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Isolation and molecular identification of tannase producing fungi from soil

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The study was conducted to isolate and identify tannase producing fungi from soil. Soil samples were collected aseptically and cultured on tannic acid agar (TAA) to screen for tannic acid hydrolysis. The observed isolates were sub-cultured to obtain pure culture and were later subjected to secondary screening in the Czapek medium. The biomass weight, tannase activity, and gallic acid concentration in the fermentation broth were determined using standard protocols. Fungal isolates with promising tannase activity were further used to produce tannase using acacia nuts and pine apple peel as sources of tannin. From the results obtained, a total of seven isolates showing good tannin degradation in both primary and secondary screening were identified by phylogenetic characterization as *Penicillium citrinum*, *Aspergillus niger* strain, *P. citrinum* isolate K9, *A. niger* strain 7806F, *Fusarium equiseti* isolate GS-WW-F14-13, *Aspergillus versicolor* isolate 777 and *A. niger* strain SCSGAF0145. Under submerged fermentation, *A. versicolor* had the highest tannase activity of 22.49±1.17 U/mL using acacia nuts as substrate. In conclusion, the isolated fungal strain is a good tannase producer which can be explored for industrial processes.

Key words: Fungi, tannase, gallic acid.

INTRODUCTION

Fungi have been exploited as a tool for manufacturing various highly important products, such as food in the form of single cell proteins, beverages, organic acids, and drugs. Bon et al. (2008) report that after antibiotics, enzymes are the main products obtained from fungi by biotechnological industries. Filamentous fungi play important role in enzyme production, because of their rapid growth rate on many substrates, ease of gene manipulation cum handling, and their ability to produce numerous biotechnological applicable metabolites. Of industrial importance is the enzyme tannase which has 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

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Penicillium (Sabu et al., 2005). Other fungal strains reported in the literature to be tannase producers are *Fusarium* and *Trichoderma* (Raghuwanshi et al., 2011).

Tannase is a hydrolytic enzyme that breaks down tannin especially tannic acid into glucose and gallic acid. Industrially, tannase is used in the clarification of fruit juice that is rich in tannins and aimed at reducing the astringency of such products (de Lima et al., 2014; Selwal and Selwal, 2012). Gallic acid released from tannin degradation is used in the production of antibiotics, trimethoprim, used in the pharmaceutical industry, and pyrogallol or ester gallates which are used as food preservatives (Malgireddy et al., 2015). Tannase is also important in controlling environmental pollution by removing tannins, mainly polyphones present in tannery effluents.

Tannins are plant secondary metabolites described as the fourth most abundant group overhauled by polysaccharides: cellulose, hemicellulose, and lignin. Apart from their astringent nature which has been explored in drug development, nutritionally they are undesirable because they possess the ability to precipitate proteins from food (Girdhari and Peshwe, 2015; Murugan and Saleh, 2010). Tannins are readily available in agro-industrial waste and enhance their resistance to microbial attack. They are known to inhibit the growth of some microorganisms which slow down the rate of biodegradation to soil organic matter, thus, constituting an environmental pollutant. Consequently, tannase producing microorganisms can use such agro-industrial waste as a substrate for secreting tannase which hydrolyses tannin to yield gallic acids in large quantities.

Waste materials such as cashew husk, rice bran, plantain flour, banana peel (Gaayathiri et al., 2020), almond and mango leaves (Ire and Nwanguma, 2020), wheat bran, eucalyptus leaves, pomegranate peel, banana peel, and guava (Ahmed et al., 2020) have been used as the source of tannins. The use of these materials helps to reduce environmental pollution and serves as the source of single cell protein and production of the enzyme tannase (Seth and Chand, 2000). The present investigation was aimed at isolating fungal tannase from soil samples and determining the ability of the isolated fungi to degrade tannin-rich agricultural wastes (groundnut husk and acacia nuts).

METHODOLOGY

Materials and chemicals

The investigation only utilized analytical-grade compounds.

Sample collection

Samples of five soil were collected from soil down different tannin-rich plants (Almond, Malay apple, Neem, Banana and Cassia) within the premises of Lagos State Polytechnic, Ikorodu, Lagos State, Nigeria and were transported to the laboratory for direct use.

Media

The main screening of fungal isolates and the isolation of tannase-producing fungi were done using tannic acid agar medium (TAA) (Pinto et al., 2001). The following ingredients were used in its preparation: 10 tannic acid; $NaNO_3$ 3; KCl 0.5; $MgSO_4{\cdot}7H_2O$ 0.5; KH_2PO_4 1.0; $FeSO_4{\cdot}7H_2O$ 0.01; agar 30. The medium was sterilized for 15 min at 121°C and adjusted to pH 4.5 +/- 0.2. Tannic acid solution was added to the medium after being sterilized separately using a membrane filter with a 33 mm diameter and 0.22 m pore size from Millipore in France.

For secondary screening of tannase-producing fungus, the modified Czapek-Dox minimum medium was employed (Bradoo et al., 1997). The following components (g/L) were used to create it: 10 tannic acid; $NaNO_3$, 6; KCl, 0.52; $MgSO_4{\cdot}7H_2O$, 0.52; KH_2PO_4, 1.52; $FeSO_4{\cdot}7H_2O$, 0.01; $ZnSO_4{\cdot}7H_2O$, pH 4.5 0.2; Cu $(NO_3)_2{\bullet}3H_2O$, 0.01. As specified in the preparation of the TAA medium, sterilization and pH correction were completed.

For the maintenance of fungal isolates, potato dextrose agar (PDA) supplemented with 0.01% tannic acid was utilized (Bajpai and Patil, 1996). The following components, g/l, were used to create it: agar 15, glucose 20, potatoes (200 g), infusion 0.1 tannic acid, and pH 5.6-0.2.

Isolation of tannase-producing fungi

With sterile distilled water, each soil sample was serially diluted (10^{-1} to 10^{-6}). Using the pour plate method, 1 mm from each dilution was plated into TAA medium. The plates were incubated at 30°C for 72 h under aerobic conditions. Selection and purification of fungi capable of growing and creating clearing zones around their colonies (Murugan et al., 2007). The acquired cultures were cultivated on PDA with 0.01% tannic acid supplementation, slants, and kept at 4°C (working cultures). Cultures of stock were kept in paraffin oil.

Screening and selection of tannase-producing fungal cultures

Using TAA plates in accordance with Bradoo et al. (1997) instructions, a primary screening for the highest tannase producers was completed (1996). The isolate was point injected onto the plates, and they were then incubated at 30°C. After 72 h of incubation, the diameter of clear zones (including the diameters of the colonies created as a result of the hydrolysis of tannic acid) around the fungal colonies was measured, then compared to determine the top tannase producers. Fungal cultures with strong tannase activity in the main screening were exposed to submerged fermentation for secondary screening (Batra and Saxena, 2005; Bradoo et al., 1997).

Each possible tannase-producing fungus's spore count was 5107, and 250 mL Erlenmeyer flasks containing 50 mL of sterilized modified Czapek-minimum Dox's medium (pH 4.5, 0.2) were used for inoculation. At the conclusion of the fermentation period, the extracellular tannase activity per flask was measured after cultures were grown at 30°C for 96 h.

Identification of tannase producing microorganisms

The fungal isolates were identified based on their morphological and microscopic characteristics. The molecular identification of the isolates up to species level is based on their ITS region carried out

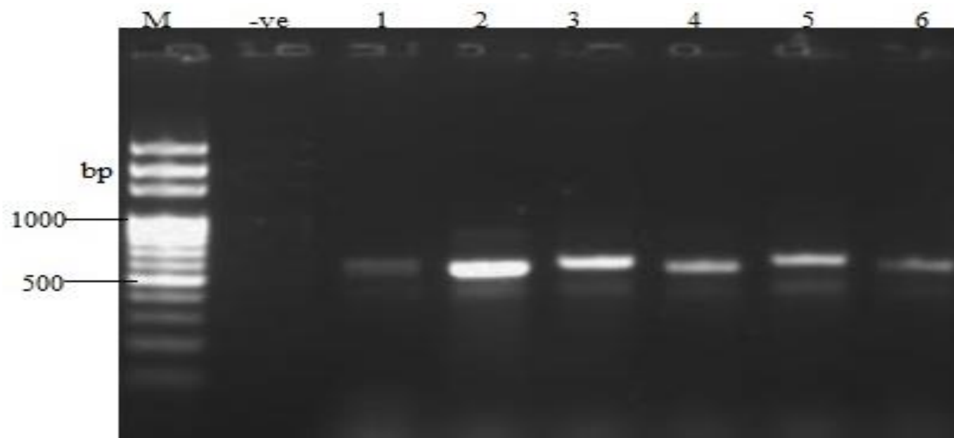

Figure 1. PCR amplication image of 18SrRNA gene bands of the fungi isolated from soil. Lane 1: DNA marker, Lane 2-6 are the 18SrRNA of the isolates.

by extracting their DNA using the Zymo Fungal/Bacteria using a DNA extraction kit as directed by the manufacturer. The ITS gene of the fungus was amplified by polymerase chain reaction using the primer pairs ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out using the Solis Biodyne 5XX Hot Firepol Blend Master mix according to the manufacturer's instructions (Figure 1).

Thermal cycling was done in an Eppendorf Vapoprotect thermal cycler (Nexus Series) for 35 amplification cycles, each lasting 30 s at 95°C, 1 min at 58°C, and 1 min 30 s at 72°C. A final extension phase lasting 10 min at 72°C was then performed. On a 1.5% agarose gel, the amplification product was separated, and electrophoresis was then done at 80 V for 1 h and 30 min. DNA bands were visible after electrophoresis, thanks to ethidium bromide staining. As a benchmark for DNA molecular weight, a 100 bp DNA ladder was employed. Exo sap was used to clean up the PCR products before they were delivered to Epoch Life science in the USA for Sanger sequencing.

Harvesting the enzyme and enzyme assay

Through Whatman No. 1 filter paper, the fermentation medium (Czapek-minimum Dox's medium) was purified. The resulting filtrate was used to measure extracellular tannase according to the described procedure by Libuchi et al. (1972). The reaction mixture was made by adding 2 mL of 0.3% (w/v) tannic acid in a 0.005 M citrate buffer (pH 5.5) solution to 0.5 mL of crude enzyme in various doses. To stop the enzyme reaction, 0.1 mL of the reaction mixture was removed from the whole system and 2 mL of an ethanol solution was added. After adding ethanol, the absorbance on the UV spectrophotometer was reported as t1 at 310 nm and t2 after 10 min of incubation at 37°C. The quantity of enzyme necessary to release 1M of gallic acid/min under specific circumstances is referred to as one unit of tannase activity. In U/mL units, enzyme activity was expressed.

Estimation of gallic acid

Using Bagpai and Patil's (1996) approach, the amount of gallic acid in the cultured broth was estimated. 1 mL of the culture supernatant was dissolved in 9 mL of citrate buffer at pH 5.5, and the absorbance was determined using UV spectrometric methods at 254.6 and 293.8 nm. The equation was used to determine the concentration:

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

Estimation of total tannin

The modified Price and Butt (1977) technique was used to calculate the waste's total tannin content. The reaction mixture was produced up to 10 mL with distilled water and contained the extract (0.5 mL), potassium ferric cyanide K3Fe(CN)6 (1%, 0.1 mL), and ferric chloride $FeCl_3$ (1%, 1 mL). Tannic acid was used as the standard to measure the absorbance at 720 nm. An extrapolation of the total tannin concentration was done using the tannic acid calibration curve (R2 = 0.9984).

Protein content determination

According to Lowry et al. (1951)'s description, the crude supernatant's protein content was estimated. 0.8 mL of distilled water was added to 0.2 mL of protein extract after it was measured into tubes. As a blank, distilled water (1.0 mL) was used, and a 100 g/mL Bovine Serum Albumin (BSA) standard curve was also built up. All of the tubes received 5.0 mL of alkaline solution containing 10 to 100 g/mL, which was added, carefully mixed, and left to stand for 10 min. After adding 0.5 mL of Folin-Ciocalteu solution, each test tube was given 30 min to settle before the optical density was measured at 280 nm in the spectrophotometer. Using data derived from the protein's standard calibration, the protein concentration was approximated.

Fermentation of agricultural waste

According to the approach used by Mohapatra et al. (2006), tannase was produced by the isolated organism by submerged fermentation of crude tannin from several agro-residues. The plant samples were collected aseptically in sample bags, cleaned, and dried in an oven at 60°C before fermentation. The samples were then processed via a grinder mixer until they were powdered. The powder was utilized as a source of crude tannins in submerged

Table 1. Microscopic and macroscopic features of fungal isolates.

Isolate	Macroscopic features	Microscopic features	Species identified
$PLA3_2$	Yellowish, cotton-like	Long, erect conidiospores, round shaped	*Penicillium* spp.
$CA6_2$	Black and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$CA6_1$	Yellowish, cotton-like	Long, erect conidiospores, round shaped	*Penicillium* spp.
$PLA3_1$	Brown center and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$PLA5_1$	Brownish-yellow and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$PLA6_1$	Yellow pink colonies creamy	Cylindrical to ovoid conidia, curved septate conidiospores	*Fusarium* spp.

Table 2. Identification of fungal isolates with ITS region of rRNA gene sequence.

Isolate	Species identified	Length (bp)	Identity (%)
$PLA3_2$	*Penicillium citrinum*	537	100
$CA6_2$	*Aspergillus niger*	575	99
$CA6_1$	*Penicillum citrnum*	523	91
$PLA3_1$	*Aspergillus niger*	570	94
$PLA5_1$	*Aspergillus vesicolor*	671	96
$PLA6_1$	*Fusarium equiseti*	570	92

fermentation and kept in a dry location in sterilized bottles at room temperature. The distilled water (200 mL) was combined with the powdered samples (50 g) and left at room temperature overnight. The mixture was simmered for 10 min after soaking. Crushed tannin was obtained from the filtered solution. After sterilization, the medium's pH was adjusted to 5.0. Crude tannin was fermented and submerged at 35°C in 250 mL Erlenmeyer flasks containing 50 mL medium and 1% (v/v) fresh inoculum. The enzyme was obtained from the cell-free fermented broth. We measured the dry weight of the biomass to track the organism's development in culture conditions (mg). By centrifuging the biomass, the biomass was separated, and the supernatant was used for tannase test.

RESULTS AND DISCUSSION

Soil inhabits diverse species of microorganisms including fungi and bacteria that have been predominantly used for the industrial production of enzymes. The biodiversity and variation of fungi isolated from soil depend on factors such as geographical locations, salinity, soil pH, moisture content, organic carbon, nitrogen sulfur, and potassium content affects (Sharma and Raju, 2013; Yu et al., 2007). Different fungi have been isolated and identified from soil including *Aspergillus, Penicillium*, and *Mucor* (Chandrashekar et al., 2014; Gaddeyya et al., 2012). In this study, tannase producing fungi were isolated from soil samples down tannin-rich plants including plantain, cocoyam, Malay apple, yam, pawpaw, cashew, almond, cassava, and neem. The fungal isolates were firstly identified to a genus level using morphological and microscopic features by considering the color of colonies formed at both sides, the top and reverse of the fungal cultures, and the shape of the spore-producing structures as shown in Table 1 (The suspected organism to the genus *Aspergillus*, *Fusarium,* and *Penicillium*). This method of fungal identification is limited to the family or genus level (Wang et al., 2016; Lutzoni et al., 2004). Therefore, molecular identification utilizing DNA barcoding and ITS region sequencing was done for authentication and identification down to the species level. One of the most crucial sequences is that of the ITS rDNA region and widely used instruments for identifying fungal species because they are widely dispersed, consistently functioning, and sufficiently preserved to offer a thorough understanding of evolutionary connections (Anderson and Parkin, 2007; Madigan et al., 2012). The sequence analysis of the ITS regions of the nuclear-encoded rDNA showed significant alignments of 90 to 100% with the isolated fungal species. The isolates were identified to be *Penicillium citrinum*, *Aspergillus niger* strain, *Penicillium citrinum* isolate K9, *A. niger* strain 7806F, *Fusarium equiseti* isolate GS-WW-F14-13, *Aspergillus versicolor* isolate 777, and *A. niger* strain SCSGAF0145 (Table 2). All the fungi isolated in this study have been previously reported to be tannase producers viz: *Aspergillus versicolor* (Batra and Saxena, 2005), *P. citrinum* (Lekha and Lonsane, 1997), *A. niger* (Wkil et al., 2020) except *F.equiseti*.

The identified fungi presented in Table 2 are good tannase producers based on the results presented in Table 3. The diameter of clear zones around the isolated colonies depicts their ability to degrade tannic acid to gallic acid and glucose. The *Aspergillus* strains produced higher diameter of clearance zone compared to *Fusarium* and *Penicillium* strains. This is substantiated considering their biomass weight, gallic acid concentration and tannase activity in the fermentation broth by utilizing

Table 3. Primary and secondary screening of isolates.

Organism	Clear zone diameter (mm)	Biomass weight (g)	Gallic acid concentration (µg/mL)	Tannase activity (IU/mL)
Penicillium citrinum	18 ± 0.1	0.11±0.01	7.62±0.18	3.24±0.14
Aspergillus niger	26 ± 0.06	0.21±0.01	8.02±0.10	4.83±0.22
Penicillum citrnum	15 ± 0.1	0.07±0.02	7.98±0.15	3.92±0.12
Aspergillus niger	25 ± 0.06	0.19±0.02	8.16±0.31	5.83±0.18
Aspergillus vesicolor	25 ± 0.1	0.13±0.01	7.98±0.23	4.77±0.02
Fusarium equiseti	17 ± 0.12	0.04±0.04	7.50±0.33	2.64±0.33

Values are expressed as Mean±SD of triple determinants.

Table 4. Concentration of tannin in plant extract.

Plant sample	Concentration (mg/mL)
Pineapple	10.336 ± 0.12
Acacia	7. 785 ± 0.08

Values are expressed as Mean±SD of triple determinants.

Table 5. Concentration of tannin in plant extract.

Organism	Substrate	Tannin concentration (mg/mL)	Biodegradation (%) of tannins	Gallic acid concentration (mg/mL)	Protein concentration (mg/mL)	Tannase activity (IU/mL)
A. versicolor	Pineapple	5.03±0.08	51.94±0.31	0.49±0.17	12.75±0.35	3.57±0.41
	Acacia	1.53±0.05	80.98±1.88	9.42±0.39	26.09±0.75	22.49±1.17
F. equiseti	Pineapple	4.43±0.41	55.87±3.95	3.75±0.27	10.70±0.67	1.53±0.17
	Acacia	2.96±0.32	63.46±1.26	4.58±0.48	21.34±1.14	12.99±1.17
P. citrinum	Pineapple	3.57±0.21	67.88±0.92	8.26±0.43	24.65±0.70	5.61±0.53
	Acacia	1.83±0.04	77.28±1.14	8.57±0.31	21.06±0.38	16.23±0.66

Values are expressed as Mean±SD of triple determinants

tannin acid as their sole carbon source. Pinto et al. (2001) reported a correlation between colony diameter (zone of clearance) and tannase production using *A. niger* strains. Thus, these isolates can be used as a source of the enzyme for tannin bioconversion process (Shete and Chitanand, 2015).

In this study, the ability of *A. versicolor*, *F. equiseti* and *P. citrinum* to secrete tannase using agricultural wastes-pineapple peel and acacia nuts as substrate was assessed. The total tannin content of the plant material was presented in Table 4. The tannin concentration of pineapple peel was higher than acacia nuts. 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, and thus, requires screening of several agro-industrial residues (Pandey et al., 1999). Acacia trees are found abundantly within the premises of Lagos State Polytechnic, Ikorodu and their nuts have constituted important environmental pollutants, thus, its selection for this project. The tannin concentration in each fermentation broth was determined. 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 implies the degradation of tannin by the organisms. The utilization of the agricultural waste was substantiated by the gallic acid concentration in each fermentation flask.

The results of biomass weight (Table 5) show 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 the highest biomass weight for acacia as substrate while *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, molds, and bacteria used as protein supplements in animal and human feed to augment their diet (Yalcın et al., 2009).

Tannase, an inducible extra-cellular enzyme produced by several animals, plants, and microbes, has wide application in tannery, alcohol industry, pharmaceuticals and beverage industries. Many researchers have reported the secretion of tannase by fungi using various agricultural materials as substrate (Reges de Sena et al., 2014). Tannase activity was highest in acacia nut extract produced by *A. versicolor* (21.146 U/mL) by submerged fermentation.

Conclusion

The present study revealed the presence of fungi in various soil samples with the ability to secrete tannase. The fungi strains identified are *A. versicolor*, *F. equiseti* and *P. citrinum* and showed good activity using pineapple peel and acacia nuts as substrates evident in the reduction in tannin concentration of various substrates and gallic acid concentration, however, *A. versicolor* showed higher activity compared to the other fungal strains used in the study. In conclusion, the isolated fungal strain is good tannase producers which can be explored for industrial processes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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