



Isolation and Primary Screening of L-Glutaminase from Agro-residual Wastes

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ABSTRACT

L-Glutaminase a therapeutically and industrially important enzyme. L-Glutaminase majorly produced by microorganisms including bacteria, fungi and yeast. The activity of L-Glutaminase has been exploited for the treatment of tumors/leukemia, as flavor enhancer and also as enzyme sensors. The enzyme mainly catalyzes the hydrolysis of γ - amido bond of L-Glutamine. The screening of L-Glutaminase producing isolates carried out by using Minimal Glutamine Agar Medium. The L-Glutaminase producers detected by the pink zone around the colony by simple plate assay method. The bacterial strains were used for glutamic acid production under optimum growth conditions. The L-Glutaminase enzyme was assayed by Nesslerization measured at 480nm using a Spectrophotometer. In the present study Solid state fermentation was carried out for the production of extracellular Glutaminase the maximum enzyme activity was observed in Cattle feed (293.4 U/gds), Tea dust (240.5 U/gds), and Green gram husk (203.6 U/gds) which is a cheap raw material and it is available abundantly.

Keywords: L-Glutaminase, Solid State Fermentation, tumor cells, biochemical characterization.

INTRODUCTION

L-Glutaminase (L-Glutaminase amido hydrolase E.C. 3.5.1.1) is the enzyme deaminating L-Glutamine to L-Glutamic acid and ammonia.¹ Glutaminase is ubiquitous in various organisms and was widely distributed in plants, animal tissues and microorganisms including bacteria, yeast, and fungi.²

In recent years Glutaminase has attracted much attention due to its wide applications in pharmaceuticals as an anti-leukemic agent.^{3,4}

Another important application of L-Glutaminase is in biosensors for monitoring the glutamine levels in mammalian and hybridoma cells.

In 1993, *Pseudomonas species* recombinant Glutaminase was patented for its activity against cancer and HIV virus therapy.⁵

Interest on amidohydrolases started with the discovery of their anti-tumor properties and since then, a lot of efforts have gone into extensive studies on microbial L-glutaminases with the intention of developing them as antitumor agents.⁶⁻⁹

On an industrial scale, glutaminases are produced mainly by *Aspergillus* and *Trichoderma* sp.¹⁰⁻¹²

Commercial production of glutaminases has been carried out using Submerged fermentation (SMF) technique.

But nowadays, solid state fermentation (SSF) has been emerging as a promising technology for the development of several bioprocesses and products including the production of therapeutic enzymes on a large scale.

The present work is aimed at isolation, screening and identification of bacterial cultures for extracellular

Glutaminase production.

The efforts are also focused on various conditions implemented for the production of Glutaminase in submerged fermentation and solid state fermentation, physicochemical characteristics and applications of L-Glutaminase.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples are collected in and around the regions of Visakhapatnam district, Andhra Pradesh, India.

All the samples are collected in sterile polythene bags and carried to laboratory.

The samples were stored at 4 °C and used for further studies.

Isolation of L-Glutaminase Producing Bacteria

The soil samples are serially diluted by taking 1gm of soil in 10ml of distilled water.

The dilutions were done from 10^{-1} to 10^{-6} and were transferred to minimal glutamine agar medium [(g/l):

KCl-0.5, MgSO₄-0.5, KH₂PO₄-1.0, FeSO₄-0.1, ZnSO₄-0.1, NaCl-25, L-Glutamine-10, Phenol red-0.012, Agar-20, Distilled water-1000ml¹³ by spread plate method.

About 50 μ g/ml of Nystatin is used to retard the growth of fungal colonies.

All the plates were incubated at 37 °C for 48-72 hrs, respectively, for adequate colony development.

Distinct colonies were picked and purified by restreaking two times on nutrient agar before storage as slant cultures at 4 °C.



Fermentation of the Medium

Five grams of the tea dust, (*Camellia sinensis*) greengram husk (*Vigna radiate*) and cattle feed were used as substrates.

These substrates were dispensed in 250ml of Erlenmeyer flask and moistened with 2ml of distilled water.

The flasks were autoclaved at 121 °C (15 lb) for 20minutes, and cooled to room temperature and inoculated with 2ml of bacterial suspension under aseptic conditions. The contents of the flask were mixed thoroughly and incubated at 35 °C for 4-5days.

Crude Enzyme Extraction

The crude enzyme was extracted by using citrate phosphate buffer. The fermented solid substrate was mixed thoroughly with 50ml of the buffer at pH-7.0 and kept in a rotator shaker at 200rpm for 30minutes. The entire contents of the flask were squeezed through a cheese cloth; the pooled extract was centrifuged at 4 °C for 20minutes at 10,000 rpm. The supernatant was collected for further analysis of enzyme assay.¹⁵

Primary Screening of the Potent Organism

For this study, Minimal Glutamine Agar Medium was prepared. About 50µl of crude enzyme was added into the plates were incubated at 37°C for 24 hours. One uninoculated plate was kept as control.¹⁴

Bacterial strain with its crude L-glutaminase enzyme showed maximum zone of color change (from yellow to pink) on minimal glutamine agar medium was selected as potential strain for further studies. Crude L-glutaminase enzyme was produced in large quantities from potential bacterial strain by solid state fermentation method described earlier. (Fig. 2)

L-Glutaminase Assay

The reaction mixture contains 0.5ml of glutamine (0.04M), 0.5ml of Tris-Hcl buffer of 0.1M at pH-8.0, 0.5ml of distilled water and 0.5ml of enzyme solution was incubated at 37 °C for 30mins.¹⁶ The enzymatic activity was stopped by the addition of 0.5ml of 1.5MTrichloroacetic acid. Then to 3.7ml of distilled water, 0.1ml of the above mixture and 0.2ml of Nessler's reagent was added and color developed after 10-15mins was measured at 450nm in UV-Visible Spectrophotometer.

One unit of L-Glutaminase was defined as the amount of enzyme that liberates 1µ mol of ammonia under optimal assay conditions. Enzyme yield was expressed as the activity of L-Glutaminase per grams dry substrate (U/gds).

Estimation of Protein Concentration

Protein concentration was measured according to¹⁷ method using bovine serum albumin as standard.

The protein concentration was expressed as mg/ml of crude enzyme.

Morphological Characterization of BT 06

Bacterial Identification

The morphological characterization of each isolate was first performed, including color and size. Gram stain test was performed for each isolate.

Characterization of Potential Bacterial Strain

The phenotypic characteristics such as microscopic appearance Gram's staining, motility, cultural and biochemical characters of the potential strain was studied by adopting standard procedures recommended by Bergey's Manual of systematic Bacteriology.¹⁸

Biochemical Characterization of Selected Microorganisms

Biochemical tests like urease production, starch hydrolysis, carbohydrate fermentation (lactose and sucrose), and catalase were performed with isolated hydrocarbon consuming bacteria (Table 1).

Starch Hydrolysis Test

Inoculated a starch plate with the organism to be tested. Incubated at for at least 48 hours. Plates were swamped with iodine solution and observed results.

Blue colour indicates no hydrolysis, while a clear zone indicates hydrolysis. The plates were detected for starch hydrolysis as when iodine added, a colour change is blue but area which shows positive result.

Catalase Test

A few drops of bacterial broth culture were placed on cavity slide. Same amount of hydrogen peroxide were released on plate. The plate was observed for bubble formation.

Hydrogen Sulphide Test

Hydrogen sulphide Agar medium was prepared and sterilized at 121 °C for 15minutes at 15psi the medium was poured into the tubes and allowed for solidification. Then the organisms were inoculated and incubated at 27 °C for 2-5days.

Urease Test

This is the positive test for the presence of urease. The test culture were inoculated heavily over the entire slope culture and incubated at 37 °C for 24 hours and the reaction recorded after 4, 8, 12 and 48 hours of incubation.

A positive urease reaction is indicated by a change in the colour of the medium from yellow to purple colour.

Indole Production Assay

The indole is detected by calorimetric reaction by p-dimethyl amino benzaldehyde (Kovac's reagent). vPeptone broth was prepared and the test culture was inoculated in the test tubes and the tubes were incubated at 37 °C for 24 hours. After incubation 0.2ml of Kovac's



reagent was added for 5 minutes. A cherry red colour in the alcohol indicates a positive reaction.

RESULTS AND DISCUSSION

In the present study, soil samples was collected and the bacterial strains were identified using minimal glutamine agar medium seven isolates were isolated and the potential isolate BT 06 is selected exhibits highest pink colour due to the change in the pH of the medium causes the breakdown of amido-bond in L-Glutamine and liberates ammonia.

The solid substrates used in Solid State Fermentation are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factor.¹⁹

The improvement in L-Glutaminase activity was recorded when the medium is supplemented with different carbon and nitrogen sources.

The potent strain was isolated as BT 06 and exhibits the maximum yield of 23.16(U/G) (Table-2).

The solid substrates such as tea dust, (*Camellia sinensis*) green gram husk (*Vigna radiate*) and cattle feed were used.

The solid state fermentation was carried out by adding the BT 06 isolate and fermentation was done for 3-4 days.

The crude enzyme was extracted and enzyme was assayed at 450nm at UV-Spectrophotometer.

The protein concentration was measured and the morphological characteristics like Gram's staining and biochemical characteristics like catalase test, starch hydrolysis test and hydrogen sulphide test were done (Table-1).

The process can be exploited for large scale production of anti-leukemic agent of L-Glutaminase and also having beneficial property for its use in food industry.

The maximum enzyme yield was observed in tea dust (240.5 U/gds), Cattle feed (293.4 U/gds), and Green gram husk (203.6 U/gds) (Figure-3).

Table 1: The Biochemical Test Result Biochemical Test BT-01 to BT-07.

Test Name	BT-01	BT-02	BT-03	BT-04	BT-05	BT-06	BT-07
Methyl red	+	+	+	+	+	+	+
Vogues provasker	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
Starch hydrolysis	+	+	-	+	+	+	-
Catalase	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+
Simmon citrate agar test	+	+	+	+	+	+	+
Gelatin hydrolysis test	-	-	-	-	-	-	-
Triple sugar iron test	+	+	+	+	+	+	+
Hydrogen sulphide test	+	-	-	-	-	-	-
Casein test	+	+	+	+	+	+	+

Bacterial Cultures



Isolates of Bacteria



Pure Culture of BT 06



Pure Culture of BT 07

Figure 1: Bacterial Cultures

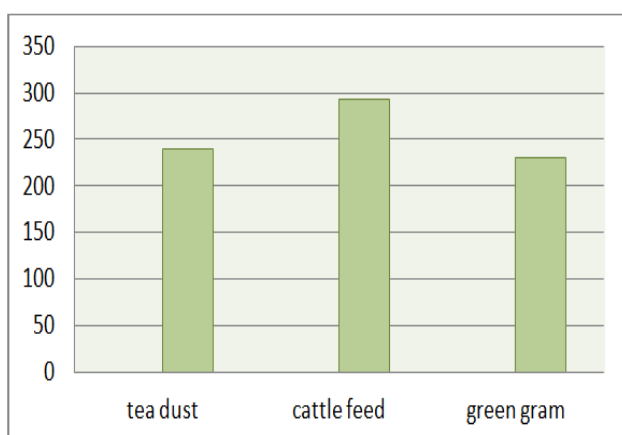


Figure 2: L-Glutaminase showing pink colour zone

Table 2: L-Glutaminase Activity Production by the Selected Isolates

Isolate Number	Yield(U/G)
BT 01	7.53
BT 02	9.02
BT 03	11.20
BT 04	11.47
BT 05	17.10
BT 06	23.16
BT 07	18.35

Glutaminase Production



Graph 1: Screening of different agro-residues for L-glutaminase production

CONCLUSION

The present study focuses on the selective isolation of the potent L-glutaminase producing soil bacteria.

The results of the preliminary study indicates that L-glutaminase producing marine bacterial strain BT-06 could be a source for extracellular L-glutaminase production with possible utilization as potential source for anti-leukemic agent and flavor enhancing agent in the food industries after the extensive therapeutic activity determination.

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