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ISOLATION AND MOLECULAR IDENTIFICATION OF L-ASPARAGINASE PRODUCING FUNGI FROM SOIL

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ABSTRACT
L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extra cellular enzyme that has received considerable attention since it is used as an anticancer agent as well as for industrial application especially to reduce the acrylamide formation in fried foods. In the present study, the fungal isolates from rhizosphere soils were screened for the L-asparaginase production by using modified Czapek Dox agar containing L-asparagine and phenol red as indicator. The strain isolated from rhizosphere soil of *Ipomoea muricata* showed the maximum zone diameter of 1.05 cm. The 16s rDNA sequence analysis indicated that the strain was most closely related to *Fusarium equiseti* (99% similarity) and the 766 nucleotide sequences has been deposited to the GenBank (National Centre for Biotechnological Information, USA, with accession number JN400528).

1. INTRODUCTION

Soil is a complex and dynamic environment in which the biological activity is mostly governed by microorganisms. The beneficial effects of soil microorganisms are manifold and range from nitrogen fixation and organic matter decomposition to breakdown of metabolic by-products and agrochemical, enhancing the bioavailability of nitrates, sulphates, phosphates and essential metals (Bridge and Spooner, 2001). Fungi are an important component of the soil microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Ainsworth and Bisby, 1995). Filamentous fungi are featured as modular organisms (Carlile 1994), which grow by the repeated iteration of modules usually to yield a branching pattern. The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in the industrialization of detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products.

Filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential.

One such enzyme is L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) which converts L-asparagine to L-aspartic acid and ammonia. The therapeutic potential of this enzyme is well established. In the study of soil fungi, particular attention is given to the rhizosphere a well-developed and diverse rhizosphere community is Screening of microorganisms for selecting suitable strains is an important preliminary step in the production of desired metabolites. Besides, a good plate screening method essentially plays a vital role in the selection of appropriate mutants during strain improvement (Rowlands, 1984). Plate screening assay is commonly used for detection of extracellular hydrolytic enzymes produced by microorganisms. Thought to play a role in the suppression of pathogens (Alabouvette, 1990; Hurek, 1997).

Fungal taxonomy is traditionally based on comparative morphological features (e.g., Lodge *et al*., 1996; Sette *et al*., 2006; Crous *et al*., 2007; Zhang *et al*., 2008). In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al*., 2006). In the 1980s, a new standard for identifying bacteria was developed in the laboratories of Woese and others; it was shown that phylogenetic relationships of bacteria, and, indeed, all life-forms, could be determined by comparing a stable part of the genetic code (Woese *et al*., 1985; Woese. 1987). Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit) and the 23S rRNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene. (Bottger, 1989; Palys *et al.,* 1997; Kolbert and Persing, 1999: Garrity and Holt, 2001; Harmsen and Karch, 2004). The 16S rRNA gene can be compared not only among all bacteria but also with the 16S rRNA gene of archaebacteria and the 18S rRNA gene of eukaryotes.

With the discovery of the polymerase chain reaction (PCR) and DNA sequencing methods, elucidation of closely related taxon with better authenticity has been made successfully in comparison to other conventional methods (Lane *et al.,* 1985; Bosshard *et al.,* 2003; Clarridge, 2004). Gene sequencing is a more accurate and reproducible method to identify microorganisms and has increased our ability to capture the diversity of microbial taxa (Clarridge, 2004). This new technology has resulted in the identification of unusual microorganisms (Drancourt, 2004). Beside this, phylogenetic analysis of 16S rRNA gene is presently an important area of evolutionary study and sequence analysis. In addition to analyzing changes that have occurred during the evolution among different organisms, the evolution of a family of sequences may be studied. On the basis of the analysis, sequences that are most closely related can be identified by the places they occupy on neighboring branches of a tree. Phylogenetic analysis of a family of related gene sequences is a method to determine how the family might have been derived during evolution. Microbial ecologists have used direct RT-PCR amplification of 16S rRNA molecules for the detection of active species of bacteria and archaea, and 18S rRNA molecules for the detection of active fungi.

The drawback to this approach for fungi is that 18S rRNA sequences often do not provide sufficient taxonomic resolution to allow identification of taxa in mixed communities to genus or species level (Anderson and Parkin, 2007). However, using 16S rRNA sequencing, these problems can be overcome by a single technology, which also facilitates the discovery of novel genera and species.

2. MATERIALS AND METHODS

2.1 Chemicals

The chemicals and media ingredients used in the study were of reputed companies from India and abroad procured from local suppliers such as Hi-media, Qualigens, Sigma Aldrich, SRL, S.D. Fine, Thomas Baker, Merck, etc. All of the chemicals were of research or analytical grade

2.2 Isolation of filamentous fungi

The soil samples were collected in sterilized polythene bags from the rhizosphere region of different plants at various locations around Karnatak University, Dharwad campus. About 1 gm of each of the above samples was taken into separate conical flasks each containing 100 ml of sterile water. The suspension was kept on rotary shaker for 30 min and kept aside to settle the suspending matter. One ml of the supernatant was serially diluted with sterile water and plated on Czapek-Dox agar plates by pour plate technique. The plates were incubated at 28+ 2^{O}C for 96-120 hours. Individual colonies were regrown on Czapek-Dox agar at 28^{O}C for obtaining pure culture. The pure culture were maintained at 4-5^{0}C and were sub cultured once in a month.

2.3 Rapid plate assay for screening of L-asparaginase producers

The isolated colonies were screened for L-asparaginase production by rapid plate assay technique as per the method described by Gulati *et al.,* (1997). The modified Czapek-Dox agar was supplemented with phenol red 0.009% (v/v). The media was sterilized, the plates were inoculated and incubated at 28+2°C for 96 h. Control plates were of modified Czapek-Dox's medium without dye and L-asparagine. The zone and colony diameter were measured after 48 h. Colonies with pink zones were considered as positive L-asparaginase producers. The isolate which has zone with maximum diameter was selected for further study. The broth studies were also carried out to compare the results obtained with positive isolates for plate.

2.4 Identification of the strain

The strain BBK9 which has produced maximum zone diameter by plate assay method was sent to Agharkar Research Institute, Pune for further identification and 16s rRNA gene sequencing. The strain was identified as *Fusarium equiseti* and the sequence obtained was analysed by using nBLAST at NCBI (http://ncbi.nlm.nih.gov/blast.cgi).

3. RESULTS

3.1 Isolation and screening of L-asparaginase producers by plate assay method (Table 1 and 2 &Fig.1)

The results revealed that out of twenty one soil isolates, the four positive potential strains show different range of zone of diameter. BBK9 has shown the colony diameter of 2.35 cm and zone diameter of 1.05 cm than compared to BBK14 which showed colony diameter of 1.57 cm and zone diameter of 0.86 cm, BBK20 has showed colony diameter of 1.61 cm and zone diameter of 0.84 cm where as BBK3 showed colony diameter of 1.34 cm and zone diameter of 0.52 cm respectively.

Table 1 Isolates with colony and zone diameter

Isolate No.	Colony diameter (cm)	Zone diameter (cm)
BBK1	1.09	-
BBK2	1.60	-
BBK3	1.34	0.52
BBK4	1.57	-
BBK5	1.72	-
BBK6	1.80	-
BBK7	0.09	-
BBK8	2.10	-
BBK9	2.35	1.05
BBK10	1.23	-
BBK11	No growth	
BBK12	1.92	-
BBK13	1.61	-
BBK14	1.57	0.86

BBK15	No growth	-
BBK16	2.12	-
BBK17	1.32	-
BBK18	1.02	-
BBK19	2.10	-
BBK20	1.61	0.84
BBK21	2.53	-

Table. 2 Colony diameter and zone diameter after 48 hrs and L-asparaginase activity with change in the pH of the culture filtrate

Isolate No.	Colony diameter cm)	Zone Diameter cm)	Enzyme Activity (IU/ml)	pH of the filtrate†
BBK3	1.34	0.52	0.04	8.10
BBK9	2.35	1.05	0.14	8.83
BBK14	1.57	0.86	0.07	8.70
BBK20	1.61	0.84	0.06	8.43

Incubation temperature 37°C † Initial pH of the medium is 6

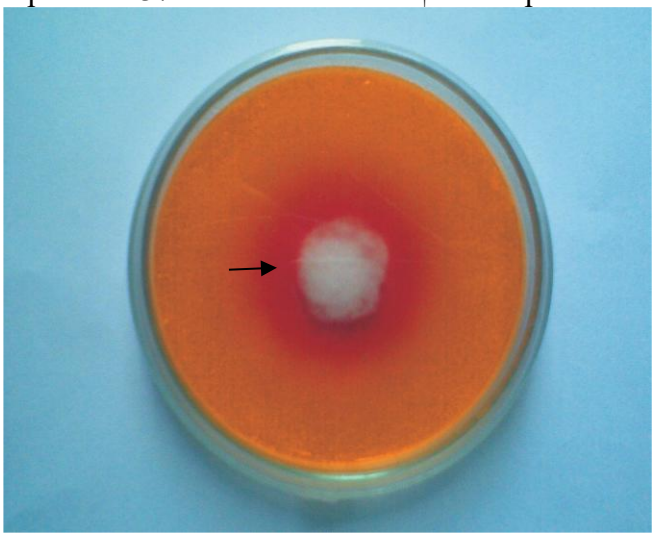

Fig 1- Rapid Plate assay for the screening of L-asparaginase producer

3.2 Identification of the strain (Fig. 4.2 and 4.3)

The strain BBK9 which has produced maximum zone diameter by plate assay method was sent to Agharkar Research Institute, Pune for further identification. Phylogenetic analysis based on 16s rRNA gene sequencing showed that the isolate was grouped into the genus *Fusarium equiseti* with Score = 1397 bits (756), Expect = 0.0 Identities = 763/766 (99%), Gaps = 1/766 (0%) Strand=Plus/Minus. The phylogenetic tree was obtained by nBLAST analysis. The sequence consisting of 766 nucleotides was submitted to the GenBank (National Centre for Biotechnological Information, USA, with accession number JN400528). The sequence data showed that the isolate BBK9 has highest sequence similarity (99%) with the genus *Fusarium equiseti.* Hence, it is concluded that the isolated strain BBK9 is *Fusarium equiseti.*

Primer NS4 (766 bases)

5’CTCGTAAGGTGCCGAACGGGTCAAAAAATAACACCGAAACGATCCCTAGTCGGCAT
AGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCCT
GATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTAGTCTTCAATAAATCCAA
GAATTTCACCTCTGACAATTGAATACTGATGCCCCCGACTGTCCCTATTAATCATTACG
GCGGTCCTAGAAACCAACAAAATAGAACCACACGTCCTATTCTATTATTCCATGCTAAT
GTATTCGAGCATAGGCCTGCCTGGAGCACTCTAATTTTTTCACAGTAAAAGTCCTGTTT

CCCCGCCACGCCCAGTGAAGGGCATGGGGTTCCACAGAGGGAAAGGCCCGGCCGAGC
CAGTACACGCGGTGAGGCGGACCGGACGGCCAGGCCCAAGGTTCAACTACGAGCTTTT
TAACCACAACAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCAC
CAGACTTGCCCTCCAATTGTTCCTCGTTAAGGGATTTAAATTGTACTCATTCCAATTAC
AAGACCCAAAAGAGCCCTGTATCAGTATTTATTGTCACTACCTCCCCGTGTCGGGATTG
GGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTAGTAGCCGTTTCTCAGGCTCCTTCTC
CGGGGTCGAGCCCTAACCCTCCGTTACCCGTTGCAACCATGTTTGGCCAATACCCAAAC
ATCG3’

NCBI Accessoion GQ505743.1/ NRRL 36478/ *Fusarium equiseti*

Score = 918 bits (497), Expect = 0.0
Identities = 504/507 (99%), Gaps = 2/507 (0%)
Strand=Plus/Plus

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Query  8    ACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGT  67
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  55   ACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGT  114

Query  68   AAAAAAGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTA  127
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115  -AAAAAGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTA  173

Query  128  AAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  AAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG  233

Query  188  AACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  AACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT  293

Query  248  GAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAAC  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  GAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAAC  353

Query  308  CCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCG  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  354  CCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCG  413

Query  368  GTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGC  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  GTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGC  473

Query  428  CACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  CACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA  533

Query  488  ACTTAAGCATATCATTAAAGCGGAGGA  514
            |||||||||||| ||| ||||||||
Sbjct  534  ACTTAAGCATATCAATAA-GCGGAGGA  559
```

Fig. 2 & 3 Phylogenetic tree obtained by N BLAST analysis of 16 S r DNA

4. DISCUSSION

The present investigation describes the isolation of filamentous fungi from different soil samples. Soil samples were collected from the rhizosphere region of different plants at various locations around Karnatak University, Dharwad campus. Soil is rich source for potential enzyme producing organisms. Four isolates out of 21 isolates collected from different rhizosphere soils showed positive L-asparaginase production by producing coloration around the colony (Gulati *et al*., 1997). The isolate BBK9 along the rhizosphere of *Ipomoea muricata* exhibited the zone of diameter 1.05 cm, activity of 0.14 IU/ml and pH of the broth was 8.83 among various filamentous fungi tested by plate assay and broth studies. BBK14, BBK20 and BBK3 have zone diameter of 0.86, 0.84 and 0.52 cm respectively. It is proposed that strain exhibiting zone of diameter above 0.9 cm are referred to as good L-asparaginase producers, those strains with zone diameter of 0.6 to 0.9 cm are referred to moderate L-asparaginase producers and below 0.6 cm zone of diameter may be referred to as poor L-asparaginase producers respectively. As per the grouping the strain BBK9 exhibited higher zone diameter and was consider as potential strain for L-asparaginase production. BBK14 and BBK20 are treated as moderate L-asparaginase producers and BBK3 was treated as poor L-asparaginase producers. Therefore, on the basis of results observed on the rapid plate assay method, it was considered that the isolate BBK9 was considered as a potential L-asparaginase producer and was

further sent to Agharkar research institute for further identification. It has been reported that number of *Fusarium* species produces L-asparaginase. They are the most active group that gave zone diameter of 1.5 to 2 cm, *Xylaria* spp and *Phomopsis* spp. are the second group showed zone diameter of 0.5 cm (Lumyong *et al*., 2010). Gulati *et al*., (1997) have also described the effect of dye on *Fusarium oxysporum*. As the dye concentration increases the density and visibility of the pink zone increased and 0.009 % was most approximate dye concentration. L-asparaginase produced extracellularly by *Fusarium* related sps. (Nakhama, 1973).

Phylogenetic analysis based on 16s rRNA gene sequencing revealed that sequence with 766 nucleotides. The sequence was submitted by Genbank (NCBI) USA, accession number JN400528. The sequence data showed that the isolated BBK9 has the highest sequence similarity (99 %) with the genus *Fusarium equiseti.*

5. CONCLUSIONS

From this study it is clearly indicated that rhizosphere soil can provide rich source of L-asparaginase producing fungi. The isolate BBK9 from rhizosphere soil of *Ipomea muricata was identified as Fusarium equiseti* (99% similarity) *has the ability to produce significant amount of* L-asparaginase. This is the first report on *Fusarium equiseti* from rhizosphere soil of *Ipomea muricata* producing L-asparaginase. However, more detailed investigation is required to characterize this microbial enzyme which may be effectively used in the large scale production for commercial and therapeutic purposes in the near future.

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