

Research Article**Study on the extraction and purification of metabolites obtained from *Trichoderma harzianum*****Bina Pani Gupta^{1*}, Abhishek Mathur², Akshma Koul², Vandana Shrivastava³**¹Dept. of Microbiology, Himalayan University, Arunachal Pradesh, India²Dept. of Research & Development, NCS Green Earth Pvt. Ltd., Nagpur, Maharashtra, India³Dept. of Microbiology, Subharti University, Meerut (U.P), India

Received: 5 December 2018

Revised: 16 January 2019

Accepted: 31 January 2019

Abstract

Background: *Trichoderma* are closely related with their ability to produce a wide range of lysing enzymes, to degrade substrates and to possess high resistance to microbial inhibitors. *Trichoderma* sp. include a number of fungal strains that are used as biocontrol agents due to their abilities to antagonize a wide range of phyto-pathogenic fungi, bacteria and oomycetes, through several mechanisms that are activated in *Trichoderma* by the pathogens. *Trichoderma* sp. antagonizes phyto-pathogens by competing for nutrients, space, by producing antibiotics as well as by inducing systemic resistance of plants. **Objectives:** The aim of present study was to identify, extraction and purification of secondary metabolites from *Trichoderma harzianum* **Materials and methods:** In the present investigation, *Trichoderma* isolate was isolated and purified from different soil samples. Further, the extraction and purification of secondary metabolites was performed by using ethyl acetate as a solvent. The solvent extract was dried in order to obtain the crude extract which is further processed for GC-MS characterization. **Results:** The GC/MS analysis of ethyl acetate extract revealed the presence of 12 compounds. These compounds were determined as 4 volatile alcohols (1-4) and fatty acid esters (5-12). The mixtures of secondary metabolites were further evaluated against the pathogenic strains for determination of antimicrobial spectrum.

Keywords: *Trichoderma harzianum*, secondary metabolites, compounds, antimicrobial activity

Introduction

Salinity can also affect plant growth because the high concentration of salts in the soil solution interferes with a balanced absorption of essential nutritional ions by the plants. The effect of salinity on plant growth and crop production results in the spread of plant pathogenic fungi which cause damping off, wilt and root-rot diseases, infertility of soil and inability of germination of seeds, physiologic drought, wilting, and desiccation of plants; Stunted growth, small leaves, short stems and branches; blue-green leaf color; retarded flowering, fewer flowers, sterility, and smaller seeds; Growth of salt-tolerant or halophilous weed plants; As a result of all these unfavorable factors, low yields of seeds and other plant parts (Parida and Das, 2005). *Trichoderma* sp. antagonizes phyto-

pathogens by competing for nutrients, space, by producing antibiotics as well as by inducing systemic resistance of plants. In addition, *Trichoderma* sp. stimulates plant growth and development by means of the production of plant growth promoting molecules (Eziashi et al., 2007; Singh et al., 2014). For controlling the infection and prevalence, chemical fungicides have been used for a long time. Intensified use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to human and environment and also builds resistance against pathogens. A reduction in chemical inputs in agriculture requires alternative methods for managing soil borne diseases for sustainable production systems. This includes the use of biological control agents (Roberts et al., 2005; Spadaro and Gullino 2005; Kamal et al., 2009). Different natural molecules/metabolites are secreted by some organisms which control the attack of plant pathogens and such pathogens which shows no resistance against insects and pests for longer period of time. In the present study, the antimicrobial metabolite was extracted and purified from

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DOI: <https://doi.org/10.31024/ajpp.2019.5.3.16>2455-2674/Copyright © 2019, N.S. Memorial Scientific Research and Education Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Trichoderma harzianum. Biological synthesis of metal nanoparticles involving the use of microbes is easy, cost-effective and eco-friendly technique. The metabolites are utilized for the synthesis of nanoparticles. These nano-particles are effective, bio-compatible and bio-degradable.

Materials and Methods

Isolation of *Trichoderma* species from soil samples

The selective medium for isolation of *Trichoderma* sp. was prepared consisting of 0.2 g $MgSO_4 \cdot 7H_2O$, 0.9 g K_2HPO_4 , 0.15 g KCl, 1.0 g NH_4NO_3 , 3.0 g D-glucose anhydrous, 0.15 g rose bengal and 20 g agar. These constituents were added to 950 ml of distilled water and autoclaved at 121°C for 30 minutes. The biocidal ingredients, 0.25g tetracycline was mixed in 50 ml of sterilized distilled water and added to the autoclaved basal medium where it cooled to 40 to 50°C. About, 10 grams of soil was suspended in 50 ml of sterile distilled water and agitated for 30 minutes at 200 rpm in a rotary shaker. Serial dilutions were made and 0.1 ml of each was spread on the selective medium plates with a sterilized glass rod. Three plates of each sample were prepared and incubated for 5 days at 30°C. *Trichoderma* isolates were collected and transferred onto potato dextrose agar (PDA) plates for maintaining pure culture (Gupta et al., 2016).

Cultivation and culture conditions

The culture was cultivated and maintained on slants of potato dextrose agar for 5 days at 28°C. Conidia was scrapped from mycelia which were grown on slants and cultivated on autoclaved CYS80 medium (sucrose 80 g/L, yellow corn meal 50 g/L, yeast extract 1g/L) as solid state fermentation (Suay et al., 2000). Three hundred milliliters flasks were incubated for 14 days at 28°C on a laboratory incubator. Crude extract was obtained by filtration and subjected to centrifugation at 5000 rpm for 15 minutes. The supernatant was pooled and designated as crude extract. The crude extract was further processed for metabolite extraction using chloroform: ethyl acetate in 1:1 ratio. The solvent was further mixed drop wise in the crude extract with slight stirring. The metabolites were further mixed in the organic layer which was further pooled out and dried to obtain the crude metabolites.

Separation and identification of antimicrobial compounds in *Trichoderma* extract by GC-MS analysis

The GC-MS spectra was obtained by using Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with Elite-1 fused silica capillary column (30 m × 0.25 mm) composed of 100 % dimethyl poly siloxane) from Perkin Pvt. Ltd., Germany. The carrier gas was helium. 70°C hold for 5 minutes and hold for 5 minutes. The control of the GC-MS system and the data peak processing were controlled by means of Shimadzu's GC-MS

solution software, version 2.21. The crude metabolites extract was injected within the column. Compound identification was verified based on the relative retention time and mass fragmentation pattern spectra with those of standards. The samples were prepared in methanol before analyzed by GC/MS.

Preparation of silver nanoparticles

For the preparation of silver nanoparticles two stabilizing agents, sodium dodecyl sulphate (SDS) and sodium citrate were used. For the synthesis of silver nanoparticles, silver nitrate solution (from 1.0 mM to 6.0 mM) and 8% (w/w) sodium dodecyl sulphate (SDS) was used as a metal salt precursor and a stabilizing agent, respectively. Hydrazine hydrate solution with a concentrate ranging from 2.0 mM to 12 mM and sodium citrate (1.0 mM to 2.0 mM) was used as reducing agents. Citrate of sodium was used as stabilizing agent at room temperature. The transparent colorless solution was converted to the characteristic pale yellow and pale red colour. The occurrence of colour was indicated by the formation of silver nanoparticles. The silver nanoparticles were purified by centrifugation. The excess silver ions were washed at least three times with deionized water under nitrogen stream. A dried powder of the nanosize silver was obtained by freeze-drying. The silver nanoparticles powder was resuspended in deionized water; and further homogenized with a ultrasonic cleaning container.

Preparation of *Trichoderma* fused Silver nanoparticles

The silver nitrate (1 mM) solution was prepared in 50 ml deionized water. The secondary metabolites were utilized as reducing agent. The metabolite solution was mixed drop wise drop with silver nitrate solution in a 200 ml Erlenmeyer flask. Further, the mixture was kept in dark at 29±1°C under continuous shaking at 200 rpm for 72 h. After 72 h of reaction time the colour change was observed.

Characterization of prepared nanoparticles via UV-absorption spectra and Transmission electron microscopy (TEM)

The formations of AgNPs and TR-AgNPs by the bioreduction of Ag^+ to Ag^0 were easily monitored using UV-Vis spectroscopy. The scanning was performed in the range of 200–700 nm. The morphology and size was determined by TEM.

Results

The present study showed the isolation and characterization of *Trichoderma* spp. culture isolated from soil. Further the culture was determined taxonomically and was determined as *Trichoderma harzianum* and was denoted as per the

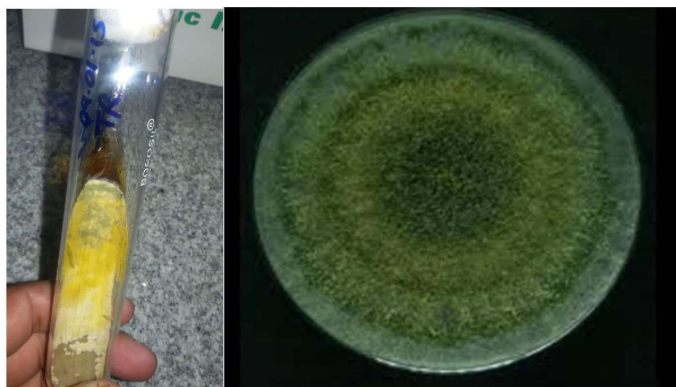


Figure 1. Isolated culture of *Trichoderma*

accession no- NCFT.9153.17. The culture photograph is shown in figure 1. The *Trichoderma* extracts were prepared and filtered. Further solvent extraction was performed in order to determine the presence of secondary metabolites if any. The solvent extract was further vacuum dried in order to evaporate the solvent and to obtain the powder having secondary metabolites for further identification and preparation of nanoparticles in fusion with silver. The GC/MS analysis of ethyl acetate extract revealed the presence of 12 compounds. These compounds were determined as 4 volatile alcohols (1-4) and fatty acid esters (5-12). The GC-MS spectra of *Trichoderma* metabolites showed the separation of fatty acids viz. Kojic acid, citric acid and acetic acid as the important components as determined by different peaks. The results showed the highest percentage of Kojic acid (65%), acetic acid (15%) and followed by citric acid (5%). The GC-MS spectrum is shown in

figure 2. In the present investigation, *Trichoderma* fused silver nanoparticles (TR-Ag Nps) were produced. The particle size was determined by SEM and their absorption spectrum was determined at 200-700 nm. It was observed that the TR-Ag Nps produced were of very fine shape and size having optimal 20 nm size. These nano particles were having slightly rough spherical structures which were observed in free and interconnected form (figure 3). The UV absorption spectra of the fused nanoparticles recorded the maximum wavelength at 415 nm.

Discussion and Conclusion

Trichoderma is known as an important and significant bio-control agent. The classical mechanisms of control have included antibiosis, myco-parasitism, and competition for nutrients. The significant combination of *Trichoderma* with synthetic salts/metallic ions can be utilized in treatment of different infections within the plants and animals both. These can be utilized as alternatives to different antibiotics etc. The present study thus concludes that, *Trichoderma* secretes some potent secondary metabolites responsible for antimicrobial activity against opportunistic and nosocomial infections causing pathogens. Both *Trichoderma* (biological) and silver (Inorganic metallic ions) are known to have significant antimicrobial potency. But fusions of both the biological and inorganic metallic ion have not been studied. The combination of secondary metabolites from *Trichoderma* and Ag ions in terms of fused nanoparticles can results in preparation of potent antimicrobial and seed

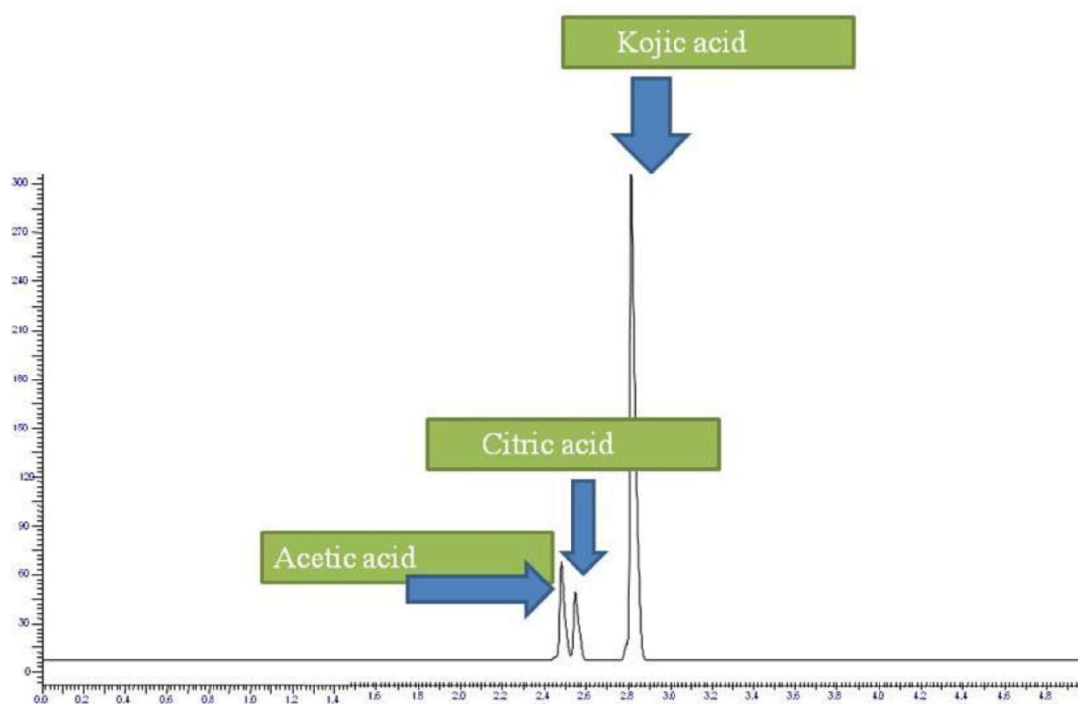


Figure 2. GC-MS spectra of *Trichoderma harzianum* metabolites extract

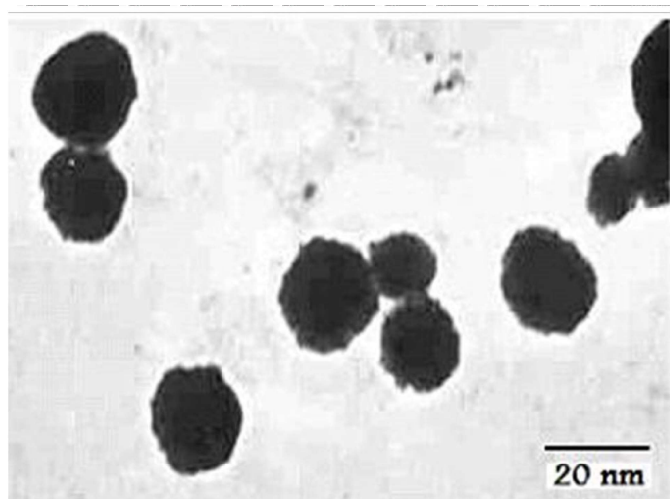


Figure 3. Particle size of Trichoderma metabolite fused silver nanoparticles

germination agents. The results of the present study correlates the findings of previous researches (Tripathi et al., 2013; Shendge, 2017; Shelar and Chavan, 2014; Devi et al., 2013). Previous studies reported by our group have already described the antifungal behavior of Trichoderma fused silver nanoparticles against fungal phyto-pathogens and antimicrobial activity against the pathogens (Gupta et al., 2016 & 2017). The present study suggests that, Trichoderma secretes some potent secondary metabolites responsible for antimicrobial activity against dreadful human pathogens. Ag⁺ nanoparticles are also potent antimicrobial agents and the combination of secondary metabolites and Ag ions in terms of fused nanoparticles can results in preparation of potent antimicrobial agents. The secondary metabolite fraction of Trichoderma was found to be Kojic acid as determined by GC-MS spectra. The nanoparticles were thus prepared by the fusion of Kojic acid and Ag⁺ ions which were found significant as per antimicrobial behavior. These nanoparticles can thus be utilized in formulating antimicrobial drug against the concerned pathogens.

Conflicts of Interest

The author declares no conflict of interest.

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