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Production and Characterization of L-Asparaginase - A Tumour inhibitor

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Abstract: This paper describes production and characterization of L-asparaginase from *Aspaergillus terreus* KLS2. This production of L-asparaginase were produced through solid state fermentation by using carob pod as a substrate. This production of L-asparaginase were achieved through optimization of fermentation parameters and it showed 6.05 IU of enzyme activity. After the production of L-asparaginase were used for purification by chromatography techniques. The purified L-asparaginase were used for the characterization. The general properties were used for characterization of L-asparaginase are effect of pH and temperature, stability of pH and temperature on L-asparaginase. Even the substrate specificity were also studied. The optimum pH 9.0 retains 100% of residual activity and 45^{0}C temperature on L-asparaginase retains 100% residual activity. Stability of pH 8.0 retains 100% activity and temperature 70 ^{0}C at 30 and 60 min. L-asparagine was used as a specific and natural substrate to the L-asparaginase and retain 100% relative activity.
Key words: L-asparaginase, solid state fermentation, carob pod, pH stability, temperature stability. Substrate specificity.

INTRODUCTION

Many enzymes have been used as drugs like wise L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic leukemia and other kinds of cancer in man[1,2,3]. L-asparaginase in the treatment of leukemia and other lymphoproliferative disorders has expanded immensely. For these reasons L-asparaginase has established itself to be an indispensable component [4].

Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine [5, 6]. Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from circulating plasma pools [6]. Clinical trials indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man [7]. It catalyses the conversion of L-asparagine to L-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions.[8]. This clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumour cells unable to synthesize this amino acids are selectively killed by L-asparagine deprivation [9].

This enzyme is widely distributed, being found in L-asparaginase is widely distributed, being found in animal, microbial and plant sources [10]. It's presence in guinea pig serum was first reported by Clementi[11]. Large number of microorganisms that include *Erwinia carotovora* [12], *Pseudomonas stutzeri* [5], *Pseudomonas aerugenosa* [13] and *E. coli*[14]. It has been observed that eukaryotic microorganisms like yeast and fungi have a potential for asparaginase production.[15,16]. For example, the mitosporic fungi genera such as *Aspergillus, Penicillium*, and *Fusarium*, are commonly reported in scientific literature to produce asparaginase[17,18,19]. This paper deals with the production of L-asparaginase from *Aspergillus terreus* through solid state fermentation by using carob pod as a substrate. There were no reports on production and characterization of L-asparaginase by using carob pod substrate.

MATERIALS AND METHODS:

MICROORGANISM:

*Aspergillus te*rreus isolated from different soil samples from various places from Gulbarga were used for the isolation of *Aspergillus terreus* strains as per the method of Seifert[20]. The isolated (Plate - 1) strains

were tentatively identified in the laboratory as described by Rapper and Fennell[21] and were maintained on potato dextrose agar (PDA).

PLATE – 1: *Aspergillus terreus*

IDENTIFICATION:

The isolated (Plate - 1) strains were tentatively identified in the laboratory as described by Rapper and Fennell[21] and were maintained on potato dextrose agar (PDA). Further confirmation was done at Agarkar Research Institute, Pune.

PRODUCTION OF L-ASPARAGINASE:

The isolated strains were screened and used potential strain for the production of L-asparaginase through solid state fermentation by using carob pod as a substrate.

FERMENTATION STUDIES:

The production of L-asparaginase was carried out by using 20 g of carob pod as a substrate under solid state fermentation. The moisture content of the flask is 65% were maintained and inoculated 1 ml of inoculum ($1x10^7$spores/ml). The content of the flask were mixed thoroughly gently beating the flasks on the palm of hand and incubated in slanting position at 35 ^{0}C for 7 days. The pH 4.5 was maintained through out the fermentation process[22].

EXTRACTION OF L-ASPARAGINASE:

The samples were with drawn periodically at 24 hrs in aseptic condition 1 gm of moldy substrate was taken into a beaker and distilled water (1:10) was added to it. The contents of flasks were allowed to have contact with water for 1 hr with occasional stirring with a glass rod. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay.

ASSAY OF L-ASPARAGINASE:

Assay of enzyme was carried out as per Imad et al.[19]. 0.5 ml of 0.04 M asparagine was taken in a test tube, to which 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit.

INTERNATIONAL UNIT (IU)

One IU of L-asparaginase is the amount of enzyme which liberates 1 μmol of ammonia per minute per ml [μ mole/ml/min].

CHARACTERIZATION OF L-ASPARAGINASE:

After fermentation process L-asparaginase were separated out by using filtration, centrifugation, and ammonium sulfate salt precipitation and purified by chromatography techniques, this partially purified enzyme were used for characterization studies.

Effect of pH on enzyme activity

The optimum pH of the purified enzyme was studied over a range of pH 4.0-11.0 with asparagine as a substrate dissolved in different buffers. The buffers used were citrate-phosphate pH 4-8 and glycine–NaOH, pH 9-11. The experiments were conducted as per the method described by Gaffar [23].

Effect of temperature on enzyme activity

The optimum temperature of L-asparaginase activity was determined with assay reaction mixture incubated at different temperature from 30 to 75^{0}C. The residual activity was measured as described by Gaffar[23].

Effect of pH on stability of enzyme activity

The pH stability of L-asparaginase was determined by pre-incubating the enzyme for 30 and 60 min for 45^{0}C in buffers of various pH values. The residual activity was measured as described by Gaffar[23].

Effect of temperature on stability of enzyme activity

The stability of enzyme to temperature was determined as per Gaffar[23]. The reaction mixture (without substrate) containing enzyme and buffer was pre-incubated for 30 and 60 min with different temperatures ranging 30-75^{0}C and cooled. The residual activity was measured as per Gaffar[23].

Substrate specificity

The reaction mixtures contained enzyme, buffer and different substrates, were used under the study as described by Imada et al.[19] and Mannan et al[-5]. L-asparagine, D-asparagine and glutamine were used as substrates in the present study.

TABLE - 1: *ASPERGILLUS TERREUS* ISOLATES FROM SOILS

Sl. No.	Sources	No. of isolates
1	Coconut plantation soil	17
2	Compost soil	08
3	Garden soil	06
4	Non compost soil	04

RESULTS AND DISCUSSION:

Production of L-Asparaginase:

The isolation pattern of *A. terreus* is presented in Table – 1. In the present study, thirty five strains of *A. terreus* were isolated and named serially from KLS1 to KLS35. The potential strains were selected on the basis of pink zone around the colony by plate assay method. Among these *Aspergillus terreus* KLS2 were used as potential strain for the production of L-asparaginase through solid state fermentation. The fermentation studies were indicated that the L-asparaginase production was maximum 6.05 IU at 72 hr fermentation period (Fig-1). Similar reports were reported by Sutthinan Khamna et al[24] reported that the maximum L-asparaginase production was observed at pH 7.0 and temperature 30^0 C at 178 hr of fermentation period.

Characterization of L-Asparaginase:

Effect of pH on enzyme activity

Fig.2 shows that partially purified L-asparaginase was active over broad pH ranges (4.0 - 11.0) with an optimum at pH 9.0. The pH optimum reported from this study is comparatively high than the results reported by De-Angeli [17] employing *A. terreus* under the submerged fermentation. This property of the enzyme makes clear that enzyme produced by *A. terreus* KLS2 under the present study has effective carcinostatic property, because the physiological pH is one of the perquisites for anti tumour activity[5]. The L-asparaginase activity below pH 8 would not be expected to be very effective for the treatment of tumour patients [25]. Triantafillons et al.[26] have studied that membrane bound L-asparaginase from *Tetrahymena pyriformis* acts optimally at pH 9.6. Mesas et al.[27] have found the optimal L-asparaginase activity at pH 7.0. The enzyme activity was slightly lowered at pH values of 7.5 or 8.0. Similarly, the pH 9.2 is optimum was reported by Pritsa and Kyriakidis [28]. Thus our result coincides with the results of Trintafillons et al.[26] and Pritsa and Kyriakidis [28].

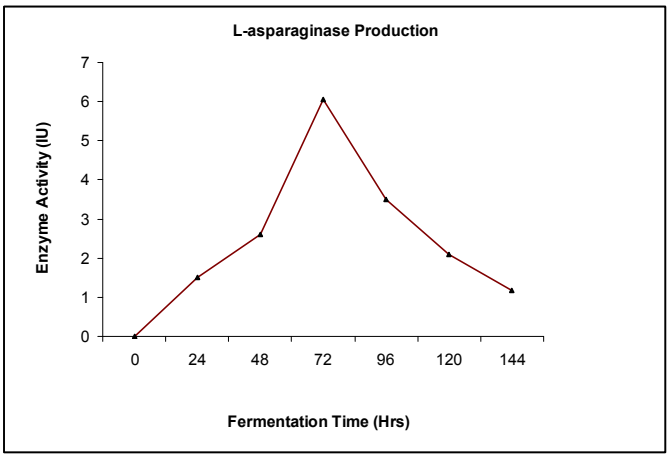

Fig-1. L-asparaginase production under solid state fermentation by using carob pod as substrate

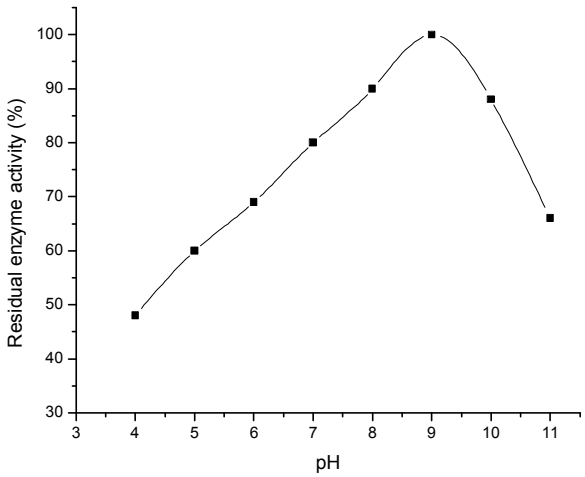

Fig. 2: Effect of pH on enzyme activity

Effect of temperature on enzyme activity

The temperature optimum of L-asparaginase from *A. terreus* KLS2 is given in Fig.3. The optimum temperature for L-asparaginase activity was found to be 37^{0}C. It is active at a wide range of temperature condition from 30^{0}C to 75^{0}C. Beyond this temperature the enzyme becomes unstable. This property of enzyme makes most suitable for complete elimination of asparagine from the body when tumour patient treated with L-asparaginase *in-vivo*. Mannan et al[-5] have found 37^{0}C to be the optimum temperature for the enzyme activity.

Effect of pH on stability of enzyme activity

The enzyme was stable at alkaline pH 8.0 and retains 100% activity even after incubation for 30 and 60 min at 37^0 C (Fig. 4). The enzyme retains 67% and 46% activity at pH 11 for 30 and 60 min respectively. It is observed that the enzyme was more stable at alkaline pH than the acidic. The similar findings were reported by Mannan et al.[5] using *Pseudomonas stutzeri* MB–405. The enzyme obtained by *P. stutzeri* MB 405 was more stable at alkaline pH than at the acidic one. The enzyme was maximally stable at pH range from 7.5 to 9.5.

Effect of temperature on stability of enzyme activity

The results on the effect temperature on enzyme stability are presented in Fig.5. The data indicated that no significant enzyme activity was lost when it is pre incubated at 70^{0}C for 30 and 60 min. The residual activity is 100% at this temperature. At 80^{0}C it retains 69% and 60% activity for 30 min and 60 min respectively. Similar results were reported by Pritsa and Kyriakidis [28], they found 100% activity of enzyme at 77^{0}C.

Substrate specificity of enzyme

The substrate specificity of the enzyme is presented in Table – 2. The results revealed that the enzyme was 100%, 5% and 4% active towards L-asparagine, D-asparagine and L-glutamine respectively. The data indicated that the enzyme extracted from *A. terreus* KLS2 is very much specific to its natural substrate asparagine. This property of the enzyme is very essential on the treatment of patients where incomplete removal of asparagine is required. The observations reported under the present study are in good agreement with the findings of Campbel and Mashburn [29] and Mannan et al.[5].

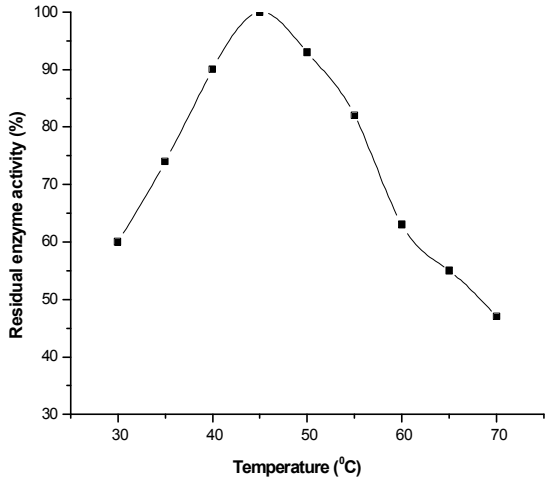

Fig.3: Effect of temperature on enzyme activity

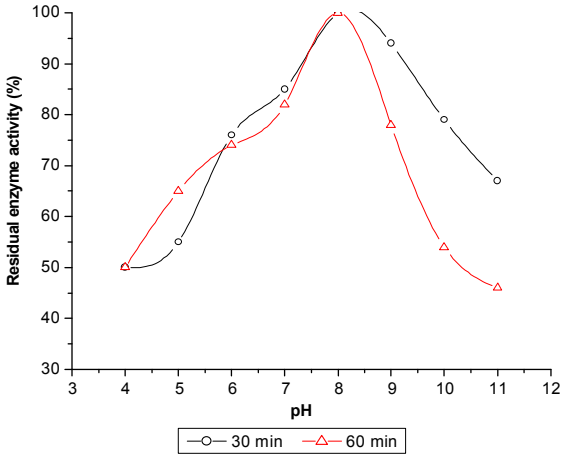

Fig. 4: Effect of pH on stability of enzyme activity

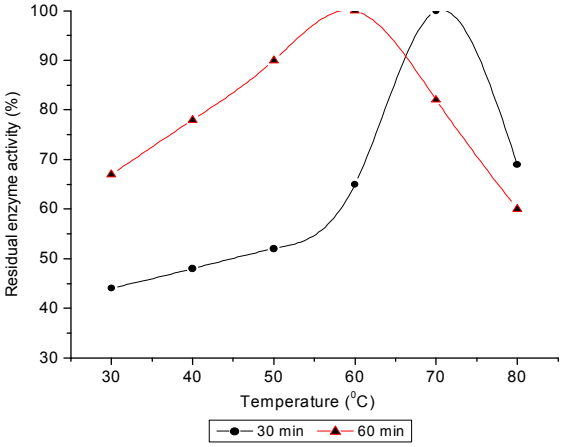

Fig. 5: Effect of temperature on stability of enzyme activity

TABLE – 2: SUBSTRATE SPECIFICITY OF ENZYME

Substrate added	Concentration (mM)	Relative activity (%)
L – Aspargine	10	100
D – Aspargine	10	5
L – Glutamine	10	4

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