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Original Research

Molecular Identification of a Potent Amylolytic Fungal Isolate of Western Ghat Forest Soil by ITS Sequencing

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Abstract

A fungus was isolated from the soil of Western Ghat forest and during the study it was screened for amylase production. The isolate MO 43 was identified up to genus level as *Aspergillus* based on cultural and microscopic characteristics. Further, the isolate was characterized up to species level by PCR amplification and sequencing of partial ITS sequences of rDNA. The phylogenetic analysis showed that the isolate was *Aspergillus sydowii*. The sequence was submitted to NCBI public database and accession number was obtained.

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INTRODUCTION

Fungi represent the greatest eukaryotic diversity on earth and they are among the primary decomposers in ecosystems. It is conservatively estimated that 1.5 million species of fungi exist (Tsui *et al.*, 2011). Fungi are used in many industrial processes, such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids, and glycolipids. Some of these products are produced commercially while others are potentially valuable in biotechnology (Kamalam *et al.*, 2012). Enzymes are one among the important metabolites produced by fungi. In particular, amylases are one of the most important industrially used enzymes. They are used in starch liquefaction to produce glucose, fructose and maltose. Amylases are also used in brewing, baking, textile, paper, detergent and sugar industries (Crueger and Crueger, 1990). Screening of fungi with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta *et al.,* 2003; Wanderley *et al.,* 2004; Sidkey *et al*., 2010).

Generally filamentous fungi are identified mainly using morphological characteristics (Bakri *et al*., 2009). However, these methods of identification are often problematic as there can be different morpho/biotypes within a single species. They are also time consuming and require a great deal of skill and expertise (Bakri *et al*., 2009). Today, comparative sequence-based identification strategies can be considered the new "gold standard" for fungal species identification (Balajee *et al*., 2009). The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. Post transcriptional processes split the cistron, removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are usually referred to as the ITS region (Schoch *et al*, 2012). The ITS region satisfies most of the requirements of a "universal" marker since this region can be reliably amplified for most fungi, is conserved, is present as multiple copies in the fungal genome, yields sufficient taxonomic resolution for most fungi, and has the additional advantage that the GenBank, European Molecular Biology Laboratory nucleotide sequence database and DNA Data Bank of Japan contain a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown isolate (Balajee *et al*., 2009).

Western Ghats of India are considered to be one of the global biodiversity hotspots. Western Ghats houses a number and a variety of plants, animals and microorganisms. Many species are endemic to Western Ghats. Microbiological studies have been carried out using soils of Western Ghats of Karnataka and the results obtained were promising in terms of ability of isolates to produce bioactive metabolites such as antibiotics and enzymes. Hence the soils from Western Ghats can be a potential source of bioactive metabolites (Mukunda *et al*., 2012; Gautham *et al*., 2012; Kekuda *et al*., 2013). In our previous study, we had isolated a potent amylolytic fungal strain MO 43 from a soil sample collected at Agumbe, Karnataka, India. The purpose of the present study was to identify this industrially important isolate MO 43 by molecular methods using ITS sequencing.

MATERIALS AND METHODS

Isolation of Isolate MO 43 from Soil Sample

The fungal isolate MO 43 was isolated on Potato dextrose agar medium during a screening program for amylase producers from the soil samples collected at Agumbe region of Karnataka, India. The isolate was characterized morphologically (both cultural and microscopic) and was identified as a species of *Aspergillus*. The isolate MO 43 performed well in primary screening by revealing wider zones of hydrolysis on starch agar. In secondary screening, the crude enzyme from this isolate showed high specific activity (Mukunda *et al.*, 2012).

Characterization of Isolate MO 43 by Molecular Methods

The fungal isolate MO 43 was characterized up to species level by partial sequencing of ITS 1 and ITS 2 regions of rDNA sequence. DNA was extracted from mycelium and spores of MO 43 by using the DNA extraction kit (Bioserve Biotechnologies India Pvt. Ltd, Hyderabad, India). The amplification of ITS region of rDNA was carried out using PCR. The PCR amplification was carried out on ABI 9700 thermocycler. Amplification of the ITS rDNA region was carried out using primers designed in the conserved region with universal primers; ITS 1 5'(TCCGTAGGTGAACCTGCGG)3' as forward primer and ITS 4 3'(TCCTCCGCTTATTGATATGC)5' as reverse primer. The reaction mixture (total 100µl) consisted of 1µl of template DNA, 2.5µl of 10X PCR buffer, 2.5µl of 25 mM $MgCl_2$, 2.0 µl of 2.5 mM dNTP, 14 µl PCR grade water, 1.0µl of forward primer 10 pm/µl, 1.0µl. of reverse primer 10 pm/µl, 1.0µl. of *Taq* DNA polymerase enzyme (1 unit/ µl.). A total of 35 cycles were carried out and PCR conditions consisted of an initial denaturation at 95^{o}C for 30 seconds, annealing at 55^{o}C for 1 minute followed by extension at 72^{o}C for 1 minute. Initial denaturing at 95^{o}C was extended for 5 minutes and the final extension was for 7 minutes at 72^{o}C. The amplified ITS regions were purified by ethanol/EDTA precipitation method and were sequenced by ABI 3730 Genetic Analyzer using big dye terminator version 3.1 cycle sequencing kits (Applied Biosystems). The sequencing results were trimmed and assembled. The analysis of the partial ITS sequence of 671 nucleotides of isolate MO 43 was carried out using NCBI BLAST. The sequences were also compared with the public nucleotide databases using the BLAST algorithm to identify the ITS sequences with high degree of similarity. The phylogenetic tree was constructed to identify the closest relative of the isolate (Viaud *et al*., 2000; Kim *et al.,* 2011). The partial ITS sequences obtained were deposited in Gene Bank for getting the accession numbers for the sequences.

RESULTS AND DISCUSSION

With the advent of new frontiers in biotechnology, the amylase family enzyme finds potential application in a number of industrial processes (Pandey *et al.,* 2000; Alva *et al.,* 2007; Sidkey *et al*., 2010). Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence (McTigue *et al*., 1995). Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Gupta *et al.,* 2003; Wanderley *et al.,* 2004; Sidkey *et al*., 2010).

The identification of fungal species by classic taxonomy is based mainly on the use of morphological markers. However, the number of these markers available is generally low, which makes difficult the classification and/or identification of related species (Magnani *et al*., 2005). Molecular methods are rapid with a turnaround time of about 24 h from the time of DNA extraction, yield results that are objective with data portable between labs, and could be more economical in the long run. PCR amplification with universal primers targeted to conserved regions within the rRNA complex and subsequent DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Sathiyavathi and Parvatham, 2011).

The colony of MO 43 was green, cottony and rough with green colored aerial mycelia. The colony was surrounded peripherally by whitish zone. Upon prolonged incubation red colored pigmentation surrounding the colony and back pigmentation was observed. The colonies were furrowed with reddish metabolites on the colony. Microscopic observation showed smooth, columnar, colorless Conidiophores (Figure 1). Vesicles were typically globose and fertile over the entire surface. Sterigmata typically in two series and the conidiospores were echinulate. With the above observations the isolate MO 43 was identified as *Aspergillus*. The isolate caused marked hydrolysis of starch on starch agar plate (Figure 2) as indicated by maximum zone of hydrolysis (16mm). The isolate was also shown to produce other enzymes viz., protease, cellulase and CMCase (Mukunda *et al*., 2012).

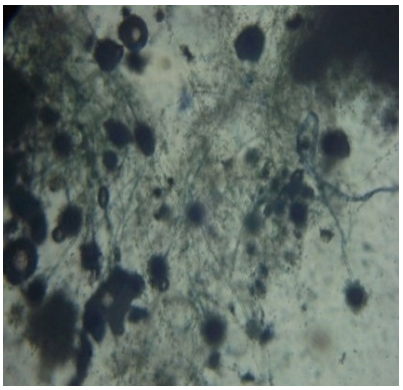

Figure 1: Microscopic view of the isolate MO 43 (Mukunda, 2013 thesis)

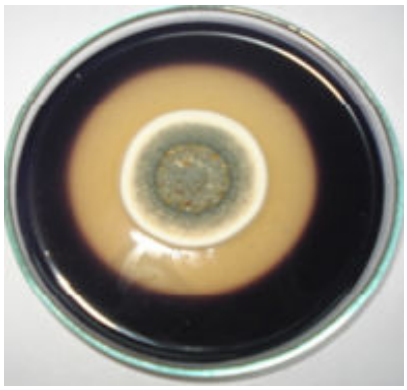

Figure 2: Amylolytic activity of isolate MO 43 on Starch agar (Mukunda, 2013 thesis)

In the earlier study, the isolate MO 43 was identified only at the genus level as *Aspergillus* by colony morphology and microscopic observation (Mukunda *et al.*, 2012). Multiple studies have demonstrated that comparative sequence- based identification using the nuclear ribosomal ITS region (ITS1, 5.8S rRNA, and ITS2) located between the nuclear small- and large-subunit rRNA genes could be employed for species complex-level identification of *Aspergillus* (Balajee *et al*., 2009). In this study, we have identified the isolate MO 43 by ITS sequencing of rDNA. The sequence of 671 nucleotides obtained is as below.

cactctcggctgaatttcgtagtcccggcgctcgcccccaggggtttcccgccgaagcacattagtattc
acggtgggaggttgggcgcctggaggcagcccgcactcagtaatgatccgtaggggttgacctgcgg
aaggatcattactgagtgcgggctgcctccgggcgcccaacctcccacccgtgaatacctaacactgtt
gcttcggcggggaacccccctcgggggcgagccgccggggactactgaacttcatgcctgagagtgat
gcagtctgagtctgaatataaaatcagtcaaaactttcaacaatggatctcttggttccggcatcgatgaag
aacgcagcgaactgcgataagtaatgtgaattgcagaattcagtgaatcatcgagtctttgaacgcacatt
gcgccccctggcattccgggggggcatgcctgtccgagcgtcattgctgcccatcaagcccggcttgtgt
gttgggtcgtcgtcccccccgggggacgggcccgaaaggcagcggcggcaccgtgtccggtcctcg
agcgtatggggctttgtcacccgctcgactagggccggccgggcgccagccgacgtctccaaccatttt
tcttcaggttgacctcggatcaggtagggatacccgctgaacttaagcatat

On obtaining the consensus sequence, it was queried against sequences with the public nucleotide databases using the BLAST algorithm for species identification. The isolate MO 43 was identified as *Aspergillus sydowii*. Figure 3 depicts the Phylogenetic tree arrangement of the isolate *Aspergillus* MO 43 based on the above sequence. The species identification can be performed by generating dendrograms, examining percent similarity/ percent dissimilarity, or executing more sophisticated phylogenetic analyses. A phylogentic analysis of the isolate was performed to determine how the 18S rRNA sequence of the isolate and related strain might have been derived during evolution (Krishnaveni and Raghunathan, 2014). The nucleotide sequence of the isolate was deposited in GenBank and the accession number given was KC442245.The systematic position of the isolate is as follows:

Domain:	Eukarya
Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Eurotiomycetes
Order:	Euratiales
Family:	Trichocomaceae
Genus:	*Aspergillus* Michaeli, 1729
Species:	*sydowii*

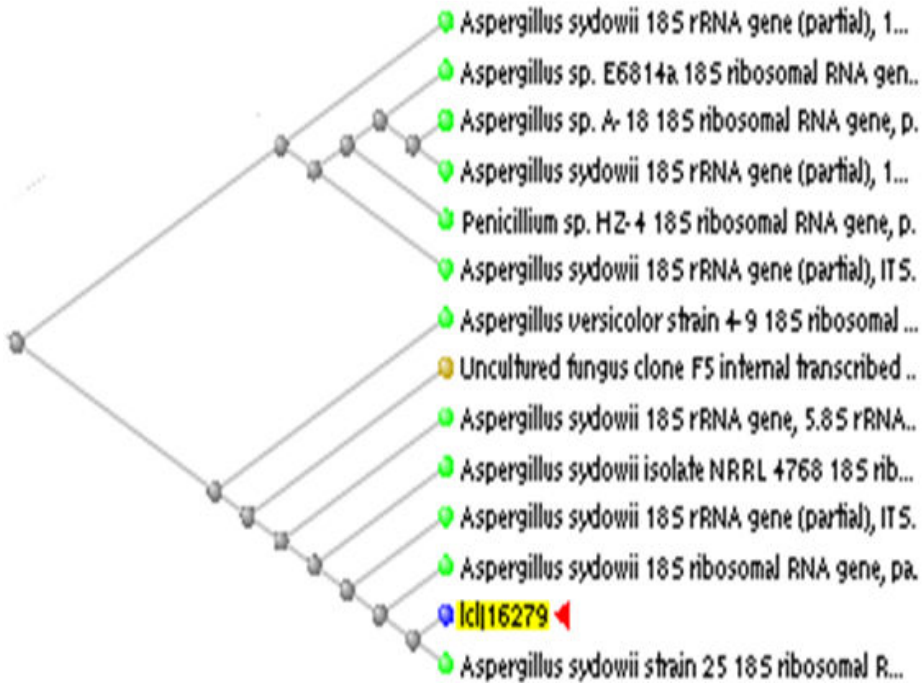

Figure 1: Phylogenetic tree arrangement of *Aspergillus* sp. MO 43

CONCLUSION

In the present work, the approach of morphological and homology analysis of partial sequences ITS regions were used for the identification of an industrially important fungal isolate MO 43 from the soils of deep and unexplored forest of Western Ghats of Karnataka, India. Hence the molecular identification methods are very helpful in identifying fungal isolates up to species level and sub species levels also.

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Conflict of Interest

Authors declared no conflict of interest.

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