MUTATIONAL STUDIES ON L-GLUTAMINASE - A TUMOUR INHIBITOR FROM MICROBIAL ORIGIN

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ABSTRACT

Glutaminase is widely distributed in microorganisms including bacteria, fungi and yeast. The enzyme mainly catalyzes the hydrolysis of amino bond of l-glutamine. This activity of L-glutaminase has been exploited for the treatment of tumors. In addition to this it will catalyzes transfer reaction. A highly savory amino acid, L-glutamic acid and a taste-enhancing amino acid of infused green tea; theanine can be synthesized by employing hydrolytic or transfer reaction catalyzed by glutaminase. Therefore, glutaminase is one of the most important flavor-enhancing enzymes in food industries. *Aspergillus* wentii KGSD4 strain were used for L-glutaminase production. The potential strains were kept for mutational studies. UV radiation is used as mutagenic agent. The mutant obtained i.e *Aspergillus wentii* KGSD4 mu were used for screening of L-glutaminase production by plate assay and it showed 1.7 cm diameter of pink zone around the colony. Screened mutated strain was employed for the production of L-glutaminase through submerged fermentation and it showed 3.21 IU after 72 hr of fermentation. It showed better results compared with *Aspergillus wentii* KGSD4 parent strain.

Keywords: UV radiation, L-glutaminase, Plate assay, *Aspergillus wentii* KGSD4 mu and Antitumour agent.

INTRODUCTION

L-glutaminase (EC 3.5.1.2) is an amidase enzyme that has a significant contributory role in cellular nitrogen metabolism in all living cells[1–4]. The use of amidases deprives neoplasms of essential nutrients and causes selective death of glutamine dependent tumor cells by depriving them of glutamine[5, 6]. L-glutaminase has attracted much attention due its applications in several fields. The L-glutaminase widely used in pharmaceutical and food industries. L-glutaminase is very much significance in enzyme therapy of cancer[7], especially acute lymphocytic leukemia[8] and HIV[7]. However, one of the major uses of microbial glutaminase in food industry, where it is used as a flavor enhancing agent[7]. L-Glutaminase is generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauce[9].

Another important application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid[10].

Although almost all living cells produce L-glutaminase, microbial L-glutaminase has received the greater attention because of its apparent advantages in production at large scale in addition to its antitumor property. However, source-dependent biochemical properties variations, productivity yields, medium component requirements, etc., play a vital role in its economic production and application potential at pharma sector level. Hence, several scientific organizations are constantly screening for potential microbial strains and developing different fermentation strategies to achieve improved productivity[3]. It is well established that effective exploitation of fermentation and other growth parameters would offer an edge over conventional processes[11].

There were scanty reports on mutational studies. Therefore we made an attempt on comparative studies of parent *Aspergillus wentii* KGSD4 and mutant *Aspergillus wentii* KGSD4 mu (mu-mutant) strain for L-glutaminase synthesis by using a cost effective fermentation medium.

MATERIALS AND METHODS

Microorganism:

Aspergillus wentii strains were isolated as per the method of Seifert[12].Soil samples were collected from various places of Bangalore University Campus. The isolated *Aspergillus wentii* KGSD4 (Plate – 1) strain were tentatively identified in the laboratory as described by Rapper and Fennell[13] and were maintained on Potato dextrose agar (PDA) slants. For Further confirmation cultures were sent to Agarkar Research Institute, Pune.

Mutational studies:

The strain *Aspergillus wentii* KGSD4 (Plate-1) was subjected to UV irradiation to induce mutation for better yield of L-glutaminase. Spore suspensions of the *Aspergillus wentii* KGSD4 (Plate – 1) were irradiated using a 15W Phillips UV lamp at varying distances (5, 10, 15 and 20 cm) for 15 min. The irradiation was performed in a

dark room and the irradiated suspensions were protected from light until plating was done on Czapek Dox agar in order to minimize any photo-reactivation effects as described by Gardener et al.[14] and Banik[15]. Under the present study mutant strain was isolated and labeled as *Aspergillus wentii* KGSD4 mu (Plate–2) and used for screening and production of L-glutaminase.

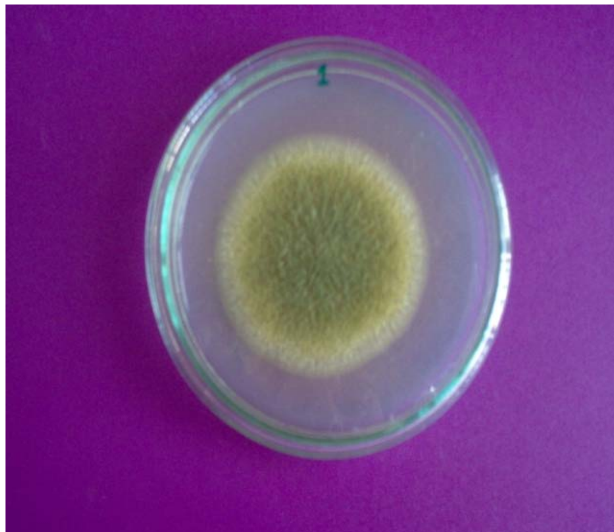

Plate 1: *Aspergillus wentii* KGSD4

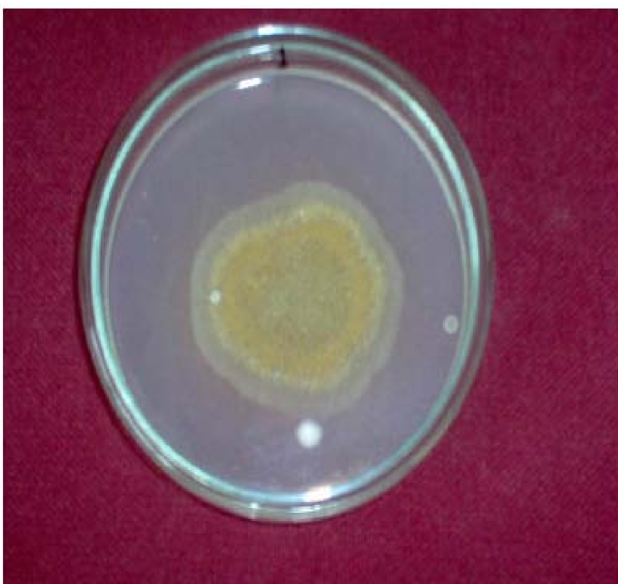

Plate 2: *Aspergillus wentii* KGSD4 mu

Screening of Mutant for L-glutaminase synthesis by plate assay

The mutant strains obtained from the above steps were subjected to rapid screening of L-glutaminase production by Plate assay (Plate – 3). This is a slight modified method as per Gulati et al.,[16].

The organisms were grown and kept on slants of solid modified Czapek Dox's medium containing (g L distilled water) glucose, 2; L-glutamine 10; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_2O$, 0.52; $CuNO_3.3H_2O$, trace; $ZnSO_4.7H_2O$, trace $FeSO_4$, trace; agar, 20.0 Modified Czapek Dox's medium was supplemented with different concentrations of the dye. A 2.5% stock of the dye was prepared in ethanol and the pH was adjusted to 7.0 using 1 mol L-1 NaOH. The stock solution of the dye ranging from 0.04 ml to 0.3ml was added to 100 ml of modified Czapek Dox's medium, giving final dye concentration of 0.2% with a final pH of 7.0. The media were autoclaved and plates prepared, control plates were modified Czapek Dox's medium (i) without dye and (ii) without glutamine. The plates were then inoculated with 96 hr cultures of *Aspergillus wentii* KGSD4 mu for rapid screening of glutaminase. The zone and colony diameters were measured after 48 hr for *Aspergillus wentii* KGSD4 mu.

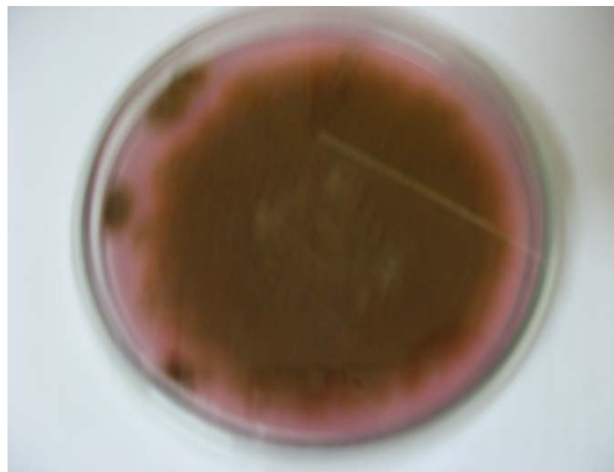

Plate 3: Plate Assay *Aspergillus wentii* KGSD4 mu

Comparative Fermentation studies on Parent and Mutant Strain of *Aspergillus wentii* KGSD4

Preparation of inoculum

Spore suspensions were prepared form 168 hr old cultures grown on PDA slants by adding 10 ml of sterile distilled water containing 0.01% Tween-80 and suspending the spores with a sterile loop. One ml of the spore suspension containing about 1 x 10^7 spores/ml was used to inoculate experimental media in the flasks[17].

Production medium for L-glutaminase

The production medium containing (g/L distilled water) glucose, 20; Malt extract, 10; Yeast extract, 4; K_2HPO_4, 2; $MgSO_4$, 0.1 and pH.6 for the synthesis of L-glutaminase.

Submerged fermentation methodology

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks. The flasks containing 100 ml of the above said fermentation medium were autoclaved at 121^{0}C for 20 min and cooled to room temperature. Then the flasks were inoculated with 1 ml of spore suspension and the contents were thoroughly mixed and incubated for a period of 3-5 days at 35^{0}C.

Extraction of L-glutaminase from fermentation medium

The samples were withdrawn periodically at 24 hr aseptically and filtered through Whatman filter paper No.1 and centrifuged at 5000 rpm for 10 min. Then the

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

Assay of L-glutaminase for crude extract

Assay of L-Glutaminase was carried out as per Imada et al.,[18]. 0.5 ml of 0.2 M glutamine was taken in a test tube, to which 1 ml of 0.2 M buffer (acetate buffer pH 5.4), and 0.5 ml of enzyme preparation was added and the reaction mixture was incubated for 15-20 min. After the incubation period the reaction was stopped by adding 1 ml of 10% TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture, to this 3.7 ml distilled water and 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The absorbance was measured at 450 nm against suitable blank. The enzyme activity was expressed in International units.

International Units (IU)

One IU of L-glutaminase is the amount of enzyme which liberates 1 μmol of ammonia per minute per ml [μ mole/ml/min].

RESULTS AND DISCUSSION

In this study, the selected twenty one isolates were used to screen for L-glutaminase production by plate assay. The *Aspergillus wentii* KLSD4 were selected as potential L-glutaminase producer. It exhibited 1.5 cm zone of diameter (pink) around the colony. This potential strain used for mutational studies, than mutant *Aspergillus wentii* KGSD4 mu were also kept for screening of L-glutaminase synthesis by plate assay (Plate-3) and it also showed comparatively higher zone of diameter to parent strain. *Aspergillus wentii* KLSD4 were confirmed at Agarkar Research Institute, Pune.

Now both the parent and mutants were used to produce L-glutaminase in fermentation medium. The mutants were obtained from the strains of *Aspergillus. Wentii* KGSD4 by UV irradiation, produced mutants were used for L-glutaminase production (Fig 1). The mutants strain *Aspergillus wentii* KGSD4 mu was showed production of L-glutaminase of 3.21 IU at 72 hr fermentation periods in submerged fermentation and it compared to the parent strain *Aspergillus wentii* KGSD4 which could yield only 2.731 IU of L-glutaminase at 72 hr fermentation period.

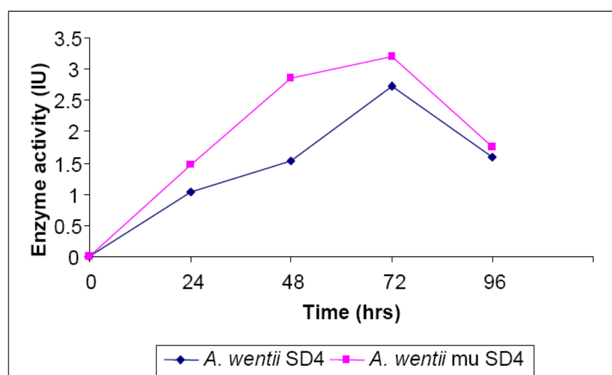

Figure 1: Effect of mutation on production of L-glutaminase by *Aspergillus wentii* KGSD4

Irradiations mutagens were used for mutagenic study when the mold is target organisms. Generally X-ray, gamma rays and UV rays were frequently used. Of these X-ray and gamma rays have high penetrating power cause, greater unclear damages as well as lethal effects in the organisms. Hence, the powerful mutagens causing damage to nuclear are generally avoided. On the other hand, UV rays with shorter wave length than the visible light are mild mutagens. Hence they are considered to be ideal for induction of mutations[19-21] (Elander, 1969; Hopwood, 1970; Thoma, 1971).

It is also apparent that induction of mutation helps in getting better strains from the point of product synthesis.

Sukumaran et al[22], showed highest L-asparaginase production in glycerol-peptone medium by two colorless mutants *Serratia marcescens* WF and 933 these organisms produced about 24 IU/10ml and 20 IU/ml of enzyme respectively.

Similarly, Singh and Sukumaran[23] reported that the L-asparaginase production of 9.8 IU/ml by mutant strain of *E. coli*. Under the present study the data revealed that the mutant strain *Aspergillus wentii* KGSD4 mu also showed increased production of L-glutaminase upto 3.21 IU at 72 hrs fermentation period when compared to wild strain *Aspergillus wentii* KGSD4 (6.05 IU) on carob pod substrates. Therefore, our results agree very close to the results of Singh and Sukumaran[23].

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