Zakirullah et al | Article 11833
J Pure Appl Microbiol. 2026. doi: 10.22207/JPAM.20.4.28
Received: 21 May 2026 | Accepted: 05 October 2026

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Isolation, Antibacterial Screening, and Molecular Characterization of Soil Fungi from Ecologically Diverse Habitats in Tirupattur District, Tamil Nadu, India

S. Zakirullah, M.A. Farook*, Abdul Jaffarali, D. Waheed and J. Rohini

Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi, Affiliated to Thiruvalluvar University, Serkadu, Vellore, Tamil Nadu, India.

Abstract

Soil environments harbor diverse fungi capable of producing biologically active secondary metabolites with antibacterial potential. In the present study, four soil samples were collected from agricultural, industrial, market, and hilly habitats, respectively, in Vaniyambadi, Tirupattur district, Tamil Nadu, India, and investigated for the isolation and screening of antibacterial-producing fungi. A total of 35 morphologically distinct fungal isolates were recovered and differentiated based on macroscopic characteristics, including colony colour, texture, growth pattern, pigmentation, and microscopic features such as hyphal and reproductive structures. The antibacterial activity of the isolates was evaluated against two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, and two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, selected as representative bacterial test organisms for preliminary screening. Among the 35 fungal isolates, four isolates, designated ICBT-FZAF1, ICBT-FZAF7, ICBT-FZHF14, and ICBT-FZIF28, exhibited antibacterial activity against one or more of the tested bacterial pathogens. Molecular identification based on ITS sequence analysis revealed that ICBT-FZAF1 and ICBT-FZAF7 were closely related to *Torulaspora globosa*, ICBT-FZHF14 was closely related to *Aspergillus candidus*, and ICBT-FZIF28 was closely related to *Aspergillus fumigatus.* Phylogenetic analysis further supported the taxonomic placement of these isolates, and the corresponding ITS sequences were deposited in the GenBank database. The study demonstrates that fungi recovered from diverse soil habitats, including *Aspergillus* species, exhibit antibacterial potential and warrant further investigation for the production and characterization of bioactive metabolites.

Keywords: Antimicrobial Resistance, Soil Microorganisms, Antibiotic Producing Fungi, *Torulaspora* and *Aspergillus* Species, 16S rRNA Gene Sequencing

***Correspondence:** farook.aim@gmail.com

Citation: Zakirullah S, Farook MA, Jaffarali A, Waheed D, Rohini J. Isolation, Antibacterial Screening, and Molecular Characterization of Soil Fungi from Ecologically Diverse Habitats in Tirupattur District, Tamil Nadu, India. *J Pure Appl Microbiol.* Published online 27 November 2026. doi: 10.22207/JPAM.20.4.28

© The Author(s) 2026. **Open Access**. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License which permits unrestricted use, sharing, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

INTRODUCTION

Antibiotics are naturally occurring bioactive compounds produced by microorganisms that inhibit the growth of or eliminate pathogenic microorganisms. They have played a fundamental role in modern medicine by reducing the morbidity and mortality associated with infectious diseases. A substantial proportion of clinically important antibiotics have been derived from secondary metabolites produced by soil-associated microorganisms, including actinomycetes, bacteria, and fungi. In their natural habitats, these metabolites contribute to microbial competition and ecological interactions, while their biological activities have provided important opportunities for pharmaceutical development. The discovery of penicillin from *Penicillium* spp. represented a major milestone in antimicrobial chemotherapy and established microorganisms as an important source of therapeutically valuable compounds. Subsequently, microorganisms belonging to genera such as *Streptomyces, Bacillus, Penicillium,* and *Aspergillus* have contributed to the discovery and production of numerous antimicrobial agents, including cephalosporins, erythromycin, tetracyclines, and vancomycin.[1-5]

The increasing prevalence of antimicrobial resistance (AMR), however, has considerably reduced the effectiveness of many conventional antimicrobial agents and has created an urgent need for the discovery of new antimicrobial compounds. The inappropriate and excessive use of antibiotics in human medicine, agriculture, and livestock production has contributed to the selection and dissemination of resistant microorganisms. Clinically important multidrug-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, and drug-resistant *Pseudomonas aeruginosa*, pose significant challenges to the treatment of bacterial infections.[6] Consequently, the exploration of microorganisms capable of producing antimicrobial metabolites with novel or complementary mechanisms of action has become an important area of pharmaceutical and microbiological research.

Soil represents one of the most diverse and complex microbial ecosystems and serves as an important reservoir of microorganisms with biotechnological and pharmaceutical potential. Soil physicochemical characteristics, including pH, temperature, moisture, nutrient availability, and organic matter content, influence the distribution and composition of microbial communities. The intense competition among microorganisms inhabiting soil environments can promote the production of secondary metabolites that provide ecological advantages to the producing organisms. These metabolites include antibiotics, extracellular enzymes, pigments, and other biologically active compounds likes hydrolytic enzymes, NagA, NagB, L-Asparaginase, L-Glutaminase, ChpC, ChpG, glutamine synthetase I (GSI), chitinolytic enzymes, etc., with potential applications in medicine, agriculture, and industry.[7-11] Therefore, the investigation of microorganisms from different soil habitats may provide opportunities for identifying strains with previously unexplored antimicrobial activities.

Although actinomycetes, particularly members of the genus *Streptomyces*, have traditionally received considerable attention as prolific antibiotic producers, fungi are also important sources of structurally diverse secondary metabolites. Genera such as *Aspergillus, Penicillium, Trichoderma,* and *Fusarium* have been reported to produce metabolites exhibiting antibacterial, antifungal, antiviral, anticancer, and other biological activities. The metabolic diversity of filamentous fungi and their ability to produce chemically diverse secondary metabolites make them valuable candidates for antimicrobial discovery and pharmaceutical research. Accordingly, the systematic exploration of soil-derived fungi may contribute to the identification of strains with promising antimicrobial properties.[12]

The isolation of antibiotic-producing fungi generally involves systematic soil sampling, serial dilution, cultivation on suitable fungal growth media, and purification of morphologically distinct colonies. The antimicrobial potential of isolated fungi can subsequently be evaluated using diffusion-based assays against selected bacterial pathogens. Fungal isolates exhibiting

antimicrobial activity can be further characterized using morphological and molecular approaches. Molecular identification based on internal transcribed spacer (ITS) region sequencing, combined with phylogenetic analysis, provides a reliable approach for species-level identification and supports the characterization of potentially valuable metabolite-producing fungal strains.[4] Such approaches facilitate the integration of microbial diversity studies with the screening of microorganisms for pharmaceutical and industrial applications.

Different ecological habitats can harbor distinct microbial communities because variations in environmental conditions influence microbial distribution and metabolic activity. Agricultural soils, hilly regions, market-associated environments, and industrially influenced soils may therefore contain fungal populations with different ecological and functional characteristics. Investigating such diverse habitats can increase the likelihood of recovering fungal isolates with distinct antimicrobial properties. In view of the continuing emergence of antimicrobial-resistant pathogens and the need for alternative sources of antimicrobial agents, systematic exploration of soil-derived fungi remains relevant to contemporary antimicrobial research.

Therefore, the present study investigated soil samples collected from agricultural, industrial, market, and hilly habitats of Vaniyambadi, Tirupattur district, Tamil Nadu, India, for the isolation and characterization of fungi exhibiting antibacterial activity. The isolated fungal strains were screened against selected Gram-positive and Gram-negative bacterial pathogens, and promising isolates were further characterized using morphological and molecular approaches. This investigation provides information on the fungal diversity associated with different soil habitats and identifies fungal isolates with potential antibacterial activity, thereby providing a basis for subsequent studies on the characterization of their bioactive metabolites.



Figure 1. Soil sampling locations in the Vaniyambadi region of Thirupathur district, Tamil Nadu, India, representing: (a) Agricultural, (b) Hill, (c) Market and (d) Industrial soil environments for isolating antimicrobial-producing fungi

Table 1. GPS Coordinates of Soil Sampling Sites in Vaniyambadi, Tamil Nadu, India

No.	Sample ID	Location Description	Geographic Coordinates	
			Latitude (°N)	Longitude (°E)
1	AF	Agriculture	12°40'28.948''N	78°38'58.017''E
2	HF	Hill	12°37'27.4''N	78°46'21.6''E
3	MF	Market	12°40'52''N	78°37'2''E
4	IF	Industrial	12°42'54.542''N	78°38'57.343''E

AF-Agriculture Fungi, HF-Hill Fungi, MF-Market Fungi and IF-Industrial Fungi

Table 2. Description of soil collected from diverse sites and isolates from Vaniyambadi region of Thirupathur district, Tamil Nadu, India

No.	Sample Sites	Specific Soil sample area	Soil depth (cm)	Numbers of isolates and their codes
1	Agriculture	Lala Eri	12	AF1, AF2, AF3, AF4, AF5, AF6, AF7, AF8, AF9, AF10, AF11, AF12 & AF13
2	Hill	Kambath Hill, Alangayam	12	HF1, HF2 & HF3
3	Market	Vaniyambadi	12	MF1, MF2, MF3, MF4, MF5, MF6, MF7, MF8 & MF9
4	Industry	Vadacheri	12	IF1, IF2, IF3, IF4, IF5, IF6, IF7, IF8 IF9 & IF10
Total				35

* AF-Agriculture Fungi, HF-Hill Fungi, MF-Market Fungi and IF-Industrial Fungi

MATERIALS AND METHODS

Sampling strategy

Soil samples were collected from agricultural, market, industrial, and hilly environments across the Vaniyambadi region of Tirupattur district, Tamil Nadu, India. Sampling locations and their geographical coordinates are presented in Table 1 and Figure 1. After removal of surface debris, soil was aseptically collected from approximately 12 cm depth using sterile spatulas, transferred into sterile labelled containers, and transported to the laboratory for microbiological analysis. Sampling and handling procedures were performed following established methods for soil microbial diversity studies.[13,14]

Preparation of soil sample

Collected soil samples were processed under strictly aseptic conditions for microbiological analysis. Approximately 1 g of each soil sample was accurately weighed using a sterile analytical balance and transferred into a sterile test tube containing 9 mL of sterile distilled water to obtain the primary dilution (10^{-1}). Prior to use, all dilution tubes and distilled water were sterilized by autoclaving at 121 °C for 15 min. The soil suspension was homogenized using a vortex mixer for 2-3 min to ensure uniform distribution of soil particles and microorganisms. Serial dilutions were subsequently prepared by transferring 1 mL from the previous dilution into tubes containing 9 mL of sterile diluent, resulting in dilution series up to 10^{-6}.[15,16]

Isolation of antibiotic producing fungi

Fungal isolates were obtained from soil samples using the serial dilution–spread plate technique on Potato Dextrose Agar (PDA) along with antibiotic Chloramphenicol (250 µg/100 ml of media). Appropriate soil dilutions were aseptically inoculated onto PDA plates and uniformly spread using a sterile spreader. The plates were incubated aerobically at 25 °C for 72

hrs. Following incubation, morphologically distinct and well-isolated fungal colonies were selected, purified, and maintained for further phenotypic characterization and antimicrobial screening. The isolation procedure was performed following standard microbiological methods.[17]

Subculturing of fungal isolates

Distinct fungal colonies were selected based on their morphological characteristics and aseptically subcultured onto fresh Potato Dextrose Agar (PDA) plates. The cultures were incubated at 25 °C for 72 hrs, and successive subculturing was performed until pure, contamination-free cultures were obtained. The purified isolates were maintained on PDA slants under suitable storage conditions for subsequent morphological characterization and antimicrobial screening. Standard microbiological procedures were followed for fungal purification and maintenance.

Extraction of secondary metabolites

Pure fungal isolates were cultured in Potato Dextrose Broth under suitable conditions to facilitate growth and secondary metabolite production. Following incubation, the cultures were separated from the fungal biomass by filtration and centrifugation. The resulting culture filtrates were subjected to ethyl acetate extraction to recover extracellular secondary metabolites. The organic phase was collected and concentrated by solvent evaporation, and the resulting crude extracts were preserved for subsequent antimicrobial activity assays. The extraction procedure was performed following previously described methods.[18]

Initial screening of isolates for antibiotic production

The fungal isolates were screened for antimicrobial activity by evaluating their inhibitory effects against selected bacterial pathogens. The test organisms included two Gram-positive bacteria, *S. aureus* and *B. subtilis*, and two Gram-negative bacteria, *E. coli* and *P. aeruginosa*, to determine the broad-spectrum antibacterial potential of the isolates. Fresh overnight cultures of the test organisms were prepared in nutrient broth and uniformly spread onto sterile nutrient agar plates to obtain confluent bacterial growth. Antibacterial activity was assessed using the agar well diffusion method. Approximately 50 µL of crude fungal extract was aseptically introduced into wells prepared in the agar medium. The plates were then incubated at 37 °C for 24 hrs to allow diffusion of the metabolites and interaction with the test organisms. Following incubation, the diameters of the clear inhibitory zones surrounding the wells were quantitatively measured to assess antimicrobial efficacy. Isolates exhibiting prominent zones of inhibition were identified as prospective antibiotic-producing fungal strains and were subsequently selected for detailed investigation and further bioactivity analysis.[19,20]

Identification of most potential isolate
Genomic DNA extraction

Genomic DNA was isolated from the selected bioactive fungal strain employing the BioBee Spin EXpure Microbial DNA Isolation Kit (BioBee Stores Pvt. Ltd.) following the optimized procedures recommended by the manufacturer. Freshly grown microbial cells were harvested and subjected to cell lysis using the extraction buffer provided in the kit to facilitate disruption of the cell wall and membrane, thereby releasing intracellular genomic DNA. Following lysis, the suspension was processed through a series of purification steps to remove proteins, cellular debris, and other contaminants. Under optimized buffer conditions, the released DNA selectively bound to the silica membrane of the spin column, while residual impurities were eliminated through repeated washing steps. The purified genomic DNA was subsequently eluted using the elution buffer supplied with the kit. The quality and purity of the extracted DNA were evaluated prior to downstream molecular applications. The isolated DNA showed satisfactory integrity and was therefore used for polymerase chain reaction (PCR), sequencing analysis, and other molecular characterization studies.[21]

PCR protocol

PCR amplification of the extracted genomic DNA was performed using universal fungal ITS primers, namely ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), to facilitate molecular identification of the isolate. These

primers specifically target the internal transcribed spacer (ITS) region located between conserved ribosomal RNA genes and are commonly used for fungal identification and phylogenetic analysis. PCR amplification was carried out in a thermal cycler using a reaction mixture containing template DNA, primers, nucleotides, reaction buffer, magnesium ions, and thermostable DNA polymerase. The amplification process consisted of an initial denaturation step followed by repeated cycles of denaturation, primer annealing, and extension under optimized conditions. The amplified PCR products were analyzed and subsequently purified to remove residual reaction components before sequencing. The purified amplicons were subjected to sequencing analysis for molecular characterization, phylogenetic assessment, and species-level identification of the selected fungal isolates.[22,23]

PCR product purification

Prior to nucleotide sequencing, the PCR-amplified products were purified to remove residual primers, excess dNTPs, buffer components, enzymes, and other impurities that may compromise sequencing fidelity. Purification of the amplified fragments was achieved using the Montage PCR Clean-Up Kit (Millipore) following the protocol prescribed by the manufacturer. The purification step ensured enhanced template quality and improved the accuracy of downstream sequence analysis. The purified amplicons were subsequently processed for cycle sequencing using the corresponding ITS primer pairs employed during PCR amplification. Sequencing reactions were conducted with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit coupled with AmpliTaq® DNA polymerase (FS enzyme) from Applied Biosystems to obtain high-quality nucleotide sequence data. The sequencing chemistry is based on the incorporation of fluorescently labeled dideoxynucleotide terminators during DNA strand elongation, enabling automated detection of nucleotide incorporation events. Following completion of the sequencing reactions, the extension products were analyzed using an automated capillary DNA sequencer to obtain high-quality nucleotide sequence data for molecular identification and phylogenetic analysis.

Sequencing protocol

Purified PCR amplicons were subjected to single-direction sequencing using universal primers targeting the fungal ITS region to obtain nucleotide sequence information for molecular identification of the isolates. Sequencing reactions were performed in a unidirectional manner, providing reliable sequence coverage and acceptable read quality for taxonomic characterization. The sequencing chemistry involved the incorporation of fluorescent dye-labeled dideoxynucleotide triphosphates (ddNTPs) during DNA strand synthesis, generating fragments of varying lengths terminated at specific nucleotides. Upon completion of the sequencing reaction, Following cycle sequencing, the extension products were purified using a standard ethanol precipitation procedure to eliminate unincorporated dye terminators, residual salts, and other reaction-associated impurities that could interfere with sequence detection and analysis. This purification process minimized background interference and improved sequence clarity. The purified sequencing products were air-dried, resuspended in nuclease-free distilled water, and denatured prior to electrophoretic separation. The purified sequencing samples were subsequently analyzed using an ABI 3730xl Genetic Analyzer (Applied Biosystems), employing high-resolution capillary electrophoresis technology for accurate nucleotide sequence determination. During electrophoresis, DNA fragments were separated according to fragment size, and fluorescent signals emitted from the labeled terminators were automatically detected for accurate base calling. The generated chromatograms provided high-quality ITS sequence data suitable for fungal identification and phylogenetic analysis.

Bioinformatics analysis procedure

The acquired ITS nucleotide sequences were analyzed through the National Center for Biotechnology Information (NCBI) BLAST algorithm to identify closely related fungal species by assessing sequence similarity. Homologous nucleotide

sequences exhibiting high sequence identity and significant alignment scores were retrieved from the database for comprehensive phylogenetic assessment. Multiple sequence alignment of the selected datasets was performed using MUSCLE version 3.7,[24] a robust algorithm recognized for its high alignment accuracy and computational efficiency in comparative genomic analyses. To enhance alignment reliability, poorly aligned regions, highly divergent segments, and ambiguous nucleotide positions were systematically excluded using Gblocks version 0.91b, thereby reducing analytical noise and improving the precision of downstream evolutionary interpretations. Phylogenetic relationships were subsequently inferred employing the maximum-likelihood (ML) method implemented in PhyML version 3.0. The HKY85 nucleotide substitution model was selected to accommodate unequal base frequencies and differential rates of transition and transversion substitutions. The statistical robustness of the inferred phylogenetic topology was evaluated using the approximate likelihood ratio test (aLRT), which provides rapid and reliable branch support estimation.[25] The resulting phylogenetic tree was finally visualized, edited, and annotated

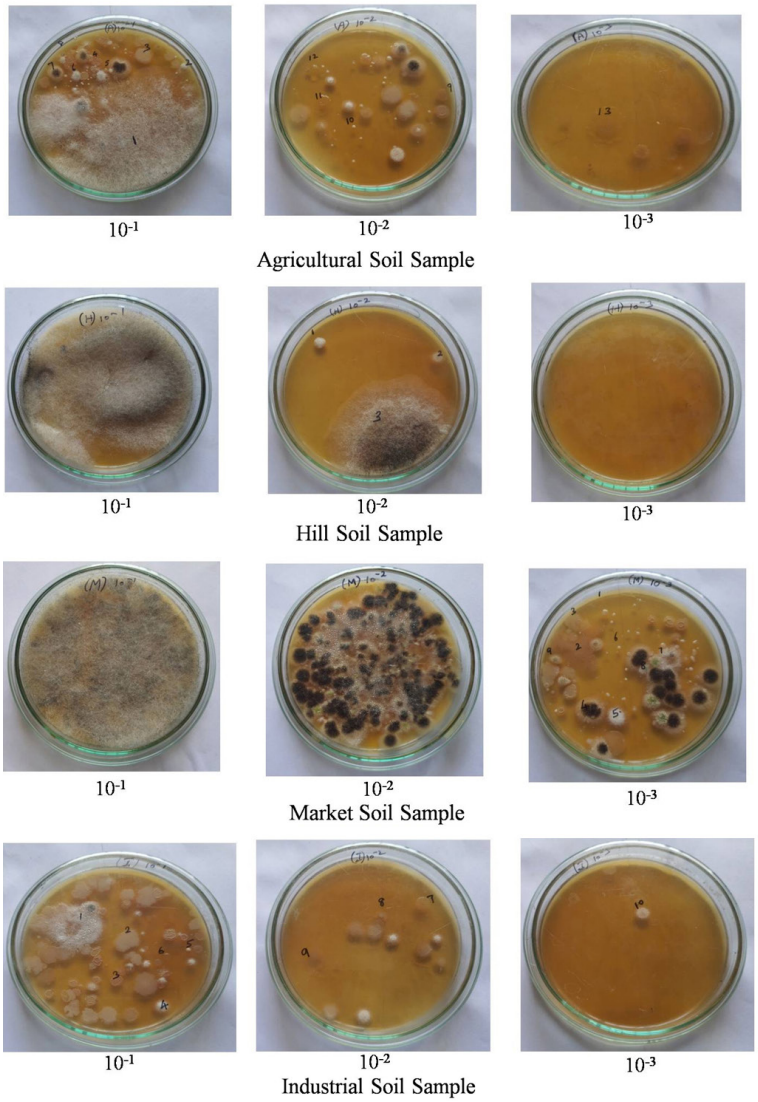

Figure 2. Isolation of morphologically distinct fungal colonies from four different soil samples using the serial dilution technique

using TreeDyn version 198.3 to facilitate accurate interpretation of the evolutionary affiliations and taxonomic positioning of the fungal isolates.[26]

RESULTS AND DISCUSSION

Soil sampling

Soil samples were collected from multiple ecologically diverse locations in and around the Vaniyambadi region of Tirupattur district, Tamil Nadu, India. The selected sampling sites represented different environmental habitats, including agricultural fields, marketplace regions, industrial areas, and hilly terrains. Inclusion of these varied ecological niches was intended to increase the probability of recovering diverse fungal populations with potential antimicrobial properties. Soil ecosystems are recognized as

Table 3. Assessment of the antimicrobial efficacy of fungal isolates against a panel of selected pathogenic bacterial strains

No.	Isolates	Inhibition zones (mm)			
		Gram-positive		Gram-negative	
		S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*
1	PC	15.6 ± 0.3	12.6 ± 0.3	10 ± 0	16 ± 0
2	AF1	11.3 ± 0.3	10.3 ± 0.3	9.6 ± 0.3	28 ± 0.5
3	AF2	0 ± 0	0 ± 0	0 ± 0	14 ± 0.5
4	AF3	0 ± 0	0 ± 0	0 ± 0	14 ± 0.5
5	AF4	0 ± 0	0 ± 0	0 ± 0	14 ± 0
6	AF5	0 ± 0	0 ± 0	0 ± 0	20 ± 0.5
7	AF6	0 ± 0	0 ± 0	0 ± 0	22 ± 0
8	AF7	10 ± 0	19.6 ± 0.6	21.3 ± 0.3	35 ± 0.5
9	AF8	0 ± 0	0 ± 0	0 ± 0	14 ± 0.5
10	AF9	0 ± 0	0 ± 0	0 ± 0	18 ± 0.5
11	AF10	0 ± 0	0 ± 0	0 ± 0	19.3 ± 0.3
12	AF11	0 ± 0	0 ± 0	0 ± 0	0 ± 0
13	AF12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
14	AF13	0 ± 0	0 ± 0	0 ± 0	18 ± 0.5
15	HF1	9.6 ± 0.3	12 ± 0	13.3 ± 0.3	31 ± 0.5
16	HF2	0 ± 0	0 ± 0	5.6 ± 0.3	25 ± 0.5
17	HF3	0 ± 0	0 ± 0	0 ± 0	10 ± 0.5
18	MF1	0 ± 0	0 ± 0	3 ± 0	12.6 ± 0.3
19	MF2	0 ± 0	0 ± 0	0 ± 0	19 ± 0
20	MF3	0 ± 0	12.3 ± 0.3	13 ± 0	25.6 ± 0.3
21	MF4	0 ± 0	9.6 ± 0.3	11 ± 0	25 ± 0.5
22	MF5	0 ± 0	0 ± 0	0 ± 0	23 ± 0.5
23	MF6	0 ± 0	0 ± 0	0 ± 0	23.3 ± 0.3
24	MF7	0 ± 0	0 ± 0	12.3 ± 0.3	23 ± 0.5
25	MF8	0 ± 0	11 ± 0	12.3 ± 0.3	22 ± 0.5
26	MF9	0 ± 0	0 ± 0	0 ± 0	0 ± 0
27	IF1	0 ± 0	10 ± 0	4 ± 0	31 ± 0
28	IF2	0 ± 0	0 ± 0	0 ± 0	10 ± 0.5
29	IF3	9.6 ± 0.3	11 ± 0	4 ± 0	22 ± 0.5
30	IF4	0 ± 0	0 ± 0	0 ± 0	23 ± 0.5
31	IF5	10 ± 0	0 ± 0	0 ± 0	24.6 ± 0.3
33	IF6	0 ± 0	0 ± 0	0 ± 0	0 ± 0
33	IF7	0 ± 0	0 ± 0	0 ± 0	13 ± 0.5
34	IF8	3 ± 0	0 ± 0	0 ± 0	24 ± 0.5
35	IF9	0 ± 0	0 ± 0	0 ± 0	18.3 ± 0.3
36	IF10	0 ± 0	0 ± 0	0 ± 0	29 ± 0.5

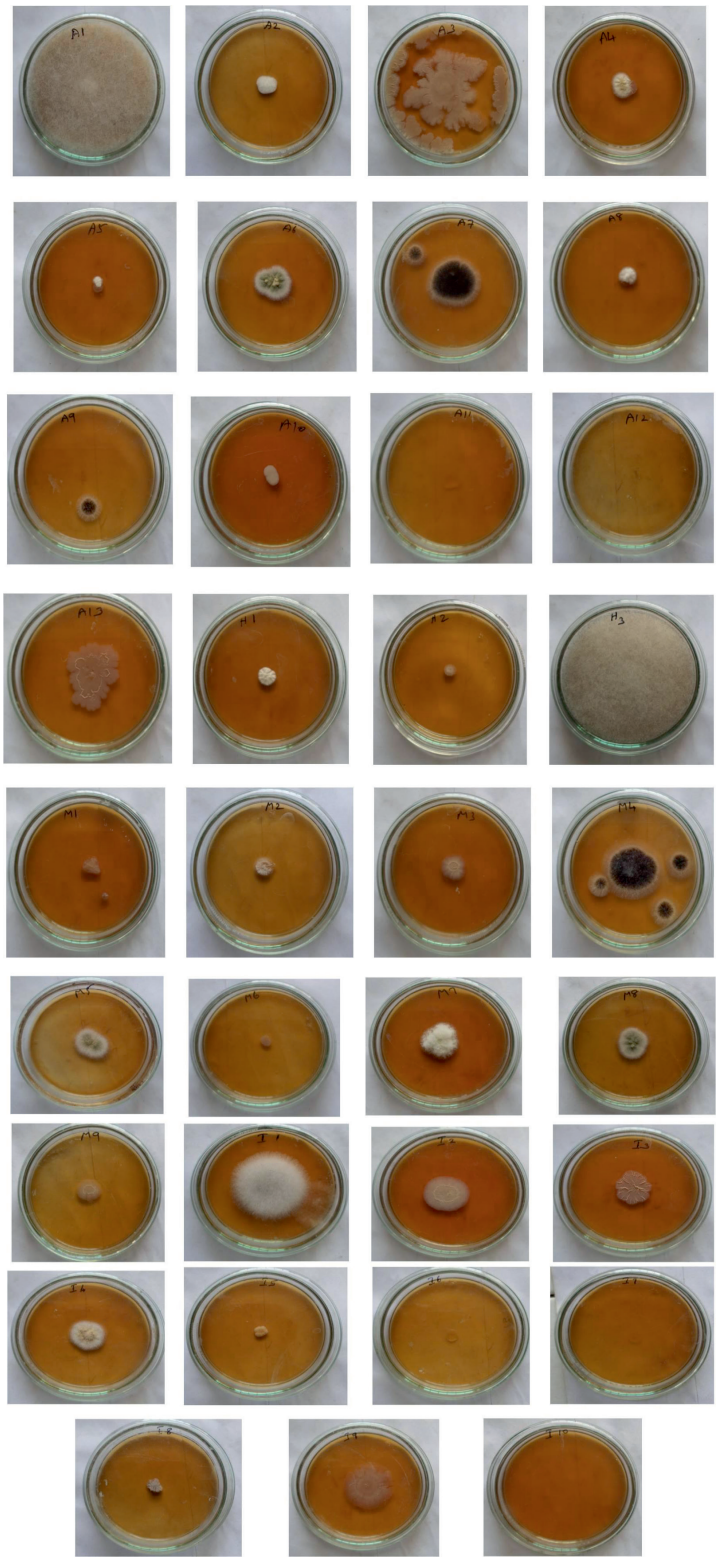

Figure 3. Purification of 35 morphologically distinct fungal isolates from four soil samples by repeated sub culturing

important reservoirs of metabolically active fungi, particularly filamentous genera such as *Aspergillus*, *Penicillium*, *Trichoderma*, and related taxa known for producing biologically significant secondary metabolites. Earlier studies have reported that soil-derived fungi possess considerable antibacterial, antifungal, and other pharmacologically important activities, emphasizing their relevance in the discovery of novel bioactive compounds.[27-29] Therefore, soils from environmentally distinct regions were incorporated into the present investigation to maximize fungal diversity and improve the likelihood of isolating antagonistic fungal strains with promising antimicrobial

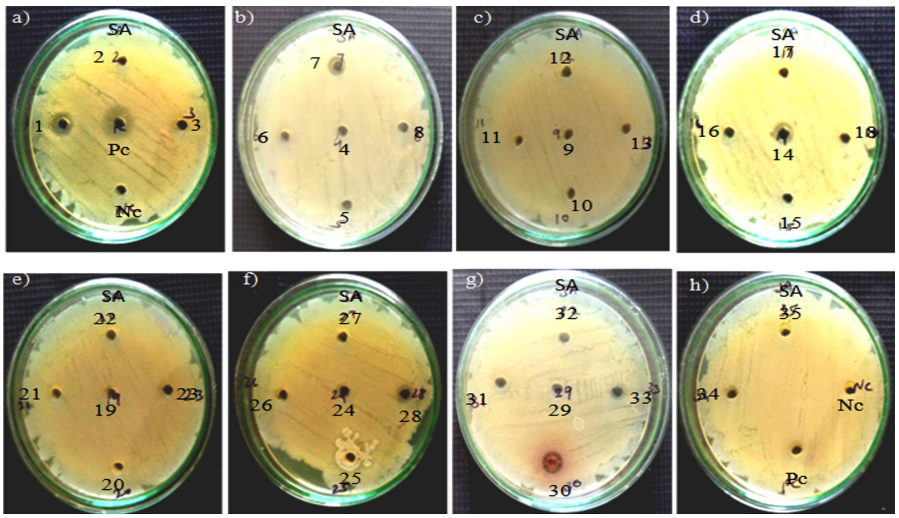

Figure 4. Zones of inhibition produced by fungal isolates against *S. aureus*. (a) Pc - Positive Control, Nc – Negative Control,1- AF1,2- AF2,3- AF3; (b) 4- AF4,5- AF5,6- AF6, 7- AF7,8- AF8; (c) 9- AF9,10- AF10,11- AF11, 12- AF12,13- AF13; (d) 14- HF1,15- HF2,16- HF3, 17- MF1,18- MF2; (e) 19- MF3,20- MF4,21- MF5, 22- MF6,23- MF7; (f) 24- MF8,25- MF9,26- IF1, 27- IF2, 28- IF3; (g) 29- IF4,30- IF5,31- IF6, 32- IF7,33- IF8; (h) 34- IF9 & 35- IF10

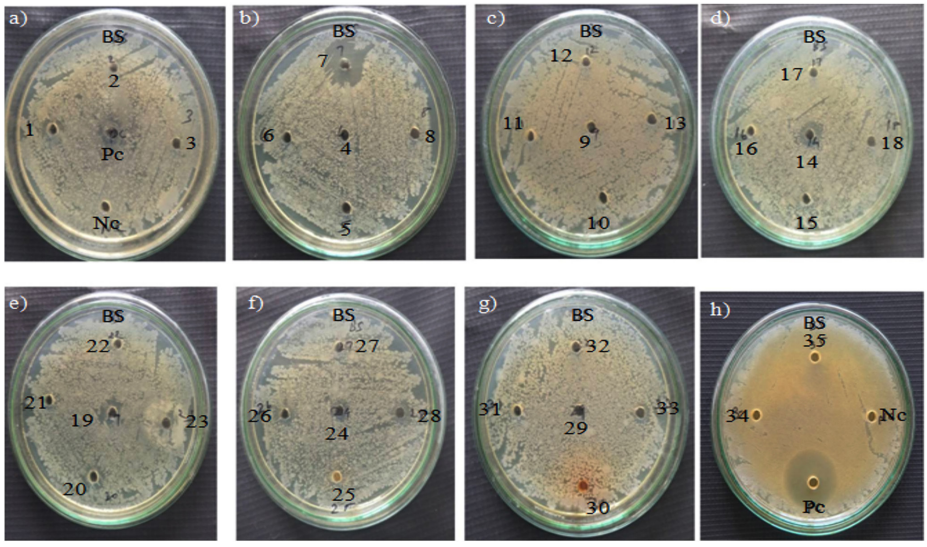

Figure 5. Zones of inhibition produced by fungal isolates against *B. subtilis*. (a) Pc - Positive Control, Nc – Negative Control,1- AF1,2- AF2,3- AF3; (b) 4- AF4,5- AF5,6- AF6, 7- AF7,8- AF8; (c) 9- AF9,10- AF10,11- AF11, 12- AF12,13- AF13; (d) 14- HF1,15- HF2,16- HF3, 17- MF1,18- MF2; (e) 19- MF3,20- MF4,21- MF5, 22- MF6,23- MF7; (f) 24- MF8,25- MF9,26- IF1, 27- IF2,28- IF3; (g) 29- IF4,30- IF5,31- IF6, 32- IF7,33- IF8; (h) 34- IF9 & 35- IF10

activity. Detailed information regarding sampling locations, including geographical coordinates and environmental characteristics, is presented in Table 1 and Figure 1 to ensure reproducibility of the study and to establish the spatial framework of the sampling strategy. In addition, Table 2

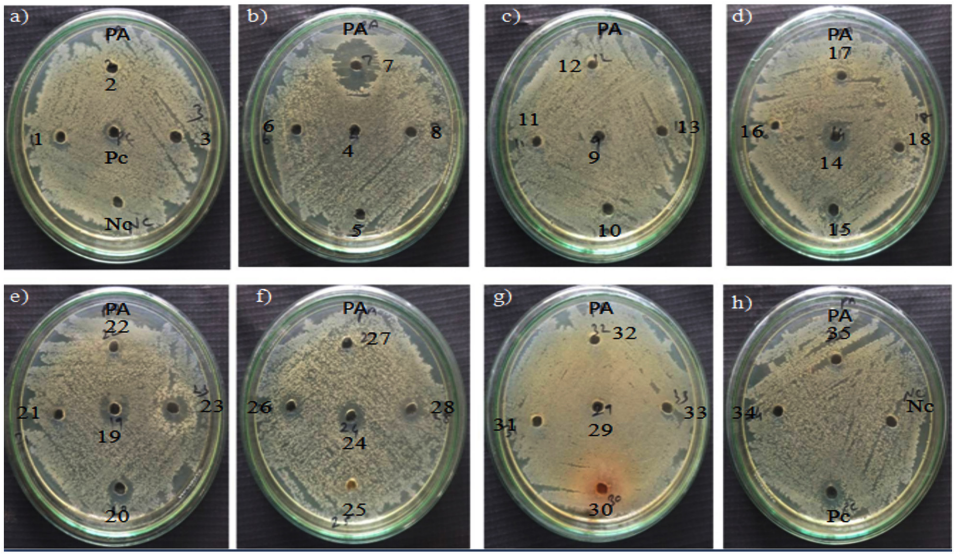

Figure 6. Zones of inhibition produced by fungal isolates against *P. aeruginosa*. (a) Pc - Positive Control, Nc – Negative Control,1- AF1,2- AF2,3- AF3; (b) 4- AF4,5- AF5,6- AF6, 7- AF7,8- AF8; (c) 9- AF9,10- AF10,11- AF11, 12- AF12,13- AF13; (d) 14- HF1,15- HF2,16- HF3, 17- MF1,18- MF2; (e) 19- MF3,20- MF4,21- MF5, 22- MF6,23- MF7; (f) 24- MF8,25- MF9,26- IF1, 27- IF2,28- IF3; (g) 29- IF4,30- IF5,31- IF6, 32- IF7,33- IF8; (h) 34- IF9 & 35- IF10

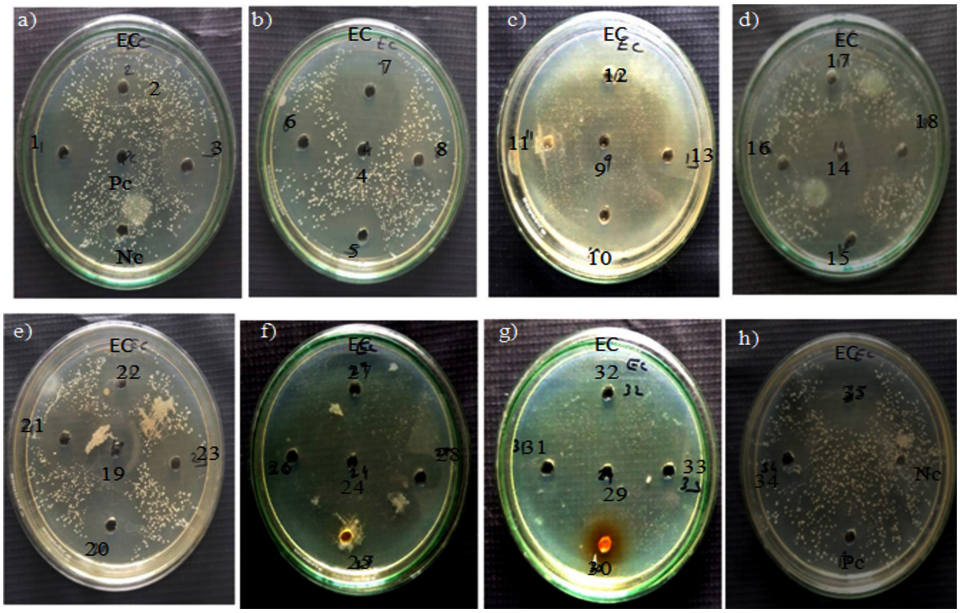

Figure 7. Zones of inhibition produced by fungal isolates against *E. coli*. (a) Pc - Positive Control, Nc – Negative Control, 1- AF1,2- AF2,3- AF3; (b) 4- AF4,5- AF5,6- AF6, 7- AF7,8- AF8; (c) 9- AF9,10- AF10,11- AF11, 12- AF12,13- AF13; (d) 14- HF1,15- HF2,16- HF3, 17- MF1,18- MF2; (e) 19- MF3,20- MF4,21- MF5, 22- MF6,23- MF7; (f) 24- MF8,25- MF9,26- IF1, 27- IF2,28- IF3; (g) 29- IF4,30- IF5,31- IF6, 32- IF7,33- IF8; (h) 34- IF & 35- IF10

summarizes the characteristics of the collected soil samples, including sampling category, geographical coordinates, and notable environmental features associated with each location.

Isolation of Fungi and its antagonistic activity

A total of 35 morphologically diverse fungal isolates were successfully recovered from four soil samples obtained from ecologically heterogeneous environments. Preliminary phenotypic characterization of the isolates was performed based on macroscopic colony morphology, including parameters such as colony diameter, pigmentation, surface texture, margin configuration, elevation profile, and overall growth characteristics (Figure 2). Morphologically distinct colonies were subsequently subjected to repeated subculturing on Potato Dextrose Agar (PDA) to establish axenic and stable fungal cultures for downstream experimental investigations (Figure 3).

The antibacterial activity of the purified fungal isolates was subsequently assessed against selected pathogenic bacteria, including the Gram-positive species *S. aureus* and *B. subtilis*, as well as the Gram-negative species *E. coli* and *P. aeruginosa*. Antimicrobial efficacy was evaluated using the agar well diffusion assay in accordance with established standard methodologies.[30] The antagonistic potential of each fungal isolate was quantitatively determined by measuring the diameter of the clear inhibition zones surrounding wells containing the respective fungal extracts.

Antimicrobial activity was expressed as mean zone of inhibition (mm) ± standard deviation. Chloramphenicol (10 µg/50 µL) was used as the positive control throughout the experiment. Among the thirty-five fungal isolates examined, most did not exhibit measurable inhibitory activity against the tested bacterial pathogens and produced inhibition zones of 0 ± 0 mm. Detectable antagonistic activity was observed only in selected isolates, and the degree of inhibition varied depending on the bacterial test organism among the fungal isolates evaluated (Table 3 and Figures 4-7).

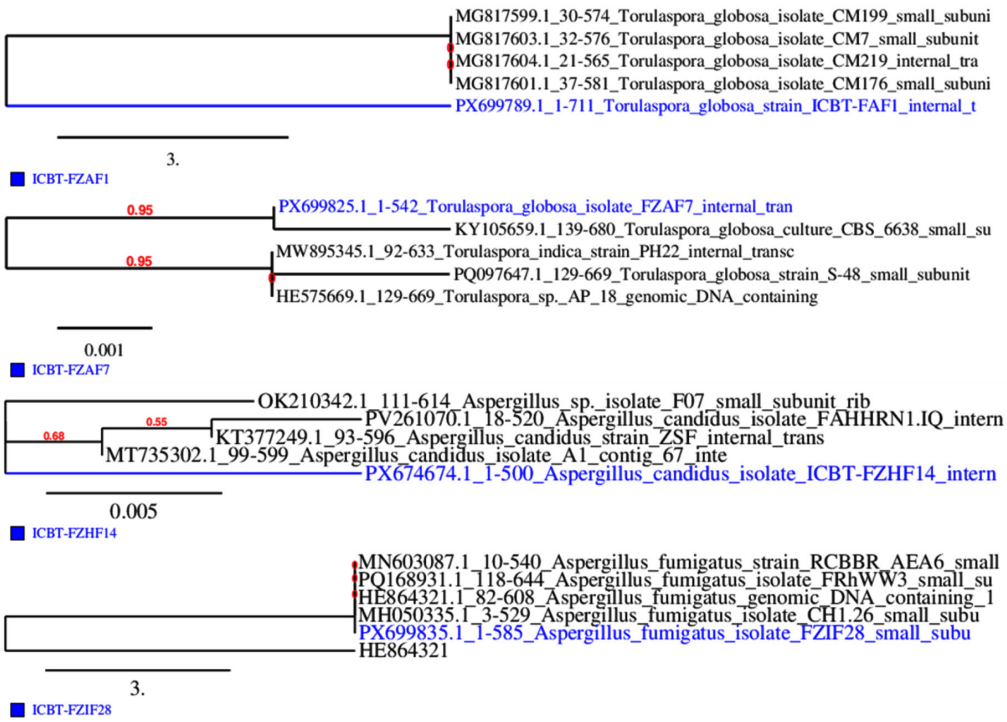

Figure 8. Phylogenetic tree of isolates based on 16S rRNA gene sequences, aligned using MUSCLE, Gblocks, PhyML, and TreeDyn showing their evolutionary relationships with reference strains

Overall, nearly 40.7% of the fungal isolates evaluated exhibited detectable antibacterial activity under the experimental conditions applied. The inhibitory responses varied among the tested organisms, indicating that the antagonistic activity of the isolates was selective and organism-dependent. These observations suggest that only certain soil-derived fungal isolates possess significant antibacterial potential against specific pathogenic bacteria. The observations documented in the present study are consistent with earlier reports demonstrating that soil-derived fungal isolates possess significant antagonistic activity against clinically relevant bacterial pathogens, particularly *S. aureus* and other related microorganisms.[31,32] Collectively, the results highlight the ecological importance and pharmaceutical relevance of soil fungi as promising reservoirs of pharmacologically important bioactive secondary metabolites with significant potential for antimicrobial applications (Table 3).

Genomic DNA isolation from fungal isolates

High-quality genomic DNA was successfully extracted from four antagonistic fungal isolates designated as ICBT-FZAF1, ICBT-FZAF7, ICBT-FZHF14, and ICBT-FZIF28 using a standardized extraction protocol optimized for fungal molecular studies. The procedure yielded purified and intact genomic DNA from all selected isolates, which was suitable for subsequent molecular characterization. The extraction process involved effective disruption of fungal cell walls and cellular membranes to release intracellular nucleic acids, followed by centrifugation to remove cellular debris and insoluble components from the lysate. The released DNA was subsequently precipitated, recovered as a pellet, and purified to eliminate proteins, polysaccharides, and other contaminating biomolecules. The extracted genomic DNA exhibited satisfactory integrity and purity, indicating its suitability for downstream molecular applications. The purified genomic DNA samples were subsequently utilized as template material for polymerase chain reaction (PCR) amplification targeting the fungal ribosomal RNA (rRNA) gene region, a highly conserved molecular marker extensively employed for taxonomic characterization and phylogenetic inference. Successful amplification of the target region enabled reliable molecular characterization and evolutionary assessment of the antagonistic fungal isolates.

Molecular identification of fungal isolates by rRNA gene amplification and sequencing

Molecular characterization based on ribosomal RNA (rRNA) gene sequencing is widely recognized as a robust and highly reliable strategy for fungal identification, offering superior taxonomic resolution compared with conventional morphological and biochemical approaches.[22] In the present investigation, sequence-based analysis demonstrated that isolates ICBT-FZAF1 and ICBT-FZAF7 shared high phylogenetic affinity with *Torulaspora globosa*, whereas isolate ICBT-FZHF14 exhibited significant nucleotide homology with *Aspergillus candidus*. Likewise, isolate ICBT-FZIF28 showed close sequence similarity to *Aspergillus fumigatus*. Species-level identification of the fungal isolates was further validated through BLAST analysis against reference nucleotide sequences available in the NCBI GenBank database. The partial ITS rRNA gene sequences generated in this study were subsequently deposited in GenBank and assigned the accession numbers PX699789 (ICBT-FZAF1), PX699825 (ICBT-FZAF7), PX674674 (ICBT-FZHF14), and PX699835 (ICBT-FZIF28). To elucidate the evolutionary relationships among the obtained isolates and closely related reference taxa, comprehensive bioinformatics analyses were performed. Multiple sequence alignment was conducted using MUSCLE, followed by the elimination of ambiguously aligned and highly variable regions through Gblocks refinement. Phylogenetic reconstruction was subsequently achieved using the maximum-likelihood algorithm implemented in PhyML, and the resulting phylogenetic tree was visualized and annotated using TreeDyn (Figure 8). The inferred phylogenetic topology strongly supported the evolutionary relatedness of the isolates with established reference species. The outcomes of the present study corroborate earlier investigations reporting that *Aspergillus* species represent dominant fungal taxa in soil ecosystems and constitute prolific producers of structurally diverse bioactive secondary metabolites.[33-35] Notably, *A. fumigatus* and *A. candidus* have previously been recognized

for their ability to synthesize antimicrobial compounds, extracellular enzymes, and other metabolites of considerable pharmaceutical importance.[36] Moreover, species belonging to the genus *Aspergillus* are well documented for producing a broad spectrum of antibacterial and antifungal metabolites, which may account for the pronounced antagonistic activity observed among the isolates examined in the current investigation.[37]

Fungi represent an important source of antibacterial secondary metabolites, and recent studies have demonstrated considerable antibacterial activity among fungal isolates and their extracts against both Gram-positive and Gram-negative bacteria.[38,39] Further demonstrated that fungal extracts could inhibit *P. aeruginosa* and *E. coli*, highlighting their potential against clinically relevant Gram-negative pathogens. Recent investigations have also combined phenotypic characterization with molecular approaches to accurately identify bioactive fungal isolates.[40,41] These findings support the present strategy of isolating fungi from environmental samples, screening them for antagonistic activity, and subsequently identifying promising isolates using ribosomal DNA/ITS sequencing.[40,41]

CONCLUSION

The present study demonstrates that soil ecosystems represent an important and sustainable source of fungi capable of producing biologically active antimicrobial metabolites. Comprehensive investigation of soil samples obtained from ecologically heterogeneous sites across the Vaniyambadi region facilitated the recovery of diverse fungal populations with potential biological significance resulted in the isolation of several fungal strains, among which a notable proportion exhibited antibacterial activity against selected pathogenic microorganisms. Antimicrobial screening identified four potent fungal isolates that showed inhibitory effects against both Gram-positive bacteria, including *S. aureus* and *B. subtilis*, and Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. Molecular characterization based on ITS rRNA gene sequencing revealed that the active isolates were closely related to *Torulaspora globosa*, *Aspergillus candidus*, and *Aspergillus fumigatus*. Phylogenetic analysis further confirmed their taxonomic identity and evolutionary relationship with previously reported bioactive fungal species. The observed antagonistic activity suggests that these fungal isolates may possess the ability to synthesize secondary metabolites with potential pharmaceutical significance. In view of the increasing global prevalence of antimicrobial resistance, the outcomes of the present study underscore the scientific importance of exploring diverse soil ecosystems as promising reservoirs for the isolation and identification of novel antimicrobial-producing fungal strains. The results also provide a foundation for future investigations involving purification, structural characterization, and pharmacological evaluation of fungal bioactive compounds for potential therapeutic and biotechnological applications.

ACKNOWLEDGMENTS

The authors sincerely acknowledge the Management and the Principal of Islamiah College (Autonomous), Vaniyambadi, for their continuous encouragement, institutional support, and provision of laboratory facilities necessary for the successful completion of this research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Berdy J. Bioactive Microbial Metabolites: A Personal View. *J Antibiot*. 2005;58(1):1-26. doi: 10.1038/ja.2005.1
2. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev*. 2003;67(4):491-502. doi: 10.1128/MMBR.67.4.491-502.2003
3. Aly AH, Debbab A, Proksch P. Fungal endophytes: unique plant inhabitants with great promise. *Appl Microbiol Biotechnol.* 2011;90(6):1829-1845. doi: 10.1007/s00253-011-3270-y
4. Nicoletti R, Fiorentino A. Plant bioactive metabolites and drugs produced by endophytic fungi of Spermatophyta. *Agriculture*. 2015;5(4):918-970. doi: 10.3390/agriculture5040918
5. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Diversity.* 2010;41(1):1-16. doi: 10.1007/s13225-010-0034-4
6. Ventola CL. The Antibiotic Resistance Crisis Part 1: Causes and Threats. *PT.* 2015;40(4):277-283.
7. Barka EA, Vatsa P, Sanchez L, et al. Taxonomy, Physiology, and Natural Products of Actinobacteria. *Microbiol Mol Biol Rev*. 2016;80(1):1-43. doi: 10.1128/MMBR.00019-15
8. Swiatek MA, Tenconi E, Rigali S, van Wezel GP. Functional analysis of the *N*-acetylglucosamine metabolic genes of *Streptomyces coelicolor* and role in control of development and antibiotic production. *J Bacteriol.* 2022;194(5):1136-1144. doi: 10.1128/JB.06370-11
9. Dreier J, Meletzus D, Eichenlaub R. Characterization of the plasmid encoded virulence region pat-1 of phytopathogenic *Clavibacter michiganensis* subsp. michiganensis. *Mol Plant Microbe Interact*. 1997;10(2):195-206. doi: 10.1094/MPMI.1997.10.2.195
10. DeJong PJ. L-Asparaginase Production by *Streptomyces griseus. Applied Microbiology*. 1972;23(6):1163-1164. doi: 10.1128/am.23.6.1163-1164.1972
11. Balagurunatha R, Radhakrishnon M, Somasumdarom ST. L-glutaminase Producing Actinomycetes from Marine Sediments –Selective Isolation, Semi Quantitative Assay and Characterization of Potential Strain. *Aust J Basic Appl Sci.* 2010;4(5):698-705.
12. Aderem A. Systems Biology: Its practice and Challenges. *Cell*. 2005;121(4):511-513. doi: 10.1016/j.cell.2005.04.020
13. Keller NP. Fungal secondary metabolism: regulation, function and drug discovery. *Nat Rev Microbiol*. 2019;17(3):167-180. doi: 10.1038/s41579-018-0121-1
14. Fierer N, Schimel JP, Holden PA. Variations in microbial community composition through two soil depth profiles. *Soil Biol Biochem*. 2003;35(1):167-176. doi: 10.1016/S0038-0717(02)00251-1
15. Wollum AG II. Cultural methods for soil microorganisms. In: Page AL, ed. *Methods of Soil Analysis: Part 2—Chemical and Microbiological Properties*. 2nd ed. Agronomy Monographs. Vol 9.2.2. American Society of Agronomy; 1983. doi: 10.2134/agronmonogr9.2.2ed.c37
16. Brown AE. *Benson's Microbiological Applications: Laboratory Manual in General Microbiology*. 9th ed. McGraw-Hill Higher Education; 2004
17. Waksman SA. A method for counting the number of fungi in the soil. *J Bacteriol*. 1992;7(3):339–341. doi: 10.1128/JB.7.3.339-341.1922
18. Gill H, Sykes EME, Kumar A, Sorensen JL. Isolation of Bioactive Metabolites from Soil Derived Fungus-*Aspergillus fumigatus*. *Microorganisms*. 2023;11(3):590-601. doi: 10.3390/microorganisms11030590
19. Panigrahi S, Rath CC. In vitro Characterization of Antimicrobial Activity of an Endophytic Bacterium *Enterobacter cloacae* (MG001451) Isolated from *Ocimum sanctum*. *S Afr J Bot*. 2021;143:90-96. doi: 10.1016/j.sajb.2021.07.044
20. Al-Rubyee SS, Al-Barhawi NI. Antibacterial effect of Bacillus subtilis extract on the growth of pathogenic bacteria and analyzed by GC-MS. *Journal of Education and Science.* 2022;31(1):111–122. doi: 10.33899/edusj.2022.132296.1203
21. Rani PEH, Jayaprakash J, Milton DJ. Molecular Profiling of Isolated Bioluminescence Strain and its Optimization Studies. *J Chem Health Risks.* 2024;14(2):3302-3307.
22. White TJ, Bruns T, Lee S, Taylor J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, Academic Press. 1990:315-322. doi: 10.1016/B978-0-12-372180-8.50042-1
23. Schoch CL, Seifert KA, Huhndorf S, et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci.* 2012;109(16):6241-6246. doi: 10.1073/pnas.1117018109
24. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004;32(5):1792–1797. doi: 10.1093/nar/gkh340
25. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol.* 2007;56(4):564-577. doi: 10.1080/10635150701472164
26. Dereeper A, Guignon V, Blanc G, et al. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*. 2008;36 (Web Server issue):W465–W469. doi: 10.1093/nar/gkn180
27. Hyde KD, Xu J, Rapior S, et al. The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity.* 2019,97:1-136. doi: 10.1007/s13225-019-00430-9
28. Frac M, Hannula SE, Belka M, Jedryczka M. Fungal Biodiversity and Their Role in Soil Health. *Front Microbiol.* 2018;9:707. doi: 10.3389/fmicb.2018.00707
29. Bills GF, Gloer JB. Biologically Active Secondary Metabolites from the Fungi. *Microbiol Spectr*. 2016;4(6). doi: 10.1128/microbiolspec.FUNK-0009-2016
30. Geetanjali G, Jain P, Pundir RK. Isolation, Purification and Characterization of Antimicrobial Metabolites from *Aspergillus ibericus*. *Biosci Biotechnol Res Commun.* 2020;13(1):84-90. doi: 10.21786/bbrc/13.1/15

31. Wadetwar RN, Patil AT. Isolation and characterization of bioactive actinomycetes from soil in and around Nagpur. *Int J Pharm Sci Res*. 2013;4(4):1428-1433. doi: 10.13040/IJPSR.0975-8232.4(4).1428-33
32. Oskay M, Tamer AU, Azeri C. Antibacterial activity of some Actinomycetes isolated from farming soil of Turkey. *Afr J Biotechnol*. 2004;3(9):441-446. doi: 10.5897/AJB2004.000-2087
33. Keller NP, Turner G, Bennett JW. Fungal secondary metabolism—from biochemistry to genomics. *Nat Rev Microbiol.* 2005;3(12):937-947. doi: 10.1038/nrmicro1286
34. Singh AP, Singh RB, Mishra S. Studies on isolation and characterization of antibiotic producing microorganisms from industrial waste soil sample. *The Open Nutraceuticals Journal*. 2012;5(1):169-173. doi: 10.2174/1876396001205010169
35. Abbas S, Senthilkumar R, Arjunan S. Isolation and molecular characterization of microorganisms producing novel antibiotics from soil sample. *Eur J Exp Biol.* 2014;4(5):149-155.
36. Arias CA, Murray BE. Antibiotic-resistant bugs in the 21st century: A clinical super-challenge. *N Eng J Med.* 2009;360(5):439-443. doi: 10.1056/NEJMp0804651
37. Pelaez F. The historical delivery of antibiotics from microbial natural products—Can history repeat? *Biochem Pharmacol.* 2006;71(7):981-990. doi: 10.1016/j.bcp.2005.10.010
38. Ortega HE, Torres-Mendoza D, Cubilla-Rios L. Antibacterial Compounds Isolated from Endophytic Fungi Reported from 2021 to 2024. *Antibiotics*. 2025;14(7):644. doi: 10.3390/antibiotics14070644
39. Rosdee S, Wisessombat S, Tayeh M, Malakul R, Phanaksri T, Sianglum W. Antibacterial activity of the endophytic fungal extracts and synergistic effects of combinations of ethylene diamine tetra acetic acid (EDTA) against *Pseudomonas aeruginosa* and *Escherichia coli. Peer J.* 2025;13:e19074. doi: 10.7717/peerj.19074
40. Mendieta-Brito S, Sayed M, Hamza FA, et al. Identification, characterization, antimicrobial activity and biocontrol potential of four endophytic fungi isolated from Amazonian plants. *Sci Rep*. 2025;15(1):39361. doi: 10.1038/s41598-025-26865-6
41. Yurchenko E, Krasowska M, Kowczyk-Sadowy M, Zapora E. Investigation of the Possible Antibacterial Effects of Corticioid Fungi Against Different Bacterial Species. *Int J Mol Sci.* 2025;26(7):3292. doi: 10.3390/ijms26073292