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Research article

Extraction, purification and characterization of L-asparaginase from isolated *Penicillium citrinum* Thom. (Strain FMG 181)

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Abstract: Mass scale production of fungal culture was done by using a Glucose-asparagine medium of 4.5 pH under submerged shake culture conditions. The fungal strain opted 4.5 pH and 10 days for good growth and enzyme production. The purification of the enzyme from mass culture developed in growth media was done by following salt precipitation, sephadex G-100 filtration and ion-exchange chromatography by DEAE cellulose columns. We obtained the purified protein and enzyme preparation that was matched with standard (66 kDa) and exhibited approximately 37 k Dawith 2.5 × 10^{-3} M. M K_m value. Partially purified enzymes prefer the alkaline pH (4–8), active at 45–50 ºC whereas DEAE separated enzyme was gradually decreasing its activity at higher temperature. It preferred aspartic acid, arginine and histidine besides L-asparagine for substrate requirement whereas DEAE purified enzyme exhibited good activity with L-arginine and L-aspartic acid. Sephadex purified enzyme was active at 45–50 ºC whereas DEAE separated enzyme was gradually decreasing its activity at the higher temperature. The present study was done considering Glucose-aspargine medium as basal medium. Further modifications of nutritional and cultural parameters may impact the enzyme production towards the higher side. Hence, more experimentations on C:N requirement and additional P, K and mineral sources is required before use this fungus for large scale production of the enzyme.

Keywords: *Penicillium citrinum* - L-asparaginase - Purification profile - Anticancer properties.

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INTRODUCTION

Several groups of microorganism have the potential of asparaginase production and enzymes derived from them are important for practical clinical use (Geckil & Gereer 2004, Sarquis *et al.* 2004, Shah *et al.*, 2010). Fungal L-asparaginase is known as a better therapeutic agent against leukemic cancer as compared to the bacterial source (Lapmark *et al.* 2010, Venil *et al.* 2009, Mohapatra *et al.* 1997). It is a fact that tumour cells are deficient innon-essential L-asparagine amino acid in absentia of aspartate-ammonia ligase enzyme synthesis and production. Hence, these cells depend upon circulating L-asparagine available and grow. In such circumstances, L-asparaginase plays a vital role in depleting the L-asparagines (Aghaiypour *et al.* 2001). The large scale production of this enzyme needs optimization of nutritional requirement and culture conditions for which several efforts have been made by many researchers (Abdel-Fattah & Olama 2002, Baskar *et al.* 2009, Warangkar & Khobragade 2009, Chandra *et al.* 2010, Patro & Gupta 2014). However, our fungal strain preferred Glucose asparagines medium for better production of L-asparaginase under submerged culture conditions. Hence, directly, we have used the basal medium for large scale production of fungal culture, the enzyme was precipitated and purified through gel filtration and ion exchange protocols and characterized.

MATERIALS AND METHODS

Previously screened fungal isolate *Penicillium citrinum* Thom. from the Microbiology Laboratory of Regional Plant Resources Centre having potentiality for L-asparaginase production was selected. The isolate was identified on the basis of their morphology and confirmed at the molecular level from Agharkar Research Institution, Pune. Pure cultures were maintained on Czapeckdox (Cz) agar slants and preserved at 4ºC with sub-

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culturing in regular intervals. 5–6 days old cultures grown on Cz agar plates were used as inoculum. The chemicals used were of analytical grade. Glucose-aspargine broth with pH 4.5 and sterilized at 121ºC for 15–20 minutes was used. 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 30ºC for 10days in shake culture condition. The enzyme L-asparaginase was assayed by estimating the amount of ammonia released in the reaction (Patro & Gupta 2014). 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^{-1}). Protein was estimated according to Bradford's method. The crude extract was precipitated with finely powdered Ammonium sulphate with 80% saturation. It was left at 4ºC overnight followed by centrifugation at 8000 rpm for 20 minutes. The pellet was collected and was dissolved in 0.05 M Tris-HCl buffer, pH 8.5.

Ammonium sulphate precipitated samples were tested for enzyme activity as well as protein and were further subjected to gel filtration-using sephadex G-100-120, with bead size 40–120 µ. Finally, pooled peak fractions collected from sephadex filtration were applied to the ion exchange column (BIORAD BP system). Samples were analyzed for enzyme activity and protein; fractions having better enzyme activity were pooled together and stored in deep freezer for later use. Gel electrophoresis was performed for determining the homogeneity of the pure enzyme and to estimate the molecular weight. The molecular weight of the protein was measured concerning the standard marker used. Substituting L-asparagine with different substrates were used in the assay mixture to determine the substrate specificity of the enzyme. The enzyme was kept at a varying temperature of 30ºC, 37ºC, 45ºC and 50ºC before adding in to the reaction mixer for assay. The Km 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 (Patro *et al.* 2014).

RESULTS

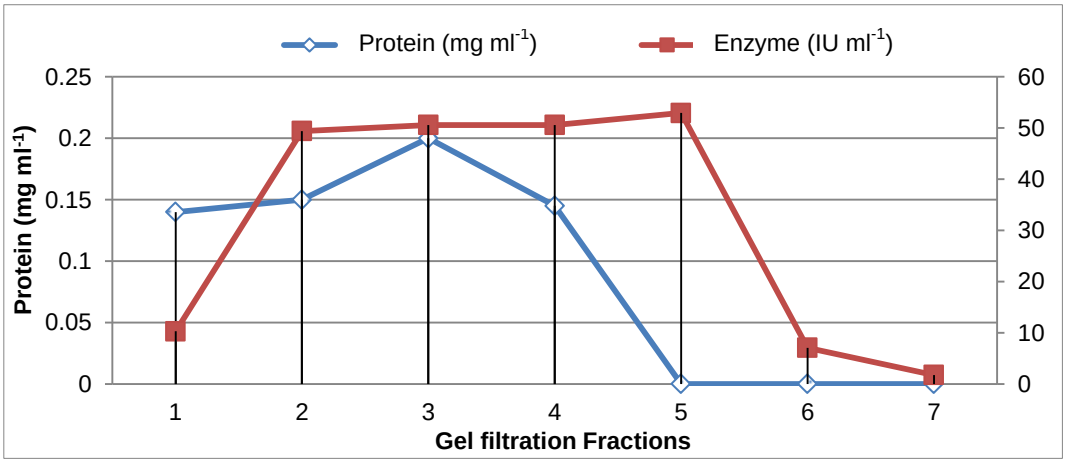

Figure 1. Purification of L-asparaginase from the *Penicillium citrinum* (Strain FMG-181). [Gel filtration chromatography - active fractions collected from the Sephadex G-100 gel filtration column]

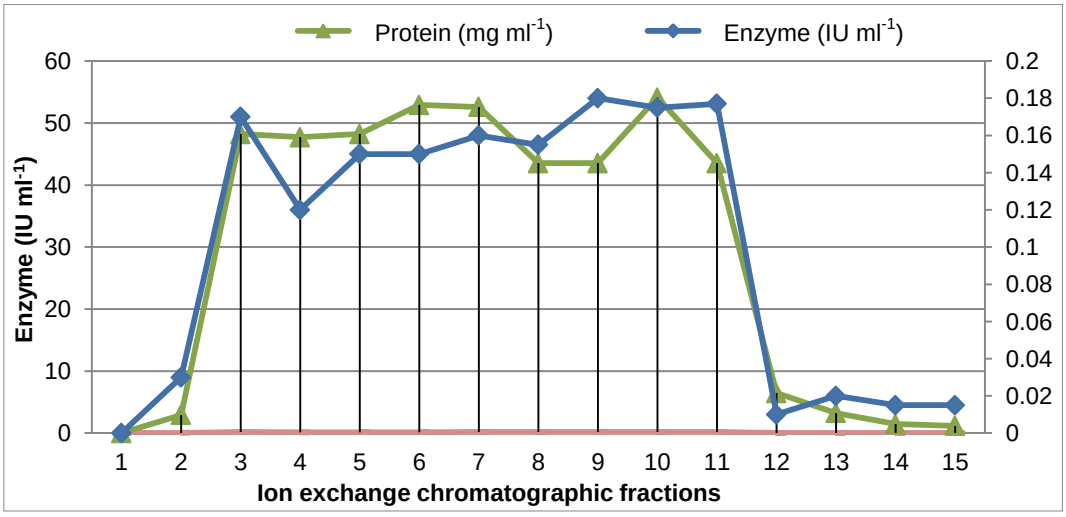

Figure 2. Purification of L-asparaginase from the *Penicillium citrinum* (Strain FMG-181). [Anion Exchange Chromatography - active fractions collected from the DEAE cellulose chromatography column]

Table 1. Purification profile of L -asparaginase from Fungal isolate FMG 181.

Stepts	collected volume (ml)	Total activlty (IU)	Total protein (mg)	Specific activity (IU mg^{-1})	Purification (Fold)	Yield (%)
Crude extract	460.000	1623.340	184.000	8.822	0.000	100.000
Ammoniumsulphateppt	90.000	4976.460	18.900	268.304	29.846	306.556
Gel filtration Sephadex G 100	65.000	320.715	9.750	329.486	37.339	197.846
DEAE Cellulose	45.000	2156.850	7.155	301.446	34.169	132.864

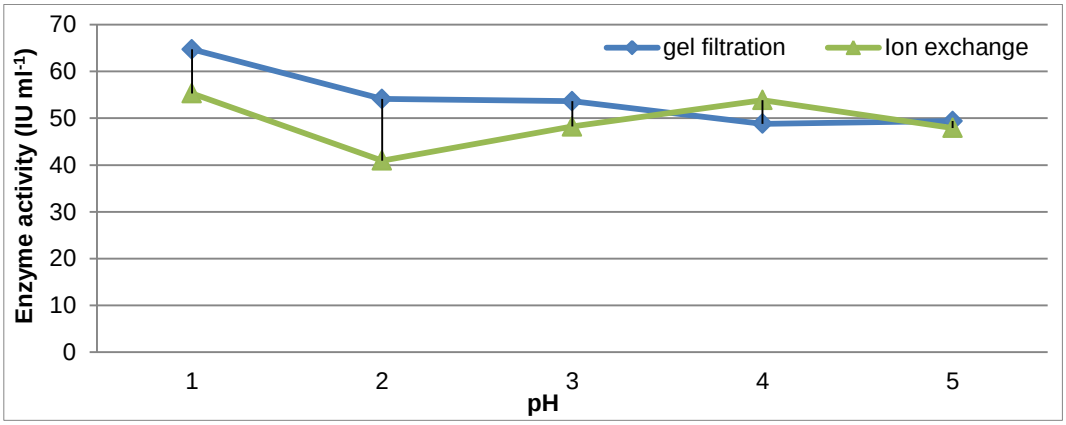

Figure 3. Effect of pH of supporting buffer on enzyme purified through gel filtration and ion exchange chromatography (1.0–4.0, 2.0–6.0, 3.0–8.0, 4.0–10.0, 5.0–7.2).

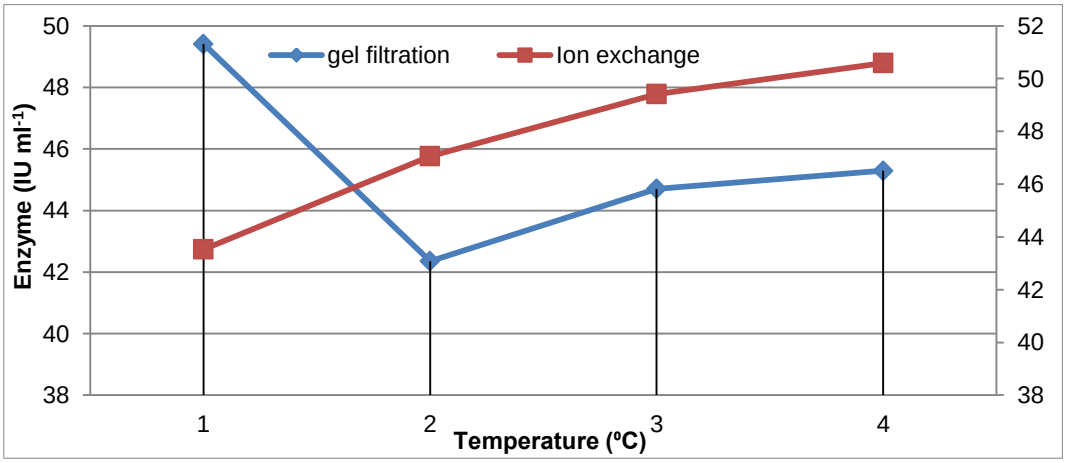

Figure 4. Effect of temperature on enzyme purified through gel filtration and ion exchange chromatography (1–30 ºC, 2–37 ºC, 3–45 ºC, 4–50 ºC).

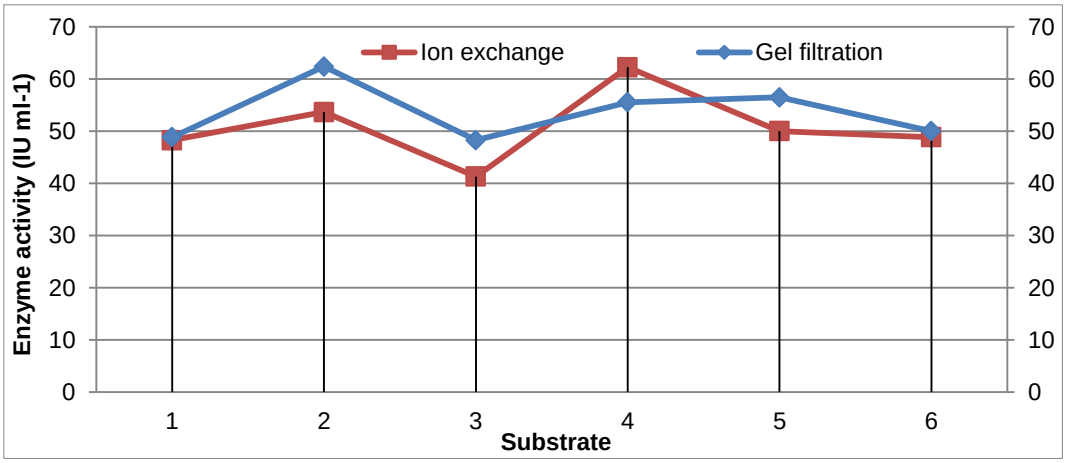

Figure 5. Effect of substrate on enzyme purified through gel filtration and ion exchange chromatography (1-asparagines, 2-aspartic acid, 3-phenylalanine, 4-arginine, 5-histidine, 6-glutamic acid)

The profile of the ammonium sulfate fraction purification on Sephadex (G100-120) gel filtration column chromatography is mentioned in figure 1. Although the fraction contained different protein molecules, fraction 5 to 20 showed the highest enzyme activity. Similarly, fractions of sephadex G 100 gel filtration on the DEAE column mentioned in figure 2 showed the most possible presence of the enzyme infraction no 2–9. Gradually the protein as well enzyme content of further fractions were decreasing. 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 184 mg to 18.9 mg in the ammonium sulfate precipitation steps. The specific activity increased to 268.304 IU mg^{-1} and 329.486 IU mg^{-1} after ammonium sulfate and sephadex gel filtration steps, respectively (Table 1). Partially purified enzymes obtained from sephadex G-100 and DEAE column chromatography was characterized for the effect of pH, temperature and substrate specificity. It prefers the wide range of pH ranged *i.e.* 4–8. The enzyme activity was lowered at high pH (Fig. 3). Sephadex purified enzyme was active at 45–50 ºC whereas DEAE separated enzyme was gradually decreasing its activity at higher temperature (Fig. 4). The partially purified enzyme (sephadex-G-100-120) exhibited preference to aspartic acid, arginine and histidine besides L asparagne for substrate requirement. Whereas DEAE purified protein exhibited good activity with L-arginine and L aspartic acid (Fig. 5). A Lineweaver-Burk analysis of the enzyme showed the K_m of 2.5×10^{-3} M. and the molecular weight of L-asparaginase- SDS-PAGE of the enzyme showed the presence of the peptide chain of ~37.00 kDa.

DISCUSSION

Several fungi are reported as producer of L-asparaginase enzyme (Raha *et al.* 1990, Mohapatra *et al.* 1997, Siddalingeshwara & Lingappa 2010). The fungal strain showed good enzyme activity in cell biomass. The fungus preferred Glucose Asparagine medium which support the positive glucose effect of glucose on enzyme metabolism (Kumar *et al.* 2009) and contradictory to *Penicillium citrinum* that indirectly supported the negative effect of glucose in enzymatic metabolism of the organism (Patro & Gupta 2014). The purified enzyme was thermally stable that ensures its suitability for long term preservation. However, L-asparaginase from this source showed a preference for 37°C make them suitable for large scale production and drug development. Large scale production of L-asparaginase may depend upon the nutritional and cultural parameters (Barnes *et al.* 1977). The present study was carried out in batch culture, continuous fermentation process, further optimization of C:N and addition of different nitrogenous compounds may elicit the synthesis and production of this enzyme by *Penicillium* sp. in liquid culture conditions. It is a preliminary report on a novel source of fungal L-asparaginase that may be exploited for mass scale production, purification and subsequent evaluation for its anticancer properties.

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