

Isolation, Characterization, Cultivation of *Trichoderma* Species and its Applications as Biopesticide

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Article Info:



Article History:

Received 12 July 2022
Reviewed 17 August 2022
Accepted 24 August 2022
Published 15 Sep 2022

Cite this article as:

Agadi HVS, Pratima S, Isolation, Characterization, Cultivation of *Trichoderma* Species and its Applications as Biopesticide, Journal of Drug Delivery and Therapeutics. 2022; 12(5):28-33

DOI: <http://dx.doi.org/10.22270/jddt.v12i5.5624>

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Abstract

Trichoderma is a genus of anamorphic fungi that are mostly found in rhizosphere soil. and can be used to combat fungal phytopathogens (*Fusarium* spp which cause the wilt stem in chilli plant). *Trichoderma* species have long been employed as a biopesticide in agriculture. It also provides the plant with growth-promoting chemicals, such as Indole acetic acid and ammonia. The molecular mechanisms underlying this very desirable favourable effect of plant growth promotion, which includes improved nutrient availability and uptake for the plant, are not entirely understood. Extracellular enzyme activity of cellulase, lipase, laccase, protease, and amylase in *Trichoderma* spp. In vitro antagonistic potential of *Trichoderma* spp. against *Fusarium* spp. was investigated.

Keywords: *Trichoderma*, Phytopathogens, Biopesticide, *Fusarium*

INTRODUCTION

Trichoderma is a genus of anamorphic fungi isolated from degrading organic waste in the soil. It is a system that works against fungal infections. Secondary metabolites produced by *Trichoderma* have antibacterial properties. It has long been used in agriculture as a biocontrol agent and as inoculant to promote plant development. The presence of *Trichoderma* in plants causes resistance to develop, which is often localized or systematic. In comparison to pathogen-triggered immunity, effective *Trichoderma* strains can induce a peculiar response in the plant. *Trichoderma* species isolates were chosen for their potential to thrive in the roots, as endophytes, or in the rhizosphere, to protect plants from pathogens or to promote plant growth. ¹

Agriculture has long been the most important sector in terms of food production, employment, and GDP contribution (Gross domestic product). In addition high-yielding seeds and adequate fertilizers, a large amount of high-quality insecticides have been employed to preserve the massive amount of agriculture production. Pesticides are used excessively, resulting in large levels of pesticides accumulating in soil and water, posing a threat to the food chain and drinking water. ²

By selecting isolates with a high capacity to secrete extracellular lytic enzymes *chitinase* and *B-1,3-glucanase*, it is crucial to isolate *Trichoderma* spp. with potentially higher antagonistic efficiency. The lytic enzymes break down cell wall

polysaccharides into small oligomers, making it easier for the hyperparasite to enter the target fungi's cytoplasm. ³

Fungal illnesses are the most debilitating of all diseases. In terms of management, *Fusarium* is the most difficult. Chili wilt is caused by *F. oxysporum*. It is a soil-borne fungus that can be found in all types of soil. *Fusarium oxysporum* is saprophytic, meaning it can thrive on organic substances for long periods of time. Drooping and yellowing of leaves, reduced growth, and short internode distances are all signs of *Fusarium* wilt in chilies. Plants that have been subjected to severe attacks seem dry and eventually die. Disease management strategies can be defensive in nature since they use a variety of control methods to limit the spread of pathogens. *Trichoderma*, which has long been acknowledged as a strong antagonist against plant pathogenic fungus, is one of the most often employed fungal biological control agents. ⁴

Chili is one of the most popular horticulture products. Plant pest species, such as plant diseases, have disrupted the production of chilli plants on numerous occasions. The use of synthetic chemical fungicides to manage plant disease on chilli. Because synthetic chemical fungicides are difficult to disintegrate or degrade naturally, they can cause a variety of issues. Pathogen resistance and the presence of residues in carcinogenic dietary items are two issues. Because of the concern for human and environmental safety, the best alternative strategy is to use biological control agents. *Trichoderma* sp. is a good biological controlling agent (BCA)

for overcoming chilli plant diseases and is one of the antagonistic microorganisms. The ability of *Streptomyces* sp. and *Trichoderma* sp., two biological control agents, to control plant diseases. The biological control agent's ability to control plant disease is still confined to one type of biological control agent. Combining several biological control agents can improve the biological control agent's ability to control plant diseases. The synergy between the use of controlling agents is what makes the combination possible. "Controlling chilli plant disease with a combination of biological control agents *Streptomyces* sp. and *Trichoderma* sp."⁵

Because of their negative side effects, chemical pesticides used in crop protection to limit the damage caused by pathogens and pests in agricultural areas pose various long-term concerns and risks to living beings. They are known to cause cancer and foetal abnormalities, and they can last for years in the environment. Biopesticides are naturally occurring substances or agents derived from animals, plants, and microorganisms like bacteria, cyanobacteria, and microalgae that are used to control agricultural pest diseases. Biopesticides are "produced from natural materials such as animals, plants, microbes, and certain minerals," according to the US Environmental Protection Agency. These biocontrol agents' products, such as genes or metabolites, can be employed to prevent crop damage. Biopesticides are considerably superior than their chemical equivalents in terms of effectiveness. Traditional chemical insecticides are used because they are both environmentally friendly and host-specific. Biopesticides can considerably improve the usage and application of agro-based chemicals in the agricultural sector to protect crop plants against invading and infecting pests. Their value as a kind of protection would eventually lead to commercial acceptance. Biopesticide production involves a variety of potential sources and technologies.⁶

Trichoderma species is a heterotrophic saprobion bacterium that competes with other microbes and prevents their growth. This fungus has several modes of action, including antibiotic and metabolite synthesis, as well as hyperparasitism, which promotes the formation of systemic resistance in plants. Strains of *Trichoderma* were recovered from agricultural land. *Trichoderma* spp. Success, and the rhizosphere is attributed to its ability to reproduce as well as its efficiency in utilizing soil nutrients; the genus' effectiveness has been observed against a number of plant pathogenic fungi. Some *Trichoderma* species have the ability to synthesize and release enzymes (cellulase, laccase, lipase, amylase, protease). Auxin and gibberellin-type growth regulators have been identified as promoters of the growth of several agricultural crops by *Trichoderma* spp. *Trichoderma* has been shown to colonize the root epidermis and outer layers of the corticle, releasing volatile substances like ethylene, alcohol, aldehydes, ketones, and non-volatile compounds such as peptides and enzymes that limit fungus growth. Abiotic variables, such as the collection site where they were isolated, reduce its efficiency. In the country, only a few investigations have focused on the identification of native *Trichoderma* spp. chemicals. In addition, the influence on *Fusarium oxysporum* inhibition, as well as the effect on plant germination and growth, was assessed. The goal of this work was to discover secondary metabolites produced by native *Trichoderma* spp. strains in vitro, as well as to assess the effects of *Trichoderma* extracts on *F. oxysporum* growth and seed germination.

MATERIAL AND METHODS:

Sample collection:

Fresh soil of *Trichoderma* species were isolated from the fields of Sorab, Dist-Shivamogga from Karnataka.

Isolation and Characterization of Fungus:

The dilution Plate Technique was used to isolate soil samples taken from 5 cm depth around the root zone of chilli plants. A representative sample was taken from them once they were pooled. Following the serial dilution plate approach, the bioagents were separated from the representative sample. 10⁻³ were collected and used for fungal bioagent isolation. 1 mL of suspension from each dilution was aseptically transferred to a Petri plate. For uniform distribution, the plates were manually rotated, and the suspension in the medium was allowed to solidify. For the growth of fungal colonies, the plates were incubated at 25°C for seven days. Colonies with *Trichoderma* species-like growth patterns were isolated and subcultured. Lactophenol cotton blue stain was used to study the separated pure colonies under the microscope. On potato dextrose agar (PDA) slants, pure colonies were subcultured.

Isolation and Characterization of Fungus involves the below procedures

- a) Preparation of Potato dextrose agar.
- b) Serial dilution of Rhizosphere soil
- c) Inoculation of a sample using Spread and Streak Plate Method
- d) Observation Morphological Characteristics of Organisms
- e) Microscopic view of *Trichoderma* species after addition of Lacto phenol cotton blue.
- f) Transfer of *Trichoderma* species to the broth for subculture.
- g) Transfer the *Trichoderma* species to the prepared potato dextrose agar slants for storage.

Analysis of Growth promoting substances by *Trichoderma* Sp

The following procedure was used to assess growth-promoting chemicals and extracellular enzymes generated by *Trichoderma* Sp.:

- A) Demonstration of Indole Acetic Acid Production
- B) Ammonification

A) Demonstration of Indole Acetic Acid

Fungus cultures were cultured in tryptophan-added Potato Dextrose Broth (PDB) (5 mm). For 20 minutes, cultures were centrifuged at 10,000 rpm. 2 mL supernatant, 2 drops orthophosphoric acid, and 4 mL Salkowski reagent were combined. The tubes were incubated at room temperature. For a total of 23 minutes, the temperature was raised. At 540 nm, the pink color intensity was measured spectrophotometrically.

B) Ammonification

As a substrate, Potato Dextrose Broth (PDB) was made with organic nitrogen. In each tube, 0.5 gram of soil was added after the fungal culture was inoculated and incubated for 24 hours. The tubes were filled with Nessler's reagent. The yellow to brown precipitated after adding Nessler's reagent indicates the presence of ammonia, which is recognized by the presence of ammonia.

Assay for Extra Cellular Enzyme Activity:

1. Preparation of media for enzyme activity

After incubation of 3-5 days, add 2% carboxymethylcellulose (CMC) to potato dextrose agar media enriched with 0.2% cango red and detained with 1M sodium chloride solution.

2. Preparation of media for Lipase activity

0.5ml of 1% olive oil and 0.5% phenol red indicator are added to PDA plates. The lipase enzyme breaks down oil to release free fatty acids, changing the media's pH and color from red to yellow.

3. Preparation of media for Protease activity

White spots with a clear zone were detected after 0.5gm of casien was put into potato dextrose agar media.

4. Preparation of media for laccase

Tannic acid (0.5 gm) was added to potato dextrose agar media, which was incubated for 3-5 days. Tannic acid is oxidised by laccase, resulting in a reddish-brown hue.

5. Preparation of media for Amylase activity

Add 1% starch to Potato dextrose agar media, then add an iodine solution and wait for the organisms and media to get stained.

a. Analysis of Amylase activity through Colorimeter

After inoculating the broth with prepared potato dextrose agar, take the culture and centrifuge it for 15 minutes. 1ml of buffer solution to 0.5ml of culture, incubate for 15 minutes, then add 1ml of DNS, boil for 10 minutes, cool for 10 minutes, and then add 7ml of water. At 540nm, the colour obtained is measured in a colorimeter.

Presence of Secondary Metabolites

The following approach was used to determine the presence of secondary metabolites in *Trichoderma* species.

Thin Layer Chromatography

Thin Layer Chromatography was used to evaluate the presence of secondary metabolites in *Trichoderma* species.

TLC Plates were exposed to a Mobile Phase containing Benzene, N-Butanol, and Acetic Acid in the following proportions: (70:25:5)

Gas chromatography-mass spectroscopy (GC-MS)

In order to confirm secondary metabolites in *Trichoderma* species, more research is needed. Gas chromatography is a technique for analyzing the secondary metabolites. The researchers used gas chromatography-mass spectroscopy (GC-MS).

Isolation and Identification Pathogen (*Fusarium* species)

The pathogen was isolated from the wilted stem of a sick chilli plant and then inoculated on potato dextrose agar plates. White cottony mass pathofungi were observed after 3-4 days. The pathogen was then validated by examining the characteristics under a microscope. It shows the *Fusarium* species characteristics.

Treated between *Trichoderma* species and Pathogen (*Fusarium* species)

Isolated both *Trichoderma* species and Pathogen will be inoculated on the same potato dextrose agar plates by using the dual culture method. Then check observation after 5-6 days. *Trichoderma* species created the clear zone around the hyphae. It indicates the resistance of *Trichoderma* species.

RESULTS:

Isolation and characterization of *Trichoderma* species;

After 3-5 days, a number of colonies were visible on the PDA plate. When the samples were serially diluted and plated on PDA medium. Using the spread plate approach, colonies were

detected in 10-3 serial dilutions, demonstrating microfungus proliferation. In addition, employing the spread plate method, a 10-4 serial dilution demonstrated the increase of microfungus growth. Lactophenol cotton blue stain was used to view a colony that generated a green-colored, irregular-shaped, and white cottony mass under a microscope. The *Trichoderma* species was identified by microscopic examination of the mycelium containing spores, which revealed that it was branching and produced conidia. The isolate was subcultured and kept at 20°C in PDA slants.

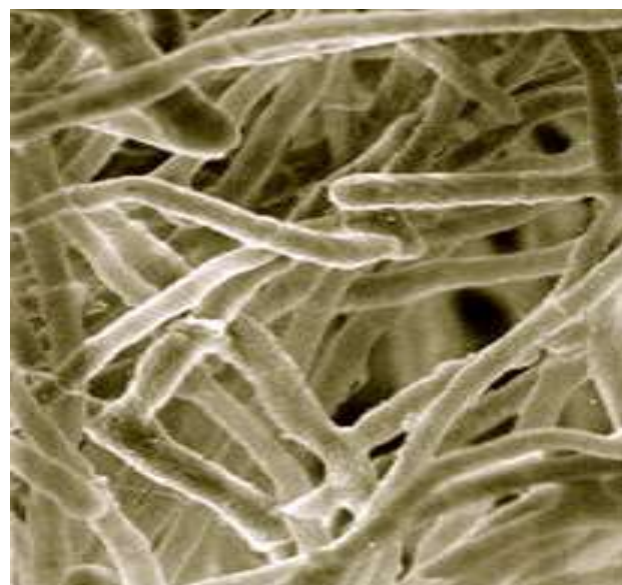


Figure 1: Represents *Trichoderma* Spp

Isolation and Confirmation of Pathogen (*Fusarium* spp.)

The pathogen was isolated from a wilt chilli stem that was inoculated on PDA media and microfungus development was seen after 4-5 days. It displayed white cottony morphological features. The microfungus was then stained by lactophenol cotton blue under the microscope. The isolate pathogen was identified as *Fusarium* species after microscopic examination. The mycelium of *Fusarium* spp. has a branching separate, filamentous structure.

Analysis of Growth Promoting Substances by *Trichoderma* species

Demonstration of Indole Acetic Acid Production

On Potato Dextrose Agar containing Tryptophan, the fungus was cultivated (5mm). For 20 minutes, the culture was centrifuged at 10,000 rpm. The 2 mL supernatant was then combined with 2 drops of orthophosphoric acid and 4 mL salkowski reagent. The tubes were incubated for 23 minutes at room temperature. Spectrophotometrically, the intensity of the pink color was measured at 540nm. Then, using a graph, compare the Standard IAA and the Culture IAA.

Standard IAA

Table 1 Represents Values obtained during the Colorimeter

Absorbance	Co concentration
0.2	0.2234
0.4	0.3121
0.6	0.5291
0.8	0.7720

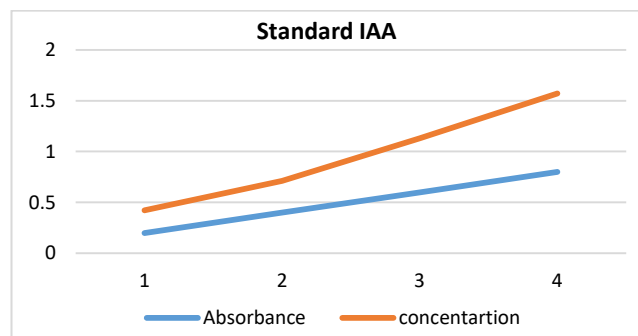


Figure 3: Graph plotted against Absorbance and concentration

Culture IAA

Table 2: Represents Values obtained during Colorimeter

Ab	Absorbance	Co	Concentration
	0.2		0.065
	0.4		0.185
	0.6		0.181
	0.8		0.193

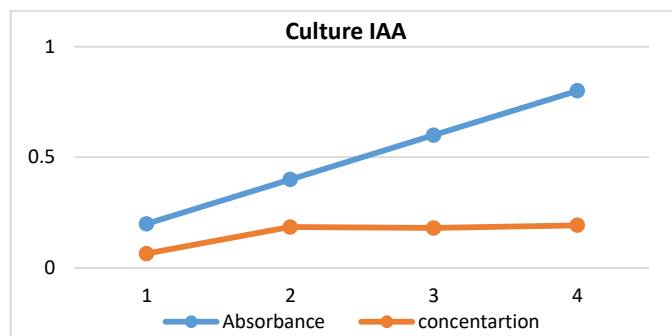


Figure 4: representation of concentration v/s absorbance of Culture IAA

Ammonification;

On PDA media, fungal growth was discovered. Then it was transferred to PDB, centrifuged, and the supernatant was removed, along with a few drops of Nessler's reagent. The presence of ammonia in the culture solution was confirmed by colour. The culture solution developed yellow to brown precipitate, indicating the presence of ammonia in the culture solution. Because of oxidation, it changes to a greenish colour after a few seconds.



Figure 5: Represents ammonification

Extracellular Enzymatic Activity on *Trichoderma* spp;

The PDA media for Cellulase enzyme was produced with 2% Carboxyl Methyl Cellulose (CMC). The presence of cellulase was demonstrated by the formation of a clear zone around the colonies. After a 3-5-day incubation period, it was supplemented with 2% congo red and dyed with a 1M NaCl solution.

The PDA was supplemented with 1 percent olive oil and 0.5 percent phenol red indicator for Lipase enzyme. The lipase enzyme destroyed the oil and released free fatty acids, causing the medium pH to change and the colour to shift from red to yellowish brown.

For laccase enzyme, incubate for 3-5 days at 28° C in PDA medium with 0.5 percent tannic acid. The medium turns a reddish colour when tannic acid is oxidised by laccase.

For the protease enzyme, PDA media with 1% casein was incubated for 3-4 days, resulting in a white spot with a hydrolysis circle.

PDA media with 1% starch incubated for 3-4 days for Amylase enzyme, then colonies were inspected and treated with iodine solution, which resulted in colonies with a brown colour look.

Amylase enzyme activation was evaluated by adding 1 ml Buffer solution to a 0.5 ml culture and allowing it to sit for 10 minutes before adding 0.5 ml of starch and allowing it to sit for 15 minutes before adding 1 ml DNS and finally adding 7 ml distilled water after 5 minutes of boiling. The intensity of the reddish brown colour was measured spectrophotometrically at 540 nm.

Table 3: Represents the values obtained through colorimeter

Absorbance	T%
0.26	0.54
0.23	0.58
0.19	0.64
0.17	0.67

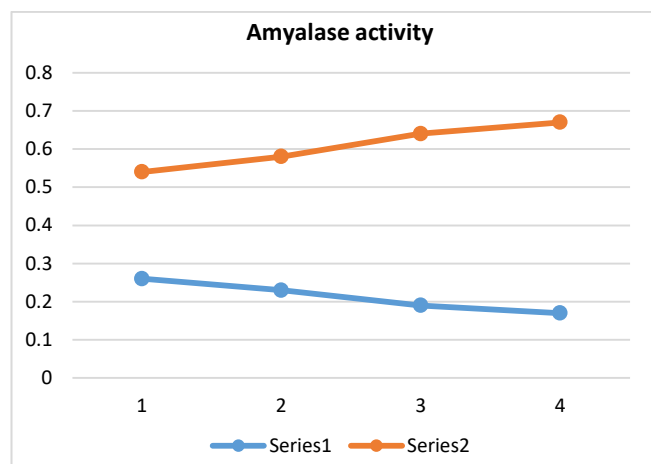


Figure 6: Graphical presentation of amylase activity

Presence of Secondary Metabolites

Thin Layer Chromatography: To check for the presence of secondary metabolites, TLC plates are utilised. The mobile phase, contains Benzene: N-Butanol: Acetic acid in the ratio (70:25:5). The presence of Secondary Metabolites was established after the TLC plates were exposed to UV light in a UV chamber.



Figure 7: Organisms subjected to UV Chamber

DISCUSSION

The *Trichoderma* species were successfully isolated from the ground soil. The soil was serially diluted and the streaks were spread on potato dextrose media plates. According to Ashish S. Ram et al (2019), the genus *Trichoderma* species consists of anamorphic organic matters with teleomorphs and its decomposing organic matters with teleomorphs. It works against fungal phytopathogens through this mechanism. This *Trichoderma* species takes more than a week for its growth after absorption of colonies with characteristics which were viewed under a microscope by staining with lactophenol cotton blue stain later the characterized *Trichoderma* colonies

were subcultured on agar slants. The isolated species were observed under the microscope. According to Krishna Kumar et al, the *Trichoderma*'s morphological and cultures were studied in four different methods which are OMA, CMO, PDA, and TSM. The morphological characters were observed and recorded. Phialide width, length, conidium presence width, and, length were observed. Growth-promoting substances and extracellular enzymes are produced by the *Trichoderma* species. The culture was prepared with tryptophan and centrifuged to obtain a supernatant layer. To this supernatant layer, salkoswki reagent, orthophosphoric acid was added. Production of IAA was observed by the pink-colored intensity and the intensity was measured using UV-spectroscopy at 540nm

Growth promoting substances for Ammonification:

PDB was prepared to contain organic nitrogen at a substrate. The fungal culture was centrifuged and then add 2-3 drops of Nessel's reagent then the culture turns to yellowish brown color after some time it looks greenish color because of oxidation.

Assay of extracellular enzyme activity

Cellular enzyme activation: preparation of PDA media with 2% carbonyl methyl cellulose with culture it forms clearing zone then it is supplemented with 0.2% congo red and destained with M NaCl solution after incubation of 3-5 days.

Lipase enzyme activation

PDA plates were prepared then it was supplemented with 0.5mL 1% olive oil and added with 0.25mL 0.5% of phenol red indicator then sterilized and inoculated. Lipase enzyme degrades oil to release free fatty acids which changes medium colour i.e., yellowish brown.

Laccase enzyme activation

Prepared PDA was supplemented with 0.5% of tannic acid then inoculated the culture on media incubate for 3-5 days at 36°C. Oxidation of tannic acid by laccase thin medium changed to reddish brown

Protease enzyme activation

Prepared PDA plates supplemented with 0.5g of 1% of casein white inoculated

Amylase enzyme activation

Prepared PDA plates with 1% of starch then inoculated the culture on plated clear zone with sp observed then iodine solution was poured and colour changed to brown. For amylase activity, 0.5mL culture was taken then add 1mL of buffer then kept for 10 minutes for incubation then add 0.5mL of starch after that it was kept aside for 15 minutes for inoculation finally add 1mL of DNI (coloring agent) kept for 5 minutes soil then add 7mL of water after some time solution settles then it is allowed to colorimetry at 540nm

TLC: The collection and surface sterilization of samples (chilly stem) which is inoculated on the PDA plates after 2-3 days. The white colony mass was observed under the microscope, after staining it with cotton blue, the fungi appeared like hairy and branched.

Treatment for pathogen by Trichoderma spp;

Isolated *Trichoderma* species and Isolated pathogen (*Fusarium* species) both are incubated on PDA media by the dual culture plate method. After 5-6 days Growth was observed. And *Trichoderma* species colonies were surrounded by clear zone formation, which indicates that the *Trichoderma* species are resistant to the isolated pathogen (*Fusarium* spp.)

CONCLUSION

The study proposed the isolation characterization and cultivation of *Trichoderma* species and its application as biopesticide. The *Trichoderma* species were successfully isolated from the rhizosphere soil and stored on agar slants. The *Trichoderma* species produced the growth-promoting substances, and it concluded. i.e., ammonia was produced, proved by ammonification method, and also produced indole acetic acid its presence checked pink color intensity by colorimetry. The species' extracellular activities presence checked i.e., Cellulase was checked by cellulose with culture, it produces clear zone formation Culture supplemented with olive oil and phenol. red. Lipase presence shows medium turns to red to yellowish-brown. Laccase also shows its presence by turning the medium to brown color, protease showed its presence by a clear zone. Finally, amylase presence is checked by starch when added the iodine it turns brown... Secondary metabolism presence checked by TLC. The pathogen is isolated by chilly plant stem and inoculation on media. The pathogen showed similar characteristics to the fusarium. Treat both *Trichoderma* and pathogen (fusarium) which showed a clear zone around. The *Trichoderma* species has Biocontroller, and Biopesticides characteristics.

Conflict of interest: Authors declare that there is no conflict of interest

Funding: Nil

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