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# Isolation and Molecular Identification of Asparaginase Producing Thermophilic Fungi from Decomposing Wastes

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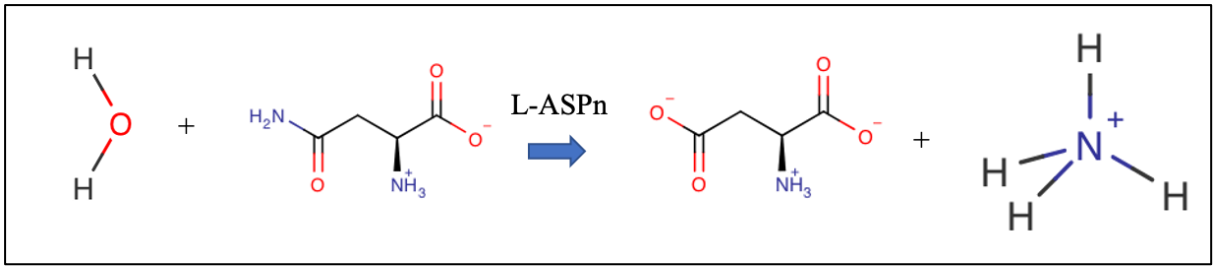
**ABSTRACT**

L-asparaginase (L-ASNase) is an enzyme of significant biomedical and industrial interest, primarily used in the treatment of acute lymphoblastic leukaemia and as a food processing aid to reduce acrylamide formation. While microbial sources are common, there is growing interest in novel sources, especially thermophilic fungi, which can offer enzymes with enhanced stability at high temperatures. This study aimed to isolate, screen, and molecularly identify potent L-asparginase producing thermophilic fungi from decomposing organic wastes. Fungal strains were isolated from various decomposing waste samples using Dilution plate technique and plated on YESA medium. Then they were screened for asparginase production using modified Czapek Dox agar media supplemented with different conc. of phenyl red dye. The most potent producers were further identified using both morphological characteristics and phylogenetic analysis of the DNA sequence data. The most potent producers were identified as Thermomyces lanuginosus strain (ATCC 200065) and Aspergillus oryzae (RIB40).

## Introduction

In recent years enzymes have gained much importance in clinical research. L-asparaginase

Is one of them which are widely present in nature. L-Asparaginase (E.C 3.5.1.1) catalyzes the hydrolysis of the non-essential amino acid L-asparagine into L-aspartate and ammonia. L-ASNase is commonly used to treat hemopoietic illnesses such as ALL (acute lymphoblastic leukemia) and Hodgkin's lymphoma [1,2]. Asparaginase is an enzyme that is employed in both medicine and food processing [3,4]. It is administered via injection into a vein, muscle, or under the skin.




L-asparaginases have wide distribution among microbes, plants, and animals [5]. Microbes are preferred over other sources of production of L-asparaginase because they have some advantages, such as easy upstream bioprocessing and convenient downstream processing, both of which facilitate industry-scale production. Microbial L-asparaginase has been extensively studied in recent years for its potential applications in the pharmaceutical and food industries [6,7,8,9].

Though L-asparaginase production by mesophilic fungi was investigated by large number of workers only limited information is available on thermophilic fungal asparaginase. It is necessary to explore thermostable L-asparaginases with ideal characteristics from thermophilic fungi.

**Isolation of thermophilic fungi**

Thermophilic fungi were isolated from different substrates such as self-heating compost soils, municipal waste and wood chip piles, zoo waste and decomposing plant materials. Substrate were collected in sterilized polythene bags and brought to the laboratory for analysis of thermophilic fungi. Dilution plate method suggested by Waksman (1922) was employed for the estimation of mycoflora. Thermophilic fungal colonies appearing on the medium were sub cultured and identified based on morphological characters with the help of standard manuals (Ref). The isolated fungi were purified by using YES medium.

The isolation was carried out using the following techniques.

**Dilution Plate Technique** For the detection and estimation of mycoflora dilution plate method was employed [10,11]

**Paired Petri plate Technique** For isolation of thermophilic fungi, we have used this method which provides moisture and suitable environment for the growth of thermophilic fungi [12].

**Humid Chamber Technique** This method is also employed for isolation of Thermophiles. The dung samples are taken and directly placed in a glass chamber which is previously arranged with sterile wet filter paper and sterile glass slide on it [13,14]. Identification of Thermophiles was made by referring relevant literature and monographs [12,15,16].

**Screening of fungi**

The experiment was carried out in modified Czapeck dox medium ( glucose 2gms,$KH_2PO_4$ 1.52 ,KCl 0.52,$MgSO_4$.7H20 ,and traces of $Cu(NO_3)_2.3H_2O$,$ZnSO_4.7H_2O$,$FeSO_4.7H_2O$,agar 20gms ,dist. Water 1000ml) with 1% L asparagines and the ph was adjusted to 6.2 with the help of 6N NaOH/6N HCL and media supplemented with different conc.of phenyl red dye (2.5 gms of phenyl red dissolved in 100ml of ethanol and pH was adjusted to 7.0 using 1M NaOH the prepared media was sterilized, and inoculated with 7 days old cultures of different isolates .medium without dye and

sodium nitrate as a nitrogen source in place of L asparagines served as control. The experiment was carried in duplicates and incubated at $45^0$C. The plates thus inoculated were incubated for further 7 days. At the end of incubation period, zone of pink coloration was measured in mm as positive for qualitative assessment and area of coloration was taken as the quantitative measurement and the isolates with highest zones were selected for asparaginase production [17].

**Molecular identification**

The thermophilic fungi identified by microscopic observations were confirmed with the help of modern analytical techniques. The molecular analysis based on DNA sequences, recognized as the most reliable methods to reveal genetic relationships between the strains, could therefore, be unambiguously used to identify and evaluate the isolates at any taxonomic rank (Bruns et.al.1991).Various molecular approaches that can be used in the studies of evolutionary relationships and identification of filamentous fungi are ribosomal RNA(rRNA)sequence comparison, amplified rDNA restriction analyses (ARDRA)using restriction fragment length polymorphism (RFLP) and DNA –DNA complementaries. Ribosomal DNA ( rDNA) is one of the most conserved regions in the genome yet interspersed with variable regions that can be useful in deciphering phylogenetic divergence.

Genomic DNA extraction from *the culture* was performed following optimal growth at 72 hours. Approximately 100 mg of mycelia were cryogenically pulverized in liquid nitrogen using a mortar and pestle. Genomic DNA was subsequently extracted employing the DNeasy Plant Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed using a Nanodrop Spectrophotometer (Thermo Scientific, USA), and the DNA was stored at -20 °C. Integrity of the extracted DNA was confirmed via 1% agarose gel electrophoresis.

**Results and Discussion**

As shown in Table 1 a total of 14 isolates from various substrates were isolated and identified based on morphology and microscopic observation (Fig 1). The potential L-asparaginase producing thermophilic fungal isolates were identified based on their micro- morphological features according to the universal identification keys (Raper & Fennell, 1965) (Chen et al., 2016).

All isolates were screened for their asparaginase production. Based on zone of pink colouration, the potential L-asparaginase producing thermophilic fungal isolates with highest zones were selected for further study and were identified by molecular method.

A single discrete PCR amplicon band of 650 bp was observed when resolved on Agarose Gel (Gel Image-1) (Figure 2).

**Table 1: Isolates showing various zones of pink colouration at different concentrations of phenyl red dye**

| Isolate No | Zone of pink colouration at different concentrations of phenyl red dye | | | |
|---|---|---|---|---|
| | 0.03 | 0.06 | 0.09 | 0.1 |
| Isolate 1 (Aspergillus) | 2.0 | 3.0 | 4.0 | 8.0 |
| Isolate 2 (Thermomyces) | 4.0 | 2.0 | 8.0 | 12.0 |
| Isolate 3 (Aspergillus) | 5.0 | 10.0 | 12.0 | 14 |

| | | | | |
|---|---|---|---|---|
| Isolate 4 (Aspergillus) | - | - | 3.0 | 4.0 |
| Isolate 5 (Mucor) | 2.0 | 4.0 | 4.0 | 5.0 |
| Isolate 6 (Mucor) | 1.0 | 2.0 | 5.0 | 4.0 |
| Isolate 7 (Thermomyces) | 3.0 | 3.0 | 5.0 | 6.0 |
| Isolate 8 (Chaetomium) | 1.0 | 2.0 | 2.0 | 3.0 |
| Isolate 9 (Aspergillus) | 2.0 | 8.0 | 7.0 | 8.0 |
| Isolate10 (Thermomyces) | 3.0 | 4.0 | 5.0 | 6.0 |
| Isolate 11 (Pencillium) | 2.0 | 4.0 | 4.0 | 6.0 |
| Isolate 12 (Aspergillus) | 3.0 | 6.0 | 7.0 | 7.0 |
| Isolate13 (Thermomyces) | 4.0 | 5.0 | 10.0 | 7.0 |
| Isolate 14 (Aspergillus) | 2.O | 5.0 | 8.0 | 10.0 |

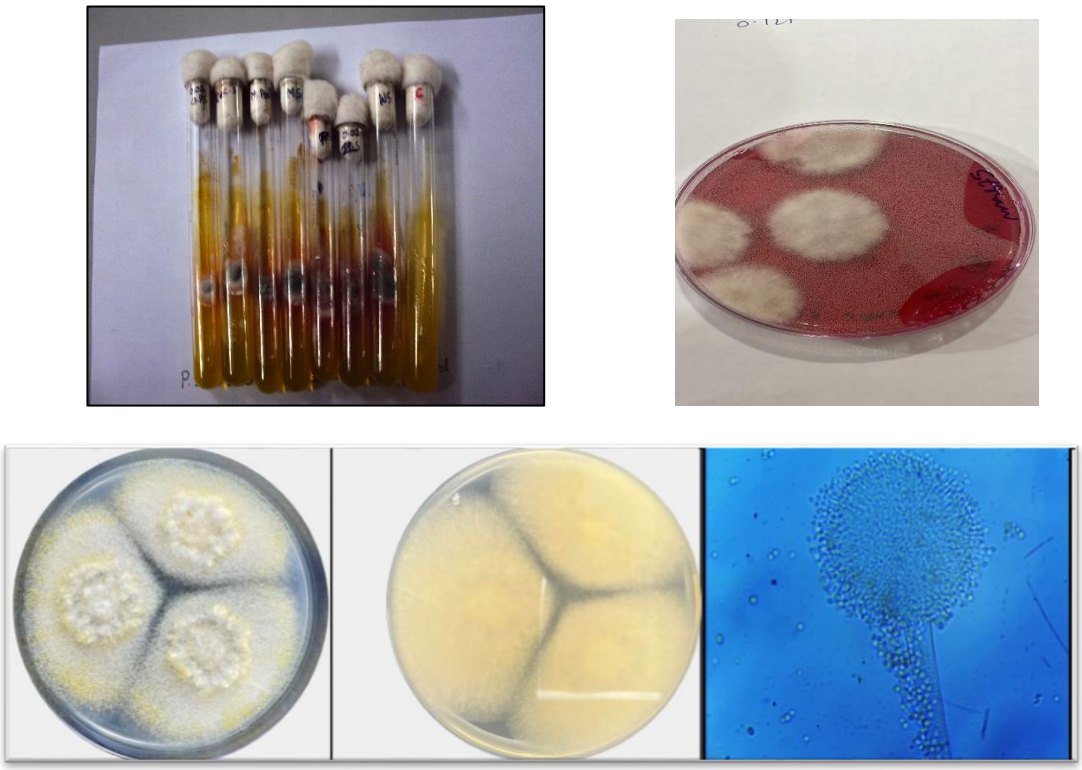


**Figure 1.** Thermophilic fungi isolated from various decomposing wastes

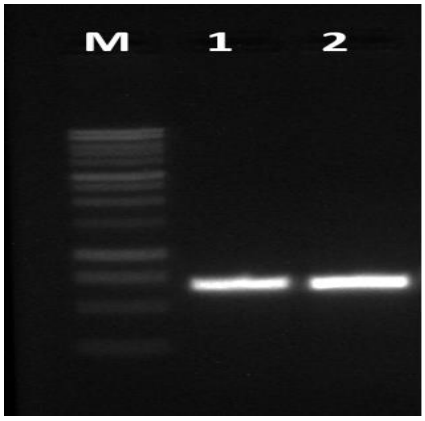



**Figure 2.** *Aspergillus oryzae RIB40* DNA was confirmed via 1% agarose gel electrophoresis

The culture, which was labeled as Sample-2 (Isolate 2) was found to be *Thermomyces lanuginosus* strain ATCC 200065 (GenBank Accession Number: EF468715.1) and Sample-3 (Isolate 3) was identified as *Aspergillus oryzae RIB40* based on nucleotide homology and phylogenetic analysis

## Conclusion

These findings suggest that decomposing wastes are a rich and viable source of novel, potent L-asparginase-producing thermophilic fungi, which have potential applications in both the pharmaceutical and food industries.

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