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CHAPTER 2

Production of L-Asparaginase enzyme by *Trichoderma Harzianum* under Solid-State Fermentation

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Abstract

The present research work describes the production of L-Asparaginase using *Tectona grandis* leaves as substrate by *Trichoderma harzianum* in a solid-state fermentation. Asparaginase is an enzyme, often referred to as L-asparaginase, used in chemotherapy, particularly for acute lymphoblastic leukaemia (ALL), by breaking down asparagine, an amino acid crucial for cell growth, thus inhibiting cancer cell proliferation. Asparaginase is an enzyme that catalyses the hydrolysis of asparagine, an amino acid vital for protein synthesis and cell growth. In present research, Asparaginase enzyme production was carried out by observing different parameters like time, temperature, moisture content, inoculum level, pH, carbon source and nitrogen source were noted. Different carbon supplements were checked for their influence on enzyme production; they are glucose, sucrose, maltose, lactose and fructose. The incubation time of 72 hrs, the temperature of 25 °C, pH 8.0, inoculum level of 90% v/w and moisture content 70% v/w were observed for asparaginase enzyme production. Among the carbon source, lactose gave better production when compared to other carbon supplements. Nitrogen source like asparaginase with different concentration were checked and 0.3% w/w gave best enzyme production. Final conclusion is that *Tectona grandis* leaves could be a promising substrate for industrial application since it produces a significant asparaginase (74.32 IU/ml) activity in solid state fermentation.

Keywords: Tectona grandis, Trichoderma harzianum, solid state fermentation.

1. Introduction

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 number of water, under the action of fermenting agent. L-asparaginase, an enzyme that hydrolyses asparagine, is produced through biotechnological methods like submerged fermentation (SmF), solid-state fermentation (SSF), and recombinant DNA technology, using various microbial sources. L-asparaginase can be used in food processing to reduce acrylamide formation. The mechanism of action of, Asparaginase hydrolyzes serum asparagine to nonfunctional aspartic acid and ammonia, depriving tumor cells of a required amino acid; thus, tumor cell proliferation is blocked by the interruption of asparagine-dependent protein synthesis. Asparaginase manufacture was carried out throughout the world by solid state fermentation and submerged fermentation. Asparaginase is used as part of a multi-agent chemotherapy regimen for the treatment of acute lymphoblastic leukaemia. Therefore, the aim of the present research work is to discovery of a new asparaginase producer that is serologically different from the previously reported ones, but one that have similar therapeutic effects.

2. Materials and Methods

Substrate: *Tectona grandis* leaves were collected from our college garden, Sathupally and dried naturally, powdered, packed and stored until further use.

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Microorganism: *Trichoderma harzianum* (NCIM 1347) procured from National Collection of Industrial Microorganisms (NCIM), Pune was used for the production of Asparaginase enzyme using *Tectona grandis* leaves as substrate. Potato dextrose agar medium was used for sub culturing and maintenance of microorganisms.

Preparation of Inoculum: streaking was done from the old cultures of *Trichoderma harzianum* on pure potato dextrose agar medium and incubated them at 360 °C for 3 days.

Development of Inoculum: 10 ml of sterile distilled water were mixed to 3 days old culture slant, from that 1 ml of suspension that contains approximately, 10^7 cells/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-12.5 g, ammonium nitrate-0.26 g, ferrous sulphate-0.01g, L-asparagine-0.5g, potassium chloride-0.5g, potassium hydrogen phosphate- 1 g. The pH medium was adjusted to 8.0. solid state fermentation was accomplished by taking 10 g of substrate in 250 ml Erlenmeyer flask, moistening it with 5 ml of production medium, mixed thoroughly and placed in an autoclaved at 15lb pressure, 121°C for 15min for sterilization. After pasteurize, it was cooled; then the flasks were inoculated with 1 ml of inoculum and incubated in an incubator.

3. Determination of Enzyme Activity

Enzyme Extraction: The enzyme extraction was carried out at a temperature of 36°C for 24 hrs interval. The solid-state fermentation material corresponding to one Erlenmeyer flask was mixed with 10 ml of sodium phosphate buffer and rotated for 45 mins with the help of Rotator shaker. After 45 min the extraction was filtered in What man filter paper, from that 2 ml of the extract was placed in centrifugal tube and centrifuged at 10,000rpm for 10 mins.

Enzyme Assay: Asparaginase enzyme activity was detected by measuring the amount of ammonia formed by nesslerization. The free suspension in centrifugal tube of 0.5ml was taken into centrifugal tube and add with 0.5ml of L-asparagine and followed by the addition of 1 ml sodium borate. It was incubated for 10mins to liberate the ammonia, 0.5 ml of 15% trichloroacetic acid was added to the centrifugal tube and centrifuged for 10mins at 10,000rpm. From the supernatant liquid 1.0 ml was taken and mixed with 1.0 ml of Nessler's reagent to detect liberated ammonia at 480 nm in UV equipment. One unit (U) of asparaginase was the amount of enzyme which liberates the 1 micromole of ammonia in 1 min at 37°C.

4. Results and Discussion

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

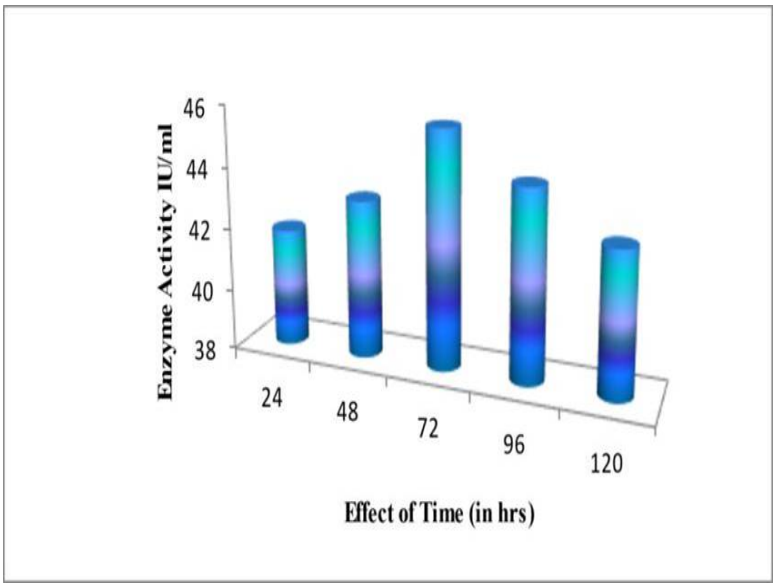

Figure 1: Effect of time on enzyme production

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 72 hrs, enzyme extraction process was done. The maximum production of asparaginase enzyme was noted at 25°C temperature Figure 2.

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 nutrientmedium was adjusted to different pH ranges 6, 7, 8, 9 and 10. The maximum enzyme productionof L-asparaginase was noted at pH 8 Figure 3.

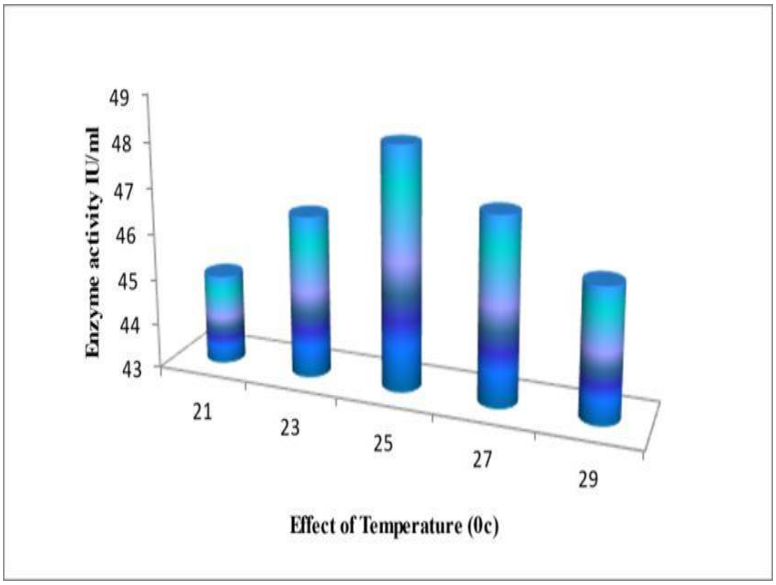

Figure 2: Effect of temperature on enzyme production

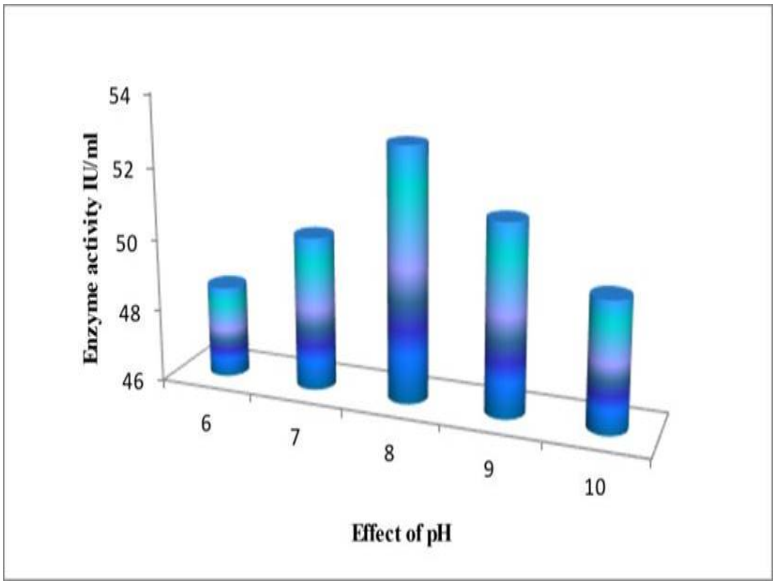

Figure 3: Effect of pH on enzyme production

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 60%, 70%, 80%, 90% and 100%, v/w. The maximum enzyme production was noted at 90% v/w of inoculum Figure 4.

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 turn reduction in penetration, it may cause contamination. To determine the moisture content on the enzyme production, various moisture content was prepared like 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 Figure 5.

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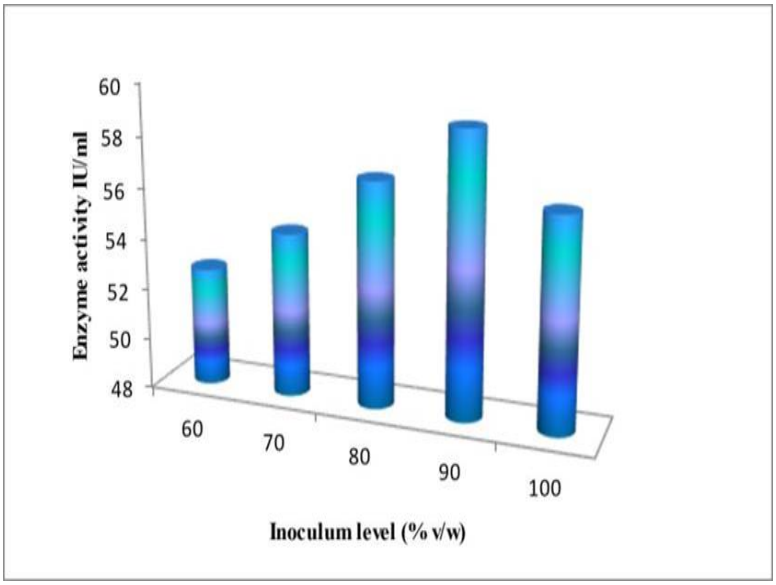

Figure 4: Effect of pH on enzyme production

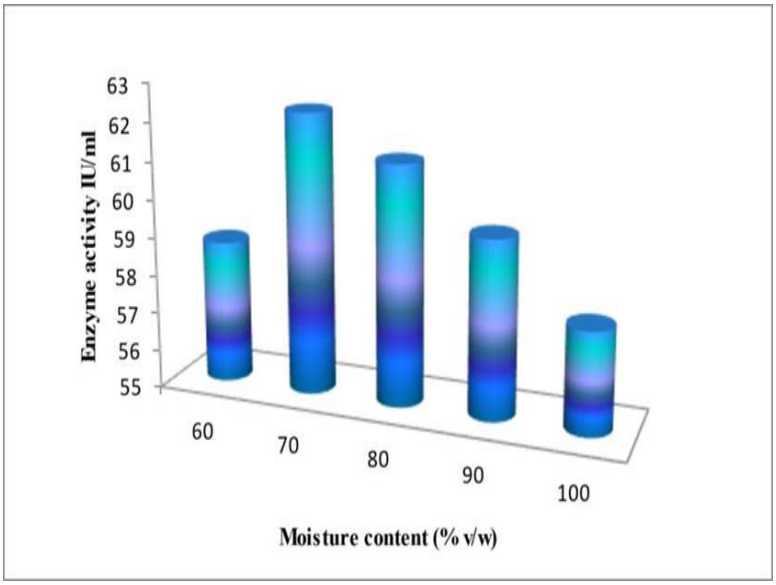

Figure 5: Effect of moisture content on enzyme production

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 lactose supplementation gave better improved enzyme production than other supplementations Figure 6.

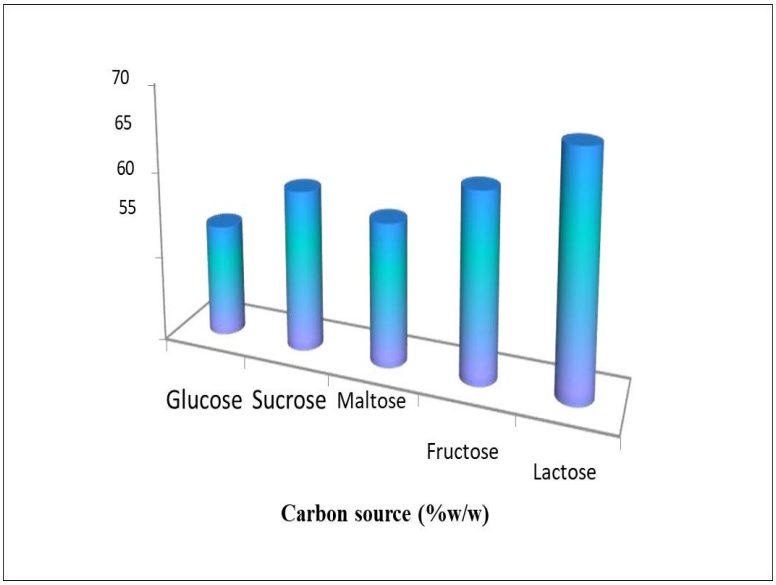

Figure 6: Effect of moisture content on enzyme production

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

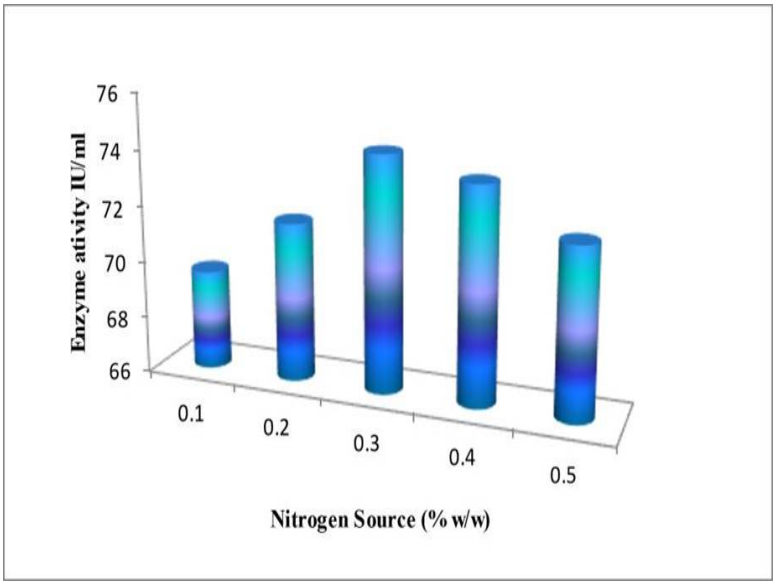

Figure 7: Effect of nitrogen source on enzyme production

5. Conclusion

We conclude that *Tectona grandis* leaves were a promising agent for the production of asparaginase enzyme. Asparaginase production research highlights its potential as a therapeutic agent, particularly in leukaemia treatment, while also exploring its applications in food processing to reduce acrylamide formation, with ongoing efforts focused on optimizing production and identifying novel sources. It gave a significant asparaginase enzyme production (74.32 IU/ml) in solid state fermentation using *Trichoderma harzianum*. *Tectona grandis* leaves is low-cost substrate and easily available in our local areas and showing suitability for the solid-state cultivation of microbes, it was suggested as a potential substrate for optimizing the parameters of asparaginase production under solid state fermentation.

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