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Production of tannase and gallic acid by three fungal strains under submerged fermentation using agricultural waste

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Abstract

Fungi are the predominant source of tannase for industrial production though other microorganisms are being exploited for industrial production and applications. In this study the ability of *A. versicolor*, *F. equiseti* and *P. citrinum* to secrete tannase using pineapple peel and acacia fruits was assessed. The pineapple peel and acacia fruits were used as source of tannins for tannase and gallic acid production under submerged fermentation. The tannin concentration in pineapple peel was higher than acacia. After fermentation, acacia was better utilised by the three fungal strains producing the least tannin concentration, higher biomass weight, gallic acid concentration, protein concentration and tannase activity. However, *A. versicolor* best utilized the two substrate compared to *F. equiseti* and *P. citrinum*. In conclusion pineapple peel and acacia nuts are good substrate for tannase and gallic acid production using the fungi strains *A. versicolor*, *F. equiseti* and *P. citrinum*.

Keywords: Agricultural wastes; Tannase; Gallic acid; Fungi and Fermentation

1. Introduction

Agricultural processes generate solid organic wastes such as tree trimmings, grass clippings, manure, crop residues such as groundnut straws and shells, rice husk and straws, maize stalk, husk and cobs, cassava stalk and peels, beans pods, cotton stalk, sugarcane bagasse and leaves, various fruit peels and seed [1]. These farm organic wastes constitutes up to 80 percent of the total solid wastes generated in Nigeria with manure amounting up to 5.27 kg/day/1000 kg live weight, on a wet weight basis [2]. These wastes contains many reusable substances of high value but they are usually underutilized by a majority of Nigeria farmers. These large volumes of biomass to a large extent are used as energy and raw materials depending on the availability of adequate technology. However, they can be used as raw materials for development of new products of industrial and economic importance [3]. In addition to the waste generated on farms, consumers in highly populated cities in Nigeria also generate agricultural waste dumped on landfills. These wastes are often left to decay in the open, resulting to environmental pollution and pose as hazard to health. They can also be burnt and this contributes greatly to global warming. Thus, efforts are being geared towards the waste to wealth program by utilization of these solid wastes as economical raw material for the production of important products of great significance and impact industrially. For example, agricultural solid wastes can be recycled as unusual ingredients in feed production to enhance food security by boosting animal protein production [4]. In the past, these waste materials have been exploited for the microbial production of enzymes with industrial applications including cellulase [5], tyrosinase [6], laccase [7] and tannase and so on. These enzymes are produced by the action of fungi or bacteria on these wastes under controlled experimental conditions in submerged or solid state fermentation.

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Tannin acyl hydrolase or tannase, is an inducible enzyme that catalyzes the hydrolysis of ester bonds and hydrolysable tannins in peptide such as tannic acid, releasing glucose and gallic acid [8, 9]. The filamentous fungi are recognized as great producers of this enzyme, and species of the genera *Aspergillus* and *Penicillium* stand out in this respect [10]. The filamentous fungi are featured in fermentation processes, as they can secrete substantial quantities of proteins in culture media [11]. Tannase utilize tannins as their sole carbon source.

Tannins are found abundantly in plants and their presence in food produces bitter taste. It has great economic value especially in photography and printing inks, production of an anti-microbial drug trimethoprim, in manufacturing propyl gallate used as antioxidants in fats and oils. The hydrolysis of tannins by tannase produces gallic acid as by product. Gallic acid exhibits wide range of biological activities, such as anti-tumor, anti-bacterial, anti-diabetes, anti-obesity, anti-microbial and anti-myocardial ischemia [12, 13, 14, 15]. Other than gallic acid production, tannase is used extensively in the preparation of instant tea, wine, beer, and coffee-flavored soft drinks and also as an additive for detannification of food. A potential use of tannase is in the treatment of waste water contaminated with polyphenolic compounds such as tannic acids [16]. The yearly demand for gallic acid is very large and amounts to 8,000 tons [17]. At present gallic acid is produced industrially by acid hydrolysis of naturally occurring gallotannins, however, due to high cost, low yield of desired product and production of large toxic effluent by acid hydrolysis, an enzyme based eco-friendly technology for gallic acid production is urgently required. Microorganisms are known to degrade tannic acid by producing tannases [18, 19]. Tannase applications are diversified among food, pharmaceuticals and rations industries. Other uses of tannase might include leather tanning, instant tea manufacturing and clarifying agent for juices, beers, some wines and sodas that have coffee as component [20].

The objectives of this study were to evaluate the production of tannase and gallic acid from *A. versicolor*, *F. equiseti* and *P. citrinum*a using pineapple peel and acacia fruits.

2. Material and methods

2.1. Collection preparation of substrate

Pine apple peel and acacia dried fruits were obtained on the campus of the Lagos State Polytechnic, Ikorodu. The plant samples were washed with sterile distilled water, drained and dried at 50 ˚C. The dried materials were ground to powder before soaking (50 g) in sterile distilled water overnight. The soaked sample was filtered and the filtrate was used as crude tannin and immediately used for production of tannase.

2.2. Production of Tannase in Submerged Fermentation (SMF)

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 hyphae fragments. Approximately, 5×10^6 spores were inoculated in 100 mL of plant tannins in 250 mL Erlenmeyer flasks. The flasks were now incubated at 30 ˚C with intermittent shaking for five days. After incubation for the desired period, the fungal mycelia were removed by centrifugation and filtration through Whatman No.1 filter paper. The supernatant was treated as crude enzyme and used to determine protein concentration, gallic acid concentration and tannase activity.

2.3. Tannase Activity

The extracellular tannase activity in the crude enzyme extract was determined using the method of Libuchi *et al*., [21]. 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 0.3% (w/v) tannic acid in 0.005M citrate buffer (pH 5.5) solution. 0.1 mL 0f 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 10 minutes 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.

2.4. Protein assay

The total protein content in the culture filtrate was estimated by Lowry's method [22], using bovine serum albumin (BSA) as standard. The protein concentration was expressed as mg of protein per mL of sample used.

2.5. Estimation of Total Tannin

The total tannin content of the agro waste was determined using the method of Onwuka [23]. The grounded sample (0.5 g) was shaken constantly for 1 min with 3 mL of methanol in a test tube and then poured into a Buchner funnel with the suction already turned on. The tube was quickly rinsed with an additional 3 mL of methanol and the content poured at once into the funnel. The filtrate was mixed with 50 mL of water and analyzed within an hour for aqueous extraction, 5 mL of water was used for the extraction and for the rinse and the filtrate was added to 50 mL of water 3 mL of 0.1M $FeCl_3$ in 0.1N NH_4Cl was added to 5 mL of the extract and followed immediately by timed addition of 3 mL of 0.008M potassium ferrocyanide. Absorbance was then taken at 720 nm spectrophotometrically.

2.6. Estimation of Gallic Acid

The gallic acid concentration in the cultured broth was estimated using the method of Bagpai and Patil [18]. The culture supernatant of 1 mL was dissolved in 9 mL of acetate buffer at pH 5.0 and absorbance was measured at 254.6 nm and 293.8 nm using UV spectrophotometer. The concentration was deduced using the equation below:

$$\text{Gallic acid } (\mu g/mL) = 21.77(A_{254.6}) - 17.17(_{A293.8})$$

2.7. Statistical analysis

All data was presented as the mean of three separate experiments using Microsoft excel 2013.

3. Results and discussion

Tannins, rated as the fourth most abundant component of plants contributes greatly to environmental pollution due to their resistant to microbial attack and inhibiting growth of some microorganisms involved in biodegradation of soil organic matter [24, 25]. The choice of a substrate for enzyme and subsequent product formation by fermentation depends on the cost, availability and suitability of the substrate for obtaining the desired product of fermentation, thus, requires screening of several agro-industrial residues [26]. Acacia tree are found abundantly within the premises of Lagos State Polytechnic, Ikorodu and their dried fruits have constituted environmental pollution, thus, its selection for this project. The tannin concentration in pineapple peel was higher than acacia (Table 1) while the tannin concentration in each fermentation broth and percentage biodegradation was presented on table 2. It was observed that there was a decrease in tannin concentration in all the fermentation flasks but *A. versicolor* showed the least tannin concentration using acacia nut extract as substrate. The reduction in tannin concentration indicates the degradation of tannin by the organisms.

Table 1 Concentration of tannin in plant extract

Plant Samples	Concentration (mg/mL)
Pineapple	10.15 ± 0.28
Acacia	9. 76 ± 0.08

After fermentation, biomass weight, gallic acid concentration was determined alongside tannase activity and protein concentration (Table 2). The results of biomass weight shows that the organisms used in this study *A. versicolor*, *F. equiseti* and *P. citrinum*a utilized the tannin extract obtained from the agricultural waste for growth. *A. versicolor* had highest biomass weight for acacia as substrate while for *F. equiseti* had the least biomass weight for both pineapple and acacia extract. This shows that the fungi can be grown abundantly on acacia nuts for the production of single cell protein. Single cell protein are dried cell mass of fungi, moulds and bacteria used as protein supplement in animal and human feed to augment their diet [27].

Gallic acid is found naturally in plants but due to their wide application and demands industrially they are produced by microbial hydrolysis of tannin using the enzyme tannase. *A. versicolor* produced the highest gallic acid concentration of 9.42±0.39 mg/mL using acacia as substrate while *F. equiseti* had the least gallic acid concentration. This further confirms the secretion of the enzyme tannase by the fungi and the suitability of the substrate for these organisms.

Table 2 Characteristics of the fermentation broth

Organism	Substrate	Tannin concentration (mg/mL)	Biodegradation *of* tannin (%)	Biomass weight (mg)	Gallic acid (mg/mL)	Protein concentration (mg/mL)	Tannase activity (U/mL)
A. versicolor	Pineapple	5.03±0.08	51.94±0.31	0.19±0.03	0.49±0.17	12.75±0.35	3.57
	Acacia	1.53±0.05	80.98±1.88	0.23±0.04	9.42±0.39	26.09±0.75	22.49
F. equiseti	Pineapple	4.43±0.41	57.87±3.95	0.07±0.01	3.75±0.27	10.70±0.67	1.53
	Acacia	2.96±0.32	63.46±1.26	0.11±0.02	4.58±0.48	21.32±1.14	12.99
P. citrinum	Pineapple	3.57±0.21	67.88±0.92	0.14±0.04	8.62±0.43	24.65±0.70	5.61
	Acacia	1.83±0.04	77.28±1.14	0.17±0.04	8.57±0.31	21.06±0.38	16.23

Tannase is an inducible extra-cellular enzyme produced by a number of animals, plants and microbes, has wide application in tannery, alcohol industry, pharmaceuticals and beverage industries. Many researchers has reported the secretion of tannase by bacteria and fungi using various agricultural materials as substrate. Tannase utilization can be carried by direct contact of enzymatic extracts with the tannin rich substrate or growing tannase-producing fungal strains on tannin-rich materials, degrading them to simpler compounds [28, 29]. In this study, the latter method was utilized. Tannase activity was highest in acacia nut extract produced by *A. versicolor* (22.49 U/mL). The tannase activity obtained in this study was relatively higher than that obtained in the work of Lima et al., [30] using Mangaba leaf, Acerola leaf, Residue mangaba and Residue acerola as substrate for *A. vesicolor* and *P. citrinum*. However, there exist similarity in the higher activity observed in *A. vesicolor* than *P. citrinum*.

4. Conclusion

From the results obtained in this study, it can be concluded that pineapple peel and acacia nuts are good substrate for tannase and gallic acid production using the fungi strains *A. versicolor*, *F. equiseti* and *P. citrinum,* however, *A. versicolor* showed higher activity compared to the other fungal strains used in the study.

Compliance with ethical standards

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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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