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SCREENING AND BIOSYNTHESIS OF L-ASPARAGINASE FROM *FUSARIUM SP* THROUGH SUBMERGED FERMENTATION

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ABSTRACT

L-asparaginase production using microbial systems has attracted considerable attention due to its cost-effective and eco-friendly nature. Although the enzyme has been found in a variety of bacteria, fungi and actinomycetes and mammals, few of the purified preparations have shown to possess anti-tumor or anti-leukemic activity. Ten *Fusarium sp* were isolated from soil samples from different regions in Bangalore. Isolated fungal strains were screened for l-asparaginase production by plate assay. *Fusarium sp* D 05 were selected as best L-asparaginase producer (1.03 cm) and were confirmation carried out by Thin layer chromatography (Rf value is 0.80). The *Fusarium sp* D 05 were employed for further fermentation kinetics studies. The different pH (6), temperature (35^0C) and inoculum size (1 ml) were optimized and it showed 119.7 IU of L-asparaginase activity. Further studies were carried out by Extraction and partial purification of L-asparaginase by salt precipitation (ammonium sulphate) by 80%. The partially purified enzyme were used for characterization of L-asparaginase and antitumour properties study.

KEYWORDS: *Fusarium sp*, L-asparaginase, Plate assay and submerged fermentation.

INTRODUCTION

Cancer is defined as uncontrolled division of cells. Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells displays *uncontrolled growth* (division beyond the normal limits), *invasion* (intrusion on and destruction of adjacent tissues), and sometimes *metastasis* (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize.

L-asparaginase (L-asparagine aminohydrolase, E.C. 3.5.1.1) belongs to an amidase group that hydrolyses the amide bond in L-asparagine to L-aspartic acid and ammonia. L-asparaginase is the first enzyme with anti-leukemic activity which has been thoroughly researched by many researchers throughout the world. It is an effective antineoplastic agent, used in the acute lymphoblastic leukemia (ALL) chemotherapy. Acute lymphoblastic leukemia (ALL) is a major pediatric cancer in developed countries. Although treatment outcome has improved owing to advances in chemotherapy (Pritsa A. A. and Kyriakidis D. A. (2001).

The characteristic, L-asparaginase enzyme has become a necessary drug in treating acute lymphoblastic leukaemia. For the synthesis production of L-asparaginase enzyme, bacteria, filamentous fungi and yeast have proved to be beneficial source. Microorganisms are the major sources for production of L-asparaginase as they can be cultivated easily, extraction, purification and processing steps from them is appropriate (Kavitha A. and Vijayalakshmi M. (2010).

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

MATERIALS AND METHODS

COLLECTION OF SOIL AND ISOLATION OF FUNGI

Soil sample was selected for the isolation of L-asparaginase producing fungi. Four different soil samples were collected from different regions from in and around Bangalore city, such as soil from Garden, near Playing ground, Near Bangalore university campus and cultivated soil were selected for isolating L-asparaginase producing fungi and Samples were stored at 4^0C.

MEDIA PREPARATION

The fungi 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.

SCREENING OF L-ASPARAGINASE 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-asparagine-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). The plates (without agar) were inoculated with 96-hr cultures of *Fusarium sp* for screening of L-asparaginase. Subsequently the pink colour was observed around the colony in the plate (Fig-1) (Jayaramu et al., 2010).

Figure-1: *Fusarium* sp.

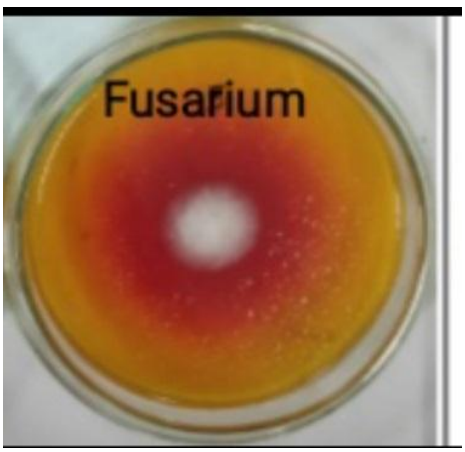

Figure-2: Plate assay by *Fusarium sp.*

FERMENTATION MEDIUM COMPOSITION

The selected *Fusarium sp* D 05 were cultured on production medium. The production medium consists (1L of distilled water) 30.0g of Sucrose; 2.0g of Sodium nitrate-; 1.0g of K_2HPO_4, 0.5g of $MgSO_4.7H_2O$; 0.5g of KCl and 0.01g of $FeSO_4$ with pH 6.8 for 96 -120h.The selected *Fusarium sp* D 05 were cultured on production medium.

OPTIMIZATION OF FERMENTATION KINETICS FOR BIOSYNTHESIS OF L- ASPARAGINASE

EFFECT OF INITIAL pH ON L-ASPARAGINASE

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.

EFFECT OF TEMPERATURE ON L-ASPARAGINASE

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.

EFFECT OF INOCULUM SIZE ON L-ASPARAGINASE

The inoculum was prepared by 168h freshly prepared culture of *Fusarium* sp D 05 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. As it was mentioned before, the media was extracted during every condition of pH or incubation temperature or inoculum size to prepare crude enzyme for each and was used to assay the L-asparaginase activity.

EXTRACTION OF L-ASPARAGINASE 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-asparaginase.

ASSAY OF L-ASPARAGINASE CRUDE EXTRACT

Assay of enzyme was carried out as per Imada *et al.*, (31). 0.5 ml of 0.04 M asparagine 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–asparaginase is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µmole/ml/min].

RESULTS AND DISCUSSION

In the present study, ten potent fungal strains were designated, out of ten isolates were isolated from soil samples collected at varying environmental stress conditions. They were isolated from in and around Bengaluru Karnataka, India. The eco-stressed soil was selected as a source for isolation of fungal L-asparaginase producers for current studies.

The results of plate assay for screening of L-asparaginase producers from the isolates were highlighted. Plate assay method was used to select potential strains by observing clear pink zone around the colony. The results from plate assay were presented in Figure 2.

The L-asparaginase producers i.e *Fusarium sp*. were segregated as poor L-asparaginase producers have less than 6mm of zone of diameter, which were strains D 04, D 06, D 07 and D 10 belong to medium L-asparaginase producers have 6 to 9 mm of zone of diameter, which were D 03, D 09, D 01 and D 02, were good L-asparaginase producers have above 9mm of zone of diameter which were D 04 and D 06. According to above segregation, *Fusarium sp* D 05 showed greater zone of diameter 1.04 cm and considered as potential strain for L-asparaginase production among the strains isolated from the soil. So the fungal strain D 05 was selected for the further studies (Plate 2). It showed 1.04 cm of cleared zone around the colony. Swathi Nageswara et al., (2014) were reported plate assay of L-asparaginase producers. Our results are good agreement with Swathi Nageswara *et al.*,(2014).

EFFECT OF INITIAL pH ON L-ASPARAGINASE BIOSYNTHESIS

The results obtained in the present study on the effect of initial pH in submerged fermentation of alkaline L-asparaginase production by *Fusarium* D 05 is represented in Table-3. It reveals that the L-asparaginase production rose with the increasing of pH of the medium from pH 3.0, up to pH 6.0 and then further increase in initial pH caused the declining of L-asparaginase yield. These increasing peaks were observed up to 96 hours of fermentation period and thereafter the decreased yield as fermentation period increased. The maximum L-asparaginase activity 27 IU was obtained at pH 6.0 for 72 hours of fermentation period. The least L-asparaginase activity was obtained at pH 3.0 with *Fusarium* D 05 strain and it showed 10.5 IU at 96 hours of fermentation period.

The effect of different initial pH of the fermentation media on the yield of L-asparaginase. The maximum L-asparaginase production was found at pH 6.0 with the activity of 27 IU, there was the constant increase was noticed in the pH range of 3-6 and thereafter enzyme production was shrunken. The results obtained in this particular are quite comparable with the reports on the production of L-asparaginase. Gulati *et al.*, (1997) have reported 6.2 was the optimum pH for L-asparaginase producing *A. terreus* strains. Similarly, Sarquis *et al.*, (2004) have reported highest L-asparaginase production of 58U/L. Baskar and Ranganathan (2009) highlighted the effect of pH ranges from 3-8 on L-asparaginase synthesis by using *Aspergillus terreus* and it showed that pH 6.0 was the optimum for the maximum production of L-asparginase and it showed 24.10 IU/ml. Our results were close agreements with Baskar and Ranganathan (2009).

EFFECT OF INCUBATION TEMPERATURE ON L-ASPARAGINASE BIOSYNTHESIS

The results obtained in the present study on the effect of temperature in submerged fermentation of L-asparaginase production by *Fusarium* sp D 05 is represented in Table 4. It reveals that the L-asparaginase production was increased along with the increase of temperature of the medium from 25^0C, up to temperature 30^0C with optimized constant pH of 6.0. These increasing peaks were observed up to 96 h of fermentation period and thereafter the decreased yield as temperature levels and fermentation period increased. The maximum L-asparaginase activity 23.8 IU was obtained at temperature 30^0C for 96 h of fermentation period. The least L-asparaginase activity was obtained at temperature 25^0C with *Fusarium* sp D 05 strain and it showed 13 IU at 96 h of fermentation period.

Sutthian Khamna *et al.*, (2009) have reported on *Amycolatopsis* CMUH002 was showed that the temperature 30^0C will be the optimum for high yield of L-asparaginase. Sarnya *et al.*, 2012 have reported that 35^0C temperature maximum L-asparaginase activity as 8.3 IU from *Aspergillus terreus* and 7.75 IU by *Aspergillus flavus*. Baskar and Renganathan (2009) highlighted the effect of temperature ranges from 25-45^0C on L-asparaginase synthesis by using *Aspergillus terreus* and it was reported that temperature 35^0C was the optimum for the maximum production of L-asparginase and it showed 24.10 IU/ml. Our results were coincides with Sutthian Khamna *et al.*, (2009).

EFFECT OF INOCULUM SIZE ON L-ASPARAGINASE BIOSYNTHESIS

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-asparaginase production by *Fusarium* sp D 05 is represented in Table-5. Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 and 1.25 mL), 1.0 mL inoculum was found to be the most suitable for high production of L-asparaginase by *Fusarium* sp D 05 in submerged fermentation at 72 h of fermentation and it showed 119.7 IU. It is clear that the L-asparaginase 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-asparaginase with high inoculum size could be observed.

The Inoculum size (spores/mL) is one of the important criteria in fermentation process. Optimization of this factor is mandatory in solid state fermentation. Because too elevated density of spores (high inoculum size) and too low density of spores (low inoculum size) bring adverse effects. Like, too high inoculum size may lead to quick and dense biomass production, then proceeding faster depletion of nutrients of media and to end with reduction of end product quality.

Jayaramu *et al.*, (2010) have reported that the inoculum size 1.0 ml showed optimum for the maximum 1.11 IU of L-asparaginase by using Czapek Dox's medium under submerged fermentation. Baskar and Renganathan (2009) were reported that the effect of inoculum size ranges from 0.5-2.5% on L-asparaginase synthesis by using *Aspergillus terreus* and it showed that 1.5% was the optimum for the maximum production of L-asparaginase and it showed 24.10 IU/ml. Amena *et al.*, (2010) also showed that inoculum size $1x10^{8}$ spores/ml and showed were the optimum for maximum production of L-asparaginase i.e. 9.8IU. Our results are good agreement with Baskar and Renganathan (2009).

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

pH	L-asparaginase Enzyme activity (IU)
3	10.5
4	11.8
5	13.6
6	27
7	14.4
8	14.3

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

Temperature in ^{0}C	L-asparaginase Enzyme activity (IU)
25	13
30	23.8
35	22.9
40	16.9
45	13.1

Table 3: Effect of Inoculum size on L-asparaginase production.

Inoculum Size (in ml)	L-asparaginase Enzyme activity (IU)
0.25	113.9
0.50	116.2
0.75	115.6
1.00	119.7
1.25	119.1

CONCLUSSION

L-asparaginase synthesis by *Fusarium sp* D 05 was evaluated under different fermentation parameters employing submerged fermentation. *Fusarium sp* D 05 is the potential strain among the fungal isolates and L-asparaginase synthesis increased after the optimization of fermentation parameters. The optimum conditions were pH 6; temperature 30°C and inoculum size 1.0 mL recorded 119.7 IU/mL. To conclude, this fermentation conditions may be used to increase the enzyme yield for biotechnological applications.

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