Research Article

PRODUCTION OF A TUMOUR INHIBITORY ENZYME, L-ASPARAGINASE THROUGH SOLID STATE FERMENTATION USING *FUSARIUM OXYSPORUM*

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ABSTRACT

L-asparaginase is a therapeutic enzyme which plays a vital role in treating different forms of cancer. The main objective of the present study is to optimize the production of L-asparaginase by *Fusarium oxysporum* using wheat bran under solid state fermentation (SSF). The maximum yield of L-asparaginase (8.14 IU) was achieved with the following optimized fermentation parameters: wheat bran (5g), fermentation time (120 hrs), initial moisture content (60% v/w), initial pH 7.0, temperature (30°C), supplemented with glucose (0.3% w/v) and malt extract (0.8% w/v). The results indicate that both the fungal strain and wheat bran can be utilized for the industrial production of L-asparaginase.

Keywords: L-asparaginase, *Fusarium oxysporum*, Process parameters, Solid state fermentation, Optimization.

INTRODUCTION

The enzyme, L-asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) is an amidase that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia[1]. It had received a great attention in recent years because of its tumour inhibitory property especially in treating acute lymphoblastic leukemia (ALL)[2]. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine[3]. Hence, they are not capable of producing L-asparagine, and mainly they depend on external L-asparagine from the circulating blood plasma[4].

Commercially, L-asparaginase is produced throughout the world by submerged fermentation (SmF). This methodology has many disadvantages such as the low concentration product formation and consequent handling, reduction and disposal of large volumes of water during the downstream processing etc. Therefore, the SmF methodology is a cost intensive, highly problematic and poorly understood unit operation[5]. An alternative solution to SmF is solid state fermentation (SSF) which is offering a wide range of advantages compared to SmF. SSF methodology is a very effective technique, as the yield of the product is many times higher when compared to that in SmF[6], and it also offers many other advantages[7].

Production of L-asparaginase using different microbial systems has attracted much attention, owing to the cost-effective and eco-friendly nature. A wide range of microorganisms including fungi, yeasts and bacteria have proved to be the beneficial sources of this enzyme[8,9,10]. Among the various microbial groups used in SSF, filamentous fungi are the most widely exploited because of their ability to grow on complex solid substrates and to produce a wide range of extra-cellular enzymes[11]. Bacterial systems are now being increasingly investigated for the production of enzymes and metabolites by SSF[12].

The main aim of the present study is to evaluate the suitability and utility of wheat bran as the substrate for the optimization of vital fermentation parameters like fermentation time, moisture content, initial pH, incubation temperature etc. for the production of L-asparaginase employing the fungal strain *Fusarium oxysporum*.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Fusarium oxysporum NCIM 1008 procured from NCIM, Pune was used in the present study. The culture was maintained on Potato Dextrose Agar (PDA) medium, incubated at 28 °C for 7 days, stored at 4°C and sub-cultured monthly.

Conidial suspension was prepared from a freshly raised seven day old culture of *F.oxysporum* on potato-dextrose agar slants by suspending in 10 ml of 0.85% sterile saline solution.

Fermentation medium

Wheat bran, obtained from the market yard of Mangalagiri, Guntur district of Andhra Pradesh was used as the substrate for the production of the enzyme, L-asparaginase by *Fusarium oxysporum*. Five grams of wheat bran substrate having the particle size of 0.354µm was taken in 250 ml Erlenmeyer flasks and moistened with 10ml of salt solution containing glucose 0.6%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.05% and KCl 0.05%. The thoroughly mixed flasks were autoclaved at 121°C (15 lb) for 20 min, cooled to room temperature and inoculated with 2 ml of the fungal conidial suspension. The contents

were mixed thoroughly and the flasks were placed in an incubator at 30°C for desired time period.

Optimization of fermentation parameters under SSF

The different physico-chemical parameters to maximize the yield of L-asparaginase by *F.oxysporum* under solid state fermentation (SSF) were investigated. The influence of fermentation time (24-144 hrs), initial moisture content (30-90 % v/w), initial pH (4-9), incubation temperature (24-36°C), and inoculum volume (0.5-3ml) were evaluated first. Later, the effect of glucose concentration (0.1-0.5% w/v) and malt extract concentration (0.2-1% w/v) were also studied. All the experiments were conducted in triplicate and the mean values of enzyme activity are considered.

L-asparaginase extraction from fermented substrate

The samples were withdrawn periodically at 24hrs in aseptic condition. One gram of moldy substrate was taken into a beaker and distilled water (1:10) was added to it. The contents of flask were allowed to have contact with water for about one hour with occasional stirring with the glass rod. The extract was filtered through Whatman filter No.1 paper and then was centrifuged. The obtained supernatant was used as enzyme preparation for quantitative analysis.

Quantitative assay for the estimation of L-asparaginase activity

L-asparaginase activity was determined using L-asparagine as substrate and the product, ammonia, released during catalysis was measured using Nessler's reagent according to the method of Imada *et al*[13]. The enzyme activity was expressed in terms of International unit (IU).

International Unit (IU)

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

RESULTS AND DISCUSSION

In SSF, the selection of a suitable substrate for the fermentation process is a critical factor. In the present study, wheat bran has been selected as the solid substrate due to its excellent particle size properties and nutritional composition required for the fungal growth and enzyme formation by the culture. The optimization studies of the fermentation parameters were carried out using wheat bran as the substrate for the production of L-asparaginase throughout the entire study.

Effect of incubation time

SSF was carried out for a period of 144 hrs. The fermented flasks were taken at regular intervals of every 24 hrs and the enzyme extraction was done as described earlier. The enzyme production showed growth relatedness as the fermentation time progressed and the maximum enzyme production (3.47 IU) was observed after 120 hrs (i.e. 5days) (Fig.1).

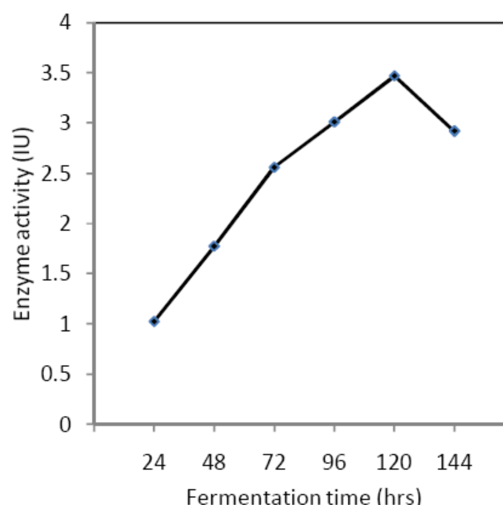

Figure 1: Effect of fermentation time on the production of L-asparaginase

After five days of fermentation time, the enzyme production started to decrease as the growth of the organism might have reached a stage, from which it could no longer balance its steady growth with the available nutrient resources[14].

Effect of initial moisture content

Different initial moisture levels ranging from 30-90% v/w were established in the wheat bran system and the fermentation was carried out for 5 days. Results obtained were shown in Fig.2.

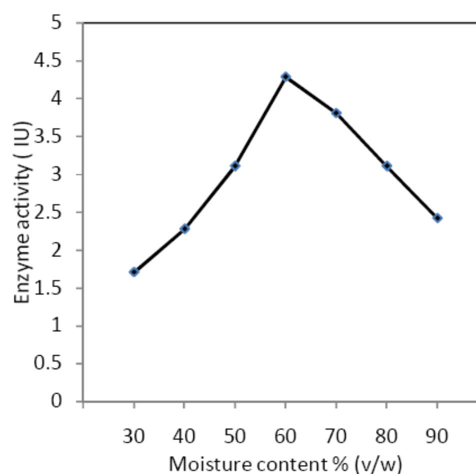

Figure 2: Effect of moisture content on the production of L-asparaginase.

The maximum L-asparaginase yield (4.29 IU) was achieved with the moisture level of 60% (v/w). The reduction in L-asparaginase production in low moisture levels might be due to the reduction in solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension. Likewise, the higher moisture levels decreased porosity, stickiness, and reduced air volume and diffusion that reduced oxygen transfer[15]. Moisture optimization

can be used to regulate and to modify the metabolic activity of the microorganism.

Effect of inoculum volume

To evaluate the effect of inoculum volume on the production of L-asparaginase, different inoculum volumes (0.5-3 ml of seven day old culture) were added to different flasks. Fermentation was carried out for seven days and the results were shown in Fig. 3.

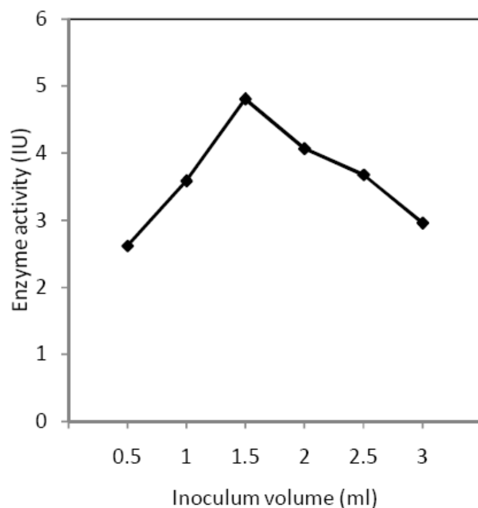

Figure 3: Effect of Inoculum volume on the production of L-asparaginase.

The maximal L-asparaginase production (4.81 IU) was achieved when an inoculum volume of 1.5 ml of 7 day old *F.oxysporum* culture was added. A low inoculum volume may give insufficient biomass causing low product formation, whereas a higher inoculum volume may produce too much biomass and can deplete the substrate nutrients or accumulation of some non-volatile self inhibiting substances inhibiting the product formation[16].

Effect of initial pH

For these experiments, the fermentative medium pH was adjusted accordingly with 1N HCl/NaOH from 4-9. Fermentation was carried out for 5 days. Maximum production of L-asparaginase (5.36 IU) was found with a pH of 7.0 as shown in Fig. 4.

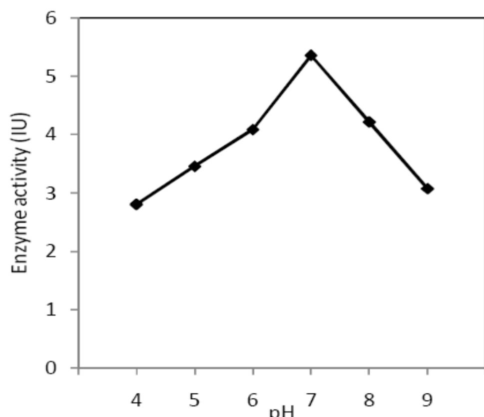

Figure 4: Effect of pH on the production of L-asparaginase.

This may be due to the balance of ionic strength of the fungal plasma membrane[17]. Generally, agro-residues possess excellent buffering capacity and that their use offers advantage for SSF processes. Microbial enzymes are produced in higher yield at a pH near to the maximal for enzyme production. Fungal strains are noted for their best performance in the range of 3.5-7.0 and also low pH avoids the contamination by other microbes especially bacteria.

Effect of incubation temperature

Fermentation was carried out at different temperatures ranging from 24-36°C. Samples were extracted after 5 days of fermentation. The organism exhibited a better growth as well as enzyme production at 30°C and it was 6.07 IU (Fig. 5).

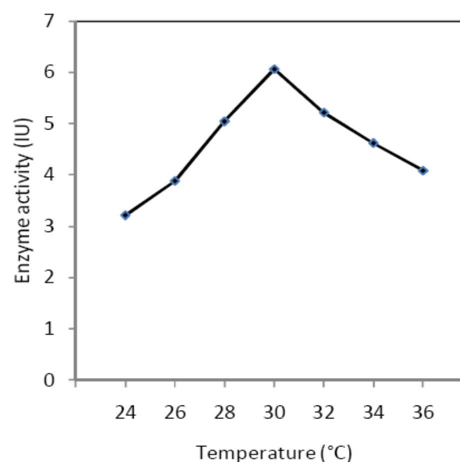

Figure 5: Effect of incubation temperature on the production of L-asparaginase.

The significance of incubation temperature in the development of a fermentation process is such that it could determine the effects of enzyme inhibition, cell viability and death. In SSF, there is a general increase in the temperature of the fermenting medium due to respiration. Heat built-up is in fact a drawback in solid state fermentation process. However, problems of heat and mass transfers are generally severe during the scale-up of SSF. In the present study, we have not encountered such difficulty.

Effect of carbon source

Incorporation of additional carbon sources such as glucose enhanced the enzyme yield from 6.07 IU to 6.92 IU (Fig. 6). The enhanced production of L-asparaginase by the incorporation of glucose may be attributed to the positive influence of additional carbon sources on enhanced biosynthesis. Optimal glucose concentration was studied by varying the glucose concentration (0.1-0.5% w/v) in the medium and the maximum production of L-asparaginase was observed at the concentration of 0.3% (w/v).

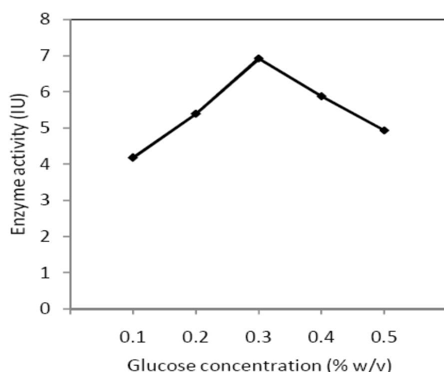

Figure 6: Effect of glucose concentration on the production of L-asparaginase.

Effect of nitrogen source

As we know that nitrogen source is the limiting factor in any fermentation process. So, the addition of nitrogen sources like malt extract was found to be the best source for maximal L-asparaginase production (8.14 IU) as shown in Fig.7. The effect of malt extract concentration was studied by varying its concentration from 0.2-1% w/v in the medium and the maximum L-asparaginase production was observed at a concentration of 0.8% (w/v).

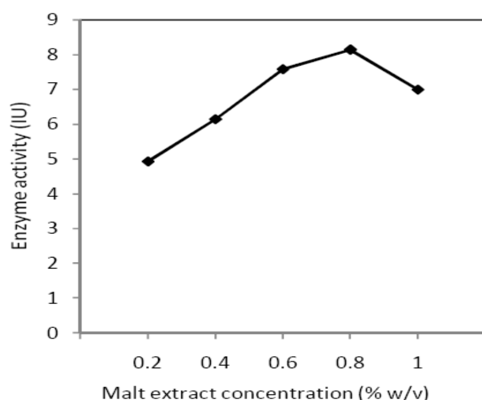

Figure 7: Effect of malt extract concentration on the production of L-asparaginase.

CONCLUSION

In the present study, the maximum value of L-asparaginase activity obtained after the optimization of all the fermentation parameters was 8.14 IU, which clearly demonstrated the scope for exploring filamentous fungi for the production of therapeutic enzymes like L-asparaginase using SSF. Moreover, the usage of cheaper substrates in SSF had made it an economically viable bioprocess for the production of microbial metabolites of biotechnological importance on a large-scale.

REFERENCES

1. Hill J, Roberts J, Loeb E, Kahn A and Hill R, L-asparaginase therapy for leukemia and other malignant neoplasm, Journal of the American Medical Association, 202, 1967, 882.
2. Verma N, Kumar K, Kaur G and Anand S, L-asparaginase: a promising chemotherapeutic agent, Critical Reviews in Biotechnology,27, 2007, 45-67.
3. Mannan S, Sinha A, Sadhukhan R and Chakrabarty SL, Purification, characterization & antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405, Current Microbiology, 30,1995; 291-298.
4. Swain AI, Jaskolski M, Housset D, Mohana Rao JK and Wlodawer A, Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy, Proceedings of National of Academic Sciences, 90, 1993, 1474-1478.
5. Datar R, Economic of primary separation steps in relation to fermentation &genetics engineering. Process Biochemistry,21,1986,19-26.
6. Arima K, Microbial enzyme production in Global Impact of Applied Microbiology, John Willey, New York, USA, 1964; 279-299.
7. Lonsane BK, Ghildyal NP, Budiatman S and Ramakrishnan SV, Engineering aspects of solid-state fermentation. Enzyme and Microbial Technology, 7, 1985, 228-256.
8. Elzainy TA and Ali TH, Detection of antitumor glutaminase-asparaginase in the filamentous fungi, Journal of Applied Science, 6, 2006, 1389-1395.
9. Ferrara MA, Severino NMB, Mansure JJ, Martin AS, Oliveira EMM, Siani AC, Torres FAG and Bon EPS, Asparaginase production by a recombinant *Pichia pastoris* strain harbouring *Saccharomyces cerevisiae ASP3* Gene, Enzyme and Microbial Technology, 39, 2006, 1457-1463.
10. Borkotaky B and Bezbaruah RL, Production and properties of asparaginase from a new *Erwinia* sp. Folia Microbiology, 47, 2002, 473-476.
11. Lee H, Song M and Hwang S, Optimizing bioconversion of deproteinated cheese whey to mycelia of Canoderma lucidum, Process Biochemistry, 38, 2003, 1685-1693.
12. Chandrasekharan M, Harnessing marine microorganisms through SSF, Journal of Scientific and Industrial Research, 55, 1996, 468-471.
13. Imada A, Igarasi S, Nakahama K and Isono M, L-asparaginase and glutaminase activities of microorganisms. Journal of General Microbiology, 76, 1973, 85-99.
14. Kashyap P, Sabu A, Pandey A, Szakacs G and Soccol CR, Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation, Process Biochemistry, 38, 2002, 38, 307-312.
15. Zadrazil F and Punia AK, Studies on the effect of particle size on solid state fermentation of sugar cane bagasse into animal feed using white rot fungi. Bioresource Technology, 54, 1995, 85-87.
16. Bilgrami KS and Verma RN, Physiology of fungi, 2nd ed, Vikas Publishing, Pvt. Ltd, 1981; 313-315.
17. Van Sumere CF, Van Sumer-de Peter C, Vining LC and Ledingham GA, Coumarins and phenolic acids in the uredospores of wheat stem rust. Canadian Journal of Microbiology, 3, 1957, 847-862.

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