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Biosynthesis of gallic acid and tannase from *Dacryodes edulis* (African Pear) seeds using *Aspergillus niger* SCSGAF0145 by submerged fermentation

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Abstract

Aspergillus niger SCSGAF0145 previously isolated and identified by our group as a tannase producer from soil was grown under submerged fermentation using pear seed as source of tannic acid. Crude tannase was precipitated out of the cell free broth and purified by gel filtration gave a purified enzyme with specific activity of 6.65 U/mg, with a final yield and purification fold of 42.08% and 5.94 respectively. The deduced V_{max} of the purified tannase was 17.57 U/mL and K_m was 1.59 mg/mL. The optimal temperature and pH of the enzyme using tannic acid as sole carbon source was 40 ˚C and 5.5 respectively. Conclusively, pear seed is a good substrate for gallic acid and tannase production by *Aspergillus niger* SCSGAF0145 and tannase secreted can be explored in various industrial processes.

Keywords: *Aspergillus niger* SCSGAF0145; Tannase; Gallic acid; Pear seed

1. Introduction

Gallic acid or trihydroxybenzoic acid is a phenolic acid found abundantly in various plants species existing free or as constituent of polyphenolic compounds, tannins. This compound is of great economic importance due to their medicinal and industrial uses. Gallic acid has been widely reported to possess antioxidant activity and have been packaged as supplements to meet human needs. Aside being an antioxidants, it possesses antimicrobial, anticancer, antioxidant, antiviral and antitumor agent. [1,2]. It has also been documented to protect against neurodegenerative disease [3], anaphylaxis [4] and peptic ulcers [5]. Industrially, gallic acid is the precursor of trimethoxy benzaldehyde, a compound used in the ink and dye industry while in the pharmaceutical industry, 3, 4, 5 trimethoxy benzaldehyde is converted to trimethoprim, a broad spectrum antibiotic [6]. It also serves as a raw material for manufacturing an intermediate for anti-oxidants, preservatives like propyl gallate [7]. From the afore mentioned uses of gallic acid the global demand for gallic acid exceeds 8,000 tons annually and its extraction from plants would be tedious and expensive, thus, alternative methods have been employed viz acid and enzymatic hydrolysis of tannic acid [8]. However, the enzymatic hydrolysis via bacterial or fungal fermentation of tannins in agricultural waste materials is preferred for gallic acid production due to low cost, high yield and high purity of the products obtained [9,10,11]. This is achieved by the enzyme tannase or tannin acyl hydrolase (EC 3.1.1.20).

Tannase catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid and gallic acid esters, releasing glucose and gallic acid [12]. It is an important enzyme with various industrial applications especially

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in the elimination of water-insoluble precipitates (called "tea cream") during the manufacture of instant tea [13], to improve the flavour of grape wine, clarification of beer and fruit juices, in coffee-flavoured soft drinks and production of gallic acid [14]. The most important method to obtain tannase is via microbial process, by majorly using the filamentous fungi of the *Aspergillus* and *Penicillium* genus [15]. They are the most promising microorganisms reported as capable of producing tannase through submerged and solid state fermentation [16]. However, the use of submerged fermentation (SmF) is beneficial because of its enhanced process control and relatively simple sterilization method [17,18]. Microbial production of tannase is also environmental friendly because it reduces pollution by breaking down tannins found abundantly in tannin rich agricultural wastes [19,20].

Tannin are found abundantly in plants and with the help of tannase producing microorganism, they serve as means of producing gallic acids in large quantity. Waste materials such as cashew husk, rice bran, plantain flour [21], Pomegranate rind [22] have been used as source of tannins. Use of these materials help to reduce environmental pollution, serve as the source of single cell protein and production of the enzyme tannase [23]. In this present study, *Dacryodes edulis* seed was used as source of tannin for the production of tannase by *Aspergillus niger.*

2. Material and methods

2.1 Collection and preparation of substrate

Pear fruits were obtained fresh and flesh was removed. The seeds were washed with sterile distilled water, drained and dried in hot air oven at 50˚C. The dried materials were ground to powder and kept in sterile air tight bags until needed for further experiments.

2.2 Source and standardization of Inoculum

The fungal strain *Aspergillus niger* used in this study was previously isolated from soil and identified to species level using molecular identification based on their ITS region. The fungal spores were harvested from 72 hrs 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 3×10^6 spores used for further analysis.

2.3 Fermentation of agricultural waste

Tannase was produced by submerged fermentation of crude tannin of pear seed. 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 were 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.

2.4 Enzyme assay

The obtained filtrate was used for extracellular tannase determination using the method reported by Libuchi *et al*., [24]. Into a clean dry test tube reaction mixture was prepared by taking 0.50 mL of crude enzyme of different concentration and 2 mL of 0.35% (w/v) tannic acid in 0.05M 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 t_1 at 310 nm immediately after adding ethanol at t_2 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.

2.5 Estimation of Gallic Acid

The gallic acid concentration in the cultured broth was estimated using the method of Bagpai and Patil [25]. 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 spectrometric. The concentration was deduced using the equation below:

$$\text{Gallic acid } (\mu g/mL) = 21.77(A_{254.6}) - 17.17(A_{293.8}).$$

2.6 Estimation of Total tannin

The total tannin content in the waste was determined using modified method of Price and Butt [26]. The reaction mixture containing the extract (0.5 mL), Potassium ferric cyanide $K_3Fe(CN)_6$ (1%, 0.1 mL) and Ferric chloride $FeCl_3$ (1%, 1 mL) was made up to 10 mL with distilled water. The absorbance was measured at 720 nm using tannic acid as standard. The total tannin content was extrapolated using a calibration curve (R^2 = 0.969) for tannic acid.

2.7 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 6hrs 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 measure at 280nm. Peak fractions were pooled and analyzed for protein concentration and tannase activity.

2.8 Protein content determination

Protein was estimated in the crude supernatant as described by Lowry *et al*. [27]. 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, 5.0 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.

2.9 Enzyme kinetics

The fraction with highest tannase activity obtained from column chromatography was used to determine the K_m and V_{max} 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.

2.10 Identification of gallic acid using Thin Layer Chromatography

Ethylacetate was added to the crude filtrate at ratio 1:1 in a separating funnel, mixed thoroughly and was allowed to stand for 10 mins to allow separation. The organic phase was collected and reduced to half the volume before spotting on TLC plates. The plate was developed in the solvent system consisted of ethyl acetate, chloroform, formic acid and methanol at ratio 3:3:5:0.5 after developing, the plate was air dried, the spots on the plate was identified with iodine vapour.

3. Results and discussion

The choice of a substrate for enzyme and subsequent product formation by fermentation depends largely on the cost, availability and suitability of the substrate to achieve the desired product. Tannins are the fourth most abundant compound in agro-industrial residues especially plants [28]. In this study, pear seed was utilized used as substrate for tannase and gallic acid production. Tannin concentration before and after fermentation was deduced to be 3.78±0.04 mg/mL and 2.41±0.07 mg/mL respectively amounting to 36.14% loss (Table 1).

Table 1 Biomass weight, tannin and gallic acid concentration

	Biomass weight (mg)	Tannin (mg/mL)	Gallic acid (µg/mL)
Before fermentation		2.41± 0.07	0.51±0.01
After fermentation	38.25±0.01	3.78±0.04	1.28±0.02
% yield		-	60.16±0.21
% decrease		36.14±2.01	-

Values are expressed as Mean ± SD of three determinants

The reduction in tannin concentration after fermentation indicates degradation of tannin to gallic acid and glucose. This was confirmed by growth of organism in the media evidence in biomass weight of 38.25±0.01 mg (Table 1). The enzymatic hydrolysis of tannin produces gallic acid. In this study a 60.16±0.21% yield of gallic acid was obtained and confirmed by their identification in the fermentation broth using TLC (figure 1). Two spots were observed for the sample, with spot 1 having same Rf value of 0.46 with gallic acid standard.

Figure 1 TLC analysis of Crude Enzyme and gallic acid standard

Table 2 Purification of tannase produced by *Aspergullus niger* SCSGAF0145

Fractions	Tannase activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude filtrate	7.900	7.06	1.12	100	1
(NH4)2SO4 precipitation	4.583	1.84	2.49	58.01	2.22
Sephadex G 150	3.325	0.50	6.65	42.08	5.94

Tannase produced under submerged fermentation is a mixture of proteins, thus was purified by precipitation with ammonium sulphate and gel filtration using Sephadex G-150. Quality of each stage was quantitatively measured and presented on table 2. From figure 2, the absorbance at 280nm of fractions obtained from gel filtration was presented. Peaks were observed, however, the peak with highest tannase activity was used for tannase characterization. Tannase is an inducible enzyme secreted in the presence of its substrate. The tannase activity in the crude enzyme was 7.90 U/mL, however, as purification progressed, the tannase activity reduced to 3.325 U/mL in the purified tannase with specific activity of 6.65 U/mg with a final yield and purification fold of 42.08% and 5.94 respectively. 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 indicates the purity of the enzyme. The purification fold is the increase of purity of the enzyme while the yield is the amount of activity retained after each purification stage. The lower the purification fold and yield the better the quality of the purified enzyme.

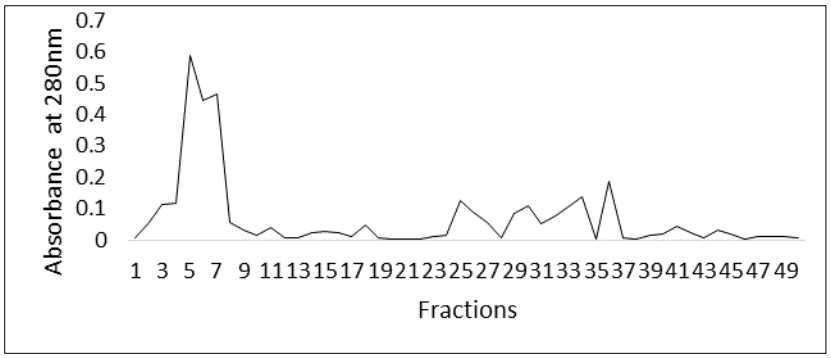

Figure 2 Chromatogram of gel filtration for tannase by *Aspergillus niger* SCSGAF0145

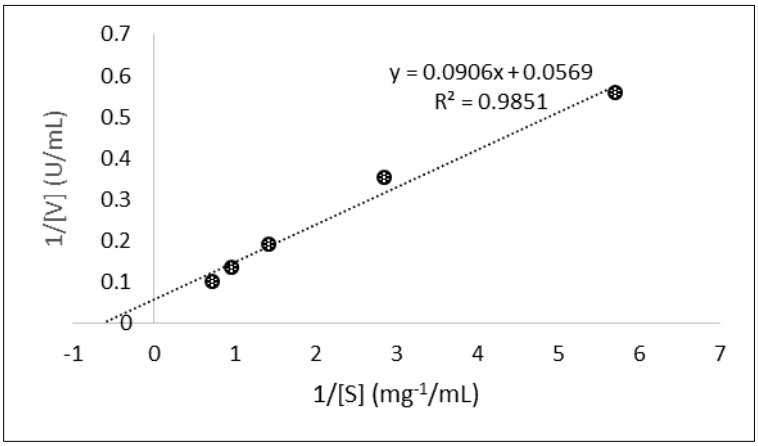

Figure 3 Line weaver-Burk plot of tannase

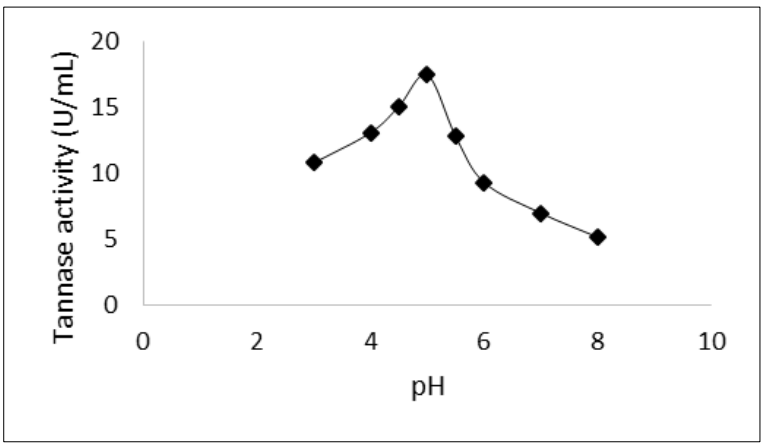

Figure 4 Effect of pH on tannase activity

The effect of substrate concentration, pH and temperature on tannase activity were presented on figure 3, 4 and 5 respectively. The maximum velocity V_{max} and Michealis constant K_m of tannase was extrapolated from Line weaver-Burke plot to be 17.57 U/mL and 1.59 mg/mL respectively. V_{max} is the maximum reaction velocity at which all enzymes become saturated with substrate while K_m is the affinity an enzyme has for its substrate. The lower K_m value the higher the affinity of the enzyme for the substrate. Though the K_m observed in this study is low, a lower value of 0.1133 M was recorded by *Aspergillus niger* MTCC5889 produced tannase from cashew testa [29]

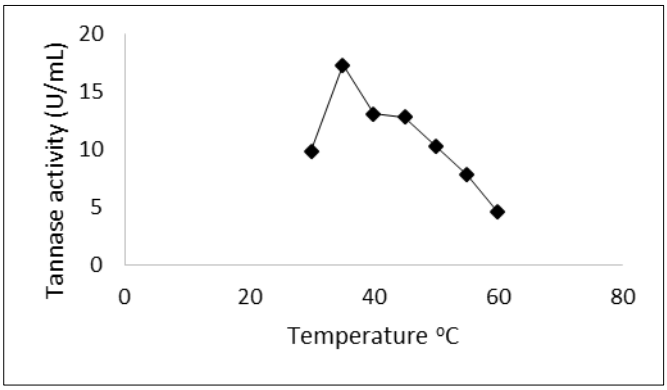

Figure 4 Effect of temperature on tannase activity

The optimum pH for tannase activity in this study is 5.5 with enzymatic activity reaching 17.52 U/mL. The results of the current study conforms to the reports of various researchers who observed optimal tannase activity in the neutral or acidic pH range *Aspergillus* species [30, 31, 32]. In this study, maximal tannase activity was observed at a temperature of 35˚C under submerged fermentation. This conforms to the work of Al-Mraai et al., [33], however, many researchers have reported optimum temperature to be 30˚C for various *Aspergillus* species *A. niger* [34, 35], *A. aculeatus* DBF9 [36] and *A. tamarii* [37] under similar fermentation process.

4. Conclusion

Aspergillus niger SCSGAF0145 used in this study secreted tannase by SmF using pear seed waste as a substrate for the production of this enzyme. Purified tannase also showed good characteristics suitable for optimization and utilization for various industrial processes especially food industry.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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