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Extraction, characterization of L-Asparaginase-Producing Fungi isolated from Diverse Soil Samples and exploring its antimicrobial activity: Emphasis on Aspergillus fumigatus for L-Asparagine Production

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Abstract: The objective of this study was to extract and characterize L-asparaginase from a thermophilic fungus, *Aspergillus fumigatus* which was isolated from different soil samples, and explore its antimicrobial activity. Six strains of fungi were isolated from soil samples at Mansoura University, identified, and screened for their ability to produce L-asparaginase using a modified Czapek-Dox medium with L-asparagine. Among the isolated fungi, *Aspergillus fumigatus* exhibited the highest L-asparaginase activity, identified through screening on Czapek's agar media. The organism was cultivated under submerged fermentation conditions, yielding L-asparaginase at its maximum activity at 40 °C and pH 8.0 after 30 minutes. The findings suggest that *Aspergillus fumigatus* from the soil samples has significant L-asparaginase activity and could be further explored for commercial L-asparagine production, given the enzyme's effective use as an antibacterial agent.

Keywords: Aspergillus fumigatus; L-Asparaginase, Optimization; Purification

Introduction

L-asparaginase (EC 3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) has gained increased awareness of its anticarcinogenic potential [1].

Furthermore, current chemotherapeutic drugs often result in harmful effects on non-target tissues. Conversely, medicinal plants are utilized to address ailments and promote balance by targeting specific biomolecules and pathways [2]. These natural remedies have evolved through extensive experimentation. The widespread use of herbal extracts in local communities is attributed to their effective biological responses and cost-effectiveness [3]. More than 80% of the global population relies on traditional medicinal remedies for healthcare support, as they are less prone to side effects and prove to be more effective [3].

Pharmaceutical enzymes serve as effective medications for a range of diseases, including cancer and myocardial infarction [3]. L-asparaginase (l-ASNase) stands out as a potent anticancer agent, demonstrating effectiveness in treating certain lymphomas and leukemia in both human and animal trials. When used in conjunction with other agents, l-ASNase is employed for the treatment of various conditions, including acute lymphoblastic leukemia (particularly in children), reticle sarcoma, Hodgkin's disease, acute myeloblastic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, and melanosarcoma as part of chemotherapy [4]. This enzyme, categorized within the homologous family of amylohydrolases, plays a crucial role in converting l-asparagine to aspartic acid and ammonia [5]. The anticancer potential of l-ASNase has garnered significant attention in recent years [6].

Commercially available antitumor enzymes for clinical use are obtained from *Escherichia*

coli and *Erwinia carotovora* and are usually Elspar, Oncaspar, Ervinase, and Hydrolase. It is sold under the name (Hydrolase) and causes therapeutic reactions and side effects [7-10]. To prevent anaphylaxis harmful and therapeutic enzymes, L - Asparaginase has been identified from plant medicinal sources. Members of the Solanaceae family are potent sources of L-asparaginase for antiproliferative/antitumor activity against hepatocellular carcinoma, colon cancer, gastric cancer, lung cancer, bladder cancer, and breast cancer cell lines [11]. Regarding enzyme concentration, significantly higher concentrations of L-asparaginase were also reported in *Vigna radiata* and *Tamarindus indica* (Fabaceae).

Solanum nigrum, [12] a medicinal plant source known as "Black Nightshade" and "Makoh" belongs to the genus Solanum, family Solanaceae. S. nigrum is widely used worldwide for a variety of conditions, including convulsions and epilepsy, pain, and ulcers. and inflammation., diarrhea, some eye infections, and jaundice. SN (fruit and leaf parts) contain flavonoids, phenolic compounds, tocopherols, polysaccharides, glycoalkaloids, and glycoproteins that have antiproliferative effects due to their immunomodulatory properties. Flavonoids and glycoalkaloids also provide antifungal and anti-inflammatory activities by activating pro-apoptotic factors or inhibiting transcription factors that play a critical role [13]. Numerous studies have demonstrated the in vitro antitumor efficacy of SN in hepatocellular carcinoma cells, human [14] ovarian carcinoma cells, human colon carcinoma cells, and human endometrial carcinoma cells. [15-16].

The increasing commercial demand for therapeutic enzymes in cancer treatment and the production of natural antibiotics and antifungal medications present new research opportunities. Utilizing plants as a cost-effective and readily available source of enzymes has become advantageous, particularly in achieving high concentrations of L-asparaginase with significantly increased activity and to avoid severe hypersensitivity reactions, it is preferable to extract L-asparaginase from medicinal plants or edible sources rather than relying on microbial or fungal sources. So, our context was conducted to extract and characterize L-asparaginase from thermophilic fungi isolated from different soil samples and explore its antimicrobial activity.

2. Materials and methods

Collection of soil samples and isolation of thermophilic fungi:

In this study, the fungi used were isolated from soil samples collected from different areas of Mansoura University. Soil fungal strains were isolated by using the dilution plating technique [17]. The modified Czapek Dox medium containing (g/L of distilled water): 2.0 glucose, 10.0 L-asparagine, 2.0 K2HPO4, 0.52 NaCl, 0.2 MgSO4·7H2O, 0.005 CacCl2.2H2O, 6.0 Na2HPO4,0.09 Phenol 0.07 BTB, and 20.0 Agar at pH 5.5 was used for maintaining isolated organisms.

Microscopic identification of the isolated fungi:

The resulting fungal colonies were subcultured onto Czapeck-Dox agar medium for identification and then each pure culture was characterized and identified based on its morphological and microscopic characteristics, sporulation, and colony color. The examination was done as described by [18], using needle mount preparation. The fragment of the sporing surface of the culture was teased out in a drop of alcohol on a clean glass slide using a needle. The fragment was stained by adding a drop of lactophenol and a cover slip was applied carefully to avoid air bubbles, then the preparation was examined under a light microscope. The purified isolated fungi were identified to the species level whenever possible. The identification of fungal genera and species was done with the help of the following universally accepted keys for identification of different isolated fungi [19 - 20].

Screening of L-asparaginase producing fungi:

The fungi isolated from the above steps were subjected to rapid screening of L-asparaginase production by plate assay [21]. Modified Czapek Dox's (mCD) medium with pH 6.2, used for fungi, contained 0.2% glucose, 1% L-asparagine, 0.152% KH2PO4, 0.052% KCl, 0.052% MgSO4.7H2O, 0.003% CuNO3.3H2O, 0.005% ZnSO4.7H2O, 0.003% $FeSO4.7H_2O$,

and 1.8% agar. The initial pH 6.2 was supplemented with 0.009% phenol red as an indicator [22]. Control plates were mCD medium containing a nitrogen source ($NaNO_3$) instead of asparagine. The six chosen fungal isolates were plated and incubated at 30°C for 48 hours. To determine the enzyme activity, the isolates that had a pink zone around the colonies indicated the production of the L-asparaginase enzyme.

L-asparaginase production by submerged fermentation:

Using modified Czapek Dox's liquid media, submerged fermentation was used to produce L-asparaginase. Using a primary screened organism, an Erlenmeyer flask was inoculated with 100 mL of the proper medium. The flasks were incubated for different incubation times (24–168 hours) at 30°C. Controls include inoculated media. By filtering through the Whatman No. 1 filter paper, the cultures were collected. To estimate enzyme activity, the culture filtrate was used as a crude enzyme.

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Assay of L-asparaginase activity:

The amount of ammonia released during nesslerization was used to calculate the extracellular L-asparaginase activity by the method of Wriston and Yellin [23]. The reaction mixture has a total volume of 2 mL and is composed of 1.5 mL of 0.04 mL-asparagine prepared in a 0.05 M Tris-HCl buffer, pH 8.6, and 0.5 mL of the enzyme. For 30 minutes, the tubes were incubated at 37 °C. The reaction was terminated by the addition of 0.5 mL of 1.5 M trichloroacetic acid (TCA). The blank was run by adding TCA followed by enzyme preparation. The contents of the reaction were centrifuged at 10,000 g for 5 min. The filtrate was then collected. 0.5 mL of filtrate was diluted to 7 mL with distilled water, and 1 mL of Nessler's reagent was then added to the resulting mixture. Before measuring the absorbance at 480 nm using a spectrophotometer, the color reaction was allowed to continue for 20 min. By comparing the absorbance with a standard curve made from solutions of ammonium chloride as the ammonia source, the amount of liberated ammonia in the test sample was calculated. One unit (U) of L-asparaginase is the amount of enzyme which generates 1μmole of ammonia in 1 min at 37 °C and pH 8.6.

Microbial susceptibility testing:

Agar well diffusion method:

The agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 9 mm is punched aseptically with a sterile cork borer or a tip, and a volume (100 μL) of sample at the desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending on the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested [24].

Tested bacterial strains:

Bacillus cereus, Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Staphylococcus aureus and Staph. epidermidis.

Determination of Minimum Inhibitory Concentration (MIC):

Serial dilutions of bacterial suspension in different concentrations were used to determine MIC in a nutrient broth medium. The control contained only inoculated broth and incubated for 24 h at 37 °C. The MIC endpoint is the lowest concentration of bacterial suspension where no visible growth is seen in the tubes. The visual turbidity of the tubes was noted, both before and after incubation to confirm the MIC value and O.D was measured at 600 nm to confirm the result [25]. The tested bacterial strain was *Bacillus cereus.*

3. Results and Discussion

Isolation and screening of microorganisms

Six distinct fungal cultures were isolated

from the soil sample. The cultures were identified morphologically as Aspergillus fumigatus (**Figure 1**), Penicillium chrysogenum, Penicillium digitatum, Penicillium purpurogenum, Rhizomucor miehei and Rhizomucor pusillus.

The identified fungi displayed L-asparaginase activities. A qualitative, straightforward, and quick method of evaluating L-asparaginase production is the plate method. Plate assay technology was used to test the fungus that produces the enzyme L-asparaginase. By using the plate assay method, the potential strains were chosen based on the pink zone surrounding the colony as shown in **Figure 2**. It is made clear that strains with zones larger than 15 mm in diameter are thought to be high L-asparaginase producers. **Figure 2** depicts the pink zone surrounding the colony of *Aspergillus fumigatus*. The pH of the culture filtrates generally rises in tandem with L-asparaginase production. By including the pH indicator phenol red in a medium containing asparagine (the only nitrogen source), the plate assay was created using this theory. A pink zone forms around microbial colonies that produce L-asparaginase because phenol red is yellow at acidic pH and turns pink at alkaline pH. This culture was subsequently submitted for submerged and solid-state fermentation. The plate assay has the benefit of being quick and allowing for the direct visualization of L-asparaginase production from the plates without the need to perform time-consuming assays [19-21].

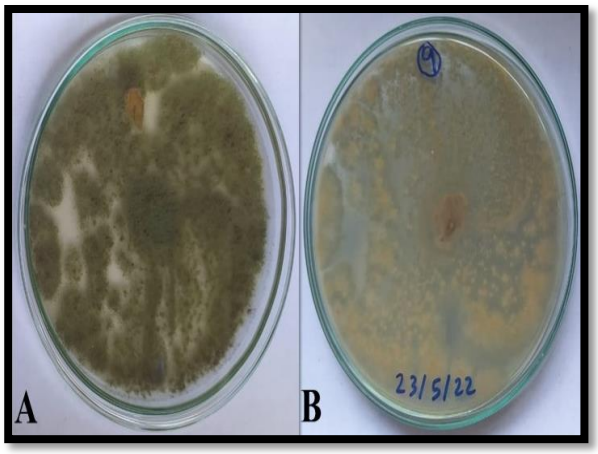

Figure 1: Plates showing the cultures of *Aspergillus fumigatus* on modified Czapek Dox's (mCD) medium without an indicator (phenol red) (A. Forward and B. Backward)

Figure 2: Plate showing pink color zone around the culture of *Aspergillus fumigatus* on modified Czapek Dox's (mCD) medium with indicator (phenol red)

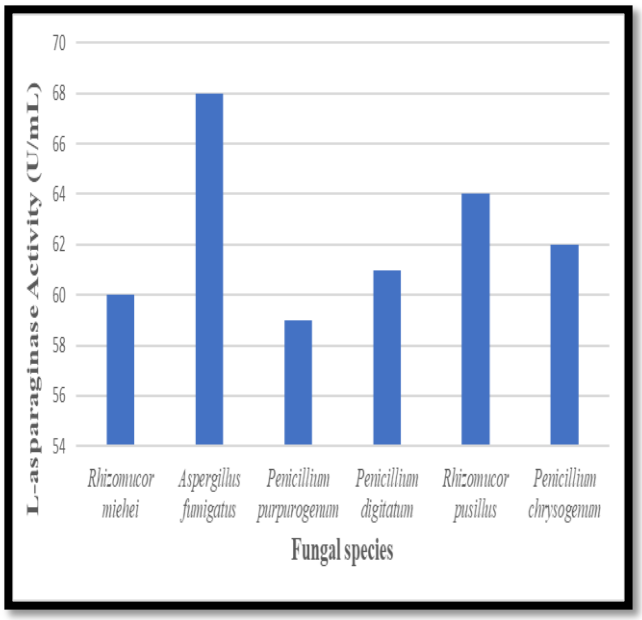

Figure 3: L-Asparginase activity of the isolated fungi

Submerged fermentation:

L-asparaginase production peaked at 80 U/ml in 30 minutes of time of incubation **(Figure 4).** Further extensions of the incubation time resulted in a decline in enzyme production rather than any discernible increase. Therefore, 30 minutes was determined to be the ideal time for enzyme synthesis. following immunization.

Fungal asparaginases are utilized as food additives. Due to fungi's capacity to use inexpensive media and produce the enzyme extracellularly, which facilitates the purification procedure, they are significant. Out of all the tested fungal isolates, *Aspergillus fumigatus*

produced the most L-asparaginase in the current study. *Aspergillus fumigatus* L-asparaginase production was identified in the current study by the formation of a pink zone around the colony. The pink zone was produced when L-asparaginase broke down the amide bond in asparagine in the growth medium to produce aspartate and ammonia, which caused phenol red to turn pink [29].

As the incubation period was extended up to 50 minutes, *Aspergillus fumigatus* L-asparaginase activity gradually increased. However, a longer incubation period with the substrate led to a decrease in L-asparaginase activity, which may have been caused by the product's inhibition. At 40 minutes of incubation, *Aspergillus fumigatus* L-asparaginase which had been purified exhibited its peak activity [32]. L-asparaginase isolated from *Pseudomonas aeruginosa* 50,071 also reached its peak activity after 30 minutes [33].

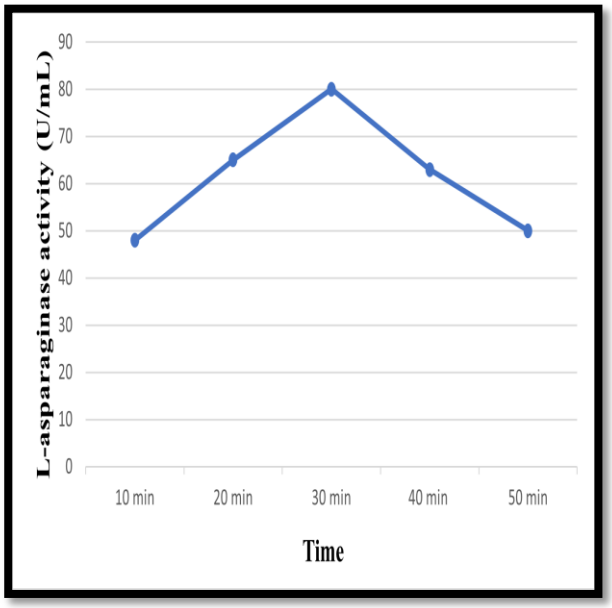

Figure 4: Effect of time on L-asparaginase activity extracted from Aspergillus fumigatus (U/mL)

The antimicrobial test by disc diffusion assay of L-asparaginase extracted from *Aspergillus fumigatus* against 8 different pathogenic bacteria with Tetracycline as +ve control showed significance inhibition zones (mm) against *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staph. epidermidis* as shown in **Figure 5** and **Table 1**. So it has been chosen to carry out the next experiments.

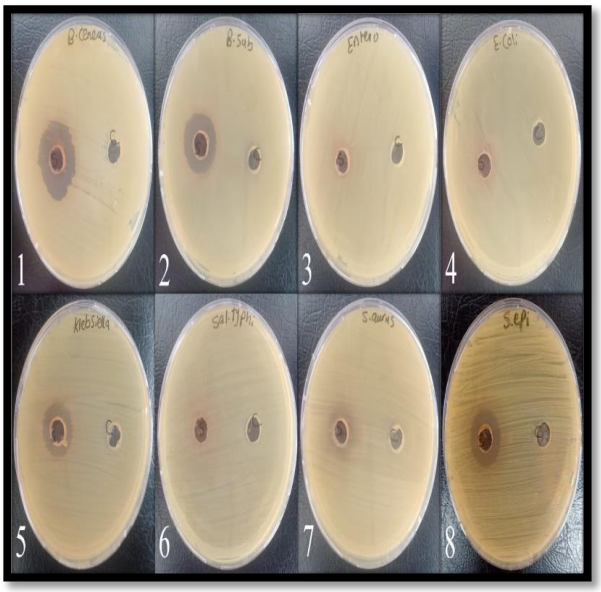

Figure 5: Antimicrobial activity of L-asparaginase extraction against 1. *Bacillus cereus*, 2. *Bacillus subtilis*, 3. *Enterobacter cloacae*, 4. *Escherichia coli*, 5. *Klebsiella pneumoniae*, 6. *Salmonella typhi*, 7. *Staphylococcus aureus* and 8. *Staph. Epidermidis*.

Table 1: Antimicrobial activity of L-asparaginase extraction against different bacterial species

Bacterial species	Diameter of clear zone by L-asparaginase extraction (mm)	Control
Bacillus cereus	25	-ve
Bacillus subtilis	20	-ve
Enterobacter cloacae	-ve	-ve
Escherichia coli	-ve	-ve
Klebsiella pneumoniae	19	-ve
Salmonella typhi	-ve	-ve
.Staphylococcus aureus	12	-ve
Staph.epidermidis	19	-ve

The MIC value was taken as the minimum concentration of L-asparaginase extracted from *Aspergillus fumigatus* against bacterial growths of *Bacillus cereus* at which no microbial growth was observed.

The MIC of L-asparaginase was 0.0125 g/mL against bacterial growths of *Bacillus cereus*. The tube's turbidity was noted after incubation to confirm the MIC value.

Flavonoids and phenolics (phytochemicals) interaction with a murine component of bacterial cell wall and Chitin monomer 2-acetamido-2-deoxy-beta-D-glucopyranose prove the effective interaction with murine and chitin, respectively. Additional validation studies are necessary to ascertain the extracts' cytotoxicity and the optimal dosage of therapeutic formulations.

Conclusions:

This study not only highlights the abundance of L-asparaginase-producing fungi in soil but also sheds light on the exceptional capabilities of *Aspergillus fumigatus* in terms of enzyme production and its potential applications in the medicine and food industries. Aspergillus fumigatus is an important source of L-asparaginase that has the potential to be used in medicine because it is easy to clean and has strong antimicrobial properties.

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