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TUMOR INHIBITORY L-ASPARAGINASE PRODUCTION BY ASPERGILLUS CERVINUS

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ABSTRACT
L – asparaginase is an important therapeutic enzyme used for the treatment of acute lymphoblastic leukemia, lymphosarcoma, lymphoproliferative disorders and acute lymphocytic leukemia. The present research work discribes the production of L-asparaginase using *Artocarpus heterophyllus* as substrate by *Aspergillus cervinus* in a solid state fermentation. Normally L-Asparaginase enzyme existed in many animal tissues, plant tissues and bacterial cell, but not in human cells. In the present research, L-asparaginase enzyme production was carried out by observing different parameters like incubation time, incubation temperature, pH, inoculum level and moisture content were noted. Different carbon supplements were checked for their influence on enzyme production; they are glucose, sucrose, maltose, fructose, and lactose. The incubation time of 72hrs, the temperature of 36^{0}C, pH 5.0, inoculum level of 70% v/w and moisture content of 70% v/w were observed for L-asparginase enzyme production. Among the carbon source, sucrose gave better production when compared with other carbon supplements. Nitrogen source like L-asparagine with different concentrations were checked and 0.4% w/w gave best enzyme production. Final conclusion is that Artocarpus heterophyllus could be a promising substrate for industrial application since it produces a significant L–asparaginase (89.36 IU/ml) activity in solid state fermentation.

KEYWORDS: Artocarpus heterophyllus, *Aspergillus cervinus*, Solid-state fermentation.

INTRODUCTION
L-asparaginase is an important therapeutic enzyme used for the treatment of acute lymphoblastic leukemia, lymphosarcoma, lymphoproliferative disorders and acute lymphocytic leukemia. The mechanism of action of L-asparaginase is to inhibit the growth of tumor cells.[1-5] Cells are able to produce the amino acid L-asparagine, which is important for its cell function, because presence of the enzyme asparagine synthetase (EC 6.3.5.4). Tumor cells lack the enzyme asparagine synthetase, being not able to produce L-asparagine for their growth and development. Solid-state fermentation is a process that takes place on a non-soluble material that acts both as support and a source of nutrients, with a reduced among of water, under the action of fermenting agent.[6-9] A major amount of researches were conducting research on the biosynthesis of L-asparaginase demonstrating the anticancer activity. L-asparaginase production was carried out throughout the world by Solid-state fermentation and submerged fermentation.[10-15] Solid-state fermentation is a very effective fermentation technique that yields high production in many times when compared to submerged fermentation. Submerged fermentation technique has many disadvantages, like low concentration production, and consequent handling, reduction, and disposal of large volumes of water during the downstream processing. Therefore, the submerged fermentation technique is a cost intensive, highly problematic, and poorly understood unit operation L-Asparaginase (E. C. 3. 5. 1. 1) is present in many animal tissues, bacteria and plants, but not in mankind. Microbial asparaginases have been particularly studied for their applications as therapeutic agents in the treatment of certain types of human cancer.[16-23] L-asparaginase from two bacterial sources (*E. coli* and *Erwinia carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukemia. It is also used for the treatment of various carcinoma and bovine lymphomosarcoma. Therefore, the aim of the present research work is to discovery of a new L-asparaginase producer that is serologically different from the previously reported ones, but one that has similar therapeutic effects.

MATERIAL AND METHODS
Substrate: *Artocarpus heterophyllus* leaves were collected from our college garden, Sathupally and dried naturally, powdered, packed and stored until further use.

Microorganism: *Aspergillus cervinus* (NCIM 1356) procured from National Collection of Industrial Microorganisms (NCIM), Pune was used for the production of L-asparginase enzyme using *Artocarpus heterophyllus* leaves as substrate. Potato dextrose agar medium was used for sub culturing and maintenance of the microorganism.

Preparation of inoculum: Streaking was done from the old cultures of *Aspergillus cervinus* on pure potato dextrose agar medium and incubated them at 36^0C for 3 days.

Development of inoculum: 10ml of sterile distilled water were mixed to 3 days old culture slant; from that 1ml of suspension that contains approximately, 10^7cells/ml was used as the inoculums.

Solid state fermentation: Solid state fermentation was carried out in 250-mL erlenmeyer flask by taking production medium containing (in g/L): Glucose- 12.5g, NH_4NO_3- 2.66g, $FeSO_4.7H_2O$ - 0.01g, L-asparagine - 0.5g, KCl- 0.5g, K_2HPO_4-1g. The pH of the medium was adjusted to 6.0. Solid state fermentation was accomplished by taking 10g of substrate in 250ml Erlenmeyer flask, moistening it with 5ml of production medium, mixed thoroughly and placed in an autoclaved at 15lb pressure, 121^0C for 15min for sterilization. After sterilization, it was cooled; then the flasks were inoculated with 1ml of inoculum and incubated in an incubator.[24-30]

Determination of enzyme activity

Enzyme extraction: The enzyme extraction was carried out at a temperature of 36^0C for 24hrs interval. The solid state fermentation material corresponding to one Erlenmeyer flask was mixed with 100ml of Sodium Phosphate buffer and rotated for 45mins with the help of Rotator shaker. After 45min the extraction was filtered in Whatman filter paper, from that 1ml of the extract was placed in to a centrifugal tube and centrifuged at 10000rpm for 10mins.

Enzyme assay: L-asparaginase enzyme activity was detected by measuring the amount of ammonia formed by nesslerization. The free suspension in centrifugation tube of 0.2ml was taken into a centrifugal tube and mixer with 0.04ml L-Asparine and followed by the addition of 0.8ml of 0.1M Sodium borate. It was incubated for 10mins to liberate the ammonia, 0.5ml of 15% trichloroacetic acid was added to the centrifugal tube and centrifuged for 10mins at 10000rpm. From the supernatant liquid 1.0ml was taken and mixed with 1.0ml Nessler's reagent to detect liberated ammonia at 480nm in UV equipment. One unit (U) of L-asparaginase was the amount of enzyme which liberates 1μmole of ammonia in 1 min at 37^0C.[31-36]

RESULTS AND DISCUSSION

To determine the effect of fermentation time on enzyme production, the medium incubate at different time intervals, after completion of every 24hrs, enzyme extraction process was done and the maximum L-asparaginase activity was noted at 72hrs. After 72hrs, it was decline due to depletion of nutrients in the medium. L-asparaginase enzyme production at different time intervals was shown in the fig.1.

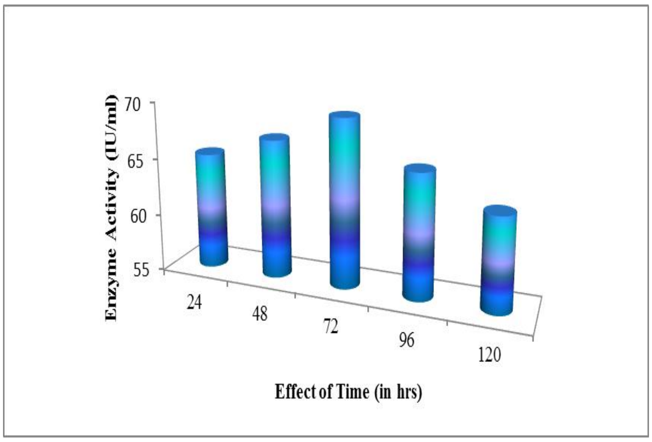

Fig. 1: Effect of time on enzyme production.

The temperature was crucial in Solid state fermentation because it ultimately affects the growth of the microorganism. To determine the effect of fermentation temperature on enzyme production, the medium was incubated at different temperatures, after completion of 72hrs, enzyme extraction process was done. The maximum production of L-asparaginase enzyme was noted at 36^0c temperature Fig.2.

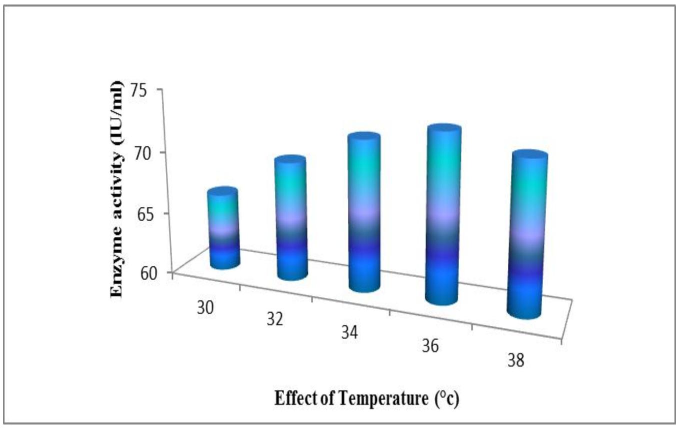

Fig. 2: Effect of temperature on enzyme production.

Every enzyme has an optimum pH when it was more effective. An increasing or decreasing pH reduces enzyme activity by changing the ionization. To determine the effect of pH, the nutrient medium was adjusted to different pH ranges 3, 4, 5, 6 and 7. The maximum enzyme production of L-asparaginase was noted at pH 5.0 fig.3.

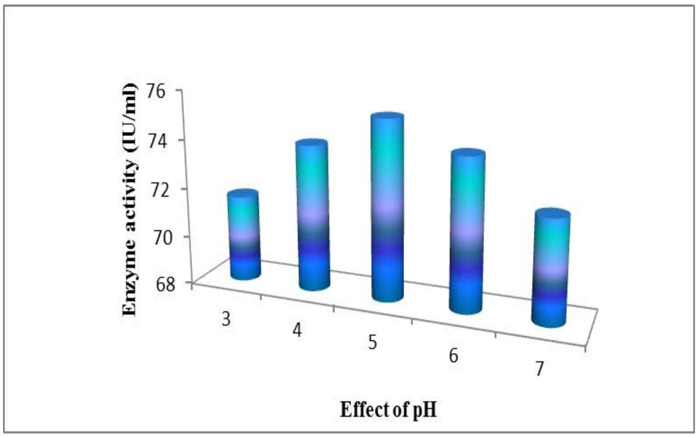

Fig. 3: Effect of pH on enzyme production.

When inoculum size was increased from 5 to 10% there was increase in enzyme production but thereafter the enzyme activity was decreased, because depletion of nutrients by the enhanced biomass, which resulted diminishing in metabolic activity. To determine inoculum size, different inoculum levels were prepared for the production of enzyme 40%, 50%, 60%, 70%, 80%, 90% and 100%, v/w. The maximum enzyme production was noted at70% v/w of inoculum fig.4.

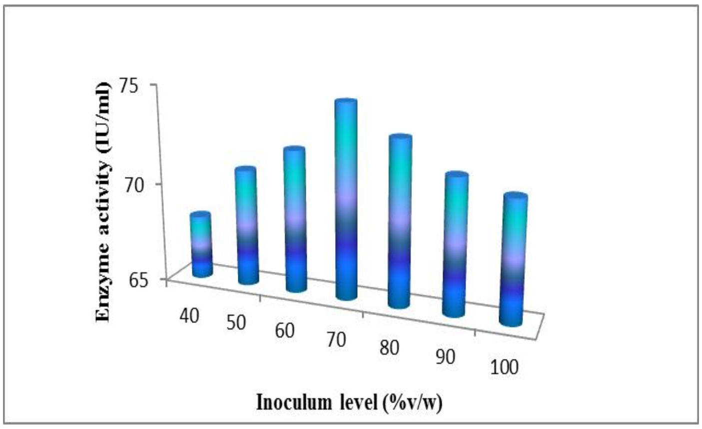

Fig. 4: Effect of inoculum level on enzyme production.

Moisture content in solid state fermentation is plays crucial role in the production of enzymes. High moisture content results in decreasing the substrate porosity, which may turns reduction in oxygen penetration, it may cause contamination. To determine the moisture content on the enzyme production, various moisture content were prepared like 40%, 50%, 60%, 70%, 80%, 90%, and 100% v/w were taken in different conical flask.. The maximum activity was noted at 70% v/w of the moisture content fig.5.

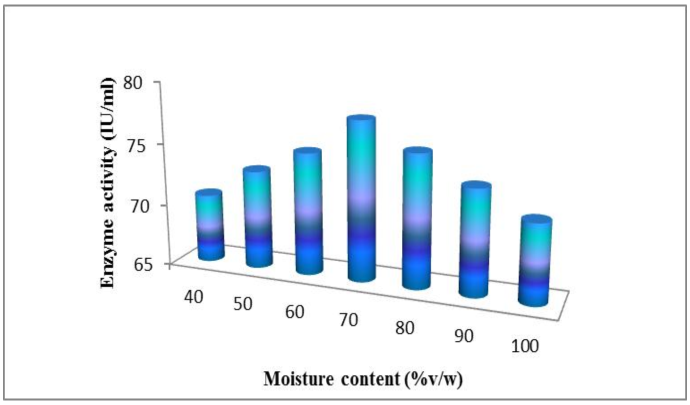

Fig. 5: Effect of moisture content on enzyme production.

To determine the effect of carbon source on enzyme production, five different carbon supplements were screened for the production of L-asparaginase enzyme which is sucrose, maltose, glucose, fructose, and lactose. The nutrient medium was enriched with different carbon concentrations % w/w. The result noted that sucrose supplementation gave better improved enzyme production than other supplementations fig.6.

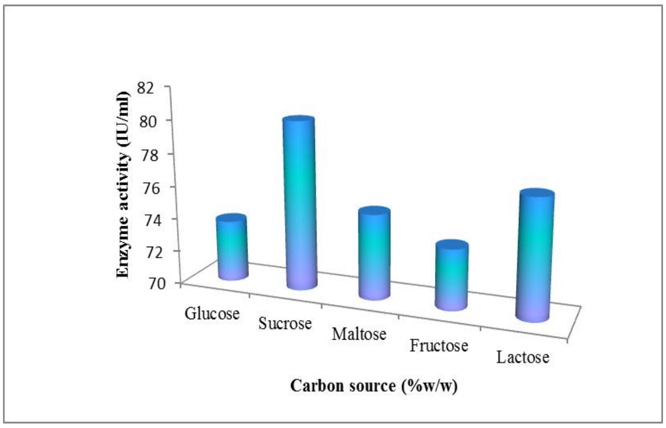

Fig. 6: Effect of carbon source on enzyme production.

To determine the effect of nitrogen source on the production of enzyme, the production medium was made with different concentrations of L-asparagine like 0.2%, 0.3%, 0.4%, 0.5%, 0.6% and 0.7% w/w were dispersed in 250ml conical flasks. The results indicate that maximum enzyme production was noted at 0.4% w/w of L-asparagine concentration fig.7.

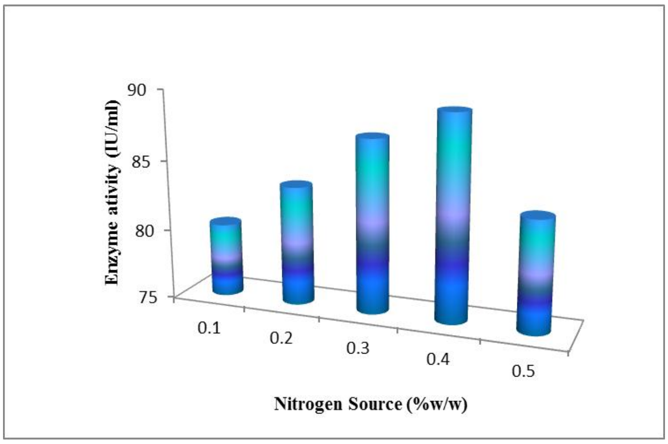

Fig. 7: Effect of nitrogen source on enzyme production.

CONCLUSION

Finally we concluded that *Artocarpus heterophyllus* is a promising agent for the production of enzyme which is having industrial application, and important therapeutic use for the treatment of acute lymphoblastic leukemia, lymphosarcoma, lymphoproliferative disorders and acute lymphocytic leukemia. It gave a significant L –asparaginase enzyme production (89.36 IU/ml) in solid state fermentation using *Aspergillus cervinus*. *Artocarpus heterophyllus* is low cost substrate, easily available raw material and showing suitability for solid state cultivation of microbes, it was suggested as a potential substrate for L –asparaginase production in solid state fermentation.

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REFERENCES

1. Vimal, A., and Kumar, A. In vitro screening and in silico validation revealed key microbes for higher production of significant therapeutic enzyme L-asparaginase. Enzyme Microb. Tech, 2017; 98: 9–17.
2. Brizzio, S., Turchetti, B., de García, V., Libkind, D., Buzzini, P., and van Broock, M. Extracellular enzymatic activities of basidiomycetous yeasts isolated from glacial and subglacial waters of northwest Patagonia (Argentina). Can. J. Microbiol, 2007; 53: 519–525.
3. Brumano, L. P., Silva, F. V. S., Costa-Silva, T. A., Apolinário, A. C., Santos, J. H. P. M., Kleingesinds, E. K. Development of L-asparaginase biobetters: current research status and review of the desirable quality profiles. Front. Bioeng. Biotechnol, 2019; 6: 212.
4. Cachumba, J. J. M., Antunes, F. A. F., Peres, G. F. D., Brumano, L. P., Santos, J. C., and Silva, S. S. Current applications and different approaches for microbial l-asparaginase production. Braz. J. Microbiol, 2016; 47: 77–85.
5. Flores, C.-L., Rodríguez, C., Petit, T., and Gancedo, C. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol. Rev, 2000; 24: 507–529.
6. Garcia-Ochoa, F., and Gomez, E. Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnol. Adv, 2009; 27: 153–176.
7. Jin, M., Slininger, P. J., Dien, B. S., Waghmode, S., Moser, B. R., Orjuela, A. Microbial lipid-based lignocellulosic biorefinery: feasibility and challenges. Trends Biotechnol, 2015; 33: 43–54.
8. Kerssemakers, A. A. J., Doménech, P., Cassano, M., Yamakawa, C. K., Dragone, G., and Mussatto, S. I. Production of itaconic acid from cellulose pulp: feedstock feasibility and process strategies for an efficient microbial performance. Energies, 2020; 13: 1654.
9. Couto SR, Sanroman MA: Application of solid-state fermentation to food industry – A Review, Journal of Food Engineering, 2006; 76: 291-302.
10. Mashburn LT and Wriston JC. Tumor Inhibitory Effect of L-asparaginase from Escherichia coli. Archives Biochemistry Biophysics, 1964; 105: 450-452.
11. Datar R. Economic of Primary Separation Steps in Relation to Fermentation and Genetics Engineering. Process Biochemistry, 1986; 21: 19-26.
12. Arima K. Microbial Enzyme Production in Global Impact of Applied Microbiology, M. P. Starr (eds.) John Willey, New York, USA, 1964; 279-299.
13. Gallogher MP, Murshall RD, Wilson R. Asparaginase Drug for Treatment of Acute Lymphoblastic Leukemia. Essays Biochemistry, 1989; 24: 1- 40.
14. Keating MJ, Holmes R, Lerner SH. L-asparaginase and PEG Asparaginase Past, Present and Future. Leuk Lympmoma, 1993; 10: 153-157.
15. Yunis AA, Arimures GK, Russin DJ. Humane Pancreatic Carcinoma (MIA PaCa-2) in Continues Culture Sensitivity to Asparaginase. International Journal of Cancer, 1977; 19: 218-235.
16. Mosterson MA, Hull BL, Vollmer LA. Treatment of Bovine Lymphomsarcoma with L-asparaginase. Journal of American Veterinary Medical Association, 1988; 192: 1301-1306.
17. Ashraf A. El-Bessoumy, Mohamed Sarhan and Jehan Mansour. Production, Isolation, and Purification of L-Asparaginase from Pseudomonas Aeruginosa 50071 Using Solid-state Fermentation. Journal of Biochemistry and Molecular Biology, 2004; 37: 387-393.
18. Praveen Kumar Dasari, Usha Kiranmai Mangamuri, Vijayalakshmi Muvva, Sudhakar Podha. Production and Optimization of L- asparaginase by Musa paradisiaca in solid state fermentation. World Journal of Pharmacy and Pharmaceutical Sciences, 2016; 5(10): 859-866.
19. Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol, 1994; 14: 4135–4144.
20. Alcaíno, J., Cifuentes, V., and Baeza, M. Physiological adaptations of yeasts living in cold environments and their potential applications. World J. Microbiol. Biotechnol, 2015; 31: 1467–1473.
21. Braunwald, T., Schwemmlein, L., Graeff-Hönninger, S., French, W. T., Hernandez, R., Holmes, W. E. Effect of different C/N ratios on carotenoid and lipid production by Rhodotorula

glutinis. Appl. Microbiol. Biotechnol, 2013; 97: 6581–6588.
22. Rigoulet, M., Aguilaniu, H., Avéret, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X. Organization and regulation of the cytosolic NADH metabolism in the yeast Saccharomyces cerevisiae. Mol. Cell. Biochem. 2004; 256: 73–81.
23. Sampaio, J. P. Leucosporidium Fell, Statzell, I.L: Hunter & Phaff. In the Yeasts, a Taxonomic Study, Kurtzman, C. P. (Amsterdam: Elsevier), 2011; 5: 1485–1494.
24. Serrano, N. F. G., Rodrigues, L., Hokka, C. O., Sousa, C. P., Teixeira, J. A., and Mussatto, S. I. Optimal glucose and inoculum concentrations for production of bioactive molecules by Paenibacillus polymyxa RNC-D. Chem. Pap, 2012; 66: 1111–1117.
25. Spencer, J., Ragout de Spencer, A., and Laluce, C. Non-conventional yeasts. Appl. Microbiol. Biotechnol, 2002; 58: 147–156.
26. Chang, H. N., Jung, K., Choe, J., Lee, J. C., and Woo, H.-C. Multi-stage continuous high cell density culture systems: a review. Biotechnol. Adv, 2014; 32: 514–525.
27. Costa, I. M., Schultz, L., Pedra, B. A. B., Leite, M. S. M., Farsky, S. H. P., Oliveira, M. A. Recombinant L-asparaginase 1 from Saccharomyces cerevisiae: an allosteric enzyme with antineoplastic activity. Sci. Rep, 2016; 6: 36239.
28. Duarte, A. W. F., Dayo-Owoyemi, I., Nobre, F. S., Pagnocca, F. C., Chaud, LCS., Pessoa, A. Taxonomic assessment and enzymes production by yeasts isolated from marine and terrestrial Antarctic samples. Extremophiles, 2013; 17: 1023–1035.
29. Klein, M., Swinnen, S., Thevelein, J. M., and Nevoigt, E. Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities. Environ. Microbiol, 2017; 19: 878–893.
30. Knothe, G. Improving biodiesel fuel properties by modifying fatty ester composition. Energy Environ. Sci, 2009; 2: 759–766.
31. Liu, Z., van den Berg, C., Weusthuis, R. A., Dragone, G., and Mussatto, S. I. Strategies for an improved extraction and separation of lipids and carotenoids from oleaginous yeast. Sep. Purif. Technol, 2021; 257: 117946.
32. Margesin, R., and Schinner, F. Properties of cold-adapted microorganisms and their potential role in biotechnology. J. Biotechnol, 1994; 33: 1–14.
33. Narta, U. K., Kanwar, S. S., and Azmi, W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Crit. Rev. Oncol. Hematol, 2007; 61: 208–221.
34. Kumar, S., Dasu, V. V., and Pakshirajan, K. Purification and characterization of glutaminase-free L-asparaginase from Pectobacterium carotovorum MTCC 1428. Bioresour. Technol, 2011; 102: 2077–2082.
35. Li, E., and Mira de Orduña, R. A rapid method for the determination of microbial biomass by dry weight using a moisture analyser with an infrared heating source and an analytical balance. Lett. Appl. Microbiol, 2010; 50: 283–288.
36. Liu, Z., Feist, A. M., Dragone, G., and Mussatto, S. I. Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. J. Clean. Prod, 2020; 249: 119308