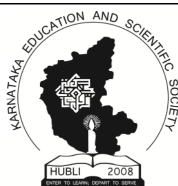

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Research Article

Anticerous Activity of L-Asparaginases Enzyme Produced from *Aspergillus Niger* by Solid State Fermentation

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ABSTRACT

Today a number of research works are carried out in production of enzymes from an isolated organism, and evaluating its effect on animal studies. L-Asparaginase is now known to be a potent antineoplastic agent in animals and has given complete remission in some human leukemia's. Extensive clinical trials of this enzyme, however, were not possible in the past because of inadequate production. Strain improvement of organisms is one alternative method that can be implemented to tackle this problem. Strain improvement can enhance the production, and increase the activity of enzymes produced. L-asparaginases are found to be responsible for catalyzing the deamination of Asparagines to yield Aspartic acid and an ammonium ion, resulting in depletion of free circulatory Asparagines in plasma. Its use in therapeutics is found to be remarkable, especially for those specific cases where blood cells become cancerous, such as in acute lymphoblastic leukemia. The commercial availability of L-asparaginase has revolutionized the molecular therapy of acute lymphocytic leukemias and melanosarcoma. Therefore, highly purified and effective Asparaginase is of great importance for today's practical interests. The present study deals with successful isolation and purification of L-asparaginase enzyme from *Aspergillus niger* by solid state fermentation. L-asparaginase produced was purified and characterized. The anticerous activity of the enzyme was studied on human cancer cell line by MTT Assay method and the results showed clear positive results.

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INTRODUCTION

L-asparaginases are responsible for the catalysis of L-asparagine into L-aspartate and Ammonia. The common sources for L-asparaginases are organisms including Proteus *vulgaris*, *Erwinia carotovora*, *Acinatobacter*, *Serratia marcescens*, *Mycobacterium bovis*, *Streptomyces griseus*, *Achromobacteraceae* and *Pisum sativum*. Recent studies on asparaginases obtained from *Escherichia coli* and *Erwinia chrysanthemi* suggests that they are useful as anti-leukemia agents. [1] L-asparaginase production in most part of the world is carried out mainly by submerged fermentation (SMF). Even though it's used widely across the world due to its reliable characteristics it has many disadvantages including its cost intensiveness and low product concentration.

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Solid state fermentation (SSF) is one of improvised and effective technique that can be implemented for enzyme production at low capital cost, low energy input using simple fermentation media, and low water content. [2] The substrate affinity of the enzyme plays an important role in determining the antitumor property of any enzyme production. Studies report highest yield of enzyme (L-asparaginase) when it's aerobically grown in corn steep liquor medium. Good enzyme production was also observed with culture media containing L-glutamic acid, L-methionine, and lactic acid. [3-5] Sodium ion also appeared to suppress L-Asparaginase production. Research works described for isolation of biologically active L-Asparaginase from fungi were successful in obtaining stable L-Asparaginase preparations with a specific activity of 1200 IU per mg of protein (2540-fold purification with 50% total recovery).[6,7]

Anticancerous studies done across the globe suggest that L-Asparaginase can be used as a potent antineoplastic agent in animals and has given complete remission in some human leukemia's. However fully fledged research works on this enzyme were not possible in the past because of its inadequate production. Recent studies reports that strain improvement may be best technique that can be employed to enhance the production, and increase the activity of enzymes. [8-10] In this study we attempted to produce L-asparaginase from *Aspergillus niger* by solid state fermentation (SSF) using three different substrates (Sugar cane, Dry Coconut, Mixed) and the produced enzyme was purified by techniques comprising of Ammonium salt precipitation, dialysis and ion exchange chromatography. Enzyme assays were carried out for calculating the enzyme activity. SDS PAGE method was used to determine the molecular weight of the enzyme produced. Attempts were made for strain improvement of the organism standardizing different parameters affecting its growth. The Anticancerous activity of the enzyme was studied on human cancer cell line (HeLa cells) by MTT Assay method. The MTT results suggest that the isolated L-Asparaginase shows anticancerous activity.

MATERIALS AND METHODS

Isolation and Identification of Microorganisms and Screening of Micro-Organisms

About 5 g of the protein rich soil was collected from places around Bangalore. Serial Dilution of the sample was performed in order to determine the number of micro-organism per unit volume in the original culture and for determination of the culture density in cells per ml. The diluted culture was spread on agar plates. Agar plates allowed the individual bacterial cells to be separate uniformly. Pour plate method was used for quantifying microorganisms that grow in solid medium and colonies formed within agar matrix. Pure cultures were maintained by pouring the molten Potato Dextrose Agar into the sterile test tubes and were allowed to solidify as slants. Once the agar got solidified, the inoculation loop was immensely heated in the Bunsen flame and pinch of the culture was streaked into the solidified Agar. This was followed by incubation at room temperature for 24hours. Screening was performed as per methodology described by Gulati *et al.* with Phenol red in a stock solution prepared in ethanol with L-asparaginase incorporated in the medium for the selection of the micro-organisms that has ability to produce L-asparaginase. [5]

Production and Purification of Asparaginase Enzyme

A seed culture for the fermentation process was prepared using Potato Dextrose broth for three different substrates and three pure cultures were inoculated in it. The culture was then incubated at room temperature for 48 hours. The production media for three different substrates was prepared. The media and the fermentor were sterilized by autoclave. The pH and temperature was set at normal levels. Fresh overnight seed culture was inoculated and incubated for 3-4 days at 30°C. The fermentation broth was then centrifuged at 6000rpm for10 minutes. The supernatant was taken and heavy particle such as proteins was removed using 100mM Trichloroacetic acid. Equal volumes of TCA and supernatant of the previous centrifuge was mixed and incubated at room temperature for 15 minutes. It was then centrifuged at 12000rpm for 10 minutes and supernatant was taken for further purification after estimating the presence of amino acids. Solvent precipitated by equal volumes of methanol and supernatant was centrifuged at 10000rpm for 15 minutes. [9] The pellet containing the enzyme (L-asparaginase) was dissolved in alkaline phosphate buffer of pH 8.6. [4] The enzyme sample was purified by Ammonium salt precipitation, dialysis and ion exchange chromatography. First the production media was centrifuged at 6000 rpm for 10 min. The supernatant was collected and pellet discarded. Then the crude enzyme was subjected to precipitation and again the precipitated sample was centrifuged at 10000 rpm for 10 min. The pellet was dissolved in Tris HCl buffer and this sample was subjected to dialysis followed by ion-exchange chromatography. [3] Enzyme assay was done for both crude and purified enzyme, using Nesselerization method and Lowry's method for determining Ammonia and protein concentrations respectively. SDS page was employed to determine the molecular weight of the enzyme produced.

Characterization of the enzyme

Characterization of enzyme was done by evaluating the effect of different parameters influencing the enzyme activity. The parameters included for the study were pH, temperature, activator, inhibitor and substrate concentration.

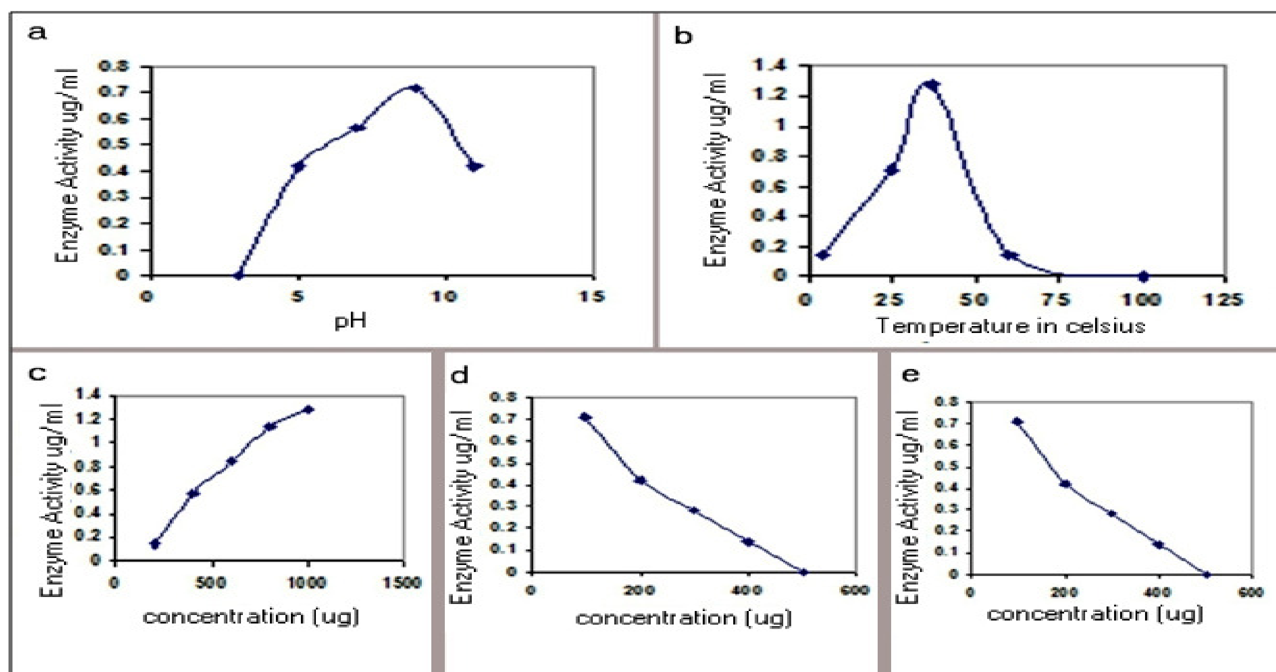

Figure 1: (a) represents the effect of pH on enzyme activity (L-asparaginase); **(b)** represents the effect of temperature on enzyme activity (L-asparaginase); **(c)** represents the effect of activator on enzyme activity (L-asparaginase); **(d)** represents the effect of inhibitor on enzyme activity (L-asparaginase) and **(e)** represents the effect of substrate concentration on enzyme activity (L-asparaginase).

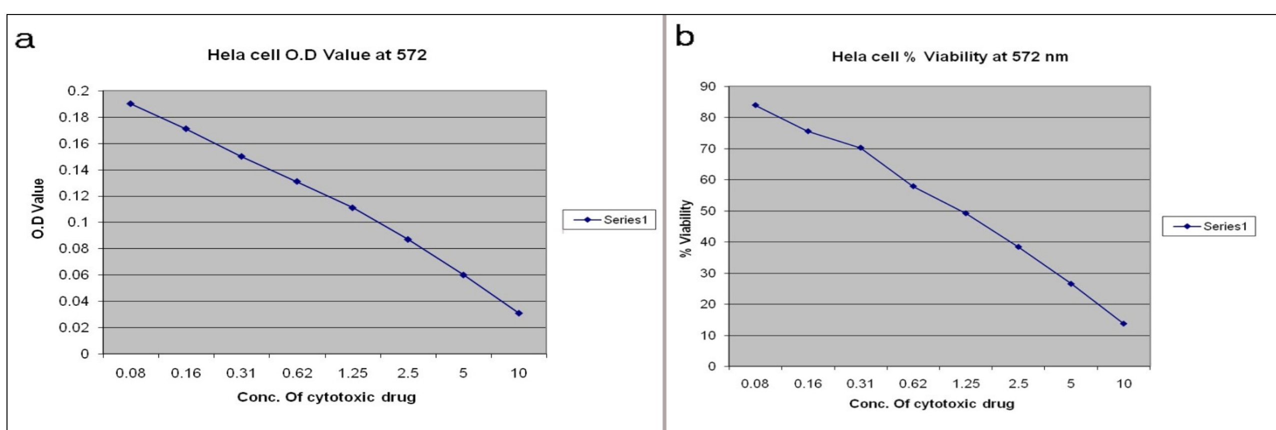

Figure 2: (a) O.D value Vs concentration on drug (for HeLa cells at 24 hours of incubation after MTT Assay) **(b)** % Viability Vs concentration on drug (for HeLa cells at 24 hours of incubation after MTT Assay).

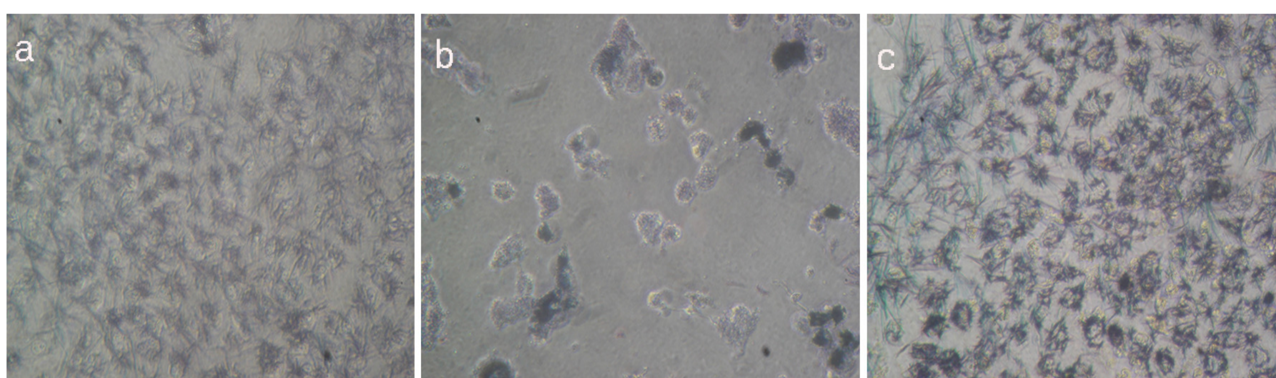

Figure 3: (a) Crystal formation in vehicle control; **(b)** Crystal formation at 10% drug and **(c)** represents the Crystal formation at 0.08% drug**.**

Anticancerous activity by MTT Assay method

MTT assay technique was employed to study the anticanceorus activity of the purified L-asparaginase enzyme. The drug stock was diluted in media (DMEM/F 12) (Gibco), 10% FBS (Gibco) as follows: - 10%, 5%, 2.5%, 1.25%, 0.625%, 0.31%, 0.15%, 0.075%. On the first day, HeLa cells were cultured in 96 well plate at a density of 1000 cells per well, incubated at 37°C, 5% CO_2 for 24 hours. On the second day, the above drug dilutions were added to the plate and incubated further at 37°C, 5% CO_2 for 24 hours to expose the cells completely to the drug. On the third day, drug was removed from all the wells and 100µl of media and 20 µl of MTT reagent were added to each of well. The culture was incubated further at 37°C, 5% CO_2 for 4 hours. After the incubation, 100 µl of crude DMSO was added to each of the well to dissolve the formazan crystals. The Optical Density (OD) readings were taken using Cytation™ microplate reader.

RESULTS AND DISCUSSION

MTT Assay- Test for cellular proliferation (Anticancerous activity)

Table 1: Represents the O.D (at 650 nm) value and % viability of HeLa cells at 24 hours incubation on MTT assay

Concentration of drug	O.D value at 572nm	% viability of HeLa Cells
10%	0.031	13.7
5%	0.060	26.5
2.5%	0.087	38.4
1.25%	0.111	49.2
0.62%	0.131	57.9
0.31%	0.150	70.3
0.16%	0.171	75.6
0.08%	0.190	84

Effect of MTT Assay - Crystal formation (HeLa cell lines)

Microorganisms (*Aspergillus niger*) were isolated from soil samples by serial dilution method. The isolated organisms were identified on the basis lactophenol cotton blue staining. The enzyme asparaginase was produced by solid state fermentation using different substrates with culture of *Aspergillus niger.* This project reports the production of L-asparaginase from *Aspergillus niger* in solid state fermentation (SSF) using three different substrates (Sugar cane, Dry Cocunut, Mixed). A 96-hour fermentation time under aerobic condition with moisture appeared optimal for enzyme production. The optimum temperature and pH for enzyme activity were found to be 37°C and 9 respectively. The enzyme activity was checked at all stages of purification. The molecular weight of the enzyme was found to be 33 kDa based on SDS PAGE analysis.

The enzyme was further characterized with respect to pH, temperature, activator, inhibitor and different substrate concentrations. The anticancer effect of L-Asparaginase was studied on human cancer cell line (HeLa) by MTT assay method. The MTT results were positive and the IC 50 (inhibitory concentration) was achieved at 1.25% of the drug dilution with the concentration of 1.56µg/µl.

CONCLUSION

Present study indicates that the local soil isolates *Aspergillus niger* can be used as potential source of L-asparaginase. Furthermore, the purified L-asparaginase showed significant anticancer activity against HeLa cell line and should be considered for further pharmaceutical use as anticancer agents.

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