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RESEARCH ARTICLE

Production, Purification and Characterization of L-Asparaginase from *Pseudomonas aeruginosa* 2488 Using Fermentation

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Abstract

The main objective of the present work is the discovery of new L-asparaginase different from the reported literature but has similar therapeutic effects. The L-asparaginase enzyme was purified to homogeneity from *Pseudomonas aeruginosa* 2488 cells that were grown on solid-state fermentation. Different purification steps (including ammonium sulfate fractionation followed by separation on Sephadex G-200 gel filtration) were applied to the crude culture filtrate to obtain a pure enzyme preparation and the characterization was followed. The enzyme was purified and showed a final specific activity of 63.8 IU/mg. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme revealed it was one peptide chain with Mr of 42 kDa. The enzyme showed maximum activity at pH 7 when incubated at 37ºC for 30 min. The amino acid composition of the purified enzyme was also determined. So, it can be extremely used in the chemotherapeutic treatment of leukemia by conjugating with PEG Asparginase which is used in cancer chemotherapy..

Introduction:-

Enzymes are biomolecules or proteins that catalyze chemical reactions. In enzymatic reactions, the molecules at the beginning of the process are called substrates, which are converted into different molecules, called products. They are more important than vitamins and minerals for general health [1]. L-Asparaginase (L-Asparagine amino hydrolase, E.C. 3.5.1.1,) belongs to an amide group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonium ion. It is a high value enzyme with intensive chemotherapeutical uses against a wide variety of tumors especially acute lymphoblastic leukemia [2,3]. Recently it is also gaining much importance in food industries for producing acrylamide free food products [4]. This enzyme is composed of four identical subunits. The active sites grip asparagine and use a well-placed threonine amino acid to perform the cleavage of reaction. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate. The enzyme L-asparaginase is widely distributed in nature from bacteria to mammals and plays a vital role to modify amino acid metabolism and is one of the most effective chemotherapeutic means of inducing remission in acute lymphoblastic leukemia [5]. Presently, L-asparaginase is purified from two microbial sources viz. *Escherichia coli* and *Erwinia carotovora* which is extensively utilized in chemotherapy of leukemia. However, their prolonged administration induces immunogenic side effects like allergic reactions, anaphylaxis, pancreatitis and neurological seizures. The anti-asparaginase antibodies so formed, abolish the enzyme activity from circulating plasma [6,7]. The aim of the present work is the discovery of a new L-asparaginase producer that is serologically different from the previously reported ones, but one that has similar therapeutic effects. Thus for the production of L-asparginase, *Pseudomonas aeruginosa* (MTCC-2488) is used which is used on different type of Cancer cell lines *in vitro* as well as i*n vivo* conditions.

Materials and methods:-

Materials:-

All the chemicals and reagents were of analytical grade and were procured from IMT Chandigarh, Glaxo India Limited, Hi-media, SD Fine Chemicals, SRL and Ranbaxy.

Methods:-

Procurement and Selection of microorganisms

The microorganism *Pseudomonas aeruginosa* which is a source of asparginase was grown on specified medium (Table 1) and sterilized by autoclaving for 15 minutes at 15 psi/cm^2. The microorganism were maintained on agar slants and kept in refrigerator at 4.0± 0.5 ^{0}C for further use and was sub-cultured after every 20 days.

Table 1: Compositions of growth media (Tryptic Soy Broth)

Sr. No.	Components	Concentration (g/l)
1.	Tryptone	1.5g
2.	Peptone	0.5g
3.	Soyabean casein digest	3g
4.	Sodium chloride	0.5g
5.	Water	100ml

Production of L-Asparginase by fermentation:- .

The Enzyme was produced by fermentation using *Pseudomonas aeruginosa 2488* at 37^{0}C for 4 days. Two flasks containing Tryptic soy broth of 100 ml was taken and labeled as Control and Test. In Test flask inoculation was done with the help of culture of *Pseudomonas aeruginosa 2488* and the Control flask was left as it is. Both the flasks were then incubated at 37^{0}C for 4 days and then the growth of microorganism was noted down by the colour change.

Ammonium Sulphate Precipitation:-

Protein solution was placed on the top of a magnetic stirrer, and added 46-70 g ammonium sulphate untill saturation. In order to obtain 85% of Enzyme 69% of ammonium sulphate was used for salting out. Approximately 48.8- 70.5% , Ammonium sulphate was optimized to obtain good yield of an enzyme [8].

Determination of Enzyme activity:-

Asparginase activity can be defined as the μM of Asparagine formed per ml of enzyme solution in one minute. Asparginase activity was determined spectrophotometrically using Nessler's reagent test kit.

Table 2: Composition of Nessler's Reagent Kit

S.No	Ingredients	Composition
1	0.1M L- asparagine	1.32 g of L- asparagine in 100 ml of distilled water
2	TCA (tri-carboxylic acid)	2.45g in 100ml distilled water
3	0.1M Tris- HCl	1.23 g of tris in 100 ml of distilled water and its pH was adjusted to 8.6 with Dilute HCl
4	Nessler's reagent	--

Different volume ranging from 0-5 ml was taken in test tubes and final volume was made to 5ml by adding distilled water. The blank was maintained by using distilled water. In one test tube 100 µl of enzyme was added and labeled as Test. 1.2 ml of Tris- Hcl was added to all the test tubes including blank and were kept at 37 ^{0}C in an incubator for 15 minutes. 5ml of Nessler's reagent was added to all the test tubes. To the above solution, 0.4 ml of TCA was added and Optical density was taken at 480 nm. The Concentration of ammonium ions formed was observed from the Standard curve of Ammonium ions .

Determination of Protein content:-

9 test tubes were taken and 0.1 ml enzyme sample with 2.9 ml distilled water was added. Absorbance at 260 and 280 nm was taken for the given samples. Protein content was determined by the formula:
Protein concentration=1.55A260-0.76A280 [9]

Purification of Enzyme:-
Purification of enzyme was done by Gel filtration chromatography (sometimes referred to as Molecular exclusion chromatography.
Preparation of Column: 2g of Sephadex G-200 was dissolved in 50mM Phosphate buffer pH 7.2. Size of the column was noted as 11.7×1.6 cm.

Procedure: The slurry was added to the column by mixing with glass rod, keeping the funnel on the top of column, matrix was uniformly added to the column, and the equilibration of column was done with the Equilibration buffer (50 mM Potassium phosphate buffer, pH-7.2). 2 ml of the dialyzed ammonium sulfate fraction was added to the column. Mobile phase was Potassium phosphate buffer. Fractions of 3ml were collected with the help of gravity. The enzyme activity and the protein content were determined [10].

Molecular weight determination by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) [11]
SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed to determine the molecular weight and the purity of the sample. SDS was performed in 10% to check the molecular weight and purity of enzyme. The protein staining was done by using Commassive brilliant blue. For sample buffer and electrode buffer, composition is mentioned in table 3 and 4 respectively, other solutions include:
Separating (4x) gel buffer: Tris-HCl (18.3g) was dissolved in 100 ml of distilled water and pH was adjusted 8.8 with 1M HCI (table 5).
Stacking (4x) gel buffer: Tris-HCl (6.055g) was dissolved in 100 ml of distilled water and pH was adjusted 6.8 with 1M HCl (table 6).
Bisacrylamide (30%): 29.2 g acrylamide was mixed with 0.8g of bis-acrylamide and mixture was dissolved in total 100 ml of distilled water.
Sample buffer:

Table 3: Composition of Sample buffer

Component	Quantity
Tris-HCI (pH 6.8) buffer	0.4 ml
SDS (10%)	2.5%
2-mercaptoethanol	0.4ml
Glycerol	2.0 ml
Bromophenol blue	0.002 gm
Distilled water	4.7ml

Electrode buffer:

Table 4: Composition of Electrode buffer

Component	Quantity
Tris-HCI	6.05 g
SDS	2 gm
Glycine	28.8 gm
Distilled water	2.0 L

Separating gel:

Table 5: Composition of separating gel

Component	Quantity
Distilled water	19.5 ml
Bisacrylamide (30%)	10 ml
4x separating gel buffer	10 ml
SDS (10%)	0.8 ml
Glycerol (10%)	0.35 ml
TEMED	20 μl
APS (2%)	0.6 ml

Immediately the whole mixture was poured in a vertical mould and saturated butanol was added and the gel was allowed to polymerize. After ½ hr butanol was removed and upper portion of gel was washed with deionized water.

Stacking gel:

Table 6: Composition of stacking gel

Component	Quantity
Distilled water	6.3 ml
Bisacrylamide (30%)	2 ml
4x separating gel buffer	2.5 ml
SDS (10%)	0.2 ml
Glycerol (10%)	0.15 ml
TEMED	10 μl
APS (2%)	0.13 ml

This mixture was poured in vertical moulds of plates on the separating gel. Comb was placed in it and gel was allowed to settle for 30 minutes. After the stacking gel was polymerized the comb was removed. Sample was prepared by heating in water bath for 2-3 minutes and the sample was loaded in sample wells with the help of auto pipette. Electrophoresis was carried out at 50V uptill dye front reached into the separating gel and the voltage was increased to 100V. After the run is complete the gel was taken out and washed with water. Then comassive blue staining was carried out.

The staining solution consisted of 90ml water, 90ml methanol, 10ml acetic acid and 0.25g Commassive blue dye. While the destaining solution consisted of 90ml water, 90ml methanol, and 10ml acetic acid

Procedure: Placed the gel in 100 ml of staining solution for 30 minutes for staining the protein in the gel. Then placed the gel in the destaining solution for destaining the gel for overnight.

Characterization of the Enzyme at optimized parameters:-

Optimization of pH for high L-Asparginase activity

The Enzyme solution was taken in different test tubes . The buffers of pH 3-9 were prepared and the enzyme activity was determined by the Nessler's method.

Optimization of Temperature for high L-Asparginase activity

The Enzyme solution was taken in different test tubes. Incubation of each tube was done at different temperatures ranging from 25 ^{0}C to 100 ^{0}C and the Enzyme activity was determined by the Nessler's method.

Optimization of Inhibitor concentration for high L-Asparginase activity

The Enzyme solution was taken in different test tubes. Inhibitors such as EDTA , Ferric chloride and Zinc sulphate was prepared and were added in different concentrations ranging from 0-0.5 ml and the enzyme activity was determined by the Nessler's method.

Results:-

Maintenance of Pure Culture of Pseudomonas aeruginosa MTCC- 2488:-

Colonies of Pseudomonas aeruginosa was maintained on tryptic soy agar plates as shown in fig 1 and was stored properly.

Fig 1: Colonies of Pseudomonas aeruginosa

Production of L-Asparginase by fermentation:- .

The growth of microorganism can be seen in Test flask due to the growth of *Pseudomonas aeruginosa* as compared to Control flask which remain of the same colour as shown in fig 2.

Fig 2: Comparison of Control and Test flask after inoculation of Tryptic Soy broth with *Pseudomonas aeruginosa* 2488.

Determination of Enzyme activity and Protein Content:-

A standard graph was prepared (fig 3) that depicts the amount of ammonium ions produced during the reaction.

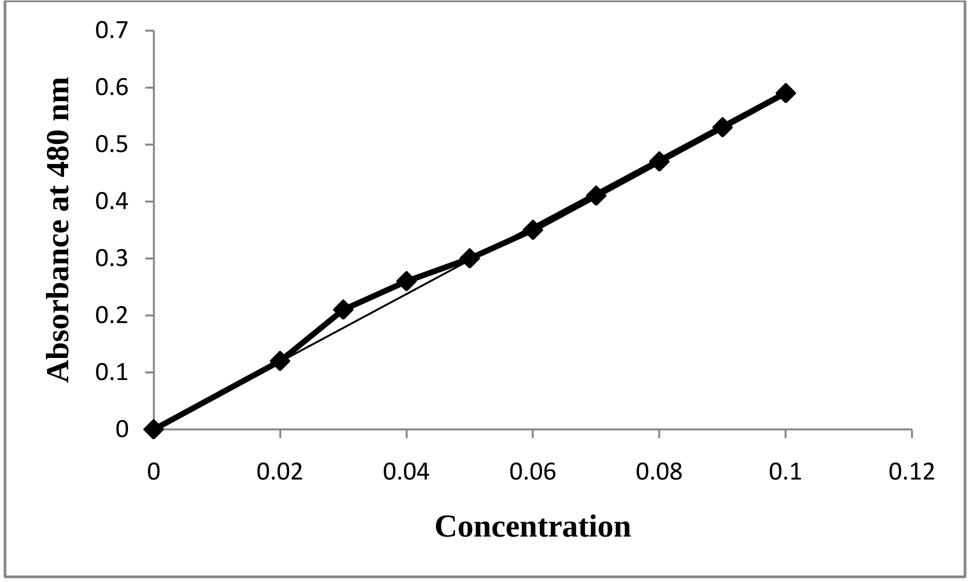

Fig 3: Standard graph showing ammonia liberation during reaction

Table 7: Purification profile of Asparaginase from *Pseudomonas aeruginosa* 2488

Step	Enzyme activity (IU)	Protein content(mg/ml)	Specific activity(IU/mg)	Purification (fold)
Crude enzyme	140	9.2	15.2	0.00
Partially purified enzyme	98	2.4	40.8	2.68
Gel filtration on Sephadex G-200 Column.	115	1.8	63.8	4.19

The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. The total protein decreased from 9.2 to 2.4mg in the ammonium sulfate precipitation step. The specific activity increased to 63.8 IU/mg after the Sephadex G-200 gel filtration chromatography. The purification fold of the partially purified enzyme was 2.68 while that of Gel filtration on Sephadex G-200 was 4.19.

The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. The total protein decreased from 4150 to 674 mg in the ammonium sulfate precipitation step. The specific activity increased to 497 and 1900 IU/mg after the Sephadex G-200.

Purification of enzyme:-

Gel filtration chromatography was performed for the purification of enzyme and it was observed in table 8 that maximum value of about 0.318 is achieved in 9th fraction i.e. Enzyme concentration in that fraction is much as compared to other fractions.

Table 8: Fractions of Gel filtration at 480 nm

Fraction Collection	Absorbance at 480 nm
1	ND
2	ND
3	ND
4	ND
5	ND
6	0.151
7	0.192
8	0.214
9	0.318

Molecular weight determination by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis):-

Electrophoresis was carried out containing 0.1% SDS. The gel was stained with Commassive blue R-250. As seen from fig 4, Lane A indicates the standard protein Ovalbumin (Mr-42 KDa), Lane B and C indicates purified L-asparagine and Lane D,E indicates Crude enzyme.

Fig 4:SDS-PAGE of L-asparginase from *Pseudomonas aeruginosa MTCC-2488*

Characterization of the Enzyme at optimized parameters:-

At optimized parameters maximum pH was obtained at 7 (fig 5) which affected the activity of asparginase thereby decreasing further production of the enzyme subsequently at all higher pH 10. Preceding studies dealing with the effect of initial pH on L-asparaginase production by other Bacillus strains such as B. circulans and Bacillus sp. (isolated from marine alga) indicated pH values 7.0 and 8.0 respectively for optimal L-asparaginase activity.

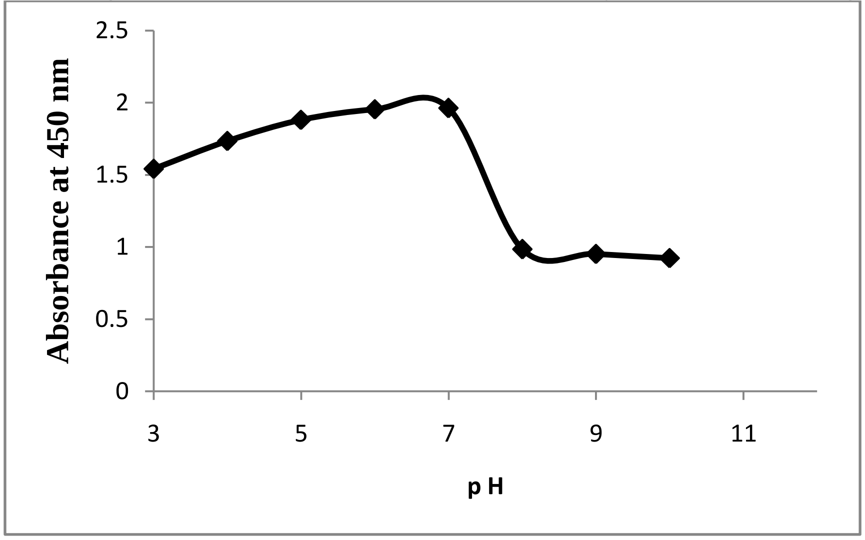

Fig 5: Effect of pH on Asparginase activity

The maximum temperature was obtained at 37^0 C (fig 6) which affected the activity of asparginase and the further production of the enzyme decreased subsequently. At high temperatures, microorganism may synthesize only a reduced number of proteins essential for growth and other physiological processes.

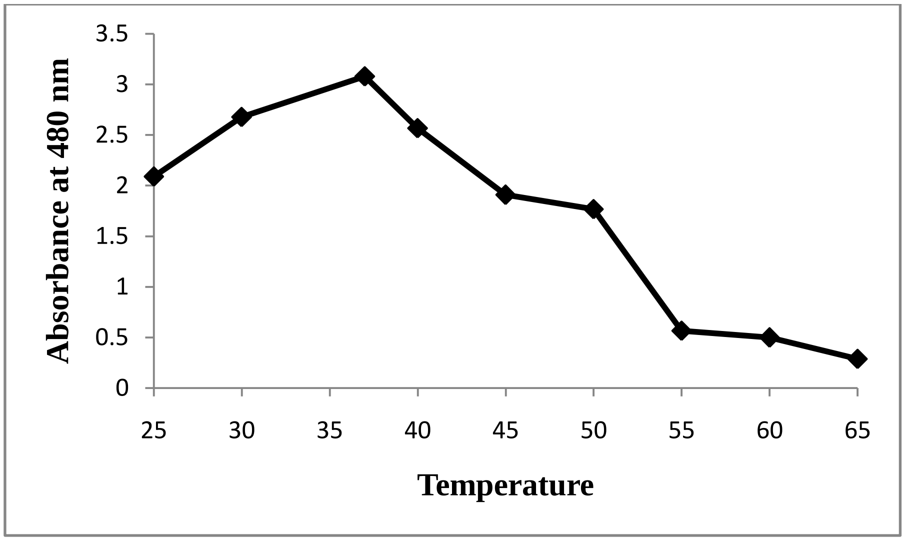

Fig 6: Effect of Temperature on Asparginase activity

At optimized parameters of Inhibitor concentration $Fecl_3$, EDTA and $ZnSo_4$ are the inhibitors which inhibits the Enzyme activity. As it is clearly seen in fig 7, if the volume of $FeCl_3$, EDTA and $ZnSo_4$ increases then absorbance decreases. Before addition of inhibitor optical density was high but after addition of these inhibitors with the maximum concentration of 4% optical density decreases, but as more concentration of these are added the optical density decreases more and more.

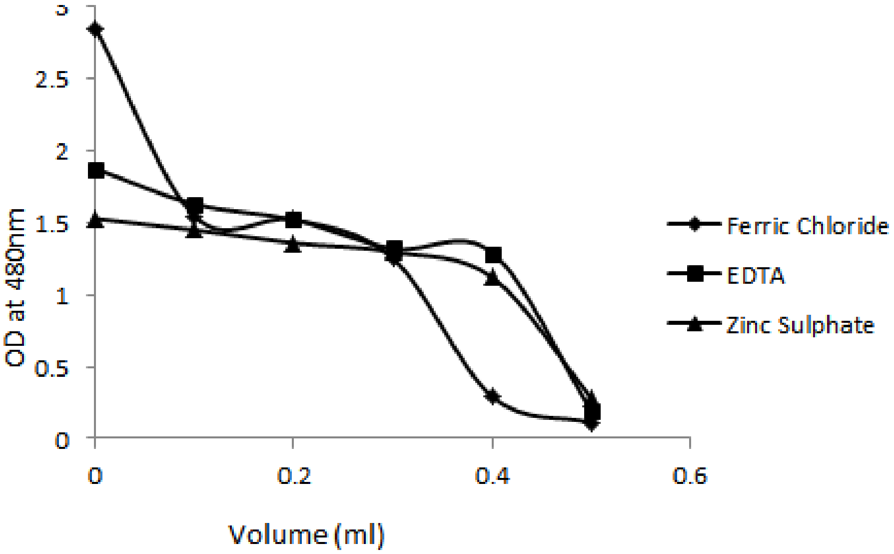

Fig 7: Effect of Inhibitors on Asparginase activity

Conclusion:-

It can be concluded the excellent properties of this enzyme, such as the activity at the alkaline pH range at 37^0C, make it extremely valuable in the chemotherapeutic treatment of leukemia by conjugating with PEG Asparginase which can be used in cancer chemotherapy. Pegasus drug which is already available in the market, so the different combination of PEG with Methoxy, Aldehyde, Carboxylic groups can be combined with the Asparagine and can be tried on different type of cancer cell lines in i*n vitro* and i*n vivo* conditions. Similarly it can be used as a food processing aid in the removal of acrylamide which is a carcinogen often formed in starchy foods when they are baked or fried. During heating the amino acid asparagine, naturally present in starchy foods, undergoes a process called the Maillard reaction, which is responsible for giving baked or fried foods their brown color, crust and toasted flavor. Asparaginase will be used during food processing prior to heat treatment. The enzyme would be subsequently inactivated by denaturation during heat processing, such as baking or frying. As a result of the catalytic activity of asparaginase, low levels of L-aspartic acid and ammonia are expected to be formed in food.

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Abbreviations:-

TCA- tricarboxylic acid
SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED- tetramethylethylenediamine
APS- ammonium persulphate
EDTA- ethylene-diamine-tetra-acetic acid
PEG- Polyethylene glycol

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