

# Production of L-Asparaginase from *Aspergillus Niger* using Agro Wastes By-Products in Submerged Fermentation Process

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## Article history

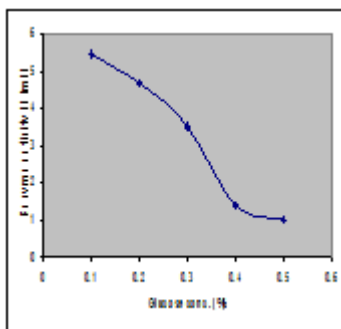
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## Graphical abstract



## Abstract

The project was carried out to obtain maximum yield of L-asparaginase from *Aspergillus niger* using by-products of agro wastes incorporated with organic salts in submerged fermentation process. The main objective of the project was to study the kinetic parameters of L-asparaginase productivity. After optimization maximum enzyme activity ( $2.83\text{U/mL} \pm 0.065$ ) was achieved using corn steep liquor as a substrate and with 4% inoculum, pH 6.5, 1% substrate concentration, 96 hrs fermentation time period and 1% glucose was used as additional supplement to the growth media to obtain better yield of L-asparaginase. This study showed that glucose concentration beyond 1% suppressed the enzymatic activity. From the results it can be concluded that L-Asparaginase production was optimized when cheap agro-waste products were used as a substrate at low concentrations and under acidic conditions. Its relative stability in acidic pH conditions make it ideal for applications in health care systems and pharmaceutical industry.

**Keywords:** L-Asparaginase; *Aspergillus niger*; corn steep liquor; submerged fermentation

## Abstrak

Projek ini dijalankan untuk mendapat hasil maksimum L-asparaginase daripada *Aspergillus niger* menggunakan produk sampingan sisa buangan agro bersama garam organik di dalam proses penapaian rendaman (submerged). Objektif utama projek ini adalah untuk mengkaji parameter kinetik produktiviti L-asparaginase. Aktiviti enzim yang optimum ( $2.83\text{U/mL} \pm 0.065$ ) telah diperolehi apabila menggunakan *corn steep liquor* sebagai substrat, 4% inokulum dengan pH 6.5, kepekatan substrat 1%, masa penapaian selama 96 jam dan 1% glukosa sebagai nutrien tambahan untuk media pertumbuhan untuk mendapatkan penghasilan L-asparaginase daripada *Aspergillus niger* yang lebih baik. Walau bagaimanapun, kepekatan glukosa 1% telah menyebabkan, aktiviti enzim mula terencat. Pada akhir kajian ini, dapat disimpulkan bahawa kehadiran produk sampingan sisa buangan agro yang murah pada kepekatan yang rendah dan stabil menghampiri pH asidik akan menghasilkan L-asparaginase yang maksimum. Enzim seperti ini adalah amat berguna dalam sistem penjagaan kesihatan dan industri farmasi.

**Kata kunci:** L-Asparaginase; *Aspergillus niger*; rendaman jagung di dalam arak; penapaian rendaman (submerged)

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## 1.0 INTRODUCTION

There are two fundamental conditions for life. Firstly, the organisms must have the ability to self-replicate and secondly, they must be able to catalyze chemical reactions efficiently and selectively. Enzymes are central to every biochemical process. Recent reasearch findings have opened a new horizon in molecular biology of microorganisms for novel applications of new enzymes<sup>1</sup>. One of the best products is L-asparaginase having a marked potential as anti-leukemic agent<sup>2</sup>. L-asparaginase has become an integral part of the modern day chemotherapy protocols, when used during the intensification

phases of acute lymphoblastic leukemia (ALL) treatment procedure, particularly for patients with high risk features<sup>3,4</sup>. L-asparaginase is widely distributed in nature from bacteria to mammals and plays a vital role in amino acids metabolism and utilization<sup>5</sup>. Production of L-asparaginase has been observed from many organisms such as *Erwinia carotovora*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas eruginosa*, *Bacillus subtilis*, *Aspergillus tamari*, *Aspergillus terreus* and *Aspergillus niger*<sup>6-11</sup>.

*Aspergillus*, *Fusarium* and *Penicillium* are generally reported in the literature as asparaginase producers<sup>12,13</sup>. Microbial L-asparaginase has efficient therapeutic values

against lymphosarcoma and acute lymphoblastic leukemia. For commercial scale production of L-asparaginase various microorganisms including *Bacillus* sp. *E. coli*, *Enterobacter aerogenes*, *Erwinia carotovora*, *Corynebacterium glutamicum* and *Candida utilis* have been utilized<sup>14</sup>.

L-aspartate and ammonia are the end products of L-asparagine hydrolysis, catalyzed by L-asparaginase. Many animals tissues, bacteria, plants and certain rodents exhibited L-asparaginase but the precise mechanism of its action is still unknown<sup>15,16</sup>. The present manuscript reports the enhanced L-asparaginase production through SmF media using waste (corn steep liquor) as substrate for growth microorganisms.

## 2.0 EXPERIMENTAL

### 2.1 Microorganisms and Culture Conditions

*Aspergillus niger* was obtained from the Enzyme Biotechnology Lab, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad. Potato dextrose agar (PDA) slants were used to culture fungi and to prepare fungal spore suspension. The preserved culture was revived on fresh PDA slants over a period of 2 weeks and was further used as inoculum for the experiment.

### 2.2 Chemical and Substrates

All chemicals used were of analytical grade and obtained from Enzyme Biotechnology Lab. (UAF). Growth supporting substrates (Corn, Corn steep liquor and red gram) were collected from grain market of Faisalabad, Pakistan. Moisture free bags were used for storage of substrates.

### 2.3 Spore Suspension

A loopful of *A. niger* culture was transferred into modified Czapek Dox medium<sup>12</sup> for preparation of fungal spore suspension. The medium contained glucose 2 g/L, L-asparagine 10 g/L,  $\text{KH}_2\text{PO}_4$  1.52 g/L, KCl 0.52 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.52 g/L,  $\text{CuNO}_3$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  in trace amounts and was kept in shaking condition at 120 rpm for four days to obtain homogenous spore suspension ( $1 \times 10^6$ - $10^8$  spores/mL).

### 2.4 Fermentation and Harvesting of L-Asparaginase

Substrate screening was carried out by inoculating 7% v/v freshly prepared *Aspergillus niger* suspension at different concentrations of Czapek dox medium. Prior to use, the media was sterilized at 121°C for 15 minutes and the medium pH was maintained at 6.2. The inoculated flasks were kept at 30°C at different time periods in shaking incubator at 120 rpm. Harvesting of samples was carried out after four days. The media was then centrifuged at 9000g for 10 minutes at 4°C to obtain the clear supernatant containing L-asparaginase enzyme. The crude extract was then used for further analytical studies<sup>18</sup>.

### 2.5 Optimization of Production Parameters

After determining the best substrate, subsequent steps were carried out to investigate the effects of substrate concentrations (0.01, 2 w/v), pH (ranging from 5-9), fermentation/ incubation time (24-120 hours), temperature (ranging from 20-40°C), various inoculum sizes (2.5-10 mL/100 mL) and different carbon sources, on L-asparaginase enzyme production.

### 2.6 L-Asparaginase Enzyme Assay

Nesslerization method<sup>19</sup> was used to check the enzyme activity. L-asparaginase hydrolyzes the L-asparagine to L-aspartic acid and ammonia. Ammonium liberated in the reaction was determined by the Nessler reaction. L-asparaginase unit of activity is defined as the amount of enzyme that liberated 1 mmol ammonia/min at 37°C<sup>2</sup>.

According to this method the reaction mixture contained 1.7 mL of 0.01 M L-asparagine and 0.1 mL enzyme. The addition of 0.01 mL trichloroacetic acid (TCA) was used to stop the reaction after 30 minutes of incubation at 37°C. The amount of ammonia released was determined calorimetrically at 480 nm by adding 0.5 mL Nessler Reagent to tubes containing 0.5 mL supernatant and 7.0 mL distilled  $\text{H}_2\text{O}$  mixture after centrifugation at (10,000 rpm, 5 min). The content in the tubes was vortex and left to stand for 10 min. at room temperature. The control that contained only TCA without the enzyme was used as the control? Protein was estimated using the Biuret method<sup>20</sup> with bovine serum albumen as the standard.

### 2.7 Statistical Analysis

Statistical evaluation of the data was carried out to determine the means and standard errors of means (Mean $\pm$ SE) for each parameter<sup>21</sup>.

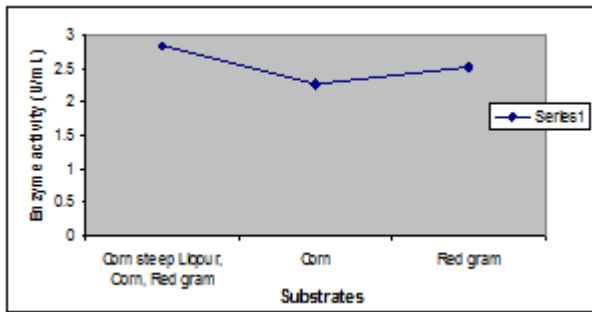
## 3.0 RESULTS AND DISCUSSION

### 3.1 Screening of Agro Waste by Products

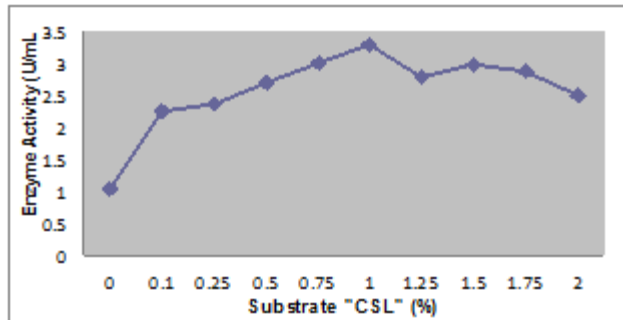
Results for screening of the best substrate showed that the yield of L-asparaginase was maximum when corn steep liquor was used as a substrate at 2% concentration. The results of the screening showed that the maximum yield of L-asparaginase (2.83 U/mL  $\pm$  0.065) was achieved after the 4<sup>th</sup> day of fermentation period as compared to corn and red gram (Figure 1). Similar results were obtained from another study when *E. coli* was grown aerobically in corn steep liquor medium<sup>22</sup>. Corn steep liquor is a well known fermented sugar source and the protein contents of corn steep liquor are 40%. The investigations showed that single amino acids supplementation to medium did not stimulate the production of the enzyme<sup>23,24</sup>.

### 3.2 Substrate Concentration

The maximum L-asparaginase production (3.31 U/mL  $\pm$  0.004) was found at 1% (w/v) of corn steep liquor. There was an increase in enzyme production by *A. niger* with the increase in substrate concentration from 0.1 to 1.0% (w/v). As the substrate concentration was increased above 1%, the enzyme activity started to decline (Figure 2). Similar results were reported using mesophilic strain of *Erwinia* sp. and mannitol as a carbon source<sup>25</sup>.



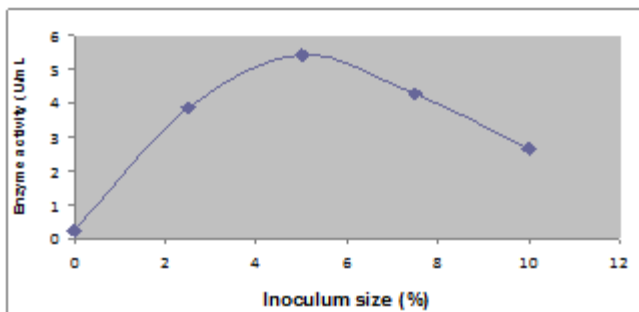
**Figure 1** Screening of agro waste by-products for the production of L-asparaginase



**Figure 2** Effect of substrate (Corn steep liquor) concentration on L-asparaginase production

### 3.3 Inoculum Size

Inoculum size was studied by inoculating set of flasks containing 1 % w/v substrate with different volumes of inoculum sizes for the preparation of L-asparaginase enzyme. It was observed that 5% inoculum gave the maximum activity ( $5.40 \text{ U/mL} \pm 0.025$ ) (Figure 3). An increase in inoculum size from 2.5 to 5% showed progressive enzyme activity reaching its highest at 5% level while further increase in inoculum size caused overcrowding of spores that decreased the enzyme activity. Similar results were obtained from the literature reported when using *Aspergillus niger*<sup>11</sup>.

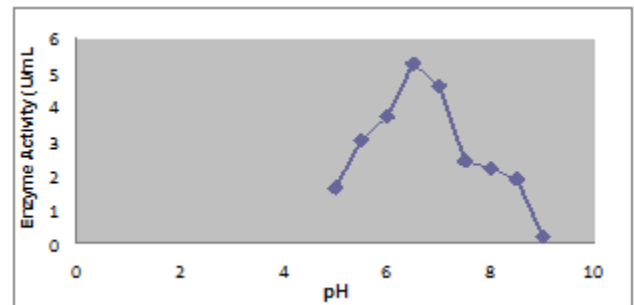


**Figure 3** Effect of inoculum size on L-asparaginase production

### 3.4 pH

The enzyme profiles revealed that after 5 mL inoculum size, 1 g substrate concentration, L-asparaginase showed maximum activity. To obtain the optimum pH, different pH i.e. 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were set for production media. The maximum yield of L-asparaginase ( $5.26 \text{ U/mL} \pm 0.59$ ) was

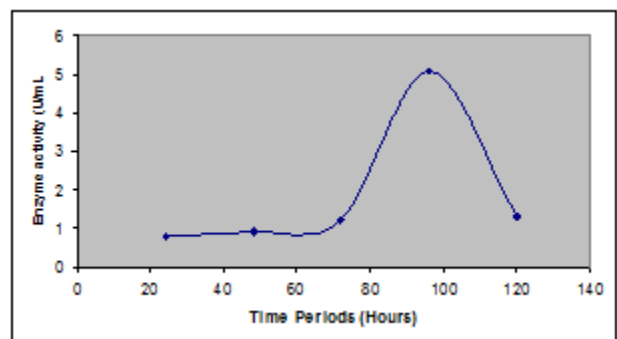
observed in the medium at pH 6.5. At low pH enzyme activity was decreased due to more acidity as well as 8.5 and 9.0 pH also showed low yield due to the alkaline conditions. These results correlated with the reported production of L-asparaginase enzyme from *A. niger* at 6.5 pH<sup>11</sup>. Literature reported that pH 9 is the optimum pH while using *Pseudomonas aeruginosa* for the production of L-asparaginase<sup>15</sup>. The optimal pH of 7.5-8.0 was observed by using *Streptomyces albidoflavus*<sup>14</sup>. Optimum activity of L-asparaginase from *A. niger* at near physiological pH makes this enzyme superior to that of L-Asparaginase from other microorganisms as a chemotherapeutic agent in the treatment of leukemia. The change in initial pH of the medium greatly influenced the enzyme secretion. Kinetic parameters for L-asparaginase production under different pH were shown in Figure 4.



**Figure 4** Effect of pH on L-asparaginase production

### 3.5 Effect of Fermentation Period on L-Asparaginase production

Figure 5 shows the role of fermentation period parameter on L-asparaginase production. The increasing trend was observed in the L-asparaginase production with increasing fermentation time and at the 96 hrs its activity was maximum ( $5.07 \text{ U/mL} \pm 0.095$ ). It was also observed that 96 hrs fermentation period produced maximum yield of L-asparaginase<sup>11</sup>. When the fermentation period was increased to 96 hrs, the enzyme activity started to decrease.



**Figure 5** Effect of fermentation period on L-asparaginase production

### 3.6 Effect of Temperature

The effect of temperature on L-asparaginase production was shown in Figure 6. The results showed that the maximum enzyme activity ( $5.31 \pm 0.06 \text{ U/mL}$ ) was achieved at 35°C. Higher temperature inhibited the production of secondary metabolites as well as growth of microorganisms and may also cause denaturation of proteins. Similar results have been

observed when using *S. Albiodifavous sp.* for the production of L-asparaginase<sup>14</sup>.

*Aspergillus niger* showed a wide range of thermal stability than the bacteria<sup>15</sup>. Temperature has a significant influence in the production of L-Asparaginase by *Aspergillus niger*. From the literature, reaction rate of L-asparaginase appeared to be optimal at 40°C and loss of activity above this temperature with 50% of the enzyme activity even at 60°C<sup>11</sup>. Higher pH and temperature inhibited cell growth of *S. albiodifavus* species as well as L-asparaginase production.

### 3.7 Effect of Carbon Sources

Figure 7 shows the effect of carbon sources on the production of L-asparaginase. Effect of carbon sources were determined by inoculating a set of flasks with different carbon sources that is, glucose, fructose, sucrose and lactose with 5% inoculum and incubated at 35°C for 96 hours at 120 rpm.

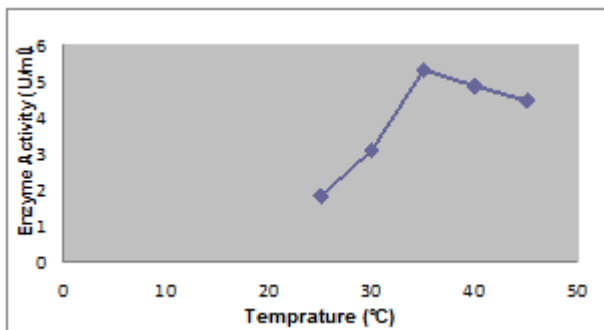


Figure 6 Effect of temperature on L-asparaginase production

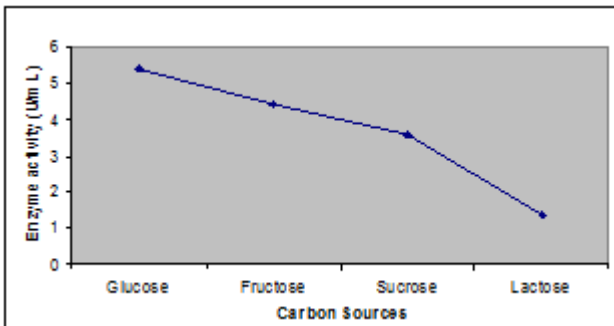


Figure 7 Effect of carbon sources on L-asparaginase production

It was observed that glucose as a carbon source has significant effect on L-asparaginase production and prove maximum enzyme activity (5.45 U/mL±0.045) while fructose also showed approximately near to maximum activity but less than glucose. It has been reported that basal medium containing maltose, starch, glucose, trehalose and glycerol as carbon source showed higher production of L-asparaginase, when the strain was cultivated in that medium<sup>14</sup>.

### 3.8 Effect of Glucose Concentration

Additional carbon source and its concentration in basal growth medium has a vital role in microbial growth and enzyme production. Optimum glucose concentration is required for the best growth of *A. niger* to yield the highest value of L-asparaginase, 0.1% glucose used in the fermentation medium as

additional carbon supplemented showed optimum results (Figure 8). Results showed that glucose was totally consumed after 72 hours. The 20 fold increase in L-asparaginase was found to be directly related to the presence of glucose during the static incubation period<sup>12,23</sup>. The addition of glucose to the medium was associated with depressed cell growth and L-asparaginase production<sup>22</sup>.

## 4.0 CONCLUSION

The present work indicated remarkable L-asparaginase production potential of *Aspergillus niger* from corn steep liquor, an agricultural by-product which has been used as for *Aspergillus niger*. Corn steep liquor is an efficient, renewable, economical and easily available carbon source. In conclusion the project was designed to find out the optimal fermentation conditions for cultivation of *Aspergillus niger*, and improved yield of L-asparaginase. However, the aptness of the enzymes for industrial applications can be investigated after purification.

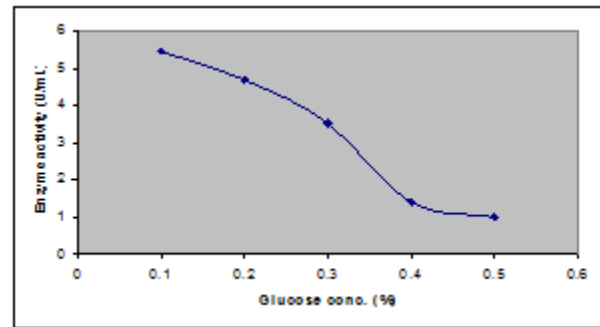


Figure 8 Effect of glucose concentration on L-asparaginase production

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