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# Enhanced recovery of L-asparaginase from isolated Penicillium sp. through modified cultural and nutritional amendments under submerged culture conditions

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## Abstract

*Culture conditions were optimized for enhancing the amount of L-asparaginase in submerged culture medium of Penicillium sp. Optimized parameters involve different C and N ratio, culture conditions and additional compounds followed by extraction, purification and characterization of the enzyme. The fungus required 8 days and a pH of 4.5 for the optimum growth and enzyme production. The maximum L-asparaginase activity was observed with fructose, ammonium sulphate and L-arginine. The enzyme production was concentration dependant and maximum activity was recorded at fructose 35g/litre with addition of 5g/litre each of L-asparagine, ammonium sulphate and L arginine. The purification of enzyme from mass culture was developed in a modified media by following salt precipitation, Sephadex G-100 filtration and ion exchange chromatography by DEAE cellulose columns. Result revealed purified protein and enzyme preparations that was matched with standard (66kDa) and exhibited approximately 97k Dawith 0901x10$^{-3}$MK$_m$ value. Enzyme purified through gel filtration was active within the fractions 4 to 7.2 and at higher temperature. The impact of nutritional and cultural conditions towards enhanced enzyme production is clearly evident in the present study. The thermal stability of the purified enzyme also ensures its suitability for long term preservation and the basic modification of the basal medium in order to achieve enhanced enzyme production may be a good support for the large scale fermentation and production of this enzyme.*

**Keywords:** L-asparaginase, Penicillium, Fungi, Gel filtration.

## Introduction

Microbial enzymes are used as an important tool in biochemical and biomedical research and in various industrial processes. Among them, L-asparaginases have major potential as a therapeutic agent for the treatment of many types of cancer especially acute lymphocytic leukaemia. The Leukaemic cells are lack of aspartate ammonia ligase enzyme and unable to perform the synthesis of L-asparagines. Hence, L-asparagine present in the surroundings of cancer cells becomes the essential amino acid for their growth. Under such circumstances, L-asparaginases degrade the surrounding asparagines and causes reduction in growth of cancerous cells[1-6]. Several microorganism have the potential of asparaginase production and enzymes derived from them are important for practical clinical use[7,8]. The L-asparaginase enzyme from *E.coli* and *Erwinia chrysanthomi* are used as chemotherapeutic agent for past many years[3,4,9]. The present study confirms that microbes are better sources of L - asparaginase because of their culture development and purification process[10-14]. Manyeucaryotic microbes like yeast and filamentous fungi are reported as producer of L-asparaginase. Microfungi such as *Aspergillus, Penicillium* and *Fusarium* are found to be good source of this enzyme[15-19]. The bioprocess development of any microbial product requires optimization of nutritional components and culture conditions[20-22]. A good effort has been done towards the enhancement of enzyme production by several microbes through cultural and nutritional modifications. The effects of culture medium, pH and aeration on L-asparaginase synthesis have been shown to vary for different organisms[23-24]. Carbon and nitrogen ratio and their sources also play an important role in high productivity of this enzyme[25-26]. Sometimes glucose may also play a vital role in gene regulation for L-asparaginase production just like in *E coli*.[27-28]. Nitrogen sources like proline, glutamic acid and sodium nitrate also affects the production of extracellular L-asparaginases[21]. In view to this, an attempt has been taken towards standardization of nutritional and cultural requirements for better enzyme production from *Penicillium* sp. and the results obtained during various experimental steps have been described and discussed herewith.

## Materials and methods

**Source, media of fungal strain:** Screened fungal isolate of *Penicillium* sp. (RF 22/G) was selected for the experiment. The chemicals used were of analytical grade. Glucose-asparagine broth with pH-4.5 and sterilized at 121ºC for 15-20 minutes was used. A single 5mm disc of inoculum derived from the culture plates was inoculated into the flask containing broth medium. The culture flasks were incubated at 30ºC for 10 days in static condition.

**L-asparaginase enzyme assay and protein estimation:** The fungus was grown in glucose asparagines medium (25ml in 150ml Erlenmeyer flasks, pH 4.5) at $30^0$C, under static condition. The cultures were harvested after the due incubation period and the mycelial mats were washed with ice cold distilled water. To evaluate the extracellular enzyme activity, culture filtrate was centrifuged at 3000rpm and supernatant was assayed for enzyme activity. The enzyme L-asparaginase was assayed by estimating the amount of ammonia released in the reaction[29,30]. The amount of ammonia released by the test sample was calculated with reference to the standard graph. The enzyme activity was expressed in terms of enzyme units (IU/ml).Protein was estimated according to Bradford's[31].

**Optimization of culture conditions:** The fungal culture was developed in basal medium containing L-aspargine 0.5%, $KH_2PO_4$ 0.1%, $MgSO_4$ 0.05% at a pH 4.5 and at 30ºC for 8 days in shake culture (50rpm) and static culture conditions. Finally, L asparaginase activity was tested in culture filtrate and biomass in order to check the best source of enzyme. Similarly, an experiment was set in same glucose–asparaginase medium for different incubation period. Enzyme activity was determined in both culture filtrate and biomass in order to select the best incubation period[26,32].

**Selection of carbon source:** Arabinose, fructose, lactose, sorbitol, starch, galactose, maltose, manitoal, sucrose, raffinose, glucose, manitol and inositol were used as carbon source and cultures were prepared by incubating them at 30ºC for 8 days. Finally, selected carbon sources (Fructose) was used for further optimization process.

**Selection of additional amino acids and other compounds:** Different nitrogen sources viz, 1-Tryptophan, 2-Glutamine, 3-Cysteine, 4-Proline 5-Threonine, 6-Methionine, 7-Phenylalanine, 8-Alanine, 9-Tyrosine, 10-Arginine, 11-Asparatic acid, 12-Guanine, 13-Xanthine, 14-Histidine, 15-Oxalic acid, 16-Citric acid, 17-Hippuric acid, 18-Potasssium Nitrate, 19-Ammonium Sulphate, 20-Pyruvic acid were added separately into the basal medium along with the selected carbon source and finally enzyme activity was tested in culture filtrate after 8 days of incubation.

**Selection of other nitrogen sources:** Fructose in combination of other nitrogen sources viz, 1-L-asparagine, 2-Ammonium sulphate, 3-L-arginine, 4-Xanthine, 5-Asparagine, Ammonium sulphate and arginine, 6-Asparagine, Ammonium sulphateand Xanthin, 7-Asparagine, Arginine and Xanthine were added separately into the basal medium along with the selected carbon source and finally enzyme activity was tested in culture filtrate after 8 days of incubation.

**Selection of Fructose concentration:** Fructose was used in different concentration (10, 15, 20, 25, 30, 35 and 40%) in basal medium to prepare fungal culture by incubating them at 30ºC for eight days. Finally, enzyme produced in culture filtrate was tested.

**Effect of L-asparagine, ammonium sulphate and L – arginine:** The basal medium having fructose were added separately with different concentration of (5,10,15,20,25g per litre) L asparagine, ammonium sulphate and L-arginine separately. Finally, 8 day old fungal culture was prepared and enzyme was estimated in culture filtrate.

**Extraction, Purification and Characterization of L-asparaginase: Inoculations and Ammonium Sulphate precipitation:** Mass scale production of fungal culture was done in optimized media in static condition at $30^0$C and enzyme extracted with 0.05M Tris-HCl buffer, pH 8.5 in the ratio 1:5. It was centrifuged at 6000rpm for 20min at 4ºC and the supernatant was collected. This was treated as the crude preparation of the enzyme. The crude enzyme was treated with ammonium sulphate (80%) and precipitate collected was dissolved in Tris-HCl buffer prior to gel filtration.

**Sephadex G-100-120 gel filtration and Ion exchange chromatography:** A chromatography column made up of glass tubing having a diameter of 2.2cm and a height of 60cm was used. Tris-HCl buffer (0.05M) with a pH 8.5 was used as eluent. For the preparation of gel slurry, 10g of sephadex was suspended in 400ml of 0.05M Tris-HCl buffer and left 24 hours to swell at room temperature. The gel slurry was slowly poured into the column without air bubbles being trapped in the column. In the packed gel, crude enzyme was loaded with Tris-HCl buffer (1:2 ratio) and fractions were collected at the rate of 5ml in glass vials. The collected fractions were tested for enzyme activity and protein randomly and the fractions showing better enzyme activity were pooled together.

Required amount of DEAE-cellulose was dissolved in Tris-HCl buffer and was left for overnight and was used to make the column. The column was packed; initially it was washed with 5N NaOH to remove ionic charges from the ion exchanger then with 5M KCl to generate desired form of weak ion exchange material. Finally, it was washed with distilled water followed by 0.05M Tris-HCl buffer (pH 8.5). The pooled peak fractions collected from sephadex filtration were applied to the ion exchange column and then 5ml fractions were collected. Samples were analyzed for enzyme activity and protein. Fractions having better enzyme activity were pooled together and stored in deep freezer for later use.

**Electrophoretic separations of L-asparaginase and its characterization:** SDS poly acrylamide gel electrophoresis (SDS-PAGE) was performed to analyse the presence and molecular weight of purified L-asparaginase. The Km of the enzyme was determined by making the reaction mixture containing fixed volume (0.25ml) of the partially purified enzyme and varying concentration of the substrate L-asparagine. The total volume of the mixture was made upto 2ml with 0.05M Tris-HCl buffer of pH 7.2 and the enzyme activity was estimated. To determine the effect of temperature, the purified

enzymes was treated at different temperature and estimated for activity.

## Results and discussions

The fungal culture produced more amount of enzyme in basic glucose asparagine medium of pH 4.5 at 30ºC for 8 days. The maximum enzyme activity was observed in nutrient medium supplemented with 1% fructose (8.68IU/mg protein) and arabinose (8.24IU/mg protein) among nine carbon sources tested (Figure-1). A higher enzyme production (16.47IU/mg protein) was exhibited in the medium containing nitrogen source, ammonium sulphate and L-arginine (15.59IU/mg protein) (0.5% w/v) as compared to other nitrogen sources (Figure-2).

Addition of pyruvic acid in the above medium also exhibited good (15.0 IU/mg protein) enzyme production. The fungus was cultured in the medium containing 1% fructose (carbon source) and 0.5% N source in different composition. Remarkable L-asparginase activity (19.70 IU/mg protein) was observed in the medium containing nitrogen source L-asparagine + ammonium sulphate + L-arginine as compared to these nitrogen sources individually or in combination (Figure-3). Although L asparagine, ammonium sulphate and L-arginine showed maximum activity as compared to other nitrogen source, the addition of fructose on enzyme production and activity was found to be concentration dependant and maximum activity was recorded at 35g/litre with addition of 5g/litre of L-asparagine, ammonium sulphate and L - arginine (Figure-4).

Figure-5 to 7 shows the enzyme activity of this fungus when grown in the media containing different concentration of selected nitrogen sources. Along with 3.5% of fructose and 05% of ammonium sulphate and L-arginine, fungus required 2.5% of L-aspargine to produced 38.383IU/mg protein (Figure-5). It was observed that changes in ammonium sulphate concentration did not show differences in enzyme activity. However, addition of ammonium sulphate at 2% concentration exhibited better enzyme activity as compared to other concentration used (Figure-6). Similaraly, L-arginine (2%) in the selected medium also exhibited good effect and enhanced enzyme activity (Figure-7).

The purification of salt precipitated enzyme through gel filtration column chromatography exhibited good enzyme activity in fractions 10 to 20. Ion exchange purification of enzymes showed good activity in fractions 2 to 6 (Figure-8a and b). The specific activity increased to 500.0IU/mg and 327.714 IU/mg through salt precipitation and gel filtration. Partially purified enzymes preferred the alkaline pH. The enzyme activity declined at alkaline pH. Enzyme purified through sephadex column was active at 45-50$^0$C whereas partially purified enzyme of DEAE cellulose colomn declined its activity at higher temperature. Partially purified enzyme exhibited activity in presence of asparagines, arginine and histidine and glutamic acid separately. On electrophoretic separation, the purified protein and enzyme preparation showed approximately 97 kDa molecular weight and exhibited $0.901 \times 10^{-3}$ M $K_m$ value.

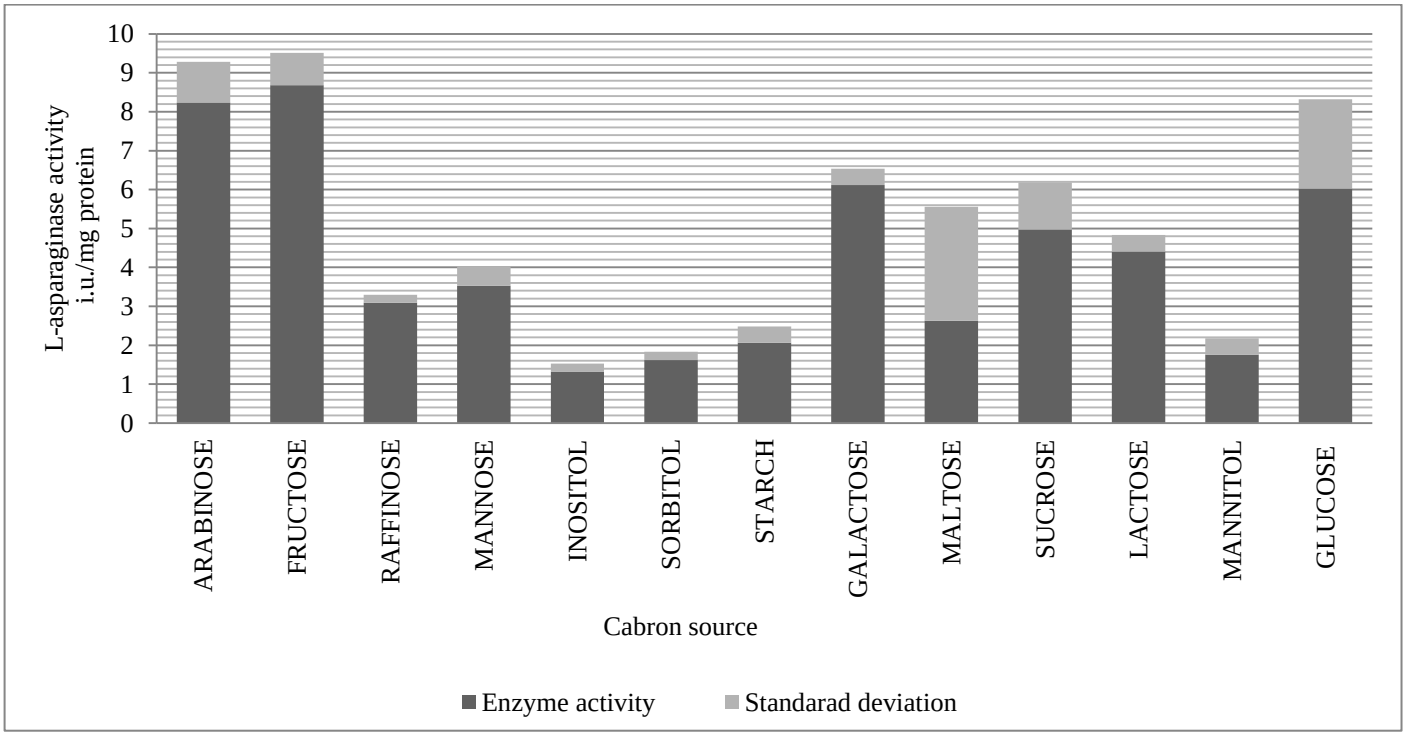


**Figure-1:** Effect of carbon sources on L-asparagines activity.

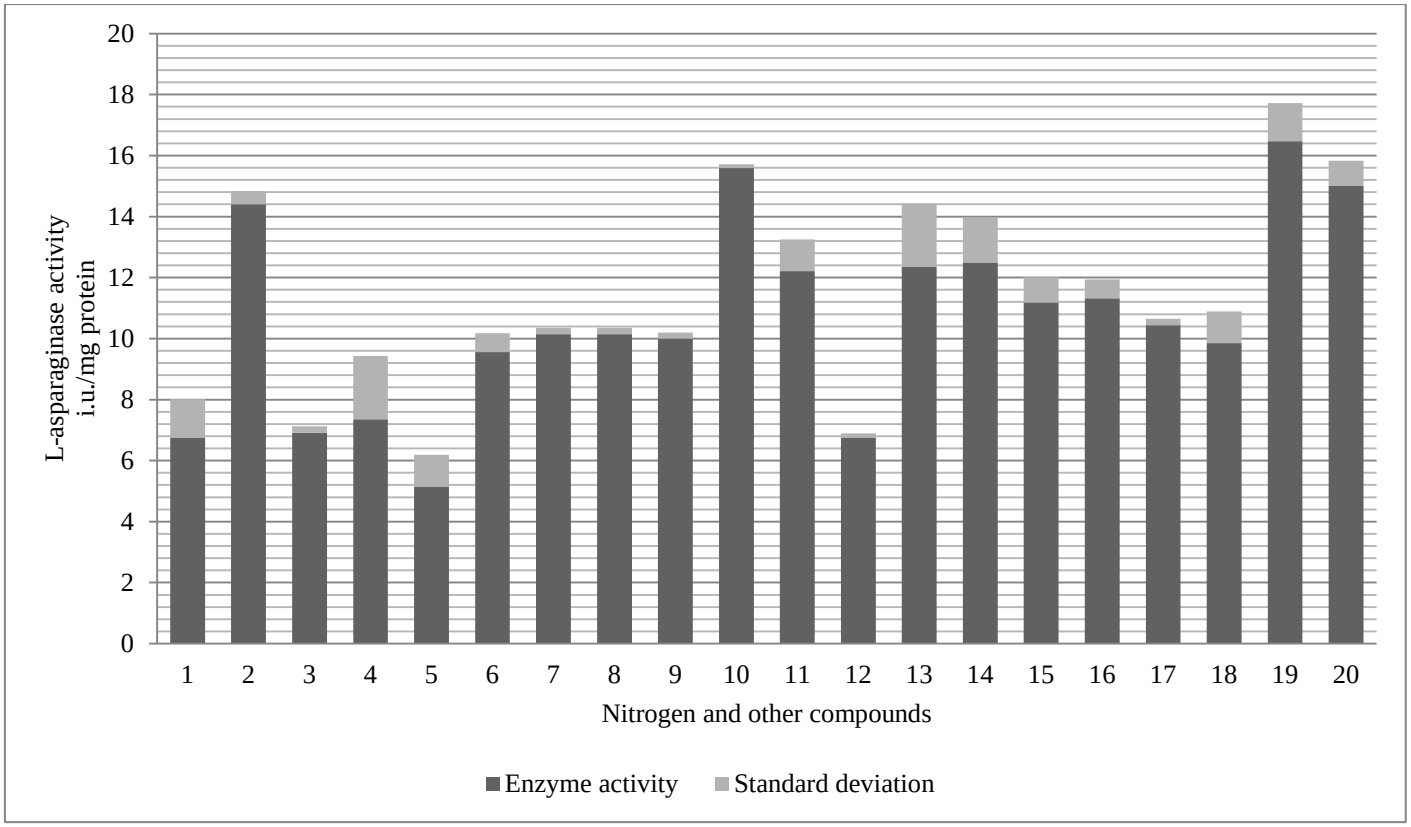


**Figure-2:** Effect of nitrogen sources and other compounds on L-asparaginse activity (1-tryptophan, 2-glutamine, 3-cysteine, 4-proline 5-threonine, 6-methionine, 7-phenylalanine, 8-alanine, 9-tyrosine, 10-arginine, 11-asparatic acid, 12-guanine, 13-xanthine, 14-histidine, 15-oxalic acid, 16-citric acid, 17-hippuric acid,18- Pot Nitrate, 19- ammonium Sulphate, 20-pyruvic acid).

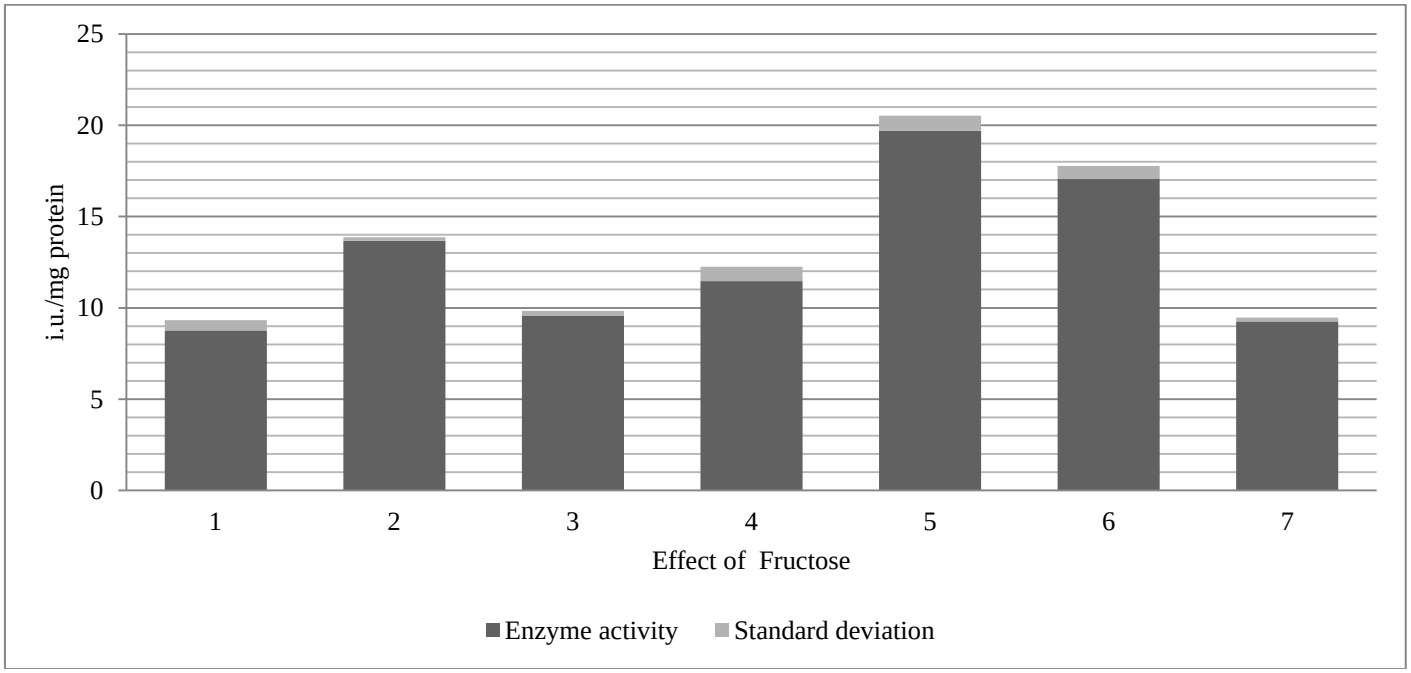


**Figure-3:** Effect of Fructose in combination of other nitrogen sources (1- L-asparagine, 2-Ammonium sulphate, 3-L-arginine, 4-xanthine, 5-Asparagine, Ammonium sulphate and arginine, 6-Asparagine, Ammsul and xanthin, 7-Asparagine, arginine and xanthine).

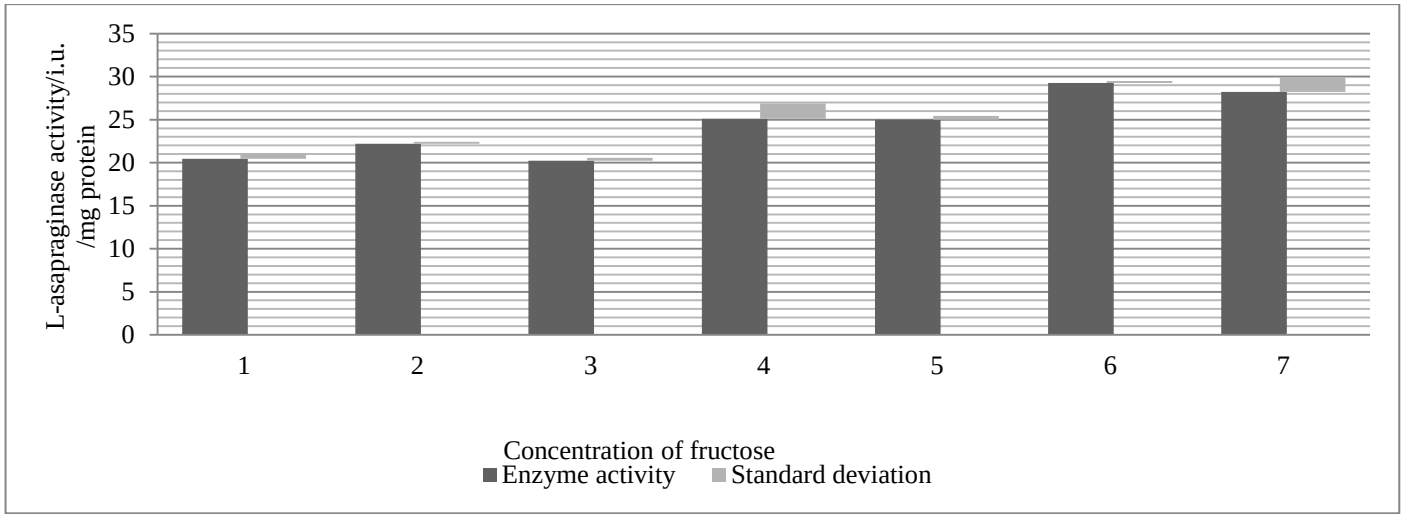


**Figure-4:** Effect of fructose concentration on enzyme activity (1-10g, 2-15g, 3-20g, 4-25g, 5-30g, 6-35g, 7-40g per litre).

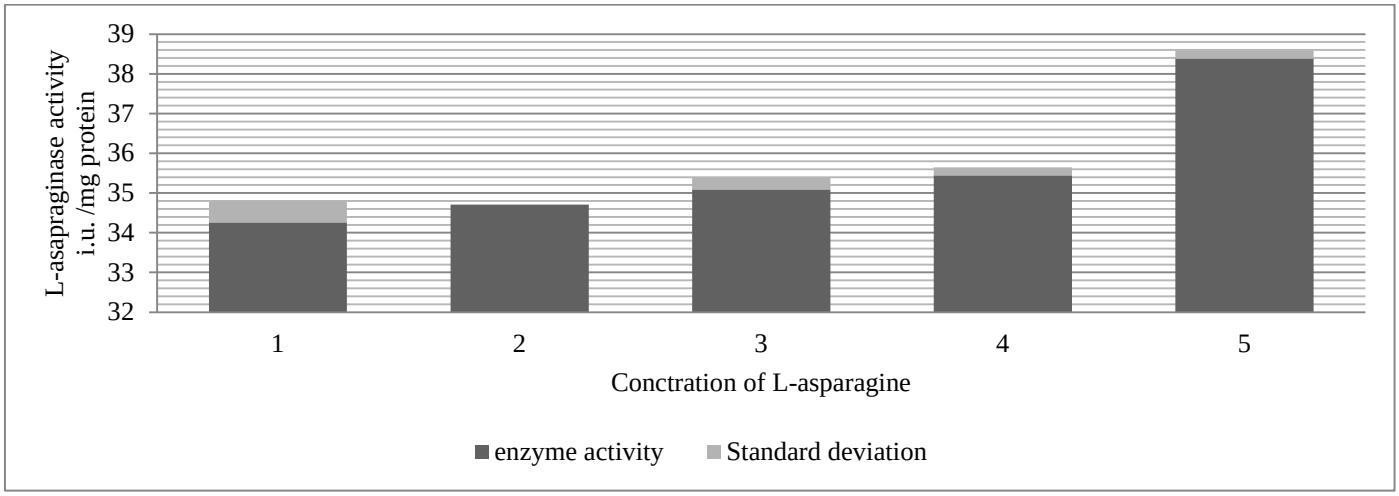


**Figure-5:** Effect of L–asaparagine on enzyme activity (1-5g, 2-10g, 3-15g, 4-20g, 5-25g, per litre).

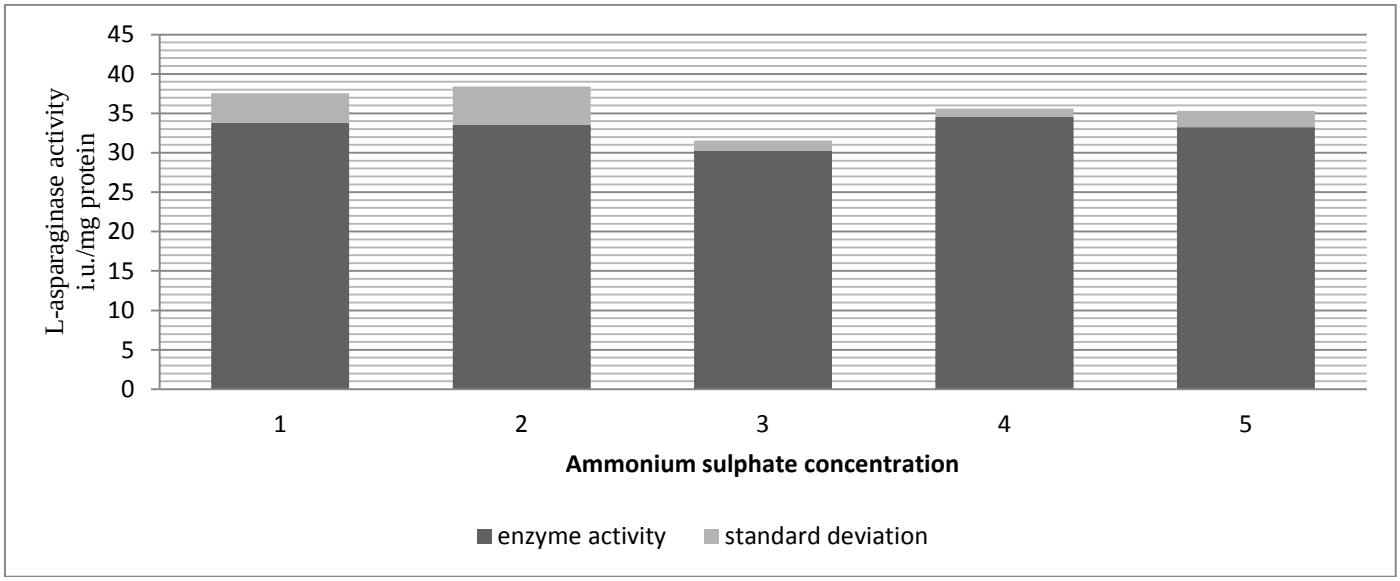


**Figure-6:** Effect of ammonium sulphate concentration on enzyme activity (1- 5g, 2-10g, 3-15g, 4-20g, 5-25g, per litre).

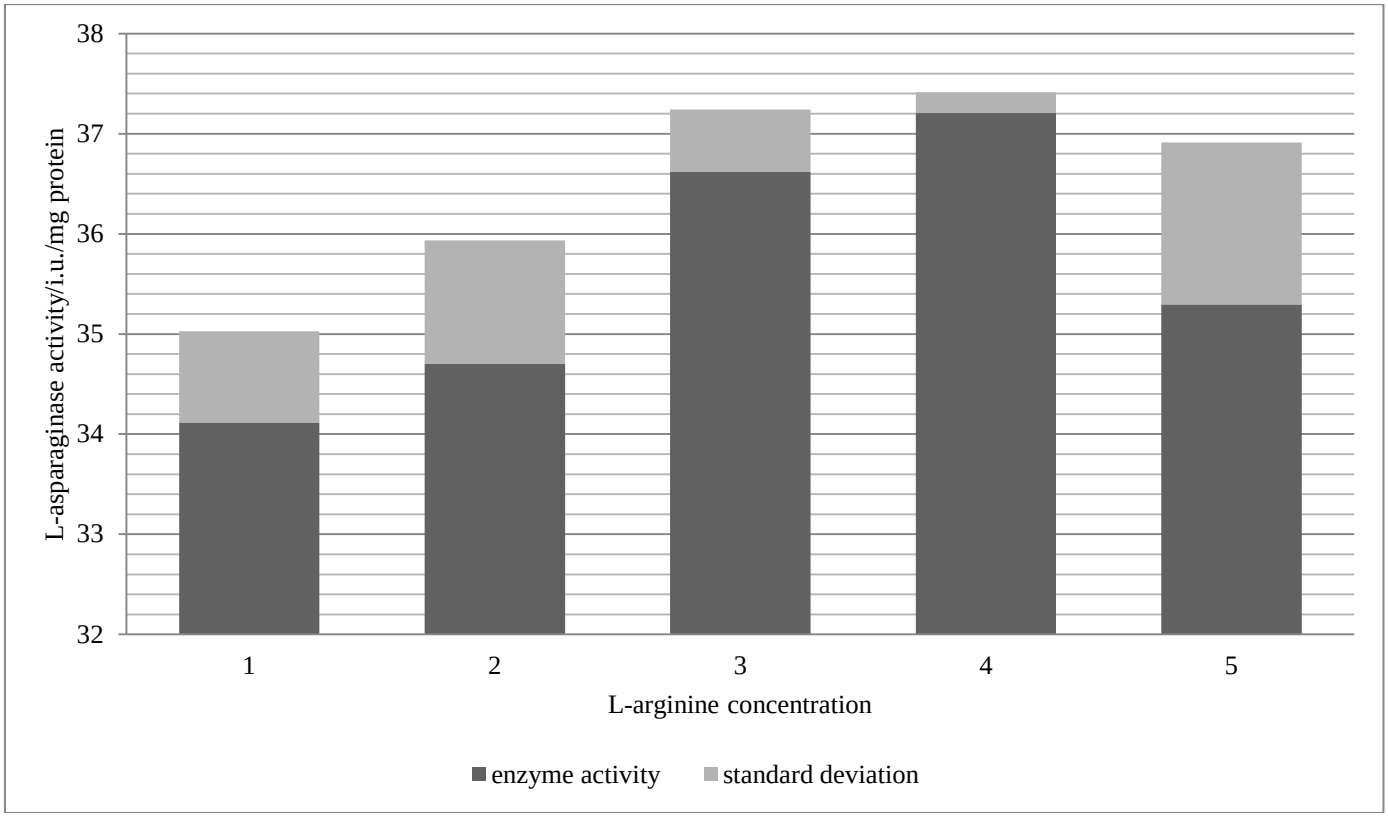


**Figure-7:** Effect of L-arginine concentration on enzyme activity (1-5g, 2-10g, 3-15g, 4-20g, 5-25g, per litre).

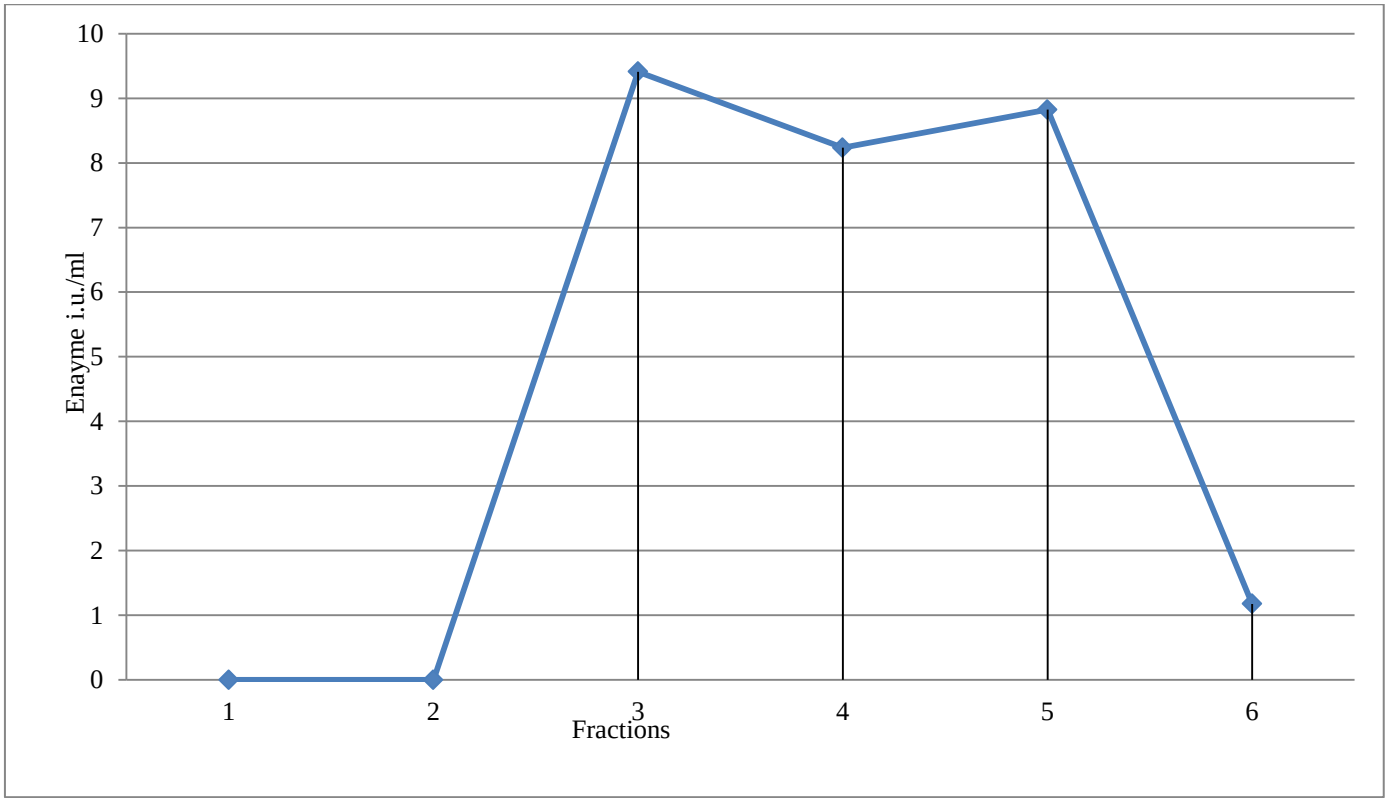


**Figure-8A:** Gel filtration chromatography - active fractions collected from the Sephadex G-100 gel filtration column.

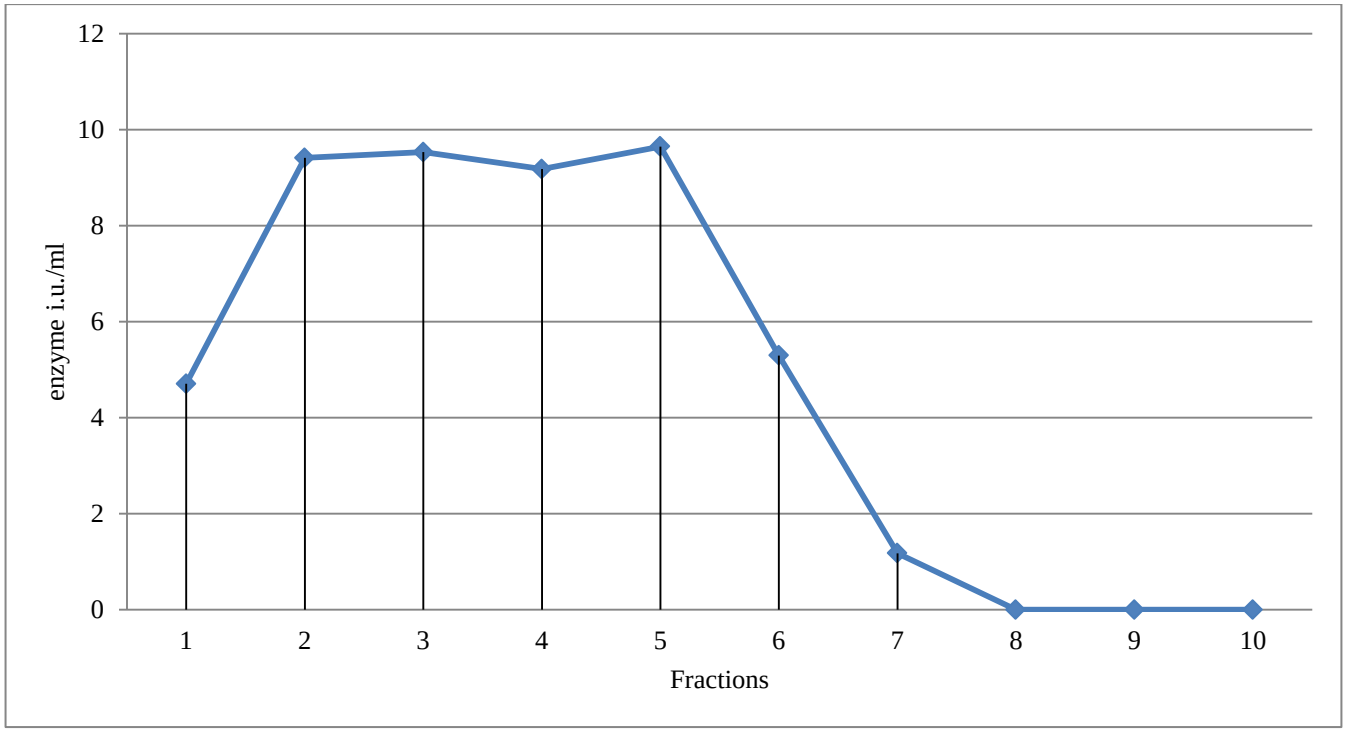


**Figure-8B:** Anion Exchange Chromatography - active fractions collected from the DEAE cellulose chromatography column.

**Discussion:** The fungal strain exhibited good enzyme activity in submerged culture condition. The fungal strain was supplied with various nutritional and growth conditions to obtain suitable protocol for improved enzyme production. *Penicillium* sp. 1 was fast growing fungus and required 8 days for maximum enzyme activity. Later the enzyme production was declined. Though several reports are available on the positive and /or negative effect of glucose on enzyme metabolism, fructose was selected as best carbon source in the present study[33]. Reported fungal strain produced enzyme of $0.901x10^{-3}$M which is comparatively higher like V*ibrio succinogens* (0.0745mM) and *Pseudomonas aeruginosa* (0.147mM). The higher Km value indicate the low substrate affinity; hence higher amount of substrate will be required to obtain adequate amount of product. However, fungal L-asparaginase is said to be better compared to bacterial L-asparaginase as L-asparaginases from bacterial origin can cause hypersensitivity in the long term use leading to allergic and anaphylaxis reactions (Reference). In this context, this fungal culture may be explored further for investigation on enhanced recovery and enzyme kinetics.

The enzyme production in the fungal culture was induced by the presence of ammonium sulphate in the batch culture medium for the enzyme production. The enhanced productivity of L – asparaginase was noted when the fungus was grown in the medium added with ammonium sulphate. The enhancement in enzyme production is due to the presence of arginine which probably supports amino acids in enzyme metabolism in microbial cells[34]. This is the reason, the partially purified enzyme favoured the presence of arginine and glutamic acid. The purified enzyme was thermally stable that ensures its suitability for long term preservation. However, L–asparginase from this source showed preference for 37°C make them suitable for large scale production and drug development. Total activity and the specific activity of L-asparaginase enzyme produced by this fungus has been decreased during ion exchange separation as compared to sephadex gel filtration. This may be due to either improper inonic saturation of the DEAE coloumn or ionic strength of protein. Extraction and purification of enzyme from this fungal source need more specific and elaborative studies prior to reach its anticancer properties.

Prior to the development of any fermentation technology optimization of culture and nutritional conditions is needed. Nutritional requirement for maximum synthesis of L-asparagine may vary in different organisms[29,30]. The rate of synthesis differs according to the culture conditions[24]. Our studies are based on batch culture experiments, further elaborative experimentation are required subsequent to large scale production of L-asparaginase. However, modification of the basal medium in order to achieve enhanced enzyme production may be a good support for the large scale fermentation and production of this enzyme that require optimization of production protocols, *in vitro* and *in vivo* trials.

## Conclusion

The present study dealt with the optimization of culture and nutritional conditions to achieve enhanced production of L-

asparaginase enzyme by submerged culture of Penicillium sp. The maximum enzyme production was obtained with the supplementation of fructose, ammonium sulfate and L-arginine in different concentrations and combination. The enzyme was active at higher temperatures as well as at different pH. The protocol developed for culture and nutritional requirements through present work may be useful for large-scale production of fungal L-asparaginase.

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