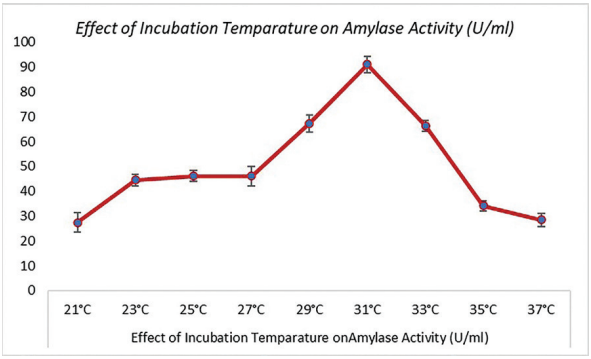


Fig. 4: Effect of Temperature on Crude Amylase Activity of *T. Harzianum*

Effect of Incubation Time

The maximum growth and activity of *T. harzianum* had been reflected as 78.21±2.59U/ml at 5 days of incubation period under SmF condition at 31˚C incubation temperature.

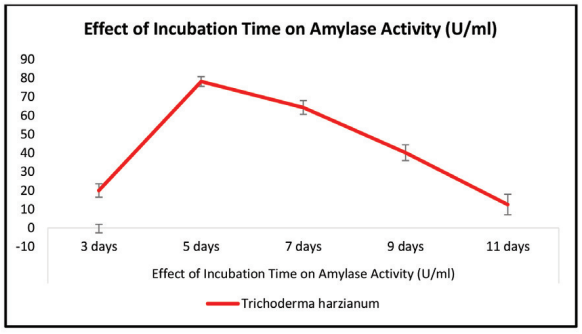


Fig. 5: Effect of Incubation Time on Crude Amylase

Activity of *T. harzianum*

Effect of pH value

Under SmF, the highest enzyme activity of *T. harzianum* (66.31±2.25U/ml) was obtained at 7.0 pH at 31°C after 5 days of incubation period.

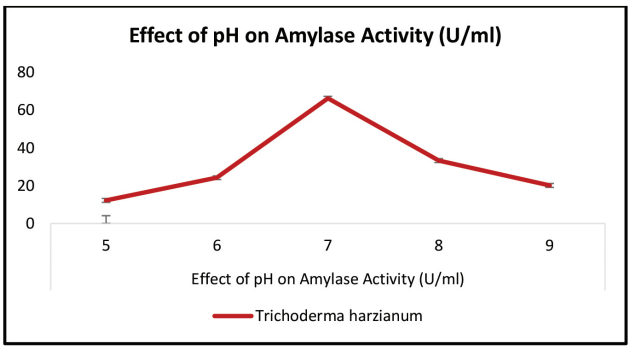


Fig. 6: Effect of pH value on Crude Amylase Activity of *T. Harzianum*

Validation of Optimized Physical Parameters for Amylase Production from *T. harzianum*

Highest amylase activity (87.69 U/ml) has been obtained by *T. harzianum* by using 1.5% Potato Dextrose broth medium at 31°C, pH 7.0 on 5 days incubation period under employing submerged condition.

DISCUSSIONS

For the growth of fungal mycelia PDA medium is the most frequently utilized culture medium by several workers who worked with it earlier, for its simple nutrient compositional formulation and likely to support inclusively for the fungal growth. (Maheshwari et al., 1999; Saha et al., 2008; XuSo et al., 1984). Our findings are in accordance with the results of Ratna et al. (2015) as the microbial diversity of different soil samples in agricultural areas of Tekkali Mandal in Srikakulam District. In their work the most dominant fungal isolates were *Aspergillus* and *Penicillium* on PDA media. All the isolates were named and characterized as *A.clavatus, A.flavus, A.fumigatus, A.granulosus, A.nidulans, A.niger, A.restrictus, A.terreus, Curvularia clavata, C.lunata, Fusarium oxysporium, F.solani, Penicillium Chrysogenum, P.frequentens, P.funiculosum, Rhizopus stolonifer, Trichoderma harzianum, T.viride*. Similarly, Noor Zaman et al. (2012) isolated fungal diversity of different samples from rainfed areas of Punjab and Pakistan. Charan et al., (2016) also reported the morphological identification of *Trichoderma harzianum* from soil samples. Here it observed the antagonistic activity of *T. harzianum* against various fungal strains. *T. harzianum* shows antagonism against all the fungal strains, it inhibits the fungal growth. Similar observation reported by the Leelavathi et al., (2014) that *Trichoderma* shows higher antagonistic activity against *Aspergillus niger, Aspergillus fumigates, Aspergillus clavatus*, *Fusarium spp., Rhizopus* and *Aspergillus terreus*. Similarly, Bardia and Rai (2007) obtained the antagonism of *Trichoderma viride and T. harzianum against Fusarium oxysporum* by mycelial growth inhibition percentage 51.15% and 58.41% respectively.

Aline et al., (2000) reported the characterization of amylase producer and antagonistic activity by *Trichoderma harzianum* against *Crinipellis perniciosa*. According to the Ramaraju et al., (2017) report amylase enzymes help to degrade the polysaccharide complex compound and convert it into simple carbon and nitrogen source which help in growth and nutrition of *T. harzianum*. Similarly, Shalini Singh (2014) observed that amylase production by *Aspergillus fumigatus* played an important role in industries. Likewise, Sakti et al., (2012) had reported the amylase activity being estimated by Dinitrosalicylic acid assay (5.9 U/ml), Dextrinizing activity (10.5 U/ml). Decrease in starch-iodine color intensity, Plate assay (18 mm) by *Aspergillus niger* under solid state fermentation was also reported in the similar documentation. Allied work has been reported by Shah et al., (2014) employing *Aspergillus oryzae* under submerged fermentation conditions. He also stressed on the enhanced productivity of amylase from *T. harzianum*, where physical parameters had played a significant role in the media optimization. Temperature is one of the most important factors in enzymatic activity, as the effect of temperature on Amylase activity was investigated during study. The maximum enzyme production was recorded at 291.05±2.20 U/ml at 32°C under submerged fermentation conditions. Similarly, *Cylindrocephalum sp.* produced amylase in liquid media and maximal amylase production was found to be at 30°C after 3-5 days of incubation period by Sunitha et al., (2012). The incubation period for the maximum amylase activity was after 5 days of incubation in SmF and highest enzymatic activity was obtained 96.37±2.98 U/ml on the 5th day although enzyme activity was decreased subsequently after the 6th day. pH plays a vital role in enzyme excretion and morphological condition of fungus at 6.5 pH the Amylase activity was found to be 87.69 U/ml in submerged fermentation conditions. Similar report has been observed by Behailu et al., (2018) & Nehad et al., (2020) in this report pH of 6.0 to 7.0, is the optimum

pH range for the attainment of amylase from various fungal isolates such as *A. niger & Trichoderma sp. Trichoderma harzianum* have adequate capability to act as biocontrol agent against fungal plant pathogens (Charan Singh et al., 2016; Viterbo and Chet 2006).

CONCLUSION

In the present context, the antagonism of *Trichoderma harzianum* against selected soil borne fungal isolates was determined by a dual culture method. *T. harzianum* is known to have the potential to inhibit the radial growth of isolated soil borne fungal strains. The present study showed that crude amylase enzymes being extracted from *T. harzianum* followed by optimization has significant activity in presence of inducible substrate starch. Amylase is well known for an efficient conversion of starch for the industrial usage & eliminating surface stains. Henceforth, the strain *Trichoderma* sp.in the current report was tried to established as a biocontrol agent along with a potent amylase producer enriching the soil nourishment and promoting plant growth. Besides that, amylase would also play a significant role in food and detergent industries for further studies as it is potentially improving the detergency and enhancing the flavors in food and it is also environment friendly.

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