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Screening and Optimization of Culture Conditions for Local Production of Tannase from Fungal Species

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ABSTRACT

Tannase is produced extensively from microorganisms such as fungi, bacteria and yeast as they are diverse naturally. The aim of this study was to isolate, screen and select local microbial strain for the production of tannase as well as optimize culture conditions for the hyper producers. Fifteen fungal species were isolated from decomposed tannin rich materials (soil under Almond leaves undergoing natural deterioration) and its surrounding soil. They were screened with mineral medium compounded with tannic acid. Selected isolates were used for tannase production under solid state fermentation and varied the fermentation conditions. All strains showed various degree of tannic acid utilization as carbon source for enzyme (tannase) production. Three isolates were identified to be potential hyper producers. The fungal species; *Aspergillus niger*, *Penicillum sp.* and *Aspergillus flavipes* yielded the highest enzyme production after incubation. Optimum temperature and pH for tannase production was recorded as 28-45°C and 5.5 respectively. The isolates were good tannase producer with high activities of the enzyme, especially *Aspergillus niger*, *Penicillum sp.* and *Aspergillus flavipes* which gave a high yield of tannase.

Keywords: Tannase, Fermentation, Culture conditions, Optimum temperature, Optimum pH, Tannin.

Introduction

The enzyme tannin acyl hydrolase (TAH) commonly referred to as tannase (EC. 3.1.1.20) catalyzes the hydrolysis of ester bonds present in hydrolysable tannins such as gallotannins, ellagitannins, and gallic acid esters resulting in the production of gallic acid and glucose.[1] Tannase is produced extensively from micro-organisms as they are very diverse naturally and their environmental and genetic manipulation is easier. The use of microorganism gives numerous advantages including generation of novel producers in reduced time period or reduced generation time. Tannase from fungal sources is reported to be highly active over a wide range of pH and temperature and can tolerate tannic acid concentrations as high as 20% (w/v) without having a deleterious effect on both the growth and enzyme production.[2] TAH is involved in biodegradation of tannins and has important applications in various industries, particularly in food and pharmaceutical sectors.

Fungal tannase is used in many industrial applications which includes clarification of fruit juices, de-tannification of food, preparation of food preservatives, high grade leather tanning, clarification of beer and wines, beer chill proofing, manufacture of coffee flavored drinks, manufacture of instant tea, production of gallic acid which is used for the synthesis of trimethoprim (antibiotic for bacterial infection). In animal feed, tannase is used to reduce the anti-nutritional effects of tannins and improve animal digestibility.[3] Utilization of industrial enzymes are on the increasing demand and are costly to purchase due to importation, as over 200 Billion Naira is spent annually for the importation of industrial enzymes including tannase in Nigeria. It is on this basis that the project is embarked on with the following objectives; to isolate, screen, identify and select locally isolated microorganisms for the production of tannase and optimize culture conditions for the identified hyper producers of tannase.

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Materials and Methods

Source of tannin

Samples rich in tannin were locally sourced from decomposed Almond fruit site, decomposed Almond leaves site and soil around almond tree within the premises of the Federal Institute of Industrial Research Oshodi, Lagos-Nigeria. Sorghum pomace which served as fermentation substrate was also obtained from the local market in Oshodi, Lagos-Nigeria.

Isolation of fungal species

Six-fold serial dilution of each samples were carried out under aseptic condition and 1 mL of each dilution was inoculated into sterilized Potato Dextrose Agar medium supplemented with 0.14 g/L streptomycin using the pour plate method.[4] These were allowed to solidify and incubated aseptically at optimum growth condition of 28°C for 120 h. After incubation, microbial growths were sub-cultured severally to obtain a pure culture, characterized and identified based on their colonial appearances including pigmentation and microscopic characteristics using a standard atlas.[5]

Screening of selected fungal isolates for tannase production

The screening methodology was carried out using the Tannase Screening Agar Medium (TSAM) containing (g/L): Tannic acid 10.0; $NaNO_3$ 3.0; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; KCI 0.5; $FeSO_4.7H_2O$ 0.01 and Agar 30.0 pH 5.5 as previously described by Pinto *et al.*[6] Point inoculation was carried out and plates were incubated at 28°C for 96 h. After incubation, the zones of tannic acid hydrolysis were measured using freshly prepared 1% $FeCl_3$ solution as indicator.

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Production of tannase in solid state fermentation

Sorghum pomace (50.0 g) was weighed and moistened with 100 mL of freshly prepared salt solution containing 0.5% NH_4NO_3, 0.1% NaCl, 0.1% $MgSO_4$.$7H_2O$ and 4% tannic acid at pH 5.5, all in a conical flask. The content was loaded in a muslin bag sterilized by autoclaving at 121°C 15 psi and a holding time of 20 min. The sterilized solid substrate was allowed to cool and then inoculated with 9 mL of inoculum spores previously prepared using sterile tween 80 solution in a sterile fermentation tray, mixed properly and incubated at 28°C for 120 h. Crude tannase was extracted by addition of 250 mL of 0.1M Sodium Acetate buffer pH 5.5, mixed well, separated using a muslin cloth and filtered using Whatman filter paper No. 1. The filtrate was centrifuged at 5,000rpm for 20 min with cold centrifuge (Sigma 4-16k). The supernatant was collected as crude tannase and kept at 4°C for further assay.[7]

Tannase assay

Tannase assay was estimated based on modified method of Sharma *et al*.[2] which is based on the formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4- ketothiazolidine) and absorbance was measured using UV-visible spectrophotometer at 520nm wavelength and the absorbance converted to enzyme units using the standard calibrated curve of gallic acid. One unit of tannase activity is defined as the amount of enzyme required to liberate 1 µmol of gallic acid/min under defined conditions. Enzyme activity was expressed as U/mL.

Statistical analysis

Experimental data were analyzed using Analysis of Variance to determine the Means. The level of significance was set at $P \leq 0.05$. The data were analyzed using Microsoft Excel.

Results and Discussion

The total culturable heterotrophic fungi count as shown on the Potato Dextrose Agar growth medium was between $2.2x10^2$ to 5.4×10^5 cfu/g. The results showed the presence of fungal isolates in the samples obtained from various sources (Table 1) which is supported by the fact that microorganisms are present in soil and decaying matters as also observed by Al-Mraai *et al.*[8] The ability of microorganisms to survive in an environment often depends on the nutritional composition of the environment or habitat and also the presence of diverse genes coding for metabolic function in their genome. In this instance, the soil sampled contained the tannin and the microorganisms utilizing the tannin had acquired chromosomal genes and /or plasmid genes for the release of the tannase in order to break down the tannin.

A total of 15 tannase producing fungal isolates were obtained from three samples with isolates 1, 2 and 3 (Table 2). These three isolates showed the highest zone of hydrolysis of 83mm each followed by isolate 4 which showed 72 mm zone of hydrolysis. The least zone of hydrolysis of 20mm was shown by isolates 14 and 15. Isolates 1, 2 and 3 were then selected for further studies based on the zone of hydrolysis. The selected fungal isolates showed their ability to hydrolyze tannins which indicates that they are good tannase producing isolates and can be used as carbon source in the production of tannase. This observation is in agreement with the work reported by Katyal and Kaur.[9]

The morphological characteristics and probable identity of the selected tannase producing isolates based on their zone of tannin hydrolysis is shown in Table 3. Isolate 1, 2 and 3 were characterized and identified as *Aspergillus niger, Aspergillus flavipes* and *Penicillum sp.* in accordance with their macroscopic and microscopic characteristics. According to Ajayi *et al.*[10], fungal isolates are good producers of tannase and are widely studied for tannase production. *Aspergillus, Penicillium, Paecilomyces* and *Rhizopus* species shows good enzyme activity as shown in this study with the fungal isolates exhibiting high tannase producing ability.

The highest tannase activity was recorded by isolate 1 (*Aspergillus niger*) with an activity of 76.632 ± 0.002 followed by isolate 3 (*Penicillum sp.*) with an activity of 73.822 ± 0.002. Isolate 2 (*Aspergillus flavipes*) showed the lowest activity of 71.345 ± 0.001. Statistical analysis showed that tannase activities of the isolates are significantly different from one another. Isolate 1 (*Aspergillus niger*) was then selected for further studies based on the activity (Table 4). This is because fungal tannase from *Aspergillus sp.* showed better activity in degrading hydrolysable tannins.[11]

The incubation temperature was observed to be optimal at incubation temperature of 37°C with activity of 73.822 ± 0.001 followed by incubation temperature of 28°C with activity of 73.346 ± 0.004. This may be as a result of individual species of microorganism and their preferred level of temperature that aids their growth. Incubation temperature of 45°C showed the lowest activity of 73.060 ± 0.0 (Table 5). Statistical analysis shows that tannase production at different incubation temperatures are significantly different from one another. Maximum tannase production was obtained at temperature 37°C however, the production decrease when the temperature was above 37°C. This is in agreement with the result of Abdal *et al.*[12] who reported 37°C as optimal incubation temperature for tannase

Table 1: Colony Forming Unit (CFU) of Fungal Isolates obtained from decomposed Almond fruit site, decomposed Almond leaves site and soil around Almond tree

Sample Code	Dilution factor	No of colonies	CFU/g
AS01	10^3	22	2.2×10^5
AS02	10^5	4	Insignificant
AS03	10^8	1	Insignificant
AF01	10^3	40	4.0×10^4
AF02	10^5	6	Insignificant
AF03	10^8	1	Insignificant
AL01	10^3	32	3.2×10^4
AL02	10^5	2	2.0×10^6
AL03	10^8	2	2.0×10^9

AS: Soil around Almond Tree. AF: Decomposed Almond Fruit. AL: Decomposed Almond Leaves.

Table 2: Screening of Selected Fungal Isolates for Tannase Production

Our reference	Zone of Tannin Hydrolysis (mm)
Isolate 1	83
Isolate 2	83
Isolate 3	83
Isolate 4	72
Isolate 5	67
Isolate 6	64
Isolate 7	60
Isolate 8	37
Isolate 9	34
Isolate 10	34
Isolate 11	34
Isolate 12	27
Isolate 13	24
Isolate 14	20
Isolate 15	20

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Table 3: Morphological Characteristics of Selected Fungal Isolates

Isolate codes	Colony appearance	Microscopy	Probable Identity
Isolate 1	Lower aerial mycelium with blackish spores against a milky background Reverse is green	Blackish spores enclosed in a sporangium which is attached to a conidiophore	*Aspergillus niger*
Isolate 2	White aerial mycelium with green spores Reverse is cream	Largely tightly packed conidia chain	*Aspergillus flavipes*
Isolate 3	Lower aerial mycelia with a light greenish with a greenish border Reverse is yellow	Conidiophore having sterigmata at the top.	*Penicillum sp*

Table 4: Tannase Production by Selected Fungal Isolates

Isolate Code	Activity (U/mL) ± SEM
Aspergillus niger	76.632 ± 0.002^{c}
Aspergillus flavipes	71.345 ± 0.001^{a}
Penicillum sp	73.822 ± 0.002^{b}

SEM - Standard Error of Mean. Mean values with different superscripts are statistically and significantly different at $P < 0.05$.

Table 5: Effect of Incubation Temperature on Tannase Production by *Aspergillus niger*

Temperature (°C)	Activity (U/mL) ± SEM
28	73.346 ± 0.004^{b}
37	73.822 ± 0.001^{c}
45	73.060 ± 0.0^{a}

SEM - Standard Error of Mean. Mean values with different superscripts are statistically and significantly different at $P < 0.05$.

Table 6: Effect of pH of Fermentation Medium on Tannase Production by *Aspergillus niger*

pH	Activity (U/mL) ± SEM
4.5	68.869 ± 0.004^{c}
5.5	72.774 ± 0.0^{d}
6.0	69.792 ± 0.0^{cd}
7.0	67.631 ± 0.002^{c}
8.0	62.582 ± 0.001^{b}
9.0	46.579 ± 0.001^{a}

SEM - Standard Error of Mean. Mean values with different superscripts are statistically and significantly different at $P < 0.05$.

Table 6 shows that pH 5.5 had the highest activity of 72.774 ± 0.0 followed by pH 6.0 with activity of 69.792 ± 0.0 while pH 9.0 showed the lowest activity of 46.579 ± 0.001. This may be due to inability of the isolates to survive at alkaline pH, however was able to survive and thrive at acidic pH thereby enhance high tannase yield. Statistical analysis shows that the pH of the production medium has significant effect on tannase production. Maximum tannase production was obtained at pH 5.5 however, the production decrease when the pH was above pH 5.5. The result is comparable to the report of Ayesha *et al.*[13] that Tannase are acidic enzymes with an optimum pH of 5.5

Conclusion

In conclusion, this study demonstrated the potential of all the locally isolated microorganisms especially *Aspergillus niger*, *Penicillum sp.* and *Aspergillus flavipes* isolated from tannin rich materials (soil under Almond leaves undergoing natural deterioration) and its surrounding soil to produce tannase. This research demonstrated the influence of the studied parameters, mainly effects of pH and temperature in the production of fungal tannase. Tannase production was studied under solid-state fermentation using tannin rich agricultural industrial waste as fermentation substrates and the wastes served as good substrates for enzyme production.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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