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Isolation and identification of *Trichoderma* species, followed by the optimization of L-glutaminase enzyme production

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Abstract

Advanced biotechnological techniques offer practical solutions to challenges related to food safety. To maintain quality control in food production and processing, the use of enzymes, antibodies, and microbes is indispensable. Isolation and identification of *Trichoderma* sp. from sugarcane soil is used for production of L-glutaminase enzyme in this study. The *Trichoderma* sp. was grown in PDA agar and then the species were isolated for huge production of L-glutaminase enzyme. The optimization of enzyme production was high in particular pH 7, temperature 30°C, Nacl concentration at 2g, 5g of substrate and ammonium nitrate shows higher enzyme production at 1g. Based on the analysis the enzymes have an average molecular weight around 14 to 20 kDa. By leveraging biotechnology, these manufacturing systems are being optimized leading to the faster, more efficient and flexible production of new or improved products.

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Introduction

Modern biotechnological approaches offer efficient strategies for resolving food safety challenges. The application of biotechnical techniques can decrease the time needed to detect foodborne pathogens, toxins, and chemical contaminants, as well as enhance the sensitivity of these findings. The use of enzymes, antibodies, and microbes is essential for monitoring quality control in food production and processing systems (Athira *et al.*, 2014). These manufacturing operations are being enhanced by biotechnology, which allows for the development of new or upgraded products with improved speed, efficiency, and flexibility. Commonly referred to as biological catalysts, enzymes enhance the speed at which chemical reactions occur (Kashyap *et al.*, 2002). The main benefit of incorporating enzymes into industrial processes lies in their efficiency, precision, specificity, convenience, and economical nature. Enzymes are generally divided into two groups: intracellular enzymes and extracellular enzymes. Intracellular enzymes are generated within the cell and facilitate reactions internally, while extracellular enzymes are secreted by cells and transported to different locations to catalyze biochemical reactions (Koibuchi *et al.*, 2000). Living microbial cells are responsible for producing enzymes. Enzymes utilized in industry are derived from both animal and plant sources, as well as from microorganisms. Despite this, the availability of animal and plant sources for enzyme production is highly constrained and can be affected by environmental influences. Consequently, there is an increasing focus on encouraging the use of microbial sources for enzyme production. Different fermentation technologies are employed for producing the majority of microbial enzymes (Coral *et al.*, 2002). Enzyme production has also benefited from other techniques, such as solid-state fermentation (SSF) with inert solid supports or the use of immobilization methods. It entails the cultivation of microbial cells in liquid media in controlled environments within large vessels referred to as bioreactors for the production of target metabolites. A variety of culture medium components consist of common, cost-effective substrates such as carbon sources (e.g., glucose, sucrose, and maltose) and nitrogen sources (e.g., ammonium salts, sodium nitrate), along with vitamins and minerals that can be provided in consistent amounts. Enzymes utilized for medical and therapeutic purposes are referred to as therapeutic enzymes (Curthoys *et al.*, 1976). One of the main prospective applications of therapeutic enzymes is in combating cancer. L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is responsible for catalyzing the hydrolysis of L-glutamine, resulting in the formation of L-glutamic acid and ammonia (Dutt *et al.*, 2010). Over the past few years, there has been a growing focus on L-glutaminase due to its suggested applications in pharmaceuticals as well as in the food industry (Iyer and Singhal, 2010). L-glutaminase has the potential to be important in treating cancer, particularly acute lymphocytic leukemia, when used either in combination with or as a replacement for asparaginase (Kumar and Chandrasekaran, 2003). Fungi serve a crucial role in generating a wide variety of products, including food items (e.g., bread and mycoprotein), alcoholic beverages (like wine and beer), as well as recombinant proteins, vitamins, and antibiotics. Various fermentation systems are utilized with fungi to attain this level of productivity, with the selection based on the type of fungus (whether filamentous or yeast), the desired product, and the production scale. In this present study is aimed to isolate and identifying the *Trichiderma* sp. from sugarcane agriculture soil sample and optimization of L-glutaminase enzyme production through fermentation process.

Materials and methods

Samples of soil were collected from a sugarcane field in Thirukkanurpatti, within the Thanjavur District of Tamil Nadu. After being well mixed, the soil was sieved using a 2mm mesh, placed in polyethylene bags, sealed securely, and stored in a specific container. Following sample collection, a serial dilution was carried out to isolate microbial colonies from the gathered specimens. PDA agar plates were inoculated with 10^{-2}, 10^{-3}, and 10^{-4} fungal dilutions and incubated at 28°C for 72 hours.

The modified Czapek Dox medium (containing 2 g of glucose, 10 g of L-glutamine, 1.52 g of monopotassium phosphate, 0.52 g of potassium chloride, 0.52 g of magnesium sulfate, 0.01 g of iron sulfate, 20 g of agar, and 1000 mL of distilled water) was used for screening fungal isolates for L-glutaminase activity in a plate assay. Additionally, 3 mL of a 2.5% phenol red stock solution in ethanol (pH 6.2) was incorporated into 1000 mL of the Czapek Dox medium.

Following 72 hours of incubation at 26±1°C, the presence of a pink zone surrounding the fungal colony in a predominantly yellow medium indicated L-glutaminase activity. The fungus demonstrating the highest productivity was identified and subsequently utilized to investigate the optimal conditions for L-glutaminase enzyme production through submerged fermentation.

The sesame oil cake was raw substrates were dried in the sun separately to decrease their moisture content, making them easier to crush. The substrates that had been crushed were individually passed through a sieve to produce a fine powder. Subsequently, the substrates were treated individually by soaking them in a 1% sodium hydroxide solution (NaOH) at a 1:10 ratio (substrate to solution) for two hours at room temperature. They were subsequently rinsed to eliminate chemical residues and autoclaved at 121°C for one hour. The substrates that had been treated were then filtered and washed with distilled water until the wash water became neutral.

The isolated culture of *Trichoderma viride* was preserved as a stock culture in Czapek-Dox agar slants. The cultures were grown at 37°C for 48 hours and subsequently kept at 4°C for routine subculturing. A 100 ml inoculum was prepared from the isolated pure culture using Czapek-Dox broth in 250 ml flasks. Before being utilized for fermentation, the inoculum flasks were maintained in a shaker at 200 rpm and 28°C for 48 hours. Fermentation medium (g/100ml) KH_2Po_4 – 0.2, $(NH_4)_2$ SO_4 – 0.14, Urea- 0.03, $MgSo_4$- 0.03, $CaCl_2$ – 0.03, $FeSo_4$-0.5, $MnSo_4$- 0.16, $ZnSo_4$- 0.14, $CaCl_2$ – 0.2 substrate (sesame oil cake) – 1g distilled water 100 ml pH-6.5-7.

Under controlled conditions, 10 ml of broth culture was added to 100 ml of the optimized culture medium, after which it was incubated in a shaker at 200 rpm and 37°C for one day.

Concurrently, a separate medium was prepared specifically for sesame oil cake substrates.

The fungus *Trichoderma viride* was individually grown in a fermented medium consisting of 100 ml of optimized media with biowaste substrate at 28 °C for 72 hours. Once the cultures had grown, the filtrates were obtained separately by means of centrifugation. The reaction mixture contained 0.5 ml of the prepared enzyme, 0.5 ml of 0.04 M L- glutamine, 0.5 ml of distilled water, and 0.5 ml of 0.1 M phosphate buffer (pH 8), and it was incubated at 37°C for 30 minutes.

After incubation, 0.5 ml of 1.5 M trichloroacetic acid (TCA) was introduced to terminate the enzymatic reaction. The blank was prepared by introducing the enzyme preparation after the TCA had been added. After taking 0.1 ml of the mixed solution, it was added to 3.7 ml of distilled H_2O, and subsequently, 0.2 ml of Nessler's reagent was incorporated. Absorbance readings were taken at 450 nm using a visible spectrophotometer. The definition of one IU of L-glutaminase (U) is the quantity of enzyme that produces 1 µmol of ammonia from glutamate when subjected to optimal assay conditions, with results expressed in U/ml.

The current research utilized sesame oil cake as a substrate to achieve the highest production of L-Glutaminase enzymes, along with an optimization study that investigated different pH levels, temperatures, sodium chloride concentrations, carbon and nitrogen sources, and various substrate concentrations (Fig. 1). The optimized media were formulated by utilizing sesame oil cake as the sole

substrate, with the pH adjusted to various levels, specifically 4, 5, 6, and 8, by incorporating 1% NaOH and concentrated HCl. Subsequently, each conical flask received an inoculation of broth culture and was set to incubate at temperatures of 25, 30, 35, 40, and 45°C, respectively. To investigate the strain's tolerance to salinity, sodium chloride was utilized at various concentrations (1%, 2%, 3%, 4%, and 5%). Different carbon sources (sucrose and lactose) and nitrogen sources (ammonium nitrate and urea) were incorporated into 100 ml of medium at various concentrations (1, 2, 3, 4, and 5 g each) in individual 250 ml flasks, which were sterilized using autoclaving.

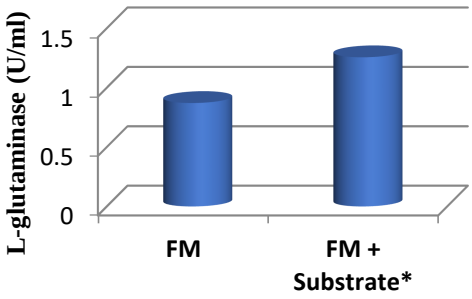

Fig. 1. Production of L-glutaminase enzyme using *Trichoderma viride*

FM–Fermented Medium

FM+ Substrate*–Fermented Medium + Sesame oil cake

The enzyme suspension was analyzed using the Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique, with 100 µg of protein loaded into each lane. The protein bands were evaluated using a Gel Documentation System (GDS) connected to a Computerized Image Analyzer (CMI), and the gels were scanned with a scanning densitometer.

Results and discussion

Fungal species in this research were isolated from a soil sample collected from a sugarcane field using the serial dilution approach with Potato Dextrose Agar. Three distinct fungal species were isolated in total, designated as IFS1, IFS2, and IFS3. The strains were characterized and identified through their cultural and morphological features. The final identification of the isolated fungi revealed them to be *Trichoderma viride, Aspergillus flavus,* and *Aspergillus niger*. L-glutaminase-producing activity in the isolated fungi was initially evaluated by growing them on Czapeck-Dox medium enriched with 1% L-glutamine. *Trichoderma viride* (IFS1) produced a clear zone around its colony in this study, suggesting positive L-glutaminase production, which was greater than that of *Aspergillus flavus* and *Aspergillus niger*.

Table 1. Effect of pH on the production of L-glutaminase using *Trichoderma viride*

Sl	pH	L-glutaminase (U/ml) (M±SD)
1	4	0.040±0.002
2	5	0.039±0.001
3	6	0.152±0.004
4	7	0.306±0.002
5	8	0.180±0.006

* Sesame oil cake; Values are expressed Mean ± Standard Deviation; n=3.

Table 2. Effect of Temperature on the production of L-glutaminase using *Trichoderma viride*

Sl	Temperature (°C)	L-glutaminase (U/ml) (M±SD)
1	25	1.029±0.014
2	30	1.256±0.016
3	35	1.132±0.012
4	40	1.163±0.017
5	45	1.128±0.014

* Sesame oil cake; Values are expressed Mean ± Standard Deviation; n=3.

L-glutaminase production was examined in this study by adding 1% sesame oil cake waste substrate to the fermentation medium. High enzyme activity, measured at 1.256±0.02 U/ml, was found in the substrate-supplemented fermentation medium, while lower productivity of 0.871±0.06 U/ml was observed in the medium without the substrate.

Given the elevated enzyme production observed in the sesame oil cake fermented medium, this substrate was selected for optimization studies focusing on various parameters, including pH, temperature, NaCl concentration, substrate concentration, and different carbon and nitrogen sources.

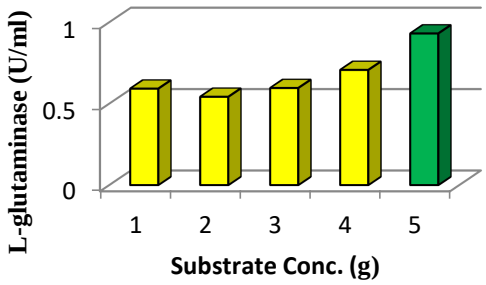

Fig. 2. Effect of different substrate conc. on the production of L-glutaminase using *Trichoderma viride*

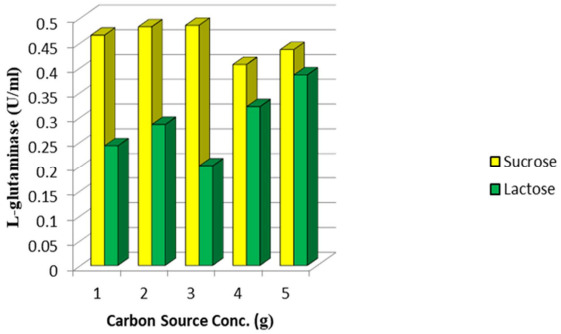

Fig. 3. Effect of carbon source different conc. on the production of L-glutaminase using *Trichoderma viride*

The productivity of L-glutaminase enzymes was optimized by supplementing media with a pH range from 4 to 8. The study found that *Trichoderma viride* had maximum enzyme productivity at pH 7, with a value of 0.306±0.002 U/ml (Table 1), in comparison to the other pH ranges tested. Optimization of L-glutaminase productivity was achieved using media at different temperature ranges, specifically from 25 °C to 45 °C (Table 2). Maximum enzyme productivity was noted at 30°C (1.256±0.012 U/ml) in this study. The productivity of L-glutaminase was optimized by utilizing media with varying NaCl concentrations ranging from 1 to 5 g individually. The study recorded maximum enzyme productivity at a concentration of 2 g NaCl in the medium (0.388±0.006 U/ml), which was higher than that in other concentrated media. L-glutaminase enzyme productivity was optimized by utilizing media with varying substrate concentrations ranging from 1 to 5 g individually. This study found that the medium with 5 g of substrate yielded the highest enzyme productivity of 0.931±0.010 U/ml. Different carbon supplementation media, such as sucrose and lactose, were used to optimize L-glutaminase enzyme productivity. Maximum enzyme productivity was found to be 0.485±0.013 U/ml with 3 g of sucrose in this study. The optimization of L-glutaminase productivity was achieved through the use of nitrogen supplements like ammonium nitrate and urea. The study found that ammonium nitrate at a concentration of 1 g yielded the maximum enzyme productivity of 1.176±0.010 U/ml compared to other concentrations (Fig. 2-4).

The enzyme's molecular weight was estimated via SDS-PAGE, where clear bands were seen in the high molecular weight area, specifically around 14-20 kDa. The findings showed that a single band was present in the SDS-PAGE, verifying that the enzyme preparation is homogeneous. The molecular weights of the purified enzymes were established by creating a graph that plots the logarithmic values of relative molecular mass against the Rf values. Based on this analysis, it was determined that the enzymes possess a molecular weight between 14.0 and 20.0 kDa.

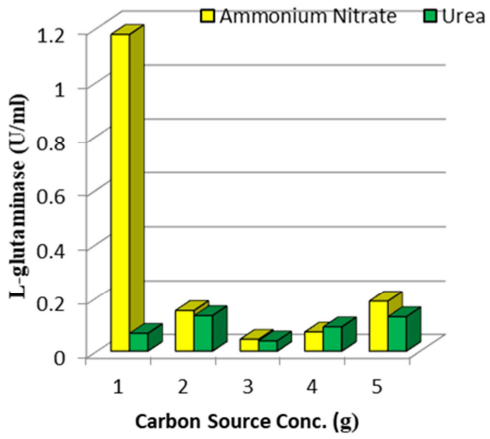

Fig. 4. Effect of nitrogen source different conc. on the production of L-glutaminase using *Trichoderma viride*

Conclusion

The present research involved isolating multiple fungal colonies from the soil sample. Identification of the L-glutaminase-producing organism was carried out based on its cultural and morphological features.

The colony was confirmed as *Trichoderma viride*, and this study found that sesame oil cake yielded the highest L-glutaminase production of 1.256±0.02 U/ml. This substrate served as the basis for additional optimization studies. In this optimization investigation, optimal L-glutaminase productivity was recorded at a pH of 7, a temperature of 30°C, a NaCl concentration of 2 g, and a substrate concentration of 5 g. The highest L-glutaminase productivity was recorded when ammonium nitrate was used as the nitrogen source.

At the same time, the greatest L-glutaminase productivity was recorded when sucrose was used as the carbon source. This study revealed noticeable bands in the high molecular weight area, specifically around 14-20 kDa. Ultimately, it was determined that the strain of *Trichoderma viride* produced significant amounts of L-glutaminase enzyme. As a result, it was evident from this study that sesame oil cake is an excellent substrate for L-glutaminase production by *T. viride*, surpassing other substrates.

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