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Research Article

Screening and Optimization of L-Asparaginase- A Tumour Inhibitor from *Aspergillus terreus* Through Solid State Fermentation

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ABSTRACT

Thirty-five filamentous fungal isolates obtained from soil samples from different regions of Gulbarga, It was identified as *Aspergillus terreus* and screened for their ability to produce L-asparaginase. Using modified Czapek Dox agar containing L-asparagine and phenol red as indicator, all thirty five L-asparaginase producing fungal isolates could be preliminary identified by plate assay method, observing pink colour formation. It was found that *Aspergillus terreus* KLS2 exhibited highest activity. The *Aspergillus terreus* KLS2 were used for optimization of fermentation parameters like pH, temperature and inoculum size for L-asparaginase production through solid state fermentation by using carob pod as a substrate. The pH 4.5, temperature 35 ^{0}C and 1x10^7 spores /ml inoculum size were found optimum for maximum 6.05 IU of L-asparaginase production.

Keywords: L-asparaginase, Solid state fermentation, Carob pod, *Aspergillus terreus*, Plate assay

INTRODUCTION

Enzymes produced by microorganisms have been used in various industries in the world. Microbial L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia[1-2]. The discovery of L-asparaginase ,a medicinal agent for the treatment of malignant tumors, was made in 1922[3].Clementi showed that guinea pig serum contained a high activity of L-asparaginase[3]. L-Asparaginase catalyses the conversion of l-asparagine to l-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions[4].Supplementation of L-asparaginase results in continuous depletion of l-asparagine. Under such an environment, cancerous cells do not survive. This phenomenal behaviour of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase[5-7].

This enzyme is also a choice for acute lymphoblastic leukemia, lymphosarcoma and in many other clinical experiments relating to tumour therapy in combination with chemotherapy. This treatment brought a major breakthrough in modern oncology, as it induces complete remission in over 90% of children within 4 weeks[1]. With the development of its new functions, a great demand for L-asparaginase is expected in the coming years.

This enzyme is widely distributed, being found in L-asparaginase is widely distributed, being found in animal, microbial and plant sources[8]. It's presence in guinea pig serum was first reported by Clementi[3]. Large number of microorganisms that include *Erwinia carotovora* [9], *Pseudomonas stutzeri* [10], *Pseudomonas aerugenosa* [11] and *E. coli*[12]. It has been observed that eukaryotic microorganisms like yeast and fungi have a potential for asparaginase production.[13-14]

There were no reports on L-asparaginase production through solid state fermentation by using carob pod as a substrate. We made an attempt to screen for L-asparaginase producing microorganisms and optimization of L-asparaginase.

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[15]. The isolated (Plate - 1) strains were tentatively identified in the laboratory as described by Rapper and Fennell[16] and were maintained on potato dextrose agar (PDA).

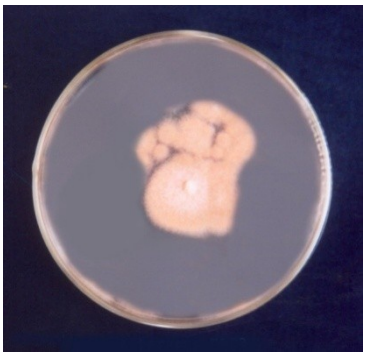
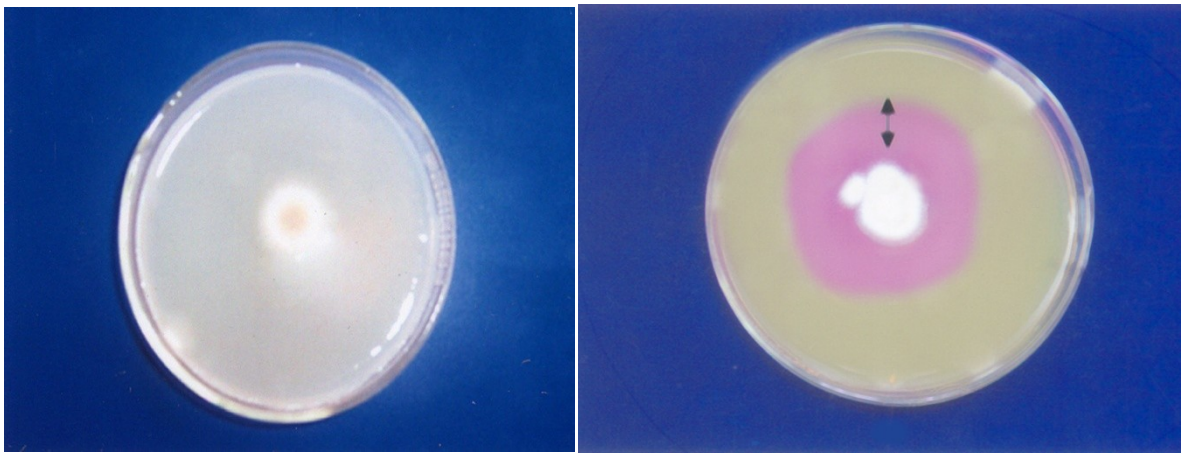


Plate – 1: Aspergillus terreus

Screening of L-Aspaerginase producer by plate assay

The strains obtained from the above steps were subjected for rapid screening of L-asparaginase production by plate assay (Plate - 2) as per Gulati et al[17].

The modified Czapek Dox's medium was supplemented with phenol red (2.5% prepared in ethanol and the pH was adjusted to 7.0) dye. The media was autoclaved and plates were prepared. Control plate was maintained without asparagine (instead containing $NaNO_3$ as nitrogen source).



A **B**

Plate – 2: Rapid Plate Assay for Screening of L-Asparaginase Producers

A: Control, B: Assay Plate

The plates were inoculated with *Aspergillus terreus* strains isolated from the soil. The zone and colony diameter was measured after 48 hrs. The isolate, which has given maximum zone of clearance, has been selected for optimization process.

Optimization of fermentation parameters for L-asparaginase production

The production of L-asparaginase under SSF mainly depends on various factors like initial pH, temperature, inoculum size. Hence, these parameters must be optimized in order to achieve higher yields of L-asparaginase. During this optimization process, once a particular parameter was optimized, the same optimum condition of that specific parameter was employed in the subsequent studies wherein another parameter is to be optimized.

Effect of initial pH on L-asparaginase production

The flasks containing 20 gm of substrate were mixed with acid/alkali moistening solution to obtain required pH. The pH was adjusted in the range of 3-7 with increments of 0.5. Thus prepared flasks were cotton plugged and autoclaved at 121^0C for 15 min.

Effect of initial temperature on L-asparaginase production

The ground substrates about 20 g were taken separately in 250 ml Erlenmeyer flasks and prepared for solid state fermentation as siddalingeshwara et al[18]., Thus prepared flasks were incubated at different temperatures like 25, 30, 35 and 40^0C.

Effect of inoculum size on L-asparaginase production

The inoculum was prepared separately at different levels i.e., 1×10^5 to 1×10^{10} spores/ml as described by Vergano et al[19]., and then fermentation studies were carried out.

Extraction of L-asparaginase from fermented substrate

The samples were withdrawn 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. The supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay.

Assay of L-asparaginase from crude extract

Assay of enzyme was carried out as per Imad et al[20]., 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].

RESULTS AND DISCUSSION

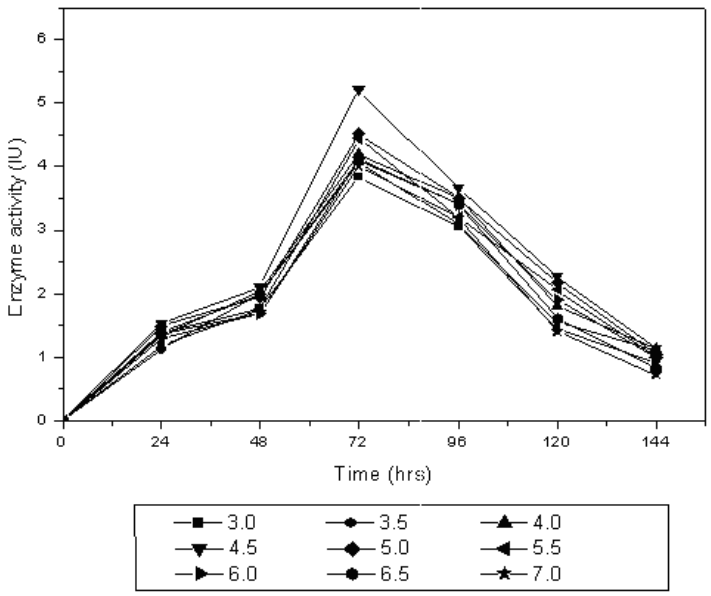
Isolation and Screening

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.

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

Table - 1: *Aspergillus terreus* isolates from soils

The results from plate assay are presented in Plate - 2. The results revealed that all the isolates showed different range of zone of diameter. Therefore, for the convenience, the grouping of strains of *A. terreus* has been done on the basis of zone of diameter they exhibited. It is proposed that the strain exhibiting zone of diameter above 0.9 are referred as good or high L-asparaginase producers, those strains with zone of diameter 0.6 to 0.9 and those having below 0.6 zone of diameter may be referred to as moderate and poor L-asparaginase producers respectively. As per this grouping the strains *A. terreus* KLS2 exhibited higher zone of diameter and considered as potential strain for L-asparaginase production among the strains isolated from the soil. As such, strain KLS3, KLS5, KLS7 can be treated as moderate L-asparaginase producers and remaining isolates treated as poor L-asparaginase producers.



Optimization of Fermentation Parameters

The effects of different initial pH on deseeded carob pod substrate on L-asparaginase production by *A. terreus* KLS2 are presented in Fig. 1. The effect of initial pH on carob pod fermentation revealed that the yield of L-asparaginase increased with the increase in the initial pH of the substrate upto 4.5 units these increasing peaks were observed upto 72 hrs of fermentation period and thereafter the yield decreased as pH levels and fermentation period increased. The maximum L-asparaginase activity 5.210 IU was obtained at pH 4.5 at 72 hrs of fermentation period. The least L-asparaginase activity was observed at pH 3.0 with *A. terreus* KLS2 strain.

Fig.1. Effect of pH on production of L-asparaginase by *Aspergillus terreus* KLS2

De Angeli et al[21]., and Ali et al[22]., have reported pH 7 and 4.5 were optimum for the maximum production of L-asparaginase under SmF process respectively. Gulati et al[17]., have reported 6.2 was the optimum pH for L-asparaginase producing *A. terreus* strains. Similarly, Sarquis et al[23]., have reported highest L-asparaginase production of 58 U/L when *A. terreus* strains cultivated in medium having pH of 6.2. In our study the data

revealed that the pH of 4.5 was found as suitable for maximum production of L-asparaginase with *A. terreus* KLS2 strain on deseeded carob substrate under solid state fermentation.

These variations in pH optima for L-asparaginase production may be due to the strain of the organism used, chemical composition of the substrate, fermentation system and finally the conditions under which fermentation takes place[24]. Thus from the above studies it clearly indicates that the production of L-asparaginase mainly depends on the strains employed during the fermentation. As such our findings are in close agreement with the findings of Ali et al[22].

The effect of different initial temperature levels on the production of L-asparaginase by *A. terreus* KLS2 strain on deseeded carob pod substrate are presented in Fig.2.

The perusal of data indicated that the L-asparaginase production increased significantly with increase in temperature from 25^{0}C - 35^{0}C. The decrease in L-asparaginase production was observed above 35^{0}C in all days of fermentation period on carob pod substrate. The maximum enzyme production of 5.28 IU was observed at 35^{0}C, whereas lowest enzyme production of 3.026 IU was observed at 40^{0}C after 72 hrs of fermentation period. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature[25,26]. Sarquis et al[23]., reported 30^{0}C is the suitable for L-asparaginase production through submerged fermentation by using *A. terreus* and *A. tamarii*.

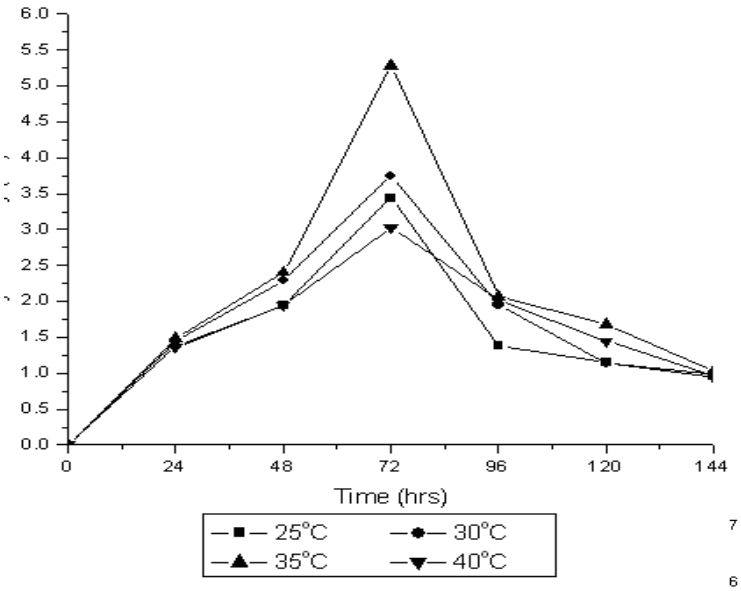


Fig.2. Effect of temperature on production of L-asparaginase by *Aspergillus terreus* KLS2

The results of studies on the effect of inoculum size on the production of L-asparaginase by employing *A. terreus* KLS2 strain using carob pod as substrate are presented in Fig.3.

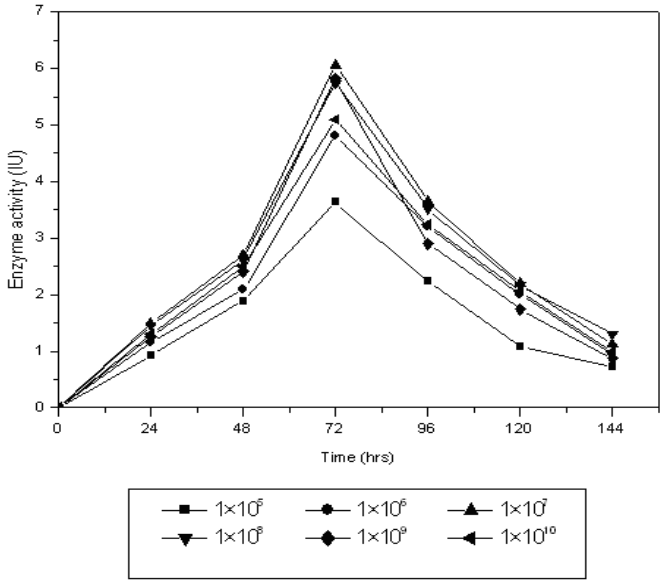


Fig.3. Effect of inoculum size on production of L-asparaginase by *Aspergillus terreus* KLS2

The data revealed that the production of L-asparaginase by *A. terreus* KLS2 increased as the inoculum size increased upto 1 x 10^7 spores/ml for 72 hrs fermentation periods. Further increase in the inoculum size has not yielded significant increase in the

production of L-asparaginase. The inoculum size of 1 x 10^7 spores/ml showed maximum production of L-asparaginase 6.05 IU at 72 hrs fermentation period. The lowest amount of L-asparaginase production of 3.64 IU was observed at inoculum size of 1 x 10^5 spores/ ml. Ashraf A. El-Bessoumy et al[27]., reported that 3ml/ flask (50ml of fermentation medium) of standard inoculum were used from *Pseudomonas aeruginosa* through solid state fermentation.

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