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Research Article

Optimization of culture conditions for the production, antifungal activity and characterization of secondary metabolites of *Trichoderma longibrachiatum*

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ABSTRACT: The present study was intended to optimize the culture conditions for secondary metabolite production by endophytic fungi. Based on the morphology and phylogeny, the fungus was identified as *Trichoderma longibrachiatum* isolated from brinjal leaf based on morphological characterization. The antifungal activity was evaluated against phytopathogens such as *Macrophomina phaseolina*, *Phytopthora infestans, Colletotrichum falcatum* and *Colletotrichum gloeosporioides* through the overlapping method, culture filtrate and organic fraction from Potato dextrose both as a growth medium. The organic fraction exhibited a significant antifungal activity, while modifications in medium composition may possess a major impact on the quantity and quality of secondary metabolites production. To achieve maximum metabolite production, the growth of the culture was optimized with screening of basal media, carbon, nitrogen, pH, trace elements and incubation period. The final optimized fermentation conditions were Minimal ereavis broth as basal media; glucose and sucrose as carbon source; Peptone and Yeast extract as nitrogen source, sodium nitrate as precursor; pH as 6; and incubation period as 7 days at 28°C. This optimization resulted in antifungal activity of 47.19-60.67% against *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides* which was higher than that before optimization (43.80%). GCMS revealed distinct metabolites of *T. longibrachiatum*, comprising antifungal metabolites and molecules with additional bioactivities. These results strengthen ongoing research on disease control in agriculture by emphasizing the biocontrol potential of *T. longibrachiatum* isolated from brinjal phyllosphere against plant pathogenic fungi.

KEYWORDS: Antifungal activity, bioactive compounds, endophytic fungus, optimum conditions, *Trichoderma longibrachiatum*

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INTRODUCTION

The environment and the health of living things are being harmed more and more by the excessive use of chemical pesticides. Plant pathogens cause significant economic and production losses, resulting in a serious global threat they attack plants separately and occasionally more than one pathogen-producing complex and more severe diseases in the leaf, stem, root, vascular system and fruit. Some of the prevalent fungal phytopathogens belong to genera *Macrophomina, Phytophthora, Colletotrichum*, *Alternaria, Botrytis, Cochliobolus, Fusarium, Geotrichum, Penicillium*, and *Sclerotina sp.* The application of microorganisms to control plant diseases is an environmentally friendly substitute to pesticides, and hence biological control has grown in popularity (Morais *et al.,* 2022). These approaches frequently demand coordinated use of several approaches, or Integrated Disease Management (IDM). Several strategies comprise the use of disease-resistant cultivars, adequate water and soil management, fertilization, crop rotations, and Biological Control Agents (BCA) to maintain or boost agricultural productivity by applying fewer chemical pesticides. (Salim *et al.,* 2017) The study of alternate approaches to managing fungal infections has grown significantly (Villa *et al.,* 2017; Das & Pattanayak, 2020). Among various beneficial categories of microorganisms, endophytic microorganisms exist without ever inflicting plant diseases on healthy plant tissues. They generate a spectacular variety of secondary metabolites that offer their host plants significant ecological advantages. The insecticidal, nematicidal, plant growth-promoting and plant-strengthening bioactivities of endophytic microorganisms are additionally being investigated for their ability to protect their hosts from environmental threats. (Soltani *et al.,* 2014). *Trichoderma* sp. have been identified as potential sources of bioactive metabolites owing to their opportunistic, avirulent plant symbionts that act as parasites and antagonists of

many phytopathogenic fungi, protecting plants from disease through parasitic behaviour, nutrient competition, and antibiotic synthesis. (Sood *et al.,* 2020; Medeiros *et al*., 2017; Harman *et al*., 2004) by producing hormone and biostimulant-like compounds, and enhanced nutrient uptake (Li *et al.,* 2017; Jangir *et al*., 2019). Secondary metabolite production, both volatile and non-volatile that might prevent pathogen development is an intriguing characteristic of *Trichoderma* sp. Although their production can differ from strain to strain, little is known about it (Stracquadanio *et al*., 2020). Secondary metabolites are metabolic pathway branching points that lead to the production of an end product and are made up of intermediates or precursors produced by primary or intermediate metabolism. As a result, the influence and interaction of nutrients is one of the primary factors driving alterations in secondary metabolism. Precursors, carbon and nitrogen supplies, phosphate, trace elements, and other factors also control secondary metabolism (Pournejati & Karbalaei-Heidar, 2020; Demain & Sanchez, 2009). The current study's objectives were to improve the cultural conditions and the production of secondary metabolites from *Trichoderma* sp., and evaluate their antifungal activity isolated from the phyllosphere of brinjal plants.

MATERIALS AND METHODS

Strain used

Endophytic fungi were isolated from fresh, healthy branches of *Solanum melongena*. The surface-sterilized leaf segments were evenly placed into Petri dishes containing medium potato dextrose agar (PDA), and the Petri dishes were observed for the emergence of endophytic fungi colonies from the leaf segments and identified based on their cultural and microscopic characteristics. Conidiophore structure and morphology were studied after conidia matured within 4-7 days of incubation. (Sahar & Zafari, 2018). Hyphal tips from developing single spores were transferred to potato dextrose media and used for further investigation. (Macias-Rodríguez *et al*., 2020; Evans *et al.,* 2003). The fungal strain was phylogenetically identified by employing DNA extraction, PCR amplifications, and sequencing (ITS).

Evaluation of fungal metabolites against phytopathogens

Effect of *Trichoderma* spp. volatile compounds on the mycelial growth of plant pathogens

The impact of volatile chemicals of *Trichoderma* spp. on the mycelial growth of plant pathogens was assessed using the method of Olivier and Germain (1983) (Volatile compound (VC)-mediated inhibition by *Trichoderma* sp. against phytopathogen-overlapping plate method). Plant pathogens (*Macrophomina phaeseolina*, *Phytophthora infestans*, *Colletotrichum falcatum*, and *Colletotrichum gloeosporioides*) and the antagonistic *Trichoderma* sp. with a diameter of 8 mm were taken from 5-day-old cultures and placed in the centre of separate petri dishes containing PDA media. By superimposing two petri dishes without covers, *Trichoderma* sp. *w*as placed on the bottom and the plant pathogens were placed individually on top. The interface was sealed with parafilm tape to prevent the loss of volatile compounds. The pathogen on top in the PDA without the antagonist on the bottom was used as a control. For each combination, three replicates were used. After 120 hours of incubation, the plant pathogens' growth was observed. For volatile inhibitors assessment, the growth inhibition percentage was determined using the pathogenic fungi's colony diameter with antagonistic treatment and control. The proportion of inhibition of endophytic fungal secondary metabolites on the growth of pathogenic fungi was calculated using the formula below:

$$P\ (\%) = (DK-DP)/DK*100$$

P = growth inhibition percentage of pathogenic fungi, DK = pathogenic fungi colony diameter on control plate, DP = pathogenic fungus colony diameter on treatment (Marques *et al.,* 2022; Hammad *et al.*, 2021; Utami *et al.*, 2019; Anees *et al*., 2018).

Effect of *Trichoderma* sp. culture filtrate on phytopathogen growth

For this experiment, 5 days old actively growing mycelial discs of *Trichoderma* sp., were added to a 250 ml conical flask with 100 ml of Potato Dextrose broth medium and cultured at 28°C for 7 days with continuous agitation. The mycelium in the culture broth was then initially filtered through normal filter paper and was removed, and the spores in the culture filtrate were then filtered again using 0.20 m diameter Millipore membranes and then stored at 4°C.

To investigate the antifungal activity of *Trichoderma* sp. culture filtrates on phytopathogen mycelial growth, the combination of *Trichoderma* sp. culture filtrate with *M. phaeseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides* individual suspensions (10^5 spores/ml) were used. To sterilise 80ml of Czapek Dox Broth (CDB), add 10 ml of *Trichoderma* sp. culture filtrate, 10ml of the pathogens culture broth and a final volume made to 100mL. Control without pathogens contained 80ml of CDB, 10ml of *Trichoderma* sp., culture filtrate, and 10 ml of distilled water. Control with phytopathogens contained 80ml of CDB, 10ml of phytopathogens spore suspension and 10 ml distilled water incubated for 120 hours at 120RPM at 28±2°C. Four independent replicates were carried out. Following incubation, the phytopathogens' growth was assessed by the serial dilution method and enzyme activities for chitinase and -1, 3-glucanase were immediately determined. (Hammad *et al.*, 2021; Kumar *et al.*, 2012).

Optimization of fermentation medium for the secondary metabolite production

Screening of basal medium

Different essential liquid growth media, including defined and undefined broths, were selected to reflect a variety of nutrition sources. We used nine different medium such as Malt extract broth, Minimal Davis broth with Dextrose, Potato carrot broth, Potato Dextrose broth, Sabraoud's dextrose broth, Czapek Dox broth, Yeast Sucrose broth, Wickerham's broth and Tryptone broth (Sharma & Sharma, 2021) to determine the ideal medium for secondary metabolite production. The carbon and nitrogen sources combination in each medium were different. The different types of fungal media were examined for maximum secondary metabolite generation via antifungal activity against plant pathogens. Endophytic fungi from an isolated *Trichoderma* sp. were diced into three pieces of about 1 cm by 1 cm and inoculated into the 100 ml of different media in a 250 ml Erlenmeyer flask. The cultures were grown at 28±2°C with 150 rpm and the fermentation process continued up to seven days, based on the growth phase of endophytic fungi *Trichoderma* sp., (Utami *et al.,* 2019). After incubation, the filtration was done through centrifugation at 10000 RPM for 5 mins and with Whatman filter paper, respectively. The secondary metabolites were collected from the culture broth and tested for antifungal activity to determine the optimal supportive media for maximum secondary metabolite production.

Designing of the medium by optimization of carbon and nitrogen sources to produce secondary metabolites

Carbon, nitrogen, pH, and incubation period sources were used to screen the important nutritional variables for the fermentation of *Trichoderma* sp.

Effect of carbon sources

Glucose, starch, sucrose, fructose, lactose, mannitol, carboxymethylcellulose, and maltose were employed to investigate the effect of various carbon sources. The carbon sources were added one at a time while replacing the carbon source with the screened Minimal Davis broth at 1%. The medium was then inoculated and cultured for 7 days at 28±2°C. Following incubation, the biomass was recorded as mg/100ml and studied antifungal activity of the culture filtrate containing secondary metabolites (Hateet *et al*., 2021)

Effect of nitrogen source

Beef extract, yeast extract, peptone, ammonium sulphate, urea, malt extract, and sodium nitrate were employed to investigate the effect of various nitrogen sources. The nitrogen source sources were added one at a time at a concentration of 1% while replacing the nitrogen source with the screened Minimal Davis broth. The medium was then inoculated and cultured for 7 days at 28±2°C. Following incubation, the biomass was recorded as mg/100ml and studied antifungal activity of the culture filtrate containing secondary metabolites (Hateet *et al*., 2021).

Effect of pH

The influence of pH on the isolate's growth and bioactive metabolite synthesis was studied in basal media Minimal Davis broth at varied pH levels (pH 4-9) with screened carbon and nitrogen. By adding 0.1N NaOH or 0.1N HCl, the medium pH was brought to the required level. The medium was then inoculated and cultured for 7 days at 28±2°C. Following incubation, the biomass was recorded as mg/100ml and studied antifungal activity of the culture filtrate containing secondary metabolites (Hateet *et al*., 2021; Shuwu *et al*., 2020).

Effect of concentration of screened carbon sources and nitrogen sources

The optimum concentration of the screened carbon (Glucose and Sucrose) and nitrogen sources (Beef extract and Peptone) was studied in the basal medium as Minimal Davis broth with concentrations of 0.5%, 1.0%, 1.5% and 2.0% respectively. Following incubation, the biomass was recorded as mg/100ml and studied antifungal activity of the culture filtrate containing secondary metabolites (Hateet *et al*., 2021).

Effect of precursors on secondary metabolites induction

With the concluded set of conditions to induce the production of SM in the growth medium some of the inducers/ precursors are incorporated into the media. Precursors by inducing the biosynthetic enzyme (synthase) or by the addition of an amount of limiting precursor frequently induce secondary metabolites production. Usually, amino acids and small molecules can act as inducers. Preferably metal ions (organic/inorganic metal salt, amino acids (Sodium nitrate, Ferric citrate, Manganous sulphate, Copper sulphate, Ferric chloride, Valine, Manganous chloride Cyanaocobalamin, Calcium D Pantothenate), antibiotics (Cefotaxime), oxidative compounds (Hydrogen peroxide) and RO generators (sodium salt of Menadione and Menadione) were incorporated with screened C and N in basal medium as Minimal Davis broth medium. The precursors such as with a constant concentration of 1mM and added to the optimized basal medium. (Spragg & Karen, 2011). Following incubation, the biomass was recorded as mg/100ml and studied antifungal activity of the culture filtrate containing secondary metabolites.

Influence of incubation duration on the production of biomass and bioactive metabolites

The optimum incubation duration was also determined with the screened concentrations of C and N sources

incorporated in the basal medium as Minimal Davis broth with optimized pH for different days i.e., 3, 5, 7, 9, 12, 15 and 18 days, respectively. Following incubation, studied antifungal activity of the culture filtrate containing secondary metabolites (Hateet *et al*., 2021; Shuwu *et al*., 2020; Choez-Guaranda *et al*., 2023)

Antifungal activity

The technique of poisoned culture medium was employed to study the antifungal activity of the optimized fermentation medium culture as described by Guerrero-Rodriguez *et al.,* 2007. The culture filtrate extracted at the concentration of 5% was added to cooled Potato Dextrose Agar (PDA) medium, mixed well, then poured and allowed to solidify. The 5 mm mycelia disc of the plant pathogens was placed on PDA and blank as PDA along with metabolite without fungal disc and incubated at 28±2°C. The antifungal activity was measured through the colony diameter of the plant pathogens. The PDA medium without any culture filtrate inoculated with pathogen served as control (Cherkupally *et al*., 2017)

Statistical analysis

In all of the aforementioned experiments, the percentage of growth inhibition was computed using the mean colony diameter and the following formula:

$$\text{Mycelial Growth Inhibition MGI (\%)} = (C - T)/C \times 100$$

where, MGI%- growth inhibition percentage, C = pathogenic fungi growth in control and T = pathogenic fungi growth in treatment (Cherkupally *et al*., 2017; Vincent 1947).

Characterization of secondary metabolites from optimized culture medium

The metabolites in the culture filtrates were subjected to GC-MS analysis to discover active biomolecule components. A single quadrupole mass spectrometer (GC-MS) detector was used to identify the substances in fungal metabolites. The chemical identification of the substances was determined by injecting standard compounds into GC-MS or comparing them with NIST library mass spectra. The electron impact energy was set at 70eV, while the ion source temperature was set to 250°C. In fully scan acquisition mode, the Electron Impact (EI) mass scan (m/s) range was 40-450 Da. (Muhammad *et al*., 2023).

RESULTS

Strain used

The isolated fungal strain from the *Solanum melongena* plant leaf belonging to the genus *Trichoderma* which was morphologically and phylogenetically identified as *Trichoderma longibrachiatum* (TL-RD-01) (Priya *et al.,* 2022) was used.

Effect of *Trichoderma longibrachiatum*'s volatile substances on plant-pathogenic mycelial growth

The isolated endophytic *T. longibrachiatum* was studied for its capacity to generate volatile compounds that were useful in inhibiting the plant pathogen's radial growth. The recorded results (Table 1) showed a significant difference in the mycelial growth of the tested plant pathogens such as *M. phaseolina*, *P. infestans, C. falcatum* and *C. gloeosporioides* by the volatile substances produced by *T. longibrachiatum*. Volatile substances emitted by *Trichoderma longibrachiatum* reduced mycelial growth of *C. falcatum* by about 60.76% followed by *P. infestans* by about 41.25 %, respectively.

Effect of culture filtrate of *T. longibrachiatum* on mycelial growth of phytopathogens

The plant pathogens' mycelial development was significantly inhibited by the *T. longibrachiatum* culture filtrate. The plant pathogen showed good mycelial growth while the culture filtrate incorporated with the plant pathogen showed less mycelial growth. The mycelial growth was recorded in terms of colony-forming units. On incubation, the *M. phaseolina* alone grew about $2x10^6$ while the *T. longibrachiatum* culture filtrate was observed with $1x10^3$

Table 1. Antifungal activity of volatile compounds of *T. longibrachiatum* on the mycelial growth of plant pathogens by overlapping plate method

S. No.	Phytopathogens	% of growth inhibition
1	*Macrophomina phaseolina*	46.99
2	*Phytophthora infestans*	41.25
3	*Colletotrichum falcatum*	60.76
4	*Colletotrichum gloeospioroides*	58.33

Table 2. Effect of *Trichoderma longibrachiatum*' culture filtrate on the growth of phytopathogens

S. No.	Treatments	CFU/mL
1	TLCF+MP	$1x10^3$
2	MP	$2x10^6$
3	TLCF+PI	$4x10^3$
4	PI	$3x10^6$
5	TLCF+CF	$2.6x10^2$
6	CF	$1x10^7$
7	TLCF+CG	$1x10^2$
8	CG	$2.6x10^7$
9	TLCF	<10

TLCF- **Trichoderma longibrchiatum* culture filtrate
MP- *Macrophomina phaseolina*, PI- *Phytophthora infestans*, CF- *Colletotrichum falcatum* and CG- *Colletotrichum gloeospioroides*

CFU/ml. The *P. infestans* observed with $3x10^6$ *with TLCF* observed with $4x10^3$ CFU/ml. The growth of *C. falcatum* was observed with $1x10^7$ while the pathogen incorporated with TLCF recorded $2.6x10^2$ CFU/ml. The *C. gloeosporioides* was observed with $2.6x10^7$ when incorporated with TLCF the growth was recorded with $1x10^2$ CFU/ml (Table 2). The results revealed the growth inhibition of the plant pathogens by the *T. longibrachiatum* culture filtrate incorporated into the growth medium.

The Chitinase and β-1,3-glucanase production were summarized (Tables 3 and 4). The *T. longibrachiatum* culture filtrate showed chitinase and β-1,3-glucanase activities with the addition of fungal plant pathogens. The maximum chitinase enzyme activity was observed with *M. phaseolina* at about 31.3U, followed by *C. gloeosporioides* at about 30U, *C. falcatum* at about 28U and *P. infestans* at about 25.3 U, respectively.

The β-1,3-Glucanase enzyme activity was recorded with 21 U of *C. gloeosporioides* followed by 19 U of *C. falcatum,* 18 U of *M. phaseolina* and 16 U of *P. infestans* respectively.

Optimization of fermentation medium for the production of secondary metabolite

Among nine different growth media tested the growth of the tested phytopathogens was found to be inhibited to a greater extent in the minimal Davis broth medium with Dextrose followed by Potato Dextrose broth and yeast extract sucrose broth. The *T. longibrachiatum* grown in the Minimal broth Davis with dextrose broth medium showed the highest inhibition rate of 13%- 28% against *C. falcatum, C. gloeosporioides, P. infestans* and *M. phaseolina*. The Minimal Broth, Davis with dextrose medium was followed by Yeast extract sucrose broth with 4.49-23.60%, Tryptone broth with 8.99-21.35%, Czapek dox broth with 7.87- 21.35% of growth inhibition (Table 5). Hence Minimal Broth, Davis medium was employed as a base medium to ascertain the ideal conditions for this bioactivity of *T. longibrachiatum*. 250 ml Minimal Broth, Davis in 500mL of Erlenmeyer flasks as basal medium added with 1% (w/v) of different carbon or nitrogen sources were used while replacing the carbon and nitrogen sources, and other parameters such as pH, medium ingredients, and incubation period were optimized.

Table 3. Chitinase enzyme activity of *T. longibrachiatum* (pkat/ml)

Treatments	CDB	CDB + *Macrophomina phaseolina*,	*Phytophthora infestans*	*C. falcatum*	C. *gloeosporioides*
TLCF	19	31.3	25.3	28	30

TLCF- **Trichoderma longibrachiatum* culture filtrate

Table 4. β-1,3-Glucanase enzyme activity of *T. longibrachiatum*

Treatments	CDB	CDB + *Macrophomina phaseolina*,	*Phytophthora infestans*	*C. falcatum*	C. *gloeosporioides*
TLCF	17	18	16	19	21

TLCF- **Trichoderma longibrchiatum* culture filtrate

Table 5. Screening of fermentation medium for the secondary metabolite production

S. No.	Medium	% of Antifungal activity of the culture filtrate			
		Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
1	Malt extract broth	10.11±0.01	11.39±0.15	10.96±0.45	21.05±0.98
2	Minimal Davis broth with Dextrose	25.73±0.11	26.66±0.09	26.85±0.52	25.79±0.15
3	Potato carrot broth	21.35±0.73	10.11±0.27	6.74±0.69	4.49±0.14
4	Potato Dextrose broth	19.09±0.47	21.35±0.27	13.48±0.05	15.48±0.15
5	Sabraud's dextrose broth	17.98±0.63	14.61±0.10	2.25±0.03	6.74±0.49
6	Czapek dox broth	21.35±0.36	21.35±0.07	14.61±0.11	7.87±0.61
7	Yeast extract sucrose broth	23.60±0.03	16.85±0.79	4.49±0.23	16.85±0.16
8	Wickerham's broth	28.09±0.12	6.74±0.41	13.48±0.46	12.36±0.20
9	Tryptone broth	21.35±0.37	20.22±0.66	8.99±0.08	11.24±0.19

Influence of carbon sources on biomass and production of bioactive metabolite

Various carbon sources (Glucose, Fructose, Sucrose, Maltose, Lactose, Mannitol, Carboxy methyl cellulose and starch) have been amended separately into the basal medium as Minimal Broth, Davis medium at 1% (w/v). Growth and bioactive metabolite production were determined as biomass/100 ml and antifungal activity through plant pathogen's radial growth in the food poisoning method respectively. Among the carbon sources, glucose was recorded with a maximum biomass of about 0.385gm/100ml and the percentage of growth inhibition was about 22-29% against the *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides*. Sucrose also gave a similar pattern with Glucose with little higher biomass of about 0.413 gm/100mL and slightly lower antifungal activity with 21-28% (Table 6).

Nitrogen source's impact on the production of bioactive metabolites and biomass

The impact of different nitrogen sources on the synthesis of bioactive metabolites in *T. longibrachiatum* was studied. Maximum antimicrobial activity was recorded in peptone with biomass of about 0.343gm/ml and growth inhibition of about 20.22-22.47 % against *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides* followed by beef extract with 0.34gm/100ml and % of inhibition ranges from 21.35-24.72% (Table 7).

Influence of pH on biomass and bioactive metabolite production

The influence of pH on the production of secondary metabolites was summarized. in the table. The optimal pH for the production of secondary metabolites was 6.0 recorded with biomass of about 0.453gm/100ml and the growth inhibition of plant pathogens such as *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides* of about 21.35% -25.84% followed by pH 7 with biomass 0.394gm/100mL and inhibition about 20.22-23.60% (Table 8).

Effect of screened carbon source and nitrogen source concentration on the production of bioactive metabolites

Among the screened carbon and nitrogen sources, the concentration of C and N has been studied. The carbon sources such as glucose and sucrose at 1% and 1.50% have showed

Table 6. Carbon sources' influence on biomass and bioactive metabolite production

Carbon sources (1%)	Biomass (g/100ml)	% of Antifungal activity of the culture filtrate			
		Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
Glucose	0.385±0.02	29.41±0.05	29.11±0.46	23.29±0.38	22.37±0.30
Fructose	0.213±0.05	10.52±0.05	16.46±0.01	15.07±0.17	14.47±0.22
Sucrose	0.413±0.02	28.09±0.12	23.60±0.85	21.35±0.42	23.60±0.13
Maltose	0.211±0.01	11.24±0.22	19.10±0.45	13.48±0.07	16.85±0.39
Lactose	0.186±0.00	16.85±0.16	14.61±0.08	11.24±0.04	12.36±0.23
Mannitol	0.284±0.01	15.73±0.10	17.98±0.23	13.48±0.05	17.98±0.34
CMC	0.156±0.01	13.48±0.12	16.85±0.34	14.61±0.31	15.73±0.07
Starch	0.216±0.06	15.73±0.29	16.85±0.84	15.73±0.15	14.61±0.07

Table 7. Influence of nitrogen source on biomass and production of bioactive metabolite

Nitrogen sources (1%)	Biomass (g/100ml)	% of Antifungal activity of the culture filtrate			
		Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
Tryptone	0.295±0.02	16.47±0.50	17.72±0.35	16.44±0.43	18.42±0.17
Ammonium sulphate	0.21±0.01	11.76±0.31	15.19±0.20	17.81±0.30	13.16±0.34
Malt Extract	0.235±0.03	16.85±0.14	17.98±0.10	10.11±0.09	17.98±0.17
Peptone	0.343±0.03	22.47±0.10	23.60±0.26	20.22±0.05	23.60±1.56
Sodium Nitrate	0.165±0.01	17.98±0.12	17.98±0.15	14.61±0.08	12.36±0.27
Yeast Extract	0.25±0.01	16.85±0.10	16.85±0.10	15.72±0.02	17.98±0.13
Beef Extract	0.34±0.00	21.35±0.26	21.35±0.27	23.60±0.23	24.72±0.24

Table 8. Influence of pH on the biomass and bioactive metabolite production

Different pH adjusted	Biomass (g/100ml)	% of Antifungal activity of the culture filtrate			
		Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
4	0.128±0.00	14.12±0.20	16.46±0.05	13.70±0.05	14.47±0.09
5	0.256±0.02	12.94±0.28	17.72±0.13	15.07±0.05	18.42±0.10
6	0.453±0.00	24.72±0.20	25.84±0.40	21.35±0.14	22.47±0.14
7	0.394±0.04	23.60±0.03	23.60±0.01	20.22±0.06	20.22±0.06
8	0.312±0.00	20.22±0.16	16.85±0.08	17.98±0.09	19.10±0.35
9	0.245±0.02	15.73±0.19	14.61±0.07	15.73±0.08	16.85±0.10

Table 9. Effect of screened carbon and nitrogen concentrations on biomass and bioactive metabolite production

Screened carbon sources and nitrogen sources	Concentration	% of Antifungal activity of the culture filtrate			
		Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
Peptone	0.5%	34.83±0.07	38.20±0.34	33.71±0.14	34.83±0.28
	1%	28.09±0.23	29.21±0.25	26.97±0.26	50.56±0.61
	1.50%	28.09±0.11	30.34±0.32	24.72±0.21	41.57±0.26
	2%	26.97±0.08	32.58±0.34	24.72±0.25	44.38±0.07
Beef extract	0.5%	32.58±0.39	24.72±0.52	30.34±0.17	34.83±0.28
	1%	39.33±0.21	38.20±0.05	48.31±0.29	50.56±0.61
	1.50%	26.40±0.09	33.71±0.10	35.96±0.06	41.57±0.26
	2%	24.72±0.18	32.58±0.06	33.71±0.23	44.38±0.07
Glucose	0.5%	39.33±0.16	26.97±0.85	37.08±0.02	40.45±0.33
	1%	44.94±0.43	31.46±0.12	41.57±0.05	57.30±0.22
	1.50%	24.72±0.31	28.09±0.06	38.20±0.22	41.57±0.45
	2%	23.60±0.30	29.21±0.43	38.76±0.18	43.82±0.32
Sucrose	0.5%	35.96±0.45	32.58±0.10	30.34±0.03	40.45±0.08
	1%	32.58±0.40	30.34±0.05	44.94±0.09	48.31±0.05
	1.50%	48.31±0.27	37.08±0.16	52.81±0.24	61.80±0.21
	2%	43.82±0.10	36.52±0.06	53.37±0.50	47.19±0.34

the highest antifungal activity in the range of 31.46-57.30% and 37.08-61.80% against *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides* respectively. The nitrogen sources such as Peptone and beef extract showed the highest antifungal activity at a concentration of 0.5% and 1% with antifungal activity of about 39.33%-50.56% and 33.71-38.40% against the plant pathogens, respectively (Table 9).

Effect of precursors on secondary metabolites induction

The medium with the ideal carbon and nitrogen sources was supplied independently with different minerals, each at a concentration of 1mM, to evaluate the influence of minerals on growth and bioactive metabolite production. The effect of inducers on the isolate's bioactive metabolite synthesis is presented in the table. Among the different compounds tested Sodium nitrate with a concentration of 1mM was recorded with the highest bioactive metabolite production studied through antifungal activity recorded with 56.18-69.62 % of plant pathogens growth (Table 10).

Effect of incubation time on bioactive metabolite production

The isolates' production of bioactive metabolites was evaluated for up to 18 days. The effect of the incubation duration on the isolate's bioactive metabolite synthesis was summarized (Table 11). The bioactive metabolite production started after 72 hours. The highest antifungal activity was observed in 7 days with a percentage inhibition of about 47.19-60.67% against *M. phaseolina*, *P. infestans*, *C. falcatum*, and *C. gloeosporioides*. The amount of metabolite produced increased after seven days, and then progressively declined.

Table 10. Effect of precursors on secondary metabolites induction

Inducers incorporated into the medium	% of Antifungal activity of the culture filtrate			
	Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gleosporoides*
Copper sulphate	43.82±0.10	49.37±0.28	52.05±0.73	55.70±0.19
Cefotaxime	42.79±0.58	49.37±0.12	54.79±0.38	60.76±0.09
Calcium D Pantothenate	34.82±0.24	41.57±0.54	38.20±0.87	51.69±0.19
Cyanaocobalamin	40.45±0.32	42.70±0.58	42.70±0.79	52.81±0.13
Ferric chloride	39.33±0.40	40.45±0.39	50.56±0.53	55.06±0.10
Ferric citrate	43.82±0.73	51.69±0.65	42.70±0.18	55.18±1.05
Hydrogen peroxide	44.94±0.34	40.45±0.41	51.69±0.59	53.93±0.08
Manganous chloride	41.57±0.06	44.94±0.72	43.82±0.12	47.19±0.14
Manganous sulphate	40.45±0.93	39.33±0.20	49.44±0.26	48.31±0.13
MD-Sodium salt	43.82±0.44	51.94±0.37	58.90±0.23	58.23±0.06
MD	40.45±0.77	58.23±0.33	54.79±0.17	60.76±0.46
Sodium nitrate	56.18±0.10	62.03±1.00	65.75±0.83	69.62±0.13
Valine	43.82±0.11	50.63±0.28	56.16±0.12	58.23±0.12

Table 11. Effect of incubation period on bioactive metabolite production

Different incubation periods in days	% of Antifungal activity of the culture filtrate			
	Macrophomina phaseolina	*Phytophthora infestans*	*Colletotrichum falcatum*	*Colletotrichum gloeospioroides*
3	16.47±0.37	11.39±0.16	10.96±0.24	11.84±0.11
5	23.53±0.27	21.52±0.06	27.40±0.39	26.32±0.13
7	47.19±0.15	55.06±0.06	61.80±0.28	60.67±0.19
9	43.82±0.14	53.93±0.27	61.24±0.15	61.24±0.06
12	41.82±0.09	50.56±0.46	60.67±0.38	60.11±0.20
15	41.57±0.07	51.12±0.20	59.55±0.04	59.55±0.11
18	40.45±0.25	52.81±0.01	58.99±0.05	58.99±0.60

Characterization of secondary metabolites

The GC-MS analysis of culture filtrate with optimized fermentation medium of *T. longibrachiatum* showed presence of compounds such as Lauric acid, 2-methylbutyl ester; 3-Diethoxy phosphonyl-demethylthiocolchicine; 2-Butenal, 2-methy l-4-[3a,4,5,7-tetrahydro- 8-hydroxy-3,3,11,11-tetramethyl-13-(3-methyl-2-butenyl)-7,15; 5-Methyl(pentamethylene)silyloxypentadecane; 4-Methyldocosane; Oxiranemethanol, 3-methyl-3-(4-methyl-3-pentenyl); 3,7-Cycloundecadien-1-ol, 1,5,5,8-tetramethyl; Cyclopropylpyrrol4-[3-(1H-imidazol-4-yl) propoxy] phenyl zmorphomethanone oxime; Cycloheptane, 1-methyl-4-methylene; 3-Bromo-4-hydroxy-2,3'-dimethyl-5,5',8,8'-tetramethoxy-1,2'-binaphthalene-1',4'-dione; Cyclopropane, 1-(1,2-dimethylpropyl)-1-methyl-2-nonyl; 2- Chloropropionic acid, octadecyl ester; Cyclotetracosane; 3-Diethoxyphosphonyl-demethylthiocolchicine; 3-Diethoxyphosphonyl-demethylthiocolchicine; 2-Butenal, 2-methyl-4-[3a,4,5,7-tetrahydro-8-hydroxy-3,3,11,11-tetramethyl-13-(3-methyl-2-butenyl)-7,15; Cobalt, (Beta.5-2,4-cyclopentadien-1-yl)[(1,2,3,4-.eta.)-tris(1,1-dimethylethyl)azete]; 3-Methoxy-2,4,5-trifluorobenzoic acid, eicosyl ester; 3-Methoxy-2,4,5-trifluorobenzoic acid, nonadecyl ester; 1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl nonyl ester; Methyl 3-bromo-1-adamantaneacetate; 2-Chloroaniline-5-sulfonic acid and 2,3,5,6-Tetrafluorophenyl isothiocyanate. The aqueous crude extracts of the *Trichoderma longibrachiatum* (Tl-01) were investigated to determine their metabolites using GC–MS, which revealed twenty-three (Table 12) compounds. Among the twenty-three compounds, the maximum antimicrobial activity was exhibited by cyclotetracosane, chloropropionic acid, and phytostimulant activity by octadecyl ester Lauric acid, 2-methyl butyl ester, 3-diethoxy phosphonyl-demethyl thio colchicine and 5-Methyl (pentamethylene) silyloxy pentadecane (Figure 1 and Table 12).

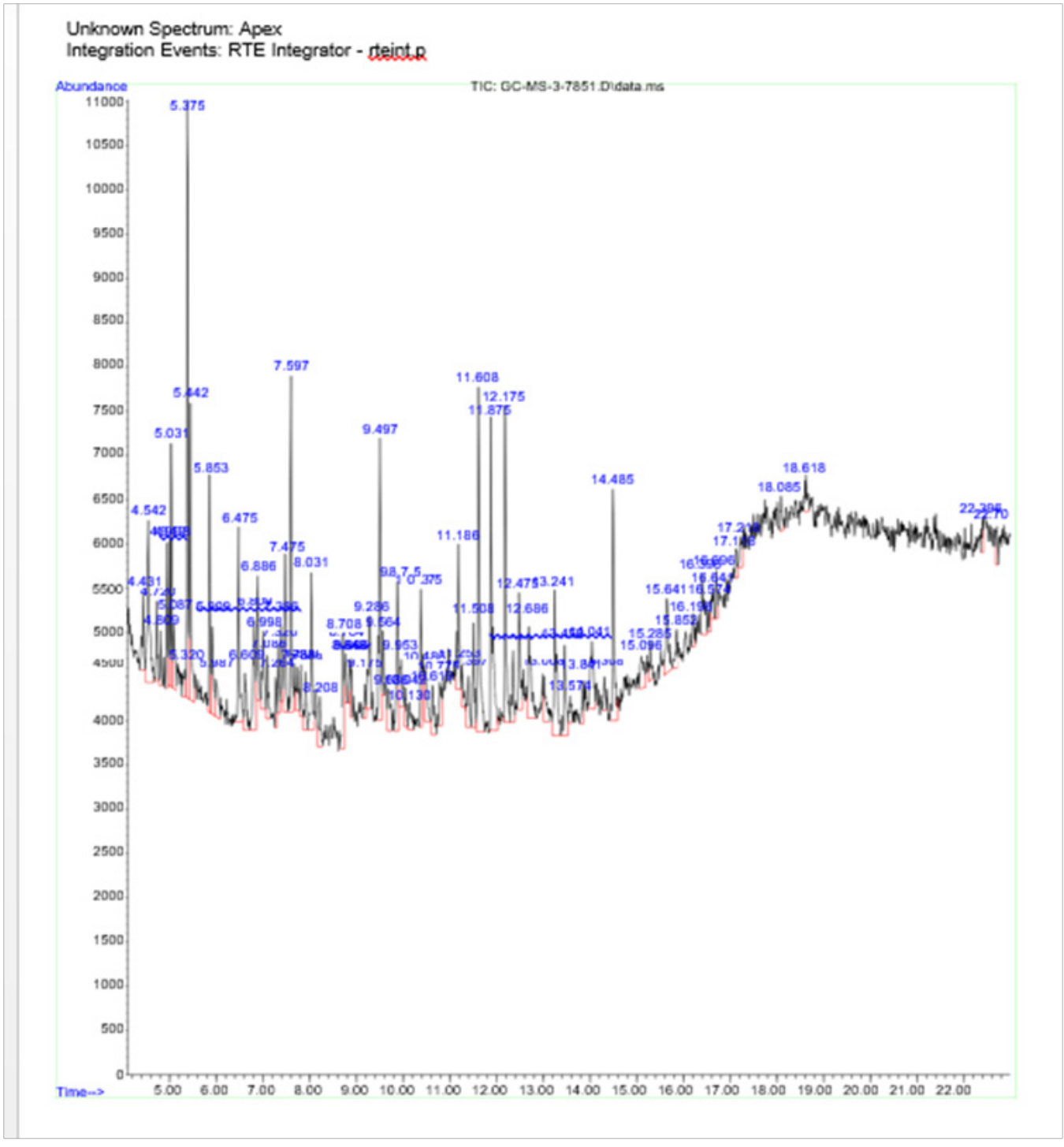

Figure 1. GCMS analysis of Secondary metabolites produced in the optimized fermentation medium.

Table 12. Secondary metabolites produced in the optimized fermentation medium

S. No.	List of compounds analysed in GCMS
1	Lauric acid, 2-methylbutyl ester
2	3-Diethoxyphosphonyl-demethylthiocolchicine
3	2-Butenal, 2-methyl-4-[3a,4,5,7-tetrahydro-8-hydroxy-3,3,11,11-tetramethyl-13-(3-methyl-2-butenyl)-7,15
4	5-Methyl(pentamethylene)silyloxypentadecane
5	4-Methyldocosane
6	Oxiranemethanol, 3-methyl-3-(4-methyl-3-pentenyl)-
7	3,7-Cycloundecadien-1-ol, 1,5,5,8-tetramethyl
8	Cyclopropylpyrrol4-[3-(1H-imidazol-4-yl)propoxy] phenylmorphomethanone oxime
9	Cycloheptane, 1-methyl-4-methylene-
10	3-Bromo-4-hydroxy-2,3'-dimethyl-5,5',8,8'-tetramethoxy-1,2'-binaphthalene-1',4'-dione
11	Cyclopropane, 1-(1,2-dimethylpropyl)-1-methyl-2-nonyl-
12	2- Chloropropionic acid, octadecyl ester
13	Cyclotetracosane
14	3-Diethoxyphosphonyl-demethylthiocolchicine
15	3-Diethoxyphosphonyl-demethylthiocolchicine
16	2-Butenal, 2-methyl-4-[3a,4,5,7-tetrahydro-8-hydroxy-3,3,11,11-tetramethyl-13-(3-methyl-2-butenyl)-7,15-
17	Cobalt, (.eta.5-2,4-cyclopentadien-1-yl)[(1,2,3,4-.eta.)-tris(1,1-dimethylethyl)azete]-
18	3-Methoxy-2,4,5-trifluorobenzoic acid, eicosyl ester
19	3-Methoxy-2,4,5-trifluorobenzoic acid, nonadecyl ester
20	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl nonyl ester
21	Methyl 3-bromo-1-adamantaneacetate
22	2-Chloroaniline-5-sulfonic acid
23	2,3,5,6-Tetrafluorophenyl isothiocyanate

DISCUSSION

Trichoderma's ability to control pathogenic fungi has been attributed to several mechanisms, including competition, antibiotic and this was in line with our findings where the growth inhibition of soil-borne fungal plant pathogens (*Macrophomina phaseolina*) and three foliar fungal plant pathogens (*Phytophthora infestans, Colletotrichum falcatum* and *Colletotrichum gloeosporioides*) was associated with the production of the volatile compounds by the endophytic fungi *Trichoderma longibrachiatum* isolated from *Solanum melongena*. The inhibitory potential of the volatile metabolites was evaluated between the phytopathogens by overlapping plate method and effectively inhibited the pathogens radial growth.

Volatile substances emitted by *T. longibrachiatum* reduced the mycelial growth of agriculturally important one soil fungal pathogens and three foliar fungal pathogens. In nature, *Trichoderma's* organic compounds mediate interactions among the fungus and its surroundings, which include other microorganisms and plant roots. The results made it evident that the liquid growth medium's inclusion of *T. longibrachiatum* culture filtrate inhibited the growth of the plant pathogens Previous studies have reported the efficacy of *T. longibrachiatum* in inhibiting the growth and development of two soil fungal pathogens, *Sclerotium rolfsii* and *Macrophomina phaseolina* through volatile organic compounds (Prabhakaran *et al.,* 2015; Sridharan *et al*., 2020).

The chitinase and β-1,3-Glucanase enzyme activity in the soil-borne and foliar pathogens treated with culture filtrate of *T. longibrachiatum* showed higher compared to the culture filtrate without exposure to the pathogens. Cherkupally *et al*. (2017) and Mukherjee *et al*. (2013) studies reported the enzymes chitinase, β-1,3-glucanase, and acid protease are related to *Trichoderma*'s mycoparasitism. It also facilitates *Trichoderma* to parasitize the host fungi.

Different concentrations of organic fractions (ethyl acetate) of *T. longibrachiatum* were used. The results of this study showed that *Trichoderma longibrachiatum* possesses the innate ability to cause an antagonistic effect on the rate of proliferation of pathogenic fungi. As a result, it was discovered that the effectiveness of the extracts was connected with the resistance or susceptibility provided by various *Trichoderma* species (Hanada *et al.*, 2009; Murtaza *et al.*, 2012). Additionally, it has been observed that a variety of processes,

such as competition, antibiotic resistance, and metabolite synthesis, contribute to the suppression of pathogenic fungi. These findings were consistent with our observations that the production of volatile compounds was a key factor in the growth inhibition of plant pathogens like *M. phaseolina*, *P. infestans, C. falcatum and C. gloeosporioides*. However, metabolite extracted with ethyl acetate substantially inhibited the development of 4 distinct plant diseases at various doses than metabolite extracted with PDB alone.

To increase the productivity of metabolites (for example, antibiotics), researchers explored the nutritional needs for secondary metabolite formation and discovered that the nutrient requirements varied from strain to strain (Shih *et al*., 2002; Singh *et al*., 2012). In our study 9 different culture media were used to screen the fermentation medium for secondary metabolites production. Minimal Broth Davis medium with Dextrose showed maximum inhibition against soil-borne and foliar phytopathogens compared to other media studied. It has previously been documented that the production of secondary metabolites is influenced by process variables like pH, temperature, incubation time, nitrogen, and carbon sources (Wang *et al*., 2011). In this study, the culture conditions such as carbon, nitrogen, precursors, pH, and incubation period were optimized. Among the various carbon sources studied, glucose and sucrose were revealed to be the optimum carbon source for the fungi's secondary metabolite production, with a maximum biomass against soil-borne and foliar-borne phytopathogens. Among the various nitrogen sources studied Peptone and Beef Extract showed the highest antimicrobial activity. Carbohydrates have been shown to interfere with the synthesis of secondary metabolites. Simple carbohydrates like glucose and dextrose alter metabolic pathways that produce intermediates that produce primary and secondary metabolites in addition to CO_2, water, and energy. (Hateet *et al*., 2021). However, initiatives have begun to manipulate dietary components to encourage the microorganism's production of secondary metabolites. (Kumar *et al*., 2012).

The pH, precursors, and incubation time are a few more variables that can affect the formation of bioactive molecules. A large number of microorganisms can produce pH-based secondary metabolites in the range of 5.5 and 8.5 (Thongwai & Kunopakarn, 2007; Hateet *et al*., 2021). Our study results showed that media supplemented with optimized pH, carbon and nitrogen sources with sodium nitrate showed the maximum antifungal activity by producing secondary metabolites.

The aqueous crude extract of the *Trichoderma longibrachiatum* (Tl-01) was investigated to determine their metabolites using GC–MS, which revealed twenty – three compounds. Among the twenty-three compounds, Cyclotetracosane, Chloropropionic acid, and octadecyl ester showed antifungal activity. Naragani *et al.* (2016) reported that secondary metabolites of the strain *Streptomyces cheonanensis* VUK-A (octadecane, 5-eicosene, 1-nonadecene and cyclo tetracosane) compounds recorded antimicrobial activity against medicinally and agriculturally important bacteria and fungi. Lauric acid, 2-methyl butyl ester, 3-Diethoxy phosphonyl-demethyl thio colchicine and 5-Methyl (pentamethylene) silyloxy pentadecane showed phytostimulant activity.

This study contains data regarding the isolation and identification of endophytic strain *Trichoderma longibrachiatum* (Priya *et al.,* 2022) and its antifungal activity through the production of effective antimicrobial compounds that could aid in the fight against plant pathogenic diseases and plant growth promoting-compounds. Through the development of straightforward culture media, we purposefully aimed to keep the SMs production as easy as possible. The optimization of the fermentation medium for the maximum production of secondary metabolites is then formulated. *In vitro* and *in vivo* efficacy of the formulation showed the optimized fermentation medium produced significant compounds which are very effective in controlling the plant pathogens and also stimulating plant growth.

CONCLUSION

Trichoderma-derived Natural Products (NPs) are being researched to develop fungicides and fertilizers for use in agriculture. The current work focused on the design, analysis, and optimization of fermentation parameters for secondary metabolite production of the beneficial fungus, *T. longibrachiatum*. This study provides a desirable alternate approach of utilizing the secondary metabolites (SMs) of bioagents to reduce chemical inputs. The *Trichoderma*'s SMs approach appears to be an encouraging and affordable technology for farmers to maintain the productivity, environmental sustainability, less reliance on chemical fertilizers, and availability of food production while respecting the environment and soil properties.

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