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Research Article

STUDY ON PRODUCTION, PURIFICATION AND CHARACTERISATION OF L-ASPARAGINASE FROM *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Water sample microbes were isolated and screened for the potential producers of L-asparaginase using modified M-9 medium plate method. The single discrete colonies which have exhibited clear pink zone surrounding microbial colonies after 48 hours incubation indicate L-asparaginase producing cultures. This can easily be detected by the change in pH of the medium using phenol red. These colonies (L-Asparaginase positive cultures) were picked up and grown in the modified Czapeck Dox agar. *E coli* and *Pseudomonas aeruginosa* are optimised for comparative study and are inoculated in modified M-9 medium as production media. The largest quantity of L-asparaginase was produced when *pseudomonas aeruginosa* was grown in the modified M-9 medium for 52 hrs. Enzyme activity and specificity was calculated for all the crude and purified samples of the two different organisms. The maximum enzyme activity and specific activity was observed in *Pseudomonas aeruginosa* by using L-Aspargine as substrate. The activity of the *Pseudomonas* increased to 602 U/g as compare to specific activity of L-asparaginase from *E coli* 520 U/g. The molecular weight of the protein was found to be 75KDa. Characterisation of the enzyme showed optimum pH 7.0 for both *E coli* and *Pseudomonas aeruginosa*. Optimum temperature was 55^0C.

Keywords: Ammonium sulphate, Ion-exchange chromatography, modified M-9 medium.

INTRODUCTION

The discovery of L-asparaginase (L-asparaginase aminohydrolase, E.C.3.5.1.1), a medicinal agent for the treatment of malignant tumors, was made in 1922. While Mashburn and Wriston[1] successfully purified *Escherichia coli* L-asparaginase and demonstrated its tumour-inhibitory activity. 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. Effective depletion of L-asparagine results in cytotoxicity for leukaemic cells but thus far, tumour inhibitory activity has been demonstrated only with asparaginases 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. Therefore, the use of new serologically different L-asparaginase with a similar therapeutic effect is highly desirable[2]. Asparaginase is marketed under the brand name *Elspar* Unlike other chemotherapy agents; it can be given as an intramuscular, subcutaneous, or intravenous injection without fear of tissue irritation. Administration of L-asparaginase has been found to reduce L-asparagine levels in the

blood and to selectively inhibit malignant growth[3]. This is explained by the fact that certain tumours, especially acute lymphoblastic leukaemia (ALL) tumour cells, have a decreased or completely absent activity for asparagine synthase and hence are nutritionally dependent on an external supply of l-asparagine for growth. The finding by Mashbum and Wriston[1] that L-asparaginase derived from Escherichia coli antitumor activity similar to that of guineapig serum opened up the possibility of large scale production of the enzyme for ultimate clinical trial. Subsequently, Roberts *et al*[4] and Campbell *et al*[5] demonstrated that *E. coli* B L-asparaginase exists in two forms, one active and the other inactive against animal tumors. The two enzymes differ markedly in the pH-activity profiles, their solubility, and chromatographic behavior. E. coli K-12 was also shown to produce a biologically active and inactive form of L-asparaginase. The active form, which is produced only under anaerobic conditions and is located near the cell surface, differs from the inactive form in its affinity for substrate, its solubility in ammonium sulfate solutions, and its sensitivity to thermal inactivation. Increasing availability of larger quantities of sufficiently purified L-asparaginase has made possible limited trials of therapy in larger animals and man. However, extensive clinical trials of L-asparaginase therapy against a variety of human neoplasms have not been possible. The present communication reports practical methods for the growth of *E. coli* with high asparaginase yields and purification of this enzyme in sufficient quantity for human therapy. H. F. Oettgen *et al* 1969[6], M. H. Bilimoria 1969[7] describes Conditions for the Production of L-Asparaginase 2 by Coliform Bacteria-Of 28 coliforms, five strains of *Escherichia coli* were particularly active in elaborating L-asparaginase 2, the form of the enzyme useful in the treatment of some forms of cancer. Since it is advantageous to start purification of the enzyme from highly active cells, cultural conditions necessary for good growth and high enzyme yield have been studied. Gentle aeration proved suitable for good growth as well as high enzyme content.

In the present investigation is to enhance the production, purification and charactesation of L-asparaginase from *Escherichia coli* and *Pseudomonas aeruginosa*

MATERIALS AND METHODS

Isolation of L-asparaginase producing organism from water sample

Water sample was collected from the Bangalore city. 1ml of water sample was serially diluted and the samples were inoculated into modified M-9 medium[8]. Water sample microbes were isolated and screened for the potential producers of L-asparaginase using modified M-9 medium plate method. The single discrete colonies which have exhibited clear pink zone surrounding microbial colonies after 48 hrs incubation indicate L-asparaginese producing cultures. This can easily be detected by the change in pH of the medium using phenol red. These colonies (L-Asparaginase positive cultures) were picked up and grown in the modified Czapeck Dox agar. It was identified by morphological and biochemical characteristics[9].

Inoculums preparation

The organism was grown on modified Czapeck Dox agar slants at 28 °C .The two different bacterial colony were scrapped off and transferred into a 250ml Erlenmeyer flask containing 25 ml of inoculum medium that is modified Czapeck Dox. The flasks were incubated at 28°C in rotary shaker at 150 rpm for 52 hours for *Escherichia coli* and 128 hours for *Pseudomonas aeruginosa*.

Production and Purification of L-asperaginase by Submerged Fermentation

100 ml of production media was prepared and autoclaved at 121^{o} C for 15min and the loop full organism was inoculated. The inoculated media (Czapeck Dox media) was kept in shaker incubator at 100 rpm at 37^{o}C for the 52 hours for *Escherichia coli* and 128 hours for *Pseudomonas aeruginosa*. After incubation the culture broth was centrifuged at 10,000rpm for 10min at 4°C. The supernatant was stored at 4^{o}C.

Purification of L-asperaginase

The efficiencies of the crude enzyme were analyzed. All the purification procedures were carried out at 4^{0}C. The crude enzyme was subjected to 70% saturated ammonium sulphate precipitation. The precipitate was collected by centrifugation at 9,000rpm for 15min and dissolved in minimal amount of 0.1M Tris Hydrochloric acid buffer of pH7.0 and then dialysed extensively against the same buffer[10]. The enzyme activities in the dialysate were assayed. The dialyzed fraction was applied to a Diethylaminoethyl cellulose (DEAE-C) column, an anion exchanger, pre-equilibrated with Tris-Hydrochloric acid buffer, pH 8.6. The enzyme was eluted (1 ml/min) with sodium chloride gradient (0.1 - 0.5 M) and 0.025M Tris buffer, pH 7.0[11].

Enzyme assay

Enzyme assay was done for both crude and after each stage of purification by Colorimetric

method. L-asparagine is used as substrate. The reaction mixture containing 0.2ml of tris hydrochloride buffer, 1.7ml of L- asparagine and 0.1ml of enzyme solution. The control containing boiled inactivated enzyme (at 100^{o}C for 5min) was treated similarly. Then centrifuge at 10,000rpm for 10 min. Take 7ml distilled water in the test tube and add 0.5 ml supernatant. Then add 1 ml Nessler's reagent in each tube and incubate at room temperature for 10 min. Take Optical density at 480nm.

Calculation for enzyme activity

Enzyme activity= micromoles of ammonia released/min/ml

Calculation for specific activity

Specific activity= enzyme activity/protein concentration

Calculation for %yield

Yield= total enzyme activity of fraction (x) /total enzyme activity in starting material

Purification factor = (specific activity of purified extract) / (specific activity of crude extract)

Estimation of protein

The amount of protein was estimated by the method of Lowry *et al.*,[12] using bovine serum albumin as the standard.

Characterisation

The optimum activity of the enzyme was determined by assaying at different temperatures 4, 28, 37, 55 and 100^{0}C and pH 3, 5, 7, 9, 11. The activity was determined in different concentration of the metal ion Calcium chloride ($CaCl_2$) and metal ion chelator Ethylenediamine tetra acetic acid (EDTA) in the concentration of 1 to 9µM and 0.5 to 3µM respectively in the reaction mixture.

SDS PAGE

SDS PAGE was performed according to the method of[13], with a separating acrylamide gel of 12.5% 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 4:1:5[11]. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers phosphorylase b (97.4KDa), Bovine serum albumin (66KDa), Ovalbumin (43KDa), carbonic anhydrase (29KDa), lactoglobulin (18.4KDa) and lysozyme (14.3KDa).

RESULTS AND DISCUSSION

After Gram's staining the microorganisms was observed under microscope at oil immersion. Pink colour rods were observed. Hence *Escherichia coli* and *Pseudomonas aeruginosa* are Gram negative bacteria's. Two strains of gram-negative organisms were screened for L-asparaginase production. Of the organisms tested, *Escherichia coli* and *Pseudomonas aeruginosa* contained measurable quantities of L-asparaginase. Effect of culture conditions on L-asparaginase production by *Escherichia coli* and *Pseudomonas aeruginosa*. On the basis of antitumor property, *Escherichia coli* and *Pseudomonas aeruginosa* was chosen as a preferred L-asparaginase-producing bacterium and the culture conditions for production of the enzyme were studied in detail. The culture was grown in media like modified M-9 medium, and L-asparaginase activity produced in the cells was measured. The largest quantity of L-asparaginase was produced when *Pseudomonas* was grown in the modified M-9 medium for 52 hrs.

Biochemical investigations for taxonomic identification of bacteria producing L-asparaginase

Taxonomic characterisation of the isolated bacterial strains was carried out by applying series of standard Biochemical tests. All the below biochemical tests confirmed that our wild organism may be *Escherichia coli* and *Pseudomonas aeruginosa*. Table 1.

Table 1: Biochemical test was performed for the identification of organism

Biochemical test	*E.coli*	*Pseudomonas*
Indole	-	-
Methyl Red	+	-
Voges Proskauer	-	-
Citrate utilization	-	+
Urease	-	-
Catalase	+	+
Gelatin	-	+
Glucose fermentation	+	-
Lactose fermentation	+	-
Hydrogen sulphide test	-	-

Production, Purification and Characterization of L-asparaginase

Enzyme activity and specificity was calculated for all the crude and purified samples of the two different organisms. The maximum enzyme activity and specific activity was observed in *pseudomonas* by using L-Aspargine as substrate showed in Table: 2, Figure: 1.

Table 2: Specific activity and fold purification for test organisms

	Organism	Conc. Of protein mg/ml	Activity U/ml/min	Specific Activity U/gm	Yield %	Fold purification
Crude extract	*E.coli*	1.976	0.104	52.8	100	1
	Pseudomonas	1.736	0.095	54.4	100	1
After heating	*E.coli*	0.392	0.086	218.6	82	4.14
	Pseudomonas	0.348	0.069	198	73	3.64
Ammonium-sulphate precipitation	*E.coli*	0.136	0.057	419	54	7.93
	Pseudomonas	0.156	0.059	378	62	6.94
Ion exchange chromatography	*E.coli*	0.078	0.039	510	37	9.66
	Pseudomonas	0.072	0.044	602	45	11.06

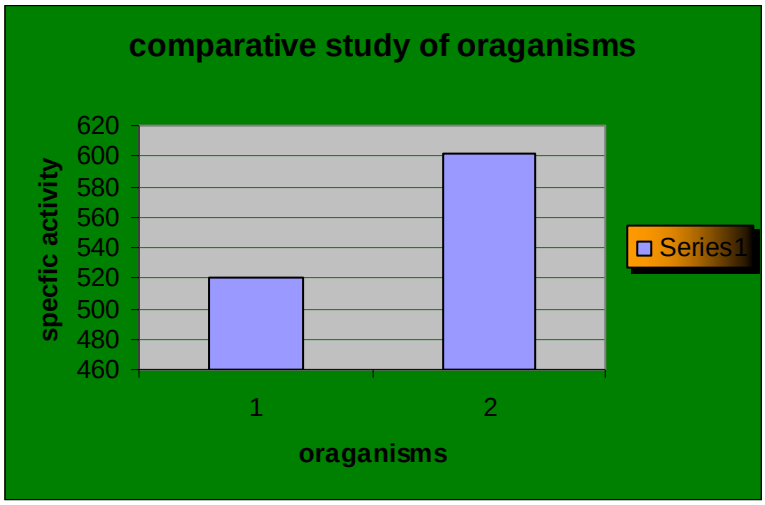

Fig. 1: Comparison of different organisms producing L-Asparaginase

According to this work specific activity of L-asparaginase from *E coli* was 510 U/g. whereas specific activity (U/g) of *Pseudomonas* was 602 which were more than *E. coli*.
According to Joseph Roberts *et al*[14] work Specific activity of *E.coli* was 496, Specific activity (IU/mg) 0f *Pseudomonas Aeruginosa* was 49[15].

SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis)
The isolated enzyme samples (pure sample and crude sample) from *Escherichia coli* and *Pseudomonas aeruginosa*.. The molecular weight of the enzyme is found to be 75KDa. Approximately by using marker in SDS-PAGE electrophoresis.

Characterization of enzyme
Effect of pH on enzyme activity
The optimum pH for *Escherichia coli* and *Pseudomonas aeruginosa* is 7 Fig: 2. A gradual increase in L-asparaginase production was observed from pH 5.0 to 6.5 followed by a gradual decrease of enzyme yield beyond pH 6.5. Higher or lower than this pH (6.5) resulted in lower yield of L-asparaginase. Regarding the pH stability, the enzyme retained more than 80% of the activity in the pH range of 7-10[16]. According to my work the optimim pH of L-asparaginase is 7-8, so at this pH the enzyme precipitates at higher concentration and highly active. The nearest physiological pH (6.5) makes this enzyme superior to that of bacterial origin as a chemotherapeutic agent in treatment of leukemia 2[17]. Our results are in good agreement with those of Amena S[16].

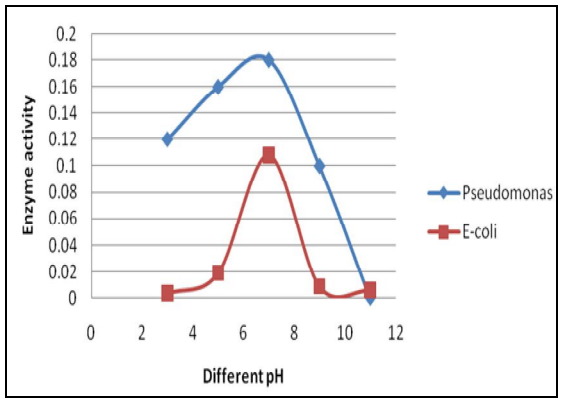

Fig. 2: Effect of pH on enzyme activity
Effect of temperature on enzyme activity

The optimum temperature of the enzyme from the different sources was found to be 55[0]C Fig: 3. Regarding the thermal stability, at 80°C, the enzyme retained 55% of the activity[16]. According to our results the enzyme was found to be

maximally active at 55°C, so it is thermostable and enzyme retained 98% activity.

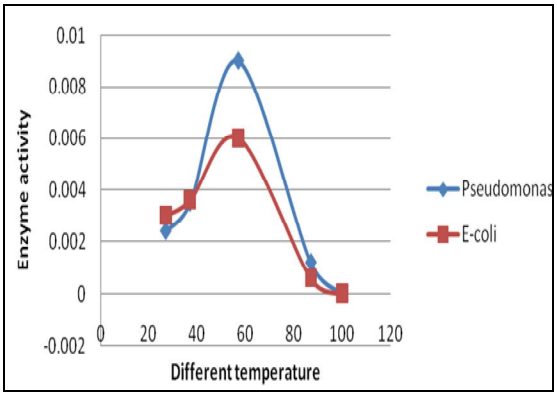

Fig. 3: Effect of temperature on enzyme activity

Effect of activator on enzyme activity

In the presence of 9µM $CaCl_2$ the enzyme activity was increased Fig: 4. Addition of activator showed increased enzymatic activity in all the samples. However at certain point the enzyme activity become stationary.

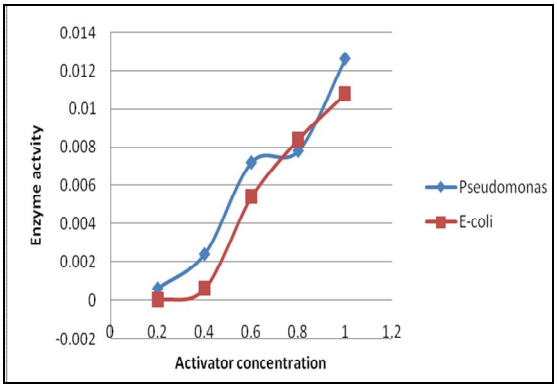

Fig. 4: Effect of activator on enzyme activity

Effect of Inhibitor on enzyme activity

Addition of inhibitor decreased enzyme activity in all the samples Fig. 5.

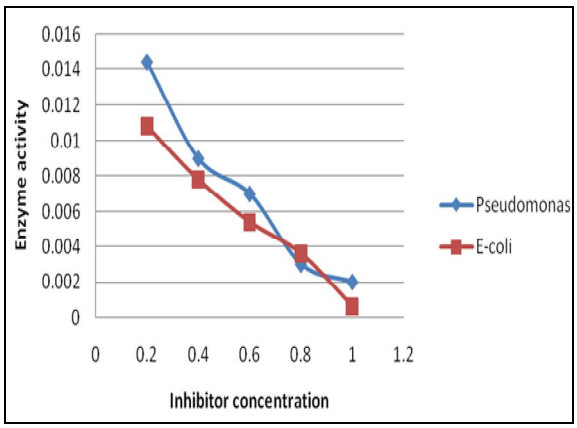

Fig. 5: Effect of inhibitor on enzyme activity

CONCLUSION

In conclusion two strains of gram-negative organisms were screened for L-asparaginase production. *Escherichia coli* and *Pseudomonas aeruginosa* was chosen as a preferred L-asparaginase-producing bacterium and the culture conditions for production of the enzyme were studied in detail. The largest quantity of L-asparaginase was produced when *pseudomonas* was grown in the modified M-9 medium for 52 hrs and the maximum enzyme activity and specific activity was observed in *pseudomonas*. Hence further investigations using more experimental paradigms are warranted for further confirmation.

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