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Production, purification and characterization of L-Asparaginase from *Aspergillus nidulans* by Solid State Fermentation

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Abstract
L-asparaginases are anti-cancer agent used in the lymphoblastic leukemia chemotherapy. L-asparaginase is hydrolytic enzyme and is produced by a large number of microorganisms. In the present study, L-Asparaginase produced by *Aspergillus nidulans* isolated from a soil sample collected from Nallamala forest, India, was purified and characterized. The enzyme was purified by ammonium sulphate precipitation, dialysis, DEAE-cellulose ion-exchange chromatography and sephadex G-100 gel filtration. The molecular mass of the purified chitinase was estimated to be 46 kDa by SDS-PAGE. It was optimally active at pH of 6 and at 40 °C. The enzyme was stable from pH 5 to 8, and up to 50 °C. Among the metals that were tested, the Fe^{2+}, Hg^{2+}, Mn^{2+} and Co^{2+} completely inhibited the enzyme activity. The enzyme was less sensitive to Al^{3+}, Ca^{2+}, Cu^{2+}, and Zn^{2+}. The purified L-Asparaginase showed superoxide and hydrogen peroxide scavenging capacity which enables anticancer property.

Keywords: L-Asparaginase; SSF; *Aspergillus nidulans, Cajanus cajans* (red gram).

1. Introduction

L-Asparaginases

L-Asparaginases catalyse the hydrolysis of L-asparagine into L-aspartate and ammonia. The asparaginases isolated from *Escherichia coli* and *Erwinia chrysanthemi* are useful anti-leukemia agents. Production of this enzyme has also been reported from *Corynebacterium glutamicum, Cylindrocarpon obtusisporum, Pseudomonas stutzeri, Rhodosporidium toruloides, Tetrahymena pyriformis, Pseudomonas aeruginosa* 50071, *Aspergillus tamarii,* and *Aspergillus terreus.* 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 reaction. Solid state fermentation (SSF) has emerged as an effective technique to increase the product yield at low capital cost, low energy input, simple fermentation media, and low water use, and offers simple operational control. Low water use in SSF results in low production environment friendly. In addition, the substrates in SSF simulate the natural habitat of filamentous fungi and serve as an anchorage for fungal hyphae. For the commercial production of the enzyme, selection of a superior strain and substrate is a crucial step. The most important requirement for an L-Asparaginase to be of therapeutic use are that it should be relatively easy to isolate, it should be static at the physiological pH and temperature, the enzyme should have low Km value and that it should not be inhibited by its products. The substrate affinity of the enzyme is considered to be the most important factor responsible for the antitumor property of an L-Asparaginase preparation. L-Asparaginase is present in a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings. This enzyme is widely used in chemotherapy of acute lymphoblastic leukemia.

Aspergillus nidulans (also called *Emericella nidulans* when referring to its sexual form, or teleomorph) is one of many species of filamentous fungi in the phylum Ascomycota.

The *Aspergillus nidulans* genome was sequenced in collaboration between Monsanto and the Broad Institute. A sequence with 13-fold coverage was publicly released in March 2003; analysis of the annotated genome was published in *Nature* in December 2005.

It is 30 million base pairs in size and is predicted to contain around 9,500 protein-coding genes on eight chromosomes. Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. *Cajanus cajan* (L) Millsp. (Sanskrit: Adhaki, Hindi: Arhar, English: Pigeon pea, Bengali: Tur) (family: Fabaceae) is the most important grain legume crop of rain-fed agriculture in semi-arid tropics. It is both a food crop and a cover/forage crop with high levels of proteins and important amino acids like methionine, lysine and tryptophan. It has many antimicrobial activity, antibacterial activity, antidiabetic effects, neuroactive properties, antioxidant activities, anti-cancer activity, hepatoprotective effects, anthelmintic activity etc.

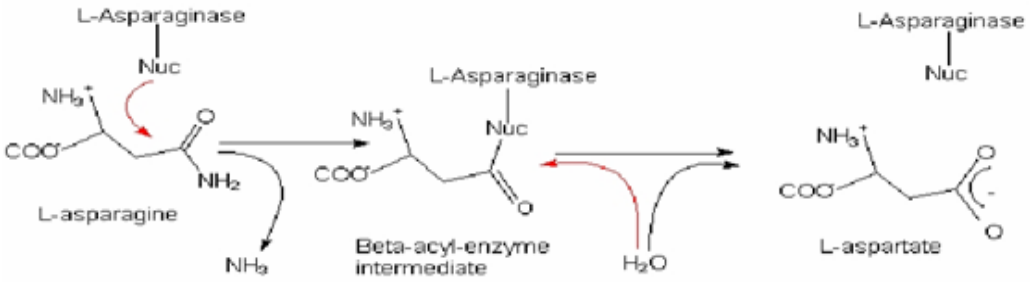

Fig 1: Conversion of L-Asparagine to Asparatate by L-asparaginase

2. Materials and Methods

2.1 Glassware and chemicals

In all the experiments acid washed (0.4N HCl) Pyrex glassware rinsed with double distilled water was used. For the preparation of culture media (HiMedia Laboratories Ltd) and other chemical reagents analytical grade chemicals (Qualigens Fine Chemicals Ltd. India and Merck and Co. Inc., USA) were used.

Sterilization

All the glassware used in the experiments was sterilized in hot air oven at 160 ºC for 4 hours. The media used in the experiments were sterilized in an autoclave at 15 lbs for 15 minutes.

Mounting liquids

For microscopic observation of fungi, Lacto phenol (equal quantities of phenol, glycerol, lactic acid and distilled water) and lacto phenol cotton blue mixture were used as mounting media and stains for semi-permanent microscopic preparations and then the slides were sealed with Canada balsam.

Collection of soil sample

The soil samples were collected from Nallamala forest, India. The samples were collected in sterilized bottles for isolation of Asparginase producing *Aspergillus nidulans*.

2.2 Composition of media employed

For isolation and the study of fungi, potato dextrose agar medium were used in the present work.

Potato dextrose agar medium

Potato dextrose agar medium was performed as the medium for the culture of the fungi. It was prepared with the following composition.

Potato	-	200 g
Dextrose	-	20 g
Agar	-	20 g
Distilled water	-	1 lit.

After preparation, it was mixed well until the contents are dissolved and then autoclaved at 121 ºC for 15 minutes.

2.3 Plate Assay for Evaluation of L-Asparaginase Production

Modified Czapek Dox's medium was supplemented with 0.3 mL of 2.5% phenol red dye prepared in ethanol at pH 6.5 with L-asparagine incorporated in the medium for evaluation of L-asparaginase activity. The media was autoclaved and the plates were inoculated with 3-d-old culture of *A. nidulans*. The clear zone appeared after 48 h of growth. Uninoculated media served as control.

Maintenance of pure cultures

The identified colonies were sub cultured on potato dextrose agar slants and preserved at 4 ºC. Sub-culturing was performed at one month interval.

Morphological identification of fungal culture

Micro slides of fungal culture were prepared in lactophenol-cotton blue, examined under microscope, observed their morphological characters and identified with the help of the standard keys provided by Rifai, (1969), in their representative manuals besides consulting relevant published literature. The measurements of the spore forms and vegetative structures were taken with the help of an ocular micro meter. The macro/micro photograph of selected fungus was also taken wherever it was found necessary. The identified fungus was stored on potato dextrose agar slants in the refrigerator at 4 ^{0}C prior to use.

2.4 Preparation of substrate for L-Asparaginase production:

10 gms of *Cajanus cajan* was weighed and powered to obtain a particle size of 1.2 to 1.4mm. To this 70% of 0.01M phosphate buffer (pH 6.5) was added and used as solid state fermentation media (76).

Production of L-Asparaginase

Fermentation was carried out following the method described in Mishra and Das. The fermentation media was sterilized by autoclaving for 30 min (referred in the text as cooking time) at 15 psi and 121 °C. The bottles were inoculated with 3 mL of fungal spore suspension and incubated under stationary condition at 37 °C for 96 h.

2.5 Crude enzyme extraction
For the extraction of crude enzyme, weighed quantity of fermented substrate was mixed with phosphate buffer (1:5). The mixture was homogenized and filtered. The filtrate was centrifuged and clear supernatant used for enzyme assay.

2.6 Determination of protein content
Protein content was determined by the method of Bradford (1976) using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard.

2.7 L-Asparaginase assay
L-Asparaginase activity was assayed following Nesselerization method *(77)*. One enzyme unit was defined as the amount of enzyme, which liberates 1 µM of ammonia per gram of dry substrate per minute (U/gds) under optimal assay conditions.

3. Purification of L-Asparaginase
The supernatant from the culture were precipitated by ammonium sulfate (80%). The precipitate was collected by centrifugation at 10000 x g for 20 min, and resuspended in phosphate buffer (20 mM, pH 6.5).

3.1 Dialysis
A typical dialysis procedure for protein samples is as follows:

1. Pre-wet the membrane phosphate buffer (20 mM, pH 6.5).
2. Load sample into dialysis tubing or device.
3. Dialyze for 1-2 hours at room temperature using phosphate buffer (20 mM, pH 6.5).
4. Change the phosphate buffer (20 mM, pH 6.5) and dialyze for another 1-2 hours.
5. Finally change buffer and dialyze overnight at 4 °C.
6. Then the sample in dialysis bag is used as protein source for further work.

3.2 DEAE-cellulose column chromatography
The dialyzed sample was then loaded on a pre-equilibrated DEAE-cellulose column chromatography (2.6 x 20 cm), and washed with the acetate buffer. The proteins were eluted in a stepwise gradient on NaCl (0 - 1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer. The fractions with Asparaginase activity were combined, dialyzed against the acetate buffer (50 mM, pH 5.0), and concentration by lyophilization.

3.3 Sephadex G-100 Gel Filtration Chromatography
The protein pellet obtained after dialysis was loaded onto a column of Sephadex G-100 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with phosphate buffer (20 mM, pH 6.5). The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. The elution fraction (2.5 mL) were collected and assayed for L-Asparaginase activity and those fractions which shown high activity was collected and used for SDS-PAGE analysis.

3.4 Molecular weight determination
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
In the present study, 12% Resolving gel and 4% stacking gel were used to separate the proteins. The detail protocol for SDS-PAGE is as follows:

Buffers
Buffers were filtered through 3MM Whatman and A was stored at 4 °C, B and C were stored at RT, sample buffer was stored in small aliquots in a frozen state.

BUFFER-A ACRYLAMIDE: BISACRYLAMIDE (30:0.8): 300 g Acrylamide, 8 g Bisacrylamide, 1L H2O

1. **BUFFER B 1.5 M TRIS / 0.4%SDS:** 90.85 g Tris pH 8.8 ,2 g SDS ,500 ml H2O
2. **BUFFER C TRIS 0.5M / 0.4% SDS:** 6.06 g Tris pH 6.8,0.4 g SDS,100 ml H2O
3. **10X RUNNING BUFFER (1 X = 25MM TRIS; 190MM GLYCINE 0.2% SDS):** 28 g Tris, Could use only 10g/L,143 g Glycine,1L H2O
4. **SAMPLE BUFFER (2X):**100 mM Tris pH 6.8,4% SDS**,**200 mM DTT**,**20 % glycerol**,**0.1% Bromophenol Blue - could use 0.01%
5. **STAIN (20X):** add 25 ml/500 ml of destain or fix (Old recipe uses 1g/100 mls and 12/500), 5 g Coomassie Brilliant Blue R,90 ml methanol, 10 ml acetic acid

DESTAIN: 45% methanol, 10% acetic acid
Fix / 1L – Gels that will be fluorography are not used but give a fast stain after only 30 mins.
12g TCA, 200 ml methanol, 70 ml acetic acid

Protocol for Preparation of Laemmli Gels
The gel mould was an assembled. The solution below in order was mixed and gel was poured immediately after adding APS.

10% APS
This is the initiator and should be added immediately before pouring - old APS may appear to work but the quality of the cross-linking was reported to be compromised. TEMED speeds up the rate of polymerization but cannot start the polymerization. The rate of polymerization is also affected by temperature. The gel was over layered with water-saturated isobutanol. Polymerize for ~ 1hr. Gels can then be used immediately or stored for 2-3 days covered in saran wrap. Running gel was runned by various elution buffers and different concentrations

Stacking gel
Solutions were mixed and poured on top of stack gel and the comb was inserted to make wells in the gel. Once the gel was set, the comb was removed and immediately pockets were rinsed out to get rid of Acrylamide that set and collapsed into the pocket. Gel apparatus was assembled, running buffer was added and samples were loaded. Inserted into chamber- make certain that buffer covers the lower electrode. Samples were

boiled to fully solubilize the proteins for 5 mins then spinned to remove insoluble debris for 5 mins.
Gels were runned for 120 mA/hrs i.e. 3 hrs at 40 mA or O/N at 10-12 mA

3.5 Optional
Pre run this gel at 40mA for 1hr.This sharpens the bands but compresses them too such that for example a 7.5% gel will run like a 10% gel. The gel was removed carefully and stained in a staining solution (methanol 4ml, distilled water 5ml, glacial acetic acid 1ml, 0.2 % Coomassie Brilliant Blue) with constant shaking for 2 hr. the excess stain was removed by destaining solution (Methanol 4ml, distilled water 5ml, Glacial acetic acid 1ml).

4 Results and Discussion
4.1 Screening and Identification of L-Asparaginase Producer
The study with phenol red dye in rapid plate assay method revealed that the presence of dye did not inhibit the growth of *A. nidulans* and a clear zone appeared around the colony. A broad zone indicated that the test organism was an efficient producer of L-asparaginase. Gulati *et al. (78)* followed similar experimental protocol for screening asparaginase producing bacterial and fungal strains. *Aspergillus nidulans* isolated from soils was screened for maximum production of L-asparaginase by observing clear zone in petridishes and was selected for further studies. Preliminary study on morphology of the fungus suggested that the fungus is from *Aspergillus nidulans*.

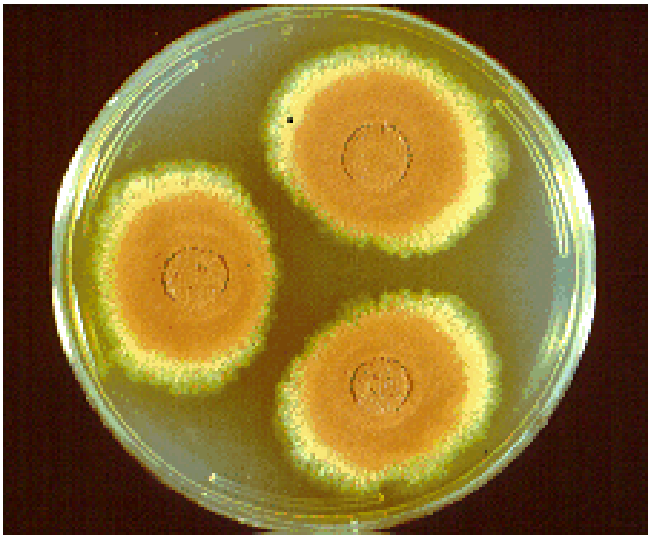

Fig 1 (A): *Aspergillus nidulans* on agar plate

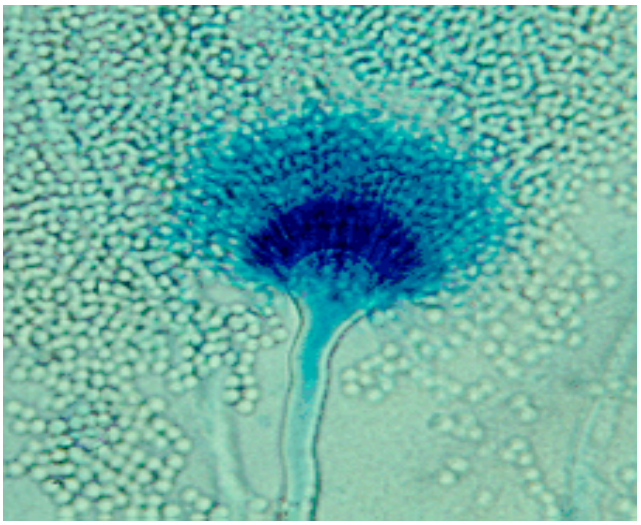

Fig 1(B): Microphotograph of *A. nidulans* (400x)

4.2 Morphological characterization
The fungus grows rapidly with immense vegetative mycelium. Colonies are typically plain green in color with dark red-brown cleistothecia developing within and upon the conidial layer (Fig. 1A). Reverse may be olive to drab-gray or purple-brown. Conidial heads are short, columnar (up to 70 x 30 um in diameter) and biseriate. Conidiophores are usually short, brownish and smooth-walled. Conidia are globose (3.0-3.5 um in diameter) and rough-walled (Fig. 1B). Based on morphological characteristics the strain was identified as *Aspergillus nidulans* (Rifai, 1969).

4.3 Purification of L-asparaginase
The L-asparaginase produced by *Aspergillus nidulans* was concentrated by ammonium sulfate (80%) precipitation and purified consecutively dialysis, gel filtration and ion-exchange chromatography. The results of the purification procedure are summarized in Table 1. The dialyzed sample was subjected to DEAE-cellulose column, and eluted at 0.1-0.3M NaCl in the buffer (Fig. 3). Following DEAE-cellulose column, the enzyme was purified 4.3-fold with a recovery of 31.6% and specific activity of 49.15 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The elution profiles of protein and L-asparaginase activity are shown in Fig. 4. After the final purification step, the enzyme was purified 6-fold with a recovery of 15.9% and specific activity of 142.7 U/mg of protein. The purified L-asparaginase appeared as a single protein band in SDS-PAGE and with molecular weight of approximately 46 kDa (Fig. 5). Similarly Akilandeswari *et al* (2012) reported the molecular weight of the L-asparaginase from the *Aspergillus niger* as 48 kDa. Different molecular masses that ranged from 33–46 kDa have been reported for other fungal L-asparaginase s (Gunaratna and Balasubramanian, 1994; Harighi *et al*., 2007; De Marco *et al*., 2004).

Table 1: Purification steps of L-asparaginase from *A. nidulans*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	423.5	1360.1	7.24	1.0	100
Ammonium sulfate	218.2	876.5	13.2	1.9	53.1
DEAE-cellulose	35.8	315.0	49.15	4.3	
Sephadex G-100	6.4	130.6	142.7	6.0	

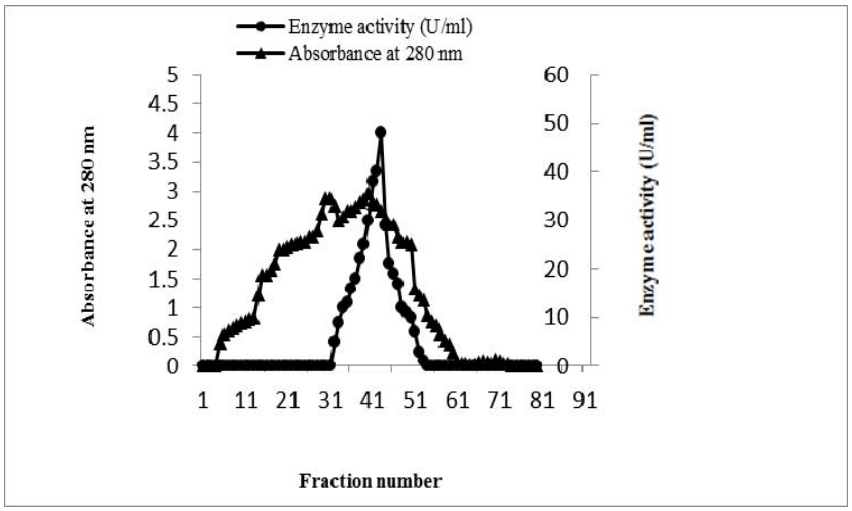

Fig 3: Chromatogram of the L-asparaginase from *A. nidulans* on DEAE-cellulose (2.6 × 20 cm). The column was eluted with NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected.

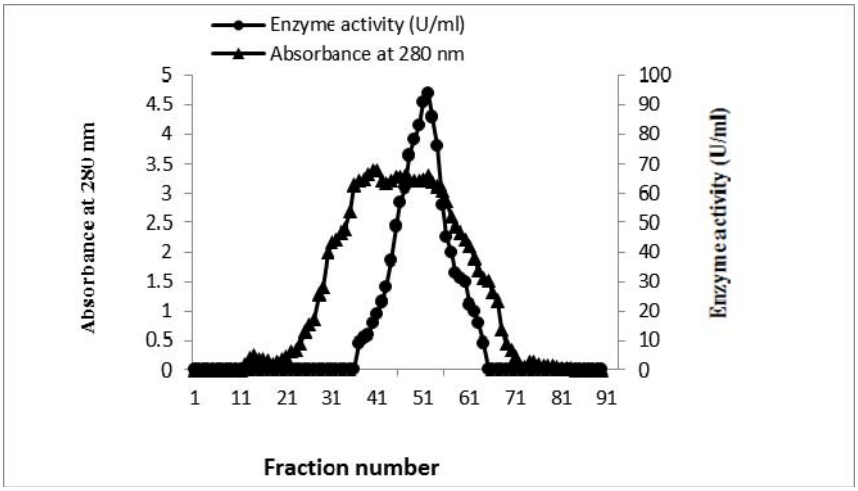

Fig 4: Chromatogram of the L-asparaginase from *A. nidulans* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 20 mM phosphate buffer (pH 6.5) at a flow rate of 15 ml/h. Fractions of 3 ml were collected

4.4 Effect of pH on enzyme activity

The optimal pHs for L-asparaginase activity and stability of the L-asparaginase were examined. The enzyme was most active between pH 4.0 and 5.0. It was relatively stable at pHs between 3.0 and 6.0. However, beyond these pH ranges, it rapidly lost its activity (Fig. 6). Compared with other *Aspergillus* L-asparaginases, similar optimal pHs were obtained; pH 4 for *Aspergillus niger* (De Marco *et al*., 2004) and pH 5.0 for *T. atroviride* PTCC 5220 (Harighi *et al*., 2007).

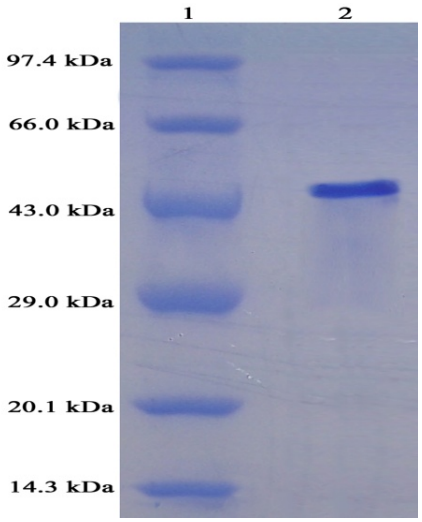

Fig 5: SDS-PAGE of purified enzyme Lane 1: Molecular weight marker protein; Lane 2: Purified L-asparaginase

4.5 Effect of Temperature

The L-asparaginase activity was most active at 40^{0}C (Fig. 7), similar to most of the other fungal L-asparaginase s (Mathivanan *et al*., 1998; Harighi *et al*., 2007; Van Nguyen *et al* 2008; De Marco *et al*., 2004). Above 50^{0}C, the activity decreased and was lost completely at 70^{0}C. When the enzyme was kept at various temperatures for 60 min in an acetate buffer (pH 5.0), it was significantly inactivated above 60 ^{0}C and completely at 80 ^{0}C. Similar thermostability was obtained below 60 ^{0}C for other *Aspergillus* L-asparaginases (De Marco *et al*., 2004; Omumasaba *et al*., 2001). The high temperature inactivation may be due to incorrect confirmation due to hydrolysis of the peptide chain, destruction of amino acid, or aggregation (Schokker and Van Boekel., 1999).

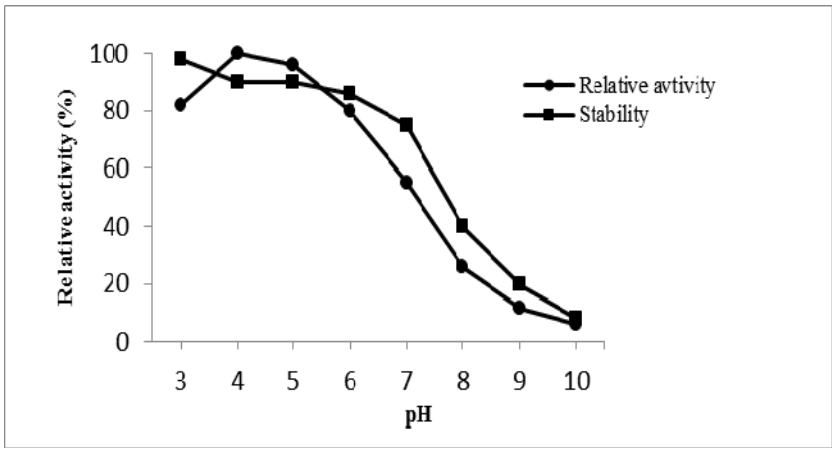

Fig 6: Optimal pH (•) and stability of pH (■) of purified L-asparaginase from *Aspergillus nidulans*.

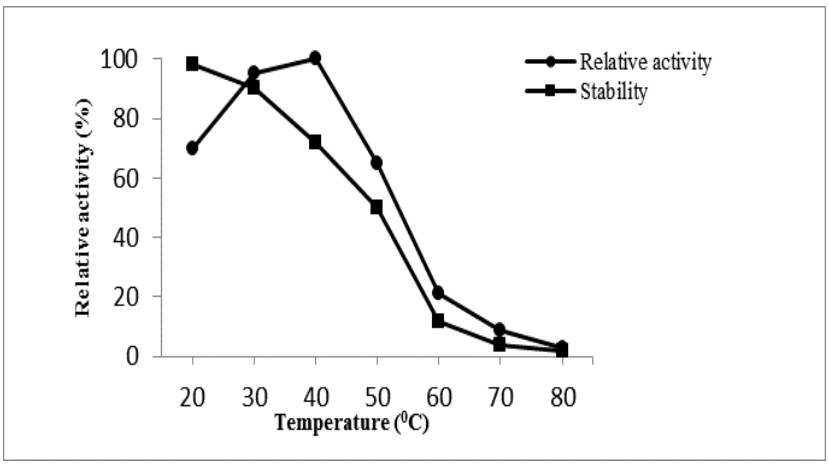

Fig 7: Optimal temperature (•) and stability of temperature (■) of purified L-asparaginase from *Aspergillus nidulans*.

4.6 Effect of metal ions

The effect of metal ions on the enzyme activity is presented in Table 2. The enzyme was strongly inhibited by Fe^{2+}, Hg^{2+}, Mn^{2+} and Co^{2+} at concentrations of 5 mM and was less sensitive to Al^{3+}, Ca^{2+}, Cu^{2+} and Zn^{2+} ions showed a slight inhibitory effect on enzyme activity. The inhibitory effect of Fe^{2+} has also been reported on L-asparaginase produced by *T. koningii* (Yuan and Jian Ju, 2011).

Table 2: Effect of metal ions on L-asparaginase activity.

Metal ions	Relative activity (%)
Control	100
Al^{3+}	89.3
Ca^{2+}	86.0
Cu^{2+}	84.5
Fe^{2+}	0
Co^{2+}	0
Hg^{2+}	0
Mn^{2+}	0
Zn^{2+}	71.6

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