ejpmr, 2020,7(10), 455-458

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

AN APPROACH ON SCREENING AND PRODUCTION OF L-GLUTAMINASE FROM ASPERGILLUS SP THROUGH SUBMERGED FERMENTATION

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Article Received on 28/07/2020 **Article Revised on 18/08/2020** **Article Accepted on 08/09/2020**

ABSTRACT

Enzymes as drugs have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes are catalytic and convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders. Totally fifteen *Aspergillus sp* were isolated from different soil samples from different areas in Bangalore. The isolated fungal strains were screened for L-glutaminase production by plate assay. *Aspergillus sp* V 08 were selected as best protease producer (1.02cm). The *Aspergillus sp* V 08 were employed for further production of L-glutaminase studies. The different pH (5), temperature (35^0C) and inoculum size (1.0 ml) were optimized and it showed 96 IU of L-glutaminase activity.

KEYWORDS: L-glutaminase, submerged fermentation, thin layer chromatography and plate assay.

1. INTRODUCTION

Biologically, glutaminases (L-glutamine amidohydrolase, 3.5.1.2) play essential roles in the biosynthesis of various nitrogenous metabolites and in maintaining the glutamine/glutamate balance in organisms. They belong to the superfamily of serine-dependent b-lactamases and penicillin-binding proteins (Irajie et al., 2016). They are ubiquitous in nature and are found in bacteria, fungi, and also higher animals (Unissa et al., 2014). Most of the commercial glutaminases currently available in the market are produced for analytical purposes; however, there are a few that are being manufactured for their use as flavor enhancers in the food industry (Vikita and Shifa, 2017).

L-glutaminase (EC 3.5.1.2) is an amidohydrolase that catalyses the hydrolytic conversion of its substrate L-glutamine to L-glutamic acid and ammonia. It can be developed as a biopharmaceutical for cancer therapy as it facilitates high l-glutamine catabolism, consequently deprives cancerous cells of a vital metabolite; L-glutamine needed for energy and growth. This in fact, results in a selective starvation and apoptosis of L-glutamine addictive tumor cells because unlike normal cells they lack L-glutamine synthetase which replenishes L-glutamine to normal cell (Nakano et al., 1989).

L-Glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes. Glutaminase activity is widely distributed in plants, animal tissues and microorganisms including bacteria, fungi and yeast (Prema Kashyap et al., 2002). L-glutaminase is widely distributed in animal and plant tissues (Roberts, 1960).

The present research work highlights on isolation and screening of L-glutaminase from *Aspergillus* sp. The effect of fermentation parameters such as pH, temperature and inoculm size for the biosynthesis of L-glutaminase through submerged fermentation process.

2. MATERIALS AND METHODS

2.1. Collection and Isolation of *Aspergillus sp* from Soil Samples

L-glutaminase producing fungi i.e *Aspergillus sp* (Plate-1) were isolated from different soil samples. Four different soil samples were collected from different places in and around Bangalore city, such as soil from near coconut tree, near playing ground, near park area soil and cultivated soil were selected for isolating L-glutaminase producing fungi and Samples were stored at 4^0C.

2.2. Media

The fungi i.e *Aspergillus sp* were isolated from the soil sample on Czapek Dox agar (CZA) medium. CZA medium composition is as follows. Glucose, 30g; NaNO3, 2g; K2HPO4, 1g; MgSO4, 0.5g; KCl, 0.5g; FeSO4, 0.010g; Agar, 15g and pH 6.5+_0.2 (1L Distilled water). The media was sterilized by autoclaving at 121^0C of temperature, 15lbs of pressure for 15 minutes, and then CZA plates were prepared for the inoculation of the soil sample.

2.3. Screening of L-Glutaminase Producers By Plate Assay

The organisms were grown and kept on slants of solid modified Czapek-Dox's medium containing (g/L of distilled water) glucouse-2g; L-glutamine-10g; Potassium Hydrogen Phosphate (KH_2PO_4)-1.52g; Potassium Chloride (KCl)-0.52; Magnesium sulphate ($MgSO_4.7H_2O$)-0.52g; Copper nitrate ($CuNO_3.3H_2O$)-trace; Zinc sulphate ($ZnSO_4.7H_2O$)-trace; Iron sulphate ($FeSO_4.7H_2O$)-trace and agar-20g. Modified Czapek Dox's medium was supplemented with different concentrations of the dye (phenol red). A 2.5% stock of the dye was prepared in ethanol and the pH was adjusted to 7.0 using 1M NaOH. The stock solution of the dye ranging from 0.04ml to 0.3ml was added to 100ml 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 were prepared. Control plates of modified Czapek-Dox's medium were (i) without dye and (ii) without glutamine (instead containing $NaNO_3$ as nitrogen source) The plates and tubes (without agar) were inoculated with 96-hr cultures of *Aspergillus sp* for screening of L-glutaminase. Subsequently the pink colour was observed around the colony in the plate (Plate-2). (Siddalingeshwara et al., 2010).

2.4. Fermentation Kinetics For Biosynthesis of L-Glutaminase.

2.4.1. Fermentation Medium Composition

The production medium consists (1L of distilled water) 30.0g of Sucrose; 2.0g of Sodium nitrate-; 1.0g of K_2HPO4, 0.5g of $MgSO_4.7H2O$; 0.5g of KCl and 0.01g of $FeSO_4$ with pH 6.8 for 96 -120h.

2. 4.2. Effect of Initial pH on L-Glutaminase

250 mL Erlenmeyer flasks contained 100 mL of fermentation media were prepared and initial pH of the media were adjusted. The adjusted initial pH of fermentation media were ranging from 3-7 with increments of 1.0. Consequently prepared flasks were cotton plugged and sterilized by autoclave at 15 lbs, 121^0C for 15 min. The flasks were aseptically inoculated with freshly prepared spore suspension and incubated.

2.4.3. Effect of Temperature on L-Glutaminase

100mL of the fermentation media were collected separately in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25^0C, 30^0C, 35^0C and 40^0C.

2.4.4. Effect of Inoculum Size on L-Glutaminase

The inoculum was prepared by 168 h freshly prepared culture of *Aspergillus sp* V 08 at different levels i.e., 0.25, 0.50, 0.75, 1.0 and 1.25 mL and then inoculated and fermentation studies were carried out.

2.5. Extraction of L-Glutaminase From Fermentation Medium

The samples were withdrawn periodically at every 24 h in aseptic condition. The extract was filtered through Whatman filter No.1. The extract was clear and was centrifuged at 2000- 3000 rpm for 15 min. The prepared supernatant was crude enzyme extract. Thus prepared crude enzyme was administered for assay of L-glutaminase.

2.6. Assay of L-Glutaminase Crude Extract

Assay of enzyme was carried out as per Imada *et al.*, (1973). 0.5 ml of 0.04 M glutamine was taken in a test tube, to which 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler' s reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in international unit.

International Unit (IU)

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

3. RESULTS AND DISCUSSION

3.1. Isolation of *Aspergillus sp*

The isolation pattern of *Aspergillus sp* is presented in Table-2. In the present study, fifteen strains of *Aspergillus sp* were isolated and named serially from V 01 to V 15. All the isolates were isolated from different places in and around Bangalore, Karnataka. The four different kinds of soil samples were used for the isolation of *Aspergillus sp* (Plate-1). Soil samples collected near coconut tree, yielded maximum number of isolates 04, soil samples collected near playing ground yielded 03 isolates, 03 isolates were isolated from near park soil and 05 isolates from cultivated soil. Soil is the exceptionally rich sources for potential enzyme producing organisms especially for fungi. Therefore, in the present study soil has been chosen as a source and the soil samples were collected from various places.

3.2. Screening of L-Glutaminase Producers By Plate Assay

After the isolation of *Aspergillus* strains, they were subjected to screening for the production of L-glutaminase by rapid plate assay. It is a slight modified method as described by Siddalingeshwara *et al*., (2010). All the fifteen isolates were exhibited the zone of diameter (Plate–2) on rapid plate assay method. For the convenience, the groupings of strains of *Aspergillus sp* isolated from the soils have been done on the basis of zone of diameter they exhibited. Therefore, it is proposed that the strains exhibiting zone of diameter above 1.0 cm are refereed as good L-glutaminase producer, those strains with zone of diameter 0.6 cm to 0.9 cm and those having below 0.6 cm zone of diameter may be referred to as moderate and poor L-glutaminase producers. As per the groupings the strain *Aspergillus sp* V 08 exhibited higher zone of diameter i.e 1.02 cm and considered as potential strain for L-glutaminase production. As such, strain V02, V05, V06,V09 V13 and V 15 can be treated as moderate L-glutaminase producers and remaining (V01,V03, V04,V07 V10,V11, V12 andV14) isolates were treated as poor L-glutaminase producers.

Therefore, on the basis of results observed on the rapid plate assay method, it was considered that the strain *Aspergillus sp* V 08 as a potential L-glutaminase producing candidate for further studies. Siddalingeshwara *et al.*, (2010) were reported on screening of L-glutaminase enzyme by plate assay by using *Aspergillus wentii*.

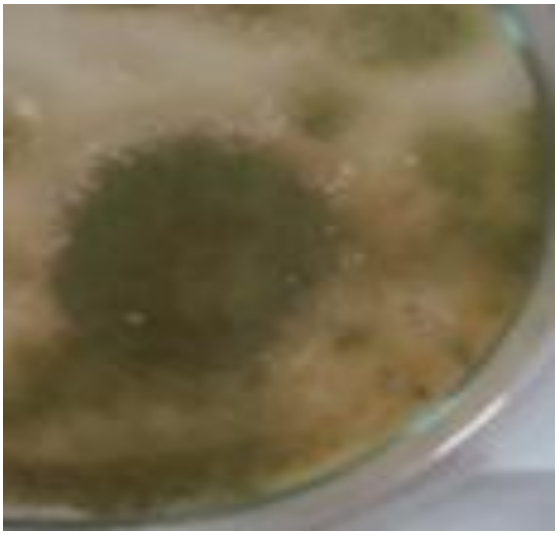

Plate: 1- *Aspergillus sp*

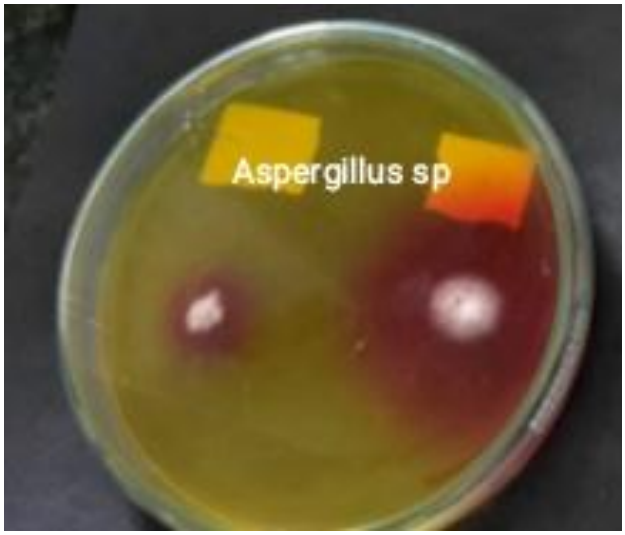

Plate-2: Plate assay

3.3. Optimization studies

3.3.1. Effect of pH on L-glutaminase production

The effects of different initial pH on L-glutaminase production by *Aspergillus sp* V 08 are presented in Table-1. The effect of initial pH on fermentation revealed that the yield of L-glutaminase increased with increase in initial pH of the fermentation medium up to 5.0 units, these increasing peaks were observed up to 72 hr of fermentation period and there after the yield decreased as pH levels and fermentation period increased. The maximum L-glutaminase activity 57 IU was obtained at pH 5.0 at 72 hr of fermentation period. The least L-glutaminase activity 23 IU was observed at pH 3.0 with *Aspergillus sp* V 08 strain.

Mahesh et al (2014) highlighted extra cellular L-glutaminase production was carried out by *Pseudomonas sp.* KLM9 strain and it showed pH 7.5 is optimum for maximum i.e 39.9 IU. Rajev kumar and Chandrasekaran (2003) reported that pH 6.0 was used for L-glutaminase production. The results obtained under the present study are coincides the findings of Rajev kumar and Chandrasekaran (2003).

Table-1: Effect of different pH on L-glutaminase production.

pH	L-glutaminase Enzyme activity (IU)
3	12
4	28
5	57
6	44
7	35
8	19

Table 2: Effect of temperature on L-glutaminase production.

Temperature in ^{0}C	L-glutaminase Enzyme activity (IU)
25	40
30	62
35	77
40	46

Table 3: Effect of Inoculum size on L-glutaminase production

Inoculum Size (in ml)	L-glutaminase Enzyme activity (IU)
0.25	**62**
0.50	**72**
0.75	**86**
1.00	**96**

3.3.2. Effect of temperature on L-glutaminase production

The results obtained in the present study on the effect of temperature in submerged fermentation of L-glutaminase production by *Aspergillus sp* V0 8 is represented in Table 2. It reveals that the L-glutaminase production was increased along with the increase of temperature of the medium from 25^0C, up to temperature 35^0C with optimized constant pH of 5.0. These increasing peaks were observed up to 72 h of fermentation period and thereafter the decreased yield as temperature levels and fermentation period increased. The maximum L-glutaminase activity 77 IU was obtained at temperature 35^0C for 72 h of fermentation period. The least L-glutaminase activity was obtained at temperature 25^0C with *Aspergillus sp* V0 8 strain and it showed 40 IU at 72 h of fermentation period.

Rajev kumar and Chandrasekaran (2003) reported 35^0C is the suitable for L-glutaminase production through submerged fermentation by using *Pseudomonas* sp BTMS-51 in packed bed reactor. Jambulingam Kiruthika and Nachimuthu Saraswathy (2013) were showed the effect of temperature on Production of L-glutaminase from a novel marine isolate *Vibrio azureus* JK-79.

3.3.3. Effect of inoculum size on L-glutaminase production

The importance of inoculum size on microbial fermentation process is widely accepted. The results obtained in the present study on the effect of inoculum size in submerged fermentation of L-glutaminase production by *Aspergillus sp* V 08 is represented in Table-3. Out of four inoculum size tested (0.25, 0.50, 0.75 and 1.0 mL), 1.0 mL inoculum was found to be the most suitable for high production of L-glutaminase by *Aspergillus sp* V 08 in submerged fermentation at 72 h of fermentation and it showed 96 IU. It is clear that the L-glutaminase production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude when enzyme productivity became maximum, thereafter no appreciable change in production of L-glutaminase with high inoculum size could be observed.

Sivakumar et al., (2006) reported 5mg/ml were inoculated for the maximum production of L-glutaminase. The 2.0 ml of the 48 hr old cell suspension of *Zygosaccharomyces rouxii* were inoculated under strict aseptic conditios in sold state fermentation (Prema Kashyap et al., 2002).

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