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ISOLATION AND SCREENING FOR L. ASPARGINASE PRODUCING FUNGI

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ABSTRACT

L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is a promising chemotherapeutic agent which plays a vital role in treatment of a variety of lymphoproliferative disorders, lymphosarcoma and acute lymphoblastic leukemia in particular. It is also used as a processing aid in the manufacture of food. Soil samples obtained from various places in and around Hyderabad, AP were screened for L-Asparginase producing fungi by a rapid plate assay. 28 Isolates were screened for L- asparginase activity by plate assay and 12 isolates were showing activity. Among the isolates *Fusarium s*p. showed high amount of L-asparaginase activity. The maximum yield of L-asparaginase was found to be 2. 72 IU.

Keywords: L- Asparginase, Anti Tumor agent, Acute Lymphoblastic leukemia (ALL)

INTRODUCTION

Asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an enzyme which converts L-asparagine, a non essential amino acid, to L-aspartic acid and ammonia. The therapeutic potential of L-Asparginase in most patients suffering from acute lymphoblastic leukemia (ALL) & lymphosarcoma is well documented [1, 2]. L-Asparginase has found use in the food industry also as it is a food processing aid to reduce the formation of acrylamide, a suspected carcinogen, in baked food products

such as snacks and biscuits. Asparagine which is normally present in starchy foods, undergoes Maillard reaction, giving the baked or fried foods their brown color, crust and toasted flavor. Certain carcinogens like acrylamide and some heterocyclic amines are also formed in this reaction. If L-asparaginase is added to the food before baking, it converts asparagine into aspartic acid, and ammonium ions. As a result, asparagine cannot take part in the Maillard reaction, and therefore the formation of acrylamide is significantly reduced [3].

Asparaginases are widely distributed in nature from bacteria to mammals and play a central role in the amino acid metabolism and utilization. In human body, L-aspartate is a precursor of ornithine in the urea cycle and has a crucial role in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose formation. L-asparaginase is the first enzyme with anti leukemic activity to be intensively studied in human beings. In healthy cells aspartate is converted to aspargine by the enzyme aspargine synthetase. Tumor cells require the amino acid Aspargine in high amounts for their growth, however these cells have very low levels of L-asparagine synthetase and cannot synthesize enough endogenous L-asparagine. They are therefore dependent on serum levels of asparagine for their proliferation and survival [4]. The enzyme L-Asparaginase hydrolyses L-Asparagine making it unavailable as a nutrient and prevents their malignant growth. Healthy cells on the other hand have normal levels of the enzyme L-asparagine synthetase and are capable of synthesizing Asparagine de novo and are not affected. Several microorganisms like bacteria, Actinomycetes, Fungi and yeasts were shown to produce L-Asparginases. To date only two native (*E.coli* and *Erwinia caratovora*) and one pegylated *E.coli* L-asparaginase preparations are available for therapy of ALL [5]. However, L-asparaginase from bacterial origin can cause hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis [6]. The search for other asparaginase sources, like eucaryotic microorganisms such as fungi, can lead to an enzyme with less adverse effects and a better therapeutic enzyme. This study was therefore taken up to screen for L-Asparginase producing Fungi.

MATERIALS AND METHODS

Sample collection: The soil samples were collected from different areas in and around Hyderabad. Telangana. The samples were collected using sterile spatula and were

transferred to new zip lock bags. The samples were transported to the laboratory for the isolation of Fungi.

Fungal Isolation: The soil samples were serially diluted and the aliquots were plated on Czapek Dox Agar (NaNO3 2.0 g, KCl 0.5 g, K2HPO4 1.0 g, MgSO4. 7H2O 0.5 g, FeSO4. 7H2O 0.01 g,); and Potato Dextrose Agar (Peeled potato200.0 g, Dextrose 20.0g) media. The plates were incubated for 24-96 h at 28^0C, and each morphologically distinct fungal colony was sub-cultured. The fungal cultures were identified based on their colony morphology, whether aerial mycelium is present or absent, colony colour, presence of wrinkles and furrows, pigment production, conidiophores, conidia etc and microscopic morphology using taxonomic guides and standard procedures.[7]

Screening for Asparginase activity: Screening of asparginase activity was done by rapid plate assay [8].Modified Czapek Dox Medium supplemented with phenol red dye , Glucose - 0.2%, L- Asparagine - 1%, Dipotassium hydrogen phosphate - 0.152%, Potassium chloride - 0.052%, Magnesium sulphate 0.052%, Copper nitrate- 0.003%, Zinc sulphate- 0.005%, Ferrous sulphate - 0.003%, Agar-1.8%, Ph- 6.2, Phenol red - 0.009%, was used for screening fungal isolates. Spot inoculation was done on modified Czapex-Dox Agar plates and incubated at 30^0C for 7 days. The appearance of pink zone around the colony in the medium, after incubation indicated L-asparaginase activity. The diameter of the colonies and that of the pink zone were measured and zone index was calculated.

Enzyme Production: The isolates showing pink zones were subcultured onto PDA slants and incubated till sporulation was seen. Spore suspension using 5 ml sterile water containing 0.1% Tween 80 were prepared from fully sporulated PDA slants.10%v/v spore suspension was added to Erlenmeyer's flask containing Modified Czapek Dox broth. The flasks were kept in rotary shaker and agitated at 150 rpm for 72-96 hrs at 30^0C. The cell mass was separated by centrifugation and the clear broth was collected and used as the crude enzyme extract.

Enzyme assay: L-asparaginase activity was determined by measuring the amount of ammonia formed by nesslerization [9] where the rate of hydrolysis of asparagine is determined by measuring released ammonia. One unit of asparginase releases one micromole of ammonia per minute at 37°C and pH 8.6 under the specified conditions.

A mixture of 0.1 ml of enzyme extract, 0.2 ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7 ml of 0.01M L-asparagine was incubated

for 10 min at 37ºC. The reaction was stopped by the addition of 0.5 ml of 1.5M trichloroacetic acid. Clarification was done by centrifugation and 0.5 ml of clear supernatant was added to 7.0 ml reagent grade water. 1.0 ml of Nessler's reagent was added and incubated at room temperature for 10 minutes. The absorbance of "Test" tube versus the respective "Blank." was read at 480 nm. Micromoles of ammonia released from an ammonium sulfate standard curve were determined. Standard was run in parallel by adding 0.5 ml of 1 µmole solution to 7.0 ml water and 1 ml of 1 umole solution to 6.5 ml reagent grade water and adding 1 ml Nesslers reagent to both.

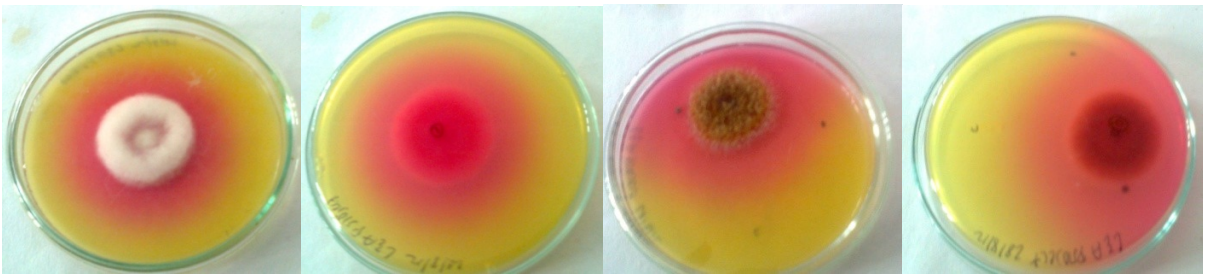
RESULTS & DISCUSSION

A total of 28 fungal isolates were obtained from 11 different samples. 12 isolates were found to be positive in primary screening (Table1 & Fig 1). The zone indexes were ranging from 1.42 to 3.33.Highest zone index was exhibited by an *Aspergillus* isolate (S104)(Fig 1c& d); however the enzyme activity of this isolate was only 1.235 IU/ml. An isolate of *Fusarium sps* (S105) (Fig 1a&b) showed an enzyme activity of 2.72 IU/ml, but the zone index was only 1.60. The Zone index and Enzyme activity of some fungal isolates did not show any relation ship. This result was similar to that reported by SoniyambyA.R *et al* .[10], Balakrishnan .K reported L.asparginase activity of different fungal & yeast isolates ranging from .002Iu to .0.884IU/ml.[11] In another study of fungal endophytes the enzyme activities were found to occur in the range of 0.006–1.136 unit/mL, and the isolates of *F. graminearum* from bark and twig exhibited high asparaginase activities of 0.950IU and 0.836IU and *F. verticillioides* showed highest activity among all the endophytic fungi with 1.136IU of enzyme. [12]. L.-Asparginase activity ranging from 0.75 to 3.75 U/ml of different fungal isolates were reported by Soniyamby A.R *et al* [10]. Many strains of Fusarium produce more than 0.5 IU/ml to 2IU/ml in their cultural filtrates [13].A maximum yield of 8.51 IU/ by *Fusarium equisetum* was obtained after optimizing process parameters in Solid state fermentation [14]. The isolate S101 which was identified as *Fusarium* sps exhibited good L- Asparginase activity (2.72IU/ml). Optimizing of process parameters and strain improvement of this isolate may give better yields and needs to be further investigated. The *E.coli* L-asparaginase is associated with significant glutaminase activity. This glutaminase activity has both advantageous

as well as deleterious effect. The main consequence of glutaminase activity is decreased synthesis of protein, which has deleterious effects. Asparaginase from several mold including *Fusarium, Penicillium, and Aspergillus* were reported. All these fungal asparaginases exhibited anti-lymphoma activity and also the enzyme was not found to act on L-glutamine [5]. Therefore Fungal Asparginases may be better alternatives to *E.coli* Asparginases.

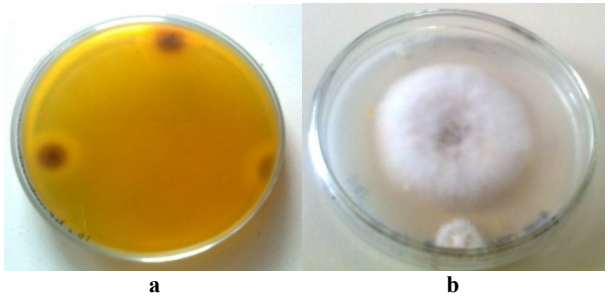
Table 1: L-asparaginase activities of various Fungal isolates

Isolate number	Isolate	Zone index	IU/ml
SI01	*Fusarium sps*	2.75	1.51
SI02	*Aspergillus sps*	1.42	1.695
SI03	*Aspergillus sps*	3	0.95
SI04	*Aspergillus sps*	4.16	1.235
SI05	*Fusarium sps*	1.6	2.72
SI06	*Aspergillus sp*	2.69	0.345
SI07	*Cladosporium sp*	3	0.37
SI08	*Aspergillus sp*	3.33	0.66
SI09	*Penicillium sp*	2.5	0.1
SI10	*Penicillium sp*	1.94	0.485
S11	*Aspergillus sps*	1.5	0.27
S12	*Fusarium sps*	2.2	1



a b c d

Fig 1: Isolates showing pink zones indicative of Asparginase production a,& b Fusarium sps; c &d Aspergillus sps



a b

Fig 2: a. Isolates not exhibiting Asparginase activity on modified Czapex Dox agar; b. Fusarium sps on plain Czapek Dox agar

CONCLUSION

The present study was done to screen for L-asparginase producing Fungi.42% of the fungal isolates screened were positive for L-asparginase activity. The isolated fungi were identified *Aspergillus* and *Fusarium sp.* showed good activity. Further studies on optimizing production and characterization of the enzyme have to be done in future to realize the potential of these isolates for production of L. Asparginase.

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