# ISOLATION AND MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI IN TIGERNUTS (*Cyperus esculentus L.*)

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## ABSTRACT

Tiger nuts (*Cyperus esculentus Lativum*) are highly valued for their sweet tubers and numerous health benefits. Like other plants, tiger nuts harbor a diverse community of endophytic microorganisms that contribute to plant survival, growth, and development. However, when tiger nut tubers are consumed raw, there is a potential for the transmission of endophytic fungi to humans. Understanding the diversity of endophytic fungi associated with tiger nuts is crucial to assess the potential consequences of their transfer to humans during tiger nut consumption. Therefore, this study aimed to isolate and identify endophytic fungi from fresh tiger nuts.

Fresh tiger nut samples were obtained from three different towns in Osun State, namely Osogbo, Oyan, and Ikirun. After aseptic rinsing and crushing of the tiger nut tubers, endophytic fungi were cultured on Potato Dextrose Agar (PDA). The molecular identification of the isolated fungi was performed using Polymerase Chain Reaction (PCR) and sequencing analysis of the internal transcribed spacer (ITS) region of the fungi using universal primers. The obtained ITS sequences were then subjected to a BLAST search on the NCBI database for identification.

Three fungi were successfully sequenced and identified in this study. They were found to share close identity with *Saccharomyces cerevisiae* isolate 27 (99.62%), *Saccharomyces cerevisiae* isolate B-NC-12-OZ03 (99.0%), and *Candida tropicalis* strain Pe1 (98.79%). The presence of *Saccharomyces cerevisiae* and *Candida* species is noteworthy, as they are relevant in fermentation processes. However, their presence as endophytes in tiger nut tubers may lead to negative fermentation or spoilage of the tubers. Furthermore, *Candida tropicalis* has been reported as a pathogen in individuals with compromised immune systems.

This study provides valuable information on the potential endophytic fungi that can be isolated from tiger nuts. Further research is recommended to explore the roles of these endophytic fungi and their implications in tiger nut quality, safety, and fermentation processes. Understanding the interactions between endophytic fungi and tiger nuts will contribute to better management practices and ensure the safe consumption of this popular plant.

**Key words:** Isolation, Identification, Tiger nuts, Endophytic Fungi, Molecular Identification, Polymerase Chain Reaction and Internal Transcribed Spacer, *Saccharomyces cerevisiae*, and *Candida tropicalis*.

## Introduction

Tigernut, scientifically known as *Cyperus esculentus*, is a perennial grasslike plant belonging to the Cyperaceae family (Coskuner, 2002). Its tubers are spherical in shape, with pale yellow-cream kernels surrounded by fibrous sheaths. The genus name Cyperus originates from the Greek word Cypeirus, while the species name esculentus signifies its edibility (Mohamed et al., 2005). Tigernuts are widely consumed in various parts of East and West Africa, including Nigeria, where they serve as a source of energy, carbohydrates, and proteins. They are consumed either in their raw form or after being fried (Abaejoh et al., 2006).

Tigernuts are cultivated in Africa, South America, Europe, and Asia, with a particular emphasis on rural farming in Northern Nigeria. They also grow naturally along riverbanks. Several African countries, such as Nigeria, Ghana, and Sierra Leone, recognize tigernuts as a valuable crop (Cyperules, 1992). In Nigeria, they are referred to as "Aya" by the Hausa ethnic group, "imumu" by the Yorubas, and "ofio" or "aki Hausa" by the Igbo people in the southern region (Omode et al., 1995). Despite being an underutilized perennial crop, tigernuts have been acknowledged for their nutritional richness and have been consumed as both food and beverage. Unfortunately, they remain largely underappreciated in Nigeria and other parts of the world (Bamishaiye and Bamishaiye, 2011).

Tiger nuts, despite their common name, are not actually nuts but rather small tubers that grow at the rhizome of the plant (Mason, 2008). This misnomer arises from their resemblance to nuts, although their chemical composition resembles both tubers and nuts (Bamishaiye and Bamishaiye, 2011). These edible tubers are sweet, creamy, and rich in protein, fiber, carbohydrates, and sugars (FAO, 1998). *Cyperus esculentus L.*, also known as chufas, rush nuts, earth nuts, and earth almond, is the scientific name for tiger nuts (Eteshola and Oraedu, 1996). The weight of fresh tiger nut tubers varies between 70mg to about 900mg, while dried tubers range from 30mg to 350mg in weight (Bamishaiye and Bamishaiye, 2011).

Tiger nuts come in two main varieties: light brown (or yellowish) and black (or dark brown), with lengths ranging from 1-3 cm and diameters from 1-2 cm (Maud, 1991). The yellow variety is the most popular due to its desirable characteristics, including larger size and appealing color (Bamishaiye and Bamishaiye, 2011). Additionally, the yellow tiger nuts yield a higher amount of milk after extraction, contain more protein and less fat, and have fewer antinutritional components compared to the black variety (Okafor et al., 2003). The tuber varieties of tigernut depicted in Figure 1 are distinguished by their tuber color (Maduka and Ire, 2018).

Tiger nuts are not only delicious but also regarded as a nutritious snack (Bamishaiye and Bamishaiye, 2011). Their probiotic properties can be attributed to the concentration of resistant starch and soluble fiber, mono-saturated fatty acids, and overall antioxidant properties (Sanchez-Zapata et al., 2012). Reports by Ezeh et al. (2014) highlight that tiger nut oil contains beneficial oleic and linoleic fatty acids, as well as fat-soluble antioxidant vitamin E (α-tocopherol and γ-tocopherol). In terms of composition, tiger nuts consist of

approximately 26-30% starch and 21-25% fat, providing around 400-450 kcal of energy per 100g (Sanchez-Zapata et al., 2012). According to Gambo and Dau (2014), dried tiger nuts typically contain 5-10% protein, 25-30% oil, 47-50% total carbohydrates, 29.5% starch, and 15.4% sugar. They are also a good source of vitamin C (Awonorin, 2014) and various minerals such as sodium, calcium, iron, zinc, phosphorus, potassium, magnesium, copper, and manganese (Sanful, 2009).

Tiger nuts have been used in the preparation of tonics and are known for their potential aphrodisiac, diuretic, and stimulant qualities in different parts of the world (Asare et al., 2020). They have also been traditionally employed for the treatment of flatulence, indigestion, diarrhea, and dysentery (Chevallier, 1996). While the awareness of their multiple uses is limited in some regions, tiger nuts are primarily consumed and recognized for their aphrodisiac properties in Ghana (Chevalier, 1998). Due to their high iron and calcium content, which promote body growth and development, they are considered an extremely nutritious crop used to supplement diets (Mason, 2008). Research suggests that consuming substantial amounts of tiger nuts may contribute to a lower incidence of health issues such as obesity, diabetes, cancer, and cardiovascular diseases (Ekeanyanwu and Ononogbu, 2010).

Although tiger nuts are sometimes processed into milk and consumed domestically or sold to the public, a significant number of people purchase these nuts from roadside vendors or while in vehicles, often consuming them without washing. This behavior stems from the common assumption that the sellers have already washed the nuts. This practice aligns with the observation made by Miller (1996) that in many parts of Africa, the desire and impatience to eat often outweigh other considerations such as food safety. Unfortunately, this tendency has resulted in a notable increase in food-borne illnesses and contamination.

While tiger nuts are recognized as an important source of nourishment, it is essential to acknowledge that they can potentially become a source of acute or chronic food-borne illnesses when contaminated with pathogenic microorganisms or microbial toxins (Negedu et al., 2011). Mordi et al. (2006) have reported the presence of contaminants such as stones, animal droppings, and other extraneous materials associated with tiger nuts.

Studies conducted in Nigeria have revealed the presence of aflatoxins at toxicologically unsafe levels on tiger nuts (Shamsuddeen and Aminu, 2016). Shamsuddeen and Aminu (2006) specifically identified the presence of *Aspergillus flavus*, a fungus known to produce aflatoxins, on freshly consumed tiger nuts in Kaduna, Nigeria. Bankole and Adebanjo (2003) also reported the contamination of tiger nuts by *Aspergillus flavus*.

Adejuyitan et al. (2011) conducted a study that identified various microbial species associated with tiger nuts, including *Staphylococcus aureus, Bacillus subtilis, Aspergillus flavus, Aspergillus niger, Fusarium solani, Saccharomyces cerevisiae, Saccharomyces fubiligera, and Candida pseudotropicalis*. Additionally, Negedu et al. (2011) reported that commodities such as groundnuts, maize, sorghum, rice, yam, cassava, tiger nuts, soybeans,

cotton seeds, and fruits can be contaminated with fungal toxins, including aflatoxins, fumonisins, ochratoxins, patulin, sterigmatocystin, and other mycotoxins, posing significant economic and health risks.

These findings indicate the potential for human exposure to mycotoxins through the consumption of tiger nuts, as they can harbor fungi capable of producing these toxins. It highlights the importance of addressing and mitigating the risk of mycotoxin contamination in tiger nuts to safeguard both economic interests and public health.

According to Sagoo et al. (2001), the lack of effective antimicrobial treatments throughout the entire process of planting, harvesting, processing, and consumption of tiger nuts raises concerns about the potential introduction of pathogens at any stage, which may ultimately be present on the final food product. This highlights the need for proper hygiene and safety measures to minimize microbial contamination.

In a study conducted by Hubert et al. (2011) to assess the microbiological safety of tiger nuts in the Cape Coast metropolis of Ghana, *Escherichia coli* and *Bacillus spp.* were found to be the most frequently encountered species. Furthermore, Onovo and Ogaraku (2007) reported the isolation of various microorganisms from exposed tiger nuts, including *Staphylococcus aureus, Fusarium solani, Bacillus subtilis, Aspergillus flavus, Aspergillus niger, Saccharomyces cerevisiae, Saccharomyces fubiligera, and Candida pseudotropicalis*, with varying frequencies of occurrence.

In various major towns across Nigeria, tiger nuts are commonly sold on the streets, often displayed in uncovered wheelbarrows, exposing them to potential environmental contaminants. Furthermore, the sellers typically use bare hands or unclean cups to dispense the nuts into polythene wrappers, which are then consumed immediately by customers. Tiger nuts have gained wide popularity among the population due to their numerous health benefits, particularly their perceived aphrodisiac properties for males and their sweet taste, making them a favored snack among children.

However, concerns have been raised regarding the hygienic practices involved in the cultivation, harvesting, and distribution of tiger nuts to the end consumers. The current state of affairs necessitates a thorough evaluation of the microbiological quality of tiger nuts sold in local markets across Nigeria. With this in mind, the objective of this study was to assess the microbiological quality of tiger nuts collected from various towns in Osun State, Nigeria. Additionally, the study aimed to isolate and identify endophytic fungi present in the nuts through PCR amplification and sequencing of the internal transcribed spacer region of the fungi.

This research is essential to shed light on the potential microbial risks associated with the consumption of tiger nuts and to provide valuable insights for improving the hygiene practices throughout the supply chain. By

understanding the microbiological quality and identifying the specific fungi present in tiger nuts, appropriate measures can be implemented to ensure the safety and integrity of this popular food item in local markets.

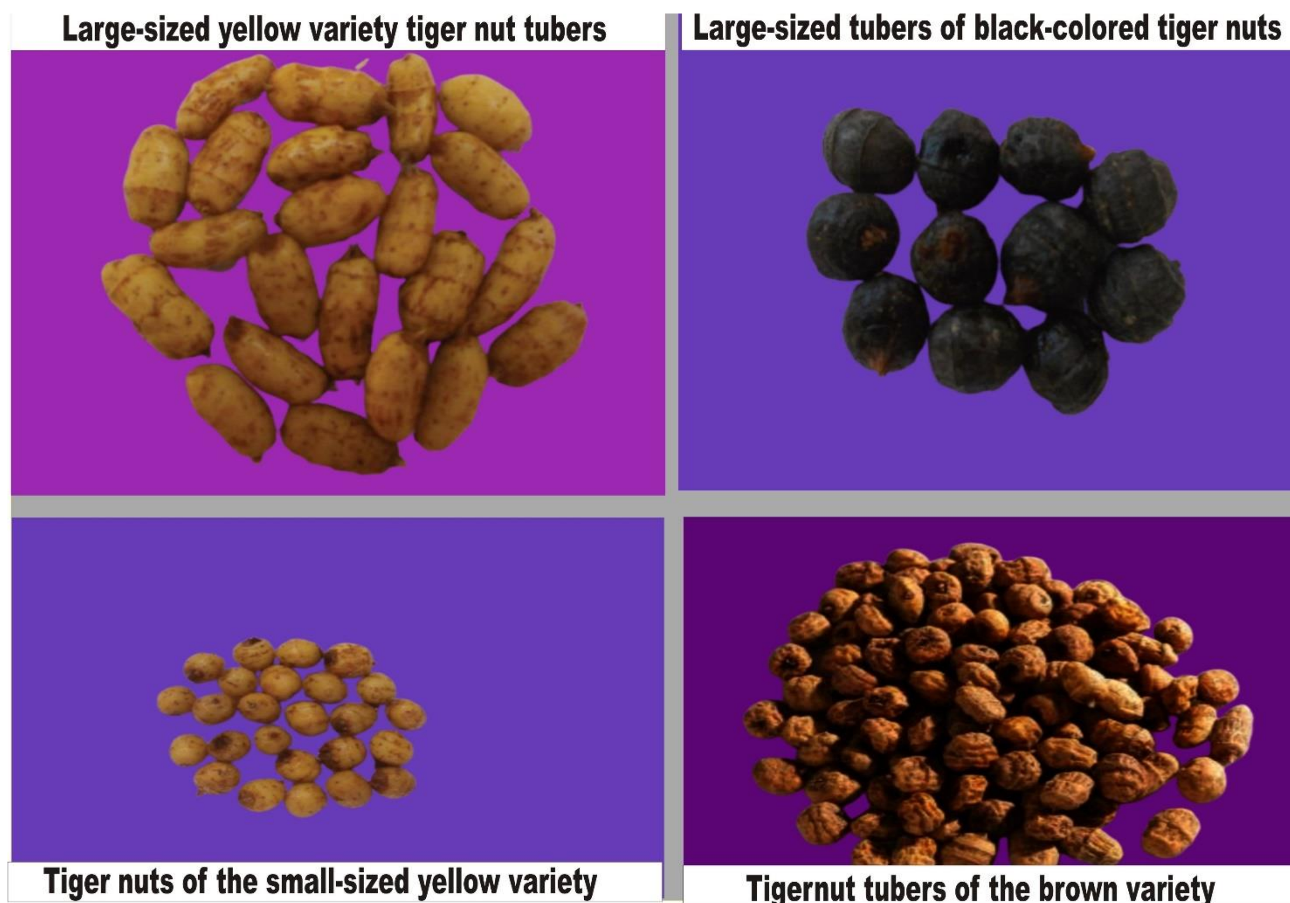


**Figure 1: Tigernut Varieties Based on the Color of Their Tubers (Maduka and Ire, 2018)**

## MATERIALS AND METHODOLOGY

The Experiment was conducted in the year 2021 at the Department of Microbiology, Osun State University, Osogbo, Osun State, Nigeria.

### Materials

Beaker, marker, glass slides, cotton wool, matches, needle and syringe, beaker, conical flask, test tube, 90% ethanol, Petri dish, wireloop, measuring cylinder, Eppendorf tube, micropipette, white tips, yellow tips, blue tips, distilled water, foil paper, cryovial, paper tape, hand gloves, ice pack and nose mask.

### Equipment

Incubator, refrigerator, drying oven, autoclave, mini centrifuge, water bath, simple light microscope, thermal cycler, vortex mixer, weighing balance and heater.

## Media and reagents

Media: Potato dextrose agar (PDA), Nutrient agar, and Peptone water.

Reagents: Sodium hydroxide, 1% agarose gel, DNA ladder, Tris-borate ethylenediaminetetraacetic acid (TBE) buffer and Ringer solution.

## Methodology

## Sampling sites

Fresh Tiger nut samples were collected from three different towns in Osun State namely Osogbo, Oyan and Ikirun.

## Sterilization of Apparatus and Work Bench:

To maintain aseptic conditions and prevent contamination, proper sterilization procedures were followed for apparatus and the work bench. Sterile containers were used to collect all samples, ensuring no external contaminants were introduced. Glassware used in the study underwent a thorough cleaning process, including washing with detergent, rinsing with distilled water, and drying using hot air. Subsequently, the glassware was subjected to autoclaving at a temperature of 121˚C for 15 minutes to achieve sterilization.

In addition to apparatus sterilization, the work bench surface was meticulously sterilized. Prior to commencing work and after each working period, the work bench surface was carefully wiped with cotton wool soaked in 75% alcohol. This step effectively eliminated any potential microbial contaminants, maintaining a sterile work environment.

## Preparation of Media:

The media were meticulously prepared in accordance with the manufacturer's instructions. Accurate measurements of the media were weighed and subsequently dissolved in the appropriate volume of distilled water. To ensure thorough dissolution, the mixture was gently heated and homogenized. Autoclaving, at a temperature of 121°C for a duration of 15 minutes, was conducted unless explicitly specified otherwise in the provided instructions. Following autoclaving, the media were allowed to cool to approximately 45°C prior to being carefully poured into petri dishes.

## Preparation of Ringers Solution

The preparation of Ringer's solution involved carefully measuring 2.5 grams of the solution, which was then added to a clean, one-liter container filled with distilled water. To ensure a thorough and uniform mixture, the container was vigorously shaken. This process facilitated the homogenization of the Ringer's solution, resulting in a well-blended and consistent solution ready for use.

### Samples Collection

Fresh samples of tiger nuts were collected from three distinct towns in Osun State, namely Osogbo (Osogbo local government area), Oyan (Odo-Otin local government area), and Ikirun (Ifelodun local government area). These samples were meticulously labeled and promptly transported using sterile bags to the Microbiology Laboratory of the Department of Microbiology at Osun State University for further analysis.

Situated in the tropical rainforest zone, Osun State spans an approximate land area of 14,875 square kilometers, located between latitude 7° 30′ 0″ N and longitude 4° 30′ 0″ E. The state capital, Osogbo, lies at 7°46′ North, 4°34′ East, encompassing a land area of 47 square kilometers. It shares borders with Ikirun, Ilesa, Ede, Egbedore, and Iragbiji. Oyan is positioned between latitude 8° 03' 0.00" N and longitude 4° 46' 0.01" E of the Greenwich Meridian. Similarly, Ikirun is located within the tropical zone, specifically at latitude 07°55′N and longitude 04°41′E of the Greenwich Meridian. It is bounded by Inisha to the north, Osogbo to the south, Iragbiji to the west, and Eko-Ende to the east (Osun State Website, 2022).

MAP OF OSUN STATE

**Figure 2: Osun State map showing different local government areas (Osun State Website, 2022)**

## Samples Preparation

The fresh tiger nuts were visually inspected, and any samples with visible surface damage were excluded. Only mature and healthy tiger nuts were selected for further analysis. The chosen tiger nuts were weighed, with each sample weighing up to 50 grams. To ensure cleanliness, the tiger nuts were thoroughly washed three times with running water to remove any unwanted particles. After washing, they were drained before undergoing surface sterilization.

For surface sterilization, the washed tiger nuts were placed in a sterile container containing 50 ml of distilled water mixed with 50% sodium hypochlorite. Using sterile forceps, the tiger nuts were soaked in this solution for 5 minutes. Subsequently, the tiger nuts were washed twice with distilled water to remove or minimize any remaining traces of the sterilizing agent.

Following the initial cleaning process, the tiger nuts were transferred to a sterile container containing 96% ethanol. They were immersed in the ethanol for three minutes, ensuring thorough coverage. Afterward, the tiger nuts underwent eight consecutive rinses with sterile distilled water in separate sterile plates. Each rinse involved transferring the tiger nuts to a fresh plate and repeating the process.

Once the rinsing procedure was completed, the tiger nuts were dried in a sterile environment. The last batch of water used in the rinsing process was collected and inoculated, then incubated for 24 hours as a sterility check measure.

After the sterilization and drying steps, the washed tiger nuts were carefully pounded using a sterile mortar and pestle until a fine consistency was achieved. This process ensured that the tiger nuts were uniformly prepared for subsequent analysis.

## Microbiological Analysis of Samples

### Serial Dilution

A ten-fold serial dilution process was performed for each sample. The serial dilution was conducted up to a dilution factor of $10^{-4}$. To initiate the dilution, 1.0 ml of each sample was transferred into a test tube containing 9.0 ml of sterile ringer solution. The test tube was then shaken to ensure proper mixing, and it was labeled as the stock solution with a dilution factor of $10^{-1}$.

From the stock solution, 1.0 ml was transferred to another test tube containing sterile ringer solution, creating a dilution of $10^{-2}$. The same procedure was repeated iteratively, using 1.0 ml from the previous dilution to prepare subsequent dilutions up to $10^{-4}$. This method involved a stepwise dilution process, transferring aliquots of the

original sample into successive test tubes containing sterile ringer solution to create a series of dilutions with decreasing concentration factors. Each dilution was appropriately labeled according to its dilution factor.

## Inoculation of Tigernut Samples

Using the pour plate method, 1.0 ml from each dilution factor test tube was aseptically inoculated onto potato dextrose agar (PDA). The inoculum was spread thinly and evenly across the surface of the agar using a sterile bent glass rod. Subsequently, the plates were incubated at a constant room temperature of 27°C for a period of 72 hours.

During the incubation period, the microbial colonies present in the inoculated samples were allowed to grow and develop on the PDA medium. This method facilitated the enumeration and isolation of viable microorganisms present in the original samples, as the dilutions allowed for the optimal growth of colonies while minimizing overcrowding on the agar plates. The incubation conditions provided a suitable environment for the proliferation and visible growth of microbial colonies over the specified period of time.

## Isolation and Identification of Fungi

Following the incubation period, the plates were carefully examined, and a diverse array of colonies exhibiting various shapes and colors were observed (Cheesbrough, 2000). Individual colonies were then subjected to identification based on their colonial morphology, which involved evaluating the growth pattern and color characteristics exhibited by each colony on the potato dextrose agar (PDA) plates. By analyzing these visual attributes, the different colonies were discerned and distinguished from one another.

## Molecular Characterization

## DNA Extraction

DNA can be efficiently extracted from different yeast species such as *Candida albicans*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Schizosaccharomyces pombe* and *Pichia pastoris* (Looke *et al*., 2011). The protocol involves lysis of yeast colonies or cells from liquid culture in a lithium acetate (LiOAc) – Sodium dodecyl sulfate solution and subsequent precipitation of DNA with ethanol (Akada *et al*., 2000).

Approximately 100 nanograms of total genomic DNA can be extracted from $1 \times 10^7$ cells. DNA extracted by this method is suitable for a variety of PCR-based applications (including DNA sequencing, real-time qPCR and colony PCR) for amplification of DNA fragments of ≤3500 bp. The DNA extraction of yeasts can also be done using the BUST n‘‘ GRAB Protocol (Harju *et al*., 2004).

### Extraction of genomic DNA from yeasts for PCR based applications

**Materials needed:** Centrifuge tubes, pipette, centrifuge, ethanol, chloroform, vortex, pipette tips

The DNA extraction was performed using the BUST n" GRAB Protocol (Harju *et al*., 2004). Seven to eight fungal colonies from an 18-24 hour old culture were inoculated into 5 ml sterile nutrient broth and incubated at 37$^{o}$C overnight. 100uL was dispensed into an Eppendorf tube and centrifuged at 10,000 x g for 5 minutes. Supernatant was discarded and 120uL of the lysis buffer was dispensed in the tube to suspend. Put in the freezer till it freezes and transferred into 95$^{o}$C water bath for one (1) minute. Froze again and boiled in water bath for another one (1) minute then spinned and vortexed for 30seconds. Then 200microlitre of chloroform was added and vortexed for 2 minutes. It was then centrifuged at 10,000 x g for 3minutes. Then the upper aqueous phase was transferred into another centrifuge tube containing 400ul of ice cold 100% ethanol and vortexed gently. It was incubated at room temperature for 5minutes and then centrifuged again at 10,000 x g for 5minutes and supernatant was removed with Pasteur pipette. The pellets were washed and suspended with 50microlitre of 70% ethanol and centrifuged at 10,000 x g for 5 minutes and supernatant was discarded. The pellets were air dried at room temperature for 5 mimutes and was re-suspended in 50microlitre of water.

### DNA Quantification of Genomic DNA

**Materials Used:** Nanodrop One, pipette, pipette tips and tissue paper.

The major equipment used for this procedure is the Nanodrop one, which works on the principle of the amount of energy absorbed by a solution is proportional to the solution"s molar absorptivity and the concentration of solute. The quality and quantity of the extracted DNA was determined using a Thermoscientific NanoDrop One Spectrophotometer, 2000C (Thermoscientific, 2022). DNA extraction method suitability is obtained by characterizing the extracted DNA"s quantity and quality. DNA quantity can be described as an indicator of extraction efficiency while DNA quality parameters (purity and intactness) indicate that the DNA is free of PCR inhibitors and appropriately sized. Before the DNA was quantified the Nanodrop one was blanked to prepare it for sample measurement, then 1.5ul of the DNA sample was dropped in the Nanodrop well and measured.

### Molecular Amplification of 16srRNA gene

**Materials Used:** Primer, polymerase enzyme (TAQ polymerase), DNA template, mastermix, sterile water.

The detection of the 16S rRNA gene in the four fungal isolates, identified based on colonial morphology, was investigated using the Polymerase Chain Reaction (PCR) technique. The PCR reaction was performed using the following materials: primers, Taq polymerase enzyme, DNA template, mastermix, and sterile water.

A pair of primers designed to amplify a 1500 bp fragment of the 16S rRNA gene was employed for the PCR reaction. The reaction was conducted in 0.2 ml PCR tubes with a final volume of 25 µl. The reaction mixture included 5 µl of the extracted DNA template, 12.5 µl of the mastermix at a final concentration of 1x, containing 1.5 units of Taq DNA polymerase and 0.2 mM dNTPs, and 0.5 µl (0.2 µM) each of the forward and reverse primers. Nuclease-free water was added to make up the final volume of 25 µl.

The PCR amplification was carried out in a thermocycler with the following parameters: initial denaturation at 95°C for 2 minutes, followed by denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, 35 cycles of amplification, extension at 72°C for 1.5 minutes, and a final extension at 95°C for 5 minutes.

To visualize the PCR products, gel electrophoresis was performed using a 2% (w/v) agarose gel run in 1x TBE buffer at pH 8.0 for 1 hour at 100V. After electrophoresis, the gel was stained with EZ vision (cleaver scientific) and then visualized. The gel analysis involved comparing the specific gene bands with the positive control and a 100 bp DNA ladder to confirm the presence of the amplified product.

**Table 1: Primers Used for DNA Sequencing**

<table>
<tr><th>Primer</th><th>Sequence</th><th>Region</th><th>Fragment</th><th>PCR Condition</th></tr>
<tr><td>Forward Primer ITS1</td><td>5ʻʻ–TCCGTAGGTGAACCTGCGG –3ʻʻ</td><td>rDNA ITS1</td><td rowspan="2">1500bp</td><td rowspan="2">Initial denaturation at 95°C for 2 min, denaturation at 90°C for 1 min, annealing at 60°C for 1 min, 35m cycles, extension at 72°C for 1.5 min and final extension at 95°C for 5 min.</td></tr>
<tr><td>Reverse Primer ITS4</td><td>5ʻʻ–TCCTCCGCTTATTGATATGC –3ʻʻ</td><td>rDNA ITS1</td></tr>
</table>

To facilitate the identification process, the Internal Transcribed Spacer (ITS) region was amplified using the ITS1 and ITS4 primers, which are commonly used general primers (Zarrin and Erfaninejad, 2016). The ITS1 forward primer has the sequence 5′-TCCGTAGGTGAACCTGCGG-3′, while the ITS4 reverse primer has the sequence 5′-TCCTCCGCTTATTGATATGC-3′. These primers specifically target the ITS regions of the ribosomal DNA (rDNA) for amplification.

## Sequencing and analysis of DNA

The process of identifying the endophytic fungal isolates was conducted by Inqaba Biotech West Africa Ltd. in Ibadan, Oyo State, Nigeria. To perform this identification, the 16S rRNA gene was sequenced using universal primers, specifically ITS1 F (5′-TCCGTAGGTGAACCTGCGG-3′) as the forward primer and ITS4 R (5′-TCCTCCGCTTATTGATATGC-3′) as the reverse primer. These primers are commonly employed to amplify the ITS regions of the ribosomal DNA. By utilizing universal primers, the 16S rRNA gene of selected isolates was amplified through PCR (polymerase chain reaction), and the resulting amplicons were then subjected to sequencing.

Subsequently, the obtained sequence data were analyzed using the BLAST (Basic Local Alignment Search Tool) tool provided by the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov). This analysis involved comparing the characteristics of the amplified sequences with known sequences in the NCBI database. By aligning the sequences against the database, the closest matches and corresponding organisms were identified. This process enabled the validation and characterization of the amplified sequences in relation to known sequences of various microorganisms.

## Results

### Characteristics of Fungi Isolates from Tiger Nuts

Four distinct fungal species were isolated from the Potato Dextrose Agar (PDA) plates based on their unique colony morphologies, including colony growth and color. The characteristics of these isolated fungal species are presented in Table 2.

**Table 2: Characteristics of Isolated Fungi Species from PDA Plates**

| S/N | Sample Code | Colony Morphology | Probable Organism |
|---|---|---|---|
| 1 | T1 | Small circular moist cream colonies | *Saccharomyces spp.* |
| 2 | T2 | Dark circular colonies | *Aspergillus spp.* |
| 3 | T3 | Small circular moist cream colonies | *Saccharomyces spp.* |
| 4 | T4 | Creamy white smooth unextensive colonies | *Candida spp.* |

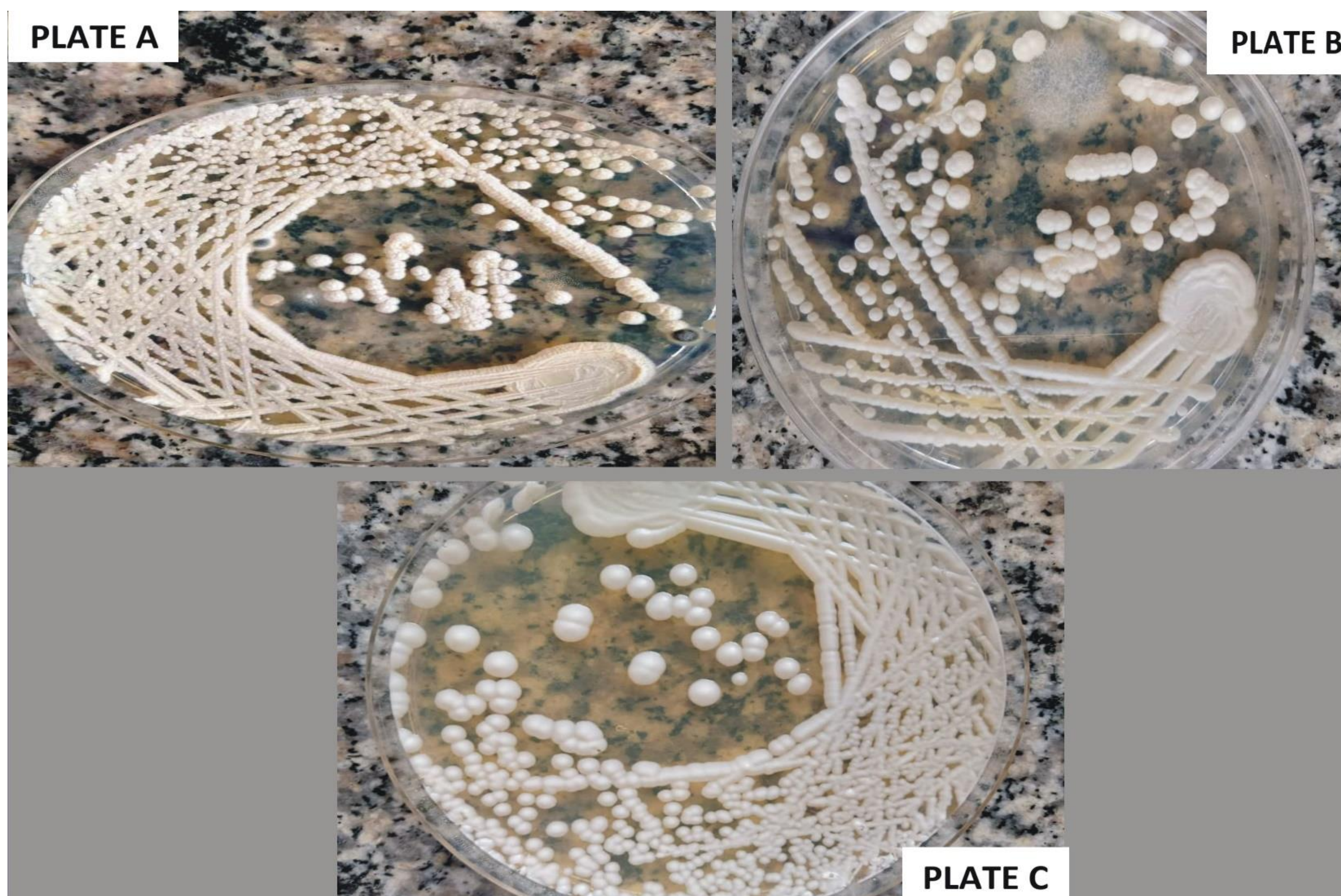


**Figure 3: Plate A, B and C - Pure Cultures of Fungi Isolated from *Cyperus esculentus* at Room Temperature (27°C) on Potato Dextrose Agar Plates**

**Identification of Fungal Isolates through DNA Sequencing and Analyses**

To identify the fungal isolates accurately, DNA sequencing and subsequent analyses were conducted. The molecular identification process involved performing Polymerase Chain Reaction (PCR) and sequencing analysis of the internal transcribed spacer (ITS) region using universal primers. This allowed for the comparison of the obtained sequences with known fungal sequences in databases.

Among the four fungal isolates, two were initially identified based on their macroscopic characteristics as belonging to the *Saccharomyces* genus. Through DNA sequencing, isolate T1 (assigned with extraction code T1_ITS-1_A03_03) was confirmed to be closely related to *Saccharomyces cerevisiae* isolate 27, exhibiting a remarkable similarity of 99.62%. Similarly, isolate T3 (assigned with extraction code T3_ITS-1_C03_09) was confirmed to be closely related to *Saccharomyces cerevisiae* strain B-NC-12-OZ03, with a similarity of 99.0%.

Isolate T2 (assigned with extraction code T2_ITS-1_H06_24) was initially macroscopically identified as an *Aspergillus spp.* but did not yield conclusive results during the analysis. On the other hand, isolate T4 (assigned

with extraction code T4_ITS-1_D03_12) was identified as *Candida spp.* macroscopically and was confirmed to be closely related to *Candida tropicalis* strain Pe1, exhibiting a similarity of 98.79%.

Table 3 summarizes the probable organisms identified based on macroscopic characterization, as well as the confirmed organisms obtained through DNA sequencing and subsequent analyses.

**Table 3: Probable Organisms Identified through Macroscopic Characterization and the Confirmed Organisms through RNA Sequencing and Analyses.**

| S/N | Sample Code | Extraction Code | Probable Organism | Confirmed Organism |
|---|---|---|---|---|
| 1 | T1 | T1_ITS-1_A03_03 | *Saccharomyces spp.* | *Saccharomyces cerevisae* |
| 2 | T2 | T2_ITS-1_B03_06 | *Aspergillus spp.* | No result |
| 3 | T3 | T3_ITS-1_C03_09 | *Saccharomyces spp.* | *Saccharomyces cerevisae* |
| 4 | T4 | T4_ITS-1_D03_12 | *Candida spp.* | *Candida tropicalis* |

**Table 4: Description of the Confirmed Fungal Species, Percentage, and Accession Number with the Greatest Similarity**

| S/N | Sample Code | Extraction Code | Description | Accession Number with greatest similarity | Percentage Similarity (%) | Fungal Species with Greatest similarity |
|---|---|---|---|---|---|---|
| 1 | T1 | T1_ITS-1_A03_03 | *Saccharomyces cerevisiae* isolate 27 | KT175188.1 | 99.62 | *Saccharomyces cerevisiae* (Baker's yeast) |
| **2** | T2 | T2_ITS-1_H06_24 | **-** | **-** | **-** | **-** |
| **3** | T3 | T3_ITS-1_C03_09 | *Saccharomyces cerevisiae* isolate B-NC-12-OZ03 | KF728786.1 | 99.00 | *Saccharomyces cerevisiae* (Baker's yeast) |
| **4** | T4 | T4_ITS-1_D03_12 | *Candida tropicalis* strain Pe1 | MK752669.1 | 98.79 | *Candida tropicalis* |

A comprehensive phylogenetic tree was constructed to elucidate the relationships between the fungal isolates and closely related species available in the GeneBank database (Figure 4). The analysis revealed that Sample T1 exhibited significant similarity to several strains of *Saccharomyces cerevisiae*. Notably, it shared 96.20% similarity with *Saccharomyces cerevisiae* isolate 27 (KT175188.1), 99.63% similarity with *Saccharomyces cerevisiae strain* YBA 08 (MN158119.1), and 99.63% similarity with *Saccharomyces cerevisiae* isolate Coyol (MZ361705.1). Sample T3 was found to be closely related to *Saccharomyces cerevisiae* isolate B-NC-12-OZ03 (KF728786.1) with a similarity of 99.00% and *Saccharomyces cerevisiae* strain XZFM7F3 (MW710205.1) with a similarity of 99.25%. Similarly, Sample T4 showed close relatedness *to Candida tropicalis* strain Pe1 (MK752669.1) with a similarity of 98.79% and *Candida tropicalis* isolate CTR206 (KX664525.1) with a similarity of 98.79%.

To generate the phylogenetic tree, a multiple sequence alignment of the fungal isolates was performed using Clustal Omega, a state-of-the-art tool developed by EMBL-EBI (Madeira et al., 2022). This alignment facilitated the comparison and identification of conserved regions among the sequences. The aligned sequences were then used to construct a Neighbor-Joining Phylogenetic Tree using Jalview Version 2, which serves as both a multiple sequence alignment editor and an analysis workbench (Waterhouse et al., 2009).

By employing these advanced tools and techniques, the phylogenetic tree provided valuable insights into the evolutionary relationships among the fungal isolates and their closely related counterparts. This information contributes to our understanding of the genetic diversity and evolutionary history of these fungi, ultimately enhancing our knowledge of their ecological roles and potential applications.

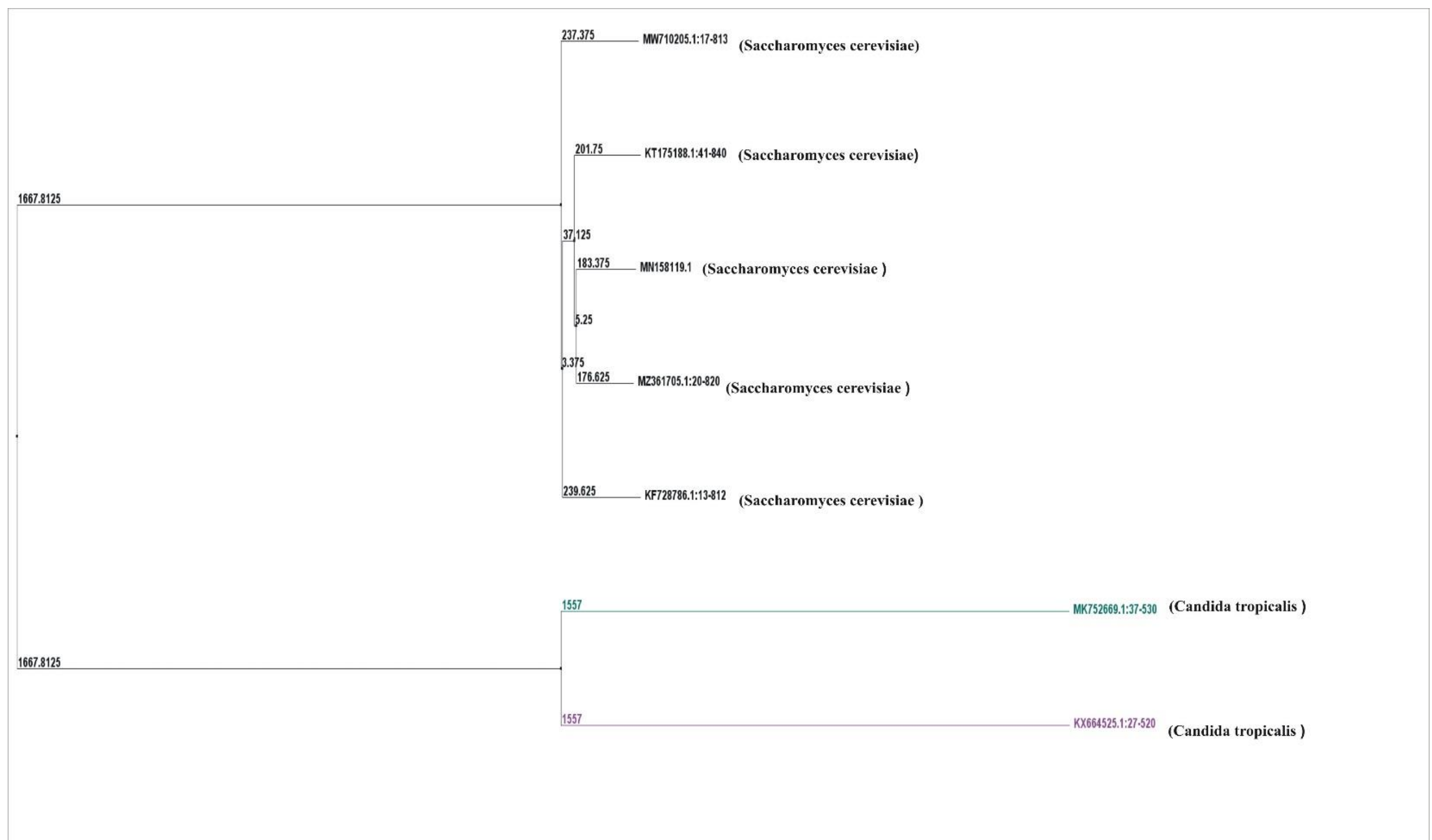


**Figure 4: Neighbour-Joining Phylogenetic Tree Based on the Sequence of ITS 1-2 Region**

To further establish the relationships between the fungal isolates investigated in this study (T1, T3, and T4) and closely related species documented in the GeneBank database, a motif analysis was conducted using the TOMTOM program (Gupta et al., 2007). The DNA sequences of the *Saccharomyces* and *Candida* strains under examination were subjected to this analysis. The results revealed the presence of five conserved motifs in the *Saccharomyces* DNA sequences (Figure 5), as well as two motifs in the *Candida* DNA sequences (Figure 5), which aligned with the findings obtained from the Clustal Omega analysis (Madeira et al., 2022). The identification of conserved sequences through motif analysis facilitates the detection of homology among various organisms and species during computational analysis (Wong et al., 2015). In terms of biological significance, the conserved sequences observed between species correspond to coding sequences that potentially preserve the structural and functional integrity of specific proteins within the organisms (Janda and Abbott, 2007).

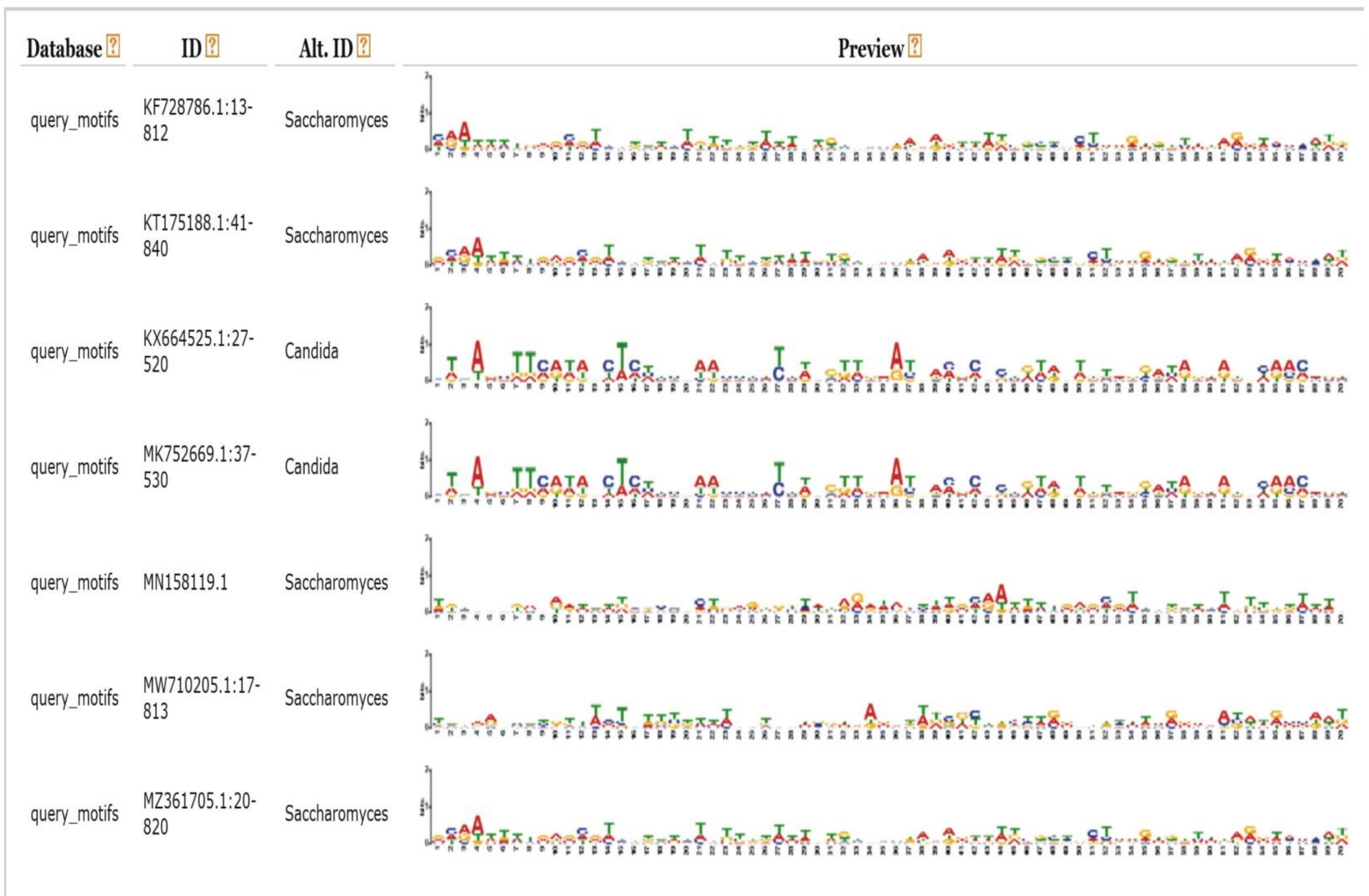


**Figure 5: Motif Analysis of the DNA Sequences of Fungal Species**

## Discussion

Despite the concerns regarding contamination, it is important to acknowledge that the consumption of fresh tiger nuts and their utilization in various products should not be discouraged due to their excellent nutritional qualities. Tiger nuts are known to be rich in minerals such as sodium, calcium, iron, zinc, phosphorus, potassium, magnesium, copper, and manganese, which contribute to their nutritional value (Sanful, 2009). In fact, regular consumption of tiger nuts has been reported to have positive effects on various health conditions, including cardiovascular disease, diabetes, cancer, and obesity (Ekeanyanwu and Ononogbu, 2010).

However, it is crucial to address the issue of contamination, as researchers have reported instances of tiger nuts being contaminated due to poor harvesting and storage techniques, as well as the distribution of contaminated nuts to consumers (Ukpabi and Ukenye, 2015). Street vendors in many Nigerian towns sell tiger nuts in unsanitary conditions, often using dirty wheelbarrows and handling the nuts with bare hands or unclean cups. Additionally, consumers frequently consume the nuts without thorough washing, leading to potential health risks. These practices align with the findings of Onovo and Ogaraku (2007), who isolated various fungal species, including

*Fusarium solani, Aspergillus flavus, Aspergillus niger, Saccharomyces cerevisiae, Saccharomyces fubiligera, and Candida pseudotropicalis*, from exposed tiger nuts.

In the present study, fresh tiger nuts obtained from three different towns in Osun State (Osogbo, Oyan, and Ikirun), Nigeria, were subjected to microbiological analysis. Endophytic fungi were isolated from the tiger nut samples, and their identification was performed using polymerase chain reaction (PCR) and sequencing analysis of the internal transcribed spacer (ITS) region of the fungi. The obtained ITS sequences were further analyzed by conducting a BLAST search on the NCBI database. As a result, three fungi were successfully sequenced and identified, showing significant similarities to *Saccharomyces cerevisiae* isolate 27 (99.62%), *Saccharomyces cerevisiae* isolate B-NC-12-OZ03 (99.0%), and *Candida tropicalis* strain Pe1 (98.79%). Detailed information regarding the identified organisms is provided in Table 2 and 4, which includes macroscopic characterization and confirmation through DNA sequencing and analysis.

The identification of the three fungal species in this study aligns with the findings reported by who isolated *Saccharomyces cerevisiae* (T1), *Saccharomyces cerevisiae* (T3), and *Candida tropicalis* (T4) from exposed tiger nuts. This similarity in the isolated fungal species reinforces the validity of the current study's findings and strengthens the link between these fungi and tiger nut samples. Furthermore, Udeozor and Awonorin (2014) conducted a separate study where they reported the isolation of *Saccharomyces cerevisiae* from a tiger nut-soy milk drink. The presence of *Saccharomyces cerevisiae* in both studies suggests a potential source of contamination in the tigernuts used for the production of various products, including the milk drink. The consistent isolation of *Saccharomyces cerevisiae* across different studies highlights the robustness of its association with tiger nuts and their derived products. These findings suggest that *Saccharomyces cerevisiae* may have established a niche in the tiger nut environment, contributing to its prevalence in both exposed tiger nuts and processed products such as tiger nut-soy milk drink. These studies collectively support the notion that the presence of *Saccharomyces cerevisiae* and *Candida tropicalis* in the current study's fungal isolates can be attributed to their association with tiger nuts and the potential contamination during processing or exposure.

The identification of *Saccharomyces cerevisiae* in the isolated fungal samples raises concerns due to its potential as an opportunistic pathogen, particularly in individuals with compromised immune systems (Cogliati, 2013). Ashiru et al. (2003) have previously suggested that the presence of *Saccharomyces cerevisiae* can be attributed to various sources of contamination, including air or dust, contaminated packaging materials, and poor hygiene and sanitation practices. The presence of *Saccharomyces cerevisiae* is worrisome due to its ability to produce mycotoxins that can lead to mycotoxicosis in humans (Umaru et al., 2014). These mycotoxins can have detrimental effects on human health. It is important to note that severe opportunistic infections caused by *Saccharomyces cerevisiae* have been reported in patients with chronic diseases, cancer, and immunosuppression. Such infections can manifest as fungemia, endocarditis, pneumonia, peritonitis, urinary tract infections, skin

infections, and esophagitis (P-Munoz et al., 2005). Considering the potential health risks associated with *Saccharomyces cerevisiae*, it is crucial to implement effective preventive measures and ensure proper hygiene and sanitation practices during the production and handling of food products, including those derived from tiger nuts. Additionally, the presence of *Saccharomyces cerevisiae* in the isolated fungal samples emphasizes the importance of rigorous quality control procedures to minimize the risk of contamination and subsequent health implications for consumers, especially those who are immunocompromised.

The isolation of *Candida tropicalis* in the current study is consistent with the findings reported by Badau et al. (2018), which confirmed the presence of *Candida spp.* in tiger nuts and its products. This observation aligns with the report by Adejuyitan (2011), indicating the isolation of *Candida tropicalis* from contaminated tiger nuts. *Candida tropicalis*, as the second most virulent *Candida* species, is commonly found colonizing and causing infections in humans, particularly on the skin, gastrointestinal tract, and female genitourinary tract (Chai et al., 2010; Wilson et al., 2015).The virulence of *Candida tropicalis* can be attributed to its ability to form biofilms, secrete lytic enzymes, adhere to epithelial and endothelial cells, and transition from buds to hyphae (Silva et al., 2011; Zuza et al., 2017). These factors contribute to its pathogenicity and ability to cause human disease. Therefore, the presence of *Candida tropicalis* in the isolated fungal samples raises concerns regarding the potential health risks associated with its presence in tiger nuts.

It is worth noting that the use of 16S rRNA gene sequencing played a crucial role in accurately identifying the endophytic fungal species and enabling phylogenetic analyses. The nucleotide sequences of the 16S rRNA gene provided species-specific signatures, allowing for precise identification of the fungal isolates. This method has been widely recognized for its rapid and accurate identification of endophytic fungal species.

The possible isolation of the fungal species from the tiger nut samples can be attributed to environmental contamination, as suggested by previous studies (Onovo and Ogaraku, 2007). This highlights the importance of ensuring proper hygiene and sanitation practices during the cultivation, harvesting, and processing of tiger nuts to minimize the risk of fungal contamination and subsequent health implications for consumers. It is crucial to raise awareness among both producers and consumers regarding hygiene and sanitation practices to reduce the risk of microbial contamination and associated health hazards.

## Conclusion

The study has revealed that the microbial quality of tigernuts obtained from the markets and the isolation of different fungal species indicates that the tiger nuts are unfit for human consumption. Therefore, tiger nut sellers should maintain adequate hygienic conditions so as to curb or reduce the potential public health risk associated with the use and consumption of contaminated tigernuts. The isolation of *Saccharomyces cerevisiae* and *Candida tropicalis* from the tigernut samples highlights the need for further research and monitoring to assess their potential impact on food safety. Although *Saccharomyces cerevisiae* is generally regarded as safe, precautions must be taken to prevent the growth of pathogenic microorganisms and aflatoxin contamination. Additionally, the presence of *Candida tropicalis* underscores the importance of proper hygiene and storage practices to minimize the risk of foodborne infections.

## Recommendation

Based on the findings and potential risks associated with the isolated organisms in tigernuts in this study, the following recommendations can be adopted to ensure food safety:

1. Quality control and sourcing: Tigernut producers and distributors should implement strict quality control measures during the production and sourcing of tigernuts. They should ensure that reputable suppliers are selected, and regular inspections are conducted to adhere to food safety standards. Regulatory agencies can also play a role by providing guidance and conducting audits to ensure compliance.
2. Good agricultural practices (GAP): Tigernut farmers should be educated and trained on GAP specific to tigernut cultivation. Agricultural extension services, research institutions, and governmental agencies can collaborate to provide training programs and guidelines for farmers, promoting proper land management, irrigation practices, and pest control to minimize fungal contamination and aflatoxin production in the field.
3. Harvesting and post-harvest handling: Tigernut producers and processors should educate workers involved in tigernut harvesting and post-harvest processes on proper hygiene practices. This includes using clean equipment, washing hands regularly, and avoiding cross-contamination with other potentially contaminated materials. Producer associations and industry organizations can facilitate training programs and disseminate guidelines.
4. Promotion of hygienic practices: It is essential to encourage and promote proper hygienic practices among tigernut sellers and those involved in the production of tiger nut-based products, such as tiger nut milk. Contaminated tiger nuts have the potential to harbor various pathogenic microorganisms, which can pose a significant risk of foodborne infections when consumed. Therefore, stakeholders in the tigernut industry, including producers, processors, distributors, and regulatory agencies, should collaborate to educate and

raise awareness about the importance of maintaining hygienic conditions throughout the tigernut supply chain. This includes implementing good manufacturing practices, providing training on proper handling and storage, and emphasizing the need for regular sanitation and hygiene practices to ensure the safety and quality of tigernut products.

5. Storage conditions: Tigernut producers, distributors, and retailers should ensure appropriate storage conditions for tigernuts to prevent fungal growth and aflatoxin production. They should provide guidance and training to staff involved in handling and storage operations, emphasizing proper temperature, humidity, and ventilation in storage facilities. Producer associations and regulatory agencies can collaborate in providing educational materials and conducting workshops for stakeholders.
6. Aflatoxin testing: Tigernut producers, processors, and regulatory agencies should collaborate to implement routine aflatoxin testing for tigernut batches, particularly those from regions or suppliers with a history of aflatoxin contamination. Regulatory agencies can set guidelines and standards for testing, while producers and processors can perform the necessary testing and reject contaminated lots. Results of testing can be shared with relevant stakeholders to ensure transparency and consumer safety.
7. Product labeling and information: Tigernut producers, distributors, and retailers should take the responsibility to provide clear and accurate labeling on tigernut products, informing consumers about potential allergens, such as *Saccharomyces cerevisiae* and *Candida tropicalis*, and the importance of proper storage and handling. They should ensure that product packaging, labels, or accompanying materials include this information. Regulatory agencies can provide guidelines and regulations for labeling requirements to ensure consistency and accuracy.
8. Consumer awareness and education: Tigernut producers, distributors, regulatory agencies, and consumer organizations should collaborate in educating consumers about the risks associated with fungal contamination and aflatoxins in tigernuts. Tigernut producers and distributors should promote consumer awareness by including information about potential risks and proper storage practices on product packaging, labels, or accompanying materials. Regulatory agencies can support these efforts by disseminating guidelines, conducting awareness campaigns, and collaborating with consumer organizations to ensure that accurate information reaches the public.
9. Regulatory measures: Regulatory agencies should establish and enforce food safety regulations specific to tigernuts. They should monitor and conduct surveillance programs to detect and prevent the distribution of contaminated products in the market. Regulatory agencies should also collaborate with industry stakeholders, such as producer associations and distributors, to ensure compliance with regulations and promote safe practices throughout the supply chain.
10. Research and innovation: Research institutions and governmental agencies should support further research on tigernuts, endophytic fungi, and aflatoxin production. This research can lead to the development of

improved cultivation techniques, innovative storage solutions, and effective control strategies to mitigate the risks associated with fungal contamination. Research findings should be shared with producers, processors, and regulatory agencies through publications, conferences, and workshops to facilitate knowledge transfer and implementation of best practices.

By implementing these recommendations, stakeholders in the tigernut industry can work together to ensure the safety and quality of tigernut products, minimize the risks of foodborne infections and aflatoxin contamination, and safeguard public health.


## Acknowledgements

The authors express their sincere gratitude to Dr. O.O. Oyedara for providing valuable supervision and support throughout the research project. Special appreciation is extended to the laboratory assistants, whose unwavering technical support greatly contributed to the successful completion of the project. The authors would also like to acknowledge the invaluable assistance received from all the dedicated staff members of the Department of Microbiology at Osun State University, Osogbo, Osun State, Nigeria. Their collaboration and assistance played a pivotal role in the overall accomplishment of this study.


## Contribution of authors:

Author Arogundade F.Q. was involved in material preparation, data collection, microbiological analysis, data analysis and interpretation, literature searches and writing of the first draft of the manuscript. Author, Olatoye T.T. was involved in sampling and microbiological analysis of samples. Both authors participated in review of the manuscript draft. All authors read and approved the final manuscript.

## Data Availability:

The data used to support the findings of this study are available from the corresponding author upon request.


## Source of funding:

No funding was received for the study.


## Conflict of interest:

Authors declare no conflict of interest

## Abbreviations

ITS Internal transcribed spacer

PDA - Potato dextrose agar

RNA – Ribonucleic Acid

rRNA - Ribosomal ribonucleic acid

DNA - Deoxyribonucleic Acid

PCR - Polymerase chain reaction

NCBI - National centre for biological information

BLAST - Basic local alignment search tool

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