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Research Article

PRODUCTION, PURIFICATION AND PROCESS OPTIMIZATION OF ASPARAGINASE (AN ANTICANCER ENZYME) FROM *E. COLI*, ISOLATED FROM SEWAGE WATER

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ABSTRACT

L-asparaginases are anti-cancer agent used in the lymphoblastic leukaemia chemotherapy. L-asparaginase is hydrolytic enzyme and is produced by a large number of microorganisms. The production of L-asparaginase enzyme was carried out by using L-asparagine as a substrate with the help of *E. coli*, isolated from sewage. The enzyme was purified by salt precipitation, dialysis and ion exchange chromatography. The enzyme activity was found to be 0.039 U/ml/min and the molecular weight was determined as 153 KDa with the help of SDS-PAGE. The Optimum temperature and pH was recorded as 55°C and 6 respectively. The study has shown the capability of the enzyme to withstand high temperature and hence can be considered as thermo stable enzyme.

Keywords: Anticancer enzyme, L-asparaginase, thermo stable, *E. coli.*

INTRODUCTION

Asparaginase (E.C.3.5.1.1) is a universally used component of childhood acute lymphoblastic leukemia (ALL) treatments, in both the remission induction phase and the post induction period. The critical role of asparaginase in the context of multi agent chemotherapy has been amply demonstrated by clinical studies showing direct relations between treatment outcome and dose intensity of asparaginase and between an inferior outcome and the use of an asparaginase preparation with a shorter half life. A recent randomized study showed a significant improvement in event-free and overall survival for children with all who were assigned to receive high dose asparaginase during continuation therapy compared with those who did not receive asparaginase [1]. The discovery of L-asparaginase, a medicinal agent for the treatment of malignant tumours, was made in 1922. L-asparaginase converts L-asparagine to L-aspartic acid. Since several types of tumour cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase [2]. Effective depletion of L-asparaginase results in cytotoxicity for leukemic cells but thus far, tumour inhibitory activity has been demonstrated only with asparaginase from *E. coli, Erwinia aroideae* and *Serratia marcescens*. The administration of such an enzyme protein for a long duration, in general, produces the corresponding antibody in the tissues, resulting in anaphylactic shock or neutralization of drug effect [3].

MATERIALS AND METHOD

Isolation and identification of E. coli from sewage water

1 ml of sewage water was collected and serial dilution was performed up to 10^{-8} dilution with 0.5% NaCl solution. 1 ml of the sample from the final dilution was spread plate on EMB (Eosin methyline blue) agar and incubated at 37°C for 24 hr (Scigenic Biotech India Pvt. Ltd.). Appearance of green shiny metallic colonies confirm the presence of *E. coli* in the sample. Pure cultures were obtained by streak plating. Presence of *E. coli* was confirmed by performing various staining methods and biochemical tests prescribed by Bergey's manual of systematic bacteriology, 4th edition [4] by using commercially available test kits KB001 and KB002 HiAssorted™ test kits (HiMedia India Pvt. Ltd.).

Production of L-asparaginase

1 ml of the overnight bacterial broth (O.D. > 0.5) was inoculated in 100 ml of production media (composition g/L: NaCl (5.0 g), L-asparagine (100.0 g), Maltose (10 g), KH_2PO_4 (0.75 g), pH-7.4), previously autoclaved at 121°C for 15 min at 15 lbs pressure in a 250 ml Erlenmeyer flask. It was then incubated at 37°C for 72 hr at 120 rpm in an orbital shaker incubator (SARTOMAT CT Plus, Sartorious Germany Pvt. Ltd.). Acidic pH after incubation confirms the production of L-asparaginase, as it converts L-asparagine in to aspartic acid and ammonia.

Purification of the crude enzyme

Modified method of Bessoumy EIAA *et al.,* (2001) [5] was used for the enzyme and purification. The Production media was centrifuged at 10,000 rpm for 12 minutes to obtain the supernatant. The supernatant after centrifugation was collected and the volume of it was measured and accordingly 70% ammonium sulphate was measured in order to conduct the salting out procedure. The supernatant was transferred into a conical flask and this flask was placed in ice cold condition on the magnetic stirrer, and the ammonium sulphate powder was added pinch by pinch until complete dissolving of ammonium sulphate takes place. After this, it was kept for overnight precipitation in the refrigerator. The pellet was collected and dissolved in 10 ml of 50 mM Tris hydrochloric acid solution. This sample was subjected to dialysis followed by ion-exchange chromatography.

Ion Exchange Chromatography

The chromatography column packed with 2% DEAE cellulose was washed twice using distilled water and kept for sonication for 15 minutes [6]. The matrix was activated using activation buffer (25 mM HCl and 25 mM NaCl). The dialyzed enzyme sample was poured into the column followed by a series of elution buffers containing 25 mM Tris HCl and increased concentration (50 mM, 75 mM…150 mM) of NaCl solution to elute the enzyme. The elutants were collected in the same test tube.

Characterization of the purified enzyme

Effect of temperature

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1ml of enzyme solution in the total volume of 2.1 ml. After incubation at 4°C, 37°C, 55°C and 80°C for 5mins, the reaction was stopped by adding 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 min [7]. The reading was taken at 660 nm in UV- Spectrophotometer to monitor the enzyme activity.

Effect of pH

The above method was repeated using 0.1M carbonate buffer of different pH (2, 4…10) and the enzyme activity was monitored spectrophotometrically.

Effect of activator

Different concentration of activator (magnesium chloride) was added to the enzyme and the enzyme assay was carried out to check

the activation and the above mentioned method was again repeated and the absorbance was taken at 660 nm.

Effect of inhibitor

Different concentration of inhibitor (EDTA-Ethylene diamine tetra acetate) was added to the enzyme and the enzyme assay was carried out to check the activity of inhibitor. The reaction mixture was prepared as mentioned above and the absorbance was taken at 660 nm.

Effect of Substrate Concentration

L-asparagine was used as substrate in different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.7 and 1.8 µg/mL) in different tubes. The enzyme activity was monitored by spectrophotometrically.

Determination of the molecular weight of the purified enzyme by SDS-PAGE

The molecular weight of the extracted enzyme was determined by performing SDS-PAGE.

Results and discussion

L-asparaginase producing *E. coli* was isolated from sewage water and identified on the basis of biochemical tests and staining techniques prescribed by Bergey's manual of systematic bacteriology, 4th edition and the results were tabulated (Table 1).

The enzyme was extracted from the production media by centrifugation and further purified by salt precipitation, dialysis and ion exchange chromatography.

Table 1: Biochemical test results for *E. coli*

Tests	Indole	Methyl red	Voges Proskauer's	Citrate	Glucose	Adonitol	Arabinose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose
Results	+	+	-	-	+	-	+	+	+	+	V	V

+ = Positive (more than 90%), - = Negative (more than 90%), V = 11-89%

The optimum enzyme activity was determined at different temperature, pH and at different concentration of activator, inhibitor and substrate concentrations. The optimum temperature for the enzyme activity was found to be at 55°C (Fig 1). The results indicate that at 30°C, L-asparaginase production was 5.4U/ml. The decrease in the yield of enzyme was observed when incubation temperature was higher or lower than optimum incubation temperature. The enzyme was found to be maximally active at 40°C. This optimum L-asparaginase activity at 40°C is similar to that of *Corynebacterium glutamicm* reported by Mesas et al.1990 regarding the thermal stability, at 80°C, the enzyme retained 55% of the activity. According to our results the enzyme was found to be maximally active at 55°C, so it is thermostable and the enzyme found to be retained 98% of its activities.

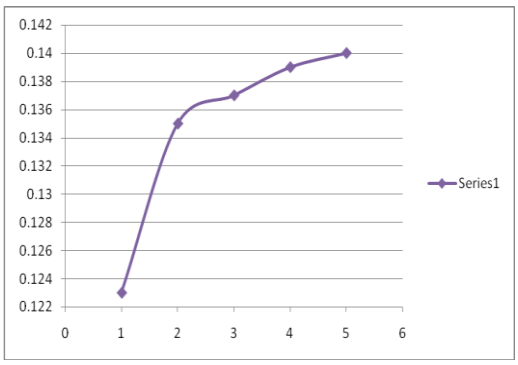

Fig.1: Effect of temperature on enzyme activity (on X-axis, 0 = 0°C, 1=10°C,...6=60°C)

The enzyme activity was found to be maximum at pH 6 (Fig.2).

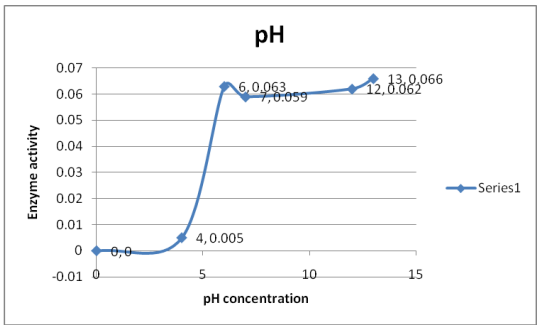

Fig.2: Effect of pH on the extracted enzyme activity

The enzyme activity was found to be maximum at an activator ($MgCl_2$) concentration of 1000 µg/mL (Fig. 3).

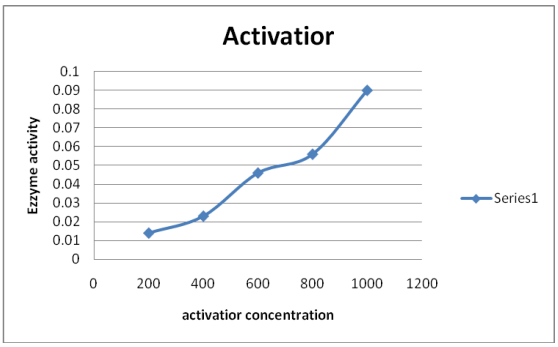

Fig.3: Enzyme activity at different concentration of activator (µg/mL)

The maximum enzyme activity was found to be declined beyond inhibitor concentration of 2 µg/mL (Fig.4) and substrate concentration of 1.2 µg/mL (Fig.5).

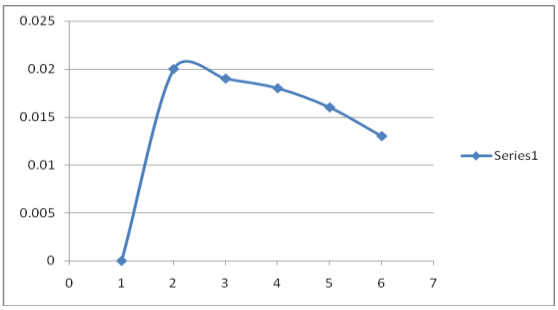

Fig.4: Effect of inhibitor concentration (µg/mL) on enzyme activity.

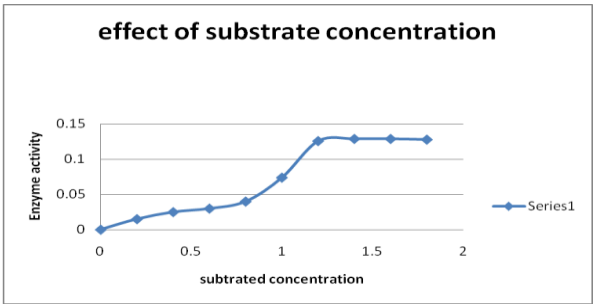

Fig.5: Effect of substrate concentration (µg/mL) on enzyme activity.

The molecular weight of the purified enzyme was determined by SDS-PAGE and found to be 153 KDa.

CONCLUSION

Our aim was the production, purification and characterization of L-asparaginase from *E.coli* and to do a comparative study based on temperature, pH, substrate concentration, inhibitor, and activator concentration. *E.coli* isolated from sewage sample and screened for the production of L-asparaginase. *E. coli* is commonly known as colon bacteria and easily available in sewage water with fecal contamination. As the isolation and screening method for *E. coli* is very easy, and the enzyme that was extracted was found to be active at higher temperature (55^{o}C). Considering all these characteristics, the production of L-asparaginase from *E. coli* may be recommended for industrial production of the said enzyme.

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