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Full Length Research paper

Extraction, purification and characterization of L-asparaginase from *Penicillium* sp. by submerged fermentation

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A fungal strain identified as *Penicillium* sp. was evaluated for its L–asparaginase enzyme production. The L-asparaginase enzyme was purified to homogeneity from *Penicillium* sp. that was grown on submerged fermentation. Different purification steps including salt precipitation, followed by separation on sephadex G-100-120 gel filtration and DEAE were applied to obtain pure enzyme preparation. The purified enzyme showed 13.97 IU/mg specific activity and 36.204% yield. The polyacrylmide gel electrophoresis of the pure enzyme exhibited one protein of 66 kDa. The enzyme showed maximum activity at 7.0 pH and 37°C and K_m value 4.00×10^{-3} M.

Key words: L – Asparaginase, fungi, *Penicillium*.

INTRODUCTION

Amino acid degrading enzymes are important chemotherapeutic agents for the cure of some types of cancers. Among them, L-asparaginase also emerged as potent health care agent for the treatment of acute lymphocytic leukemia (Bessoumi et al., 2004; Sahu et al., 2007; Gupta et al., 2009a, b; Shah et al., 2010). It is fact that tumor cells take L-asparagine from blood circulation or body fluid as it cannot synthesize L –asparagines. The presence of L-asparaginase enzyme as chemotherapeutic agents may degrade the L-asparagine present in blood circulation and indirectly starve tumor cells and lead to cell death.

Microbial enzymes are preferred over plant or animal sources due to their economic production, consistency, ease of process modification, optimization and purification. They are relatively more stable than corresponding enzymes derived from plants or animals (Savitri et al., 2003). L-asparaginase production using microbial systems has attracted considerable attention owing to the cost-effective and eco-friendly nature. A wide range of microorganisms such as filamentous fungi, yeasts, and bacteria have proved to be beneficial sources of this enzyme (Verma et al., 2007). L-asparaginase has been use as a chemotherapeutic agent for over 30 years, mainly from the bacterial strains of *Escherichia coli* and *Erwinia chrysanthemi* (Aghaiypour et al., 1999, 2001; Krasotkina et al., 2004). L-asparaginases from bacterial sources sometimes cause allergic reactions and anaphylaxis (Keating et al., 1993; Bessoumy et al., 2004; Sarquis et al., 2004). The search for other asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that some eukaryotic microbes like yeast and filamentous fungi have a potential for L- asparaginase production (Theantana et al., 2007; Sukumaran et al., 1979; Nakahama et al., 1973). Some fungi such as *Aspergillus tamari* and *Aspergillus terreus* have proved to be beneficial sources of this enzyme (Soni, 1989). For the commercial production of enzyme, selection of superior strain and harvesting protocol is a crucial step. Besides source organisms, sufficient quantity of enzyme is also important for clinical trails. To overcome such type of

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difficulty, other sources of enzyme should be explored.

MATERIALS AND METHODS

Fungal strains used

Previously screened fungal isolates from the Microbiology Laboratory of Regional Plant Resources Centre having potentiality for L-asparaginase production were selected for experimentations.

Characteristics and identification

The isolates were identified on the basis of their morphology. Identification of the fungal cultures was done from Agharkar Research Institution, Pune.

Pure culture and inoculum preparation

Pure cultures were maintained on Czapeckdox agar slants and preserved at 4°C with sub-culturing in regular intervals. 5 to 6 days old cultures grown on Czapeck dox agar plates were used as inoculum.

Media used

Glucose-aspargine broth with pH 4.5 was prepared; 25 ml of the same was distributed in 150 ml flasks. The flasks were sterilized at 121°C for 15 to 20 min.

Inoculation

A single 5 mm disc of inoculum derived from the culture plates was inoculated into the flask containing broth .The culture flasks were incubated at 30°C for 10 days in static condition.

L-Asparaginase enzyme assay and protein estimation

The enzyme L-asparaginase was assayed by estimating the amount of ammonia released in the reaction (Imada et al., 1973). 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). The protein estimation was done by Bradford's method (Bradford, 1976).

Large scale production and extraction, purification, and characterization

The organisms were inoculated in the aforementioned broth media and incubated for a period of 10 days in static condition at 30°C. They were harvested after due incubation period. The biomass was weighed and was macerated with 0.05 M Tris-HCL buffer, pH 8.5 in the ratio 1:5. It was centrifuged at 600 rpm for 20 min at 4°C and the supernatant was collected. This was treated as the crude preparation of the enzyme. It was estimated for enzyme activity and protein, and was subjected for first purification step with ammonium sulphate precipitation.

Ammonium sulphate precipitation

The crude extract was precipitated with finely powdered ammonium sulphate with 80% saturation. It was left at 4°C over night followed with centrifugation at 8000 rpm for 20 min. The pellet was collected and was dissolved in 0.05 M Tris-HCl buffer, pH 8.5.

Sephadex G-100-120 gel filtration

Ammonium sulphate precipitated samples were tested for enzyme activity and protein and were further subjected to gel filtration-using sephadex G-100-120, with bead size 40 to 120 µl.

Preparation of gel-column and application of sample

A chromatography column made up of glass tubing having a diameter of 2.2 cm and a height of 60 cm was used. 0.05 M Tris-HCl buffer, pH 8.5 was used as eluent. The eluent was stored in tightly stoppered brown bottles and brought at the same temperature as that of the gel bed, to prevent bubble formation within the gel bed. For the preparation of gel slurry, 10 g of sephadex was suspended in 400 ml of 0.05 M Tris-HCl buffer and left for 24 h to swell at room temperature. The column was packed with sephadex and stabilized. Ammonium sulphate precipitated samples were poured continuously into the column and fractions were collected in vials (5 ml each). The collected fractions were tested for enzyme activity and protein randomly, and the fractions showing better enzyme activity were pooled together.

Ion exchange chromatography

Required amount of DEAE-cellulose was dissolved in Tris-HCl buffer and was left over night and was used to make the column. The column was packed; initially it was washed with 5 M NaOH to remove ionic charges from the ion exchanger then with 5 M KCl to generate desired form of weak ion exchange material. Finally, it was washed with distilled water followed by 0.05 M Tris-HCl buffer (pH 8.5). Finally, pooled peak fractions collected from sephadex filtration were applied to the ion exchange column. 5 ml 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 separation of L-asparaginase

Gel electrophoresis was performed for determining the homogeneity of the pure enzyme and to estimate the molecular weight. The resolving gel (dist. water 3.75 ml, resolving buffer 2.5 ml, acrylamide solution 3.6, 10% SDS 100 µl, 10% APS 57.1 µl, TEMED 42.85 µl) was prepared and was pipetted into the Sandwich. 100 ml of water-saturated butanol was added to the gel and was allowed to polymerize for 30 TO 40 min. After the due time, the butanol solution was decanted out and washed with ddH_2O and soaked with tissue paper. Stacking gel was prepared (dist. water 3.0 ml, stacking buffer 0.65 ml, acrylamide solution 1.2 ml, 10% SDS 50 µl, 10% APS 50 µl, TEMED 6 µl) added above the resolving gel. The comb was inserted and allowed to polymerize for 30 to 45 min). After the polymerization, the gel was clamped in the electrophoresis unit and the comb was removed carefully after adding the tank buffer. A standard marker and denatured sample to each well were loaded; electrophoresis was run at 40 V for 1 h and then 55 V for 3 h. When the dye reaches to the bottom of the gel the supplied power was turned up and the gel unit was removed from the electrophoresis unit. The plates were gently separated with the help of a spatula and the gel was carefully transferred into the staining solution for staining overnight. Next, it was distained for 7 h or overnight. Then, it was paced in the fixer solution and the bands

Table 1. Analysis of L-asparaginase in different purification steps.

Sample	Enzyme (IU/ml)	Protein (mg/ml)
Crude	2.058	0.280
Am.Sulphate precipitate	10.000	1.220
Sephadex gel filtration f3	0.000	0.400
6	0.000	0.870
9	10.294	0.800
12	12.941	0.790
15	10.117	0.820
18	9.705	0.840
21	10.235	0.850
24	0.000	0.270
27	0.000	0.000
30	0.000	0.000
Biologic LP-Ion exchange fraction No- 4	0.588	0.006
5	17.058	0.160
6	34.117	0.320
7	10.00	0.156
8	2.058	0.010
Precipitatedsample-fraction-6 of biologic LP	33.529	2.400

were observed under Gel Doc. The molecular weight of the protein was measured with reference to the standard marker used.

Characterization of the partially purified enzyme

Substrate specificity

Substituting L-asparagine with different substrates like L-arginine, L-Phenylalanine, L-Histidine, L-glutamine and L-aspartic acid were used in the assay mixture to determine the substrate specificity of the enzyme. The substrates had a concentration of 0.04 M, keeping L-asparagine as control.

pH optima

pH of the Tris-HCl buffer added in the reaction mixture for enzyme activity was adjusted to 3, 4, 5, 6, 7, 8, 9 and 10 respectively, and the pH optima was determined by detecting the enzyme activity at each level.

Temperature tolerance

The enzyme was kept at a varying temperature of 30, 37, 45 and 50°C before adding into the reaction mixer for assay.

Determination of K_m

The K_m of the enzyme was determined by making the reaction mixture containing fixed volume (0.25 ml) of the partially purified enzyme and varying concentration of the substrate L-asparagine. The total volume of the mixture was made up to 2 ml with 0.5 M Tris-HCl buffer of pH 7.2 and the enzyme activity was estimated. A graph of the substrate concentration was plotted against the reaction velocity.

RESULTS AND DISCUSSION

The *Penicillium* sp. was grown in basal glucose asparagines medium (pH 4.5 at 30°C for 10 days in static conditions). The fungus exhibited the L-asparaginase production under submerged culture developed in basal glucose asparagine medium. The partial purification of L-asparaginases crude preparation was affected by ammonium sulphate precipitation (80%) and showed that most of the enzyme activity was preserved in the precipitate (Table 1). The total protein decreased from 126 to 14 mg in ammonium sulphate precipitation. The specific activity increased from 7.350 to 13.970 IU/mg after sephadex gel filtration and ion-exchange chromatography respectively (Table 2).

The pH optimization of the enzyme was studied using a 0.05 M Tris-HCl buffer of different pH values ranging from 3 to 10 showed maximum enzyme activity at pH 7 (Table 3). The purified enzyme showed maximum activity 37°C. The analysis of substrate specificity showed that the enzyme catalyzes L-asparagine as a substrate. A Lineweaver-Burk analysis of the enzyme showed the K_m of 4.00×10^{-3} M. Molecular weight of L-asparaginase-SDS-PAGE of the enzyme showed the presence of the single peptide chain of ~66.00 kDa (Figure 1). This is a preliminary study on L-asparaginase from *Penicillium* sp. Further exploratory research is required to optimize the nutritional and cultural amendments in order to develop

Table 2. Purification profile of L-asparaginase from *Penicillium* sp.

Steps	Collected volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	450	926.10	126.00	7.350	0.000	100.00
Ammonium sulphate precipitation	90	900.00	109.8.	8.196	1.115	97.181
Sephadex gel filtration	65	692.77	53.30	12.997	1.768	74.850
Ion-exchange chromatography	10	335.29	24.00	13.970	1.900	36.204

Table 3. Effect pH, temperature and substrate on partially purified L-asparaginase.

Parameter		Enzyme activity (IU/ml)
pH	4	20.882
	5	26.470
	6	24.705
	7	35.882
	8	16.176
	9	13.529
	10	7.941
Temperature	30	20.294
	37	33.529
	45	19.411
	50	12.352
Substrates	L-asparagine	33.529
	L-aspartic acid	0
	L-Phenylalanine	0
	L-Glutamine	0
	L-histidine	0

bioprocess technology.

Several fungi are reported as producer of L–asparaginase enzyme (Mohapatra et al. 1997; Drainas and Draines 1985; Raha et al., 1990; Gupta et al., 2009a; Siddalingeswara and Lingappa, 2010b). The *Penicillium* sp. showed good enzyme activity in cell biomass.

L-asparaginase of different microbes has different substrate affinity and species play different ecophysiological roles in the enzyme activity (Warangkar and khobragade, 2010). Our fungal strain produces enzyme of K_m 4.0 × 10^{-3} M. It is comparatively higher than other organisms like *vibrio succinogens* (0.0745 mM) and *Pseudomonas aeruginosa* (0.147 mM) (Willis and Woolfolk, 1974; Bessouny et al., 2004). Higher K_m value exhibited its high affinity with the L-asparagine as substrate. The enzyme production is the complex chain reactions and is supported and induced by suitable substrates. *Penicillium sp.* preferred L–asparagines as substrate. This characteristic phenomenon of *Penicillium sp.* was corroborated with the Dunlope and Roon (1975) who noted the increment in enzyme production due to the addition of L glutamine or glutamate in the fermentation medium. The purification steps followed for the L-asparaginase from this fungal species achieved a protein of single peptide chain of 66 KDa. The temperature tolerance of the enzyme showed that it had maximum activity at 37°C and may be quite stable at high temperature too. Enzymatic activity was optimum at pH 7. It clearly indicates that L- asparaginase from this fungal species is pH dependant. This observation is corroborated with the earlier studies done by Sahu et al. (2007). Since, the work has been done under batch culture; further experimentation for the development of fermentation technology in mega scale production of this enzyme is needed. Any drug discovery and development program necessitate the standardization of small scale protocols, their exploration and standardization for mega scale*, in vitro and in vivo* trials and clinical trials etc, the

Figure 1. Gel electrophoresis analysis of L-asparaginase. Lane 1: BSA (66 kDa), Lane 2: pure enzyme (66 kDa).

present work and its outcome may be a mile stone in drug development program.

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