

ISOLATION AND SCREENING OF L-ASPARAGINASE PRODUCING FUNGI FROM SOIL SAMPLES

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ABSTRACT

L-asparaginase has been used as anti-tumor agent for the effective treatment of acute lymphoblastic leukemia and as food processing aid to reduce the acrylamide formation during frying of starchy foods at high temperature. In the present study thirty eight fungal strains were isolated from soils which collected from different parts of India. The isolated fungi were screened for L-asparaginase production using Czapek's agar media. On the basis of pink colour zone formed, one fungal strain was selected and identified as *Aspergillus* sp (KUFS20). A comparative study was carried out to evaluate L-asparaginase production in submerged and solid state fermentation using different substrates. Among the twenty substrates used, orange peel (70.67 ± 1.14 U/g) showed maximum enzyme production.

Keywords: L-asparaginase, *Aspergillus* sp, Solid state fermentation, Antileukemia, Orange peel

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) received increased awareness in current years for its anticarcinogenic potential. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcom and melanosarcoma¹. L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. Asparagine is a nutritional requirement of both normal cells and cancer cells².

Low levels of the nonessential amino acid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirement for asparagine³⁻⁴. This is because normal cells produce enzyme asparagine synthetase, which is able to synthesize asparagine, whereas, in cancer and tumor cells this enzyme is present at low levels⁵.

The effective depletion of L-asparagine results in cytotoxicity for leukemic cells (Figure 1). The FDA has approved L-asparaginase for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma. ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are the brand names of L-asparaginase available in the market.

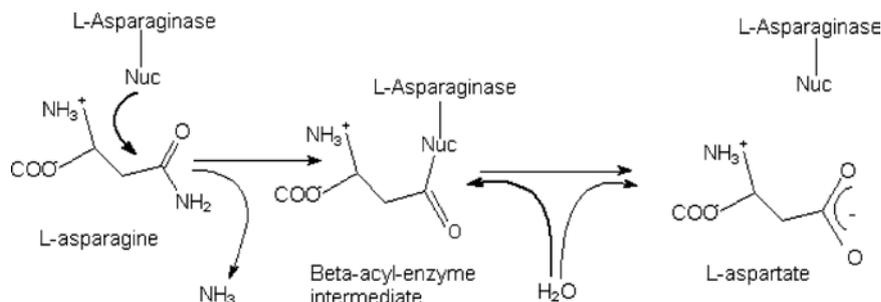


Fig. 1: Schematic illustration of the reaction mechanism of L-asparaginase.

The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack⁶.

L-Asparaginase (L-asparagine amidohydrolyses) has been widely found in biological world. Asparaginase activity is widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi. Major genera of microorganisms reported to produce asparaginase. Bacteria species include; *Erwinia carotovora*, *E. coli*, *Pseudomonas fluorescens*, *Mycobacterium phlei*, *Staphylococci*, *Thermus aquaticus*, *Tetrahymena pyriformis*, *Pseudomonas ovalis*, and *Serratia marcescens*. Yeast includes *Rhodotorula* sp, and *Candida utilis* and the fungi like *Aspergillus tamari*, *Aspergillus terreus*, *Aspergillus nidulans*, *Penicillium* sp, *Fusarium* sp, and *Helminthosporium* sp. There are only a few studies on L-asparaginase production by fungi⁷. These have established that filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium* and some yeast species produce this enzyme⁸.

The objective of this study was selection of suitable fungi for the production of asparaginase and to screen the different agro industrial byproducts as substrates for maximum enzyme production.

MATERIALS AND METHODS

Isolation of fungi

The fungi used in this study were isolated from samples of soil collected from different areas of India. The dilution plate-method was employed for the isolation of fungal strains⁹. The isolated organisms were maintained on modified Czapek Dox medium containing (g/L of distilled water): 2.0 glucose, 10.0 L-asparagine, 1.52 K₂HPO₄, 0.52 KCl, 0.03 FeSO₄·7H₂O, 0.05 ZnSO₄·7H₂O, 0.3 NaNO₃, and 15.0 Agar at pH 6.2.

Screening of L-asparaginase producing fungi

The fungi obtained from the above steps were subjected for rapid screening of L-asparaginase production by plate assay as per Gulati *et al.*¹⁰. Modified Czapek Dox's (mCD) medium¹¹, pH 6.2, used for fungi contained 0.2% (w/v) glucose, 1% (w/v) L-asparagine, 0.152% (w/v) K₂PO₄, 0.052% (w/v) KCl, 0.052% (w/v) MgSO₄·7H₂O, 0.003% (w/v) CuNO₃·3H₂O, 0.005% (w/v) ZnSO₄·7H₂O, 0.003% (w/v) FeSO₄·7H₂O, 1.8% (w/v) agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. Control plates were mCD medium containing NaNO₃ as nitrogen source

instead of asparagine. The plates were inoculated with the 38 selected fungal isolates and incubated at 30°C for 48 h. The isolates that showed pink zone around the colonies indicated L-asparaginase production and were selected for determination of enzyme activity.

L-asparaginase production by submerged fermentation

Submerged fermentation for L-asparaginase production was carried out using modified Czapek Dox's liquid media. Erlenmeyer flask containing 100 mL of appropriate medium were inoculated with primary screened organism. The flasks were incubated at 30°C at different incubation period (24-144 h). Uninoculated media served as controls. The cultures were harvested by filtration through Whatman No. 1 filter paper. The culture filtrate was used as crude enzyme to estimate enzyme activity.

L-asparaginase Assay

L-asparaginase activity was measured following method of Imada *et al.*¹². This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml supernatant into 0.5 ml 0.04 M L-asparagine and 0.5ml 0.05 M tris (hydroxymethyl) aminomethane (tris HCl) buffer, pH 7.2 and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min, and absorbance of the supernatant was read using a UV-visible spectrophotometer (UV-2450, SHIMADZU) at wavelength of 450 nm. One unit of L-asparaginase activity is defined as that amount of enzyme which catalyses the formation of 1µmol of ammonia per min under the conditions of the assay.

Screening and Selection of Solid Substrates for SSF

Twenty different agro-industrial residues were tried as substrates for L-asparaginase production. The substrate include coconut oil cake (COC), ground nut oil cake (GNOC), tea waste (TW), green gram husk (GGH), sugar cane bagasse (SCB), wheat bran (WB), cotton

seed oil cake (CSOC), rice bran (RB), green peas husk (GPH), cotton seed (CS), ground nut shell (GNS), rice husk (RH), orange peel (OP), paddy straw (PS), corn flour (CF), corn cob (CC), pine apple peel (PAP), maize straw (MS), lemon peel (LP) and tamarind seed (TS). Enzyme production was carried out in 250 mL flasks containing 10 g substrates moistened with 0.01 M Phosphate buffer pH 6.2 to a moisture level of 50%. All flasks were sterilized at 121°C for 30 min. The flasks were inoculated with 1 mL (10^6 spores/ml) and then incubated at 30°C for 96 hours.

The crude enzyme was extracted at the end of fermentation period by adding 50 mL of 0.01 M phosphate buffer pH 6.2, shaking for 30 min followed by centrifugation at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation. All values given are means of three determinations.

Statistical analysis

The SPSS software (10.0 versions) was used for the major data processing throughout this work. All results were expressed as the mean \pm SD.

RESULTS AND DISCUSSION

Isolation and screening of microorganisms

A total of thirty eight fungal cultures were isolated from soil sample and named serially from KUFS1 to KUFS38. The isolation pattern of fungi is presented in Table 1. The cultures were morphologically identified as *Aspergillus sp.*, *Fusarium sp.*, *Penicillium sp.*, *Mucor sp.* and *Basidiomycetes sp.* The plate method is a qualitative, simple and rapid screening procedure for L-asparaginase production. The fungus used for the production of L-asparaginase enzyme was screened by using plate assay method. The potential strains were selected on the basis of pink zone around the colony by plate assay method. From the total isolate 23 fungal showed L-asparaginase activities. It is exposed that the strain exhibiting zone of diameter above 15 mm are referred as high L-asparaginase producers. *Aspergillus sp.* KUFS20, isolated from garden soil, was produced 27 mm diameter pink zone surrounding the colony showed in Figure 2.

Table 1: Sources of fungi

S. No	Soil type	Place	No. of isolates
1	Kitchen waste dumping soil	Coimbatore	5
2	Dairy waste contaminated soil	Trivandrum	6
3	Garden soil	Coimbatore	7
4	Cultivated land	Nagarcoil	9
5	Coconut waste dumping soil	Nagarcoil	11

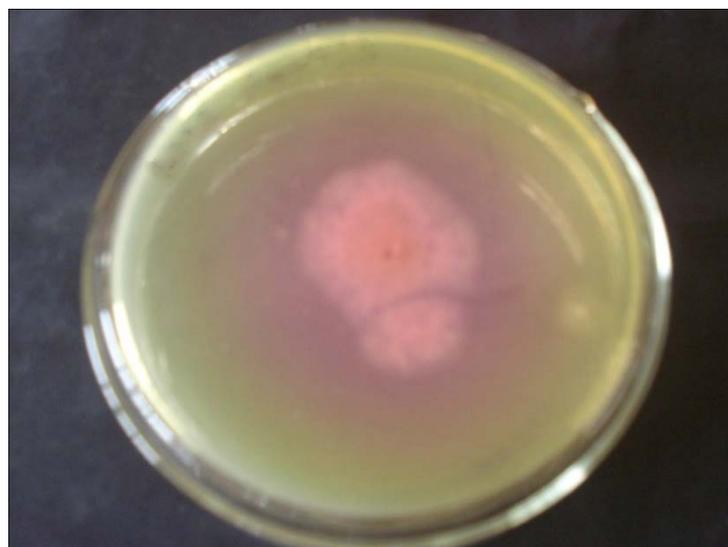


Fig. 2: Plate showing pink colour zone around the colony

It is generally observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates¹³. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase. This culture was further submitted for submerged and solid state fermentation. The plate assay is advantageous as the method is quick and L-asparaginase production can be visualized directly from the plates without performing time consuming assays¹³⁻¹⁵.

Submerged fermentation

In submerged fermentation the production of L-asparaginase was reached maximum of 3.8 U/ml at 96 h of incubation period (Figure 3). Further increases in incubation period didn't show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was to be 96 h after inoculation. Our results was different from Sarquis *et al.*⁸ who reported the highest L-asparaginase activity of *A. terreus* in liquid medium was found at 48 h while in solid medium the optimal period for enzyme production was 96 h¹⁶.

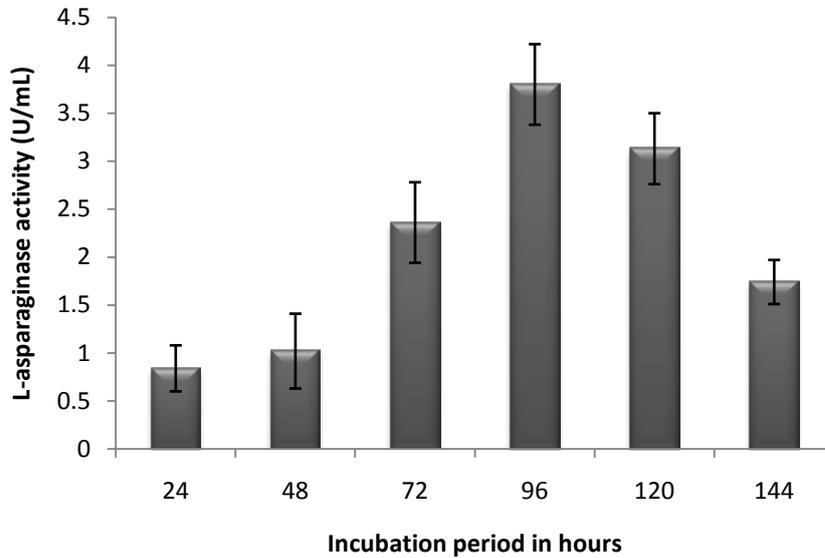


Fig. 1.2: Production of L-asparaginase by *Aspergillus sp* (KUFS20) at different time intervals

Screening of Solid substrate on L-asparaginase production

When different substrates were used in the solid state fermentation medium, the highest enzyme activity was obtained in orange peel 70.67±1.14 U/g and minimum for ground nut shell 10.27±0.40 U/g (Figure 3). The production increased several fold from the

submerged production. Bhaskar and Renganathan¹⁷ reported production L-asparaginase by *Aspergillus terreus* using ground nut oil cake as substrate. Siddalingeshwara *et al.*¹⁸ reported production of L-asparaginase using carob pod as substrate through solid state fermentation by *Aspergillus terreus*.

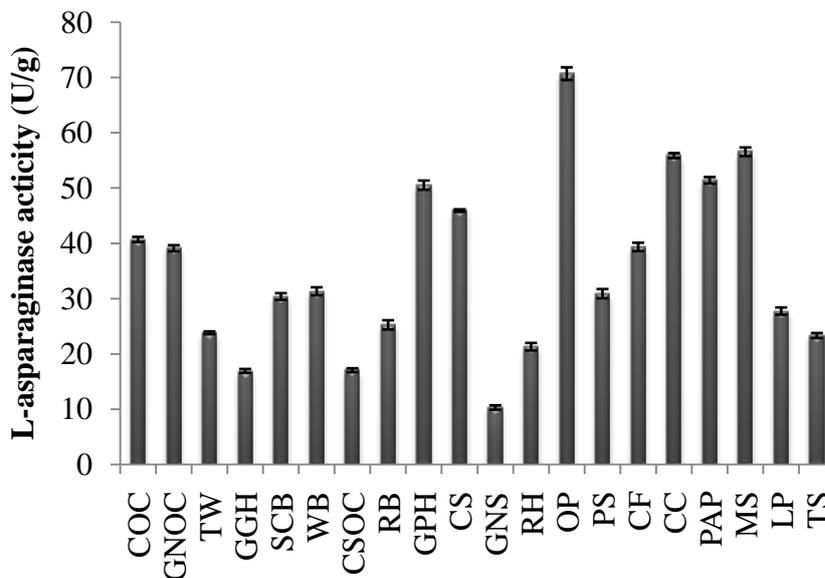


Fig. 3: Showing the effect of substrate for L-asparaginase production by *Aspergillus sp* KUFS20

L-asparaginase production in solid state fermentation (SSF) was much higher than that in submerged fermentation (SmF). L-asparaginase production throughout the world is carried out mainly by submerged fermentation. SSF offers a number of advantages over conventional submerged fermentation for enzyme production¹⁹. Mitchell and Lonsane²⁰ reported that the production enzyme is often simple, when agro-industrial by-products like wheat bran, rice bran or wheat straw are used as substrate. Because the moisture level is low, the volume of medium per unit weight of substrate is low. Hence, enzyme activity is usually very high²¹. Solid state fermentation involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganisms, especially enzymes or secondary metabolites. And SSF is very cost effective method since agro-industrial byproducts can be used as the substrate other than the costly chemicals.

CONCLUSION

From this study, it is clearly indicated that soils can provide a rich source of L-asparaginase producing fungi. *Aspergillus* sp KUF520 isolated from garden soil has the ability to produce a significant amount of L-asparaginase enzyme. However, more detail investigation is required to characterize this microbial enzyme, which may be effectively used in the large scale production for commercial and pharmaceutical purposes in the future.

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