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Isolation and molecular identification of potential mycotoxigenic fungi from someselected foods

Uloma Ezinwanne Ozoani *, Uzoamaka Ogechi George-Okafor and Ezinne Oluoma Ozoani

Department of Applied Microbiology and Brewing Enugu State University of Science and Technology Enugu, Nigeria.

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Abstract

Mycotoxin, produced by filamentous fungi is one of the major contaminants of food which is detrimental to human health and causes severe economic loss. Identification of the different fungal species responsible for food spoilage is important as it will enhance food safety. The main objective of this study was to isolate and identify the fungal food pathogens in some selected foods at molecular level. The food samples were Cake, Dry fish, Corn and Tomato. They were cultured for fungal isolation using selective media. Identification of predominant isolates was done through Phenotypic (cultural and morphological characteristics) and Genotypic characteristics (sequencing by ITS). The most predominant isolates were identified to be *Aspergillus flavus* strain 1BB_17, *Aspergillus flavus* strain Beca _67, *Fusarium fujikuroi*, *Fusarium minicarnatum*, A*spergillus flavus* clone EF_384, *Aspergillus niger* strain ND 89 respectively. The result obtained in this study is an indication of possible mycotoxin contamination in foods and its consumption can cause health hazards for humans.

Keywords: Food; Fungi; Mycotoxin; *Aspergillus*; *Fusarium.*

1. Introduction

Foods, though highly nutritious to consumers are also good sources of nutrients for microbial contamination including fungi. Globally, consumption of contaminated foods emphasizes a clear food security threat, and the central elements leading to contamination are microorganisms specifically fungi which produce low- molecular weight toxic secondary metabolites known as mycotoxins [Enyiukwu *et al.*, 2014]. Mycotoxins are poisonous chemical compounds and secondary metabolites produced by fungi [Marta and Bedaso, 2016]. They are characterized bytheir ability to withstand heat, activation at low concentration, wide spectrum of toxic effects like carcinogenic, mutagenic, teratogenic and immunosuppressive, capable of causing acute or chronic effects on human beings such as induction of cancer, birth defect, liver cirrhosis, reproductive and digestive dysfunction and premature puberty in girls [Speijers and Speijers, 2004 ; Lee and Ryu, 2017]. The fungi that produce mycotoxins in food fall broadly into two groups: those that invade before harvest called field fungi and those that occur only after harvest called storage fungi. Mycotoxins are ubiquitous and accessible in different materials including human foods, animal feeds, animal products and soil [Gizachew *et al.,*2016].Generally, the fungi that cause spoilage could be considered toxigenic or pathogenic [Al-Hindi *et al.*,2011].

About 25% of the global food and feed output is contaminated by mycotoxins which negatively affects human and animal health, productivity, livelihood, income and causes significant economic losses (Enyiukwu *et al.*, 2014). Furthermore, Nigeria has been reported to have the highest estimated number of liver cancer attributable to aflatoxin in the whole world (Liu and Wu, 2010). However , according to predictions there about 5.1 million fungal species but only about 5% of the predicted filamentous fungal species have been described (O'Brien *et al.*,2006) and over 300 mycotoxins have been identified and reported but only about 5 are regularly involved in contamination of food and feed (Liu and Wu,

*Corresponding author: Uloma Ezinwanne Ozoani

2010). Hence, the aim of this study was to isolate and identify potential fungal mycotoxin producers from selected food samples.

2. Materials and Methods

2.1. Sample collection

A total of 20 food samples, 5 each of the samples (Cake, Dry fish, Corn and Tomato) were bought from food vendors within Enugu State University of Science and Technology (ESUT) Agbani campus. The samples were packed in sterile polyethene bags, labeled accordingly and taken to the laboratory for analysis.

2.1.1. Preparation of Stock Solution of the Food Samples

Ten (10g) of each of the food sample was suspended into 5ml sterile water and mixed with agitation to obtain stock solution of the sample (Muhammad *et al*., 2017) .

2.2. Isolation of fungi

One millimeter (1ml) of each stock solution of the food samples was inoculated onto Potato Dextrose agar (Himedia, India) supplemented with 0.05%(w/v) chloramphenicol to prevent the growth of bacteria and incubated at 30^0C for 7days. The observed growths (discrete colonies) were further subcultured onto fresh PDA plates to obtain pure cultures.

2.3. Identification of Fungal Isolates

This was done via morphology and molecular assays as described by George-Okafor *et al.,*2022)

2.4. Morphological Identification of Fungal Isolates

Fungal isolates were identified based on the growth rate, mycelia appearances on the plate, color and microscopic examination using lactophenol cotton blue staining as described by George-Okafor *et al*., (2022). Slides for light microscopy were prepared by spreading pure fungal isolates using a sterile inoculating loop on a slide containing a drop of 95% alcohol. The alcohol in the smear was allowed to evaporate before staining the fungal mount with lactophenol blue. A drop of lactophenol blue was added to the slide and a cover slip carefully placed on the smear without allowing air bubbles to form. The excess stain around the slide was removed using blotting paper and the slide viewed under oil immersion objective (Fisher Scientific, USA) to observe the spores of fungi, shape of vesicles and other microscopic characteristics of the isolates. The characteristics and other features were observed and recorded.

2.5. DNA Extraction

The method of Bhutia and Tamang (2021) was used. The bacterial DNA extraction was done using ZR bacterial DNA Miniprep (Zymo Research). Two milliliters (2ml) of each bacteria cell broth was added to a ZR Bashing TM Lysis tube, followed by addition of 750µL lysis solution and secured in a bead fitted with 2ml tube holder assembly and processed at maximum speed for less than 5minutes. The ZR Bashing Bead TM Lysis was centrifuged in a micro centrifuge at 10,000 rpm for one minute, the supernatant was transferred to a Zymo-Spin TM IV Spin filter (Orange top) in a collection tube after which 800µL mixture from DNA binding buffer was added to the Zymo-Spin TM II column in a collection tube, and centrifuged at 10,000rpm for 1minute two times. Then, 200µL DNA pre wash buffer was added to the Zymo-Spin TM IIC in a new collection tube and centrifuged at 10,000rpm for 1minute after which 500µL bacterial DNA wash buffer was added to the Zymo-Spin IIC column and centrifuged for 1minute before it was transferred to a clean1.5mL Micro centrifuge tube. The 100µL DNA Elution Buffer was added directly to the column matrix, centrifuged for 30 seconds to elute the DNA.

2.6. Preparation of Agarose Gel for Electrophoresis

One gram (1g) of agarose for DNA and two grams (2g) of agarose for PCR were measured and mixed with 100mL 1XTAE in a microwavable flask and microwaved for 1-3minutes until the agarose was completely dissolved and was allowed to cool down to about 50^0C for about 5minutes. Then 10ml of EZ Vision DNA stain was added and the agarose poured into a gel tray with the well comb in place. The newly poured gel was placed at 4^0C for 10-15minutes until it was completely solidified.

2.7. Electrophoresis for DNA Concentration

This quality of the DNA was checked in 0.8% agarose gel and quantified using Nano-Drop ND-1000 spectrometer (Eppendorf, Germany).

2.8. Internal Transcribed Spacer (ITS) Gene Amplification of the Fungi

The PCR mix is made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (MO270); 1µL each of 10µL forward (ITS 1: TCC GTA GGT GAA CCT GCGG) and reverse primer (1TS4: TCCTCCGCTTATTGATATGS) ; 2µL of DNA template and then made up with 8.5µL Nuclease free water. (George-Okafor *et al*., 2022).

2.9. Electrophoresis for Amplified DNA Purification

The amplified DNA fragments were run on agarose gels and 1X TBE buffer (Tris-HCL/Boric Acid /EDTA). The gels were stained with GelRed (Nucleic Acid Gel**,** Biotium) and visualized on a MultiDoc –It (UVP). Following electrophoresis, the DNA fragments were extracted from the agarose gel using a razor blade and the gel slice was placed in a sterile1.5ml micro centrifuge tube. Thereafter , 150µl of TE buffer (10mM Tris-HCL pH 8.0 and 1mM (ethylenedinitrilo) tetra acetic acid (EDTA) was added, the gel mixture was quickly frozen using dry ice and the tubes were incubated at 720C for 3min in a Thermo block (Thermo mixer compact, Eppendorf). After incubation, the tubes were centrifuged at 10000 rpm for 30seconds at room temperature. The solubilized gel solution was transferred into a new sterile tube (1.5ml) and the DNA contained in the solution was precipitated by addition of 1/10 volume of sodium acetate (3M, pH5.2 and 2.5 vol of cold ethanol and incubated overnight at -20^{0}C after which the tubes were centrifuged at 16000 rpm for 20 minutes. The obtained pellets were washed with 600µl of 70% ethanol (v/v), dried and resuspended by adding TE buffer (10mM EDTA).

2.10. Sequencing and Phylogenetic studies

The amplified fragments were sequenced using a Genetic Analyzer 31 30XL Sequencer from Applied Biosystems using manufacturers manual while the kit used was Big Dye Terminator V3.1 cycle sequencing kit, Bio-Edit software and MEGA X were used for all genetic analysis .The obtained sequence of each isolate was subjected to a BLAST search http://www.nci.nlm.nih.gov for similar sequences of known species domiciled in NCBI (National Center for Biotechnology Information) database (Tamura *et al.,*2021).

3. Results

Table 1 Phenotypic Identification Scheme for Predominant Fungal Isolates

Isolate code	Cultural characteristics	Morphological characteristics	Suspected genera
*SC1	Yellowish-green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus*[1]
*SC2	Yellowish- green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus*[2]
SC3	Yellowish- green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus* [3]
SF1	Yellowish- green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus* [4]
* SF2	Yellowish- green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus*[5]
SC	Yellowish- green woolly colonies surrounded by a white circle that changed to dark green color on PDA	Conidiosphores had a rough texture and thick walls were non pigmented and unbranched	*Aspergillus flavus*[6]

*PCA1	Fast growing white and dark purple mycelium on PDA, spores were oval	Most of the macroconidia were slender with three septate with a curved apical cell and notched basal cell	*Fusarium sp*[1]
PCA 2	Fast growing white and dark purple mycelium on PDA, spores were oval	Most of the macroconidia were slender with three septate with a curved apical cell and notched basal cell	*Fusarium sp*[2]
*PCB 1	An initially white colonies which later changed to black after a few days producing conidial spore on PDA	Conidial heads were biserate and radiated, thick smooth walls, the conidia were brown globose to subglobose finely roughened to rough –walled	*Aspergillus niger* [1]
PCB 2	An initially white colonies which later changed to black after a few days producing conidial spore on PDA	Conidial heads were biserate and radiated, thick smooth walls, the conidia were brown globose to subglobose finely roughened to rough -walled	*Aspergillus niger* [2]
*PT1	Fast growing white and dark purple colonies aerial undersurfaces mycelium on PDA, spores were oval	Most of the macroconidia were slender with three septate, a curved apical cell and notched basal cell	*Fusarium sp*[3]
PT2	Fast growing white and dark purple colonies aerial undersurfaces mycelium on PDA, spores were oval	Most of the macroconidia were slender with three septate, a curved apical cell and notched basal cell	*Fusarium sp*[4]

Key:* = Most predominant isolates within the group.

Table 2 Molecular Characterization of Predominant Fungal Isolates

Isolate code number	Species/strain Basepair	%Similarity	Genebank accession
SC1	*Aspergillus flavus* strain IBB_17, 3-563	93.4	MH 793837.1
SF 2	*Aspergillus flavus* strain Beca_67 , 111-639	97.26	KY234273
PT1	*Fusarium fujikuroi,* 700-874	100	MT603295.1
PCA1	*Fusarium minicarnatum,* 55-548	98.61	MT563420
SC2	*Aspergillus flavus* clone EF_384, 3-564	92.80	MF459666.1
PCB 1	*Aspergillus niger* strain ND 89, 13-447	71.81	MG659683.1

Table 3 Recovery Pattern of Predominant Fungi from the Food Samples

Isolate code	Identified Fungi	No. of Assays Carried out	No. of times Recovered	% Recovery
SC1	*Aspergillus flavus* strain IBB_17	5	4	80%
SE2	*Aspergillus flavus* strain Beca_67	5	5	100%
SC2	*Aspergillus flavus* clone EF _304	5	4	80%
PSB1	*Aspergillus niger* strain ND 89	5	3	60%
PCA1	*Fusarium minicarnatum* strain JL5 [-2]	5	4	80%
PT1	*Fusarium fujikuroi*	5	5	100%

Key:% Recovery $= \frac{\text{Number of times recovered}}{\text{Number of times assayed}} \times 100$

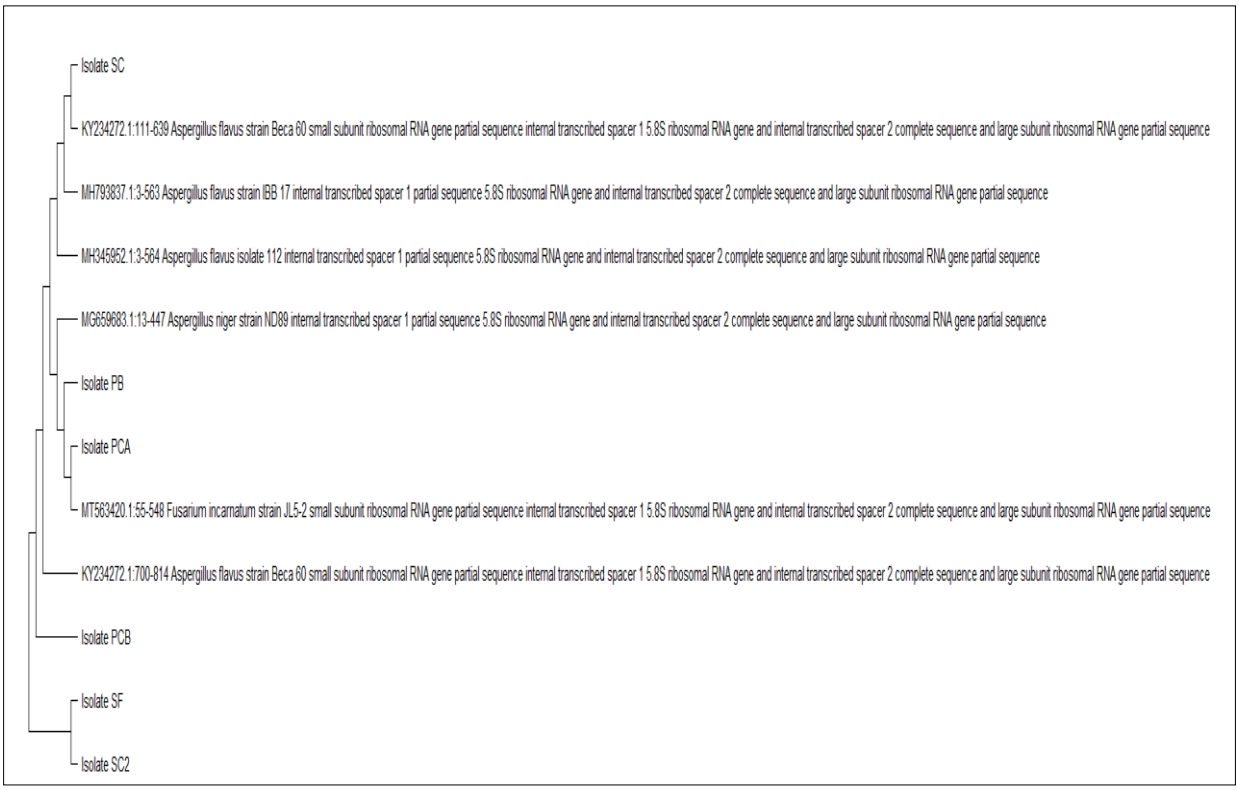

Figure 1 Phylogenetic Scheme of Predominant Fungal Isolates

4. Discussion

Table 1 and 2 shows the morphological and molecular identification of the fungal isolates , 12 most predominant fungal isolates were isolated from 4 different food samples (cake, corn ,dry fish, tomatoes) , they were morphologically identified as ; *Aspergillus flavus* , *Aspergillus niger* and *Fusarium* spp. The fungal isolates were further identified through molecular techniques by sequencing using ITS primers which identified them as; *Aspergillus flavus* strain IBB_17 with (93.4%) similarity and gene bank accession number MH793837.1, *Aspergillus flavus* strain Beca_67, (97.26%) similarity and gene bank accession number KY234273, *Fusarium fujikuroi*, (100%) similarity and gene bank accession number MT603295.1, *Fusarium minicarnatum* strain JL5-^{2}, (98.61%) similarity and gene bank accession number MT563420, *Aspergillus flavus* clone EF_385, (92.80%) similarity and gene bank accession number MF459666.1 and *Aspergillus niger* strain ND89, (71.81%) similarity and gene bank accession number MG659683.1 respectively. Their phylogenetic analysis is shown in figure 1. The recovery pattern of the predominant fungal isolates as shown in table 3 indicated their percentage recovery to be: *Aspergillus flavus* strain 1BB_17 (80%), *Aspergillus flavus* strain Beca_67 (100%), *Aspergillus flavus* clone EF_304 (80%), *Aspergillus niger* strain ND89 (60%), *Fusarium minicarnatum* strain JL5-^{2}(80%) and *Fusarium fujikuroi* (100%). The results obtained in this study are similar to the result of Sultan *et al*. (2022) who isolated *Aspergillus niger* and *Fusarium* spp as the most common genera in stored grains. *Aspergillus niger* is known to produce Ochratoxin and *Aspergillus flavus* strains produce aflatoxins , a potent carcinogen while *Fusarium* spp are known to produce zearalenone, fumonisins and deoxynivalenol which can pose a risk to consumer health (Ahmad and Jae-hyk 2017; Sultan *et al*.,2022). Devi and Gogi (2021) also reported that *Fusarium* spp is a spoilage organism in vegetables. The fungal isolates from the food samples in this study are known to be spoilage organisms associated with agricultural products including cereals, fruits, nuts and vegetables (Muhammad *et al*., 2004).

The results of this study also conforms with the reports of previous studies; Al. Masoodi *et al*. (2023) isolated *Aspergillus* and *Fusarium* as the most predominant fungal species from corn though different strains, similar observations were also recorded by (Omaima *et al.,*2018) . The variations in the presence of these fungi can be due to difference in physiological maturity in the various corn species, moisture content, the degree of infection, harvest and storage conditions (Al-Masoodi *et al*., 2023). Sudawa *et al*., (2022) isolated *Aspergillus* spp as the most predominant fungal specie from cake. The presence of these fungi is as a result of poor handling practices in the production process, food supply chain, storage conditions, distribution, marketing practices and transportation. Yusuf *et al.* (2020) ; Wogu and Ofuase (2014) ; Onuorah and Orji (2015) also isolated *Fusarium* spp from tomato which were associated with the

spoilage of tomato. The presence of *Fusarium* spp in tomato can be linked to post harvest contamination (Onuorah and Orji, 2015). *Fusarium* spp were also found to be human and feed pathogens (Placinta *et al.,* 1999), numerous *Fusaruim* spp are also considered worldwide to be the most dangerous fungi and one of the most important genera responsible for a large number of plant diseases. The occurrence of *Fusarium* spp in food represents an issue in several countries around the world (Bottalico and Perrone, 2002; Hussain *et al*., 2018). Akwuobu *et al*. (2019) also isolated *Aspergillus* spp from dry fish which is attributed to poor handling and unhygienic methods of processing. The percentage recovery of the fungal species in the various food samples is an indication that they are major contaminants of the various foods.

5. Conclusion

The result of this study shows that the various food samples contain toxigenic fungi and they pose serious threat to food safety, food security, human and animal health as well as economic development. The identification of the various fungal species especially molecular identification to strain level will be of great help to distinguish similar species as well as enhance food safety.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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