

# Production, Partial Purification and Assay of L-Glutaminase Enzyme from *Aspergillus Niger* by Solid State Fermentation

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## ABSTRACT

L-Glutaminase (L-Glutamine amidohydrolase, EC 3.5.1.2) is the important enzyme that catalyzes the deamination of L-Glutamine to L-glutamic acid and ammonium ions. Of late L-Glutaminase has received much attention with respect to its therapeutic and industrial applications. It acts as a potent antileukemic agent and has flavor enhancing property for fermented foods. Glutaminase production is widely distributed in plants, animals, bacteria, yeasts, and fungi. This study investigates the production and partial purification of extracellular Glutaminase enzyme from *Aspergillus niger* using agricultural waste by solid state fermentation (SSF). 80% ammonium sulphate fractionated enzyme exhibited maximum activity of 10.932 $\mu$ M/ml/min and a specific activity of 0.879U/mg protein.

**Keywords:** L-Glutaminase, *Aspergillus niger*, SSF, Activity, Specific activity

## INTRODUCTION

L-Glutaminase (L-Glutamine amidohydrolase EC 3.5.1.2.) is the enzyme deamidating L-Glutamine to L-Glutamic acid and ammonia. Glutaminase is ubiquitous in microorganisms and it plays a major role in the cellular metabolism of both prokaryotes and eukaryotes. In general, Glutaminases from *Escherichia coli*, *Pseudomonas* spp., *Rhizobium etli*, *Micrococcus luteus* K-3, *Bacillus* spp., *Clostridium welchii*, *Vibrio costicola*, *Zygosaccharomyces rouxii* and *Aspergillus oryzae* have been isolated and well studied.

L-Glutamine is used as obligate nitrogen donor for the biosynthesis of purine and pyrimidine nucleotides in a living cell. Tumor cells have no mechanism to synthesize L-Glutamine and hence take it as an exogenous source. L-Glutaminase causes selective death of glutamine-dependent tumor cells by depriving cells with L-Glutamine, and hence it is used as an effective agent in the treatment of acute lymphocytic leukemia and HIV. [1]

In food industry, L-Glutaminase is used as a flavour enhancer by increasing glutamic acid content in food through hydrolysis of L-Glutamine to L-Glutamic acid and ammonia. Since the sources for L-Glutaminase are limited, the search for potential microbial strains that produce the enzyme with novel properties for their industrial production is being pursued all over the world. [2] Glutaminase is also taking an important role that controls the delicious taste of fermented foods such as soy sauce and in general food products by increasing the glutamic acid content therefore, this enzyme has attracted a great attention in food industries. [3] Its commercial importance demands the search for new and better yielding microbial strains and economically viable bioprocesses for its large-scale production. [4]

Different methods of fermentation technology can be applied for the production of L-Glutaminase. Commercial production of L-Glutaminase had been carried out using submerged fermentation

(SMF) technique. [5,6] But now a days, solid state fermentation (SSF) has been emerged as a promising technology for the development of several bioprocesses and products including the production of therapeutic enzymes on a large-scale. [7] The primary advantage of SSF is the fact that many metabolites are produced at higher concentration.

*Aspergillus niger* is a species of great industrial importance being classified as Generally recognized as safe (GRAS) by the U.S. Food and Drug Administration, it is widely used in the industrial production of citric acid and a variety of industrial enzymes such as amylases, pectinases, and proteases. [8] Current study aims at the production and partial purification of L- Glutaminase enzyme from *Aspergillus niger* by solid state fermentation (SSF) using agricultural wastes namely sugar cane bagasse, vegetable peels and paddy straw.

## MATERIALS AND METHODS

### Isolation and screening for *Aspergillus niger*

*Aspergillus niger* species were isolated from different sources like rotten fruits, rotten vegetables, sugar cane waste and spoiled bread. The fungal cultures were maintained on PDB agar media, incubated at 30°C for 5 days, screened for the *Aspergillus niger* using Czapeck dox agar media supplemented with tributyrates and phenol red indicator. Morphological properties of the isolated strains were determined by Lacto phenol Cotton Blue staining (LPB). Positive cultures were then stored at 40°C until use.

### Fermentation medium for L - Glutaminase production:

The dried agricultural substrates namely paddy straw, Vegetable peels and sugar cane bagasse were separately pulverized in a blender at low speed and was taken in 3 different Erlenmeyer's flasks and moistened with 10 ml of salt solution containing glucose 0.6%,  $\text{KH}_2\text{PO}_4$  0.1%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.05% and KCl 0.05%. The contents in the flasks were rehydrated using

distilled water to get the required moisture level and autoclaved at 120°C for 20 minutes, cooled to room temperature and inoculated with 1ml of inoculums. Then the flasks were incubated at 30°C temperature for 120 hours of pH- 7. After incubation the substrates were analysed for L-Glutaminase production using Nessler's reagent.

### Enzyme Extraction and Purification:

Extraction procedure was followed as per the method of Mervat Morsy et.al. [9] The crude L-Glutaminase was extracted from the fermented solid substrate by using 25ml of 0.1 M phosphate buffer (pH - 8), centrifuged at 1500 rpm for 10 min in a cooling centrifuge. The clear supernatant was collected and subjected to 80% of Ammonium sulphate fractionation. The pellet was dialysed for desalting overnight in 0.05M Phosphate buffer pH - 8. Further the enzyme was purified by anion exchange chromatography on a Diethyl amino ethyl (DEAE)-cellulose column, pre-equilibrated with Phosphate buffer. The bound enzyme was eluted with a linear salt gradient of NaCl (0.1–0.25 M) and was used for enzyme assay.

### L - Glutaminase assay

Assay of Glutaminase was carried out by the method of Imada [10] using L- Glutamine as substrate. L-Glutamine (1 ml of 1%) in 0.01 M phosphate citrate buffer (pH 7.0) was made to react with 1 ml of dialysed Glutaminase enzyme for 60 min at 37°C. The enzymatic activity was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. The reaction mixture was centrifuged at 5,000 rpm for 5 min to remove the precipitated protein. Then to 0.1 ml of supernatant, 3.7 ml of distilled water and 0.2 ml Nessler's reagent was added to it. The reaction mixture was incubated for 15 minutes at room temperature and the absorbance of the orange red solution was measured at 480 nm using a spectrophotometer. One unit (U) of L- Glutaminase was defined as the amount of enzyme that liberates 1  $\mu\text{mol}$  of ammonia under optimal assay conditions. Protein concentration was analyzed as per the

method of Lowry *et.al.* [11] Bovine serum (1 mg/ml) was used as the standard.

## RESULTS

Screening for *Aspergillus niger*: Species identification was done using two methods- Morphological examination of the colonies on potato dextrose agar grown for 5 days at 27°C revealed the appearance of black surface, densely sporulated small, black, thin, smooth conidial heads. Further in Microscopic identification using Lactophenol cotton blue, properly teased

and observed under microscope revealed that the conidial heads are black and radiated, clearly differentiated thick mycelium with black speherical spores. The isolated fungal strain upon sub culturing with modified Czapeck dox agar media supplemented with tributurate and phenol red indicator shown the disappearance of pink colour indicating the growth of *Aspergillus niger* which was further confirmed with microscopic examination (Fig - 1 and 2).



Fig – 1, *Aspergillus niger* on czapeck Dox agar medium

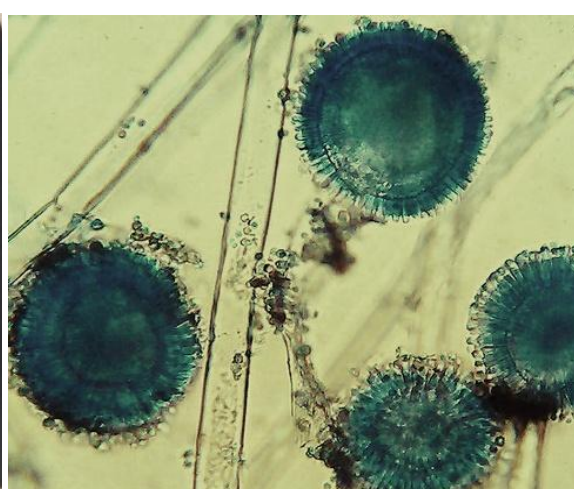


Fig – 2, Microscopic view of *Aspergillus niger*

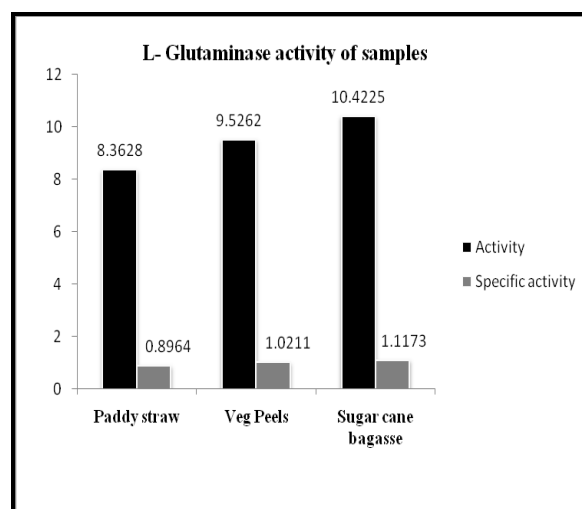
Table – 1, L - Glutaminase activity

Sample	Conc. of Ammonia released (mM)	L- Glutaminase Activity (mM/min/ml)	Specific activity U/mg Protein
Paddy Straw (A)	2.5088 ± 0.0321	8.3628 ± 0.1070	0.8964 ± 0.0114
Veg peels. (B)	2.8940 ± 0.0457	9.5262 ± 0.0436	1.0211 ± 0.0046
Sugar Cane bagasse. (C)	3.1301 ± 0.0794	10.4225 ± 0.2644	1.1173 ± 0.0283

Values are means ± SEM; n = 3

### Solid state fermentation (SSF) for L- Glutaminase production using *Aspergillus niger*

Solid state fermentation resulted in effective production of L - Glutaminase enzyme from *Aspergillus niger* using various agricultural wastes namely paddy straw, vegetable peels and sugar cane bagasse. After analysis results revealed that *Aspergillus* grown on sugar cane bagasse showed maximum enzyme activity and specific activity of 10.422 mM/min/ml and 1.117U/mg protein respectively. Vegetable peel and paddy straw sources showed slightly lesser Glutaminase activity of 9.526 mM/min/ml and 8.362 mM/min/ml respectively in comparison to sugar cane bagasse.



Graph – 1, L- Glutaminase activity and specific activity

## DISCUSSION

Fermentation technology has been widely used for the production of a wide variety of substances of industrial, medical and agricultural applications. Different agro-industrial by products have been used as solid substrates for the production of L-Glutaminase by *Trichoderma koningii*, *Vibrio azureus JK-79* and *A. fumigates*.<sup>[12-14]</sup> Extracellular enzymes are gaining importance in industrial application as they minimize the cost of production process. Glutaminase being an extracellular enzyme has an additional advantage of having easy purification steps.

In the current study *Aspergillus niger* species were isolated from different sources like rotten fruits, rotten vegetables as well as spoiled bread and were further cultured on Czapeck dox agar media supplemented with tributyrates and phenol red indicator. Morphological studies under microscope stained with Lacto phenol Cotton Blue staining (LPB) confirmed the isolate as *Aspergillus niger*. Solid state fermentation using various agricultural wastes resulted in effective production of L – Glutaminase enzyme.

Solid state fermentation uses culture substrates with low water levels (reduced water activity), which is particularly appropriate for mould. The methods used to grow filamentous fungi using solid state fermentation allow the best reproduction of their natural environment as the medium is saturated with water but little of it is free-flowing. The solid medium comprises both the substrate and the solid support on which the fermentation takes place.

## CONCLUSION

Here in the study among the three substrates used, sugar cane bagasse was shown to be an effective substrate for the Glutaminase production. Sugar cane bagasse which is a sugar-industry waste is easily available and economical. Hence this process can be exploited for the large scale production of L-Glutaminase, which has

wider application in the field of food and pharmaceutical industries.

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