Comparative Evaluation on Tannase Production by *Lasiodiplodia plurivora* ACN-10 under Submerged Fermentation (SmF) and Solid State Fermentation (SSF)

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Authors’ contributions

This work was carried out in collaboration between both authors. Author FSI designed the study. Author ACN performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FSI and ACN managed the analyses of the study. Authors FSI and ACN managed the literature searches. Both authors read and approved the final manuscript.

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**ABSTRACT**

**Aim:** Tannase (tannin acyl hydrolase, E.C. 3.1.1.20) catalyzes the hydrolysis of ester bonds from complex hydrolysable tannins with the production of gallic acid and glucose and possess broad applications in biotechnology. This study is aimed at the production of tannase by *Lasiodiplodia plurivora* ACN-10 in SmF and SSF using *Terminalia cattapa* (almond leaves) and *Magnifera indica* (mango leaves) as substrates.

**Study Design:** The design adopted to evaluate the production of tannase is submerged (SmF) and solid state (SSF) fermentation.

**Study Area:** Federal Institute of Industrial Research Oshodi, Lagos State Nigeria.

**Methodology:** Fifteen different soil samples were indiscriminately collected within Oshodi, Lagos, Nigeria and were inoculated on PDA plates at 30°C for 3-5 days. A total of 30 isolates was screened on Czapek dox minimal agar incorporated with 1% tannic acid and plates were incubated for 96 h at 30°C. Fungal isolates which were able to disintegrate tannic acid produced a clear halo

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zone around the colony diameter and were selected to be positive for tannase activity. The best isolate was identified based on its morphological, microscopic and molecular characteristics. Thereafter production and extraction of tannase was carried out in SmF for 0-120 h and in SSF for 0-144 h using *Terminalia cattapa* (Almond leaves) and *Magnifera indica* (Mango leaves) as substrates.

**Results:** The total fungal count ranged from $1.0 \times 10^4$ to $3.5 \times 10^5$ CFU/g. A total of 30 fungal isolates produced clear halo zones (ranging from 20 to 70 mm) around the colonies during the screening with tannic acid. Isolate ACN-10, which showed the highest tannic degradation was identified based on its morphological and microscopic characteristics. On the basis of 18S rRNA gene sequence studies, the isolate was identified as *Lasiodiplodia plurivora* strain ACN-10 and the sequence was submitted to the Genbank with the accession number: MG250374. Results obtained in this study indicated that both substrates can be used by the isolate for tannase production in both SmF and SSF. The result of our investigation on the use of *Terminalia cattapa* (almond leaves) and *Magnifera indica* (mango leaves) as substrates for tannase production showed that optimum yield (6.064 U/ml) was obtained at 120 h in SSF while optimum production (4.623 U/ml) was observed in SmF at 96 h using *Terminalia cattapa* as substrate.

**Conclusion:** Results obtained from this study indicated higher tannase production in solid-state fermentation compared to submerged fermentation. This is the first report to the best of our knowledge that *Lasiodiplodia plurivora* strain is implicated in tannase secretion. The result also demonstrated high production of extracellular tannases from low cost substrates which can be optimized and scaled up for industrial processes.

**Keywords:** Tannase; solid state fermentation; submerged fermentation; almond leaves; mango leaves.

1. INTRODUCTION

The tannase, equally referred to as tannin acyl hydrolase (EC 3.1.1.20) hydrolyse ester bonds and side hydrolyzable tannins such as tannic acid with the release of gallic acid and glucose. Tannases are water-soluble phenolic complex and possess chattels to blend with cellulose, protein, pectin and gelatin (Lekha and Lonsane 2013). Tannins are broken down chemically through acidification or biologically through the action of tannase [1]. This group of phenolic compound known is distinguished from other plant secondary phenolics in their chemical and biological activities. They are regarded as polyphenolic secondary metabolites of plants which are found in large arrays of herbaceous and woody plants. The formation of intricate protein and erstwhile macromolecules such as cellulose, minerals and starch demonstrate their major characteristics [2,3]. This enzyme possesses inducible ability which accelerates the decomposition of intermolecular ester bonds in different substrates in the vein of gallo tannins, gallic acid esters, epicatechin gallate; epigallocatechin-3-gallate, releasing glucose and gallic acid [4]. The degradation and catalysis of hydrolyzable tannins like methyl gallate, tannic acid, n-propylgallate ethyl gallate, and isoamyl gallate is carried out by tannase. Fungi are predominant producers of tannase besides yeast and bacteria [5]. They possess the ability to degrade tannin as sole carbon source Aguilar and Gutierrez Sanchez [3] Several fungal genera have been reported to produce tannase which include Aspergillus, Penicillium, Trichoderma, Rhizopus, Mucor Batra and Saxena, [6]; Abou-Bakr et al. [7]; Lima et al. [8]; Murad et al. [9]; Zakipour et al. [10]. Tannase has wide applications in several industrial processes such as in the manufacture of instant tea, production of coffee-flavoured soft drinks, manufacture of gallic acids, clarification of fruit juices, beer and wine, among others (Juliana et al., 2014; Murad et al. [9]; Beena et al. [11]; Agbo and Spradlin, [12]. Tannase is utilized as an important intermediary necessary for the production of trimethoprim Sabu et al. [13]. Gallic acid invariably is used in healthcare, photography, production of inks and paints (Mukherjee and Banerjee [14]. Tannase is produced industrially using either submerged (SmF) or solid state fermentation (SSF). However, SSF is preferred due to some advantages it posse over SmF which include low cost of bioprocess operations, limited use of water, ease of use of agrowaste materials as substrates as well as high yield of the product (Juliana et al., 2014). In Nigeria, most of the plant waste materials from pulp industrials such as leaves of some plants constitute a huge environmental pollution. This is because they are indiscriminately discarded or
allowed to litter or burnt without recourse to the impact it may have on the environment. This problem could be solved by utilizing some of these agrowaste materials such as Terminalia cattapa (almond leaves) and Magnifera indica (mango leaves) as sources of carbon and nitrogen for the production of tannase by certain microorganism using either SmF or SSF. Therefore, this present study reports the comparative assessment of tannase production by Lasiodiplodia plurivora ACN-10 under submerged and solid state fermentation using agricultural waste materials from Terminalia cattapa (almond leaves) and Magnifera indica (mango leaves) as substrates.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fifteen different soil samples were indiscriminately collected within Lagos (Oshodi), Nigeria. All samples were stored in sterile screw capped containers, conveyed to the laboratory and preserved at 4°C in the refrigerator till further processing.

2.2 Chemicals and Reagents

Analytical grade chemicals were obtained from Hi-media and sigma.

2.3 Isolation and Screening Medium

Potato dextrose agar (PDA) medium consisting of 1% tannic acid was utilized for the isolation. Czapek dox minimal medium was employed for qualitative and quantitative (primary and secondary screening) screening of isolates.

2.3.1 Pour plate technique

One gram of each of the soil samples were aseptically suspended in 10ml of sterile water to make microbial suspension. Serial dilutions of 10^3, 10^5 and 10^8 were made by pipetting 1ml into additional dilutions containing 9ml of sterile distilled water. Finally, 1ml aliquots of various dilutions were plated out to which 15-20 ml of sterile cool, molten PDA medium (45°C) was added. Upon solidification, the plates were incubated at 30°C for 3-5days. Fungal colonies which developed were isolated and purified by repeated sub culturing Girdhari and Peshwe [15].

2.3.2 Screening for tannase producing fungi

A total of 30 isolates was screened. Circular bits (2 mm) diameters were inoculated on czapek dox minimal agar incorporated with 1% tannic acid and plates were incubated for 96 h at 30°C. Fungal isolates which were able to disintegrate tannic acid produced a clear halo zone around the colony diameter and were selected to be positive for tannase activity. The zone diameter and colony diameter were recorded according to the method of Hamada et al. [5].

2.4 Identification of Fungal Isolates

2.4.1 Morphological identification

Tannase producing fungal isolates were studied for various morphological character of which include the macroscopic characteristics: colour, shape, texture, margins and zonation and microscopic features: conidia, conidiophores, hyphae, and phylliades.

2.4.2 Molecular characterization of fungal isolates

The molecular base of the isolate was determined using 18S rRNA gene technology. Genomic DNA of fungus isolate was amplified using universal primers for 18S rRNA gene of fungus. The PCR amplification was carried out in 0.2 ml PCR tubes with 25μl reaction volume with composition. To eliminate any risk of contamination from extraneous DNA, the reaction mixture without template was simultaneously run along with PCR reaction as control. Amplifications were done using thermal cycler (Bio rad) with a temperature profile standardized for 18S rRNA gene amplification Nisha [16].

2.5 Production of Tannase in SSF

2.5.1 Substrates

2.5.1.1 Magnifera indica (Mango leaves)

Fresh mango leaves were plucked from trees within Oshodi, Lagos, washed thrice over running water, dried in the drying oven at 50°C for 72h and ground to a fine powder. The pulverized powder was properly placed in polyethene for further use.

2.5.1.2 Terminalia cattapa (Almond leaves)

Fresh almond leaves were plucked within Oshodi, Lagos, washed thrice over running water.
water, dried in the drying oven at 50°C for 72 h and ground to fine powder. The pulverized powder was properly placed in polythene for further use.

2.5.1.3 Inoculum preparation for SSF

Inoculum were prepared according to the procedure adopted by Ramirez-Coronel et al. [17]. The fungal isolates were cultivated on PDA slants; after which they were incubated for 6 days (96-144 h) at 30°C until a clear sporulation was achieved. The spores were then scraped into a sterile 0.02% Tween 80 solution and counted using haemocytometer. Then 3 ml of spore suspension was inoculated into the autoclaved substrates (mango and almond leaves) moistened with the mineral solution and incubated at 30°C for 144 h.

2.5.2 Tannase production and extraction

Solid state fermentation was performed in 250 ml conical flask containing 50 g of substrates (mango and almond leaves) each moistened with 50 ml mineral solution in a ratio of (1:1). The contents of the flasks were autoclaved at 121°C at 15 psi for 45 min.

2.5.2.1 Extraction

At desired intervals (24 h), the flasks were taken out and contents were extracted with 20 ml of extraction buffer (0.05 M Citrate buffer, pH 5.5) and incubated in the shaker incubator for 2 h at 150 rpm. Thereafter, the content of the flask was filtered through sterile muslin cloth and centrifuged at 6000*g for 15 min. The culture supernatant was utilized as a source of crude tannase for further analysis.

2.6 Determination of Tannase Activity

The spectrophotometric procedure described by Sharma et al. [18] was applied to determine tannase activity using tannic acid as substrate and gallic acid as calibration standard. A 0.25 ml of 0.01 M tannic acid in 0.05 M citrate buffer, pH 5.0 was put in test tube and 0.5 ml of extracted enzyme was added to the substrate and incubated at 30°C for 10 min. Also, 0.3 ml of methanolic rhodanine (0.667% w/v Rhodanine in methanol) was added and left for 5 min. Thereafter, 0.2 ml of 0.5 M KOH solution was equally added along with 3.5 ml of distilled water. The mixtures were incubated at 30°C for 10 min and pink color formed were read at 520 nm wavelength using the spectrophotometer. The control which is buffer only without enzyme and blank which is without enzyme and substrate were all treated same as sample and read at 520 nm. Calculation of tannase activity was based on the change in absorbance as one unit of tannase activity is described as the quantity of enzyme required to liberate a micromole of gallic acid per minute under specific reaction condition.

2.7 Protein Determination

Protein was estimated in the crude supernatant as described by Lowry et al. [19]. 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 ug/ml) 10 mg-100 ug/ml, 5.0 ml of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 min thereafter 0.5 ml of Folin-Ciocalteu solution was added to all the test tubes and left for 30 min 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.

3. RESULTS AND DISCUSSION

Fungi are the predominant source of tannase for industrial production though other microorganisms are being exploited for industrial production and applications therefore, various literature revealed the presence of tannase.
enzyme in plants, bacteria and fungi. In this context, during this present study, Lasiodiplodia plurivora ACN-10 tannase producing fungus was isolated from samples collected from these areas rich in phenolic and tannin rich compounds which could act as natural substrates for tannases.

3.1 Isolation and Screening of Fungi Isolates

Table 1 shows total fungal count of the different soil samples. Result showed that out of fifteen different soil samples randomly collected within Oshodi, Lagos Nigeria, the total fungal count (TFC) ranged from 1.0×10⁴ to 6.5×10⁵ CFU/g. The highest TFC was observed in sample ACN 10 while the least was obtained in sample ACN 12. A total of thirty (30) fungal isolates were screened on Czapek Dox Minimal agar containing 1% tannic acid as screening medium. Screening of fungal isolate Isolates for tannase production as expressed in Table 2, indicated that isolate ACN 6³, 9² and 10¹ showed highest tannase ability with a diameter of inhibition of 70 mm, respectively, followed by isolate ACN 1² which showed an inhibition of 65 mm, while Isolate 7⁴ had the least tannase activity with an inhibition diameter of 5 mm. Thereafter, 10¹ was used for further study in both SmF and SSF. Nisha [16] isolated tannase producing fungi and bacteria from various tannin rich sources such as pine forest soil, garden soil, apple orchard soil of different areas of Himachal Pradesh India. Melo et al. [20] isolated tannase producing fungi (Aspergillus sp.) from Brazilian caves. Mario et al.[21] Isolated and evaluated tannin-degrading fungal strains from Mexican desert. Girdhari and Peshwe [15] collected and screened tannase producing fungi from various environmental sources such as tea waste dump site, agro-residue waste sites and site near tannery industries. Depanjali and Gardener [22] investigated tannase production by Aspergillus niger isolated from the bark of tannin rich Acacia nilotica.

3.2 Screening of Fungal Isolates for Tannase Production

Tannic acid was used as a sole substrate for screening of tannase producing fungus during this investigation. The screening of fungal isolates for tannase production is depicted in Table 2. The 30 fungal isolates screened showed different levels of tannase secretion. The preliminary studies conducted on agar plates showed clear halo zones due to hydrolysis around each colony after 24 h – 120 h of incubation. It was observed that fungal strains formed compact colonies and the zone of clearance around the colonies were easily measured. In case of spread colonies, the reverse side of the petri dish showed zone of clearance below the colony growth. However, Pinto et al. [23] reported correlation between colony diameter (zone of clearance) and tannase production using A. niger strains. In this present study, zone of hydrolysis and colony diameter were considered as indicator of higher tannase production. The highest zone of inhibition was observed in isolate ACN 10³ with zone of inhibition of 70 mm and a colony diameter of 30 mm followed by isolate 6³ and 9². Isolate 7⁴ had the lowest zone of clearance (15 mm). This result is in agreement with that reported by Murugan et al. [24], where a correlation was established between tannase production, diameter of colony and zone of clearance.

Table 1. Total fungal count (CFU /g) from the different soil samples

| Sample code (ACN) | CFU/g |
|-------------------|-------|
| 1                 | 1.7 × 10⁴ |
| 2                 | 3.2 × 10⁴ |
| 3                 | 4.5 × 10⁴ |
| 4                 | 3.3 × 10⁴ |
| 5                 | 3.1 × 10⁴ |
| 6                 | 6.0 × 10⁵ |
| 7                 | 2.8 × 10⁵ |
| 8                 | 4.0 × 10⁵ |
| 9                 | 3.5 × 10⁵ |
| 10                | 6.5 × 10⁵ |
| 11                | 4.5 × 10⁴ |
| 12                | 1.0 × 10⁴ |
| 13                | 2.3 × 10⁴ |
| 14                | 3.75 × 10⁴ |
| 15                | 2.4 × 10⁴ |

3.3 Identification of the Isolate

3.3.1 Macroscopic and microscopic characteristics of the fungal isolate

Table 3 shows the microscopic and macroscopic characteristics of the isolate. The result revealed the characteristics of the isolate based on its colonial morphology (macroscopic) on agar, considering differences such as colour, border, surface appearance, elevation and period of incubation. Morphological and microscopic identification of the fungal isolate was carried out using various identification keys such as shape, color zone size, margins and texture.
Table 2. Screening of fungal isolates for tannase production

| S/N | Sample code (ACN) | Diameter of inhibition (mm) | Colony diameter (mm) |
|-----|-------------------|-----------------------------|----------------------|
| 1.  | 2                 | 30                          | 25                   |
| 2.  | 2^2               | 30                          | 35                   |
| 3.  | 6^1               | 70                          | 20                   |
| 4.  | 6^2               | 62                          | 55                   |
| 5.  | 6^3               | 55                          | 47                   |
| 6.  | 5^1               | 25                          | 23                   |
| 7.  | 7^1               | 28                          | 25                   |
| 8.  | 7^2               | 25                          | 18                   |
| 9.  | 7^3               | 20                          | 13                   |
| 10. | 7^4               | 15                          | 16                   |
| 11. | 8^1               | 40                          | 17                   |
| 12. | 8^2               | 35                          | 30                   |
| 13. | 8^3               | 60                          | 40                   |
| 14. | 8^4               | 35                          | 25                   |
| 15. | 9^1               | 52                          | 27                   |
| 16. | 9^2               | 70                          | 17                   |
| 17. | 9^3               | 40                          | 15                   |
| 18. | 10^1              | 70                          | 30                   |
| 19. | 12^1              | 32                          | 25                   |
| 20. | 13^1              | 60                          | 32                   |
| 21. | 13^2              | 60                          | 35                   |
| 22. | 14^1              | 50                          | 45                   |
| 23. | 14^2              | 30                          | 20                   |
| 24. | 14^3              | 30                          | 21                   |
| 25. | 14^4              | 47                          | 32                   |
| 26. | 15^1              | 50                          | 40                   |
| 27. | 15^2              | 25                          | 17                   |
| 28. | 1^1               | 62                          | 40                   |
| 29. | 6^4               | 65                          | 60                   |
| 30. | 7^b               | 60                          | 30                   |

Microscopic characteristics such as type of conidia, conidiophore, hyphae and phyllades were all examined and observed. Mangrola [25] observed tannase producing fungal isolates microscopically after staining with lactophenol cotton blue stain when viewed. Murugan et al. [24] also screened tannase producing fungal isolates using simple agar plate and submerged fermentation (SmF) process. Inamdar [26] identified tannase producing fungal isolates based on their morphological characteristics.

3.3.2 Molecular identification

On the basis of 18S rRNA gene sequence studies, the isolate was found to be *Lasiodiplodia plurivora* strain ACN-10 (Fig. 1). The ribosomal rRNA gene sequence has been submitted to the Genbank and was allotted the accession number MG250374.1. Below are the PCR amplification image and neighbour joining phylogenetic tree of the isolate (Fig. 2).
Table 3. Microscopic and macroscopic characteristics of the selected isolate

| Isolate code | Macroscopic characteristics                                                                 | Microscopic characteristics                                                                                                  | Isolate                  |
|--------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| 10^1         | Growth: colonies growth spreading rapidly with abandoned aerial mycelium, thin velvety and matured within 3-5 days of incubation period. Colonies are wooly with dirty initially and gradually turning smoky grey which consist almost entirely of spore chains with reverse totally black or dark. | Hyaline, cylindrical, thin-walled, 1-6 septate, rarely branched, basal, middle or apical cells swollen rounded at apex. Conidiophores absent. Conidiogenous cells: holoblastic, discrete, hylaline, smooth, thin-walled and cylindrical. Conidia: hyaline, aseptate, sub-globose to sub cylindrical with granular content. Both ends rounded, wall pigmented, verruculose ellipsoid to ovoid. Septate with longitudinal striations. | Lasiodiplodia sp. |

Fig. 1. PCR amplification image of 18SrRNA gene bands of the fungi isolated from soil. Lane 1: DNA marker, Lane 2-3: 18SrRNA of the isolates

Optimum yield (4.615 U/ml) was observed at 96 h incubation in SmF using mango leaves as substrate. Murad et al. [9] and has reported optimum tannase production after 96 h of fermentation for A. niger which is in agreement with our finding for SmF but differed with 120 h reported in SSF. Paranthaman et al. [27] have also reported maximum tannase production after 96 h of incubation for A. oryzae which is in contrast with the result (120 h) of this study in SSF. The results from this study is in contrast with Lal et al. [28] who reported that maximum tannase production from A. niger was achieved at 7th day of incubation. The results obtained also differed with Cavalcanti et al. [29] who reported that optimum production of tannase by A. niger was recorded at 24 h of incubation.

Solid-state fermentation (SSF) is generally preferred for enzyme production due to the fact that it allows production of highly concentrated crude enzyme with low cost for the extraction of crude enzyme, although submerged fermentation
(SmF) is believed to offer a number of advantages over solid-state fermentation for certain enzyme production Beena, [11]. The optimum tannase yield (6.064U/ml) obtained from this study showed an improved yield using Terminalia cattapa when compared with optimum tannase yield of 4.65U/ml in SSF reported by Souza et al. [30]. The optimum yield (6.064 U/ml) reported in our study is higher than 4.97 U/ml obtained in SSF by Tan et al. [31]. Furthermore, Cruz et al. [32] and Lima et al. [8] reported higher tannase yield in SSF than in SmF. This is consistent with our observation that higher optimum yield of tannase was obtained in SSF compared to the optimum yield observed in SmF.

In the present study, the prospect of submerged and solid-state fermentation was explored and efforts were made to utilize natural substrates (Almond and Mango leaves) that contained tannin as an inducer cum substrates for tannase production. Results obtained from this study indicated higher tannase production in solid-state fermentation compared to submerged fermentation. The data also confirms the positive role played by Terminalia cattapa and Magnifera indica leaves in the production of tannase by Lasiodiplodia plurivora ACN-10. This present study further indicated that in spite of the fact that natural substrates could induce and enhance tannase production, the microorganism require tannic acid in the medium for adequate synthesis confirming the inducer role of tannic acid for tannase production. Therefore, from the study it is evident that SSF encouraged better yield of tannase than SmF and clearly reveals the potentials of this tannin rich residue (Terminalia cattapa) as one of the cost effective substrate to be explored and used in large-scale production.

Fig. 2. Neighbour-joining phylogenetic tree of isolate ACN-10 made by MEGA 6.0. Bootstrap values of >50% (based on 1000 replicates) are given in the nodes of tree. Accession Number: MG250374.1
4. CONCLUSION

This study reported the potential of tannase production by *Lasiodiplodia plurivora* ACN-10 under solid state fermentation and submerged fermentation using *Terminalia cattapa* and *Magnifera indica* leaves as substrates. The results obtained indicated higher tannase production by *Lasiodiplodia plurivora* ACN-10 in solid-state fermentation using *Terminalia cattapa* (6.06 U/g) than using *Magnifera indica* leaves (4.89 U/g) after 144 h and 96 h of incubation, respectively. Generally, higher yield of tannase was achieved under solid state fermentation compared to submerged cultivation when either *Terminalia cattapa* leaves or *Magnifera indica* leaves was used as substrate. The result of this study has revealed the potential of using *Terminalia cattapa* as an invaluable substrate to be employed in commercial production of tannase under SSF by the fungus and equally could help in minimizing the environmental pollutions caused by these agrowaste materials which are usually dumped indiscriminately in the
environment. Indeed, the result of this study has huge prospect in the conversion of waste to wealth. This is the first report to the best of our knowledge that *Lasiodiplodia plurivora* strain is implicated in tannase secretion. Hence, it is necessary to evaluate the optimization of culture conditions for tannase production by *Lasiodiplodia plurivora* ACN-10 under solid state fermentation as well as characterization of the enzyme for possible industrial implications. These researches are currently ongoing in our research laboratory.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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