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Research Paper

Isolation of amylase and cellulase producing fungi from decaying tubers and optimization of their enzyme production in solid and submerged cultures

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Abstract. The aim of this work was to isolate hydrolytic enzyme-producing fungi from decaying tubers and to optimize their enzyme production in solid and submerged cultures. Five fungal genera were isolated from decaying cassava, cocoyam, white yam, and sweet potato tubers. Amylase and cellulase production were qualitatively detected in PDA and CMC plates of the isolates using Lugol's iodine and 1% Congo red staining solution techniques respectively. The five fungal genera were identified as *Aspergillus* species I and II, *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. based on their macroscopic and microscopic features with reference to Dichotomous Key for fungi. Amylase and cellulase production by the isolates in solid state and submerged cultures were optimized using rice grains, sweet potato, white yam, cassava and cocoyam flours as substrates. *Aspergillus* species I produced the highest glucoamylase units of 572.16 ± 7.92 and 482.70 ± 2.00 µmole/g of solid substrate from rice grains and sweet potato flour mixed with 20% rice husk respectively. *Rhizopus* species produced the highest cellulase units of 263.52 ± 3.68 µmole/g from sweet potato flours in solid substrate fermentation. Cellulase, gluco- and alpha-amylase productivities were generally higher in solid state than in submerged cultures. The results of this experiment have shown that decaying tubers are good sources of amylase and cellulase producing fungi. Amylase and cellulase production capacity of the isolates of *Aspergillus* and *Rhizopus* species are quite high and they have high potentials for biotechnological applications.

Keywords: Fungi, amylase, cellulase, tubers, fermentation, diseased tubers.

INTRODUCTION

Carbohydrate materials (sugars, starch and cellulose) are valuable and natural industrial raw materials used worldwide (De Wit *et al*., 1993; Sudha *et al*., 2014). A lot of useful products can be produced from the monomeric units of these carbohydrate materials. However, in order to convert starch and cellulose to useful products, they need to be hydrolyzed into their monomeric units by either enzymes or chemicals (acids or bases). Although chemical hydrolyses are presently faster and cheaper than enzymatic method, they are not environmentally friendly and they require special (non-corroding) vessels for the reaction to take place. Bioconversion using enzymes are safer and more environmentally friendly than the use of chemicals. Amylases and cellulases are the key enzymes used for starch and cellulose hydrolysis respectively. Amylases are used in starch processing industries for the production of glucose and fructose syrups (Silva *et al*., 2010), paper and pulp, beverage and bakery industries (De Souza and Magalhaes, 2010). Cellulases catalyze the breakdown of cellulose components of plant cell wall. They are used in protoplast isolation, and bioethanol production from lignocellulosic materials (Grover *et al*., 2013).

Various microorganisms such as bacteria (Padhiar and Kommu, 2016; Fentahun and Kumari, 2017); fungi (Khokhar *et al*., 2011; Irfan *et al*., 2012a; Grover *et al*., 2013;

Egbere *et al*., 2014; Saxena *et al*., 2015), yeast (Buzzini and Martini, 2002; Yaicin and Corbaci, 2013; Carrasco *et al.*, 2016; Thongekkae and Kongsanthia, 2016). Among the various microorganisms that are employed for amylase and cellulase production, fungi are the most reliable because they can produce extracellular amylases in large scale for industrial processes (Saxena *et al.,* 2015). Amylase and cellulase-producing fungi can be isolated from various environments such as soil (Saxena *et al*., 2015), starch processing waste site (Ogbonna *et al*., 2014; Oboh, 2005), farmland, market place, food processing sites (Johnson *et al*., 2014) and palm oil waste (Shahriarinour *et al*., 2011).

Nigeria is blessed with a lot of natural resources among which are biodiversity (including microorganisms and plants). In addition, Nigerian weather is favourable for growth and metabolite production by various microorganisms. Nigerian weather is also very suitable for agriculture and about 37.33% of Nigerian soil is arable land (World Bank collection of development indicators, 2014). Various crop plants are cultivated by Nigerian farmers both at large and subsistent scales. However, food processing sector is still underdeveloped in Nigeria to the extent that a lot of food materials rot away during their seasons. Nigerian farmers produce a lot of tuber crops such as cassava, yam, cocoyam and sweet potatoes and are even the present highest producer of cassava tubers (http://www.faostat.fao.org). Tuber crops are very susceptible to microbial attack due to their high water contents (60 to 70%). Furthermore, tubers which are stored food reserve of certain crop plants are mainly composed of outer cellulose and inner starchy food. Microorganisms that can synthesize amylases and cellulases are more likely to colonize these tubers and bring about their deterioration and decay. These decaying tubers may serve as natural sources of amylase and cellulase-producing microorganisms such as fungi. However, most of the enzymes including amylases and cellulases used both in cottage and large scale industries in Nigeria are imported from other countries. Nigeria as a nation spends a large amount of national revenue on the importation of industrial enzymes. Most of the key industrial enzymes can be sourced from Nigerian bio-resources. Some of these bioresources are stored tubers which contain large amount of starch and cellulosic materials. There is therefore a need to explore the potentials of isolating high amylase and cellulase-producing fungi from decaying tubers. The aims of this study were to explore the potentials of decaying tubers as sources of amylase and cellulase enzymes, and to evaluate the healthy tubers and rice grains as substrates for production of the enzymes in both solid state and suspended cultures. We screened four different tubers (yam, cocoyam, sweet potatoes and cassava) in their early decaying process for amylase and cellulase-producing fungi and investigated the potentials of the healthy tubers as substrates for enzyme production by the isolates in both solid state and submerged cultures.

MATERIALS AND METHODS

Tubers

Four tubers, namely cocoyam (*Colocasia esculenta*), white yam (*Dioscorea rotundata)*, sweet potato (*Ipomea batatas)* and cassava (*Manihot esculenta* Crantz*)* both healthy and those in early stage of decay were procured from a nearby local market in Enugu state of Nigeria and transported to the Plant Pathology laboratory, Department of Plant Science and Biotechnology University of Nigeria Nsukka for identification and processing.

Isolation of fungi from diseased tubers

Potato Dextrose Agar medium was used for isolation of enzyme-producing fungi. The decaying tubers were cut between the healthy and infected portions to obtain the decaying parts. The cut out decaying portions were sliced into small sizes of about 5 by 7 mm with a sterile cutter. The tuber pieces were washed three times with distilled water and rinsed twice with 0.1% mercury chloride and rinsed again twice in distilled water. Each piece was picked with a sterile forceps and inoculated at the centre of Potato Dextrose Agar in Petri dish. The dishes were covered and incubated at room temperature 27 ± 2°C. The cultures were examined daily until full fungal colonies were formed within 5 to 7 days of incubation. Single colony was picked with a sterile inoculation loop and transferred to freshly prepared PDA plates. The sub-culturing was done several times until pure culture of each isolate was obtained. A total of nine fungal isolates were obtained.

Screening the nine isolates for amylase and cellulase production

The nine fungal isolates were screened for amylase production by sub-culturing them on starch peptone agar medium with the following composition (in g/L): starch, 20; peptone, 10; chloramphenicol, 0.05; and agar, 20. Cellulase-producing fungal species were screened for, using a medium composed (in g/L) of carboxyl methyl cellulose, 10; sucrose, 30; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 0.05; KCl, 0.5; FSO_4, 0.01; and agar, 20. The pH of the medium was adjusted to 5.0 with either 1 N NaOH or HCl solution before autoclaving at 121°C for 15 min at 15 lbs pressure. A cork borer with diameter of 4 mm was used to transfer a fully sporulated pure culture of each of the isolates to the starch peptone agar plate and carboxyl methyl cellulose medium. The plates were incubated at 27 ± 2°C. After 4 days of incubation, the plates with starch medium were flooded with 10 ml Lugol's iodine solution (10% potassium iodide and 5% iodine crystal) while the carboxyl methyl cellulose medium plates were flooded with 10 ml of 1 % Congo Red staining solution for

30 min. The iodine solution was decanted after 5 min and rinsed out with distilled water to expose zones of clearance around colonies that produced amylases. The Congo red solution was decanted and flooded with 10 ml of 1 M NaCl solution for 20 min and decanted to expose the zones of clearance. The diameter of the clear zone formed around each fungal colony was measured with a transparent meter rule and was taken to represent the amylolytic and cellulolytic activities of each fungal isolate. Five fungal genera produced clear zones on starch Peptone and on CMC plates. Pure cultures of the isolates that produced amylase and cellulase were sub-cultured into freshly prepared PDA test tube slants and incubated for 7 days at 27 ± 2°C. The fully sporulated test tube slant cultures were stored at 4°C in a refrigerator and sub cultured every six weeks.

Preliminary identification of the five amylollytic and cellulolytic isolates

The five isolates that produced clearance zones in soluble starch agar and CMC-agar were identified by sub-culturing in fresh PDA plates for 5 to 7 days. The growth pattern was observed visually throughout the cultivation period. For the fast growing species like *Rhizopus* a thin strand of mycelium was picked with a pair of forceps on the third day of inoculation and placed on a clean microscope slide and stained with lacto-phenol cotton blue solution. The stained mycelium was viewed under the microscope at 100 × magnification. The same process was carried out with all the isolate on either 6^{th} or 7^{th} day depending on the growth rate. A photomicrograph of each isolate was taken with a digital camera. Each fungus was identified based on macroscopic and microscopic observation of the colonies, conidia, conidiophores, sporangium and sporangiophores. A combination of dichotomous key for fungi and basic mycology text book (Alexopoulus *et al*., 2007) were used in the identification to generic name.

Preparation of tuber flour

The healthy tubers were washed with tap water, peeled, sliced to thin chips of about 8 × 5 mm and sun dried to about 10% moisture content. The dried chips were milled and sieved with a muslin cloth. The resulting flour was stored in an air tight container in a refrigerator (~4°C).

Amylase and cellulase production by the fungal isolates in solid substrate cultures (koji)

Preparation and inoculation of 'koji'

The solid substrates were prepared using flour made from healthy cocoyam, white yam, sweet potato, cassava and rice grains. All tuber flour and rice grains were supplemented with 20% rice husks for amylase enzyme production while 80% rice husk was supplemented with 20% sweet potato flour for cellulase enzyme production. The substrates were used to prepare *koji* according to the method of Ogbonna and Okoli (2010). Eighty grams (80 g) of each tuber flour was mixed with 20 g of rice husk (for amylase) and rehydrated with 60 ml of distilled water (60% moisture). In case of solid substrate for cellulase enzyme production, 80 g rice husk was mixed with 20 g of sweet potato flour and rehydrated with 60 ml of distilled water. The components were thoroughly mixed in 250 ml beaker. Rice grains (80 g) was weighed and washed three times in cold water and soaked in cold water for one hour. The rice grains were removed from water and put into a sieve to drain out the water for 30 min. Each of the tuber flours and rice grains mixed with 20% rice husks and 80g rice husk mixed with 20% sweet potato flour were steamed for 30 min over a moderate flame. Spores were harvested from 7 days old slant of each fungal isolate by adding 6 ml of sterilized distilled water. Inoculation wire loop was used to loosen the spores. The spore suspensions were decanted into sterile beakers and the spores were enumerated by counting under the microscope using a haemocytometer (BS 748, Hawksley, UK). The spore suspension was diluted to a concentration of 2×10^7 spores/ml. One milliliters (1 ml) spore suspension from each fungal isolate was used to inoculate each of the substrates. Each substrate was prepared in triplicates, wrapped in a muslin cloth and wrapped again in a sterile white towel with thermometer inserted and incubated inside a small wooden box. The culture was incubated at room temperature (27 ± 2°C) for 48 h. Each culture was mixed manually, wrapped and incubated for an additional 24 h at room temperature. During the incubation, the variation in temperature was not more than 2°C.

Harvesting and determination of the enzyme activities

One gram (1 g) each from the inoculated substrate was weighed out of the 72 h old *koji* into 10 ml of sterilized distilled water in a test tube containing 1 ml of 50 mM acetate buffer (pH 5.8) and mixed thoroughly. In case of solid substrate for cellulase, 1 g was weighed into 10 ml of sterilized distilled water in a test tube containing 1ml of 0.1 M citrate buffer (pH 4.8) and mixed thoroughly. The test tubes were kept on a rotary shaker and left to extract at 100 rpm for 30 min at room temperature. The liquid component containing the enzymes was filtered through 0.45 µm sodium acetate membrane filter and centrifuged at 7000 rpm for 10 min. The supernatant was used as crude amylase and cellulase respectively.

Amylase production in submerged fermentation

The liquid culture medium consisted of the following

components (in grams per liter of distilled water): soluble starch, 20; KH_2PO_4, 0.4; NH_4NO_3, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.1; and $FeSO_4.7H_2O$, 0.01. All the components were stirred to mix and the pH was adjusted to 6.9 with acetic acid. The culture medium was dispensed in 50 ml aliquots into 250 ml Erlenmeyer flasks and the mouths of the flasks were plugged with silicon plugs, wrapped with aluminum foil and sterilized by autoclaving at 121°C for 20 min. Spores were harvested from test tube slants as described before. One milliliter (1 ml) of spore suspension containing 2×10^7 spores/ml was used to inoculate each 50 ml medium. The flasks were placed in wooded box laid with towel with a thermometer inserted and incubated at room temperature (27 ± 2°C) with intermittent manual shaking for 7 days. The culture broth was filtered through 0.45 µm sodium acetate membrane filter and centrifuged at 7000 rpm for 10 min and used as the crude amylase.

Production of cellulase in submerged fermentation

The submerged fermentation medium was composed of the following (in g/L of distilled water): Carboxyl methyl cellulose salt, 5; KH_2PO_4, 2; NH_4NO_3, 2; $MgSO_4.7H_2O$ 1; $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 0.016; $ZnSO_4.7H_2O$, 0.345; $CoCl.6H_2O$, 0.002; and $CaCl_2$, 0.3. The pH was adjusted to 4.8 with 0.1 M citrate buffer. The medium components were stirred thoroughly to dissolve and then dispensed in 50 ml aliquots into 250 ml Erlenmeyer flasks and plugged with silicon plugs and sterilized by autoclaving at 121°C for 20 min. After cooling to room temperature, the medium was inoculated with 5 ml of spore suspension as described above and incubated at room temperature (27 ± 2°C) in a wooded box as described before for 7 days. At the end of the cultivation, the culture broth was filtered and centrifuged as described for amylase and used as crude cellulase enzyme.

Enzyme assays

Assay for glucoamylase

Glucoamylase activity was determined by the method of Miller (1959). Glucoamylase activity was defined as the amount of enzyme that liberates 1 µmole (one micromole) of reducing sugar per minute with glucose as standard under the assay conditions. The enzyme activities were calculated using a calibration curve prepared with D-glucose as standard. The values of enzyme activities were expressed as µmole/g in solid substrate fermentation and µmole/ml for the submerged fermentation. Crude amylase enzymes prepared in both solid and submerged cultures (0.5 ml each) was dispensed into clean glass test tubes (50 ml vol) containing 1 ml of 50 mM acetate buffer (pH5.8) and 0.5 ml of 1% soluble starch. The mixture was incubated for 30 min at 50°C in a water bath. The reaction was stopped by adding 1ml of 3, 5-dinitrosalycilic acid (DNSA) reagent and boiling for 10 min for color development. The mixture was allowed to cool at room temperature and the absorbance was read at 540 nm using spectrophotometer (Shimadzu UV1800, Japan). The concentration of the released glucose was calculated from a glucose calibration curve.

Assay for alpha amylase

Alpha amylase activity was determined according to the method of Varalakshmi *et al.* (2009) with modifications. Soluble starch 1.0 w/v% (0.5 ml) was added into a glass test tube (30 ml) containing 0.2 ml of 0.1 M phosphate buffer (pH 5.6.). The crude amylase enzyme (0.3 ml) was added. The mixture was incubated at 25°C for 20 min. The reaction was stopped by addition of 1 ml 3,5-dinitrosalycylic acid reagent and boiling at 100°C for 10 min in a water bath. The absorbance was read at 540 nm after cooling and the glucose liberated was estimated from glucose calibration curve.

Assay for cellulase

Cellulase enzyme activity was assayed according to the modified method of Miller (1959) by measuring the amount of reducing sugar released from low viscosity carboxymethyl cellulose (CMC) solution per minute under the assay condition with glucose as a standard. The reaction mixture consisted of 0.5 ml of 1% w/v CMC in 0.5 ml of 0.1 M citrate buffer (pH 4.8) and 0.5 ml crude cellulase enzyme. The reaction mixture was incubated at 40°C for 30 min and the reaction was terminated by adding 1 ml of DNSA reagent. The reaction mixture was cooled to room temperature and the absorbance was read at 540 nm. One unit of CMCase activity was expressed as the amount of enzyme that liberates 1 µmole of reducing sugar (glucose equivalent) per minute per milliliter of the reaction mixture under the experimental conditions.

Statistical analysis

Every Experiment was performed three times and the data obtained were subjected to one way analysis of variance (ANOVA). The means were separated using Least Significant Difference (LSD) test at $P< 0.05$.

RESULTS AND DISCUSSION

Isolation and determination of amylase and cellulase activities of fungi from decaying tubers

Out of the nine fungal species isolated from decaying

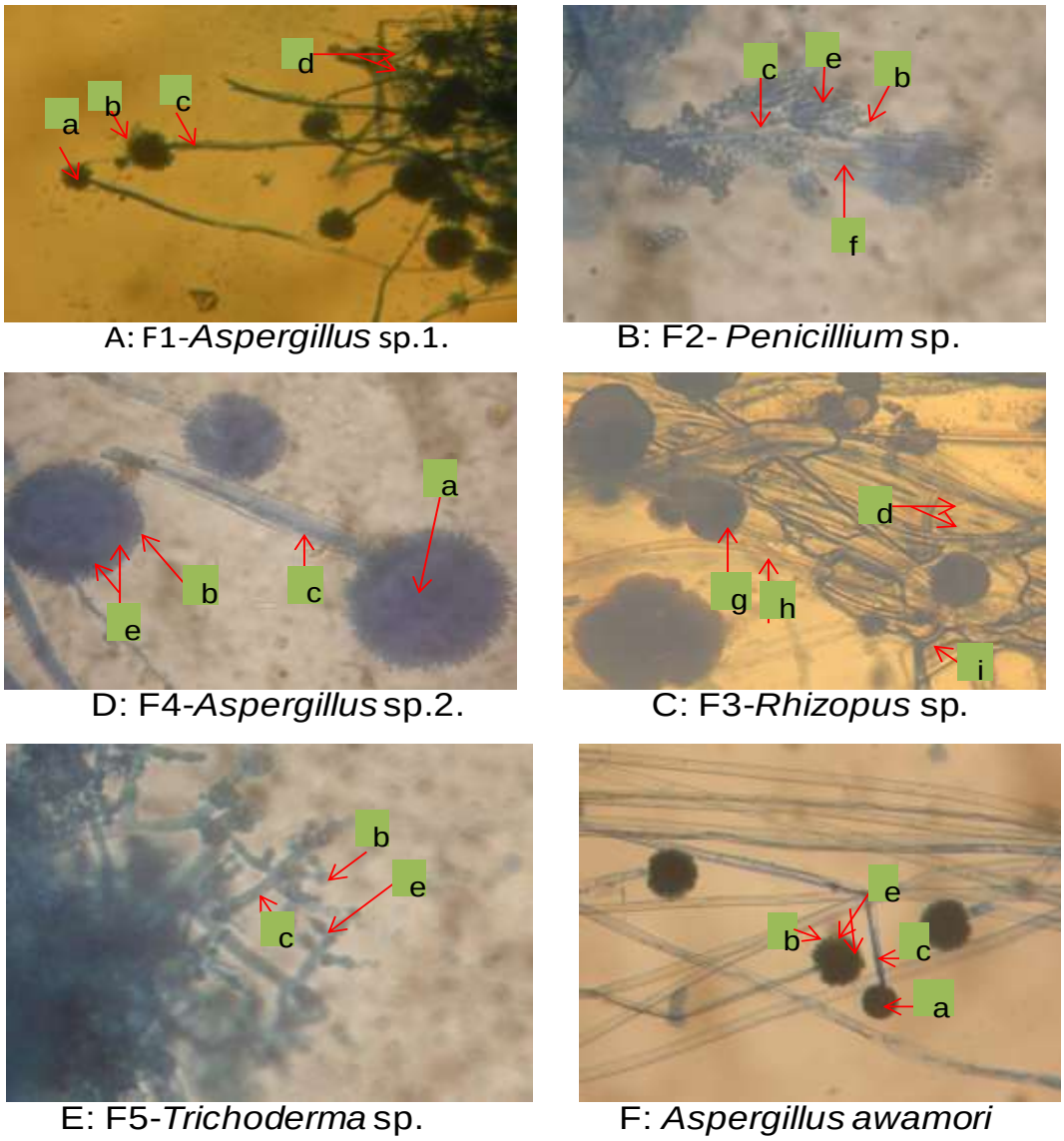

Figure 1. Photomicrographs of the fungal isolates. A: vessicle, B: conidium, C: conidiophore, D: hyphae, E: phialides, F: metula, G: sporangium, H: sporangiophore, I: rhizoid, (mg × 100).

tubers, five showed amylase and cellulase production potentials by producing clear zones around their colonies in soluble starch-peptone, and CMC agar plates respectively. The five fungal isolates were identified as *Aspergillus* species I, *Aspergillus* species II, *Rhizopus* sp., *Penicillium* sp. and *Trichoderma* sp. based on macroscopic and microscopic features with reference to dichotomous key for fungi and use of standard Mycology text (Alexopoulos *et al*., 2007). Photomicrographs of the isolates are shown in Figure 1.

Effects of solid substrates on glucoamylase production by the five fungal isolates

Out of the five fungal isolates, three fungal genera namely *Aspergillus* species I, *Penicillium* sp., and *Rhizopus* species produced appreciable amount of glucoamylase. Generally, *Aspergillus* species I produced significantly more glucoamylase from all the substrates than the other four isolates. The glucoamylase activities of *Aspergillus* species I was even higher than that of the reference fungus (*Aspergillus awamori)* (Table 1). Rice grain supported the highest glucoamylase production by *Aspergillus* species I (572.16 ± 7.92 µmole/g) and this was followed by sweet potato flour (482.70 ± 2.00 µmole/g). The second and third best strains for glucoamylase production were *Penicillium* sp., and *Rhizopus* sp. which produced 388.86 ± 5.10 and 347.86 ± 2.72 glucoamylase units from sweet potato flour. On the whole, sweet potato flour was the best substrate for glucoamylase production by all the fungal isolates.

Table 1. Effects of solid substrates on glucoamylase production (in µmole/g) by the five fungal isolates in comparison with *Aspergillus awamori*.

Fungal isolates	*Dioscorea rotundata* Flour+rice husk	*Colocasia esculenta* Flour+rice husk	*Manihot esculenta* Flour+rice husk	*Ipomea batatas* Flour+rice husk	*Oryzae sativa* grains Flour+ rice husk	Soluble starch Flour + rice husk
Aspergillus sp. I	354.06 ± 0.56	378.84 ± 1.28	409.08 ± 2.00	482.70 ± 2.00	572.16 ± 7.92	256.56 ± 1.82
Penicillium sp.	212.48 ± 1.1	290.46 ± 1.82	224.86 ± 1.46	388.86 ± 5.10	62.34 ± 0.74	82.10 ± 2.64
Rhizopus sp.	178.22 ± 1.8	133.40 ± 1.8	155.26 ± 0.72	347.86 ± 2.72	160.00 ± 2.92	143.22 ± 4.77
Aspergillus sp .II	126.10 ± 0.43	112.98 ± 0.72	131.24 ± 1.64	272.78 ± 2.00	125.38 ± 2.20	113.34 ± 3.28
Trichoderma sp.	168.56 ± 0.92	136.30 ± 1.82	224.32 ± 1.28	221.04 ± 2.38	133.40 ± 5.48	226.32 ± 0.72
Aspergillus awamori	381.02 ± 0.92	401.98 ± 3.28	354.78 ± 2.36	609.90 ± 2.38	458.1 ± 4.02	544.28 ± 2.00

Table 2. Effects of solid substrates on Alpha amylase production (in µmole/g) by five fungal isolates in comparison with *Aspergillus awamori.*

Fungal isolates	*Dioscorea rotundata* Flour+rice husk	*Colocasia esculenta* Flour+rice husk	*Manihot esculenta* Flour+rice husk	*Ipomea batatas* Flour+rice husk	*Oryzae sativa* grains Flour+rice husk	Soluble starch Flour+rice husk
Aspergillus sp. I	89.12 ± 1.18	85.12 ± 0.74	93.12 ± 1.32	83.30 ± 1.00	82.40 ± 1.08	146.58 ± 1.40
Penicillium sp.	84.08 ± 3.16	88.28 ± 0.84	85.46 ± 0.40	82.22 ± 1.00	131.58 ± 5.74	108.02 ± 0.74
Rhizopus sp.	52.34 ± 0.64	51.06 ± 0.72	53.82 ± 0.44	50.92 ± 0.98	51.76 ± 0.68	61.12 ± 0.84
Aspergillus sp. II	53.52 ± 0.54	50.12 ± 1.08	52.70 ± 0.70	59.02 ± 0.98	52.00 ± 0.10	51.52 ± 0.90
Trichoderma sp.	66.02 ± 0.70	64.30 ± 1.14	58.82 ± 0.60	51.52 ± 0.98	57.64 ± 0.90	55.66 ± 1.20
Aspergillus awamori	81.82 ± 2.28	90.74 ± 0.84	82.50 ± 1.78	82.98 ± 0.98	82.10 ± 1.68	94.12 ± 1.62

Table 3. Effects of solid substrates on cellulase production (in µmole/g) by five fungal isolates in comparison with *Aspergillus awamori*.

Fungal isolates	*Dioscorea rotundata* Flour+rice husk	*Colocasia esculenta* Flour+rice husk	*Manihot esculenta* Flour+rice husk	*Ipomea batatas* Flour+rice husk	*Oryzae sativa* grains Flour+rice husk	Soluble starch Flour+rice husk
Aspergillus sp. I	74.36 ± 2.56	79.28 ± 0.74	78.36 ± 0.72	136.30 ± 1.82	69.98 ± 1.10	78.72 ± 1.46
Penicillium sp.	81.10 ± 5.30	83.46 ± 3.46	70.72 ± 0.36	135.02 ± 1.64	69.80 ± 2.74	68.98 ± 2.38
Rhizopus sp.	177.12 ± 2.20	121.36 ± 5.46	101.68 ± 2.92	263.52 ± 3.68	130.66 ± 0.54	106.06 ± 1.10
Aspergillus sp. II	92.58 ± 3.66	91.66 ± 0.54	110.08 ± 0.72	239.26 ± 1.28	90.40 ± 0.74	96.02 ± 1.10
Trichoderma sp.	71.98 ± 2.00	148.16 ± 2.74	140.50 ± 1.64	148.34 ± 2.20	80.38 ± 2.38	129.20 ± 1.64
Aspergillus awamori	78.54 ± 2.36	78.98 ± 1.26	76.18 ± 0.38	158.00 ± 2.38	72.16 ± 1.46	98.22 ± 2.74

Effects of solid substrates on alpha amylase production by the five fungal isolates

Results of the effect of solid substrates on alpha amylase production by the five fungal species are shown in Table 2. Among the five isolates, *Aspergillus* sp. I was still the best producer of alpha amylase. The alpha amylase production by *Aspergillus* sp. I from soluble starch was significantly higher than the activities obtained from other substrates ($p < 0.05$). This was followed by cassava but there were no significant differences among the activities obtained from other substrates ($p > 0.05$). *Penicillium* species was the next best strain, producing 131.58 ± 5.74 µmole/g from rice grains. Alpha amylase produced by other strains from all the substrates was comparably low.

Production of cellulase by the five fungal isolates in solid state cultures

Results of the effects of solid substrates on cellulase production by the five fungal isolates are shown in Table 3. *Rhizophus* species was the best producer of cellulase, followed by *Aspergillus* sp. II. There were no significant

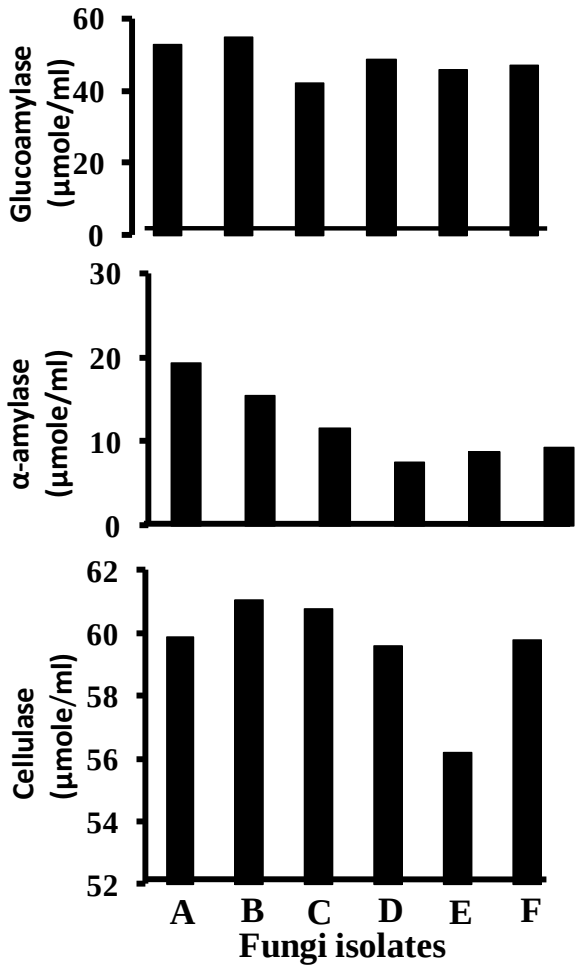

Figure 2. Comparison of glucoamylse, alpha amylase, and cellulase activities (U/ml) of some fungal isolates in submerged fermentation. The isolates were tentatively identified as A: *Aspergillus* sp. I; B: *Penicillium* sp. C: *Rhizopus* sp.; D: *Aspergillus* sp. II; E, *Trichoderma* sp. and F, *Aspergillus awamori.*

differences in cellulase production by *Aspergillus* sp. I and *Penicillium* sp. It is interesting to note that both *Rhizopus* and *Aspergillus* sp. II produced significantly higher cellulase than *Trichoderma* sp. which is traditionally used for cellulase production. Sweet potato flour was the best substrate for cellulase production by all the fungal isolates (Table 3). The units of cellulase activities produced from Sweet potato flour by all the fungal isolates were above 100 µmole/g.

Effects of submerged fermentation on gluco-amylase, alpha amylase and cellulase enzyme production by the five fungal isolates

As shown in Figure 2, *Penicillium* sp. and Aspergillus sp. I produced higher glucoamylase activities than the other three fungal isolates in submerged cultures. However, there were no significant differences in gluco-amylase production by the other isolates. In the case of alpha amylase, *Aspergillus* sp. I was the best, followed by *Penicillium* sp. while *Aspergillus* sp. II produced the least alpha amylase activity (Figure 2). The highest cellulase activity was produced by *Rhizopus* sp. while the least was produced by *Trichoderma* sp. ($P < 0.05$). However, there were no significance differences among the cellulase activities of the other isolates ($P > 0.05$). On the whole, the units of cellulase activities produced in submerged cultures (Figure 2) were lower than those obtained in solid state cultures (Table 3).

DISCUSSION

Amylase and cellulase-producing fungi were successfully isolated from decaying tubers. Previous reports (Arora *et al.*, 2017; Mahmood *et al*., 2016; Irfan *et al*., 2012a; Gupta *et al*., 2008; Pandey *et al.,* 2000) have also shown that fungi that belong to the genera *Aspergillus, Rhizopus* and *Penicillim* are amylase and cellulase producers. Sun *et al*. (2007) and Arora *et al*. (2017) used *Penicillium* spp. for amylase production while Nyamful, (2014) and Abdelwahab (2015) isolated and produced glucoamylase by *Rhizopus* MENACOII A and *Rhizopus oryzae* respectively. Negi and Banerjee (2009), Zambare (2010) and Karim *et al.* (2017) have also reported high glucoamylase production by *Aspergillus* species.

The present study has shown that sweet potato is a good substrate for enzyme production. This is in agreement with that of Ominyi (2013) who reported that sweet potato flour was the best substrate for glucoamylase production by *Aspergillus niger.* Mahmood *et al*. (2016) used potato peels as substrate for alpha amylase production by *A. niger*. Sweet potatoes contain high concentrations of both starch and sugars. Thus, they can support fast cell growth on sugars and starch-induced amylase production.

It is important to note that glucoamylase produced by *Aspergillus* sp. 1 in this study is higher than the values reported by others. Arora *et al*. (2017) obtained a maximum glucoamylase activity of 10.29 ± 0.07 u/ml from rice bran using *Penicillium* sp. and after supplementing with sucrose and yeast extracts as carbon and nitrogen sources respectively, the maximum enzyme activities increased to 13.70 ± 0.77 and 14.41 ± 0.07 units/ml respectively. *Aspergillus awamori* was used as a reference strain here because it is currently used for industrial production of amylases. It is interesting to note that amylase production by *Aspergillus* sp. I is comparable with that of *Aspergillus awamori*.

The cost of substrates represents a significant percentage of the total production costs of many bulk chemicals, yet, a lot of work has focused on the use of soluble starch, which is relatively very expensive, for

enzyme production. Mahmood *et al*. (2016) worked on the use of potato peels as substrate for alpha amylase production by *A. niger* and they concluded that soluble starch was the best carbon source for supplementing potato peel for alpha-amylase production in solid substrate culture. They reported a maximum unit of alpha amylase of 1298.12 ± 2.14 U/g of solid substrate after optimizing the culture conditions. It is therefore noteworthy that in the present study un-processed flours, especially sweet potato, supported higher glucoamylse production than soluble starch. It is also interesting to note that cassava flour is a good substrate for alpha amylase production by *Aspergillus* species I. These un-processed flours are cheap and easily available and their use as substrates for production of enzymes will significantly reduce the cost of production.

Although most reports on cellulase production are centered on *Trichoderma* species (Irfan *et al*., 2012b; Kilikian *et al*., 2014; Florencio *et al*., 2015; Irfan *et al*., 2016), the present study has shown that *Rhizopus* species is a good strain for cellulase production. A major drawback with the use of *Trichoderma* sp. for enzyme production is that the growth rate is generally lower than those of most other fungal genera. There is therefore a need to further study cellulase production by this isolate of *Rhizopus*

Most large scale fermentation processes are done in submerged cultures. Florencio *et al*. (2015) reported that submerged fermentation was the best process for cellulase production by various species of *Trichoderma.* Irfan *et al*. (2013) also investigated celluases and hemicellulases production from untreated, chemically treated and enzymatically treated sugarcane bargasse, wheat and rice straw by mono and mixed cultures of *A. niger* and *Trichoderma viride*. They reported a maximum CMCase activity in the submerged cultures of 6.8 ± 0.5 U/mg and xylanase activity of 105 ± 2.8 U/mg. Furthermore, Irfan *et al*. (2016) also optimized the culture parameter for endoglucanase production by *Trichoderma harzianum* using submerged culture with sugarcane as the substrate. However, the present study has shown that gluco-amylase, alpha-amylase and cellulase production in solid state cultures were, on the whole, higher than in submerged cultures. These results are in agreement with the work of Kilikian *et al*. (2014) who compared cellulase production in solid and submerged fermentation by various fungi and reported that more enzyme activities were produced in solid state than in submerged cultures. Mrudula and Murugammal (2011) also reported higher units of cellulase from *Aspergillus niger* in solid state than in submerged culture. Furthermore, Varalakshmi *et al*. (2009) compared alpha amylase production by some fungal isolates in solid and submerged fermentation and reported that higher enzyme activities were obtained in solid than in submerged substrate cultures. The lower enzyme activity in submerged cultures might be due to low dissolved oxygen concentration. Enzyme production is an aerobic process and d epending on the media composition and temperature, oxygen solubility in culture broths is very low. Furthermore, the strategies used to increase oxygen transfer to culture broths, such as increased aeration and agitation speeds generate high hydrodynamic stress which adversely affects filamentous fungi. Thus, Lin *et al*. (2017) worked on how to improve cellulase production by *Trichoderma reesei* in submerged fermentation by the expression of a *Vitreoscilla* hemo-globin gene. Another possible reason for higher enzyme activity in solid state cultures is the low water activity.

Aside from higher enzyme productivity, solid state culture is very simple and can be done in simple vessels without complex aeration and mixing systems. The risk of contamination is also lower and it can be done without automated temperature control systems. It is therefore more suitable for developing countries without constant and stable electricity supply.

CONCLUSION

The present study has demonstrated that decaying tubers are good sources of amylase and cellulase-producing fungi. The five fungal genera isolated from decaying tubers showed moderately high amylase and cellulase production potentials and are therefore good candidates for large scale enzyme production for various applications. We have also demonstrated that unprocessed tuber flour and rice grains are very good substrate for enzyme production. This finding has the potential of significantly reducing the cost of enzyme production. It is hoped that using the isolates, especially *Aspergillus* sp. I, crude amylase and cellulase enzymes can be produced in solid state cultures for local applications such as poultry feeds, bakery and bio-ethanol industries. There is, however, a need to identify the isolates to species level using the ITS gene.

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