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Full Length Research Paper

Isolation of *Aspergillus niger* from three varieties of Bambara nuts for simultaneous production of phytase and tannase

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The aim of this work was to isolate strain of *Aspergillus niger* for simultaneous production of phytase and tannase enzymes. In this study, *A. niger* strains were isolated from Bambara nuts (*Vigna subterranea*) of three different color seed coats. Growth of colonies in plates with tannic acid and phytic acid sodium salt-containing media indicate their ability to synthesize both enzymes. The strain of *A. niger* isolated from red color seed coat of Bambara nuts showed the highest potential for the production of both enzymes and was used to assay for phytase and tannase in three bean flours in solid-state fermentation.

Key words: *Vigna subterranea*, phytate, tannin, *Aspergillus niger*, colonies, solid-state fermentation, phytase, tannase.

INTRODUCTION

The genus, *Aspergillus*, consists of more than 180 officially recognized species and comprises a particularly important group of filamentous ascomycete species. However, most of the members are useful microorganisms in nature for degradation of plant polysaccharides (de Vries et al., 2000; 2003), and they are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes (Fawole and Odunfa, 2003; Wang et al., 2003). Indeed, *Aspergillus* sp., *Aspergillus niger* and *Aspergillus ficuum*, have most commonly been employed for commercial production of phytase (Sabu et al., 2005) and tannase (Bajpai et al., 1996). Both enzymes have potential applications in animal diets, food and pharmaceutical industries (Madeira et al., 2011).

Phytase (EC. 3.1.3.8) is capable of hydrolyzing phytate, a major storage form of phosphate in plant seeds during maturation. However, phytate is an anti-nutrient factor in animal feed where it forms insoluble complexes with protein and divalent cations. Therefore, phytase supplement can help improve the availability of phosphates, proteins and divalent cations. Tannase (tannin acyl hydrolase, EC. 3.1.1.20), on the other hand, catalyses the hydrolysis of ester bonds in hydrolysable tannins, tannic acid, which is also an anti-nutrient in animal feed thereby making tannase a good supplement for improving the bioavailability of nutrients.

Fungal phytases, such as *Aspergillus* phytases, have received more attention due to high production yields and acid tolerance for feed production (Kim et al., 1998). Currently, most commercial phytases are produced by *A. ficuum* NRRL3135 (Ullah and Gibson, 1987). Fungal tannases have received a lot of interests as well (Madeira et al., 2011). Purnama (2004) found that *A. niger* isolated from cacao pod reduced tannin levels by up to 79.3% (wt/wt). Species belonging to the *Aspergillus* and *Penicillium* genuses were reported as the best tannase producers (Pinto et al., 2001). Interestingly, *A. niger* is on the Generally Recognized as Safe (GRAS) list of the Food and Drug Administration (FDA) in the United States (Tailor and Richardson, 1979).

Tannase and phytase are extracellular inducible enzymes produced in the presence of tannic acid and phytic acid respectively by fungi, bacteria and yeast (Aguilar et al., 2002; Mondal et al., 2001; Battestin et al., 2007; Lee et al., 2005). Therefore, microorganisms such as *A. niger*, that have the ability to grow in a

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Figure 1. Mature Bambara nuts (*Vigna subterranea*) of three different color coats: (a) cream, (b) red and (c) black.

medium containing both tannic acid and phytate would have the potential to produce tannase and phytase respectively, which would give us a synergistic commercial advantage. In the present study, therefore, *A. niger* strains with the potential of producing phytase and tannase were isolated from three varieties of Bambara nuts.

MATERIALS AND METHODS

Mature Bambara nuts (*Vigna subterranea*) of three different color coats (cream, black and red) (Figure 1) were purchased from local farmers in Zaria, Kaduna State, Nigeria. They were identified and validated at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The voucher number as deposited at the herbarium unit is 1321. They were taken to the Laboratory of Biochemistry Department, Ahmadu Bello University, Zaria, Nigeria where they were picked clean of all debris and broken seeds. Three bean species, *Vigna unguiculata, Vigna racemosa* and *Phaseolus vulgaris* were also purchased from local farmers in Zaria, Kaduna, State, Nigeria for the solid-state fermentation (SSF).

The cleaned and sorted Bambara nuts were intermittently moistened and allowed to grow mouldy. *A. niger* was isolated from the mouldy Bambara seeds by standard procedures as adopted by Pang and Ibrahim (2004) and identified according to the method of Ellis (2006).

Screening for simultaneous tannin and phytic acid degrading *A. niger* isolates was carried out by simple agar plate method; an enrichment culture technique in minimum medium as carried out by Pinto et al. (2001). The selection medium (g/L); $NaNO_3$-3.00, KH_2PO_4-1.00, $M_9SO_4.7H_2O$-0.50, F_eSO_4. $7H_2O$-0.01, agar- 30.0 was autoclaved at 121°C for 15 min and supplemented with 1% phytic acid (10 ml) and 1% tannic (10 ml) previously filter-sterilized through Whatman filter paper N^o1 with pH- 4.0 adjusted using 100 mM NaOH. Strains of *A. niger* isolated were then point-inoculated and incubated at 30°C for 96 h and the diameters (mm) of the colonies were measured at 24 h intervals.

The *A. niger* spore inoculum was prepared from the isolate that exhibited highest tannin and phytate utilization potential by adding 10 ml of sterile distilled water containing 2.5% Tween 80 (polyoxyethlene sorbitan monoolate) to a fully sporulated slant culture. The spores were dislodged by vigorous shaking and spores number estimated by direct microscopic enumeration using cell-counting hemocytometer (Neubauer chamber; Merck, S.A., Madrid, Spain). The volume of spores' suspension was adjusted to 1.064×10^7 spores/ml and the harvested *A. niger* spores were centrifuged at 3000 ×g for 2 min, washed in sterile distilled water and re-centrifuged. The washed cells were then used as inoculums singly in the SSF of *Vigna unguiculata, Vigna racemosa* and *P. vulgaris* (Dapiya et al., 2010).

Here, about 250 g of each of the three (3) beans flour were weighed into 500 ml flat bottom flask and autoclaved at 121°C for 15 min. Moisture contents of the samples were adjusted to 25% before aseptic inoculation with spore suspension of *A. niger*, containing 1.064×10^7 spores/25 g of flour (Bhat et al., 1997), and incubated at room temperature (29 ± 3°C) for 48 h. After the fermentation, the fungal growth was terminated by drying at 55°C in oven for 24 h (Fadahunsi, 2009) and re-ground using kitchen blender.

The enzymatic solution was obtained from the fermented medium by the addition of 25 ml 20 mM acetate buffer pH 5.0 per 25 g of fermented medium and the flask was shaken in a shaker for 1 h at 200 rpm. The solution was then filtered and centrifuged at 10,070 × g for 30 min at 4°C. The supernatant was then used for phytase and tannase activity assays according to the methods of Lee et al. (2005) and Mondal et al. (2001), respectively. One unit of enzymatic activity was defined as 1 mM of phosphate produced per min for phytase assay and the amount of enzyme needed to release 1 mM per min of residual tannic acid for tannase assay.

RESULTS AND DISCUSSION

Different colonies (isolates) were observed on the Potato Dextrose Agar (PDA) plates that were spread with the liquid from the three types of Bambara nuts. The black-colored colonies were observed on the three Bambara nuts varieties, while red-colored colonies were observed on the cream- and red-colored seed coat Bambara nuts (Figure 2). The green colonies were observed on the black-colored seed coat Bambara nuts.

The black-colored colonies were identified as *A. niger* based on the structural morphologies as observed under the light microscope. It was observed that the isolate possessed distinct conidiophores terminated by a swollen vesicle bearing flask-shaped phialides (Pitt and Samson, 2000). The black-colored colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads (Ellis, 2006). The red and green color isolates that appeared on the PDA plates may be other *Aspergillus* sp. or other type of fungi or microorganism.

Bradoo et al. (1996) reported formation of a clear

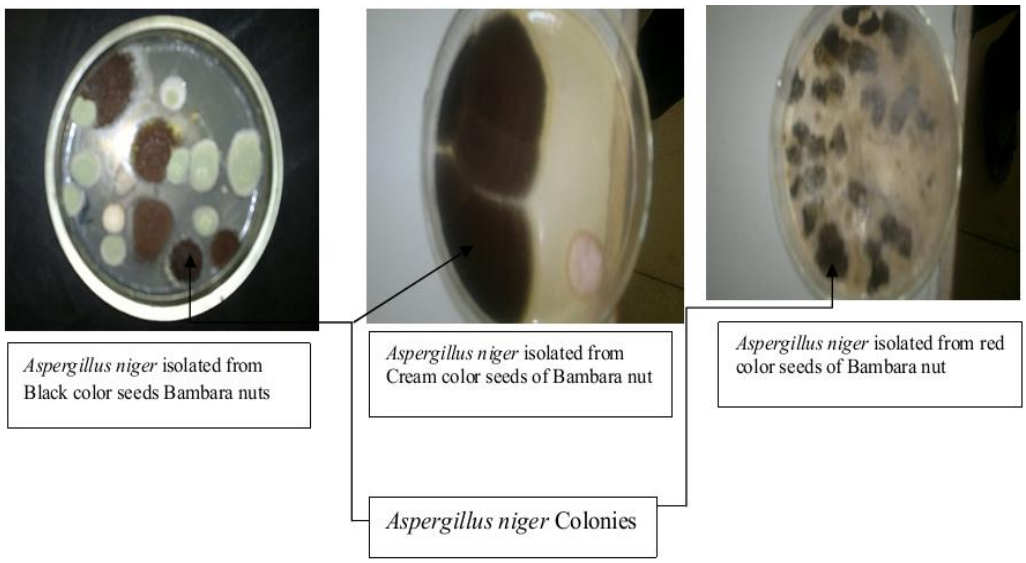

Figure 2. *Aspergillus niger* isolated from Bambara nuts of different color seed coats

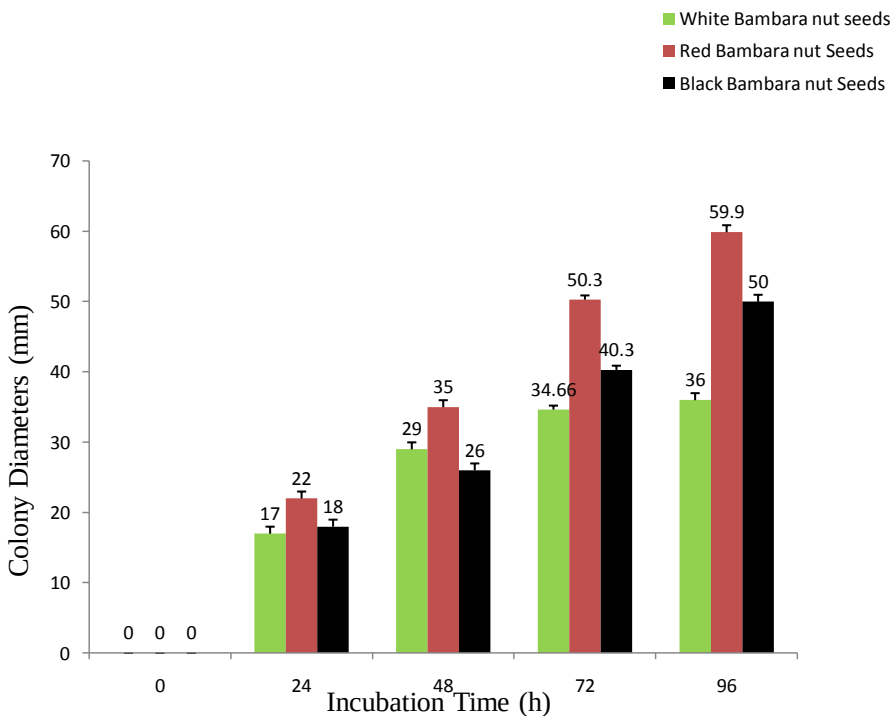

Figure 3. Colony diameters of *A. niger* strain in tannic acid and sodium phytate agar plate isolated from Bambara nut of different seed coats.

zone around the mycelium, suggesting tannase activity. However, the observation of this clear zone is difficult, therefore, measurement of colony diameter was applied since direct measurement of the colony diameter is a good indicator of the ability of tannic acid and phytic acid utilization as carbon sources which could be attributed to the tannase and phytase activities in the medium (Pinto et al., 2001). The *A. niger* isolated from the red-colored seed coat Bambara nuts showed the highest colony diameter in the selection medium and was selected as the highest simultaneous producer of phytase and tannase (Figure 3) and assayed for enzymatic activity

Table 1. Phytase and tannase activities of *Aspergillus niger* in solid state fermentation.

Organism	Phytase activity (μM.min^{-1})	Tannase activity (μM.min^{-1})
Vigna unguiculata	7.47 ± 0.084^{b}	17.41 ± 0.44^{b}
Vigna racemosa	11.81 ± 0.26^{c}	5.77 ± 0.30^{a}
Phaseolus vulgaris	5.13 ± 0.37^{a}	6.48 ± 0.17^{a}

Values are mean ± standard error of mean (SEM) for three replicates and values with different superscripts are significantly different from each other at $P < 0.05$. Data were analysed using the One-way Analysis of Variance (ANOVA) and the Duncan multiple range test was used for determination of difference between means using the SPSS 17 software package.

by SSF. The SSF was selected because of reports that the production of the enzymes increased by some folds in SSF when compared to liquid surface and submerged fermentations (Lekha and Lonsane, 1997). The assay of both enzymes using three bean flours show that phytase activity range between 5.13 and 11.81 μM/min while tannase activity range between 5.77 and 17.41 μM/min (Table 1), which is consistent with the reports of Madeira et al. (2011), Roopesh et al. (2006) and Vassilev et al. (2007).

The present work has shown that *A. niger* can be easily isolated from Bambara nuts and that the *A. niger* isolated from red-colored seed coat of Bambara nuts have better potential for simultaneous production of phytase and tannase. Therefore the isolate can be good inoculums for other legume detoxification.

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