KEY FERMENTATION FACTORS FOR THE SYNTHESIS OF
L-ASPARAGINASE - AN ANTI TUMOUR AGENT THROUGH SSF
METHODOLOGY

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ABSTRACT
The objective of this investigation was to isolate soil fungal isolates and to screen them
for L-asparaginase production. The main key objective of this paper deals with
Evaluation of fermentation process parameter interactions for the production of L-
asparaginase by Aspergillus terreus. Thirty five isolates were isolated and screened by
plate assay method, out of thirty five Aspergillus terreus KLS2 were selected for
optimization of key fermentation parameters like moisture content, bed depth and
particle size. The optimum 65% moisture content, 30 mm of bed depth and 2mm of
particle size were used to show maximum 5.63 IU ofL-asparaginase production.
Key words: L-asparaginase, Aspergillus terreus, fermentation.

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 received increased attention in
recent years for its anticarcinogenic potential. Cancer cells differentiate themselves
from normal cells in diminished expression of L-asparagine [4, 5]. Hence, they are not
capable of producing l-asparagine, and mainly depend on the l-asparagine from the
circulating plasma pools [4].

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 [6, 7, 8, 9, 10]. L-Asparaginase catalyzes the hydrolysis ofL-
asparagine into L-aspartate and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate (Fig. 1)\textsuperscript{[11]}.

L-Asparaginase is produced throughout the world by submerged fermentation (SmF). This technique has many disadvantages, such as the low concentration production, and consequent handling, reduction, and disposal of large volumes of water during the downstream processing. Therefore, the SmF technique is a cost intensive, highly problematic, and poorly understood unit operation \textsuperscript{[12]}. SSF offers numerous advantages over submerged fermentation (SmF). SSF should not be seen as a technology, which can simply replace SmF. Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SmF \textsuperscript{[13]}, and it also offers many other advantages \textsuperscript{[14]}. The aim of the present study is to evaluate the suitability and utility of carob pods as best substrate for optimization of key fermentation factors like moisture content, bed depth and particle size for the production of L-asparaginase employing locally isolated strain \textit{Aspergillus terreus}. There were no reports on solid state fermentation for L-asparaginase production by using carob pod as substrate.

\textbf{Figure1}

Schematic illustration of the reaction mechanism of L-asparaginase \textsuperscript{[11]}. 

\begin{center}
\includegraphics[width=\textwidth]{asparaginase_mechanism.png}
\end{center}
MATERIALS AND METHODS

Fungal isolates and cultural condition

Fungal isolates were isolated from soil samples collected from different regions of Gulbarga, using potato dextrose agar (PDA) medium by serial dilution method. The inoculated agar plates were incubated at 28°C for 4 to 7 days. Thirty five isolates of Aspergillus terreus were selected and tentatively identified in the laboratory as described by Rapper and Fennell [15], and were maintained on potato dextrose agar (PDA) at 4°C. Further confirmations were done at Agarkar Research Institute, Pune.

Production of L-asparaginase:

The isolated strains were screened by palate assay method [16], and used potential strain for the production of L-asparaginase through solid state fermentation by using carob pod as a substrate.

Optimization of fermentation parameters for L-asparaginase production

The production of L-asparaginase under SSF mainly depends on various factors like initial moisture content, particle size, and bed depth. 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.

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°C for 7 days. The pH 4.5 was maintained through out the fermentation process [17].

Effect of initial moisture content on L-asparaginase production

A set of conical flasks containing 20 g of substrate (2 mm size) were moistened with an appropriate amount of distilled water in order to obtain different moisture levels like 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75%. The contents were autoclaved at...
121°C for 20 min., and inoculate each flask with one ml of spore inoculum of *A. terreus* KLS2. Thus prepared flasks were mixed thoroughly and incubated in a humidity chamber (60-75%) at 35°C temperature for a period of 7 days\[^{17}\].

**Effect of initial particle size on L-asparaginase production**

The ground substrates were sieved through sieves having different sizes, like 2, 4 and 6 mm and each particle size was separately used in studies.

**Effect of initial bed depth on L-asparaginase production**

The substrate was taken in 250 ml beaker and adjusted to bed height of 10, 20, 30, 40 and 50 mm. Thus prepared media were inoculated and incubated as described earlier.

**Extraction of fermented substrate**

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

**Quantitative assay for L-asparaginase activity**

Assay of enzyme was carried out as per Imad et al.\[^{18}\]. 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

Thirty five fungal isolates from different soils in Gulbarga regions were isolated and identified as *Aspergillus terreus* and confirmed from Agarkar Research Institute, Pune. The potential strains were selected on the basis of pink zone around the colony by plate assay method. Out of thirty five strains *Aspergillus terreus* KLS2 were selected as potential strain for the production of L-asparaginase.

The moisture content in carob pod tested for maximum L-asparaginase production indicated enhanced enzyme production with increase in the substrate moisture content up to 65% beyond which it declined (Fig.2). The highest production of L-asparaginase was obtained at a moisture level of 65% and the maximum L-asparaginase activity of 4.96 IU was observed and it declined sharply at lower levels of moisture content. The lowest enzyme activity of 1.41 IU at 30% moisture level was observed.

![Figure2](image.png)

**Figure2**

Effect of moisture content on production of L-asparaginase by *Aspergillus terreus* KLS2.
The reduction in the production of L-asparaginase in reduced moisture content might be due to the reduction in solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension. Likewise, the higher moisture levels decreased porosity, stickiness, and reduced air volume and diffusion that reduced oxygen transfer. Gervais and Molin and Venil and Lakshmanaperumalaswamy and reported 75% and 50% moisture content were optimal for xylanase and L-asparaginase production in solid state fermentation respectively, by A. terreus and Serratia marcescens SB08. As such, in the present study A. terreus KLS2 has given high amount of L-asparaginase at 65% of moisture content. Therefore, our findings were in good agreement with the findings of Venil and Lakshmanaperumalaswamy.

The thickness of the substrate layer under any natural fermentation conditions plays a key role in the desired end product formation. Further, it also affects the growth and enzyme activity of the organism involved during the fermentation. The L-asparaginase synthesis by optimizing particle size using carob pod as substrate were presented in Fig.3. The production of L-asparaginase enhanced as the bed depth increases from 10 mm to 30 mm and thereafter it decreases by further increase in the bed depth of the substrate layer. The maximum production of 5.57 IU of L-asparaginase was obtained at 30 cm bed depth at 72 hrs of fermentation period. The lowest (3.20 IU) L-asparaginase production was noticed on 50mm particle size of carob pod substrate. At higher bed depths lower productions of L-asparaginase were observed, that might be attributed due to lower metabolic activity of the organism coupled with improper aeration, gaseous exchanges as well as unsuitable temperature elevations have impaired the productions.
Figure 3

Effect of bed depth on production of L-asparaginase by *Aspergillus terreus* KLS2

Amongst different initial particle sizes tested for L-asparaginase production, the substrate with particle size of 2 mm has shown maximum L-asparaginase production of 5.63 IU with particle size of 2 mm at 72 hrs of fermentation period. The lowest (3.14 IU) L-asparaginase production was noticed on 6mm particle size of carob pod substrate. The results on the activity of L-asparaginase from deseeded carob substrate at different initial particle size by *A. terreus* KLS2 are presented in Fig. 4. The particle size of the substrate greatly influences the production under solid state fermentation process \[^{23, 24}\].

Hence optimum particle size needs to be studied at which maximum productivity can be obtained. It was reported that substrates with finer particles showed improved degradation due to increase in surface area and greater growth of the fungal cultures was stimulated by smaller particle size of substrate \[^{25, 26}\].
Effect of particle size on production of L-asparaginase by *Aspergillus terreus* KLS2.

From this work we conclude that *Aspergillus terreus* KLS2 is a promising agent for industrial application since it give significant L-asparaginase production in carob pod under solid state fermentation (SSF) methodology. Hence this was the first report on carob pod, used as substrate for L-asparaginase production.

**REFERENCE**


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