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Research Paper

Isolation, Purification and Characterization of Asparaginase from Aspergillus Species

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Abstract - *The L-asparaginase enzyme was purified to homogeneity from Asparaginase has been purified from Aspergillus terreus and Aspergillus niger that were grown under submerged fermentation. Different purification steps (including Ammonium sulfate fractionation dialysis and column chromatography) were applied to the crude culture filtrate to obtain a pure enzyme preparation. In SDS PAGE, both enzymes migrated as polydisperse aggregates yielding broad and diffused bonds. These two fractions yielded one band corresponding to a molecular weight range from above 94 KDa (Asparaginase). The enzyme exhibited distinctly under different pH and temperature profiles. The optimum pH and temperature for asparaginase were found to be 7 and 35°C respectively. Such as Asparaginase is 503.3, 303.3 U/ml and 3.6%, 4.21% respectively. Hence the purified enzyme will be used in industrials applications as well as clinical trails.*

Keywords: asparaginase, *Aspergillus terreus* and *Aspergillus niger.*

Introduction

Fungi are very effective and efficient bio degraders because of the wide range of extracellular enzymes they produce, which are capable of degrading complex polymer, such as cellouse, protein and lignins[1]. Yeasts and mycelial fungi are used in a variety industrial fermentation processes. *Saccharomyces* species are used extensively in brewing beers and wines, as well as in breadmaking[2]. They are precipitated by protein precipitating reagents such as trichloroacetic acid and ammonium sulphate. Asparagine is an amino acid required by cells for the production of protein [3]. Asparagine can be produced within a cell through an enzyme called "asparagines synthetase" or it can absorbed into the cell from the outside (ie., it is consumed in the patients diet, absorbed into the body and made available to the body's cells). Towards cells, more specifically lymphatic tumour cells, require huge amounts of asparagines to keep up with their rapid, malignant growth [4]. This means they use both asparagines from the diet as well as what they can make themselves (which is limited) to satisfy their large asparagines demand. L-asparaginase is an enzyme that destroys asparagines external to the cell [6]. Normal cells are able to make all the asparagines they need internally whereas tumour cells become depleted rapidly and die [7]. Asparaginase is an enzyme and it is used as chemotherapeutic agent for a treatment of human cancer and acts as a catalyst in the breakdown of asparagines to aspartic acid and ammonia [8].

Material and Methods

Collection of microorganisum

The marine soil samples were collected from microbial Germ plasm collection unit at mic college kanchikacherla.

Potato Dextrose Agar Medium

The potato tubers were peeled and weighed for about 200g. The tubers were chopped into small pieces with the help of a sterile knife. The chopped potatoes were transferred into a conical flask containing about 100ml of distilled water. The contents were boiled for 20 minutes. The supernatant was decanted and filtered by muslin cloth and the filterate was collected, by adding dextrose (20g) and agar (15g) were transferred into the extract and shacked to dissolve the ingredients, the medium was made upto 1 litre by addition of distilled water. The pH of the medium was observed to 5.6 finally. The medium was sterilized for 20 minutes. 1ml of spoilage crust fruit sample was taken in test tube containing 10ml of sterile distilled water. The tube was then shaken for 15 minutes, the aliquot, was serially diluted to get 10^{-2}, 10^{-3}, dilution. Then 0.1ml of the final aliquots was plated on the potato dextrose agar medium was gently rotated to get the uniform distribution of the suspension. Three replicates were maintained for sample and the plates were incubated of ambient temperature for about 1 week. The fungal colonies appearing on the potato agar medium were recorded from 5th day onwards.

Lactophenol cotton blue mounting of fungi

Place a drop of lactophenol cotton blue on a clean slide. Transfer a small tuft of the fungus, probably with spores and spore bearing structures into the drop, using a flamed, cooled needle. Gently tease the material using the two mounted needles. Mix gently the stain with the mold structures. Place a cover-glass over the preparation taking care to avoid trapping air bubbles in the stain.

Preparation of the pre inoculums

The fungal cultures from Potato Dextrose Agar medium (PDA) slants were subcultured into PDA at 30 DC for 72 hrs. The pre inoculum was prepared by adding 2.5ml of distilled water to remove the *Aspergillus terreus* spores, obtaining a suspension containing 5.0 x 10^7 spores / ml.

Screening of asparginase producing micro-organisms

Modified czapek Dox s medium was supplemented with phenol red. 2.5% stock of the dye was adjusted to 7.0 using 1mol 1^{-1} NaOH. The media were autoclaved and plates prepared. Control plates ere of modified Czapek Doxs medium (a) without dye (b) without aspargine(instead containing $NaNO_3$as nitrogen source). The plates were inoculated with a 96-h culture of *Aspergillus terreus* as a test organism. The zone and colony diameters were measured after 48 h. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing aspargine(sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing asparaginase.

Growth and Asparaginase production

An inoculum was prepared by culturing each of the asparginase producing organisms into modified czapek doxs culture broth tubes at 37ºC for 48 hrs. For growth and Enzyme production 1ml of the culture was inoculated into 200 ml of czapek doxs broth. The turbidity of the culture was measured at 520 nm in a spectrophotometer at different time intervals. Then the culture broth was centrifuged at 10000g (~18,000 rpm) for 15 minutes at 4ºC to remove the cells. The resulting supernatant solutions were used as the crude cell free supernatant.

Characterization of enzymes

SDS is an anionic detergent which binds strongly to, and denatures, proteins. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein SDS complex carries net negative charge, hence move towards the anode and the separation is based on the size of the protein.

SDS – PAGE

Assemble the glass plate, sandwich using two clean glass plates and two 0.75mm spacers. Lock the sandwich to the casting stand. Freshly prepared 15% for catalase and 6% for Asparaginase were pour the prepared separating gel solution to the sandwich until the height of the solution is 11cm.Allow the gel to polymerize for 30-60 min at room temperature.

Pour the Stacking gel

Prepare the same 15% for asparginase and prepared the stacking gel solution were described in appendix. Pour the stacking gel solution into the sandwich until the height of the solution is 1cm from the top of the plates..Insert 1.5mm Teflon comb into the stacking gel solution. Allow the stacking gel to polymerize for 3-40 min. at room temperature.

Sample preparation

Dilute the protein sample to be analyzed 1:1 (v/v) with 2X SDS /sample buffer and heat 5 min. at 100°C in a sealed. Screw cap micro centrifuge tube if the sample is a precipitated protein pellet dissolves the protein in 50 to 100µl of 1 X SDS / sample buffer and soil for 5 mins. At 100°C carefully remove the Teflon comb without damaging the wells. Rinse well with 1 X SDS / electrophoresis buffer to remove the unpolymerized monomers. Using a Pasteur pipette fill the well with 1 X SDS /electrophoresis buffer. Attach gel sandwich to upper chamber. Fill lower buffer chamber with recommended amount of SDS / electrophoresis buffer. Place sandwich attached to upper buffer chamber into lower buffer chamber. Partially fill the buffer chamber with 1 X SDS/electrophoresis buffer that the sample wells of the stacking gel are filled with sample. Using a 25 or 100µl micropipette, load the protein samples into the well as a thin layer of the bottom of the wells. Add an equal volume of 1 X SDS / sample buffer to any employ wells to prevent spreading of adjoining lanes. Fill the remainder of the upper buffer chamber with 1 X SDS/electrophoresis buffer so that the upper platinum electrode is completely covered. Do slowly.

Run the gels

Connect the power supply to the cell and run at 10M A of constant current slab gel 1.5mm thick, until the bromophenol blue tracking dye enters separating gel. Then increases the current to 15MA.After the bromophenol blue tracking dye has reached the bottom of separating gel disconnect the power supply.

Disassemble and analyse the gel

Discard electrode buffer and remove the upper buffer chamber and attached sandwich. Orient the gel so that the order of the sample wells is known, remove sandwich from the upper buffer chamber and lay the sandwich on a shoot of absorbent paper or paper towels. Carefully slide one of the spacers halfway from the edge of the sandwich along the entire length. Use the exposed spacer as a level to pry open the glass plate exposing the gel.

Purification table

Measure for each purification step.
The volume of the enzyme solution (ml)
The protein content of the solution (mg.ml^{-1})
The activity of the enzyme solution (U.ml^{-1})
Total amount of enzyme (U) =Activity (U.ml^{-1}) x Volume (ml)
Specific activity (U.mg^{-1})
Activity (U.ml^{-1}) / protein content (mg. ml^{-1}).
Yield (%)
Total amount of enzyme after a purification step / total amount of enzyme

Results

Five different mold cultures were isolated and identified. Maximum zone of *Aspergillus* strains were used

for the production of enzymes such as Asparginase using liquid state fermentation. These enzymes were purified and characterized. Different purification steps including ammonium sulphate, fractionation, dialysis and column chromatography were applied to the crude culture filtrate to obtain a pure enzyme preparation.

Biodiversity of fungi

In this study, totally 5 species of fungi were isolated by plating technique. All the number of fungi were belonging to form class Deuteromycetes (4 genera, 5species) (Table 1). Among the Deuteromycetus, groups Aspergillus (2 specices) was the dominant species followed by Penicillium, Trichoderma, and Verticillium (1 species).

Table 1: Fungi isolated from marine soil

S.NO	Fungal Species
1	*Aspergillus niger*
2	*Aspergillus terreus*
3	*Penicillium funiculosum*
4	*Trichoderma viride*
5	*Verticilium species*

Table 2: Screening of asparaginase producing microorganism

Fungal species	Diameter of colony	Diameter of zone	Potency index
A.terreus	1.68	0.97	0.56
A.niger	1.97	0.70	0.35
P. funiculosum	2.0	0.51	0.25
T.viride	2.5	0.42	0.16
Verticilium sp	2.9	0.35	0.12

Inference

Though there were five colonies seen on the modified czapek doxs agar plate only one *Aspergillus terreus* had ability to produce maximum zone of clearance. Even though the other colonies had the ability to grow on the czapek doxs agar plate, they showed minimum zone around them. This might be due to the low gene expression, resulting in the poor enzyme production. So *Aspergillus terreus* was found to be good producers of Asparaginase.

Growth and enzyme production

The absorbance of the culture sample was noted at different time intervals and the P^H was also determined. From this, the enzyme activity was calculated. The values were tabulated (Table 3, Figure 2). A graph was plotted and the growth pattern was observed.

Table 3: Growth studies of Asparaginase

Growth studies	Enzyme activity (U/ml)	pH	OD at 520nm
12	0	6.5	0
24	0	6.7	0.13
36	9.1	6.8	0.31
48	11.7	6.87	0.94
60	15.9	7	1.2
72	20.8	7.2	0.80
84	19.2	7.1	0.72
96	17.3	6.9	0.65

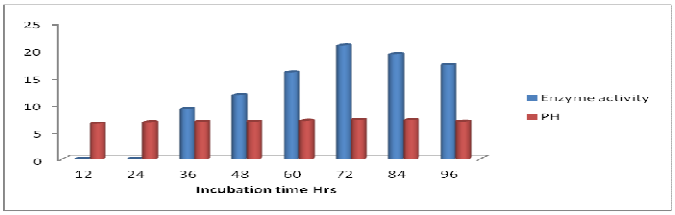

Figure 2: Growth studies of Asparaginase production

Inference

Maximum asparaginase activity (20.8 U/ml) was observed after 72 h of incubation. However, further incubation led to a decrease in the asparaginase activity .A shift in the P^H of the growth medium was also observed. The P^H increased with the increase in enzyme activity and after some time it stared decreasing. Subsequently, the P^H value of the medium increased with the increased with the increase in incubation time, reaching a maximum (P^H 7.0) after 72 h of incubation.

Optimum temperature and pH for Asparaginase activity

The effect of temperature on the activity of the Asparaginase is shown in (Table 4 and fig.3). The optimum temperature for Asparginase enzyme was at 35°C and showed maximum activity (28.7 U/ml). Further increase in temperature resulted in the lower activity of asparaginase. The enzyme activity of the asparaginase was determined at different pH value by using different buffers. The optimum pH for maximal activity of Asparaginase was 7.0 (27.5 U/ml) **(**Table 5 and Figure 3).

Table 4: Optimal temperature for Asparaginase activity

Temperature	Enzyme activity (U/ml)	OD at 520nm
30°C	18.8	0.91
35°C	20.6	1.30
40°C	19.2	0.96
45°C	17.4	0.87

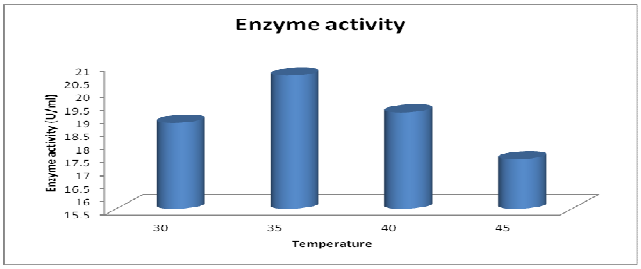

Figure 3: Optimal temperatures for Asparaginase activity

Table 5: Optimal pH for Asparaginase activity

P^H	Enzyme activity (U/ml)	OD at 520nm
5	16.7	0.72
6	18.2	0.81
7	20.3	1.20
8	19.1	1.0

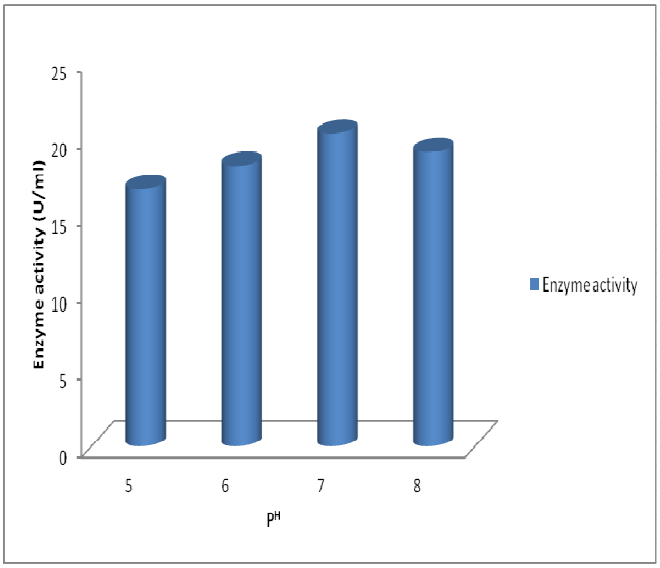

Figure 4: Optimal pH for Asparaginase activity

Purification of asparaginase by Sephadex G-200 column

The sample was passed through the sephadex column and the fractions were collected. The detection of maximum asparaginase activity in fraction 6 (Table 6)

Table 6: Elution profile of asparaginase activity on Sephadex G-200

Fraction number	Enzyme activity (U/ml)	OD at 520nm
1	5.35	0.17
2	6.59	0.21
3	10.36	0.33
4	13.39	0.42
5	16.85	0.52
6	30.20	2.02
7	17.59	0.59
8	15.39	0.49
9	12.56	0.41
10	10.05	0.32

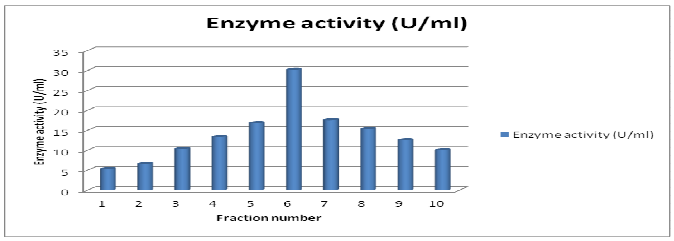

Figure 5: Elution profile of asparaginase activity on Sephadex G-200

Table 7: Purification of Asparaginase produced by *A. terreus*

S.No	Steps	Enzyme Activity (U/ml)	Total amount of Enzyme (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification factor	Yield (%)
1	Crude	20.75	4150	63	65.8	-	100
2	Ammonium Sulfate fractionation	24	2400	10	240	3.64	57
3	Dialysis	25.2	1260	3.2	393.7	5.9	30
4	Column chromatography	30.2	151	0.3	503.3	7.6	3.6

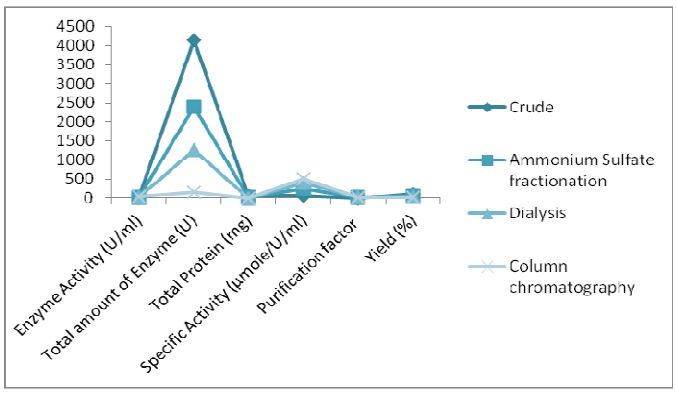

Figure 6: Purification of Asparaginase produced by *A. terreus*

Purification of asparaginase from *A. terreus*

Asparaginase was assayed by using the modified method based on that of Meister *et al.*, 1955. Ammonium sulfate fractionation and Dialysed cell extracts were prepared and were assayed at 500 nm and protein in the cell extracts was quantified. The culture supernatant of *A. terreus* containing an initial Asparaginase activity of (20.75 U/ml) was concentrated by ammonium sulphate precipitation. The optimum ammonium sulfate fractionation was (80% w/v saturation) showed that the 3.64 fold increase in specific activity compared to the unconcentrated supernatant. As shown in(Table 7 and fig 6) ammonium

sulphate precipitation resulted in specific activity of 240 (U/mg protein / ml) and yield of 57%. The Asparaginase was subjected to dialysis against Boric acid Borax buffer solution resulted in specific activity 393.7 (U/mg protein / ml) and showed a final purification factor 7.6 with a 3.6% yield.

Characterization of Asparaginase by SDS- PAGE

SDS- PAGE analysis was used to determine the molecular weight of Asparginase and Catalase. The marker proteins were phosphorylase (94 KDa), BSA (66.5 KDa), Carobonic anhydrase (30 KDa) and α-lactoalbumin (14.4 KDa).Sodium- dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli on a 0.75mm thick polyacrylamide slab gel consisting of 12% separating gel 6% for asparaginase and 4% stacking gels for enzyme. Molecular characterization of crude and partially purified enzyme showed molecular weight above 94 KDa (Asparginase). There was increase in molecular weight was noted between crude and partially purified enzymes.

Discussion

Asparaginase hydrolyze the aminoacid L-asparaginase to L-aspartic acid and ammonia. In this study, totally 5 species of fungi were isolated and identified. All species of fungi were belonging to form class deuteromycetes (4 genera, 5 species).Though there were five colonies seen on the modified czapek doxs agar plate only one *Aspergillus terreus* had ability to produce maximum zone of clearance. So *Aspergillus terreus* was found to be good producers of Asparaginase. The incubation period of fungi and bacteria was 48hrs and 18hrs, while in broth studies it is 24-48hrs for bacteria and often exceeds 96h for fungi.

The effect of pH (5-8) was studied for the activity of Asparaginase by *A.terreus*. The increase in the amount of Asparaginase synthesis was observed from p^H 5-6 and the maximum activity of enzyme was observed at pH 7.0 (20.3 U|ml). However pH of the fermentation medium beyond 7.0 resulted in a marked decreased the level of production of enzyme. Maximum growth and production of L-asparaginase by the *Bacillus* strain were obtained when the initial pH of the cultivation medium was adjusted to pH 8.0.Temperature is another critical factor that determines the growth and simultaneous production of Asparaginase. Temperature optimum was studied from known to be at 30-45°C for the *Aspergillus niger*, and the maximum production was observed at 35°C (Table7).The optimum temperature for maximum L-asparaginase activity was at 37°C which was lower than that reported earlier from a marine *Vibrio* sps. (Selvakumar *et al*., 1991).The sample was passed through the sephadex column and the fractions were collected. The detection of maximum asparaginase activity in fraction 6. The present study investigate the activity of Asparaginase, catalse enzymes were purified and characterized. Asparaginase enzyme was purified with specific activity of 240 U/mg after ammonium sulfate precipitation. The enzyme solution was then purified by using dialysis. This purification step showed 7.6 fold enzyme purification with a specific activity of 503.3 U/mg proteins. The results indicated the effectiveness of purification method applied in this study. The purification of L-asparaginase from *Serratia marcescens* yielded a 365-fold. Purification and 15% recovery of the enzyme. Enzyme preparations were found to be more stable if they were purified DEAE column before ammonium sulfate fractionation. Fraction with maximal activity was pooled for ammonium sulfate fractionation, this pool contained 0.2 to 0.6 mg of protein per ml. Approximately 80 to 90% of the enzyme activity was salted out at 55 and 65% saturation of ammonium sulfate. The precipitate was dialyzed against 0.01M sodium phosphate (pH 6.9) and the enzyme preparation was purified by gel electrophoresis with recoveries of 80 to 95%.The dialysed eluate from the gel electrophoresis was applied to a G-200 sephadex column (0.9×15cm) to remove nondialyzable gel constituents and was eluted 0.01M sodium phosphate buffer (pH 6.9). The final enzyme preparation was concentrated by dialysis against cold solids sephadex G-200, and the solution was stored was stored frozen.

Though there were five colonies seen on the DFSE agar plate only one *Aspergillus niger* had ability to produce maximum zone of clearance. Even though the other colonies had the ability to grow on the DFSE agar plate, they showed minimum zone around them. Asparaginase is intended for use as a processing aid during food manufacture to convert asparagine to aspartic acid in order to reduce the formation of acrylamide. Acrylamide is formed from asparagines and reducing sugars primarily in starchy foods that are baked or fried at temperatures above 120°C.

Conclusion

Microbial asparaginase have been particularly studied for their application as therapeutic agents in the treatment of certain types of human cancer L-asparaginase from microbial sources is currently in clinical use for the treatment of acute lymphoblastic leukemia. It has been also used for the treatment of pancreatic and bovine lymphomosarcoma.

Enzymes are among the most important product obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food Biotechnology utilize enzyme at some or the other. Developments in Biotechnology are yielding new application for enzymes.

Five fungal species were isolated from marine soil. The fungal species were belonging to form class Deuteromycetes majority of fungi were contributed by the species of Aspergillus, followed by Penicillium, Trichoderma and verticillium species. Aspergillus was the dominant genera used for the enzyme production.

In this study enzymes were produced from fungi using liquid – state fermentation. This study showed that the fungi have the ability to produce these enzymes during liquid –state fermentation at their favourable condition. The optimum temperature and pH of the enzymes were analysed by using various temperature and pH. The purified enzymes were reported by the technique SDS-PAGE.

Standard method of enzyme purification including ammonium sulfate precipitation and dialysis had a yield of Asparaginase was 57, 75%. Purification of final step Asparaginase resulted in a specific activity of (605.7, U/mg) and purification factor (9.2, 8.8). Molecular characterization

of enzymes revealed that increase in molecular weight for purified enzymes. This study concluded that fungal organisms should be used as effective source for the production of enzymes, secondary metabolites and the purified enzymes will be used to prevent diseases like cancer oxidative stress and athroscelerosis.

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