Poll Res. 44 (3–4) : 231-236 (2025)

ISSN 0257–8050

DOI No.: http://doi.org/10.53550/PR.2025.v44i03-04.013
Journal Home Page: www.envirobiotechjournals.com

ISOLATION AND IDENTIFICATION OF THERMOPHILIC FUNGI USING MORPHOLOGICAL AND MOLECULAR APPROACHES

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(Received 2 February, 2025; Accepted 10 April, 2025)

ABSTRACT

A special class of microorganisms known as thermophilic fungus prefers high temperatures, usually between 40°C and 60°C. Because of their exceptional physiological and biochemical adaptations, this fungus can thrive in harsh settings including compost piles, hot springs, and decomposing organic materials. They are extremely valuable for industrial uses in waste management, food processing, and biofuel production due to their enzymatic properties, especially in the synthesis of thermostable enzymes including cellulases, xylanases, and proteases. Furthermore, thermophilic fungi are essential to the breakdown of organic matter and the cycling of nutrients in natural environments. In the present study, isolation, identification and molecular screening carried out for the thermophilic fungi.

KEY WORDS: Thermophilic Fungi, biotechnology, Industrial Applications, Diversity, and Adaptations.

INTRODUCTION

Thermophilic fungi are a small group of eukaryotes widely distributed in natural habitats. They thrive in extreme environmental conditions, including high temperatures, pH variations, and pressure, which are significant from ecological and industrial perspectives. Temperature is a critical factor affecting their survival and growth. As defined by Cooney and Emerson (1964), thermophilic fungi require an optimum growth temperature of 45°C, with a growth range between a minimum of 20°C and a maximum of 50°C or higher. True thermophilic fungi do not grow below 20°C but thrive above 50°C, with some species capable of growing at temperatures as high as 60–62°C. Over the past four decades, many species sporulating at 45°C have been reported. These fungi are notable for their ability to produce novel metabolites and enzymes, enhancing their ecological and industrial importance. (Johri, 1999; Saroj, 2017; Singh, 2009).

Among the eukaryotic organisms, only a few species of fungi can thrive at temperatures between 45°C and 55°C. Such fungi comprise thermophilic and thermotolerant forms, which are arbitrarily distinguished based on their minimum and maximum growth temperature (Semira *et al.*, 2021). Thermophilic fungi are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products and other accumulation of organic matter where in the warm, humid and aerobic environment provides the basic condition for their development (Sujeeta *et al.,* 2017).

It is possible to cultivate thermophilic fungi in minimum media with growth yields and metabolic rates that are similar to those of mesophilic fungi. Thermophilic fungi and filamentous fungi in general have been better understood because to studies of their growth kinetics, respiration, mixed-substrate usage, food uptake, and protein breakdown rate (Morya and Yadav, 2008). Fungi comprises thermophilic and thermotolerant forms. The thermophilic fungi have growth at or above 50°C, whereas the thermotolerant forms grow at a temperature of 20°C -55°C (Pavan and Mahadevamurthy, 2021).

Humicola was established by Traaen (1914) for two species of fungi with hyaline hyphae. Fassatiova (1967) emended the diagnosis of this genus and constructed a key for the determination of *Humicola* sp. She pointed out that according to the taxonomic opinions of Hughes (1953) and Subramanian (1962) hyphal color was not a decisive feature and that only fungi with the definitely expressed character of forming a leuriospores could be included in the genus. De Bertoldi (1976) subsequently concluded that aleuriospore size was the most effective and stable diagnostic morphological character in *Humicola* (12-16). *Humicola* piriformis is a species of fungi the family Chaetomiacea. *Humicola* sp is a nematophagous fungus that can survive in many soils and plant debris. It can spread through water and wind (Fassatiová, 1967; Subramanian, 1962).

MATERIALS AND METHODS

Isolation of Fungi

The soil fungus was isolated using the dilution plate approach. Ten milliliters of sterile distilled water and one gram of dirt were mixed together in a test tube and shaken until the mixture was homogeneous. Add 9 ml of sterile distilled water to 1 ml of the homogenous solution in a test tube to create 10-2 dilutions. One milliliter of the 10-2 dilution is mixed with nine milliliters of sterile distilled water to create 10-3 dilutions. The diluted material is serially diluted and then put to the solidified agar medium using the spread plate method. The plates were cultured for 5–14 days at room temperature (27 °C) after inoculation (Fig. 1).

Pure culture Preparation

Each unique fungal colony had a small portion of the inoculum extracted, which was then reinoculated on a petri plate with solidified PDA media. The plates were then cultured for five to seven days at room temperature (27 °C).

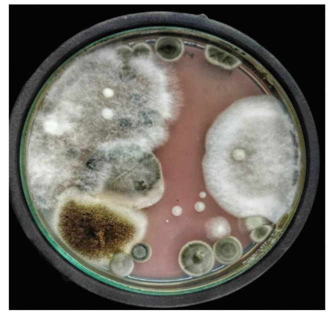

Fig. 1. Mixed culture of Fungi

Screening of thermophilic Fungi

A systematic methodology was carried out for the isolation of thermophilic fungi from soil samples. One g soil sample was suspended in 5 ml of sterile distilled water, vortexed thoroughly, and subjected to heat treatment in a water bath at 70°C for 24 hours to enrich thermophilic fungal spores. Following heat treatment, 0.5 ml of the suspension was inoculated into 100 ml of sterile nutrient broth (composition: 4 g/l yeast extract, 20 g/l glucose, 1.0 g/l KHPO, 0.5 g/l MgSO·7HO, pH 7.0 ± 0.2) and incubated in a shaking incubator at 28°C for 72 hours (O Ibraheem, 2021).

Post-incubation, 200 µl of the enriched broth culture was spread onto Potato Dextrose Agar (PDA) plates supplemented with 1% streptomycin to inhibit bacterial contamination. The inoculated plates were incubated at 50°C in the dark for 7 days, facilitating the growth of thermophilic fungal colonies (Fig.1, 2a & 2b).

Fig. 2a. *Humicola piriformis* on SDA medium (Top view)

Morphological characteristics of the emerging colonies were recorded, and selected isolates were subjected to further for microscopic observations

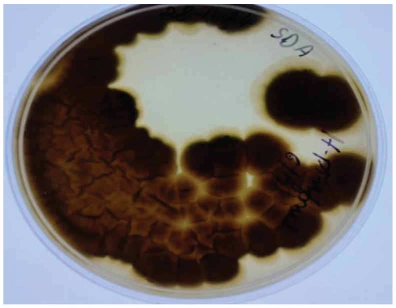

Fig. 2b. *Humicola piriformis* on SDA medium (Bottom view)

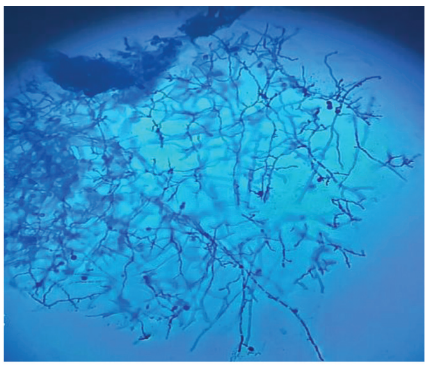

Fig. 3. Microscopic observation under 10X

and molecular identification.

Molecular Identification of fungus using ITS method

DNA isolation by CTAB Method

1000 of extraction of CTAB 0.5 g of the fungal sample were mixed with buffer, incubated for 30 minutes at 60 °C, and then homogenized. After the incubation period, centrifuge the homogenate for five minutes at 14,000 x g. Chloroform and isoamyl alcohol (1:24) are added in equal amounts. After 5 seconds of vortexing, centrifuge the sample at 14,000 x g for 5 minutes to separate the phases. The tube containing the aqueous top phase should be changed. To precipitate the DNA, add 0.7 L of cold isopropanol, and then leave it overnight at -20°C. After that, spin the DNA column for a minute at 12,000 rpm while adding 750 µl at a time. Pour in 750 µl of wash buffer and spin for 1 minute at 12000 rpm. Repeat the washing. For two minutes, dry spin the DNA column at 12,000 rpm. Add 20 µl of elution buffer, let it settle for three minutes at room temperature, and then spin it for one minute at 12,000 rpm. After adding 1 µl of RNase solution A, wait at 37°C for 30 minutes. The resulting DNA was measured using agarose gel electrophoresis (Panzer *et al.*, 2015) (Fig. 5).

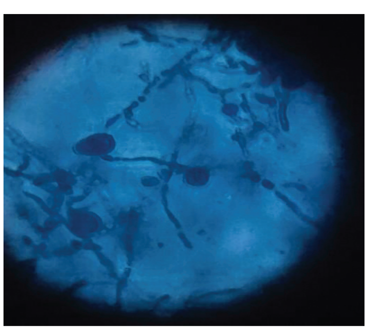

Fig. 4. Microscopic observation under 100X

Conditions for PCR

Two minutes of initial denaturation at 95 °C. 30 seconds of final denaturation at 95 °C. Annealing for 30 seconds at 50 °C. One minute of elongation at 72 °C. Do steps two, three, and four thirty times (30). The ultimate elongation at 72 °C takes 10 minutes (Fig. 5). Always keep at 4°C (Table 1).

Table 1. Primers with sequence of ITS 1 and ITS 4.

Primer	Sequence	Annealing temperature
ITS-1	5′-TCCGTAGGTGAACATGCGG-3′	53 0C
ITS-4	5′-TCCTCCGCTTATTGATATGC-3′	57 0C

Preparation of PCR reaction mixture

To construct multiplex PCR assays for DNA barcode primers and evaluate the effectiveness of amplification, each PCR reaction used two microliters of 10X reaction buffer, 0.5 microliters of $MgCl_2$ (50 pM), 1 microliter of DNA template (25 ng), 1 microliter of forward primer (10 pM), and 0.5 microliters of Taq polymerase (5 Standard primers

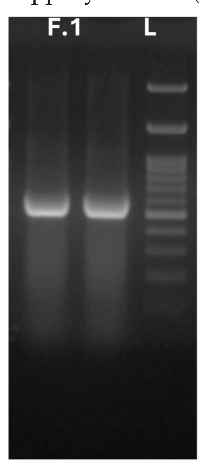

Fig. 5. Agarose (1.5%) gel electrophoresis of PCR using ITS1 and ITS4 primers fungal sample.

are provided as primers for the 18srRNA gene amplification, together with 1 µl of the dNTPs mix (10 mM) and 1 µl of reverse primer (10 pM) (30).

Gel purification protocol

Cut the desired DNA band on the gel. After adding 600 µL of a gel solubilization buffer, heat the mixture to 55 C until the gel is completely dissolved. 200 µL of isopropanol should be mixed and then added to the column. Spin at 12,000 rpm for one minute. Pour in 700 µL of wash buffer and spin for 1 minute at 12000 rpm. Run the dry spin for two minutes. Add 20 µL of elution buffer and spin for 1 minute at 12000 rpm (Abadi *et al.,* 2022).

Sanger Sequencing PCR

Two minutes of initial denaturation at 95 °C. Final denaturation for 30 seconds at 95 °C 30 seconds of annealing at 50 °C. Four minutes later, stop at 60 °C. Repeat steps two, three, and four thirty times. Keep indefinitely at 4°C (Abadi *et al.,* 2022).

Post-Sequencing and PCR Purification

Add 2.5 µL of EDTA and 125 Mm to each well, then spin rapidly. Add 35 µL of ethanol using a multichannel pipette. After vertexing for 10 minutes at 2000 rpm, centrifuge for 30 minutes at 3510 rpm. Use a tissue bed to decant ethanol at 300 rpm (invert the plate for 30 seconds). After adding 40 µL of 80% ethanol to each well, centrifuge the wells for 12 minutes at 3510 rpm. Repeat the inverted spin described above. Cover the plate with a lint-free tissue and let it air dry for 30 to 45 minutes. After adding 13 µL of HiDiFormamide, quickly spin the mixture. At 95 °C, denature for 5 minutes. In the sequencer, insert the plate (Abadi *et al.,* 2022).

Data analysis

The resulting sequencing files will be viewed using software such as FinchTV, BioEdit, Chromas Lite, Seq Scanner, etc. The quality of the sequence can be assessed using electropherogram peaks, and the BLAST server or servers linked to specific databases can be used to analyze the sequencing data.

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CCGTTGCTTCGGCGGGCGGCCCGGGTCCTGCCCGGCGCCCCTCGGCCCTC
GCGGGCGCCCGCCGGAGGAAAACCAAACTATTGCATTGTATGGCCTCTCTG
AGTCTTCTGTACTGAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTG
GCGGGCATGCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCGGGCTTGTG
TTGGGGACCTGCGGCTGCCGCAGGCCCTGAAATGCAGTGGCGACGCTCGG
AGTGACAGCGAGCCCGCCACTGCATTTCAGGGCCTGCGGCAGCCGCAGGT
CCCCAACACAAGCCCGGGGCTTGATGGTTGAAATGACGCTCGAACAGGCA
TGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATT
CACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCG
ATGCCAG
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Fig. 6. *Humicola piriformis* partial sequence of the small subunit ribosomal RNA gene.

RESULTS AND DISCUSSION

The ability of fungal strains to colonize soil and compete with native microorganisms is a critical factor in their potential industrial and biotechnological applications. To ensure the viability and purity of the isolated thermophilic fungus, it was cultivated on Sabouraud Dextrose Agar (SDA), a standard medium for fungal growth. Morphological and microscopic examination of the colonies revealed distinct characteristics associated with thermophilic fungi.

For precise identification, molecular sequencing was conducted using universal fungal primers ITS1 and ITS4, targeting the Internal Transcribed Spacer (ITS) region, a well-established genetic marker for fungal classification (Fig. 6). The sequencing results confirmed the isolated strain as *Humicola piriformis*, a thermophilic fungus (Fig. 7) known for its potential in enzyme production and industrial applications (Stefanos et al., 2018).

Several studies have highlighted the significance of *Humicola* species, particularly in the production of thermostable enzymes. Elkhateep and Daba (2022) explored the chemical and bioactive metabolites of *Humicola* and *Nigrospora*, emphasizing their potential applications. Similarly, Bosco et al. (2018) conducted characterization and screening of fungal strains for thermophilic enzymatic activity, supporting the findings that thermophilic fungi are valuable sources of industrially relevant enzymes.Ahirwar *et al*. (2017, Waill *et al.,* 2022) successfully isolated and screened thermophilic and thermotolerant fungi for hemicellulase production, demonstrating their adaptability to high-temperature environments.

Moreover, thermostable enzyme-producing bacteria have also been extensively studied. Semira *et al.* (2021) investigated thermostable amylase-producing Bacillus species, which exhibited efficient extracellular amylase synthesis. Similarly, Guta *et al.* (2024) characterized thermostable enzyme-producing bacteria from hot springs in Ethiopia, further validating the potential of thermophiles in industrial applications. Furthermore, Temporiti *et al.* (2022) highlighted the role of fungal enzymes in

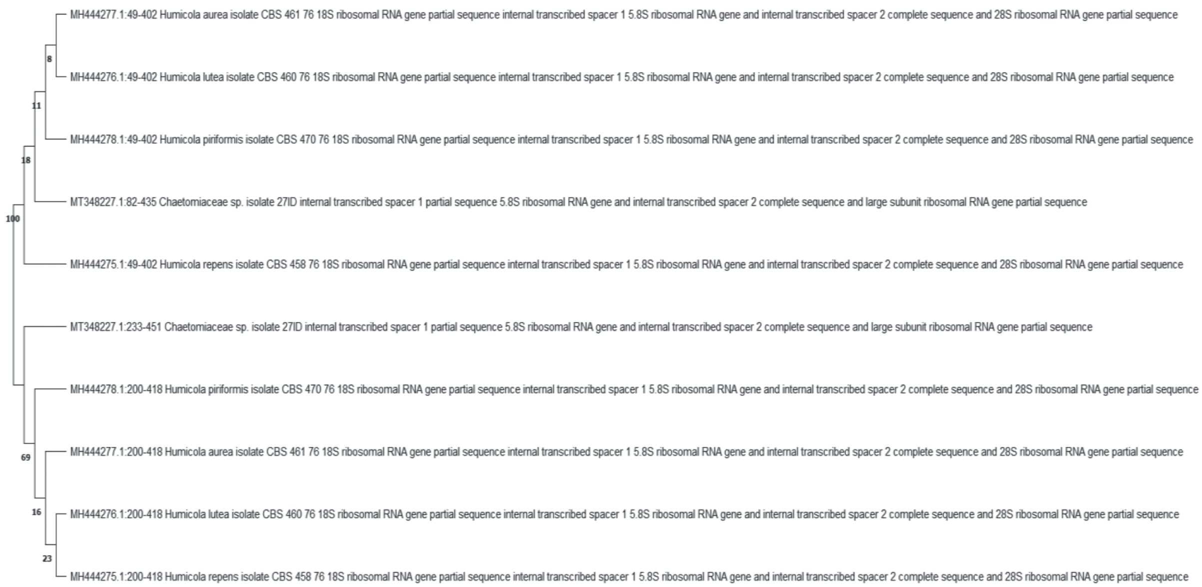

Fig. 7. Phylogenetic tree based on 18S rRNA sequence

plastic biodegradation, emphasizing the environmental significance of thermophilic fungi.

CONCLUSION

The findings of this study contribute to the growing body of research on thermophilic fungi and their potential for industrial enzyme production, biodegradation, and biotechnological applications. Further investigations will focus on optimizing enzyme production conditions and assessing the functional properties of the thermostable enzymes produced by *Humicola piriformis*.

Conflict of Interest - None

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