OPTIMIZATION OF L-ASPARAGINASE PRODUCTION BY ASPERGILLUS TERREUS MTCC 1782 USING BAJRA SEED FLOUR UNDER SOLID STATE FERMENTATION

V.Varalakshmi[1*], K. Jaya Raju[2]

[1,2]*Center for Biotechnology, Department of Chemical Engineering, A.U. College of Engineering, Andhra University, Visakhapatnam- 530003, Andhra Pradesh, India,* ***Varalakshmivummidi@gmail.com***

*******Corresponding Author*

Abstract

Enzymes are the biocatalysts synthesized by living cells. They are Complex protein molecules that bring about chemical reactions concerned with life. They are protein in nature, colloidal and thermolabile in character, and specific in their action. L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extra cellular enzyme that has received considerable attention since it is used as an anticancer agent. The present work deals with production of extracellular L-asparaginase from Aspergillus terreus MTCC 1782 using Bajra seed flour under solid state fermentation Process parameters like Incubation time(96 h), Temperature (30^0 C), Moisture content (70% v/w), pH of the medium(8.0), Inoculum Age (5 days), Inoculum volume (1 ml), carbon source (1.5% w/v glucose), nitrogen source (2% w/v ammonium sulphate), and metal salts (0.1% w/v Magnesium sulphate) were optimized and giving an overall yield of 273.3 U/gds of maximum L-asparaginase activity after optimization. The observation made in this study hold great promise for scale up production of L-asparaginase from Aspergillus terreus MTCC 1782 using Bajra seed flour as substrate under solid state fermentation.

Index terms: *L-asparaginase, Aspergillus terreus, Bajra seed flour, Solid state fermentation, Optimization*

----------***----------

1. INTRODUCTION

Many enzymes have been used as drugs like wise L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) attracted much attention because of its anticarcinogenic potential. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma. [1]. L-asparaginase belongs to an amidase group that hydrolyses the amide bond in L-asparagine to aspartic acid and ammonia. L-asparaginase is very essential amino acid for the growth of tumor cells whereas the growth of normal cell is independent of its requirement [2]. It can be produced within the cell by an enzyme called Asparagine synthetase. Most of the normal tissue synthesizes L-asparagine in amounts for their metabolic needs but the tumour cells (especially Malignant and Carcinoma Cell) require external source of L-asparaginase for their growth and multiplication [3]. In the presence of L-Asparaginase, the tumor cells deprived of an important growth factor and they may failure to survive. Thus this enzyme can be used as a chemotherapeutic agent.

L-asparaginase is relatively wide spread enzyme found in many tissues, bacteria, plant and in the serum of certain rodents, not of man. The microbial sources are very common for L-asparaginase, because they can be easily cultured and extraction, purification of L-asparaginase from them is also convenient, facilitating for the Industrial scale production. The most commonly used microorganism to produce L-asparaginase are Erwinia caratovora, Bacillus sp. Corynebacterium glutamicum, Pseudomonas stutzeri and E. coli.[4] L-asparaginase from E .coli has excellent power to inhibit the activity of tumour cells, and that from E. chrysanthemi is also pharmacologically active[5]. However, L-Asparaginase from bacterial sources causes hypersensitivity in the long term leading to allergic reactions and anaphylaxis [6]. The search for other L-Asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi have a potential for L-Asparaginase production .Yeast sources such as Rhodatorula sp., Rhodosporidium toruloides, actinomycetes such as Nocardio sp Streptomyces longsporusflavus and fungal sources such as Aspergillus tamari and Aspergillus terreus, Aspergillus nidulans, Aspergillus niger have been found to produce L-Asparaginase.

Solid state fermentation (SSF) is a very effective technique as the yield of the product is many times higher than in submerged fermentation [7].SSF offers many advantages over submerged fermentation such as lower energy requirements, less risk of bacterial contamination, less waste water generation and less environmental concerns regarding the disposal of solid waste [8]. Other advantages include ease of product extraction that does not require complicated methods of treating the fermented residues. In comparison with SmF, SSF offers better opportunity for the biosynthesis of low-volume-high cost products [9]. The present research work was carried out for the production of L-asparaginase by Aspergillus terreus MTCC 1782 under solid state fermentation using bajra seed flour as substrate and optimized the production conditions.

2. MATERIALS AND METHODS

2.1 Microorganism and Inoculum Preparation

The fungal strain Aspergillus terreus MTCC 1782 was procured from Institute of Microbial Technology, Chandigargh, India. It was maintained on Potato Dextrose Agar (PDA) slants. The microbial strain was grown at 30°C for 4 days after which, it was stored at 4°C until further use and sub-cultured after every four weeks. For preparing a spore suspension, to a well-sporulated slant of A.terreus, 10 ml of sterilized 0.1% Tween 80 solution was added. The surfaces were scrapped with an inoculating loop to suspend the spores and the spore suspension was taken as inoculum.

2.2 Solid-State Fermentation:

Fermentations were initiated from spores in 250mL Erlenmeyer flasks that contained 5 g of bajra seed flour. The media was moistened to 40% (v/w) with distilled water, autoclaved at 1210C for 15 min at 15 lb pressure and cooled to room temperature and then inoculated with 1 ml of 96 h old A.terreus spore suspension under aseptic conditions. The contents of the inoculated flasks were mixed thoroughly and incubated at 300C temperature in an incubator for 96 h of period. All experiments were carried out in duplicate [10].

2.3 Crude Enzyme Extraction

After the incubation period, the crude enzyme from the fermented substrate was extracted using 0.1M phosphate buffer (pH 8). After mixing the fermented substrate with 41 ml of buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was centrifuged at 10,000 rpm for about 10 min at 4°C in a cooling centrifuge. Supernatant was collected and used for enzyme assay.

2.4 Assay of L-Asparaginase

The activity of L-asparaginase was determined by estimating the amount of ammonia liberated from L-asparagine. The method of Imada et al., 1973 was followed. The enzymatic reaction mixture contains 0.5ml of L-asparagine (0.04M , 0.5ml of Tris-HCl buffer 0.1M (pH 8.0), 0.5ml of enzyme solution and distilled water to a total volume of 2.0ml was incubated at 37°C for 30 min. The reaction was stopped by adding 0.5ml of 1.5 M Trichloro-acetic acid (TCA). Then to 3.7ml distilled water, 0.1 ml of the above mixture and 0.2ml of Nessler's reagent was added and colour developed was read after 10-15 min at 450nm in a UV-Visible spectrophotometer. The ammonium concentration of the reaction was determined by the reference from the standard curve of ammonium sulphate.

One unit (U) of L-asparaginase was defined as the amount of enzyme that liberates 1μmole of ammonia under optimal assay conditions. Enzyme yield was expressed as the activity of L-asparaginase per gram dry substrate (U/gds).

3. RESULTS AND DISCUSSIONS

3.1 Screening of Substrates

In SSF, the selection of a suitable substrate for a fermentation process is a critical factor as they play a dual role of supply of nutrients to the microbial culture growth and anchorage for the growing cells. In the present study, seven substrates, viz. bajra flour, ragi seed flour, cassava, sugarcane baggase, groundnut shell powder, tamarind seeds, and corn cob were screened with Aspergillus terreus and the results were shown in fig.1

All the substrates promoted enzyme production with A.terreus. The maximum L-asparaginase activity of 160.38 U/gds was achieved in a medium containing bajra seed flour as the substrate followed by ragi seed flour and lowest activity of 55.66 U/gds was observed in case of corn cob.

Abha Mishra et al., 2006 reported production of L-asparaginase from Aspergillus Niger using agricultural substrates like bran of Cajanus Cajan, Phaseolus mungo and Glycine max.

Hymavathi et al., 2009 reported asparaginase production by isolated Bacillus circulans MTCC 8752 under solid state fermentation using different agricultural materials like red gram husk, Bengal gram husk, coconut, and groundnut cake.

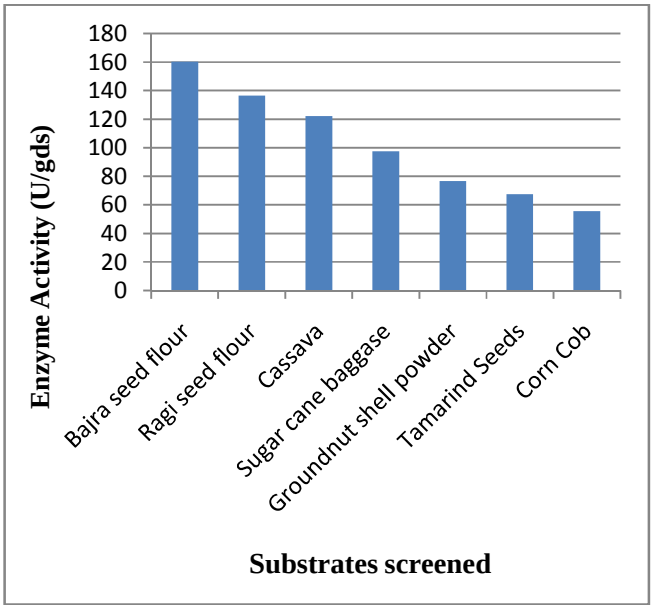

Fig-1: Screening of different substrates for L-asparaginase production by A.terreus

3.2 Optimization of Fermentation Process:

Fermentation parameters that influence the L-Asparaginase production during SSF were optimized over a wide range. The strategy adopted for standardization of fermentation parameters to evaluate the effect of an individual parameter and incorporate it at standard level before standardizing the next parameter. The Process parameters optimized were incubation time, incubation temperature, initial moisture content of the substrate, initial pH adjusted with 1N HCl or 1N NaOH, inoculum age, inoculum Volume. And also the effect of additional supplements like carbon sources, nitrogen sources and metal salts were also studied. All the experiments were conducted in duplicate and the mean values were reported.

3.2.1 Effect of Fermentation Time:

Optimum fermentation time for asparaginase production was determined by conducting experiments with the bajra seed flour as substrate using different time intervals from 24 h to 216 h with a variation of 24 h. From fig.2 it can be concluded that there were variations in enzyme level produced in culture filtrates with period of incubation. Analysis of culture supernatant showed enzyme activity rise from an initial of 49.2 U/gds at 24th h giving its peak activity of 169.46 U/gds at 96 h of fermentation. Fermentation beyond 96h showed a decrease in enzyme production, which could be either due to the inactivation of the enzyme because of the presence of some kind of proteolytic activity or the growth of the organism might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient resources. At longer incubation periods, the enzyme activity decreased which might be due to depletion of nutrients, accumulation of toxic end products.

Abha Mishra et al., 2006 reported 96 h as optimized incubation period for the production of L-asparaginase by Aspergillus niger.

Soniyamby et al., 2011 reported 96 h as optimum incubation period in case of Penicillium sp.

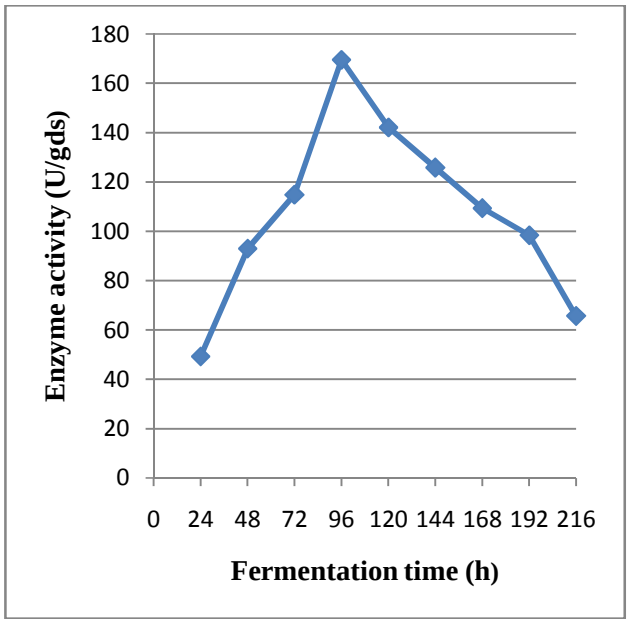

Fig-2: Effect of Incubation time on L-asparaginase production

3.2.2 Effect of Temperature

Fermentation was carried out at different temperatures such as 20, 25, 30, 35, 40, 45, 50, 55, 600C to study their effect on enzyme production. Incubation temperature has a profound effect on L-asparaginase production by A.terreus under solid cultural conditions. The maximum enzyme yield of 174.9 U/gds was obtained when SSF was carried out at 30°C.

The significance of the incubation temperature in the development of fermentation process is such that it could determine the effects of inhibition, cell viability and death. However, the enzyme production reduced gradually with further increase in incubation temperature. This may be due to heat that accumulates in the medium during mesophilic aerobic SSF, because of poor heat dissipation which could lead to a further drop in the oxygen level and there by reducing the growth of the test organism.

Sarqius et al.,(2004) have reported 30°C is suitable for L-asparaginase production through submerged fermentation by using A. terreus and A. tamarii.

K.G. Siddalingeshwara (2010) reported optimized temperature as 30OC by Emericella nidulans.

Yogendra singh et al., (2012) observed the maximum activity at 30ºC by Bacillus aryabhattai strain ITBHU02.

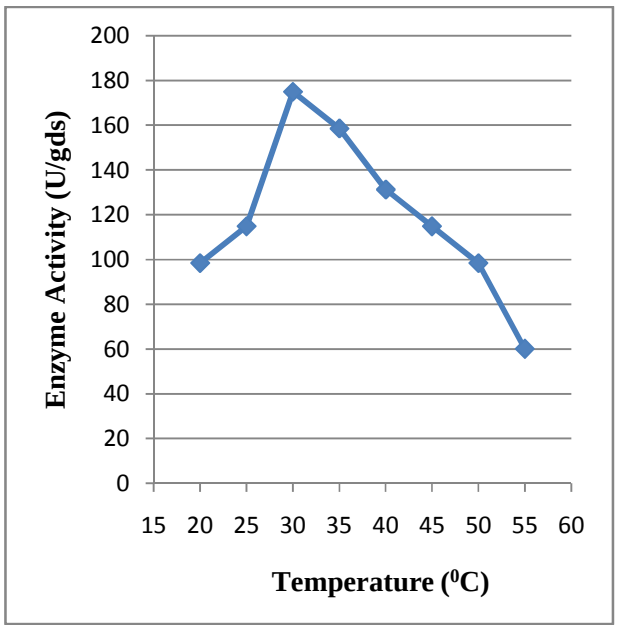

Fig-3: Effect of temperature on L-asparaginase production

3.2.3 Effect of Initial Moisture Content

Moisture content of fermentation medium is the most critical factor in SSF is determined for L-Asparaginase production by maintaining the medium with moisture content range of 10 to 100 %(v/w) with a variation of 10%(v/w) .The highest enzyme production of 185.8 U/gds was achieved at 70% initial moisture content. A further increase in the initial moisture content beyond 70% resulted in a significant reduction in the enzyme production. Moisture optimization can be used to regulate and to modify the metabolic activity of the microorganism. High moisture level of substrate leads to decreased porosity, lower oxygen diffusion, increased risk of bacterial contaminations, enhanced aerial mycelial formation, reduction in gas volume, decreased gaseous exchange and change in the degradation of the lignin. Likewise low moisture levels might lead to reduction in solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension. Therefore, initial moisture content plays an important role in enzyme production during SSF.

Abha Mishra (2006) reported 70% moisture content to be optimum by Aspergillus Niger.

Kaliwal B.B et al(2011) reported 70% moisture content to be optimum by Fusarium equiseti.

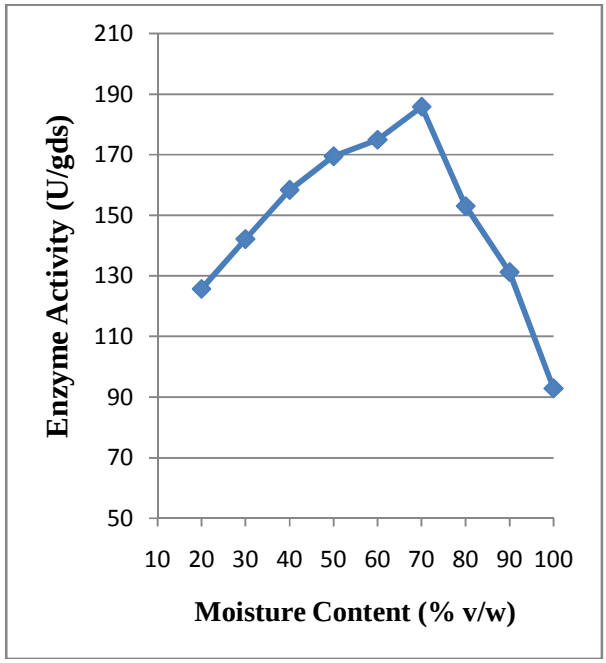

Fig-4: Effect of initial moisture content on L-asparaginase production

3.2.4 Effect of Initial pH:

Growth and metabolism along with enzyme production is governed by an important factor called pH. Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity.

Experiments were carried out to find the optimum pH in order to maintain the favourable conditions for increased L-asparaginase production. This was established by carrying out the fermentation by varying the pH from 2-10 (adjusted with 1N HCl or 1N NaOH. The maximum L-asparaginase production of 191.3 (U/gds) was obtained at pH-8.0. This may be attributed to the balance of ionic strength of plasma membrane.

G.Thirumurugan et al., 2011 reported an optimum asparaginase production at pH 8.0 by Aspergillus Terreus.

Selvakumar 2011 observed peak activity of asparaginase at pH 8.0 by Streptomyces noursei MTCC 10469.

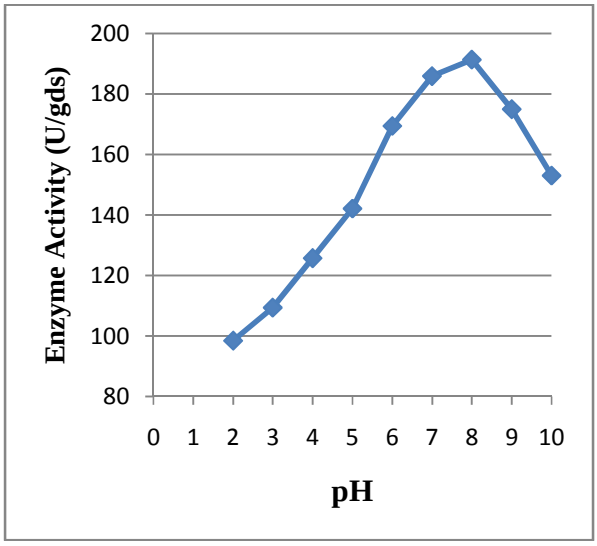

Fig-5: Effect of initial pH on L-asparaginase production.

3.2.5 Effect of Inoculum Age

The effect of inoculum age on L-asparaginase production was studied by conducting the fermentation with different inoculum ages. The substrate was inoculated with 1-day old culture to 10-day old day culture in different flasks. The substrate was incubated at 30°C for 4 days. After the completion of fermentation, the enzyme was extracted and analyzed for the L-asparaginase activity. The five day old culture gave maximum production of asparaginase 202.2 U/gds. The biosynthetic activity declines as the age of the culture increases.

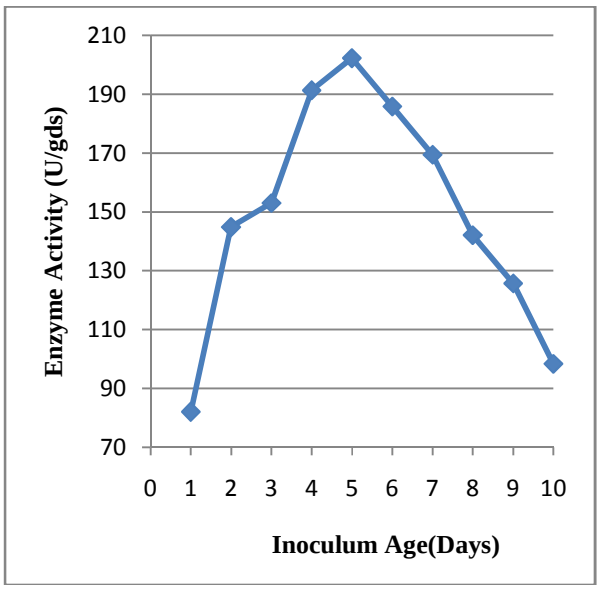

Fig-6: Effect of inoculum age on L-asparaginase production

3.2.6 Effect of Inoculum Volume

Inoculum volume is also an important factor that influences the production of metabolites under SSF. Adequate inoculum can initiate fast mycelium growth and product formation, there by reducing other organism contamination. Quantity of inoculum had a definite effect on enzyme titers. Optimization of inoculum volume is necessary in SSF because low density of spores leads to insufficient biomass and end product synthesis as well as permit the growth of undesirable contamination and too high densities of spores may cause a quick and too much biomass production thereby leading to fast nutrient depletion and ultimately reduction in the end product quality. In general lower level of inoculum may not be sufficient to initial growth whereas a higher level may cause competitive inhibition.

The effect of inoculum level on L-Asparaginase production was studied by conduction of the fermentation with different inoculum levels. The substrate was inoculated with culture of 1-10ml of inoculum level in different flasks. The substrate was incubated at 30°C for 5days. After completion of fermentation, the enzyme was extracted and analyzed for the L-asparaginase activity. 1 ml inoculum level gave maximum production of L-asparaginase 207.7U/gds.

Jayaramu et al., 2010 obtained maximum titre values of asparaginase with inoculum size of 0.75 ml using Emerricelan nidulans.

Chankya Pallem et al.,(2011) have reported the maximum production of L-asaparaginase with inoculum volume of 1.5 ml of 7 days old Fusarium oxysporum through SSF.

Optimum production values were obtained for Pseudomonas aeruginosa by Manikandan et al., 2010 with 0.5 ml of inoculum on soya bean meal.

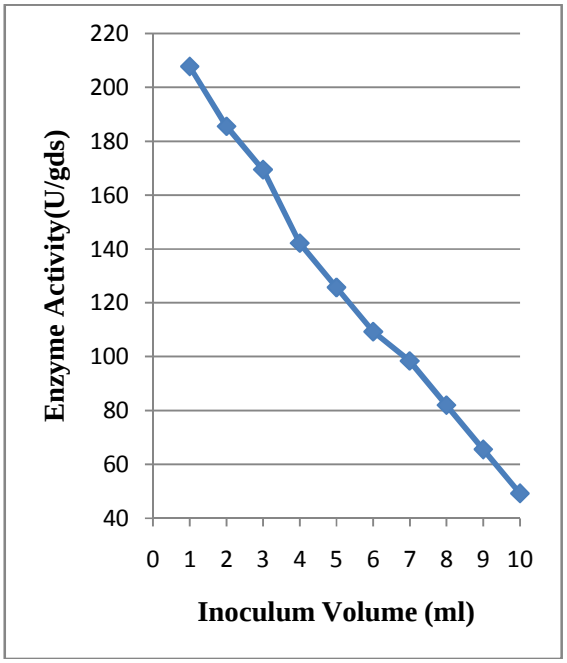

Fig-7: Effect of inoculum volume on L-asparaginase production

3.2.7 Effect of Carbon Source:

Generally carbohydrates are used as carbon sources in the microbial fermentation processes. The energy for the growth of desired microorganism during industrial fermentation derived either from the oxidation of medium components or from light .Carbon sources in media formulation are used to enhance growth and subsequently resulted in higher enzyme production, which is normally observed in the synthesis of primary metabolites, such as enzymes. Poor growth in SSF system is associated with poor nutritional level in solid substrates. The carbon concentration had a positive effect on L-asparaginase production and high titres can be obtained in a medium rich of carbon source.

To determine the effect of carbon sources on L-asparaginase yield, different carbon sources were tested which include glucose, lactose, fructose, maltose, sucrose, galactose, and soluble starch. Each of them at a concentration of 0.5% w/v with other optimized conditions was supplemented to the production medium of A.terreus and they have exerted a considerable effect on the biosynthesis of L-asparaginase.
The maximum enzyme production was promoted by glucose with a yield of 218.6 U/gds.

Baskar and Renganathan (2011) reported that glucose was found to be best carbon source for maximum L-asparaginase production using modified Czapek-dox media containing soya bean flour as substrate by Aspergillus terrus MTCC 1782.

Chankya Pallem et al.,(2011) have reported Fusaium oxysporum exhibited the maximum production of L-asparaginase production by using glucose as the carbon source.

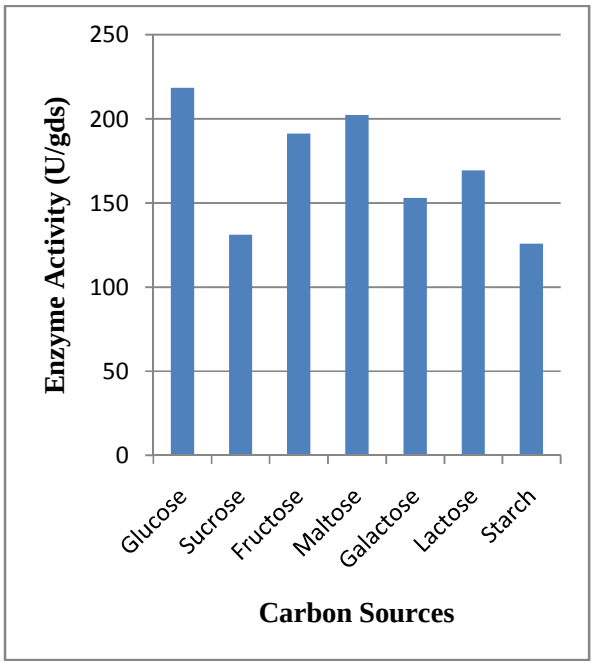

Fig-8: Effect of carbon source on L-asparaginase production

3.2.7(a) Effect of Glucose Concentration:

In order to investigate the effect of glucose concentration on the fermentation medium, SSF was carried out with different glucose concentrations varying from 0.5-3.5 % (w/v). From the data obtained, it can be concluded that the maximum production of L-asparaginase (235.0 U/gds) was obtained with the optimum glucose concentration of 1.5% (w/v). Further increase in glucose concentration resulted in the decrease of enzyme production it may be due to inhibitory affect at higher concentrations.

Baskar and Renganathan 2011 reported that 0.6% glucose was found to be best carbon source for maximum L-asparaginase production using modified Czapek-dox media containing soya bean flour as substrate by Aspergillus terrus MTCC 1782.

Chankya Pallem et al., (2011) have reported Fusaium oxysporum exhibited the maximum production of L-asparaginase production by using 0.3%glucose as the carbon source.

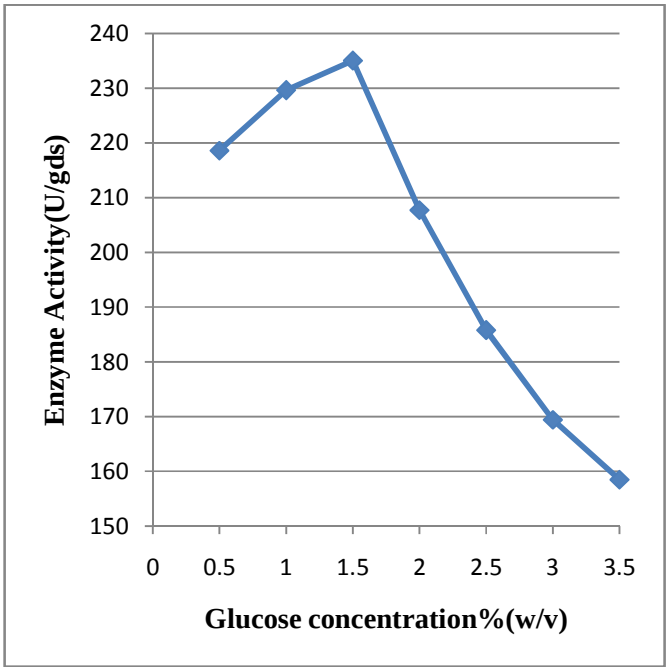

Fig-9: Effect of glucose concentration size on L-asparaginase production

3.2.8. Effect of nitrogen source:

In microorganisms, nitrogen (both organic and inorganic forms) is metabolized to produce amino acids, nucleic acids, proteins and cell wall components. Nitrogen sources have been preferred for enhancing the production of L-asparaginase. Most of the industrial enzymes utilize nitrogen source either in organic form or inorganic form sometimes both. In many instances growth will be faster with supply of organic and inorganic nitrogen source.

The supplementation of additional nitrogen sources (either organic or inorganic) such as ammonium nitrate, ammonium sulphate, sodium nitrate, malt extract, yeast extract, beef extract, tryptone, urea and peptone to the production medium had shown a profound impact on the production of L-asparaginase by A.terreus under SSF. Among the various nitrogen sources tested, ammonium sulphate in the medium promoted enhanced growth of microorganism and consequently the L-asparaginase production with an yield of 246.0 U/gds.

Gaffar and Shethna, (1977) observed the positive effect of supplementation of ammonium sulphate in the production of L-asparaginase.

Sreenivasulu et al.,(2009) have reported ammonium sulphate exhibited maximum enzyme production by the isolated fungus VS-26.

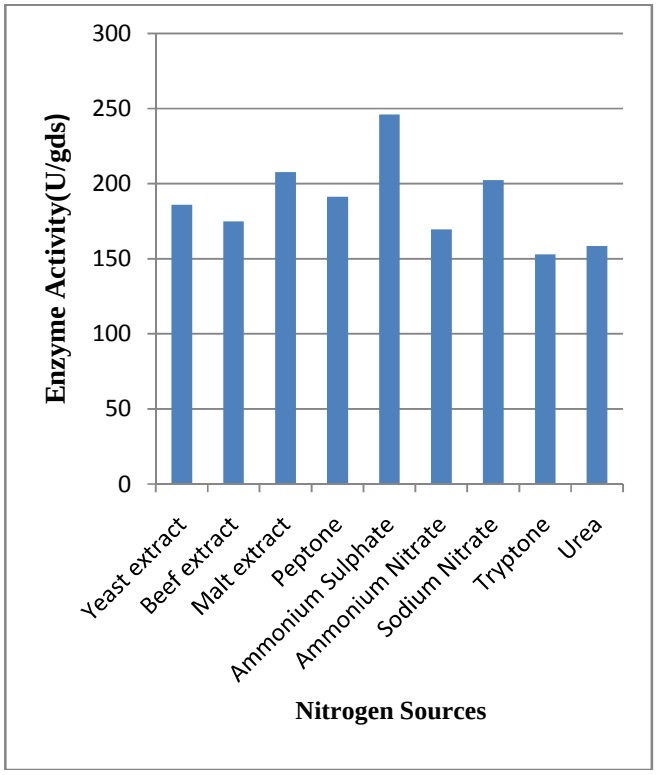

Fig- 10: Effect of nitrogen source on L-asparaginase production

3.2.8 (a) Effect of ammonium sulphate concentration:

In order to evaluate the effect of ammonium sulphate concentration on the fermentation medium, SSF was carried out with different concentrations of ammonium sulphate varying from 0.5-3.5% (w/v).

The results revealed that the maximum L-asparaginase production (267.8 U/gds) was obtained with ammonium sulphate concentration of 2% (w/v). Further increase in ammonium sulphate concentration resulted in the decrease of enzyme production due to the repressor effect of ammonium sulphate at higher concentrations.

Gaffar and Shethna, (1977) and Sreenivasulu et al.,(2009) have reported maximum production of L-asparaginase with 1.0% of ammonium sulphate.

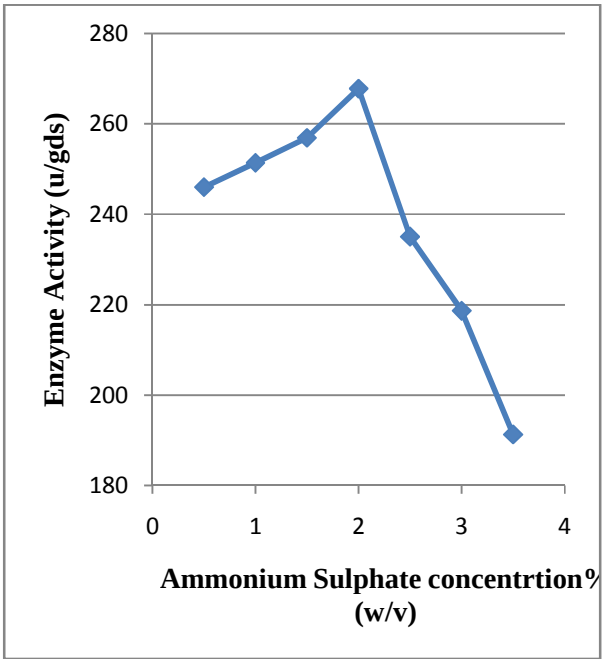

Fig-11: Effect of ammonium sulphate concentration on L-asparaginase production.

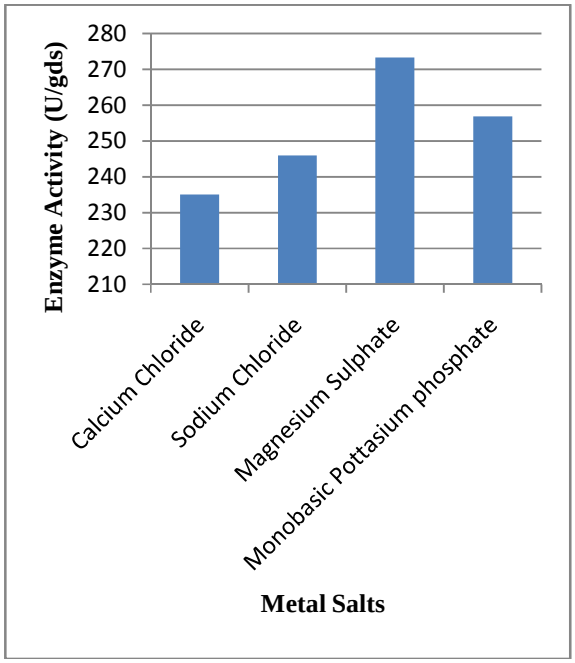

Fig-12: Effect of metal salts on L-asparaginase production

3.2.9 Effect of metal salts on L-Asparaginase production:

The effect of metal salts on L-asparaginase production was determined by adding different metal salts to fermentation medium. Metal salts provide metal ions that are essential for cell mass formation and also act as cofactor for several biosynthetic enzymes. The metal salts selected were MgSO4.7H2O, CaCl2, NaCl, KH2PO4, at 0.1% (w/v) concentration. Addition of magnesium sulphate to the fermentation medium showed high L-asparaginase activity of 273.3 U/gds.

Yasser et al 2002 reported that maximum activity was observed with MgCl2 by Pseudomonas aeruginosa using corn steep liquor.

CONCLUSIONS

The observations made in this work hold great promise for maximum production of L-Asparaginase enzyme (273.3 U/gds) after optimization of fermentation parameters such as fermentation time, temperature, initial moisture content, pH, inoculum age and inoculum volume, carbon source such as glucose, nitrogen source such as ammonium sulphate and metal salt such as magnesium sulphate by Aspergillus terreus MTCC 1782 using bajra seed flour as substrate under solid state fermentation. This clearly indicates that the Aspergillus terreus MTCC 1782 is a potential strain for L-Asparaginase production under solid-state fermentation. These studies also indicates that the bajra seed flour is an effective substrate for the production of L-Asparaginase enzyme. As the bajra seed flour has the good nutritive value, low cost, and easily available substrate has paved a way for the large scale production of L-Asparaginase enzyme, a potential antitumor agent, which has vast applications in health care and food industries.

REFERENCES

[1]. Head DR, Behm FG. Acute lymphoblastic leukemia and the lymphoblastic lymphomas of childhood. Semin Diagn Pathol. 12(4), 325-34. 1995.
[2]. Berenbaum MC, Ginsburg H, Gilbert DM. Effects of L-asparaginase on lymphocyte target cell reactions. In Vitro Nature 227, 1147–1148. 1970.
[3]. Broome JD. L-asparaginase EC-II from Escherichia coli. Some substrate specificity characteristics. Biochemistry 8, 3766-3772.1963.

[4]. Howard Cedar and James H. Schwartz.Production of L-Asparaginase II by Escherichia coli. Journal of Bacteriology: 2043-2048.1968.
[5]. James BH, Frederick HC. L-Asparaginase from Erwinia carotovora, Substrate specificity and enzymatic properties. J Biol Chem;247:1020-1030.1972.
[6]. Canddik,M.X.,Peters,O.,Platt,A.Nitrogen regulation in fungi. Antoine Van Leeuwenhook, 65:169-177.1994.
[7]. Arima K Microbial enzyme production. In: Starr MP, editor Global Impacts of Appl Microbiol 279-294.1964.
[8]. Doelle HW, Mitchell DR, Rolz CE Solid Substrate Cultivation. Elsevier Applied Science London 7-16,1992.
[9]. Balakrishnan K, Pandey A Production of biologically active secondary metabolites in solid state fermentation. J Sci Ind Res 55: 365-37, 1996.
[10]. Ashraf S.A. El-Sayed ,L-glutaminase production by Trichoderma koningii under solid-state fermentation. Indian J Microbiol.1-8, 2008.
[11]. Imada A, Igarasi S, Nakahama K and Isono M. L-asparaginase and glutaminase activities of Microorganisms. Journal of General Microbiology,76: 85-99,1973.
[12]. Abha Mishra . Production of L-Asparaginase, an anticancer agent, from Aspergillus niger using agricultural waste in solid state fermentation. Applied Biochemistry and Biotechnology: 135,2006.
[13]. Hymavathi M, Sathish T, Subba Rao Ch, Prakasham RS . Enhancement of L-Asparaginase production by isolated Bacillus circulans (MTCC 8574) using response surface methodology. Appl Biochem Biotechnol; 159(1), 191-198, 2009.
[14] . A.R. Soniyamby, S. Lalitha,B.V. Praveesh & V. Priyadarshini., International Journal of Microbiological Research, 2(1), 38,2011.
[15]. M.I. Sarquis, E.M. Oliveira, A.S. Santos & G. Costa., Man Inst Oswaldo Cruz., 99(5), 489,2004.
[16]. K.G. Siddalingeshwara & K. Lingappa., An International Journal of Pharmaceutical Sciences,
1(1), 103, 2010.
[17]. Yogendra Singh, S.K.Srivastava, L-asparaginase Production by a new isolate Bacillus aryabhattai strain ITBHU02 in solid state culture, 1st International Conference on Biosciences and Bioengineering: A collaborative Approach, 2012.
[18]. Hosamani R and Kaliwal B B, L-Asparaginase-An Anti Tumour Agent production by Fusarium equiseti using Solid State Fermentation. International Journal of Drug Discovery Vol. 3, Issue 2 : pp-88-99,2011.
[19].G.Thirumurugan,MosesJeyakumarRajesh,LeelavathyRajesh,VanapalliVSatyaveni,Rajarammohan
Sivasubramanian,Effect of Inducers and Physical Parameters on the Production of L-Asparaginase Using Aspergillus Terreus,J Bioprocess Biotechniq 1:110 doi:10.4172/2155-9821.1000110,2011.
[20]. Selvakumar Dharmaraj, Study of L-asparaginase production by Streptomyces noursei MTCC 10469, isolated from marine sponge Callyspongia diffusa. Iranian Journal of Biotechnology Vol. 9, No. 2 : 102-108,2011.
[21]. Jayaramu M , Hemalatha N B, Rajeshwari C P, Siddalingeshwara K G, Mohsin S M and Sunil Dutt P LN S N, A Novel Approach for Detection, Confirmation and Optimization of L-Asparaginase from Emericella Nidulans , Current Pharma Research Vol. 1, Issue 1: 20-24,2010.
[22]. Chanakya Pallem., Nagarjun. V. and Srikanth, M.Production of a tumor inhibitory enzyme, L-asparaginase through solid state fermentation using Fusarium oxysporum, International Journal of Pharmaceutical Sciences Review and Research,7(2),189-192,2011.
[23]. Manikandan R, Pratheeba CN, Pankaj Sah and Stuti Sah, Optimization of Asparaginase Production by Pseudomonas aeruginosa Using Experimental Methods. Nature and Science 8(2) : 1-6, 2010.
[24]. Baskar G, Renganathan S. Design of experiments and artificial neural network linked genetic algorithm for modelling and optimization of L-asparaginase production by Aspergillus terreus MTCC 1782. Biotechnology and Bioprocess Engineering ;16: 50-58, 2011.
[25]. Gaffar S. A. and Shethna Y. I.Purification and Some Biological Properties of Asparaginase from Azotobacter vinelandii. Appl Environ Microbiol.: 33(3), 508-514,1977.
[26]. Sreenivasulu V., Jayaveera K. N. and Mallikarjuna Rao P.Optimization of process parameters for the production of L-asparaginase from an isolated fungus,Research J. Pharmacognosy and Phytochemistry,1(1),30-34, 2009.
[27]. Yasser R. Abdel-Fattah, Zakia A. Olama, Process Biochemistry,38, 115-122,2002.