Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 4, 2012

Research Article

PRODUCTION OF BIOACTIVE ENZYME L-ASPARAGINASE FROM FUNGAL ISOLATES OF WATER SAMPLE THROUGH SUBMERGED FERMENTATION

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Received: 26 April 2012, Revised and Accepted: 09 Jun 2012

ABSTRACT

L-asparaginase is used in the treatment of cancer, especially for acute lymphoblastic leukemia. Fungal species were isolated from water sample and screened for its ability to produce the extracellular L-asparaginase enzyme. Effect of pH and temperature were investigated in the production of enzyme. Highest yield of enzyme was observed when the pH of the fermentation cycle raised above 8 at 35°C. Effective production was observed while using starch and proline as carbon and nitrogen source. The molecular weight of L-asparaginase was determined by SDS-PAGE & it was found to be 48 kDa.

Keywords: *Aspergillus niger,* Submerged Fermentation, Optimization and Shake culture fermentation.

INTRODUCTION

Bacterial L-asparaginases are enzymes of high potency used in treating various kinds of cancers, mainly acute lymphoblastic leukemia. Bacterial L-asparaginases are either high affinity periplasmic enzyme or low affinity cytoplasmic enzyme. A high affinity periplasmic L-asparaginase is particularly effective in certain kinds of cancer therapies [1,2].

Normal cells do not require much asparagine to survive when compared to cancer cells and able to make required quantity of the asparagine by them. Tumor cells, more specifically lymphatic tumor cells, require huge amounts of asparagines to keep up with their rapid, malignant growth by utilizing asparagine from the diet as well as from its own limited expression. On the other hand, tumor cells become depleted rapidly and die if the dietary supply is cut off, because they cannot manufacture enough asparagine internally to support their continuing growth. L-Asparaginase catalyses the conversion of l- asparagines to l-aspartate and ammonia this catalytic reaction is essentially irreversible under physiological conditions. Supplementation of L-asparaginase results in continuous depletion of asparagine[3].

Asparaginase can be produced by using variety of microbial sources which includes fungi, yeast and bacteria. Asparaginase from *Escherichia coli* and *Erwinia sp.* has been used as anti-tumor and anti-leukemia agent. The utilization of asparaginase from the above mentioned sources was initially limited because of its potential toxicity and several side effects. *Aspergillus niger* has been one of the sources of industrial enzyme that has been designated by FDA as a safe source. In our present investigation *aspergillus niger* from the water sample was isolated and screened for its activity to produce L-asparaginase. L-Asparaginase was produced by submerged fermentation and various Carbon and Nitrogen sources were optimized with respect to variations in pH and Temperature [4,5].

MATERIALS AND METHODS

Materials

Czapekdox agar, Potato Dextrose agar, Potato Dextrose Broth and Yeast extract were obtained from Himedia laboratories, Mumbai. Standard L-asparaginase was purchased from GetWellifesciences, New Delhi. L-Asparagine monohydrate and Nesslers reagent were purchased from Madras Scientific. Di-Potassium hydrogen phosphate and sodium potassium tartrate was obtained from Qualigens. All other reagents and chemicals were of analytical grade.

Isolation of microbe from water sample

A miniature volume of water sample was transferred to a petridish containing sterile potato dextrose agar for pre-enrichment of samples. The plate was incubated at 30°C for 14days in an incubator. The colony formation was clearly visible. A loopfull of the inoculum from the agar was streaked on fresh Czapekdox agar plates and the plates were incubated at 30°C for 7 days. Single discrete colonies were isolated and used for identification[6].

Screening of L-asparaginase producer by plate assay

The strains obtained from the above steps were subjected for rapid screening of L-asparaginase production by plate assay method. The modified Czapekdox's medium was supplemented with phenol red dye (2.5% prepared in ethanol and the pH was adjusted to 7.0). The media was autoclaved and plates were prepared. Control plate was maintained without asparagine. The plates were inoculated with fungal strains isolated from the water sample. The zone was observed after 48 hrs[7].

Preparation of Production Medium

For the preparation of production medium, chemical ingredients were accurately weighed and transferred into a clean 500ml conical flask containing 250 ml of distilled water. Composition of production medium was 0.7g of $MgSO_4.7H_2O$, 0.5g of K_2HPO_4, 1.0g of L-asparagine, 0.5g of KCl, 0.1g of $CaCl_2$, 0.5g of yeast extract were dissolved by gentle heating with slight agitation. After complete solidification of the component, volume was made up to 500 ml with distilled water and the pH of the medium was adjusted to pH 7.0 and sterilised in an autoclave at 121°C for 30 min [8].

Production and Extraction of L-asparaginase from fermented medium

Erlenmeyer flask (250ml) containing 100ml of production medium was inoculated with isolated fungal and incubated at 35°C in shaker incubator oscillating at 250 rpm for 72 hrs. One ml of extract was withdrawn in an aseptic condition and filtered through whatmann filter paper. The clear extract was centrifuged at 2000-3000 rpm for 15 min. The supernatant were used for enzyme estimation and tested for L-asparaginase content [9].

L-asparaginase assay

The enzyme activity was determined by calculating the amount of ammonia liberated by Nesslerization method. A reaction mixture containing 0.5ml of 0.04M L-asparagine, 0.5 ml of 0.5M tris(hydroxymethyl) aminomethane-Hcl buffer of pH 8.6, 0.5 ml of an enzyme preparation and distilled water to a total volume of 2.0 ml was incubated at 30°C for 30 min. From the above mixture 0.1 ml was transferred into a clean test tube. To this 3.7 ml of distilled water and 0.2 ml of Nesslers reagent were added, and the mixture was again incubated at 30°C for 20 min. Absorbance was measured at 450 nm using spectrophotometer. The blank was run by adding

enzyme preparation after the addition of trichloro acetic acid (TCA). The enzyme activity was expressed in International unit. One IU of L-asparaginase is the amount of enzyme which liberates 1µmole of ammonia per min per ml [mole/ml/min][10].

Optimization of the pH and temperature of the medium

Erlenmeyer flask (250ml) containing 100ml of production medium was inoculated with fungal isolate and incubated at various pH 5.0, 6.0, 7.0, 8.0 and 9.0. The pH at which maximum activity was found is considered as an optimized pH. A 100ml of production medium was prepared with the optimized pH and inoculated with the fungal isolate and incubated at various temperatures (30°C, 35°C and 37°C). The temperature at which maximum yield of enzyme was observed is considered as an optimized temperature.

Effect of Carbon and Nitrogen Substrates on production

To the production medium with optimized pH and temperature, 1.0 g of Carbon source (either Starch, Glucose or Sucrose) and 0.5 g of Nitrogen source (either DL-Tryptophan, L-Proline or L-Arginine) was added and the effect of Carbon source and Nitrogen source on the enzyme activity were studied. Temperature, pH, Nitrogen and Carbon source having maximum enzyme activity has been investigated [11].

The effect of K_2HPO_4 on the production medium

With all the maximum yielding parameters maintained in the production, Di-potassium hydrogen phosphate of various concentrations are added (0.5, 0.7, 0.9, 1.1 and 1.3mg/ml) and its effect has been studied[12,16].

Rapid confirmation of L-Asparaginase by rapid flask assay method

The modified asparagine medium was added with phenol red solution for confirmation of L-asparaginase production. Phenol red at acidic pH shows yellow colour and at alkaline pH it turns to pink. It is generally observed that L-asparaginase production was enhanced by increase in pH. Pink coloration of solution helps to identify the production of enzyme [13].

Rapid confirmation of L-asparaginase by thin layer chromatography method

Primarily produced enzyme was subjected to thin layer chromatography (TLC) for the confirmation of L-asparaginase production by using silica gel G as stationary phase and saturated phenol with water in the ratio of 1:1 v/v as a mobile phase. Ninhydrin solution was used as spraying agent for identification[14].

Determination of molecular weight

SDS PAGE was performed with a separating acrylamide gel of 10% and stacking gel of 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and de stained with a solution of methanol, acetic acid and water in the ratio of 4:1:5v/v. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers like Lysosyme (14.4 kDa), Endonuclease Bsp 981 (25kDa), Ovalbumin (45kDa), Bovine serum albumin (66kDa) and β-Galactosidase (116kDa)[15].

RESULTS AND DISCUSSION

Screening and Isolation of L-Asparaginase producers

Collected water sample was poured in to the PDA (potato dextrose agar) agar and kept for 14 days for the growth of fungal species. A loopfull of organism streaked over the plate containing Czapekdox's medium. The colonies with blackish brown and black with slight yellowish mycelia are visualized. The appearance is similar to the appearance of a*spergillus niger*[7]. This fungal isolates from water sample were inoculated in separate assay plates containing modified czapekdox's medium for screening of L-Asparaginase producers. The intensity of colour of zone formation helps in confirming the fungal species.

Determination of L-Asparaginase activity

The L-Asparaginase activity was assayed by Nesslerization method, a most common method for activity estimation. This was performed by quantifying ammonia formation in a spectrophotometric analysis at 450 nm for the respective concentrations and the observed values are shown in optimisation studies.

Effect of pH

The activity of L-asparaginase was evaluated at different pH values. The enzyme mixture was incubated with 1M Tris – Hcl (pH 8.0 – 9.0) in the pH range of buffer 5-9, under assay conditions and the amount of ammonia liberated was determined. The pre incubation was carried out for 10 minutes and then the activity was measured at 450nm.The Effect of pH on L-Asparaginase production is shown in figure 1.The maximal production of enzyme was observed at pH 8 and above. The productivity at pH 9.0 is twice that of pH 5.0. Hence pH 9.0 has been considered as optimum pH.

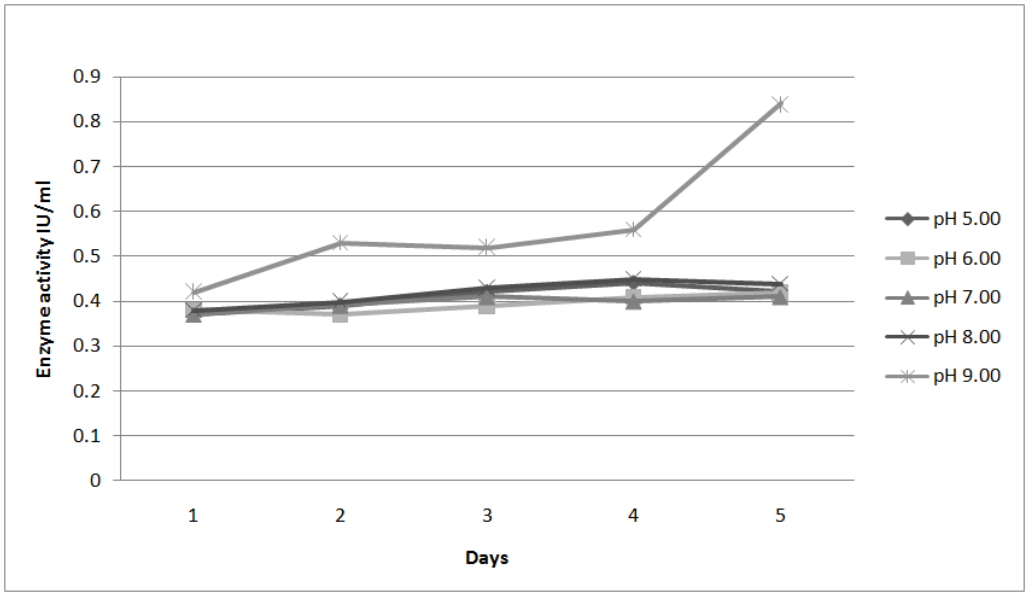

Fig. 1: Study of effect of pH on enzyme production

Effect of Temperature

The optimum temperature for the enzyme production was determined by incubating the assay mixture at temperatures ranging from to 30°C - 40°C and the amount of ammonia liberated was determined. Thermal stability studies were carried out by pre-incubating the enzyme at different temperatures for 15 min. Enzyme activity at different temperatures is tabulated in table 1.The productivity was found to be high in water isolate at temperature 35°C. This result suggests that *Aspergillus niger* adapts more active confirmation and stable at 35°C. The amount of ammonia liberated was found to be 0.75, 0.9, 0.82 IU/ml for temperature 30°C, 35°C, 37°C respectively.

Table 1: Effect of temperature on enzyme activity at optimized pH 9.0

Temperature °C	Activity IU/ml
30	0.748
35	0.991
37	0.825

Effect of Substrates

The production of L-Asparaginase was studied using different carbon and nitrogen sources at optimized pH 9.0 and Temperature 35°C.The result revealed that proline has maximal activity of 4.09 IU/ml. Glucose and starch addition showed approximately 3 times higher yield than normal production medium whereas sucrose addition showed 2 times higher which was observed 1 time lesser production than glucose and starch. L-Proline, L-Arginine and DL-Tryptophan showed good yield as such in carbon source. The amount of ammonia liberated was found to be 3.48, 2.45, 3.5, 4.09, and 3.5, 3.51 IU/ml for the substrates Glucose, Sucrose, Starch, Proline, Arginine, Tryptophan and Starch respectively. The results are shown in table 2.

Table 2: Enzyme activity for various substrates at 35 ^{0}C and pH 9.0

Substrates	Enzyme activity IU/ml
Glucose	3.48
Sucrose	2.45
Starch	3.60
Proline	4.09
Arginine	3.50
Tryptophan	3.51
Production medium	0.99

Effect of Di-Potassium hydrogen phosphate on enzyme production

Studies on nutrient requirements of *Aspergillus niger* conducted showed that change in concentration of di-Potassium hydrogen phosphate influences production as well as pH. The enzyme retains its activity 53 %, 77 %, 79 %, 100 % and 68 % at concentrations 0.5, 0.7, 0.9, 1.1 and 1.3 mg/ml of K_2HPO_4 respectively. The enzyme shows maximal activity with addition of 1.1mg/ml of K_2HPO_4.

Molecular weight of L-asparaginase

The molecular weight of the produced L-asparaginase was compared with the standard markers and it was found to be approximately 48 kDa. The comparison of molecular weight markers is shown in figure 3. Reports on production, purification and characterisation of extracellular L-asparaginase from *bacillus sp*. revealed by SDS-PAGE confirmed a peptide chain with molecular weight of 45 kDa. *Aspergillus niger* showed only one protein band on SDS-PAGE suggesting that it contained a single polypeptide chain.

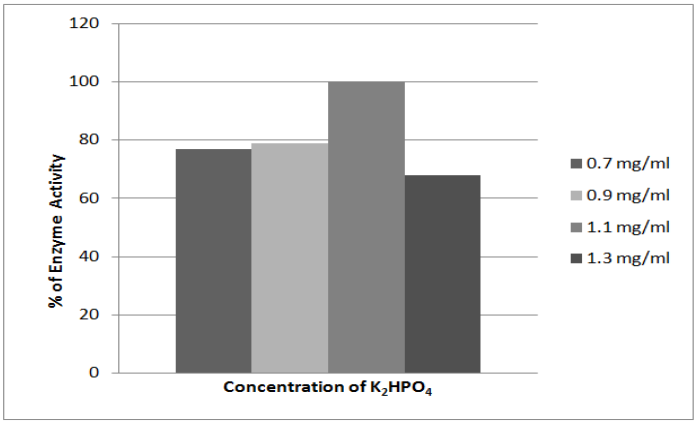

Fig. 2: Effect of Di Pottasium Hydrogen Phosphate on enzyme production

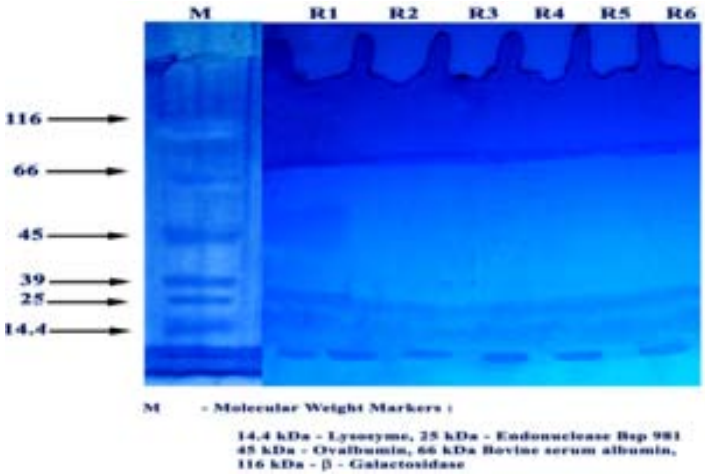

Fig. 3:

CONCLUSIONS

In our present investigation *aspergillus niger* was isolated from water and utilized for production of L-asparaginase. Optimum pH and temperature was found to be pH 9.0 and 35°C. Starch is found to be a better carbon source and proline was found to be the best `nitrogen source.

The present study revealed that the L-asparaginase may be produced by submerged fermentation from a*spergillus niger* isolated from water. The optimum pH for L-asparaginase production was found to be above 8.The enzyme had its optimum activity at a temperature of 35°C. Three different carbon sources glucose, sucrose and starch were used for the production, among this Starch was found to be the best carbon source. Three different nitrogen sources were used for the production L-Proline, L-Arginine and DL-Tryptophan. From the results proline was found to be the best nitrogen source. The L-asparaginase produced was confirmed by two methods Rapid shake flask assay method and thin layer chromatography method.

The high catalytic activity of the enzyme at physiological pH and temperature and its considerable stability over a wide range of pH and temperature makes it highly favourable to act as a potent anticancer agent. Studies on the enzyme relating to purification and characterization would open new avenues in the application of the enzyme in the healthcare industry. Consequently we suggest that enzymes which degrade amino acids should receive greater attention as potential therapeutic agents.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Pharmaceutical Technology, Anna University of Technology, Tiruchirapalli, for their support and assistance in this research.

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