**Identification of *Trichoderma* Strains isolated from Copper Mining Locations of Uttarakhand using BiOLOG Microstation System**

**Abstract**

Twenty one *Trichoderma* stains ((20 isolates of TCMS series *viz.*, TCMS-2, TCMS-4, TCMS-5, TCMS-12, TCMS-14a, TCMS-14b, TCMS-15, TCMS-16, TCMS-24, TCMS-32, TCMS-34, TCMS-36, TCMS-43, TCMS-60, TCMS- 62, TCMS-64, TCMS-65, TCMS-72, TCMS-85 and TCMS-93 were isolated and purified from the rhizosphere soil samples collected from different copper mining sites of Uttarakhand hills and one isolate of SBIT series *i.e.,* SBIT-32 was collected from the Sri Biotech limited) were characterized/identified using BioLOG Microsystem Version 4.2. Cultures were incubated for 72 hours in the BioLOG Microsystem plates and the incubated plates were analyzed using Bio-Log Microsystem Version 4.2 for species identification. The BioLOG Microsystem determine the Trichoderma species based on various carbon source (substrate) utilization patterns among the 95 BioLOG substrates. Of these, ten isolates (TCMS-4, 12, 14a 15, 32, 34, 43, 72, 85 and 93) were identified as *T. viride*, five (TCMS 16, 24, 36, 60 and 65) were *T. harzianum*, three (TCMS 5, 14b and SBIT) were *T. koningii* and three isolates (TCMS 2, 62 and 64) belonged to *Hypocrea lixii.* The identified Trichoderma species may be further screened for their antagonistic potential and successfully used for the sustainable management of crop diseases in the organic cultivation system or in the integrated disease management strategies.

***Key words***: Biological control, Characterization, Biopesticides, Sustainability, Organic farming.

**Introduction**

*“Trichoderma* is a soil inhabitant fungus which grow in diverse habitats. The fungi belonging to the genus under Deuteromycotina, Hyphomycetes, Moniliales, and Moniliaceae. This genus comprises large number of fungal species and are widely used for bio-control of plant diseases incited by fungal and bacterial pathogens. In addition, it was also found effective in increasing plant growth and development” (Harman and Bjorkmann, 1998; Singh *et al.*, 2006). “*Trichoderma* species alone or in combination with the compatible fungicides have been used for the control of several diseases like root rots, wilts, damping off, white rot, collar rots etc. in a wide variety of crops” (Samuels, 1996).

 “*Trichoderma* colonizes plant roots and grows as endophyte within the plant system; *Trichoderma* provides multifaceted benefits to its host like nutrient uptake, disease resistance, plant growth promotion, abiotic stress tolerance etc.” (Woo *et al*., 2023).

*“Trichoderma* also produces more than 100 types of secondary metabolite, such as pyrones, polyketides, terpenes, and non ribosomal peptides which are having industrial importance” (Rubio *et al.,* 2009). “*Trichoderma atroviride* secretes a LysM protein (Tal6) which binds to the chitin and disrupts the fungal cell wall” (Romero-Contreras et al., 2019). Researchers are interested in this genus because of its novel biological properties against several plant pathogens and biotechnological applications.

*Trichoderma* species exhibits various enzymatic activity such as chitinases, proteinases, cellulases etc. (Xue *et al.*, 2021). The ecological role of this genus is to play role in the decomposition of plant residues in soil. It is very difficult to identify the Trichoderma species based on morphological/microscopic or cultural characters. Keeping the above factors in view, a study was carried out on“Identification of *Trichoderma* isolates collected from copper mining areas of Uttarakhand using BioLOG Microsystem”.

**Review of literature**

“The genus *Trichoderma* was first erected by Persoon” (Cook and Baker, 1983; Bisset, 1991 a, b). “Genus *Trichoderma* have traditionally been classified as Fungi Imperfectii as they were reported to produce only asexual spores i.e., conidia” (Singh *et al.*, 2006). “With the recent advances in molecular taxonomy, many of the species have been reclassified as belonging to the genus Hypocrea of the class Ascomycetes” (Gams and Bisset, 1998; Druzhinina *et al.*, 2005). “The teleomorph of this genus is Hypocrea Fr. under Hypocreales, Ascomycotina” (Rifai and Webster, 1966a).

Rifai (1969) first time given the species aggregates in Trichoderma and grouped Trichoderma into nine aggregates. Bisset (1992) elevated Rifai’s species aggregates to species level and recognized two to several species within each of five sections of the genus.

Kullnig *et al.* (2000) examined 76 isolates from Russia, Nepal and northern India, reporting five undescribed taxa. A similar study was carried out by Kubicek *et al.* (2002) in south-east Asia, in which seven new species were found among 96 strains (Bissett *et al.,* 2003). *Trichoderma* isolates, representing different species screened for tolerance to increasing osmotic pressure and temperature while carbon assimilation was investigated using Phenotype Microarray technique with BioLog TM FF MicroPlates. Metabolic profiles based on growth and respiration on 95 carbon substrates was assessed on Biolog FF MicroPlates™. Forty six Trichoderma isolates were screened using BioLOG Microstation System and ITS Markers (ITS-1 and ITS-2) to group under different taxonomic groups.

**Material and Methods**

***Collection of soil samples, isolation, and purification of Trichoderma***

Extensive collections of soil samples were carried out in different farming situations from the copper mining areas of Uttarakhand such as Nainital, Bageshwar and Tehri Garhwal districts. Approachable locations of copper mining sites were selected to collect the soil samples. Rhizosphere soil near the healthy crop and the soil adhered to the root zone was collected. Five random samples were collected and mixed thoroughly and used as composite sample for that location. Soil samples were air dried for four hours. Isolation was done by employing serial dilution technique. Serial dilutions were carried out up to 10-3 dilution. 1.0 ml of soil suspension was during the pour plate technique to isolate the *Trichoderma* sp. on *Trichoderma* selective medium (TSM). TSM was used for isolation of *Trichoderma* spp. (Elad *et al.,* 1981; Mukherjee, 1991). One ml suspension was poured with 1.0 ml pipette to the Petri dish and then added the TSM on the suspension. The plates were slowly rotated clockwise and anticlockwise to ensure uniform distribution of the suspension in the medium. The incubation was done for five days at 28±10 C and the *Trichoderma* colonies were identified and purified using hyphal tip technique. This culture was finally maintained and used for further studies.

Out of 49 Trichoderma strains isolated from the soil, 21 potential isolates were identified using BioLOG Microstation system including the isolate (SBIT) collected from the Sri BioTech Laboratory, Hyderabad were named as SBIT Series.

***Characterization of Trichoderma isolates using BiOLOG Microstation System (Version 4.2)***

The Microstation System/Microlog is an easy-to-use yet advanced tool for identifying and characterizing microorganisms. Combined databases include over 1,900 species of aerobic bacteria, anaerobic bacteria, fungi and yeasts. Biolog’s patented microbial identification technology with 95 carbon source utilization tests in a microtiter plate format (microplate™) can recognize over 4 x 1028 possible metabolic patterns.

*Principle*

Biolog’s innovative, patented technology uses each microbe’s ability to use particular carbon sources to produce a unique pattern or “fingerprint” for that microbe. As a microorganism begins to use the carbon sources in certain wells of the microplate, it respires. For bacteria, this respiration process reduces a tetrazolium redox dye and those wells change color to purple. The end result is a pattern of colored wells on the microplate that is characteristic of that microorganism. For fungi, respiration and assimilation are detected. The color change in the wells is reddish orange due to the respiration of fungi, which reduces the dye. Assimilation or growth is detected by the turbidity of the well. A fungal pattern is readable either visually or by a fiber optic reading instrument the microstation reader. The BioLOG software searches and compare the fingerprint data its database and identifies the fungus. By developing a simple tool that allows 95 simultaneous carbon source (depicted in Plate 1) utilization tests. Biolog has accomplished its goal of producing an efficient, easy-to-use, powerful, and reliable microbe identification system (Plate 1).

*Microbial Identification Process*

Microbial identification involves four basic steps.

1. *Culturing of the fungus on Biolog media*

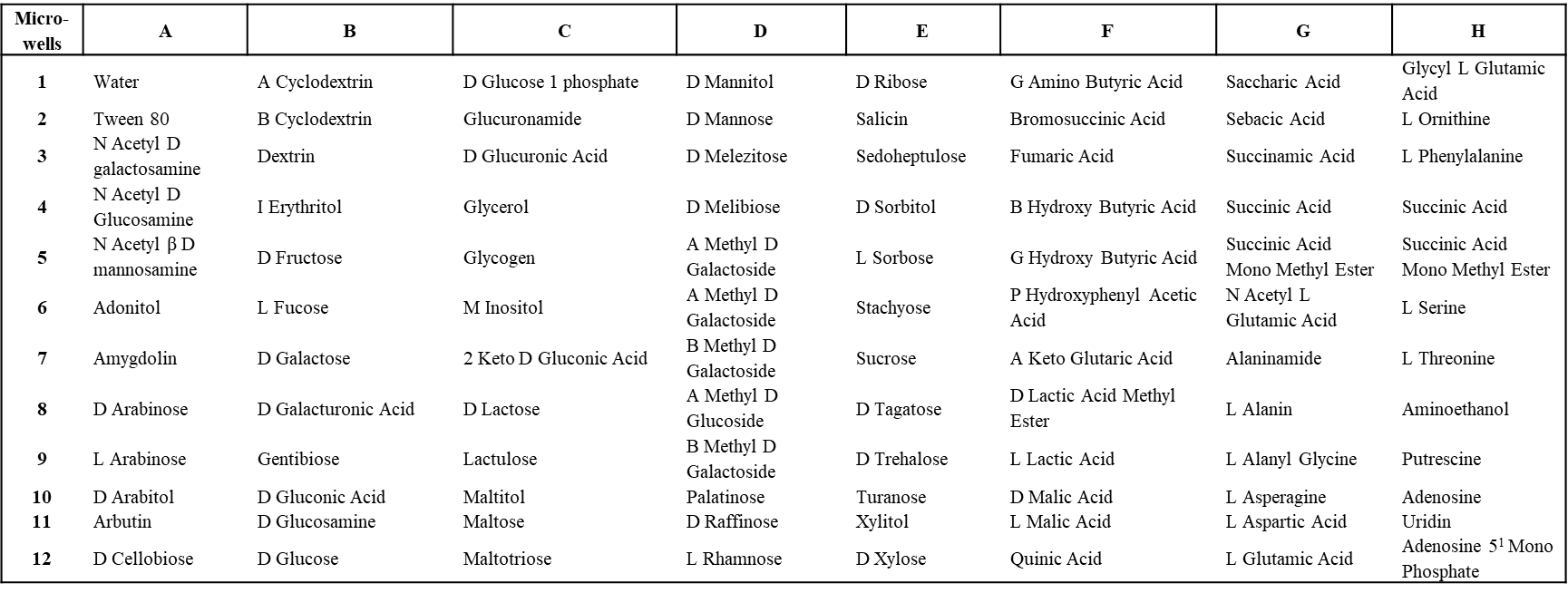
Biolog recommended media were used for the good growth of the colonies for accurate identification of the fungus.

1. *Preparation of inoculum at specified cell density*

Microbiologists are sometimes trained to prepare cell suspensions by judging cell density by eye. This practice not yields accurate and reproducible results.

1. *Microplate inoculation*

Cell suspension was pipetted out on the microplates and closed the lid of the plate and incubated at 28±10 C for 48 hours.

**Plate 1.** **Substrates of BiOLOG Microstation System (Version 4.2) culture plates**

1. *Reading the microplate and identification of the fungi*

After incubation, plates were red with the BioLOG Microstation System reader. The software matches the results with its exclusive database and identify the fungus in seconds.

*Preparing inoculum*

The fungus was isolated and cultured on PDA, a liquid inoculum was prepared with following standards.

|  |  |  |  |
| --- | --- | --- | --- |
| **Organism type** | **Inoculating Fluid** | **Turbidity Standards** | **Inoculum density** |
| Filamentous Fungi | FF-IF (Filamentous Fungi- Inoculating Fluid) | FF (Filamentous Fungi) | 75% T (Turbidity) |

*Protocol for inoculation*

Bio-Log microplates were labelled with the organism name/number and plate type (side of the microplate). Using aseptic technique, the suspension was poured into a multichannel pipette reservoir.

Eight sterile tips were firmly attached to the pipettor and filled the tips with the suspension. Primed the tips by dispensing once back into the reservoir. Filled all microplate wells by placing the tips at an angle inside the top of the wells. For filamentous fungi 100 μl inoculums suspension per well was added. Covered the microplate with its lid. Incubated at 260C for 72 Hours.

*Reading of microplate using the plate reader BiOLOG microstation system (Version 4.2, GEN II)*

* Log on to Micro-Log 4.20.05 software.
* Select the Set up tool.
* Select the reader Type: Bio-Tek and Com Port: 1.
* Then, click on the Initialize Reader.
* After initialization, fix the microplate in Bio-Log Microstation in appropriate position.
* Name the output file, where it should be saved.
* Then select the Data tool and provide details regarding Plate No., Sample Type, Plate Type, Strain No. and incubation time.
* Click on the Read this button.
* Finally, save results and identification of the sample with most matching species.

**Results and Discussion**

**Identification of selected potential *Trichoderma* isolates using Bio-Log System (BiOLOG Microstation System- Version 4.2)**

The present study was conducted at the Biological Control Laboratory, Department of Plant Pathology, G.B. Pant University of Agriculture and Technology, Pantnagar, U.S. Nagar, Uttarakhand-263145. The study was aimed to identify the *Trichoderma* isolates, which were isolated from the copper mining areas of Uttarakhand. For Identification of Trichoderma isolates up to species level based on biochemical properties to utilize the carbon source present in the BioLOG culture plates, the BioLOG Microstation System was used. The BioLOG culture plates contains 96 different Carbon sources (Plate 1), the BioLOG system reads the data of carbon utilization by the different *Trichoderma* isolates and compare it with the exclusively available data in the system and then provides the accurate identification of result up to the species level within a very short time.

In the experiment, twenty one *Trichoderma* isolates (20 isolates of TCMS series *viz.*, TCMS-2, TCMS-4, TCMS-5, TCMS-12, TCMS-14a, TCMS-14b, TCMS-15, TCMS-16, TCMS-24, TCMS-32, TCMS-34, TCMS-36, TCMS-43, TCMS-60, TCMS- 62, TCMS-64, TCMS-65, TCMS-72, TCMS-85 and TCMS-93, and SBIT-32) were characterized/identified using BioLOG Microsystem version 4.2 after 72 hour incubation time, were used for species identification. Species of *Trichoderma* strains were identified based on various carbon source (substrate) utilization patterns among the 96 biolog substrates.

Out of twenty one Trichoderma isolates used for the identification, ten isolates (TCMS-4, TCMS-12, TCMS-14a TCMS-15, TCMS-32, TCMS-34, TCMS-43, TCMS-72, TCMS-85 and TCMS-93) were identified as *T. viride*, five isolates (TCMS-16, TCMS-24, TCMS-36, TCMS-60 and TCMS-65) were *T. harzianum*, three (TCMS-5, TCMS-14b and SBIT-32) were *T. koningii* and three isolates (TCMS-2, TCMS-62 and TCMS-64) belonged to *Hypocrea lixii.* (Plates 2).

Similarly, Kullnig *et al.* (2000) examined 76 isolates from Russia, Nepal and northern India, reporting five undescribed taxa. A similar study was carried out by Kubicek *et al.* (2002) in south-east Asia, in which seven new species were found among 96 strains (Bissett *et al.,* 2003) using metabolic profiles based on growth and respiration on 95 carbon substrates assessed on Biolog FF MicroPlates™. Isolates of *Trichoderma harzianum* revealed metabolic and genetic variability that explain the broad distribution of this species aggregate in diverse habitats.

Ninety six *Trichoderma* strains were analyzed for morphological and bio-chemical characters (BioLOG Microstation system) using already established exclusive database. Out of which, 56 isolates were identified as *T. harzianum*, 16 as *T. virens*, eight as *T. spirale*, three as *T. koningii*, three as *T. atroviride*, four as *T. asperellum*, two as *H. jecorina* (anamorph: *T. reesei),* two as *T. viride*, one each as *T. hamatum* and *T. ghanense* (Christian *et al.,* 2003).

A similar study was conducted by Lilliana *et al.* (2009) for diversity analysis of 183 isolates using Biolog system. A comparatively high diversity of species was found, comprising 29 taxa: 60 isolates as *T. asperellum*, three as *T. atroviride*, five as *T. brevicompactum*, three as *T. crissum*, three as *T. erinaceum*, two as *T. gamsii*, two as *T. hamatum*, 49 as *T. harzianum*, six as *T. koningiopsis* (6), three as *T. longibrachiatum*,one *T. ovalisporum*,two as *T. pubescens*, four as *T.* *rossicum*, one *T. spirale*,three *T. tomentosum*,eight *T. virens*, seven *T. viridescens* and three *H. jecorina* along with 11 currently undescribed species.

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| **TCMS 2: *Hypocrea lixii*** | **TCMS 4: *T viride*** | **TCMS 5: *T. koningii*** |
|  |  |  |
| **TCMS 12: *T viride*** | **TCMS 14a: *T viride*** | **TCMS 14b: *T. koningii*** |
|  |  |  |
| **TCMS 15: *T viride*** | **TCMS 16: *T harzianum*** | **TCMS 24: *T harzianum*** |
|  |  |  |
| **TCMS 32: *T viride*** | **TCMS 34: *T viride*** | **TCMS 36: *T harzianum*** |
|  |  |  |
| **TCMS 43: *T viride*** | **TCMS 60: *T harzianum*** | **TCMS 62: *Hypocrea lixii*** |
|  |  |  |
| **TCMS 64: *Hypocrea lixii*** | **TCMS 65: *T harzianum*** | **TCMS 72: *T viride*** |
|  |  |  |
| **TCMS 85: *T viride*** | **TCMS 93: *T viride*** | **SBIT**: ***T. koningii*** |

**Plate 2**: **Identification of *Trichoderma* spp. (TCMS 2) using BioLog Micro-station system**

**Conclusion**

In the present study, BioLOG Microstation System was used to identify the Trichoderma strains isolated from the cooper mining sites of Uttarakhand. It was found that the BioLOG Microstation System is easy to handle, update, and is customized for specific needs. It identifies the fungi based on per cent utilization of carbon sources available in the BioLOG Microplate/s. Compared to molecular detection method, BioLOG Microstation System method is easier and didn’t require much inputs for the identification of fungi.The large number of organisms in Biolog's data base warrants ongoing verification of assigned identities with known authentic isolates. In summary, although the Biolog system offers great promise, its identifications should be viewed cautiously. BioLOG Microstation System is useful to identify the Trichoderma upto the species level. Once the Trichoderma identified with the BioLOG Microstation System, details about the species may be obtained from the data base of the Trichoderma or somewhere else and then utilized for specific purposes of the identified species after thorough screening.

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Details of the AI usage are given below:

1.

2.

3.

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