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PRODUCTION OF FUNGAL L-GLUTAMINASE THROUGH SOLID STATE FERMENTATION OF AGRICULTURAL BI-PRODUCTS

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ABSTRACT

L-Glutaminase (EC.3.5.1.2) is an amidohydrolase catalyzing the hydrolytical deamination of L-glutamine resulting in the production of L-glutamic acid and ammonia. Due to its variety of application potential in food industry, high value chemical production and therapeutic it is in much demand. The microorganisms, including bacteria, yeast, and filamentous fungi, have been found to produce L-glutaminase. Keeping in view the great demand of enzyme it is attempted to use cheep raw materials for low cost production of L-glutaminase. The mustard oil cake is used in the study has been found to be a promising raw material.

Keywords: L-Glutaminase (EC.3.5.1.2) is an amidohydrolase, amidohydrolase, L-glutamic acid

INTRODUCTION

A potent therapeutic agent and flavor-enhancing capacity of glutaminase in the fermented foods has received much attention for industrial application. The L-glutaminase can be used for the treatment of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). Apart from being used as flavor enhancer and nutraceuticals, it is also used as precursor for chemical production. Glutaminase is considered as a multifunctional enzyme because of its involvement in many metabolism i.e. energy metabolism, ammonia trafficking and regeneration of neurotransmitter glutamate

(1). Glutamine and glutamate are non-essential amino acid. Hydrolytic enzyme, L-glutaminase hydrolyses L-glutamine in L-glutamic acid and ammonia. So the enzyme occurs in all living organisms and play important role in cellular metabolism. In human there are two isoforms of glutaminase that is Kidney-type and Liver-type. The molecular weight of glutaminase, its subunit, optimum pH and substrate specificity of the isoenzyme differ with the living organisms. 40-50°C of temperature is considered optimum for this enzyme (2).

Multidisciplinary application has increased the demand of glutaminase. The available commercial glutaminase is still limiting the potential use of glutaminase. It is required to focus on high glutaminase producing microorganisms, cheap substrates, production and optimization methods, purification, immobilization or lyophilization. Since the limited sources for L-glutaminase for potential microbial strains has to be discovered which hyper produce the enzyme with novel properties for their industrial production (3).

Glutamine being as a non-essential amino acid, the enzyme L-glutaminase which utilizes it as substrate is widely distributed among plants, animals and microorganisms. It is basically a non-essential amino acid but in certain condition such as trauma, surgery and sepsis it is essential to body so considered as a 'conditionally essential' or 'semi-essential' amino acid (4). Glutaminase has been used as an anti-retroviral agent in treating AIDS as it lowers L-glutamine levels in both serume for prolonged periods which reduces the activity of HIV.

Glutamate gives 'umami' taste which is one of the basic tastes of foods. The glutaminase activity in fermented foods is desirable because it acts on L-glutamine to generate ammonium as a neutralizer of acidity and glutamate, as a flavor enhancer (5). Since the utility and application in pharmaceutical industry and in other sector such as food industry for flavor enhancer, in preparation of biosensor, for monitoring the glutamine level in mammalian and hybridoma cell cultures. In chemical industry, for the production of glutamic acid, threonine (gamma glutamyl transfer reactions) and an antioxidant glutathione, the demand gets increased.

A potent microorganism and low cost substrate can enhance the production with reduced production cost. Microbial production of glutaminase with different substrates such as wheat bran using *Serratia marcescens* (6), wheat gluten using *Lactobacillus rhamnosus* (7), Ragi straw

using *Aspergillus* (8), *Zygosaccharomyces* (9) species etc.

Microorganism such as *Aspergillus* and *trichoderma* species has been used for industrial production. In the present investigation alternative raw material and wild type potential microbial isolate have been used for production.

METHODS

Sample collection

The fungal isolates were collected from soil sample near rice bran oil industry in Bilaspur, Chhattisgarh. Cultures were maintained in Potato Dextrose Agar slant at 4°C.

Screening for L-glutaminase

Screening was performed with the modified Czapek Dox medium consisting of (g/l): L-glutamine, 10g; KH2PO4, 1.5g; KCL, 0.52; MgSO4.7H2O, 0.52g; agar; 20g; CuSO4.5H2O, trace; ZnSO4.7H2O trace; FeSO4; trace; phenol red at pH 7.0; 28°C and with minimal glutamine agar (g/l): Dextrose 0.5; KCl 0.5; MgSO4 0.5; KH2PO4 1.0; FeSO4; ZnSO4 0.1; NaCl 25; L-glutamine 10; phenol red 0.25 in which L-glutamine act as carbon and nitrogen source and phenol red act as pH indicator. Prepared plates were inoculated with the fungal isolates and incubated at 28°C for 48 hours, cultures showing utilization of glutamine and production of glutamic acid (with or without concomitant production of other organic acids), indicated as pink colour due to increased pH by liberated ammonia.

Inoculum preparation:

Inoculum was prepared in 250 ml Erlenmeyer flasks containing 50 ml of liquid media containing (g/l): L-glutamine, 10g; KH2PO4, 1.52g; KCl, 0.52; MgSO4.7H2O, 0.52g; and phenol red at pH 7.0 and incubated at 28°C for 24 hour.

Production conditions:

L-glutaminase production by fungal isolates was carries out in solid state fermentation using agro-forestes by-products such as mustard oil cake, dori oil cake and ground nut oil cake. The substrate were moistened with mineral salt solution contains glucose 0.6%; 0.1% KH2PO4; KCl 0.05%; MgSO4.7H2O, 0.05% and rehydrated using distilled water to get required moisture level. Flasks containing production medium were inoculated with 1% inoculum (v/v). Incubation was carried out at 28°C for 4-5 days. The enzyme activity was determined using the culture filtrate. Experiments were carried out in triplicate and the mean values with standard deviations were calculated.

Enzyme extraction and L-glutaminase activity of enzyme:

The glutaminase produced in the solid state fermentation were extracted by using 41 ml of 0.1 M phosphate buffer saline (pH 8.0). After mixing it on shaker for 30 min, mixture was centrifuged at 10,000 rpm. The supernatant was further used for enzyme assay (10).The reaction mixture of 0.5 ml of 0.04 M glutamine, 0.5 ml of 0.5 M Tris HCL buffer, 0.5 ml of an enzyme preparation and distilled water to a total volume of 2.0 ml. The mixture was incubated at 37°C for 30 minute. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. Control was by adding enzyme preparation after the addition of trichloroacetic acid. In 3.7 ml of distilled water, 0.1 ml of the above mixture at 15 to 20°C for 20 min, absorbance at 450 nm was measured. The liberated content was measured. One international unit of L-glutaminase is the amount of enzyme which liberates 1 µmol of ammonia under optimal condition.

Process optimization studies:

The optimization studies were carried out by observing the production at varying physiological parameters such fermentation temperature (25-45°C), pH (5.0-10), time (15-87h) and moisture content (50-100%v/v).

RESULT AND DISCUSSION

Isolation and screening

Soil sampling was from suitable niche for potential producers of glutaminase. In present study the fungal cultures were isolated from soil of rice bran oil industry from Bilaspur, Chhattisgarh. It was randomly chosen. In the screening media, pink zone containing colonies were picked and enzyme activities were measured. The production of ammonia gives basic condition to phenol red in media and brings color change to pink. After screening, green colony containing fungal isolate which was showing large zone and activity were selected for further study (Figure-1). Morphologically characterized by cotton blue staining which shows resemblance to Genus *Aspergillus* (Figure-2 (a) and (b)).

Optimization and fermentation parameter:

Three different types of oilcakes were used as substrates for the production of glutaminase i.e. mustered oil cake, Mahua (Madhuca indika) oil cake, groundnut oil cake. The highest glutaminase activity (463.33 U/ml) was obtained with the mustered oil cake fermentation. The ground nut fermentation showed 356.66 U/ml and dori oil cake fermentation has 200 U/ml glutaminase activities (Figure - 4) at optimum condition. The solid substrates rendered nutrient to the microbes and also

induce their growth. The higher growth of culture in mustered oil cake could be due to the high glutamine content and excellent particle size of mustered oil cake. Kashyap et al., 2002, have reported that by using Sesamum oil cake, at 64% moisture content, 30°C temperature and 48 hour incubation time the enzyme activity is 2.40 U/gds. Ye et al (11) has observed soyabean cake 10.6 U/gds at groundnut cake 7.8 U/gds and rapseed cake 5.4 U/gds. The maximum yield for coconut oil cake for L-glutaminase is 51.52 U/gds by *A. oryzae* at 70% initial moisture content, 30°C and pH 7.0 (Prasanna and Raju, 2012). No one has reported the enzyme activity of dori oil cake in literature. In this study it was fist time employed as substrate for glutaminase production.

The significance of temperature optimization study was to understand the effect of temperature on biological process. It affects in such a way that it could determine the effect of protein denaturation, enzyme inhibition, cell viability and death. The fermentation temperature study were carried out with 25 to 45°C and found that 30°C (Figure - 6) was most suitable condition for growth as well as enzyme production for isolated strain. Prasanna and Raju (8) have the same maximum enzyme activity at 30°C whereas Rashmi *et al*. (12) has found the maximum enzyme activity at 32 °C. In this study enzyme activity at 30°C temperature was found 473.33 U/ml, as the metabolic activity of microorganisms is highest at optimum temperature. Incubation higher than 40°C shows remarkable inactivation of L-glutaminase. In solid state fermentation, the temperature gets increased due to respiration of microorganisms. This is drawback at the time of scale-up.

The pH range from 5.0 to 10 showed that at pH 7.0 (Figure - 8), the production level is high and enzyme activity at this pH was 420 U/ml. In Prasanna and Raju (8) and Abdallah et al. (13) finding, the optimum pH is 7.0 which is same as this experimental observation. The obtained optimum pH is close to the finding of Rashmi et al (12) which is 6.5. The pH is associated with the balance of ionic strength of a fungal plasma membrane. The pH of medium strongly affects the growth and activity of microorganism and microbial enzymes are produced in higher yield at a pH near to the maximal of enzyme production. Fungal isolate are noted for their best performance in the range of 5-10. Generally the agro-industrial residue possesses excellent buffering capacity and that their use offers advantage for solid state fermentation.

Incubation time from 15h to 87h was observed and maximum production was seen in 87h (Figure-5). Rashmi et al (12) has got 72h for their experiment on *Aspergillus flavus* (FGNAS-7). The enzyme activity showed growth relatedness as the incubation period increases. The glutaminase activity also increases at 87h and it was 363.33 U/ml. After 87h the enzyme activity started getting constant value and then decreases as the fungal growth have reached the stage from where it could not balance the steady growth within the available nutrient resources or the enzyme might be inactivated by the presence of some kind of proteolytic activity or due to the denaturation of the enzyme protein.

In this study the moisture content was observed from 50 to 100 % (v/v) and 80% (Figure-7) was found optimum for production because the glutaminase activity was highest 366.66 U/ml at this moisture level. 70 % moisture content was optimum in the study conducted by Prasanna and Raju (8) and 60% was optimum in the study of *Aspergillus flavus* (FGNAS-7) by Rashmi et al (12). The initial moisture content of any substrate in solid state fermentation has utmost importance because it controls the growth, metabolite production and enzyme activity of microorganisms. It is reported that higher moisture level causes decrease in porosity, alteration in particle structure, enhancement of bacterial growth or low oxygen transfer and the due low moisture level the lower degree of swelling and reduced solubility of the nutrients of the solid substrates.

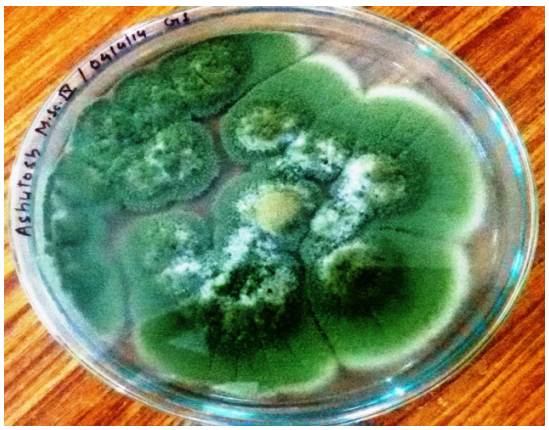

Figure 1: Green colony of fungus

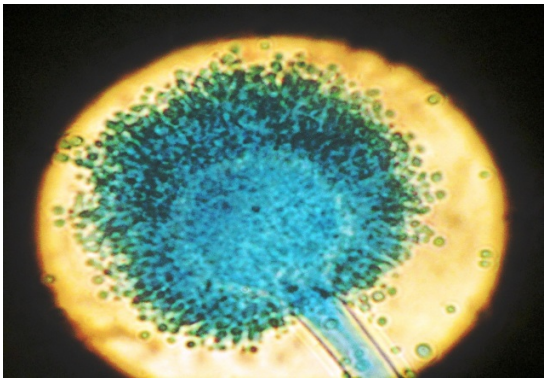

Figure 1 (a) Spore of fungus

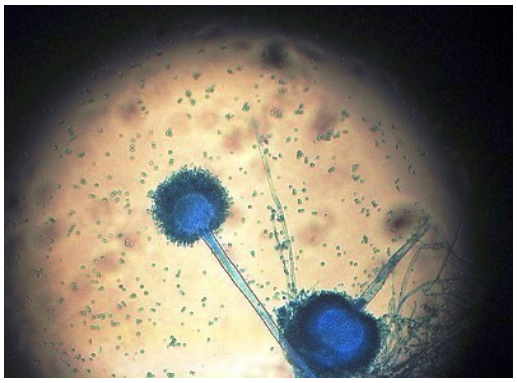

Figure 1 (b)

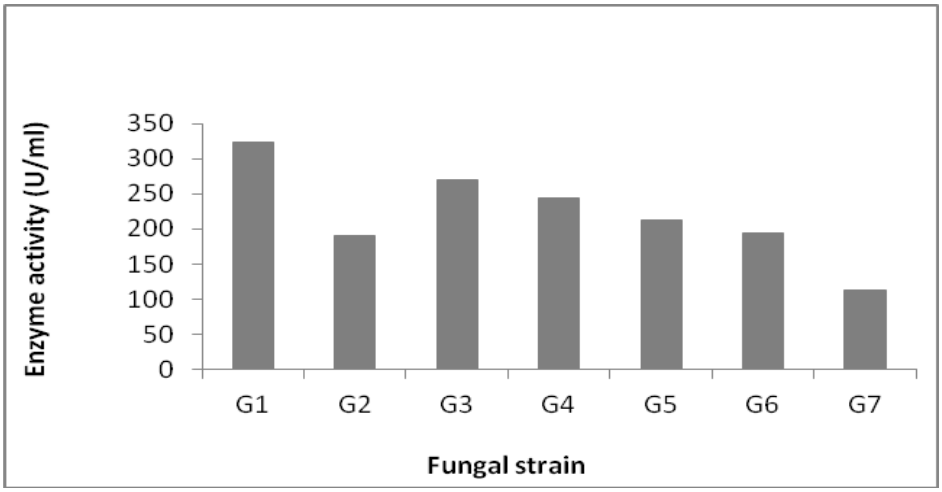

Figure 3: Enzyme activity of fungal isolates

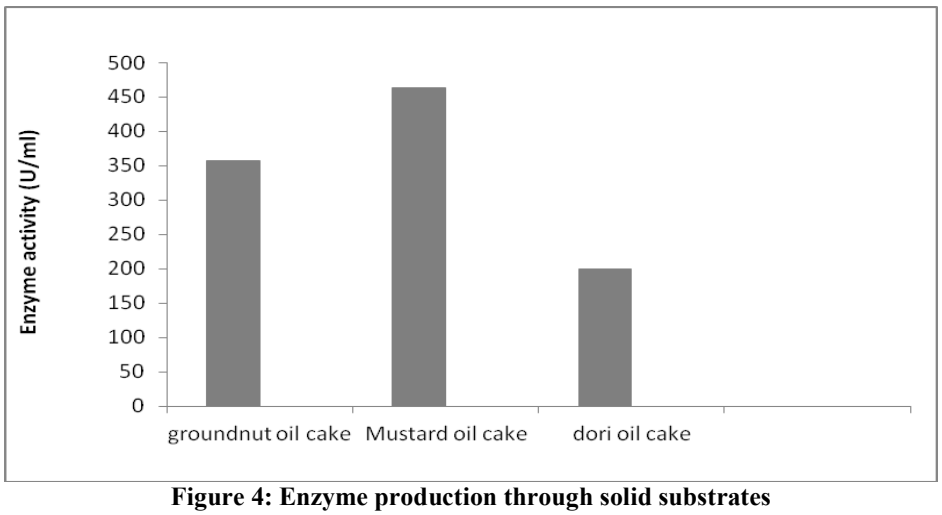

Figure 4: Enzyme production through solid substrates

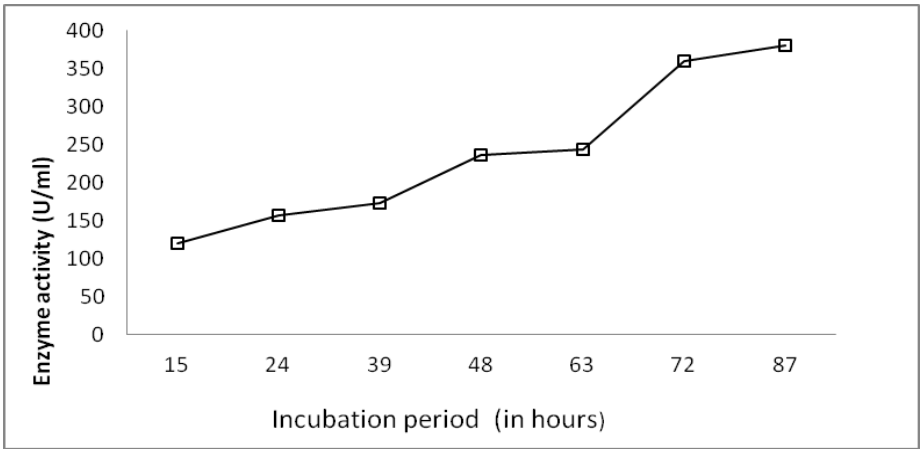

Figure 5: Effect of incubation time on Enzyme production

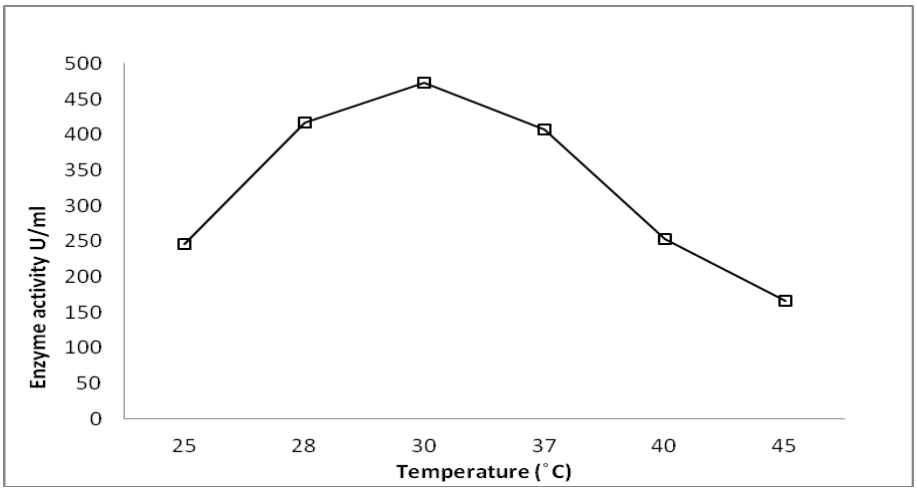

Figure 6: Effect of temperature on Enzyme production

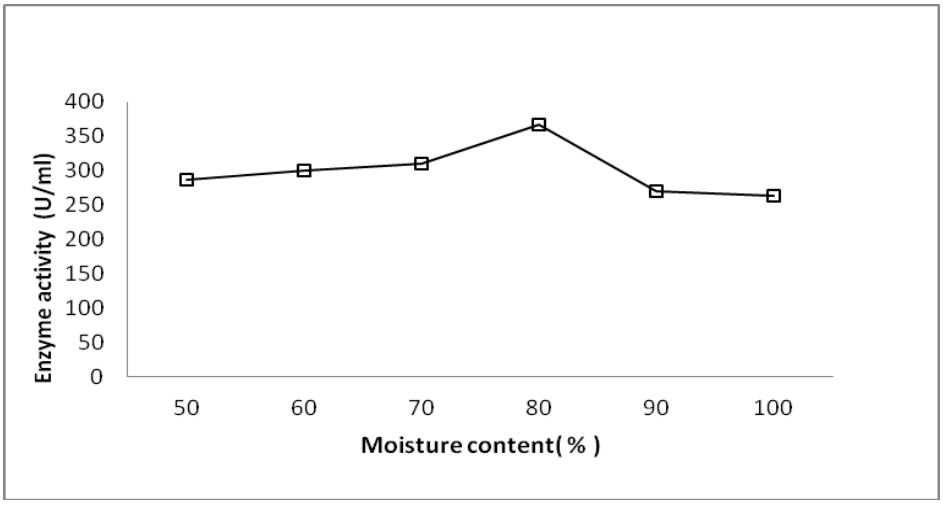

Figure 7: Effect of initial moisture content on Enzyme production

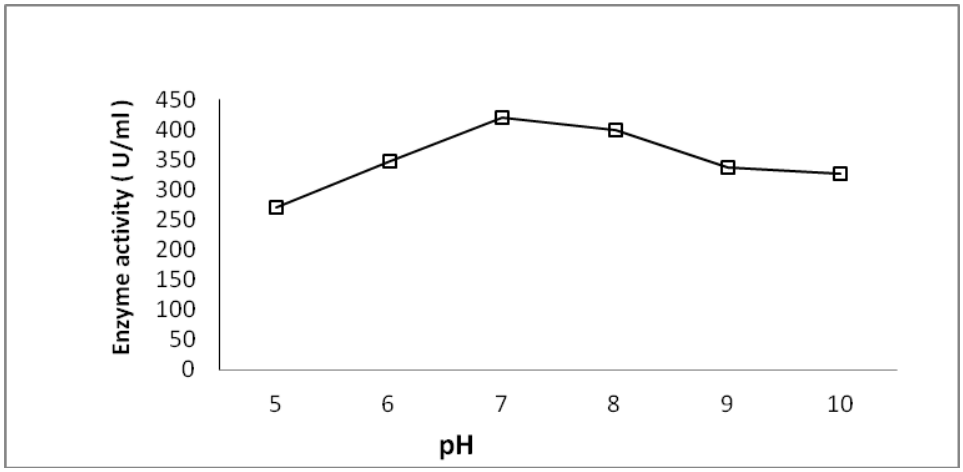

Figure 8: Effect of pH on on enzyme production

CONCLUSION:

The present study was mainly for the isolation of naturally existing glutaminase producing fungal strain and the production of glutaminase using agricultural by-products at laboratory scale to reduce the cost of production. It was a small step towards the goal of cost reduction of glutaminase in the market. The study included the assessment of the activity of the L-glutaminase enzyme on different substrates like mustard oil cake, ground nut oil cake, dori oil cake. The production has optimized by taking different parameter. The morphological identification concludes that the fungal isolate belongs to *Aspergillus* species.

The data summarized in this work that the isolated fungal strain and agriculture byproduct, mustered oil cake may be useful for low cost industrial production of glutaminase. It also illustrates the bioutillization of agricultural by products for commercial and medical application in food and pharmaceutical industry.

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