Development and Evaluation of a Liquid Formulation of *Trichoderma viride* as a Bio-pesticide for Pest Management

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**Abstract**

The use of biopesticides in modern agriculture is acknowledged as an efficient substitute for chemical pesticides because they are based on natural ingredients. Fungal biopesticides are the most widely used and have a lot of success stories among the various biopesticides. *Trichoderma* is a genus of filamentous fungi that has several promising biocontrol agents for different plant diseases. *Trichoderma viride* uses a variety of strategies to fight plant diseases, including high reproducibility, adaptability, production of enzymes and antibiotics, capacity to colonize the rhizosphere, and significant inhibitory effects against phytopathogenic fungi. The prospective *Trichoderma viride* isolates are produced utilizing various carriers either through solid or liquid fermentation technologies, however solid fermentation produces the highest levels of reproductive capacity. High cost of substrate and storage methods are major problems to accelerate the production. The aim of our research is commercially available substrates were carried out to screen out stabilization of formulations for small scale production of *Trichoderma viride*.

**Keywords:** Fungi; *Trichoderma viride*; Bio-pesticide; Formulation; Commercialization.

**Introduction**

In order to increase the production of food around the world, modern agriculture uses pesticides and chemical fertilizers. To meet the need for food demand, the plant grows quickly and effectively when Chemical fertilizers are used. The negatives of employing a bigger quantity of more chemical or synthetic fertilizers include environmental contamination, long-term changes in the ecology and physiochemistry of the soil, decreased agricultural productivity, and several health risks. Constant use of chemical pesticides degrades soil quality and fertility, increases the likelihood that heavy metals may accumulate in plant tissues, and reduces the nutritional content and edibility of fruit (Farina and Hasanpoor, 2015; Sneha et al., 2015). Climate factors increase abiotic stress on crops, reducing agricultural productivity. (Chaudhari et al., 2023). Over the years, agriculture has undergone numerous changes. Since then, a wide variety of organic fertilizers that act as organic stimulators for plant growth have been developed. A unique kind of organic fertilizers includes
products based on "bio-fertilizers," which are microorganisms that encourage plant growth. A material called bio-fertilizer contains biological microorganisms. When sprayed to plant surfaces, they promote plant development by supplying the host plant with more crucial nutrients. In addition to producing compounds that promote growth, bio-fertilizers provide nutrients through natural processes such nitrogen fixation, phosphorus solubilization, and stimulation of plant growth (Ajmal et al., 2018).

The health of the soil and plant productivity are significantly influenced by numerous interactions between plants, soil and microorganisms (Harman et al., 2020). The ecological balance of soil is maintained by soil microorganisms working in various ways with plant roots and one another to carry out a variety of vital activities (Kumar and Mohapatra, 2021). Since bio fertilizers support the natural soil micro biota, which affects nutrient accessibility and organic matter breakdown, they are a likely strategy to improve soil microbial status (Chaudhari et al., 2021). The potential of bio fertilizers to produce a high level of microbial diversity in soil may lead to more productive crops for sustainable agriculture (Agri et al., 2022).

The result will be a decreased demand for chemical fertilizers and assistance, while the significance of biopesticides is increasing. (Vaishali, 2014). Bio-pesticides are pesticides have gained a lot of interest from the scientific community, which has proposed they could replace synthetic pesticides manufactured from naturally occurring biochemical, microbes, and plants that are safe for the environment. Not all organic products function as biopesticides. If they affect the pest's nervous system, some are chemical insecticides. Expanding the use of biopesticides can help health programmes. Biopesticides have been developed as a result of the problems that chemical pesticides cause, including genetic alterations in plant populations, a decline in beneficial species, harm to the environment or water bodies, food poisoning, and health problems like cancer. The risk of chemical exposure is reduced, fertilizer runoff causes less water pollution, and there are fewer applications, harms beneficial pests less, is biodegradable, and improves nutritional quality (Aneja et al., 2016).

Bio pesticides can be used to manage pests that harm plants. They can be living things (natural enemies), their byproducts (phytochemicals, microbial compounds), or both. They pose less risk to the environment and human health. The most common biopesticides are those that are detrimental to the target pest. Contains Phytophthora herbicides and Bacillus thuringiensis biopesticides (Bhattacharjee and Dey 2014).

Similar to the use of chemical pesticides, microbial pathogens are disease-causing organisms that proliferate widely among pest populations. Through introduction or flooding applications, these pathogens are used for biological pest control. Like other living things, insects are susceptible to a number of diseases brought on by viruses, bacteria, fungus, protozoa, and nematodes, among other kinds of microorganisms. To create crop protection methods that are friendly to the environment, researchers have examined insect microbial infections in great detail. The development of chemical resistance and residues at higher nutrient levels is a significant barrier to pest control in the crop protection environment of today. To combat the negative impacts of chemicals on non-target organisms, biopesticides have recently taken the place of chemical pesticides (Kachhawa, 2017).

Numerous species of the fungus genus Trichoderma are used as industrial biopesticides (Kumar, 2017). Trichoderma is a fungus that grows quickly and is frequently used as a growth booster and biological control agent to combat soil-borne plant illnesses. Due to their various modes of action, filamentous fungi of the genus Trichoderma are among the best studied biopesticides. Direct mycoparasitism, the creation of enzymes and antibiotics, competition, and the capacity to make plants tolerant to a variety of stressors are just a few of the strategies they use to manage plant pathogens (Alwadai, 2022).

The pathogen-suppression arsenal of Trichoderma species encompasses a variety (overparasitism), antibiotics, competition, and induced resistance (Sharma, 2019). Competition between Trichoderma species and mycoparasites is frequently used to suppress soil-borne pathogens (Mukherjee et al., 2022). A putative Trichoderma species has been suggested by a recent study by Ren X et al. (2022). Inhibits the formation of aflatoxin B1 by isolates of aflatoxinogenic Aspergillus flavus. This implies that employing Trichoderma species as a biocontrol agent against A. flavus and preventing a flatoxin accumulation can be a successful biocontrol strategy, particularly when many isolates with various modes of action are combined (Ren et al., 2022).

Trichoderma sp. biomass and formulations must be produced in large quantities at low cost using locally accessible strains and inexpensive raw materials, such as diverse wastes and byproducts. With or without minor adjustments to the culture medium’s composition, sufficient biomass yields, including effective propagules (such as
chlamydospores), can be achieved utilising such inexpensive growth substrates (Kumar et al., 2014).

*Trichoderma viride* is very capable of procreating and sporulating, as well as being competitive and surviving through saprophytic growth (Howell, 2013). *Trichoderma* strains expand quickly when injected into soil due to their innate resistance to a variety of harmful substances, such as pesticides, fungicides, and herbicides (Seethapathy et al., 2017). A range of trash and byproducts that can be employed as growth substrates to produce sufficient biomass containing effective propagules with little to no media composition adjustment. This review describes his two primary techniques for producing *Trichoderma* species in large quantities: liquid fermentation and solid-state fermentation (Kumhar et al., 2014).

It has enormous potential for commercial *Trichoderma* spp. bulk production. Finding alternatives to pesticides and chemical fertilizers to control widespread plant diseases and *Trichoderma* spp. plant growth stimulants has been the focus of research (Kumhar et al., 2014). There have been numerous research on the production of *Trichoderma* spp. utilising traditional synthetic media such glucose, cellulose, soluble starch, and molasses. In order for bio control products to be successful commercially,

- Consistent and widespread action.
- Security and Stability.
- Longer shelf life.
- Low capital cost.
- Ready Availability of Carrier Materials.
- Economic and viable market demands

This research provides information on effective approach, such as biopesticides, to help rehabilitate agricultural soils and thereby promote sustainable growth (Glass et al., 2002).

**Materials and Methods**

**Isolation and Identification of Trichoderma viride**

There are a number of techniques for isolating *Trichoderma*, however, serial dilution of samples is one of the techniques that has been documented most frequently in the literature (Seethapathy et al., 2017; Glass et al., 2002; Hewavitharana et al., 2018). This method is easy, economical, and suitable for handling big samples. Take a *Trichoderma viride* sample that is commercially available in market and inject it into a maize plant. Take a soil sample from the soil of the maize plant after ten days. 10 ml of sterile, distilled water should be added to 1g of the soil sample before mixing well. Incubate the supernatant for 4 to 5 days at 25° to 30°C after spreading it over a potato dextrose agar plate. To obtain a pure culture, *Trichoderma* colonies were subculture on fresh PDA plates (Hewavitharana et al., 2018). Microscopic analysis is used to identify *Trichoderma viride*.

**Mass Production Technology**

**A) Mass Production of Trichoderma viride in Liquid State Fermentation**

Liquid state fermentation (LSF) is applied for the processes in which soluble materials in water is used for the microbial growth. Through the use of liquid fermentation technology, *Trichoderma* spp. are typically produced in large quantities using potato dextrose broth, molasses-yeast medium, and wheat bran (Srivastava et al., 2016). For the mass production black gram soak water, etc. are also employed.

Prepare 100ml of Potato dextrose broth and incubate it for 15 min at 121°C temperature and 15psi pressure and cooled it room temperature before inoculation. Add 0.1gm antibiotic in broth for inhibition of bacterial growth and Inoculate 1ml of *Trichoderma viride* suspension in 100ml of Potato dextrose broth and then incubate at 25°C to 30°C for 5 to 6 days.

**B) Mass production of Trichoderma viride in Solid state fermentation**

For mass multiplication the selected medium should be inexpensive and readily available with appropriate nutrient balance. Solid-state fermentation (SSF) is an effective method for the mass production of fungal biopesticides since it provides micropropagules with higher conidia content. Various cheap cereal grains like, sorghum, rice, millets, ragi are used as substrates (Ramanu jam et al., 2010). For mass production of *Trichoderma viride* in sorghum take 100gm sorghum in 250ml flask and soak in 2% sucrose solution for 6 hour. After that remove water from flask though filtration and sterilized in autoclave. Cooled at room temperature and next day inoculated with 10ml of *Trichoderma viride* suspension and incubated for 10 to 15 days for production of *Trichoderma viride* which produces dark green spore coating on the grains which showed in Fig. 3. (Hewavitharana et al., 2018)

**Optimization and Formulation in Liquid Carrier Material**

Although *Trichoderma* has excellent potential for treating diseases, it could not be used in the field as a suspension of spores. To facilitate application, storage, commercialization, and field use, the *Trichoderma* culture should be produced as formulations and immobilized in certain carriers. *Trichoderma* spp. were cultured on sorghum for the final product’s formulation. After the sorghum flasks have been incubated for 7 days, each one should be filled with distilled water before the liquid is extracted using filter paper. Then optimized for CFU count.

For optimization, several sugar solution concentrations of 0.1%, 1%, 10%, 15%, and 20% were created. 100 ml of an Erlenmeyer flask were filled with the prepared liquid carrier material. Prior to inoculation, all media were sterilized in an autoclave at 121 °C (15 psi) for 25 minutes and allowed to cool. Each flask inoculates with an antibiotic to prevent...
bacterial development. *Trichoderma viride* liquid solution were inoculated into all liquid carrier material under sterile conditions and incubated at room temperature (28 °C) on a shaker at 60 rpm for 180 days. According to Kumar *et al.* (2014) CFU of *Trichoderma* should be a minimum of 2 x 10^6 CFU per ml or gram in selective medium of *Trichoderma*. Other microbial contaminants should not have more than 1 x 10^4 CFU/ml, and pathogenic contaminants should not be present. The viability of the *Trichoderma* formulations should be maintained for a considerable period of time.

**Results**

**Isolation and Identification of Trichoderma viride**

The fungal population isolated from soils and spread on the PDA plates. The dilution plate method was used for the isolation of pure fungi from soil samples. Identified pure colony from mix population by its colony characteristics and then spread on new PDA plates. *Trichoderma* isolates were identified microscopically which is showed in Fig. 1.

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**Mass Production of Trichoderma viride in Liquid State Fermentation**

The results of the liquid-state fermentation revealed that locally available liquid substrates such as coconut water, rice mill effluent, and black gram soak water are used for the mass production of *Trichoderma viride*. We are using potato dextrose broth for mass production of *Trichoderma viride*. It showed high sporulation. A study revealed that sporulation of *T. viride* is high in liquid media compared to solid media of the same substrate. *Trichoderma* is grown in the liquid medium, which is mixed with demineralized water for formulation with respect to the number of CFU counts.
Mass Production of Trichoderma viride in Solid State Fermentation

There was a significant difference in the growth of *T. viride* in different solid carrier materials with respect to the number of CFU/ml produced in the selected solid carrier materials. Following the CFU/ml values, the highest stabilization potential was recorded for sorghum after 9 days of fermentation for *Trichoderma* viride. At the end of the fermentation period, the conidia in the medium are harvested. A common way of separating the conidia from the medium is by filtration or centrifugation. Active component by combining it with other active (functional) and non-active (inert) ingredients. Water, oil, polymer, or a combination of these can all be used in liquid formulations (Grewal, 2005). Demineralized water and a 0.1% sugar-based solution are used as the carrier material in the current investigation. This formulation was determined to be acceptable for *Trichoderma* spp. growth and viability during storage. Up to 120 days, viability and survival were assessed. After 120 days, *Trichoderma*’s maximum growth and higher survival stabilization are subject to change. The inoculant must have at least 2×10⁶ CFU/ml. The colony forming units of the concentrated *Trichoderma viride* solution that were obtained after filtering were displayed in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Dilution</th>
<th>No. of colonies</th>
<th>cfu/ml</th>
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<tbody>
<tr>
<td>1</td>
<td>10⁴</td>
<td>Lown growth</td>
<td>-</td>
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<tr>
<td>2</td>
<td>10²</td>
<td>35</td>
<td>3×10⁴</td>
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<tr>
<td>3</td>
<td>10³</td>
<td>20</td>
<td>2×10⁵</td>
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<tr>
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<td>10⁵</td>
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<td>6×10⁶</td>
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<tr>
<td>6</td>
<td>10⁶</td>
<td>4</td>
<td>4×10⁷</td>
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Discussion

In agriculture, fungi *Trichoderma viride* has been widely used in agriculture as a biopesticide because of its ability to protect against phytopathogens. Our results revealed that Trichoderma fungi have good potential for mass production on locally available substrates in liquid as well as solid-state fermentation. A study reported that the growth performance of *Trichoderma viride* on PDA showed maximum at 192 hours of incubation period.

In liquid state fermentation growth of *Trichodrma viride* on potato dextrose broth showed significant growth at 6th day of incubation. It is a popular method for producing large quantities. The choice of liquid medium is crucial for the growth of *Trichoderma viride*.

When using sorghum for the growth of *Trichoderma* in solid-state fermentation (SSF), the moisture content of the sorghum substrate is an important factor that can affect the growth of *Trichoderma* in SSF. The optimal moisture content for the growth of *Trichoderma* can vary depending on the specific *Trichoderma* species used, but generally, a moisture content of around 60-70% is suitable for *Trichoderma* growth on sorghum substrate. The addition of supplements to the sorghum substrate can help to promote the growth of *Trichoderma* in SSF. We are using 2% sucrose solution as additional supplements which enhance the growth of *Trichoderma viride*.

Conclusion

The use of fungi *Trichoderma* have been widely used in agriculture as bio-agents, which is the best way to replace chemical pesticides. It can reduce health, social, and environmental issues. Locally available substrates are potentially cost-effective for the production of *Trichoderma* spp. Among these, sorghum is the most effective substrate for the growth of *Trichoderma*. It produces effective molecules and secondary metabolites, which mediate *Trichoderma*’s positive interaction with plants and provide resistance to pests. Bio-agent *Trichoderma* itself being non-pathogenic to plants, it is formulated in a way that favor’s the activity and survival of microbes. It is economical to produce, has persistent storage ability, and is easy to handle.

Authors’ Contribution

All authors contributed equally at all stages of research work and preparation of the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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