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Research Article

PRODUCTION, PURIFICATION AND STANDARDIZATION OF ANTICANCEROUS ENZYME (L-ASPARAGINASES) FROM *Aspergillus niger* USING SOIL SAMPLES BY SOLID STATE FERMENTATION

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Abstract

Biotechnology techniques can provide an unlimited and pure source of enzymes as an alternative to the harsh chemicals traditionally used in industry for accelerating chemical reactions. L-asparaginase is one among them, found in various plants, animals and bacterial cells. L-asparaginase is studied 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. In this study we have made an effort to isolate, identify and screen micro-organism (*Aspergillus niger*) for the production of anticancerous enzyme (L-asparaginases) by solid state fermentation process and the produced enzyme was purified and characterized for L-asparaginases. The aim of the study was to validate the solid fermentation process in terms of its reliability and feasibility for production of L-asparaginase enzyme. This method was found to be very cost effective and reliable when compared to the other expensive techniques used around the globe for enzyme production. Even though the product yield and purity is comparatively less in comparison with the other techniques it can be still used as a reliable technique for short scale enzyme production.

Key words: Solid state fermentation process; L-asparaginase; Anticancerous enzyme; *Aspergillus niger*

Introduction

L-asparaginases are involved in catalyzing the hydrolysis of L-asparagine into L-aspartate and Ammonia. L-asparaginases are commonly found in most of the organisms including *Proteus vulgaris*, *Erwinia carotovora*, *Acinotobacter*, *Serratia marcescens*, *Mycobacterium bovis*, *Streptomyces griseus*, *Achromobacteraceae* and *Pisum sativum*. Though L-Asparaginase has been reported in many higher plants, little work has been carried out on their purification and characterization. The L-asparaginases of *Erwinia* and *E. coli* have been reported since many years as effective drugs in the treatment of acute lymphoblastic leukemia (Mishra *et al.*, 2006; Pieters *et al.*, 2011). It has been observed that eukaryote microorganisms like yeast and filamentous fungi also have a potential for Asparaginase production (Wade *et al.*, 1971). The best example for them are the mitosporic fungi genera such as *Aspergillus*, *Penicillium*, and *Fusarium* (Herbert, 1985) Studies clearly suggest that the asparaginases isolated from *Escherichia coli* and *Erwinia chrysanthemi* are useful as anti-leukemia agents (Roberts *et al.*, 1968).

L-asparaginase production throughout the world is carried out mainly by submerged fermentation (SMF). This

technique, however, has many disadvantages including its cost intensiveness and low product concentration. Solid state fermentation (SSF) is one of improvised and effective technique that is capable of increasing the product yield of enzyme production at low capital cost, low energy input using simple fermentation media, and low water content. Low water use in SSF results in low production of effluents and reduced growth of contaminating bacteria and yeasts. These in turn, help in reduction of the cost for sterilization. The use of agricultural wastes as source of energy makes the SSF environment friendly when compared to other techniques available (Sarquis *et al.*, 2004).

The most important requirement for any enzyme to be considered for therapeutic use is that it should be relatively easy to isolate, static at the physiological pH and temperature, the enzyme should have low Km value and it should not be inhibited by its products. The substrate affinity of the enzyme is considered to be the most important factor responsible for the antitumor property of any enzyme production. Studies suggest that the highest yield of enzyme (L-asparaginase) was obtained when cells were grown aerobically in corn steep liquor medium. Good enzyme production was associated with media containing L-glutamic

acid, L-methionine, and lactic acid. The addition of glucose to the medium, however, was found to be resulting in decreased production of L-asparaginase (Roberts *et al.*, 1968). Sodium ion appeared to suppress L-asparaginase production. Previous works using the procedure 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).

Recent investigation on L-asparaginase reports that it can be used as 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 will be best technique that can be employed to enhance the production, and increase the activity of enzymes. 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). 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.

Materials and Methods

Isolation and Identification of Microorganisms

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 24hrs.

Screening of Micro-Organisms

Screening was performed as per methodology described by Gulati (Gulati *et al.*, 1997) 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.

Production of Asparaginase by Solid State Fermentation Process

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.

Production of L-asparaginase in Fermentor

The production media for three different substrates was prepared. The media and the fermentor were sterilized by autoclave. The pH and temperature was set by default. Fresh overnight seed culture was inoculated and incubated for 3-4 days at 30°C. The fermentation broth was then centrifuged at 6000rpm for 10min. The supernatant was taken and heavy particles such as proteins were removed using 100mM Trichloroacetic acid. Equal volumes of TCA and supernatant of the previous centrifuge was mixed and incubated at room temperature for 15mins. It was then centrifuged at 12000rpm for 10mins 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 15mins (Prakasham *et al.*, 2007). The pellet containing the enzyme (L-asparaginase) was dissolved in alkaline phosphate buffer of pH 8.6 (Ghasemi *et al.*, 2008).

Purification of Crude Enzyme

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 (Dhevagi *et al.*, 2006).

Enzyme assay

Enzyme assay was done for both crude and purified enzyme, using Nesslerization method and Lowry's method for determining Ammonia and protein concentrations respectively.

Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE)

SDS page was employed to determine the molecular weight of the enzyme produced.

Results and Discussion

Enzyme assay

Enzyme activity studied in this study is shown in Table 1. It is evident with the result that sample 1 showed highest enzyme activity in crude and purified enzymes.

Comparison study with different substrates used for production

The comparative study with different substrates (Mixed, Coconut and Sugarcane) used for production the enzyme activity and the specific enzyme activity has been shown in Table-2. It was observed that mixed coconut and sugarcane showed better enzyme activity in respect to coconut and sugarcane alone.

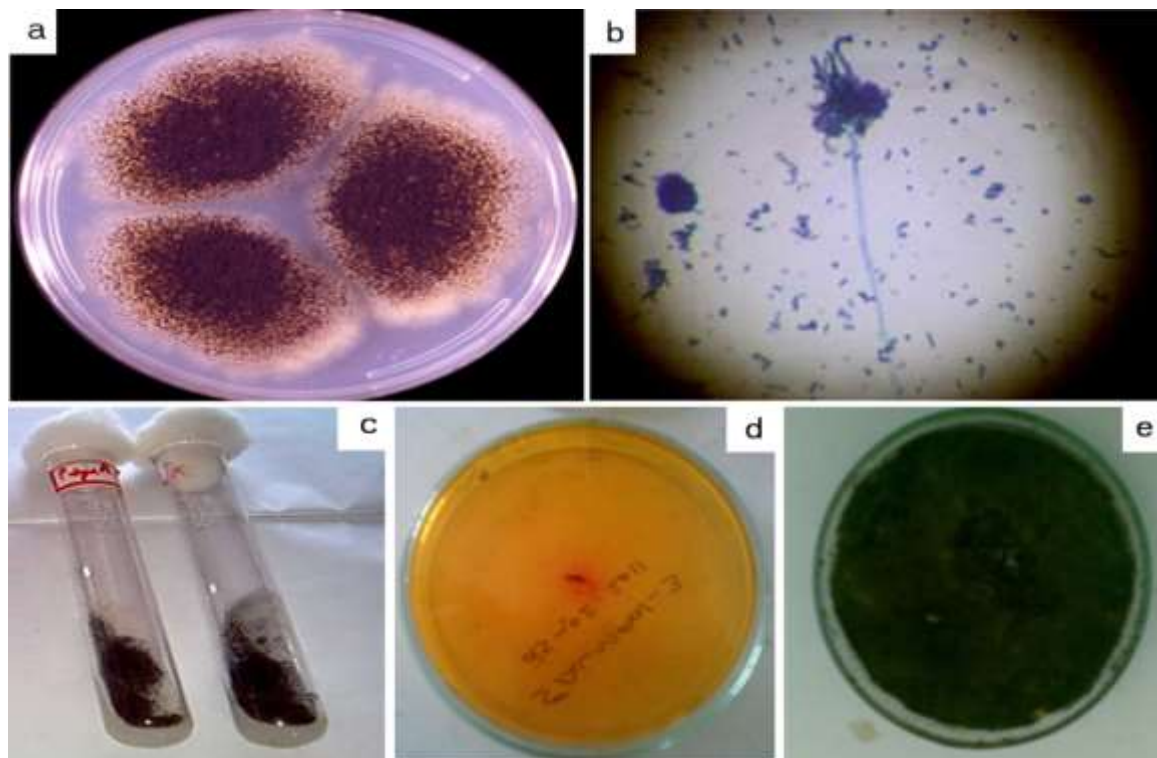


Fig. 1

Fig. 1.a: *Aspergillus niger* culture developed using pour plate method after a period of 3 day incubation; **Fig. 1.b:** The microscopic view of *Aspergillus niger* obtained as a result of screening procedure (Identification tests for fungus-Lactophenol cotton blue staining) done for *Aspergillus Niger*. **Fig. 1.c:** The slants of pure culture of *Aspergillus niger* maintained. **Fig 1.d:** The screening results for production of asparaginase enzyme (The organism *Aspergillus niger* was taken from the pure culture and screened for L-asparaginase production by Gulati *et al.* (1997) method). *Aspergillus niger* gave the positive result, media colour changed from orange to pink. **Fig 1.e:** The seed culture of *Aspergillus niger* obtained.

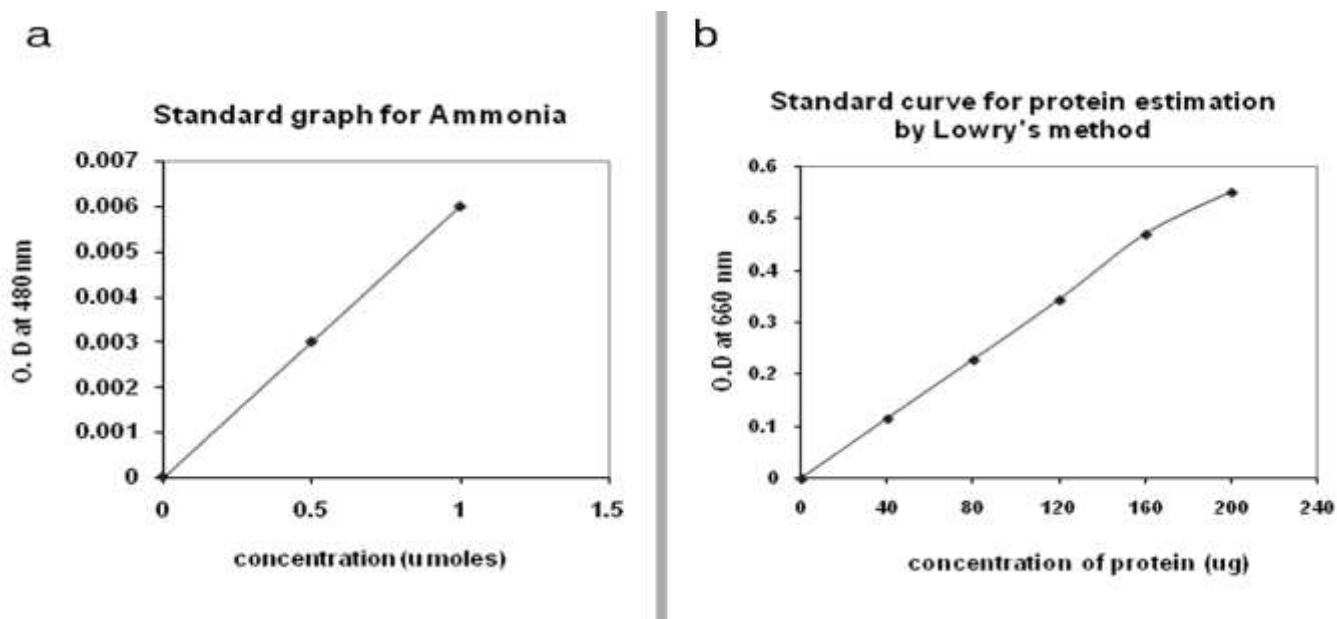


Fig 2: a The standard graph obtained for estimation of Ammonia; **b** The standard curve for protein estimation by Lowry's method.

Table 1: The enzyme activity calculated (Nesslerization method) in accordance with the standard graph of Ammonia and Protein concentration calculated from the standard curves plotted.

Enzyme activity calculated (Nesslerization method) in accordance with the Standard graph of Ammonia (Fig.2a)					
Crude enzyme:			Purified enzyme:		
Sample 1	7.7ug/ml		Sample 1	2.42ug/ml	
Sample 2	1.41ug/ml		Sample2	1.14ug/ml	
Sample 3	0.57ug/ml		Sample 3	0.42ug/ml	
Protein concentration calculated in accordance with the Standard curve for protein estimation by Lowry's method (Fig 2b)					
Crude enzyme:			Purified enzyme:		
Sample 1		0.058mg	Sample 1		0.010mg
Sample 2		0.046mg	Sample2		0.030mg
Sample 3		0.042mg	Sample 3		0.022mg

Table 2 Represents the comparative study with different substrates (Mixed, Coconut and Sugarcane) used for production the enzyme activity and the specific enzyme activity calculated from the data shown in Table 1

Enzyme	Protein(mg)	Enzyme Activity (U/ml)	Specific Enzyme Activity (U/mg)	Yield	Purification Factor
MIXED (COCUNUT AND SUGARCANE)					
Crude	0.058	7.7	132.7	1	1
After ion-exchange chromatography	0.010	2.42	242	0.31	1.82
COCUNUT					
Crude	0.046	1.14	30.6	1	1
After ion-exchange chromatography	0.030	1.41	38	0.80	1.24
SUGARCANE					
Crude	0.042	0.57	1.35	1	1
After ion-exchange chromatography	0.022	1.42	28.5	0.24	21.1

SDS-PAGE

SDS PAGE of the enzyme was performed with the standard method. It was observed that that molecular weight of the enzyme was found to be 39.6kda (PAGE photograph not shown).

Conclusion

Soil samples were collected and microorganisms were isolated by serial dilution method. The isolated organisms were identified on the basis lactophenol cotton blue staining. Fungal species *Aspergillus niger* was identified based on Lactophenol staining. The organism identified was

screened for the production of asparaginase enzyme. 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 using three different substrates (Sugar cane, Dry Cocunut, Mixed). Among different substrates used for, mixed substrate (sugarcane and coconut) showed maximum enzyme production followed by that of Dry Coconut and Sugar Cane. A 96-hour fermentation time under aerobic condition with moisture appeared optimal for enzyme production. The optimum temperature and pH for enzyme

activity were 37°C and 9, respectively. The study suggests that choosing an appropriate substrate when coupled with process level optimization improves enzyme production markedly. Developing an asparaginase production process based on Mixed as a substrate in solid state fermentation is economically attractive as it is a cheap and readily available raw material in agriculture-based countries. The enzyme thus was extracted and purified. 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.

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