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Characterizing Phytate Hydrolyzing Fungal isolate using ITS Region Profiling

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ABSTRACT: Along with the conventional methods of identification, the advance genome sequencing and computational methods are advantageous for identification of various lives. Enormous databases of nucleotide sequence are now available as a result of advance genome sequencing and computational methods. Such databases can be retrieved, assembled and analyzed for identification of various cells. Identification of a phytate hydrolyzing fungal isolate fsp-4 as *Aspergillus terreus* was carried out on the basis of macroscopic, microscopic and molecular characterization using ITS sequences. Dealing with complex and diverse fungal communities in various environmental samples was the challenge in characterizing fungal isolate using ITS region profiling, also identifying specific phytate-hydrolyzing fungi amidst the vast array of microbial species.

Even with these challenges, the study contributed significantly to our understanding of phytate hydrolyzing fungi, shedding light on their role in nutrient cycling and their potential applications in agriculture, biotechnology, and environmental remediation. The use of ITS region profiling allows for a more comprehensive and rapid identification of these fungi, enabling researchers to explore their diversity and ecological significance more effectively. The findings from this study may pave the way for developing sustainable agricultural practices and optimizing phytate degradation in various industrial processes, leading to enhanced nutrient availability and reduced environmental impact.

Keywords: Genome sequencing, computational methods, *Aspergillus terreus*, molecular characterization, ITS sequences.

INTRODUCTION

The conventional methods of identification need to cultivate the organisms on the basis of phenotypic identification, microscopic analysis and biochemical characteristics. Although morphological characteristics are helpful for identification of fungi, but it has limitations as some of the characters produced rarely and a for time (Slepecky and Starmer 2009). Certain fungal genera encompass a multitude of species with morphological characteristics that can be extremely complex and challenging to accurately place within an evolutionary framework, particularly at the species level. Even for expert mycologists, distinguishing between these species based solely on morphological traits can be difficult and sometimes inconclusive (Geiser, 2004). Several factors contribute to these challenges like hybridization (Hughes, *et al*., 2013), cryptic speciation (Matutea and Sepulveda, 2019) and convergent evolution (Burn and Silar 2010). These may be the reasons for ambiguities in morphological characters of such fungi. A number of microorganisms which are difficult to cultivate cannot be identified due to incomplete data available regarding these uncultivable organisms. Advancement in genome sequencing and computational methods provides precise and quick tool for identification of such microorganisms. The genomic tools like polymerase chain reaction, DNA microarray, metagenomic analysis allows identification starting with single molecule. Some of the techniques provides immediate results while few needs excessive computational analysis.

The field of molecular fungal identification has continued to progress rapidly. Technological advancements and the establishment of extensive sequence databases have further enhanced the reliability and speed of fungal identification using molecular data. As a result, molecular methods have now become an essential part of mycological research, significantly advancing our understanding of fungal diversity, evolutionary relationships, and taxonomy. The use of molecular data has enabled scientists to explore the vast kingdom of fungi more comprehensively than ever before, shedding light on their ecological roles, interactions, and potential applications in various fields (Raja *et al*., 2017). White *et al*. (1990) described the development of fungal nuclear ribosomal operon primers, these primers targeted specific regions of the fungal DNA, including the large subunit (nrLSU-26S or 28S), the small subunit (nrSSU-18S), and the complete internal transcribed spacer region (ITS1, 5.8S, ITS2; approximately 0.45-0.80 kilobases in length). The successful amplification and sequencing of these

regions opened the door to a new era of molecular phylogenetic sequence identification in the kingdom fungi (Burns *et al.*, 1991; Seifert *et al*., 1995; Ciardo, *et al.*, 2006). One of the key regions targeted was the ITS region, in molecular identification of fungi. The high variability in the ITS regions allowed researchers to discern even closely related fungal species, providing a powerful tool for accurate fungal identification (Chen *et al.*, 2000; Franco-Duarte *et al.*, 2019).

The identification of microorganisms with gene sequencing methods, selection of target gene is very important. During identification of bacteria 16 s rRNA molecules will be the target gene, whereas for fungi the suitable target gene for identification will be internal transcribed spacer regions ITS1 and ITS2. Comprehensive reference libraries of data bases viz GenBank, Smart Gene, Ribosomal Database Project, MicroSeq were essential for correct classification and identification of organisms upto species level (Tang, *et al*., 1998; Simmon *et al.*, 2006).

Employing MegaBLAST, a powerful sequence alignment tool available on the NCBI GenBank nucleotide database for ITS gene sequence, Dubey (2022) identified as new species of *Beltraniapseudo rhombica as Beltraniapseudo rhombica* Crous & Y. Zhang 2014.

Present study focuses on identification of efficient phytase producing fungal isolate on the basis of morphological as well as molecular identification. *Aspergillus terreus* is a saprophytic Mold found worldwide in soil and vegetation like habitats. It may function as opportunistic pathogen. *Aspergillus terreus* has subjugated the biological production of statins (Subhan *et al*., 2016), organic acid and enzymes.

Materials and Methods:

Collection of sample and isolation of fungi:

Soil and litter samples of poultry, cattle and goatshed and garden were collected in sterile polyethene bags using sterile spatula.

One gm of each sample was serially diluted at 10^{-4} -10^{-10} dilutions. Inoculation of each dilution was then carried on potato dextrose agar by means of spread plate technique and the plates were incubated for 4-5 days at 40°C. Isolated colonies were maintained as *fsp-4*, a pure culture at 4°C (Bharambe-Chaudhari and Peshwe 2022).

Identification based on morphological characters:

Macroscopic and microscopic characteristics of the fungi:

The Colony colour, diameter, margin, surface, pigmentation, texture of *fsp-4* was studied on wheat extract mineral medium. The morphology of isolate*fsp-4* was studied by using lactophenol cotton blue staining and observed under the light microscope.

Scanning Electron Microscopy (SEM): The SEM analysis of fungal strain was done at ICON Analytical, Mumbai. Six-day old culture of fugal isolate, *fsp-4* was observed through SEM to determine the size, shape and arrangement of the organism.

Identification by molecular characterization: The molecular characterization studies were carried out in collaboration with Gene Ombio Technologies, Pune Maharashtra.

Genomic DNA isolation from fungi: Genomic DNA of the fungal isolate was isolated using DNeasy Tissue kit (QIAGEN) according to the manual instructions provided by the manufacturer.

Polymerase Chain Reaction: Amplification of the fungal ITS region was carried out with the standard polymerase chain reaction protocol (Table 1). For amplification, a pair ITS1 and ITS4(Table 2) were used as primers, with 45°C annealing temperature.

Further for purification of amplified products was carried out using gene O-spin PCR product Purification kit (Gene Ombio technologies, Pune; India) and sequenced using ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The Sequencing Analysis 5.2 software was used for analysis of the sequence of amplified products. The NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) BlastN site was used for BLAST analysis of amplified sequence.

Table 1: PCR Protocol.

Fungal ITS gene PCR			
Stage	Temperature (°C)	Time (min:sec)	Cycles
Initial Denaturation	95	2:00	Hold
Denaturation	94	1:00	35 cycles
Annealing	45	0:30	
Amplification	72	1:00	
Final Amplification	72	5:00	Hold
Hold	4	Until use	Hold
Amplification product size	~400-600 bp		

Table 2: Primer sequence.

Sr. No.	Name	Primer sequence (5'-3')	Bases	Amplicon size	Reference
1.	ITS 1	TCCGTAGGTGAACCTGCGG	19	650 bp	Mitchell *et al.*, 1994
2.	ITS 4	TCCTCCGCTTATTGATATGC	20		

Agarose gel electrophoresis of PCR products for confirmation of PCR amplification: The PCR products were resolved using 2% agarose by Agarose gel electrophoresis while the reference ladder used to compare the amplicon size.

Ethidium bromide 0.5 µg/ml was added to 2% agarose Agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) prepared in 0.5X TBE buffer. 1 µl of 6X Gel tracking dye was mixed with 5.0 µl of PCR product. In one of the lanes of the gel, 5µl of gScale 100-1000bp size standard (geneOmbio technologies, Pune; India) was loaded to confirm the amplicon size using reference ladder. The amplicons resolution performed at 5V/cm till the tracking dye is left 2/3 distance from well within the gel. The UV Trans illuminator was used to detect the DNA bands The GelDocXR gel documentation unit of BIO-RAD was used to record gel images. Through this reaction 650bp sized PCR products were generated.

Purification of PCR products: One primer is needed for DNA sequencing while PCR requires two primers (forward and reverse). If sequencing is done with two primers, two sequences will be obtained, superimposed on each other and completely unreadable. Hence PCR product have to be purified before sequencing. GeneO-Spin PCR purification Kit (geneOmbio technologies, Pune; India) was used to purify the amplified PCR products. The PCR products were eluted in final volume of 20.0µl. The purified PCR products were subjected to resolution using agarose gel electrophoresis.

DNA sequencing: Using the gene specific sequencing primers (ITS1 and ITS4) and ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the purified PCR amplicons was sequenced using Machine: 3130 Genetic analyser Automated DNA sequencing machine. The Softwares used were Sequencing Analysis 5.1; ChromasPro v3.1.

RESULTS

Identification of efficient Phytase producer:

Macroscopic and microscopic characteristics: Beige to cinnamon-coloured colonies, with 4-5 cm diameter on 5-6 days incubation at 40ºC were observed on WEM medium. However, the back side of the colony showed yellow coloured pigmentation. The colony was whitish cinnamon colour on 3rd and appears finely granular cinnamon coloured with conidial production as shown in Fig. 2. *A. terreus fsp-4* showed compact, biseriate and densely columnar conidial head under light microscope (Fig. 1).

SEM Observation: The scanning electron Microscopy revealed that the *A. terreus strain fsp-4* has septate mycelium with compact conidial head. The conidia were smooth walled with a diameter of 1.807µm - 2.295µm. The SEM images are as shown in Fig. 3.

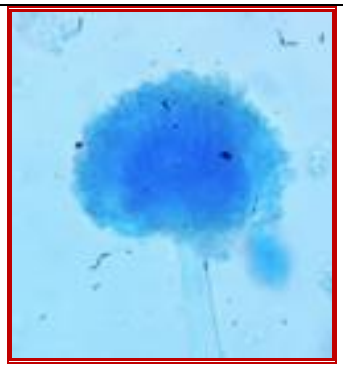
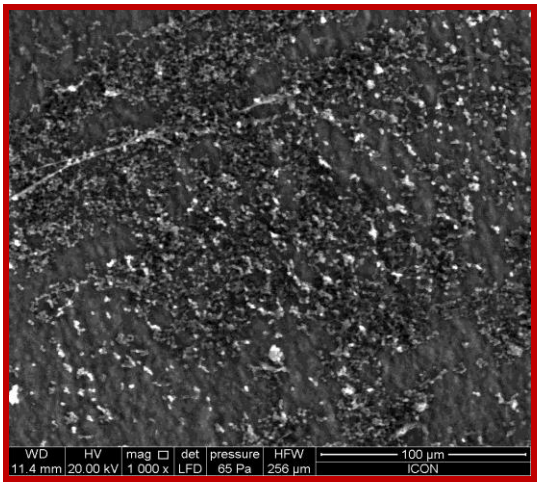
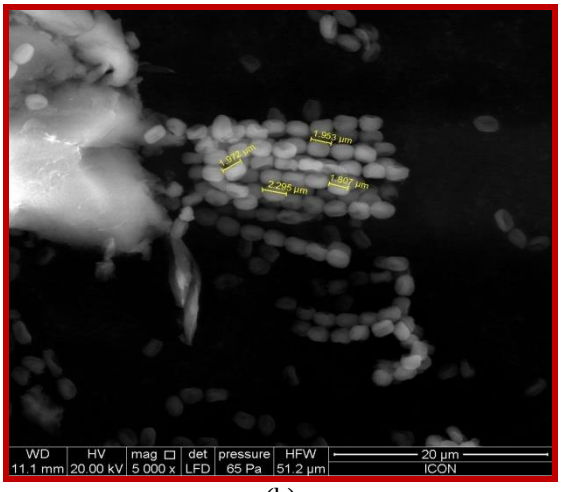


Fig. 1. *A. terreus fsp-4*under light microscope.

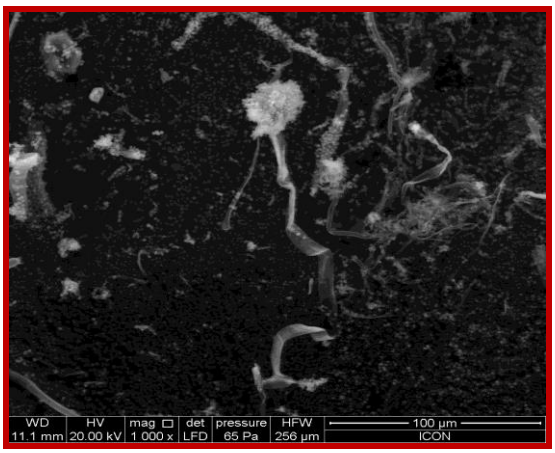
Fig. 2. *A. terreus fsp-4* colony.



(a)



(b)



(c)

Fig. 3. Scanning Electron Microscope images of *A. terreus strain fsp-4.*

Identification of efficient phytase producer by molecular characterization: The fungal genomic DNA was isolated Using DNeasy Tissue kit(QIAGEN).https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-plant-pro-and-plant-kits

The PCR reaction was performed using primers ITS1 and ITS4 for amplification morphologically as well as genomic sequencing of PCR amplified internal transcribed spacer region (ITS), along with partial β-tubulin, calmodulin as well as RNA polymerase large subunit genes of fungus ITS region. The gel image is as shown in Fig. 4. The amplified ITS sequence was sequenced after purification. Blast analysis of the sequence obtained was compared with GeneBank data base at BLASTN site at NCBI http://www.ncbi.nlm.nih.gov/BLAST). The sequence of fungal isolate fsp-4 showed 100% resemblance to *Aspergillus terreus* KY926855.1. The sequence was deposited to GeneBank with accession number MH357344.

Similar studies are reported by Badotti *et al.* (2017). They exploited ITS1 and ITS2 primers in identification of *Basidiomycota* sp. Ezeonuegbu *et al.* (2022) characterized 15 fungal isolates and reported as the 15 fungal species were belonging to *Aspergillus, Fusarium, Penicillium* and *Trichoderma* genera using the same approach.

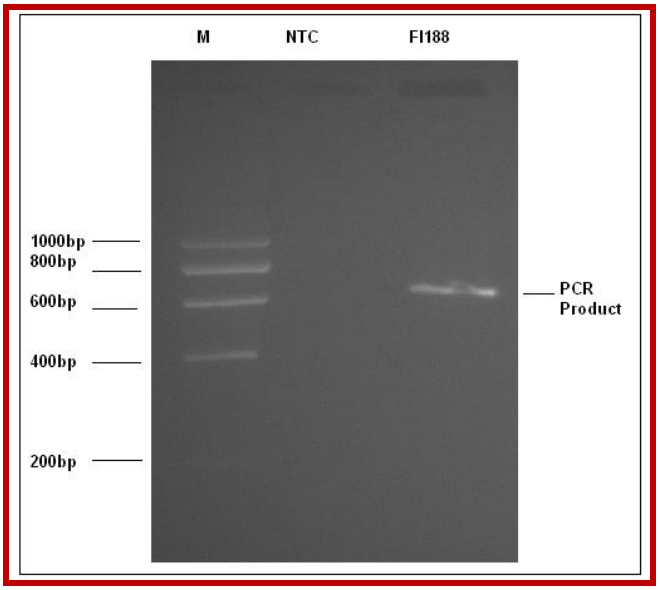


Fig. 4. ITS PCR product loaded on 2%agarose gel. Well M: 200bp molecular marker, Well NTC: Negative test control, **FI188: Sample** PCR Product.

Classification:

Scientific classification

Kingdom: Fungi

Division: Ascomycota

Class: Eurotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

Species: *A. terreus*

Construction of phylogenetic tree:

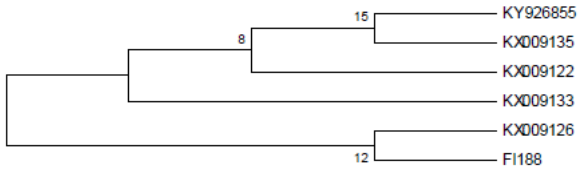


Fig. 5. Phylogenetic tree.

The phylogenetic tree of isolate fsp-4 was drawn by means of cluster algorithm with first five hits in NCBI nucleotide sequence database as shown in Fig. 5. The MEGA 6.0software used to generate the phylogenetic tree. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973). As seen in the optimal tree, the percentage of replicate trees in which the associated taxa clustered together in the 300 bootstrap replicates are mentioned adjacent to the branches (Felsenstein, 1985). The drawn to the scale tree with branch lengths in the same units showing the evolutionary distances are used to construct the phylogenetic tree. The Maximum Composite Likelihood method (Tamura *et al.*, 2004) was used tocompute the evolutionary distances. The Evolutionary analysis was computed using the MEGA6.0 software. A total of 486 positions were there in the final dataset after elimination of positions showing the gaps and missing data after analysis of six nucleotide sequences and the codon positions and noncoding regions. Goudazi *et al.* (2015) similarly studied genetic diversity of anaerobic gut fungi in buffalo employing molecular methodologies based on ribosomal ITS1 sequence and also exploits the MEGA software to generate phylogenetic tree. Comparable investigations were carried out by Dharani *et al*. (2022) for identification of Post-harvest rot causing fungi *Alternaria solani* of tomato fruits using universal primers ITS1 and ITS4 and phylogenetic analysis using MEGA software.

CONCLUSIONS

The phytate hydrolyzing fungus was identified as *Aspergillus terreus* using ITS region sequencing. Thus, utilization of ITS region profiling as a molecular tool proved effective in identifying and characterizing phytate-hydrolyzing fungus. This approach enabled a more comprehensive understanding of the fungal populations involved in phytate hydrolysis, offering potential applications in feed industry.

FUTURE SCOPE

The molecular characterization of phytate hydrolyzing fungi using ITS region profiling has broad implications across various fields, including agriculture, biotechnology, environmental science, and nutrition. As research progresses, it is likely to uncover even more potential applications and shed light on the diverse roles that these fungi play in ecosystems.

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Conflict of Interest. None.

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