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SCREENING AND ISOLATION OF THERMOPHILIC FUNGI OBTAINED FROM THREE SELECTED COMPOST WASTES SITES

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ABSTRACT

Thermostable enzymes have found applications in the improvement of products quality in various industries. Despite the discovery of several microorganisms as potential source of thermostable enzymes, more biodiversity explorations are currently been carried out for high activity enzymes producing isolates. The discovery of new isolates having these characters will allow to efficiently carry out reactions in various industrial processes that involve extreme conditions. This study was aimed to screen and isolate thermophilic fungi from three selected compost wastes sites (Palm oil mill, Wood chip piles and Abattoir dump), and identify potential producers of lipases, cellulases and proteases respectively. The thermophilic fungi were obtained from an equal depth of one meter in all the three collection sites with a temperature range of 40 to 45^{O}C and later cultured at 70 ^{O}C. Further experimental screening analysis showed the presences of lipase, cellulase and protease producing fungi present in samples collected from palm oil mill, wood chips pills and abattoir dump site respectively. The isolated fungi were used for enzyme production in submerged fermentation for 10 days at 50^{O}C. Culture filtrate obtained from the medium of production were used for the assessment of enzymatic activity. The highest lipase, cellulase and protease activities were obtained from isolates 3 A (56.56 U/mL), 3A (38.35 U/mL) and 2B (3.0 U/mL) respectively. These isolates with high enzymes activities were identified microscopically as *Rhizopus* sp., *Aspergillus flavus* and *Neurospora* sp. accordingly. We believed that these strains could be further exploited for numerous industrial applications that require thermophilic enzymes.

Keywords: *Rhizopus* sp., *Aspergillus flavus*, *Neurospora* sp., Thermophiles, Thermostable enzymes

INTRODUCTION

Dynamic application of enzymes in industrial processes has consequentially led to search for thermophilic fungi as they serve as a potential source of thermophilic enzymes such as lipolytic, proteolytic, cellulolytic, lignolytic and amylolytic enzymes which are used in the industries. Applications of these enzymes have found their ways in numerous industries such as; detergent, chemical, oil, food, brewing, pharmaceutical, leather, paper, dye and textile industries **(Gomes and Steiner, 2004; Chrisnasari *et al*., 2018; Gulmus and Gormez, 2020)**. Enzymes produced by these thermophiles are thermostable, extreme pH tolerant and also possess high activity at other extreme environmental or industrial conditions **(Ahirwar *et al*., 2017; Gulmus and Gormez, 2020)**.

Thermophiles are ubiquitously present in the environment, a number of them are found in industrial effluent, aquatic sediments, sludge, wood chip piles, composts site and other accrued organic matter that provides favorable conditions (warm, humid, and aerobic) for their growth **(Lee *et al*., 2014; Ahirwar *et al*., 2017)**. Most enzymes obtained from thermophilic fungi often exhibit higher temperatures tolerance than those produced and extracted from mesophilic fungi. Some of these thermostable enzymes are stable between 50 to 80^{O}C **(Lee *et al*., 2014)**, any temperature below 20°C has inhibited the activity and growth of true thermophilic fungi **(Maheshwari *et al*., 2000; Ahirwar *et al*., 2017)**. Several thermophilic fungi have also been isolated from environments associated with harsh conditions like high water pressure, absence of oxygen, high salinity and aridity **(Lee *et al*., 2014)**. Research has shown that many thermophilic organisms were mostly isolated from composts: prevalence of these microbes in compost sites is owing to their extreme temperatures, aerobic and humidity conditions present in the compost. In addition, compost also serve as a source of nutrients for the development of microorganism **(Lee *et al*., 2014)**.

During metabolic activities of fungi found on composts, various organic materials are broken down to smaller organic molecules. The overall process of metabolism is made possible through the thermophilic fungi ability to secrete numerous enzymes capable of degrading composts **(Raut *et al*., 2008)**. The thermophilic enzymes evaluated in this study are proteases, cellulases and lipases. These are largely required in the food industry to reduce time, energy and cost of operation **(Raveendran *et al*., 2018)**. Proteases are known to catalyze peptide bonds in proteins through hydrolysis and thus they are used in brewing, meat tenderization and for milk coagulation **(Patel *et al*., 2013)**. Proteases have also been used in improving food digestion, flavour and nutritional value as well as aiding emulsification and coagulation processes **(Aruna *et al*., 2014)**. Cellulases act on cellulose and hydrolyze β-1,4 linkages found in carbohydrates to release glucose subunits. Classes of cellulases include endo-(1,4)-β-d-glucanase (EC 3.2.1.4), exo-(1,4)-β-d-glucanase (EC 3.2.1.91) and β-glucosidases (EC 3.3.1.21) **(Schülein, 1988)**. Fungi cellulases are used to increase yield, performance, clarification and improve stabilization in juices production processes **(Dervilly *et al*., 2002)**. In addition, they are also utilized in the extraction of important phytochemicals such as phenolic and flavonoids from flowers, seeds and fruits **(Kabir *et al*., 2015)**, while lipases hydrolyze long-chain of triglycerides. These enzymes are used in improving cheese texture, the flavour development in butter, the aroma in beverages alongside increasing the shelf life of baked products **(Aravindan *et al*., 2007)**. Based on the commercial benefits of these enzymes, there is need to search for thermophiles that can produce thermophilic and thermostable enzymes that will be highly stable and resistant to product inhibition during these production processes **(Arora *et al*., 2015; Mallerman *et al*., 2015)**. Hence, in the present study, we isolated and screened thermophiles (fungi) potentially producing extremophilic lipases, cellulases and proteases, for future biotechnological applications.

MATERIAL AND METHODS

Collection of samples

Samples were collected from three different composts site (palm oil mill, wood chip piles and abattoir wastes dumping piles) of Oye-Ekiti, Ekiti State, Nigeria.. The samples were obtained from the piles at a depth of 1 meter using a shovel and then transported to the laboratory in sterile polythene bags within 2 hours

from the collection for microbiological and biochemical study **(Alsohaili and Bani-Hasan, 2018).**

Screening and isolation of thermophilic fungi

The thermophiles were isolated by taking 1 g of different samples of soil and suspending them in 5 mL of sterile distilled water. These were vigorously vortexed and subsequently placed in the water bath for 24 hours at 70 ^{O}C. Then 0.5 mL of the liquid were inoculated into flasks containing 100 mL of broth (4 g/L yeast extract, 20 g/L glucose, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H2O$, pH 7.0 ± 0.2) and then kept in shaking incubator for other 72 hours at 50^{O}C. After 72 hours, 200 µl samples were taken and inoculated separately on Potato Dextrose Agar (PDA) containing 1% streptomycin. The plates were incubated at 50^{O}C in the dark for 7 days and then colonies observed as described in **(Gaddeyya *et al*., 2012; Reddy *et al*., 2014; Alsohaili and Bani-Hasan, 2018).**

Screening for thermophilic enzymes

(i) plate screening for lipases
The fungi isolated from palm oil mill compost site were screened for lipase production by inoculation on phenol red olive oil agar plates containing 0.01% (w/v) phenol red, 0.1% (w/v) $CaCl_2$, 1% (v/v) olive oil, 2% (w/v) agar, 1% (w/v) streptomycin; the pH was adjusted to 8.0 using 0.1 N NaOH. The plates were later incubated at 50^{O}C for 5 days **(Rai *et al*., 2014).**
(ii) plate screening for cellulases
For this screening the thermophiles obtained from wood chip compost piles, were used. The ability of the isolates to secrete cellulase was tested using Czapek agar plate containing 1 g/L carboxymethylcellulose (CMC); 0.5 g/L $NaNO_3$; 1 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.001 g/L $FeSO_4 \cdot 7H_2O$; 1 g/L yeast extract; 15 g/L agar) for 5 days at 50^{O}C. The pH was regulated to 5.0. Fungi showing colonies with clear haloes were considered to be positive cellulase producers **(Kasana *et al*., 2008).**
(iii) plate screening for proteases
The fungi obtained from abattoir wastes dump site were screened for protease activity on agar medium comprising of 10 g/L casein and 20 g/L agar at pH 8.0. The inoculated plates were incubated for 5 days at 50^{O}C before observing haloes of hydrolysis **(de Veras *et al*., 2018).**

Production of thermophilic enzymes

For enzymes production fungi were inoculated in 250 mL Erlenmeyer flask containing 100 mL of the media described below.
(i) lipase production
The basal medium for lipase production consist of 0.1% yeast extract, 0.3% peptone, 0.05% $CaCl_2.2H_2O$, 0.05% NaCl, 1% olive oil and , 0.02% streptomycin; , pH was adjusted at 8.0 **(Ayinla *et al*., 2017).**
(ii) celullase production
Cellulase production in shaking flasks was carried out using **Mandels and Weber (1969)** medium, supplemented with 1% CMC and 2.5% wheat bran. The medium also consist of 0.2% KH_2PO_4 , 0.03% $CaCl_2 \cdot 2H_2O$, 0.03% urea, 0.03% $MgSO_4 \cdot 7H_2O$, 0.14% $(NH_4)_2SO_4$, 0.025% peptone, 0.01% yeast extract, 1 mL Tween-80, 0.005% $FeSO_4 \cdot 7H_2O$, 0.0016% $MnSO_4 \cdot H_2O$, 0.0014% $ZnSO_4 \cdot 7H_2O$, and 0.002% $CoCl_2 \cdot 6H_2O$, pH 5.0 in 250 mL Erlenmeyer flask **(Saroj *et al*., 2018).**
(iii) protease production
Submerged fermentation medium for protease production include the following : 1% of casein, 2.5% wheat bran, 0.1% (w/v) of each of $(NH_4)_2SO_4$, $MgSO_4.7H_2O$ and NH_4NO_3, to pH 8.0 in 250 mL Erlenmeyer flasks **(Macchione *et al*., 2008).**
Each medium was inoculated with a loopful of actively growing fungal colonies obtained from plates. Inoculated media were placed in a shaking bath and incubated at 50^{O}C and with a constant oscillation of 160 rpm. After 10 days , the supernatants were obtained by centrifugation at 5,000 rpm for 15 min at 4^{O}C and filtered through Whatman no. 1 filter paper before determine their respective enzymatic extracellular activities **(Ayinla *et al*., 2017).** The protein contents of all the analyzed supernatants (crude enzyme) were also estimated using Bradford assay **(Bradford, 1976).**

Measurement of enzyme activity

(i) assay for lipase
Lipase activity was assayed using **Yadav *et al*. (1993)** method with olive oil as substrate. Olive oil (5 mL) was vigorously mixed with 0.1 M phosphate buffer (20 mL) and pre-incubated for 10 min at 37^{O}C.. The reaction was then activated by the addition of 1 mL crude enzymes and the mixture incubated for 30 min at 40^{O}C. The reaction was finally terminated by the addition of 15 mLacetone-ethanol (1:1). Free fatty acids released during the reaction were then titrated with 0.05 N NaOH after the addition of three drops of phenolphthalein indicator. One unit of lipase activity was defined as the amount of enzyme which produces 1µmol of fatty acids per minute under assay conditions **(Lanka and Trinkle, 2017).**
(ii) assay for cellulase
Carboxymethyl cellulase (CMCase) was performed according **to Ghose (1987**) method. The assay was carried out at 50^{O}C with a reaction mixture containing 0.5 mL crude enzyme and 0.5 mL of 2% substrate (CMC) dissolved in 50 mM sodium citrate buffer (pH 4.8) and incubated for 30 min. After incubation, 3 mL of DNS (3,5-dinitrosalicylic acid) reagent was added; the mixture was heated for 5 min in boiling water to obtain a coloured reaction mixture and the absorbance measured at 540 nm. One unit of cellulose activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrate per mL per min under the assay conditions **(Saroj *et al*., 2018).**
(iii) assay for protease
Protease activity was determined using **Carrie Cupp-Enyard (2008)** method. This assay utilizes casein as a substrate where 5 mL of 0.65% casein solution was incubated for 5 min at 37^{O}C. The reaction was then activated by the addition of 1 mL of crude enzyme solution before heating in water bath for 30 min at 37^{O}C. The reaction mixture was terminated by the addition of 5 mL of Trichloroacetic acid (TCA) solution and filtered using Whatmann No 1 filter paper., 5 mL of sodium carbonate and 1 mL of 2 fold diluted Follin Ciocalteus phenol reagent were added to the filtrate before incubating in dark for 30 min at room temperature for the development of blue colour. The absorbance was measured at 660 nm against a reagent blank using tyrosine standard. One protease unit was defined as the amount of enzyme that releases 1 µM of tyrosine per minute at pH 7.5 at 37^{O}C **(Mohapatra *et al*., 2003; Chandrasekaran *et al*., 2015).**

All the experiments were done in triplicates and values are expressed as mean ± SEM (n=3). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

Microscopic examination of isolated fungi

The three fungal isolates from different sources and having the highest enzymatic activities were evaluated for their fungal morphology by observing the colony features (colour, shape, size and hyphae) by a compound microscope Harris HNB-107BN with a digital micro camera MC-D20DU using a lactophenol cotton blue-stained slide mounted with a small portion of the mycelium at 40X magnification (Gaddeyya et al., 2012; Alsohaili and Bani-Hasan, 2018).

RESULTS AND DISCUSSION

Screenings of fungal isolate for enzymatic activities

The thermophilic fungi were isolated from different compost wastes sites namely; palm oil mill, wood chip piles and abattoir wastes dump site at a temperature of 40, 40 and 45^{O}C respectively at a depth of one meter. All samples were treated at the high temperature of 70^{O}C before culturing. All strains were then screened according to the potential enzymatic activity that could occur at the site of sample collection. Thus, the isolate obtained from the palm oil mill, wood chips pills and abattoir dump site were screened for lipase, cellulase and protease activities respectively (Figure 1). The subjection of environmental samples to the high temperature of 70^{O}C was used to remove all non-thermophilic microorganisms that were present at the various sites of screening. The rise in temperature has been known to suppress the growth of mesophilic fungi leaving the thermophilic and thermotolerant that are capable of living in environment with high temperatures and thus secreting thermophilic enzymes such as lipases, cellulases and proteases. These enzymes have high potentials in many industrial process **(Moretti *et al*., 2012)**.

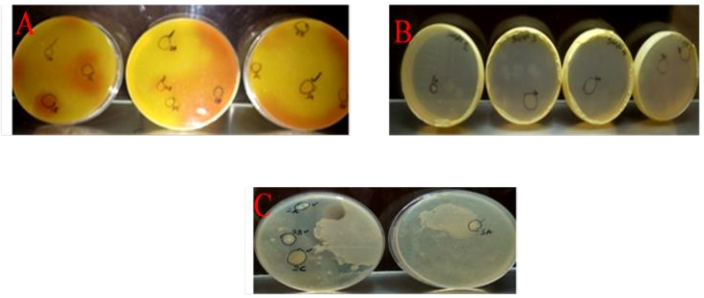

Figure 1 Thermophilic fungi isolates screen for enzymatic activities show zones of hydrolysis. A. Lipase Activity; B. Cellulase Activity; and C. Protease Activity

Quantitative enzyme activity

The enzymatic analyses were carried out on supernatants after growing newly isolated fungi in Erlenmeyer flask incubated at 50 ^{O}C. The culture filtrates were used for quantitative estimation of enzymes activities; results are shown in Tables 1-3.

Table 1 showed the lipolytic activities of isolates obtained from palm oil mill site; isolate 3 A had the most proficient lipase activity (56.562 U/mL) and isolate 2A had the least activity (41.487 U/mL).
Table 2 showed that four of the five isolates obtained from the wood chip piles had high cellulolytic activity at the end of 10 days incubation period. The cellulase activities of newly isolated thermphilic starins range from 38.352 U/mL (isolate 3A) to 3.503 U/mL (isolate 1A)
Table 3 showed the proteases activities of isolated thermophiles from abattoir dump site which ranges from 2.054 – 3.003 U/mL. isolate 2 B and 3 C had the highest and lowest protease activities respectively.

Table 1 Protein concentration and enzymes activities of lipases in culture filtrate

Samples Site	Isolates	Protein Concentration (mg/mL)	Fatty Acid Released (µg/mL)	Enzyme Activity (U/mL)
Palm Oil Mill	1 A	0.563±0.008	1375.474±0.063	45.253±0.033
	2 A	1.719±0.012	1244.343±0.034	41.482±0.027
	2 D	0.796±0.005	1538.464±0.048	51.281±0.035
	3 A	0.039±0.003	1.696.831±0.028	56.562±0.018

The experiment was performed in triplicates. Values are expressed as mean ± SEM (n=3). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results were considered statistically significant at $p \leq 0.05$.

Table 2 Protein concentration and enzymes activities of cellulases in culture filtrate

Samples Site	Isolates	Protein Concentration (mg/mL)	Enzyme Unit (µM)	Enzyme Activity (U/mL)
Wood Chip Piles	1 A	0.489±0.033	105.011±0.023	3.503±0.021
	3 A	0.622±0.022	1150.522±0.028	38.352±0.018
	4 A	0.429±0.020	375.011±0.021	12.501±0.031
	7 A	0.571±0.012	392.501±0.013	13.084±0.042
	7 B	0.616±0.028	457.028±0.016	15.233±0.023

The experiment was performed in triplicates. Values are expressed as mean ± SEM (n=3). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results were considered statistically significant at $p \leq 0.05$.

Table 3 Protein concentration and enzymes activities of proteases in culture filtrate

Samples Site	Isolates	Protein Concentration (mg/mL)	Enzyme Unit (µM)	Enzyme Activity (U/mL)
Abattoir Dump Site	2 A	0.852±0.013	6.163±0.028	2.054±0.018
	2 B	0.369±0.008	9.014±0.021	3.003±0.009
	2 C	0.537±0.011	6.474±0.013	2.162±0.012
	3 C	0.298±0.009	6.233±0.018	2.081±0.013

The experiment was performed in triplicates. Values are expressed as mean ± SEM (n=3). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results were considered statistically significant at $p \leq 0.05$.

Microscopic and morphological identification of fungi isolates

Microscopic and morphological identification were carried on fungi isolates with the highest enzymatic activities (Figure 2). Lipases producing isolate 3A obtained from the palm oil mill (Figure 2A) indicated the presence of dark colonies with root-like rhizoids and branching hyphae and identified as *Rhizopus* sp. The cellulases producing isolate 3A obtained from wood chip piles (Figure 2B) indicated massive mycelium with narrow branched hyphae and yellowish green spores and identified as *Aspergillus flavus*. The proteases producing isolate 2B obtained from abattoir wastes dumping site (Figure 2C) showed widely spread colonies with yellowish brown and darkened annular structures and identified as *Neurospora* sp.
The 3 fungal strains presently isolated in this work corroborate the results obtained by **Maheshwari *et al*. (2000)** who reported that few species of thermophiles have been identified and described out of 75,000 known fungi. After qualitative tests performed on plates, the quantitative enzymatic tests that were also conducted using the supernatants obtained from the liquid cultures in aerated Erlenmeyer flasks, corroborate that these strains are efficient producers of thermophilic enzymes.
Previous studies showed that that aerated shake flask cultures compared to static cultures are essential for the production of enzymes with high activity **(Papagora *et al*., 2013)**. **Salleh *et al.* (1993)**, **Essamri *et al.* (1998)** and **Mukhtar *et al.* (2016)** reported that *Rhizopus oryzae* are excellent producer of high activity lipase . This corroborate our microscopic analysis showing that of the isolate with the highest lipase activity was *Rhizopus* sp. *Aspergillus flavus* was identified as the isolate with the highest cellulolytic activity, confirming results obtained by **Chandra *et al*. (2007)** who found that secreted cellulolytic enzymes from *Aspergillus flavus* are responsible for the degradation of cellulosic material. Furthermore, the extracellular production of proteases by isolates obtained from abattoir wastes dump site showed growth and enzyme production at pH 8.0 and 50 ^{O}C. This result is similar with what was obtained from *Thermomyces lanuginose* P134 (**Li *et al*., 1997)** and *Tritirachium albumlimber* **(Samal *et al*., 1991)** that also grew optimally at 50 ^{O}C as *Neurospora sp*. obtained from this study.
Microscopic analysis allowed the identification of the thermophilic isolates with the highest enzymatic activities. Screening for thermophiles at a depth of 1 meter below ground surface facilitated obtaining viable isolates, however the temperature and moisture content obtained at this depth was assumed be adequate enough for their survival and optimum growth **(Rajasekaran and Maheshwari, 1993; Pedersen, 2000)**.

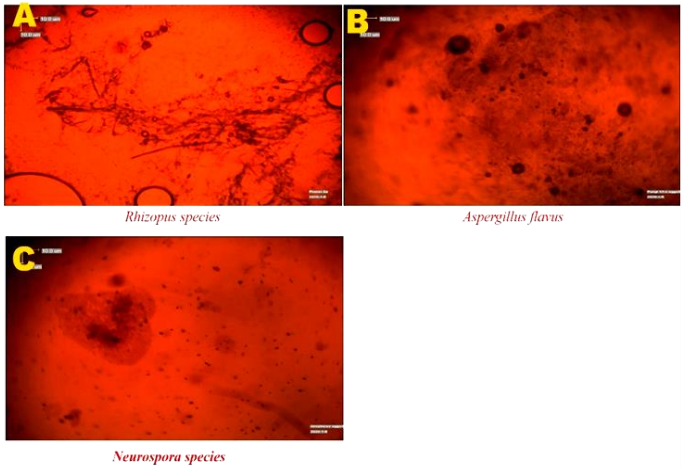

Figure 2 Microscopic and morphological analyzes of thermophilic fungi with the highest enzymatic activities. [A] *Rhizopus sp*. from isolate 3A obtained from palm oil mill; [B] *Aspergillus flavus* from isolate 3A obtained from wood chip piles; [C] *Neurospora sp*. from isolate 2B obtained from abattoir dumping site.

CONCLUSION

Enzymes are liable to denaturation at high temperature thereby causing inhibition of growth and other metabolic activities in microorganism **(Pundir *et al*., 2012)**. Thus, the need for desired good level of activity and enhanced stability at higher temperature are factors to be considered for enzyme selection **(Moretti *et al*., 2012)**. This study therefore allowed the isolation and identification of thermophilic fungi (*Rhizopus* sp., *Aspergillus flavus* and *Neurospora* sp.) that can withstand high temperature alongside producing thermostable enzymes with highly significant activities. The obtained result indicate that all the isolates are potent producer of extracellular thermophilic hydrolytic enzymes (lipase,

cellulase and protease) working at a temperature of 50 ^{O}C. This evidence suggests that these thermophilic fungi could be of great advantage in various industrial processes. Thus, more further studies are needed for the biochemical and molecular characterization of these isolates to strengthen our understanding of their metabolic activities. Detailed knowledge of the catalytic and biophysical properties of these thermophilic enzymes are very critical towards bioengineering of these isolates to withstand extreme industrial processes or environmental conditions.

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