Purification and Partial Characterization of Tannase produced by *Aspergillus niger* SCSGAF0145 using *Arachis hypogaea* (Groundnut) Shell

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**ABSTRACT**

Tannase have been widely secreted industrially by fungi using tannin rich agricultural waste as substrate. *Aspergillus niger* SCSGAF0145, was used for the secretion of tannase using groundnut shell as substrate under submerged fermentation. The crude tannase secreted was precipitated out using ammonium sulphate and purified on Sephadex G-150. Kinetic parameters of the secreted tannase were determined using tannic acid as substrate. The estimated biomass weight, tannin concentration, gallic acid concentration and tannase activity of the crude enzyme after fermentation was 81.00±0.08 mg, 1.31±0.01 mg/mL, 0.67±0.03 µg/mL and 6.04 U/mL respectively. The purification fold and enzyme yield after purification were 13.44 and 47.86%. The purified enzyme showed maximal activity at a pH of 5.5 and temperature of 35°C while producing $K_m$ and $V_{max}$ of 0.43 moles/L and 21.55 U/mL respectively using tannic acid as its sole carbon source. In conclusion, *Aspergillus niger* SCSGAF0145 secreted tannase of high activity using groundnut shell as source of tannic acid which could be utilized for various industrial purposes.

**Keywords:** *Aspergillus niger*, gallic, groundnut shell, tannase.

**I. INTRODUCTION**

Enzymes are important organic compounds required in various industrial processes for the production of a wide range of edible and non-edible materials to meet human needs. One of such enzyme is tannase, which catalyses the hydrolysis of ester bonds in hydrolysable tannins (tannic acid) yielding glucose and gallic acid (Yao et al., 2014; Belur & Mugeraya, 2011). Consequently, it has been used extensively in various industries for the clarification of wine, beer, coffee flavored soft drinks and in the manufacture of instant tea (Lekshmi et al., 2021). Tannase is also environmental friendly because it can break down tannins found abundantly in agricultural wastes to gallic acid. Tannase is produced widely by microorganisms (fungi or bacteria) via fermentation of suitable tannin rich substrates (Prakash & Deedi, 2020; Dhiman et al., 2018).

Tannins are a group of water soluble polyphenolic compounds naturally found in plants and has been reported to be the fourth most abundant plant constituents after cellulose, hemicellulose and lignin (Ire & Nwanguma 2020). Nutritionally, tannins are anti-nutrients considered undesirable because their presence in food causes reduction in nutritional value by forming complexes with protein especially digestive enzymes and starch (Thakur et al., 2019).

Their astringent nature also poses as problems in food production. They are also found in industrial effluent and agro industrial wastes which may result to serious environmental problems. Thus, reduction of tannins by hydrolysis to its constituent is needed. This has been achieved by both acid and enzymatic hydrolysis (Radenkovs et al., 2018). However, it has been widely reported that the enzymatic hydrolysis preferable to acid hydrolysis because it requires less energy and less pollution (Hadi et al., 1994).

Gallic acid or trihydroxybenzoic acid is phenolic acid found abundantly in plant parts free or as part of tannins. It can be isolated from plants or produced by acid hydrolysis of tannins or via fermentation of tannins present in plants. However, the fermentation process is more preferred due to low cost, high yield and high purity of gallic acid obtained (Saeed et al., 2021). Apart from being a good antioxidant (Gao et al., 2008), it is used as a raw material for manufacturing of the preservatives, propyl gallate (Malgireddy et al., 2015). In pharmaceutical industry it is a precursor of 3, 4, 5-trimethoxy benzaldehyde which is converted to trimethoprim, a broad spectrum antibiotic while finding relevance in the ink and dye industry (Mohapatra et al, 2020).

Groundnut shell is the outermost layer of the groundnut seed which accounts for 25-35% weight of groundnut pod (Sada et al., 2013). Globally, Nigeria is having been rated the
fifth-largest producer of groundnut of about 2.4 million tons on 2.8 million ha of land (FAOSTAT, 2019). Thus, a large quantity of ground nut shells is generated annually which constitutes environmental nuisance before they are non-biodegradable, however, the presence of macromolecules such as cellulose, hemicellulose, and lignin qualifies them for use as source of biofuel (Duc et al., 2019), animal feed (Jekayinfa et al., 2020). Other reports on the utilization of groundnut shell includes production of oil (Radhakrishnan and Gnanamoorthy, 2015), bioethanol (Olafimihan et al., 2015; Nayachaka et al., 2013) and production of briquettes (Oyelaran et al., 2015). The study is aimed at using groundnut shell as a substrate for the production of the enzyme, tannase using Aspergillus niger.

II. METHODOLOGY

A. Collection and Preparation of Substrate
Groundnut shells were obtained on the campus of the Lagos State Polytechnic, Ikorodu. The shells were washed with sterile distilled water, drained, and dried at 50 °C. The dried materials were ground to powder and kept in sterile airtight bags until needed for further experiments.

B. Source and Standardization of Innoculum
The fungal strain Aspergillus niger used in this study was obtained from Microbiology laboratory of Lagos State Polytechnic, Ikorodu. It was previously isolated from soil sample and identified to species level using 18s rRNA analysis.

The fungal spores were harvested from 72-hour old cultures grown on PDA/tannic acid agar slants by adding 10 mL of sterilized normal saline and a few drops of sterilized Tween-80 followed by vortexing. The spore suspension obtained was filtered through sterile cotton to ensure the removal of hyphal fragments and standardized to approximately 3×10⁶ spores used for further analysis.

C. Fermentation of Agricultural Waste
Tannase was produced by submerged fermentation of crude tannin of ground nut shell according to the method of Mohapatra et al. (2006). The powdered samples (50 g) were mixed with distilled water (200 mL) and kept at room temperature overnight. After soaking, the mixture was boiled for 10 mins. The filtered solution was used as source of crude tannin. The pH of the medium was adjusted to 5.0 after sterilization. Fermentations were carried out through submerged fermentation of crude tannin at 35 °C in 250 mL Erlenmeyer flasks containing 50 mL medium with 1% (v/v) fresh inoculum. The cell-free fermented broth was used as the source of the enzyme. The growth of the organism in culture media was monitored by measuring dry weight of the biomass (mg). The biomass was separated by centrifugation and supernatant was used source of crude tannase.

D. Enzyme Assay
The obtained filtrate was used for extracellular tannase determination using the method reported by Libuchi et al. (2000). Into a clean dry test tube reaction mixture was prepared by taking 0.5 mL of crude enzyme of different concentration and 2 mL of 0.35% (w/v) tannic acid in 0.05 M citrate buffer (pH 5.5) solution. 0.1 mL of the reaction mixture was withdrawn from the total system and 2 mL of ethanol solution was used to stop enzyme reaction. Absorbance on UV spectrophotometer was noted as t₁ at 310 nm immediately after adding ethanol at t₂ after 30 mins of incubation at 37 °C. One unit of tannase activity is defined as the amount of enzyme required to liberate 1M of gallic acid/min under defined conditions. Enzyme activity was - expressed as U/mL.

E. Estimation of Gallic Acid
The gallic acid concentration in the cultured broth was estimated using the method of Bagpai and Patil (1996). The culture supernatant of 1 mL was dissolved in 9 mL of citrate buffer at pH 5.0 and absorbance was measured at 254.6 nm and 293.8 nm using UV spectrometer. The concentration was deduced using the equation below:

Gallic acid (µg/mL) = 21.77(A₂₉₃.₈) – 17.17(A₂₉₀₃.₈)  

F. Estimation of Total Tannin
The total tannin content in the waste was determined using modified method of Price and Butt (1977). The reaction mixture containing the extract (0.5 mL), Potassium ferricyanide K₃Fe(CN)₆ (1%, 0.1 mL) and Ferric chloride FeCl₃ (1%, 1 mL) was made up to 10 mL with distilled water. The absorbance was measured at 720nm using tannic acid as standard. The total tannin content was extrapolated using a calibration curve (R² = 0.969) for tannic acid.

G. Enzyme Extraction and Purification
The crude tannase was purified from cell free medium and precipitated out up to 40% with ammonium sulphate and allowed to precipitate for 6 hrs at 4 °C. This precipitate was discarded, and the supernatant was saturated with ammonium sulphate up to 80%, which was kept overnight for residual enzyme precipitation. This precipitate was dissolved in 20 mL of 1 mM citrate buffer (pH = 6.0) and was further purified by Sephadex G-100 column chromatography. The protein was eluted at a flow rate of 0.5 mL/min. The fractions were collected, and absorbance was measured at 280 nm. Peak fractions were pooled and analysed for protein concentration and tannase activity.

H. Protein Content Determination
Protein was estimated in the crude supernatant as described by Lowry et al. (1951). Protein extract, 0.2 mL was measured into tubes and 0.8 mL of distilled water was added to it. Distilled water (1.0 mL) was utilized as blank, while Bovine Serum Albumin (BSA) standard curve was equally set up (100 µg/mL) 10 -100 µg/mL, 50 mL of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 mins thereafter 0.5 mL of Folin-Ciocalteu solution was added to all the test tubes and left for 30 mins after which the optical density was read in the spectrophotometer at 280 nm. The protein concentration was estimated using the values extrapolated from the standard calibration of the protein.

I. Enzyme Kinetics
The fraction with highest tannase activity obtained from column chromatography was used to determine the Km and Vmax of tannase. This was determined by Lineweaver-Burk plots of reciprocal reaction velocities versus reciprocal substrate concentrations (0.05 -2.0 mg/mL) tannic acid in
citrate buffer (pH 6.0). The activity of purified tannase was determined at temperatures ranging from 20 to 60 °C under standard conditions while the determination of the optimum pH, the enzyme was assayed with citrate buffer ranging from pH 4.0 to 7.0.

III. RESULTS

The biomass weight of the organism was estimated to be 81.00±0.08 mg. From Table I, there was increase in gallic acid concentration in the broth after fermentation while a decrease was observed in tannic acid.

Table II shows the characteristic of each purification stage. The enzyme was purified by ammonium sulphate precipitation at 80% and components were separated on column chromatography. It was observed that the tannase activity and protein concentration increased down the purification steps while an increase was observed for specific activity, yield and purification fold.

![Fig. 1. Chromatogram of gel filtration for tannase by Aspergillus niger.](image)

From Fig. 1, above, three peaks were observed, however, that fraction 3 has the highest tannase activity.

![Fig. 2. Line weaver-Burk plot of the purified tannase.](image)

From the Line- weaver Burk plot (Fig. 3), $K_m$ was deduced to be 0.43 moles/L while $V_{max}$ is 21.55 U/mL.

![Fig. 3. Effect of pH for tannase activity.](image)

IV. DISCUSSION

Filamentous fungi play important role in the production of many industrial enzymes due their rapid growth rate on many substrates including agro wastes and ease of gene manipulation. Tannase have been reported to be secreted by various fungal strains (Aguilar-Zárate et al., 2014), such as Aspergillus which is considered to be the best producer, followed by Penicillium (Sabu et al., 2005), Fusarium and...
Trichoderma (Raghuvanshi et al., 2011). In this study, Aspergillus niger SCSGAF0145 was employed for tannase production using ground nutshell. From Table I, the tannase secreted utilised tannins in groundnut shell to produce gallic acid. This is evident in the reduction of tannin concentration by 54.95±0.03% after fermentation and concomitant increase in gallic acid concentration by 75.89±0.21%. The biomass weight obtained in this study further confirms the suitability of groundnut shell as substrate for tannase secretion. It is worthy to note that tannase is an inducible enzyme secreted only in the presence of suitable substrates, tannic acid (Reges de Sena et al., 2014). The tannase activity of the crude enzyme obtained in this study is accord with the study of Sherief et al., (2011) who reported tannase activity of 5.20 to 16.41 IU/g by various species of Aspergillus using green tea as substrate under solid state fermentation.

The purity of the enzyme purification was quantitatively measured and presented on Table II. The crude enzyme is a mixture of various protein and minerals, thus, as purification progresses, there is a decrease in total tannase activity and protein concentration due to loss of some proteins. However, the loss results to increase in specific activity of the purified enzyme. The percentage yield and purification also indicate the purity of the enzyme while the yield is the amount of substrate concentration on activity. The purity of the enzyme purification was quantitative after each purification stage. The lower the percentage yield and purification also indicate the quality of the purified enzyme.

Kinetic parameters, maximum velocity \( V_{\text{max}} \) and Michaelis constant \( K_{\text{m}} \) of tannase was extrapolated from Line weaver-Burke plot obtained by considering the effect of varying substrate concentration on activity. \( K_{\text{m}} \) is the affinity an enzyme has for its substrate. In this study, \( K_{\text{m}} \) is estimated to be 0.43 moles/L which indicates strong affinity of the enzyme to its substrate because, low \( K_{\text{m}} \) value corresponds to high affinity for substrate. \( V_{\text{max}} \) estimated to be 22.55 U/mL is the maximum reaction velocity at which all enzymes become saturated with substrate. In this study, \( V_{\text{max}} \) and temperature are pH important parameters that affect enzyme activity. In this study, maximal tannase activity was observed at 35°C under submerged fermentation, however, researchers have reported tannase optimum temperature to be 30°C for various Aspergillus species A. niger (Cruz-Hernández et al., 2006; Treviño-Cueto et al., 2007), A. aculeatus DBF9 (Banerjee et al., 2007) and A. tamarii (Costa et al., 2008) under similar fermentation process. The effect of pH on tannase activity presented on figure 4, showed optimum pH of 5.5. This is in accord with reports of various researchers who observed optimal tannase activity in the neutral or acidic pH range (Bradoo et al., 1997; Lokeswari & Jaya Raju, 2007).

V. Conclusion

Aspergillus niger SCSGAF0145 used a novel agrowaste, groundnut shell as sole source of tannic acid for gallic acid via simultaneous secretion of tannase. Further partial purification and characterization of the enzyme substantiates its high specificity and activity in the utilization of tannic acid, thus, can be utilized for various industrial purposes.

Conflict of Interest

Authors declare that they do not have any conflict of interest.

References


DOI: http://dx.doi.org/10.24018/ejbio.2022.3.5.399

Vol 3 | Issue 5 | September 2022


