

# Isolation and Screening of L-Asparaginase Producing Fungi from Kadalundi Mangrove Forest Soil



## Microbiology

**KEYWORDS :** L-asparaginase, *Mangrove*, *Aspergillus oryzae*, *Byssoschalymus niveus*

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### ABSTRACT

*L-asparaginase (E.C.3.5.1.1) has chemotherapeutic property and has been used in the treatment of acute lymphoblastic leukemia and lymphosarcoma. Microorganisms such as bacteria and fungi produce asparaginase and have wide application in medical and food industry. In the present study, eight fungal isolates were obtained from the soil samples collected from Kadalundi mangrove forest. The isolated fungi were further screened for production of L-asparaginase using Czapek's Dox Agar medium using plate assay and among them 2 isolates showed significant enzyme activity. The isolates were identified as Aspergillus oryzae and Byssoschalymus niveus and their L-asparaginase production was carried out under submerged fermentation. Aspergillus oryzae showed maximum enzyme production.*

### INTRODUCTION

L-asparaginase (E.C 3.5.1.1) is a tetrameric protein that hydrolyzes free L-asparagine to aspartic acid and ammonia (Goodsell D S, 2005; Verma *et al.*, 2007). L-asparagine is an essential aminoacid for the production of protein in tumor cells whereas the growth of normal cell is independent of its requirement. L-asparaginase can be produced within the cells by an enzyme called asparagines synthetase and can be absorbed from the outside (Soni Yadav *et al.*, 2014). Lymphatic tumor cell require huge amount of asparagines to keep up their malignant growth. In the presence of L-asparaginase tumor cell get deprived and cannot survive (Ali *et al.*, 1994; Berenbaum *et al.*, 1970; Box *et al.*, 1978). L-asparaginase is an enzyme drug which is used to reduce the circulatory L-asparagine level, stopping tumor cell growth which is widely used in the treatment of acute lymphoblastic leukemia (Granda *et al.*, 2004). L-asparaginase are among the largest among group of therapeutic enzymes as they account for about 40% of the worldwide sale of anti leukemic and antilymphoma agents (Warangkar *et al.*, 2009).

L-asparaginase production using microorganisms have received great awareness, as the demand for this therapeutic enzyme is increasing several folds every year (Gurunathan and Sahadevan, 2011). A wide range of microorganisms such as filamentous fungi, yeasts and bacteria have proved to be beneficial sources of this enzyme. Bacterial enzymes have shown high potential against children's acute lymphoblastic leukemia but shows hypersensitivity reactions. Hence the search for new potential L-asparaginase by eukaryotes is getting wide attention (Sarquis *et al.*, 2004). The presence study is aimed to isolate asparaginase producing fungi from mangroves and to screen suitable fungi for large scale application.

### MATERIALS AND METHODS

#### COLLECTION OF SAMPLE

Soil samples were collected from Kadalundi mangrove forest located in Calicut district of Kerala. The surface soil is composed of silt and clay loam and is about 3-4 m in depth. The central portion of the top 2cm sediment soil sample was collected in sterile polythene bag and transported to the laboratory.

#### ISOLATION OF FUNGI

Soil samples were serially diluted and spread on Czapek dox agar medium. The inoculated plates were kept for incubation at 28°C for 2-3 days.

### SCREENING FOR L-ASPARAGINASE PRODUCTION

All the isolates were tested for L-asparaginase activity using modified Czapek dox agar containing glucose-2.0, L-asparagine-10, KH<sub>2</sub>PO<sub>4</sub> -1.52, KCl - 0.52, MgSO<sub>4</sub>.7H<sub>2</sub>O - 0.52, CuNO<sub>3</sub>.3H<sub>2</sub>O - trace, ZnSO<sub>4</sub>.7H<sub>2</sub>O - trace, FeSO<sub>4</sub>.7H<sub>2</sub>O - trace (g/L of distilled water) at pH 6.2 and 0.09% (v/v) phenol red as indicator to the media. Control plates were of modified Czapek dox medium containing NaNO<sub>3</sub> instead of L-asparagine as nitrogen source and phenol red dye. The plates were inoculated with the 8 selected fungal isolate and incubated at 30°C for 72 hrs. Development of pink colour zone around the colonies indicates L-asparaginase production and isolates that developed pink colour zone were selected for submerged fermentation.

### L-ASPARAGINASE PRODUCTION BY SUBMERGED FERMENTATION

L-asparaginase production was carried out by submerged fermentation using primary screened organisms in modified Czapek dox liquid medium. The enzyme production was carried out by inoculating the fungal strains in 100 ml of production media supplemented with 0.1% L-asparagine. The inoculated fungal cultures were incubated at 30°C. The biomass harvested after growth was filtered using Whatman No: 1 filter paper and crude enzyme was separated. Estimation of enzyme production was carried out at different time intervals (24 – 120 hrs) using the crude enzyme.

### PROTEIN ESTIMATION

Estimation of protein was carried out by Lowry *et al.*, (1951) method using Bovine Serum Albumin as standard.

### DETERMINATION OF ENZYME ACTIVITY

L-asparaginase activity was measured using the method of Mashburn & Wriston. In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reaction. A mixture of 0.1ml of enzyme extract, 0.2 ml of 0.05M Tris-HCl buffer (pH 8.6) and 1.7 ml of 0.01 M L-asparagine was incubated for 10 min at 37°C. The reaction was stopped by addition of 0.5ml of 1.5 M trichloroacetic acid. After centrifugation at 10000rpm at 4°C, 0.5 ml of the supernatant was diluted to 7 ml with distilled water and treated with 1ml of Nessler's reagent. The color reaction was allowed to develop for 10 min and the optical density was read at 480nm with spectrophotometer. The ammonia liberated was entrapped from a curve derived with ammonium sulphate as standard. One unit (IU) of L-asparaginase was defined as the amount of enzyme

which liberates 1  $\mu\text{mol}$  of ammonia per minute under the assay conditions. (David and David, 1974).

### IDENTIFICATION OF FUNGI

Fungal isolates that showed higher activity were chosen and identified based on morphological and microscopical features. Microscopical identification was carried out by lactophenol cotton blue staining. Fungi was identified from National Centre for Fungal Taxonomy, New Delhi.

### RESULTS AND DISCUSSION

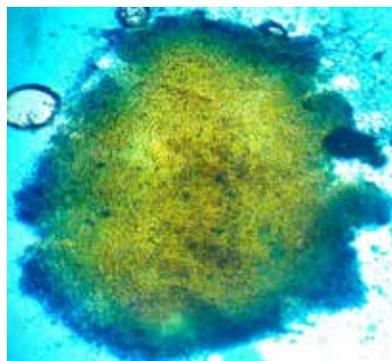
A total number of eight fungal isolates were obtained from the soil samples collected from Kadalundi mangrove forest and were screened for L-asparaginase production using Czapek dox agar medium. All the eight isolates showed positive for L-asparaginase activity by developing pink colour zones around the colony in plate assay method. Monica *et al.*, (2014) have reported the isolation of L-asparaginase producing fungi *Mucor hiemalis* from the soil of Karnataka. Lynette *et al.*, (2014) reported L-asparaginase producing fungi from marine soils of Mangalore beach. Nagarajan *et al.*, (2014) isolated L-asparaginase producing fungi from endophytic region of plants. The fungal isolates were further subjected to secondary screening for enzyme activity by Mashburn and Wriston method. Among the eight isolates, KMG-F4 and KMG-F6 showed higher activity of 74.5 IU/ml and 69.3 IU/ml respectively (Table 1). The remaining isolates showed activity ranging from 12.4 IU/ml to 31.3 IU/ml. Ram kumar *et al.*, (2016) have reported 111 IU/ml of L-asparaginase production from *Fusarium* sp, Sarquis *et al.*, (2004) reported 58 IU/ml of L-asparaginase production from *Aspergillus terreus*. Enzyme activity of 278 IU/ml and 167 IU/ml by *Penicillium* sp. were reported by Patro and Gupta (2014) and Mushtaq *et al.*, (2012) in optimized submerged fermentation conditions.

The fungal isolates KMG-F4 and KMG-F6 were identified as *Aspergillus oryzae* and *Byssoschlamys niveus*. Mangroves provide a good source of microorganisms and these fungal isolates from mangroves will be a potent source for L-asparaginase production. Further optimization of bioprocess parameters will enhance the level of enzyme activity and these two fungi and can be used in large scale production for therapeutic purpose.

**Table 1: L-asparaginase activity of fungal isolates**

S.No	Fungi	Enzyme activity (IU/ml)
1	KMG-F1	12.4
2	KMG-F2	7.6
3	KMG-F3	28.7
4	KMG-F4	74.5
5	KMG-F5	31.3
6	KMG-F6	69.3
7	KMG-F7	14.3
8	KMG-F8	19.7

**Fig 1: Microscopic observation of *Aspergillus oryzae* and *Byssoschlamys niveus*.**



### REFERENCES

1. Ali S. S., Rai V., Soni K., Kulshrestha P. and Lai S. K., 1994. A fungal L-asparaginase with potential antitumor activity. *Ind. J. Microbiol.*, 34: 73-76, 1994.
2. Asparaginase Assay. Worthington enzyme manual 1993, Published by Worthington Biochemical corporation. Available from: <http://www.worthington-biochem.com/index/manual.html>
3. Berenbaum M. C., Ginsburg H. and Gilbert D. M., 1970. Effects of L-asparaginase on lymphocyte target cell reactions In vitro. *Nature*, 227: 1147-1148. PMID: 4915992.
4. Box G. E. P., Hunder W. G. and Hunder S. J., 1978. Statistics for experiments. John Wiley & Sons Inc, New York.
5. David K. and David G., L-Asparaginase production by the rumen anaerobe *Vibrio succinogens*. 1974. *J. Appl. Microbiol.*, 27: 206-209.
6. Goodsell D.S., The molecular perspective L-asparaginase, 2005. *Stem cells* 23: 710-711.
7. Granda C., Moreira R. G. and Tichy S. E., Reduction of acrylamide formation in potato chips by low-temperature vacuum frying, 2004. *J of Food Sci.*, 69 (8); 405 - 411.
8. Gurunathan B. and Sahadevan R., 2011. Optimization of media components and operating conditions for exogenous production of fungal L-asparaginase. *Chiang Mai J Sci.*, 38: 270-279.
9. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J., 1951. Protein measurement with the folin-phenol reagent. *J. Biol.Chem.*, 48: 17-25
10. Lynette Lincoln and Sunil S More, 2014. Isolation and production of clinical and food grade L-asparaginase enzyme from fungi. *J. Pharm Phytochem.*, 3(3): 177-183.
11. Mohsin S. M., Sunil Dutt PLNSN., Siddalingeshwara K. G., Karthi J., Jayaramu M., Naveen Mani, Vishwanatha T and Prathibha K. S., 2012. Optimization of fermentation conditions for the biosynthesis of L-asparaginase by *Penicillium* sp. *J. Acad. Indus. Res.*, 1(4): 180-182.
12. Monica Thakur, Lynette Lincoln, Francois N. N. and Sunil S M., 2014. Isolation, purification and characterization of fungal extracellular L-asparaginase from *Mucor hiemalis*. *J. Biocatal Biotransformation*, 2(2): 1000108.
13. Nagarajan A., Thirunavukkarasu N., Suryannarayanan T. S. and Sathyanarayana N. G., 2014. Screening and isolation of novel glutaminase free L-asparaginase from fungal endophytes. *Res. J. Microbiol.*, 9: 163-176.
14. Patro K. K. R. and Gupta N., 2014. Impact of cultural and nutritional conditions on L-asparaginase production by *Penicillium citrinum* thom. *Int. J. Phram. Med & Bio. Sci.*, 3(3): 114-120.
15. Ram Kumar, Vijay Kumar Sedolkar, Triveni A. G., Mendem Suresh Kumar, Channappa T S. and Subhaschandra M Gaddad, 2016. Isolation, screening and characterization of L-asparaginase producing fungi from medicinal plants. *Int. J. Phar. Phar sci.*, 8 (1): 281-283.
16. Sarquis M. I. M., Oliviera E. M. M., Santos A. S. and G L da-Coasta, 2004. Production of L-asparaginase by filamentous fungi, *Mem Inst Oswaldo Cruz.*, 99: 489-492.
17. Soni Yadav, Sitansu Kumar Verma, Jitendra Singh and Ajay Kumar, 2014. Industrial production and clinical application of L-asparaginase: A Chemotherapeutic Agent. *Int. J. Med. H. Bio. Bioeng. Pharma Eng.*, 8(1): 54-60.
18. Verma N., Kumar K., Kaur G. and Anand S., L-asparaginase : A promising chemotherapeutic agent, 2007. *Crit Rev Biotechnol.*, 27: 45-62
19. Warangkar S. C., Khobragade C. N., Dawane B. S. and Bhosale R. B., 2009. Effect of dihydropyrimidine derivatives on kinetic parameters of *E. carotovora* L-asparaginase. *Int J Biotechnol Appl.*, 1: 5-13.