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ARTICLE

Molecular Identification of Organisms from Bread Mold

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ABSTRACT

The study focused on the molecular identification of organisms associated with bread mold with the aim of isolating, characterizing, and accurately identifying fungal contaminants responsible for bread spoilage. One gram (1 g) of a contaminated bread sample was serially diluted and cultured on Potato Dextrose Agar (PDA). The pure isolates were coded as GBMF1, GMBF2, GBMF3, and GBMF4, and incubated for another three days. Pearson correlation was used to determine if there is a difference in the genetic distance between the isolates at $p = 0.05$. After incubation, the fungal structures were observed under the ×40 objective lens for microscopic characterization. Morphological and microscopic characterization suggested that the isolates belonged to the genera *Aspergillus*, *Cladosporium*, *Penicillium*, and *Fusarium*. Genomic deoxyribonucleic acid (DNA) was successfully extracted from two of the isolates (GBMF1 and GBMF4) with purity ratios ranging from 1.70 to 1.80 and concentrations between 24 and 31 ng/µL. Polymerase Chain Reaction (PCR) amplification of the internal transcribed spacer (ITS) region produced clear bands of approximately 600 bp, confirming the presence of fungal DNA. Sequencing and Basic Local Alignment Search Tool for nucleotides (BLASTn) analysis revealed that isolate GBMF1 had 100% similarity with *Aspergillus niger*, while isolate GBMF4 showed 75–88% similarity with *Fusarium equiseti* and related taxa. The findings demonstrate that combining morphological and molecular techniques provides accurate and reliable identification of fungi in spoiled bread.

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Practically, the study emphasizes the need for strict hygiene in bread production, proper storage, and routine molecular surveillance to ensure food safety and reduce public health risks associated with mycotoxigenic fungi.

Keywords: Bread; Contaminants; Fungi; Gene; Mold; Polymerase Chain Reaction

1. Introduction

Bread is one of the most widely consumed staple foods worldwide, providing essential carbohydrates, fiber, and energy to millions of people. Among various types of bread, the fermentation process that involves *Saccharomyces cerevisiae* (baker's yeast) is pivotal for leavening dough, contributing to the texture, flavor, and volume of the final product. Bakeries maintain specific environmental conditions such as optimal temperature and humidity to promote yeast activity, which, unfortunately, also creates an environment conducive to the growth of unwanted microorganisms, particularly molds. Mold contamination in bread is not only detrimental to product quality but also poses significant health risks due to the potential production of mycotoxins [1]. Molds such as *Aspergillus*, *Penicillium*, and *Fusarium* are responsible for the production of harmful secondary metabolites, which can have severe toxicological effects on human health [2,3]. Mycotoxins, such as aflatoxins, ochratoxins, and trichothecenes, are of particular concern in food safety, as they can accumulate in bakery products and pose long-term health risks when consumed over time. These mycotoxins can lead to diseases including liver cancer, immune suppression, and neurological disorders [4,5]. Therefore, accurate identification of mold species is crucial in evaluating the associated health risks and implementing appropriate quality control measures to mitigate these risks in bakery products. Mold contamination in bread not only affects food safety but also reduces the shelf life of the product, as molds thrive in moist, warm environments typically found in bakery production areas [6].

Historically, mold species in bakery products have been identified based on their phenotypic characteristics, such as colony morphology, color, and texture. However, phenotypic identification methods have significant limitations. Many mold species exhibit similar morphological traits, making them difficult to differentiate with high accuracy. Additionally, such methods require specialized expertise and are often prone to misidentification due to the overlapping characteristics between species [6]. Moreover, traditional methods are time-consuming, which makes them less suitable for routine monitoring of mold contamination in bakery settings.

In response to these challenges, molecular techniques have emerged as reliable alternatives for fungal species identification. Among these methods, DNA barcoding has become a widely adopted approach for the identification of fungal species, including molds. DNA barcoding involves amplifying specific regions of the fungal genome, followed by sequencing and comparison with known databases. The internal transcribed spacer (ITS) region of the ribosomal ribonucleic acid (RNA) gene is the most used genetic marker for fungal barcoding due to its high variability between species and relative conservation within species [7]. This method allows for more precise identification of fungal species compared to traditional phenotypic methods. Research on the improvement of shelf life, hardness, chewability and gumminess of bread was demonstrated by using ancestral enzymes through bioinformatics analyses [8]. This helps to improve activity and thermal stability. This bioinformatics and silico method offers a breakthrough in bread research. Similarly, bread being a staple food and the most common to every class of people in Nigeria, the application of molecular analyses and the use of bioinformatics analyses is significant to help keep a database of bread mold-causing microbes that may be injurious to public health. The study is important because there are few data on bread mold in Nigeria.

However, despite the advantages of DNA barcoding, several challenges remain in accurately identifying mold species, particularly those that are closely related. Many species of molds share highly similar or identical ITS sequences, making it difficult to distinguish them using this marker alone. Additionally, variations in primer-binding sites can lead to incomplete or inaccurate amplification, further complicating the identification process [9]. To address these limitations, researchers have advocated for the use of multiple genetic markers in combination with the ITS region to enhance species-level discrimination. Targeting other regions of the fungal genome, such as the large subunit (LSU) and small subunit (SSU) ribosomal RNA genes, has shown promise in improving the resolution of fungal identification [2,4].

Recent studies have highlighted the importance of incorporating multiple primer pairs to enhance the accuracy of mold species identification. By amplifying different regions of the fungal genome, a combination of primers can increase the specificity and sensitivity of mold detection, allowing for the identification of a broader range of species [1,2]. This approach is particularly beneficial in identifying molds that produce different mycotoxins but are genetically similar, which is crucial for risk assessment in food safety [5]. Moreover, the use of multiple primer pairs can improve the reliability of molecular techniques in routine quality control testing, particularly in the food industry, where accurate mold identification is essential for product safety. This study aims to improve the molecular identification of organisms from bread mold.

This study is limited to the molecular identification of fungal organisms isolated from bread mold. It involves the culturing and isolation of fungal species from contaminated bread, the extraction of genomic DNA from pure isolates, and molecular identification through Polymerase Chain Reaction (PCR) and sequencing.

The aim of this study is to identify fungal organisms associated with bread mold using molecular techniques.

The objectives of the study are to:

1. Culture and isolate pure molds from the contaminated bread sample.
2. Extract genomic DNA from the isolated fungal cultures.
3. Carry out molecular identification for accurate species-level identification of bread molds.

2. Materials and Methods

2.1. Research Design

This study was designed as a laboratory-based experimental investigation aimed at isolating and identifying fungal species responsible for bread mold (**Figure 1**). The design combined both morphological and molecular methods to ensure accurate and reliable results. A contaminated bread sample was collected aseptically and labeled accurately. In the laboratory, the sample was serially diluted using normal saline and cultured on Potato Dextrose Agar (PDA) prepared according to the manufacturer's standard (39 g/L). Distinct fungal colonies were observed, isolated, and sub-cultured to obtain pure cultures. The pure isolates were first examined for morphological and microscopic characteristics using Lactophenol Cotton Blue stain. Thereafter, the isolates underwent molecular analysis, which involved DNA extraction using the ZymoBIOMICS DNA Microprep Kit (Zymo Research, California, USA), PCR amplification of the ITS gene region using ITS1 and ITS2 primers, and agarose gel electrophoresis to confirm successful amplification [10]. The PCR products were purified and subjected to Sanger sequencing for molecular identification. Sequence data were analyzed using ChromasLite and BioEdit for editing, while BLAST search on the National Center for Biotechnology Information (NCBI) database was used to identify related species [10]. Phylogenetic analysis was carried out using MEGA11 software to determine evolutionary relationships between the isolated fungi and closely related reference species.

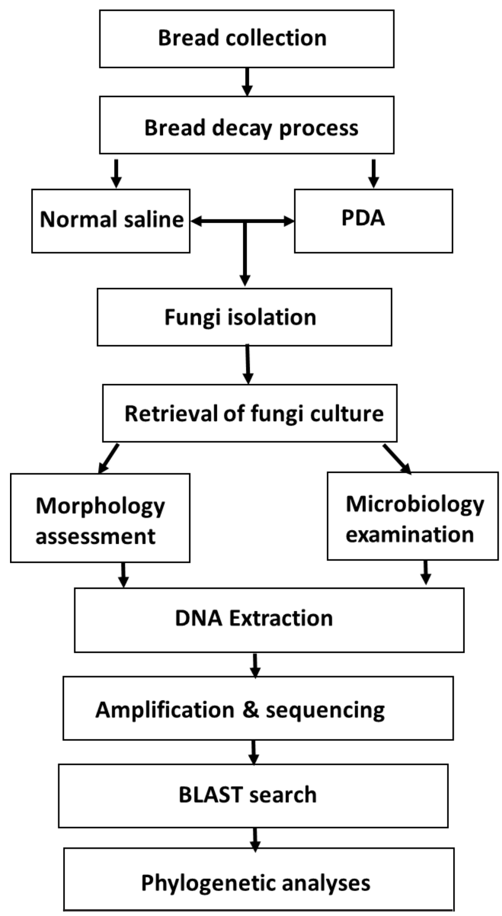


Figure 1. Flow chart of DNA sequencing of bread mold contaminated at the Biology and Biotechnology Laboratory, University of Port Harcourt, Nigeria.

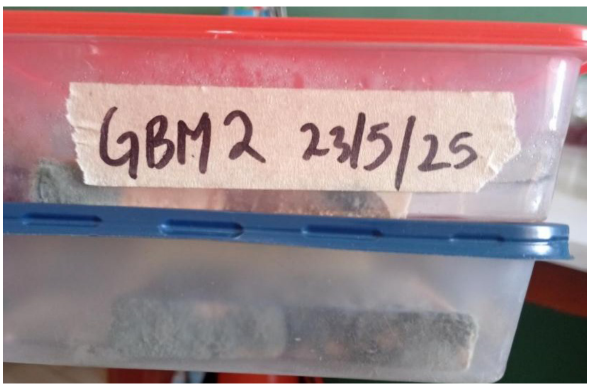
2.2. Materials

The materials used for this study include Petri dishes, test tubes, test tube rack, measuring cylinder, conical flasks, beakers, weighing balance, inoculating loop, syringe, glass slides, cover slips, microscope (×40 objective), autoclave, vortex mixer, micropipettes, Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) [11], ZymoBIOMICS DNA Microprep Kit, centrifuge, gel electrophoresis apparatus, and ultraviolet (UV) transilluminator. The reagents include Lactophenol Cotton Blue (used for staining and viewing fungal structures on wet mount slides), Lactic acid (added to Potato Dextrose Agar (PDA) after cooling to inhibit bacterial growth), Normal saline (Analytical Sodium Chloride) 0.9 g per 100 mL, used as a diluent for serial dilution, Safe Green dye (used for gel visualization during electrophoresis), Tris-Borate-EDTA (TBE) buffer (for gel preparation and electrophoresis), primers (ITS1 and ITS2), master mix, sterile nuclease-free water, Ethanol (70%) (used for sterilization and cleaning of surfaces) and Agarose powder (for gel electrophoresis).

All materials used throughout the analysis were properly labelled for easy identification and traceability throughout the study.

2.3. Sample Collection and Preparation

A loaf of bread was purchased in a geo-referenced location in Port Harcourt using a Garmin Global Positioning System (GPS) (etrex, USA), and a slice was aseptically removed and placed into a sterile container (**Figure 2**, Plates 1A and 1B), then covered with a little part open and left at ambient room temperature (28–30 °C) for seven days. A contaminated bread sample was transported to the laboratory in a sterile polyethylene bag after visible mold growth appeared on the bread surface. In the laboratory, one gram (1 g) of the moldy area was aseptically collected using a sterile spatula, placed on a sterile aluminum foil, weighed on a digital weighing balance, and then transferred into 9 mL of Normal saline to make a ten-fold serial dilution for culture and isolation.



(**a**) Plate 1A: Picture of Contaminated Bread after Seven Days In the laboratory of the Department of Biology and Biotechnology, University of Port Harcourt, Nigeria.

(**b**) Plate 1B: Contaminated bread from where fungi isolates were derived.

Figure 2. Bread contamination and decay process.

2.4. Sterilization Procedures

All glassware, reagents, and equipment used in this study were properly sterilized to maintain aseptic conditions and prevent cross-contamination. The culture media and glassware were sterilized in an autoclave at 121 °C for 15 min under a pressure of 15 psi. Work surfaces and instruments such as inoculating loops were wiped with 70% ethanol before and after use, while the loops and needles were sterilized by flaming in a Bunsen burner before each inoculation.

2.5. Media Preparation (Potato Dextrose Agar)

Potato Dextrose Agar (PDA) was used for culturing and isolating fungi from the contaminated bread. The standard preparation ratio according to the manufacturer's specification for PDA is 39 g/L. Accordingly, 39 g of

PDA powder was weighed using an analytical balance and dissolved in 1 L (1,000 mL) of distilled water in a conical flask. The mixture was heated gently until completely dissolved. The medium was autoclaved at 121 °C for 15 min to sterilize it. After cooling to about 45 °C, lactic acid was added aseptically to the medium to inhibit bacterial growth. The sterilized medium was then poured into sterile Petri dishes and allowed to solidify.

2.6. Serial Dilution and Culture Technique

A ten-fold serial dilution was carried out to reduce microbial load in the bread sample prior to culturing. Six sterile test tubes (10^{-1}–10^{-6}) were arranged on a rack, each containing 9 mL of sterile normal saline (0.9 g NaCl per 100 mL distilled water). One gram of the moldy bread sample was homogenized in 9 mL of sterile normal saline to prepare the stock solution (10^{-1} dilution). A 1 ml aliquot from the first tube was transferred sequentially to the next test tube, up to the sixth tube, to achieve dilutions up to 10^{-6}.

Using the spread plate method, 0.1 mL from appropriate dilutions was transferred onto the solidified PDA plates. The inoculum was evenly spread using a sterile glass spreader. The plates were incubated in an inverted position at room temperature (25–28°C) for 3 days. Distinct colonies showing different morphological features were sub-cultured onto fresh PDA plates to obtain pure isolates. Pure isolates were then obtained, coded as GBMF1, GBMF2, GBMF3, and GBMF4, and stored in sterile conditions for further morphological and molecular analyses.

2.7. Microscopy and Identification of Fungal Isolates

Each pure colony was examined macroscopically for colony color, texture, and surface morphology. A small portion of the fungal growth was mounted on a clean glass slide with a drop of Lactophenol Cotton Blue stain. The preparation was covered with a coverslip and examined under a compound microscope using the ×40 objective lens. Microscopic features such as spore shape, septation, and arrangement of conidia were observed and compared with standard fungal identification keys to determine the probable genera.

2.8. Molecular Analysis and Fungal Isolation

Molecular analysis was carried out to identify the fungal isolates at the genetic level through DNA extraction, amplification, and sequencing. The process involved fungal DNA isolation, barcoding through PCR, gel electrophoresis, and Sanger sequencing

Genomic DNA extraction was performed using the Zymo BIOMICS DNA Microprep Kit (Zymo Research, USA) with ZR Bashing Beads at MyAfroDNA Laboratory, Port Harcourt, Nigeria. Each fungal culture was scraped and transferred into a bead-bashing tube. Mechanical disruption was carried out using a vortexer for optimal cell lysis. The remaining steps: lysis, DNA binding, washing, and elution, followed the manufacturer's instructions. The eluted DNA was stored at −20 °C until further use. DNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Absorbance readings at 260 nm were used to calculate DNA concentration, while the 260/280 nm ratio assessed purity. DNA samples were either processed immediately or stored at −20 °C for downstream applications.

2.9. Fungal Barcoding (ITS PCR Amplification)

Molecular identification of fungal isolates was performed by amplifying the Internal Transcribed Spacer (ITS) region using universal primers:

ITS1 Forward: 5'-CTT GGT CAT TTA GAG GAA GTA A-3'

ITS2 Reverse: 5'-GCT GCG TTC TTC ATC GAT GC-3'

The PCR reaction mixture contained 2 µL of DNA template, 1 µL of each primer, 4 µL of PCR master mix, and nuclease-free water to make up the final volume. The amplification was carried out under the following thermal conditions: initial denaturation at 95 °C, followed by 35 cycles of denaturation, annealing, and extension, with a final extension at 72 °C. The PCR products were analyzed by agarose gel electrophoresis to confirm successful amplification.

2.10. Gel Electrophoresis and Sanger Sequencing

A 2% agarose gel was prepared by dissolving 0.6 g of agarose in 30 mL of 1X Tris-Borate-EDTA (TBE) buffer. The solution was heated to dissolve the agarose completely and allowed to cool to about 45 °C. Safe Green dye (30 µL) was added to the gel solution for DNA visualization. The gel was poured into a casting tray with pre-inserted combs and allowed to solidify. After solidification, 4 µL of each PCR product and a molecular marker (100 bp ladder) were loaded into the wells. Electrophoresis was performed, and the gel was viewed under a UV transilluminator. Positive amplification was indicated by visible DNA bands of approximately 600 base pairs.

PCR products were purified and subjected to Sanger sequencing using the BigDye™ Direct Cycle Sequencing Kit. Sequencing reactions were prepared with M13-tagged ITS primers, purified, and run on a genetic analyzer via capillary electrophoresis. The sequence data obtained were analyzed using chromatogram visualization software to determine accurate base calls. The generated nucleotide sequences were compared with reference sequences in the NCBI GenBank database using the BLASTn algorithm for species identification.

2.11. Sequence Analysis and Phylogenetic Relationship

The DNA sequences generated were viewed and edited using ChromasLite and BioEdit software. Sequence similarity searches were performed using BLASTn against the NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related fungal species. The aligned sequences were analyzed using ClustalW, and phylogenetic trees were constructed using the Neighbor-Joining method [12] implemented in MEGA version 11 [13]. Bootstrap analysis with 500 replicates was performed to evaluate the reliability of the tree branches. The evolutionary relationship was inferred between the isolates and closely related taxa, showing *Aspergillus niger* clustering closely with its reference strain, while the Fusarium-related isolate showed a weak association due to low sequence similarity.

Statistical Analysis

Pearson's product–moment correlation was done to compare whether there was any significant difference between percent genetic identity and distance between bread molds. All analysis was done in *R* statistical environment 4.2.2 (R Development Core Team).

3. Results

3.1. Fungal Plate Count from Bread Mold Sample

Table 1 shows the colony-forming units (CFU) obtained from bread mold samples cultured on Potato Dextrose Agar (PDA) at different dilutions (10^{-4}, 10^{-5}, 10^{-6}). The highest colony counts were recorded at the 10^{-4} dilution (89 and 73 CFU), while the counts decreased progressively at higher dilutions (26 and 29 CFU at 10^{-5}; 24 and 5 CFU at 10^{-6}). This trend indicates a high fungal load in the original bread sample, reflecting heavy contamination. The consistency between replicate plates shows good experimental reliability. High CFU values at lower dilutions are typical of heavily colonized bread samples, where molds such as *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium* thrive under warm and humid conditions.

Table 1. Fungal Plate Count from Bread Mold Sample Retrieved from Biology Biotechnology Laboratory, University of Port Harcourt, Nigeria (±SE).

Sample ID/Dilution	Colony Count (CFU)	
	Plate 1	Plate 2
GBM PDA 10^{-4}	89	73
GBM PDA 10^{-5}	26	29
GBM PDA 10^{-6}	24	5

3.2. Morphological and Microscopic Characterization of Fungal Isolates from Bread Mold

Table 2 presents the macroscopic (colony morphology) and microscopic (spore and hyphal features) characteristics used to presumptively identify fungal isolates. GBMF1 showed dense black aerial mycelia with globose spores and non-septate hyphae, typical of *Aspergillus* species. GBMF2 exhibited grey colonies with septate hyphae, consistent with *Cladosporium* species. GBMF3 had blue-green colonies and chain-forming conidia, identifying it as *Penicillium*. GBMF4 displayed white fluffy mycelia with macroconidia, typical of *Fusarium* species. These observations align with established fungal identification guides, confirming that bread molds can be accurately identified through cultural and microscopic analysis before molecular confirmation.

3.3. Macroscopic and Microscopic View of Fungal Isolates from Bread Mold

Table 3 visually complements **Table 2**, providing photographic documentation of the isolates. The macroscopic view illustrates colony color, texture, and growth pattern on PDA, while the microscopic view highlights spore and hyphal morphology. These images serve as a reference for correlating visual traits with molecularly confirmed genera (*Aspergillus*, *Cladosporium*, *Penicillium*, and *Fusarium*). The consistency between morphological and molecular data reinforces the reliability of classical mycological techniques in preliminary fungal identification.

3.4. Mean DNA Concentration and Purity of Fungal Isolates

Table 4 shows the purity and concentration of genomic DNA extracted from fungal isolates GBMF1 and GBMF4. Purity ratios (A260/A280) of 1.70–1.80 indicate good-quality DNA, suitable for PCR amplification. The mean DNA concentrations ranged from 25 to 30 ng/μL, sufficient for downstream molecular analyses. The reproducibility between duplicate samples (GBMF1: 24–26 ng/μL; GBMF4: 29–31 ng/μL) demonstrates consistent DNA extraction performance. These results confirm that the extraction and purification methods effectively isolated intact DNA with minimal protein contamination. Also see **Appendix A**, **Table A1** for further details of purification process.

Table 2. Morphological and Microscopic Characterization of Fungal Isolates from Bread Mold Retrieved from Biology Biotechnology Laboratory, University of Port Harcourt, Nigeria.

Isolate ID	Macroscopic Features	Microscopic Features	Probable Genera
GBMF1	Black aerial dense mycelia with spore, white margin, cracked yellow reverse.	Presence of globose black spores in cluster. Presence of thick-walled nonseptate hyphae	*Aspergillus*
GBMF2	Grey, black reverse, non-crack, cream margin, slow-growing circular, domed-shaped, dry mycelia	Presence of septate-bound hyphae, no space seen	*Cladosporium*
GBMF3	Blue-green, cream reverse, non-crack, white circular margin with dry rough mycelia	Presence of greenish spores in chains	*Penicillium*
GBMF4	Fast growing, white non-crack reverse, white margin, white fluffy dense mycelia, smooth surface	Presence of thick walled, macroconidia	*Fusarium*

Table 3. Macroscopic and Microscopic View of Fungal Isolates from Bread Mold Retrieved from Biology Biotechnology Laboratory, University of Port Harcourt, Nigeria.

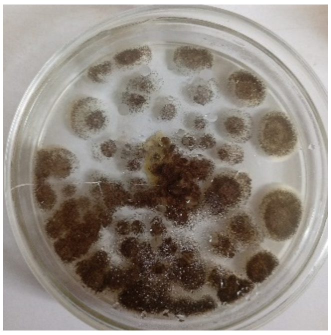
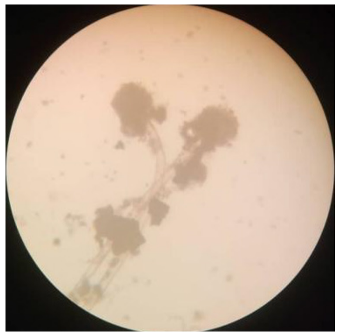
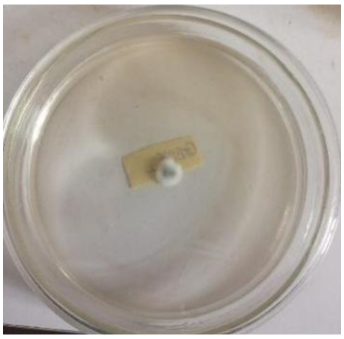
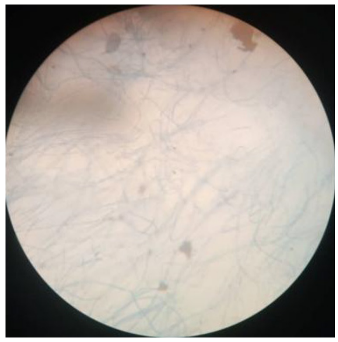
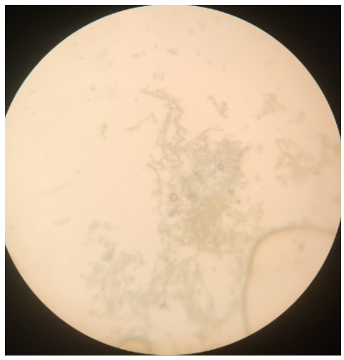
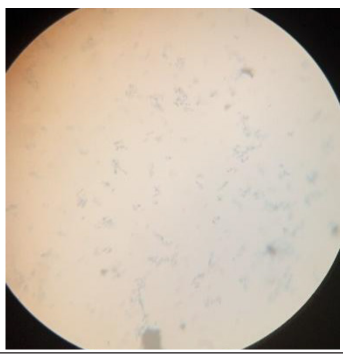
Isolate ID	Macroscopic View on PDA	Microscopic View	Probable Genera
GBMF1			*Aspergillus*
GBMF2			*Cladosporium*
GBMF3			*Penicillium*
GBMF4			*Fusarium*

Table 4. Mean DNA Concentration and Purity of Fungal Isolates from Bread Mold Retrieved from Biology Biotechnology Laboratory, University of Port Harcourt, Nigeria (±SE).

Isolate Code	PCR	Purity (A260/A280)	DNA Concentration (ng/µL)
GBMF1	ITS	1.80	25
GBMF4	ITS	1.70	30

3.5. Fungal DNA Detected Using PCR

The ITS PCR reactions were successful in amplifying the targeted fungi's ITS region across all isolates. The agarose gel electrophoresis result shows distinct DNA bands at approximately 600 base pairs, corresponding to the internal transcribed spacer (ITS) gene region (Plate 2, as shown in **Figure 3**). The presence of clear, sharp bands in lanes representing isolates GBMF1 (S6) and GBMF4 (S7) confirms successful PCR amplification. The uniform band intensity indicates good DNA template quality and efficient amplification by the ITS primers. This result validates the molecular detection of fungal DNA and supports subsequent sequencing analysis.

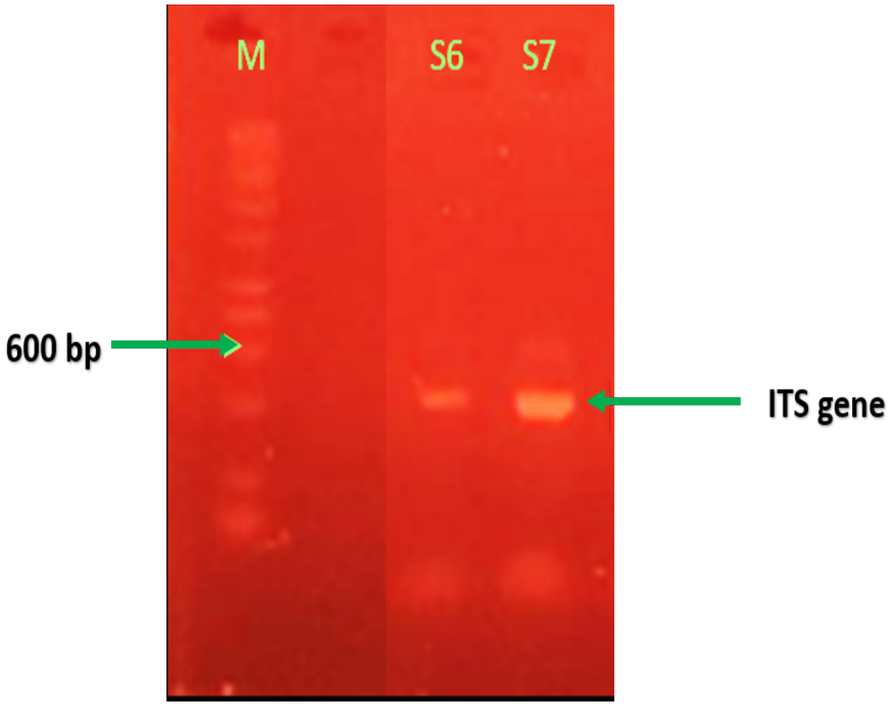


Figure 3. Plate 2: Representative Gel of Detected Fungal DNA (ITS Gene Amplification) retrieved from bread mold at the Laboratory of Biology and Biotechnology, University of Port Harcourt, Rivers State, Nigeria.

3.6. Molecular Identification of Fungal Isolates Using Sanger Sequencing

The molecular identification of the fungal isolates using GenBank revealed distinct levels of similarity with known sequences (**Table 5**). Isolate GBMF1 showed a 100% match with *Aspergillus niger* strain Ct201, indicating that this isolate can be confidently identified as *Aspergillus niger*. This perfect match suggests that the genetic sequence of GBMF1 is identical to the reference strain in the database. In contrast, isolate GBMF4 exhibited an 88% match with *Fusarium equiseti* strain KolF-557, suggesting that while GBMF4 is closely related to *Fusarium equiseti*, it may not be the same species. The lower percentage identity could reflect genetic variation within the species, differences between strains, or the possibility that the isolate belongs to a closely related species. The results confirm the identification of GBMF1 as *Aspergillus niger*, while GBMF4 is likely *Fusarium equiseti*, but with some genetic divergence.

Table 5. Fungal Species Identified Using Sanger Sequencing (±SE).

Isolate Code	Closest GenBank	Name of Species	% Identity
GBMF1	*Aspergillus niger* strain Ct201	*Aspergillus niger*	100
GBMF4	*Fusarium equiseti* strain KolF-557	*Fusarium equiseti*	88

3.7. BLAST Analysis and Phylogenetic Tree Construction for Isolate GBMF1

BLASTn analysis of the ITS sequence of isolate GBMF1 showed 100% query coverage, an E-value of 0, and 100% sequence identity with several *Aspergillus niger* strains in the NCBI database. The top BLASTn hits included *Aspergillus niger* strain Ct201 (GenBank Accession: PQ787748.1), isolate DNik7 (MZ474945.1), and strain VT66 (OR625091.1), among others. All the closest matches displayed perfect sequence alignment, confirming that the internal transcribed spacer (ITS) region of GBMF1 is identical to that of known *A. niger* isolates. Based on these molecular characteristics and sequence homology, isolate GBMF1 is conclusively identified as *Aspergillus niger* (GenBank Accession: PQ787748.1). The phylogenetic tree (**Figure 4**) was generated using the Neighbor-Joining (NJ) method with 500 bootstrap replications in MEGA. Isolate GBMF1 clusters closely with *Aspergillus niger* (GenBank Accession: PQ787748.1) with strong bootstrap support, confirming its taxonomic placement within the *Aspergillus niger* clade. The tree was rooted using an appropriate outgroup sequence to enhance phylogenetic resolution.

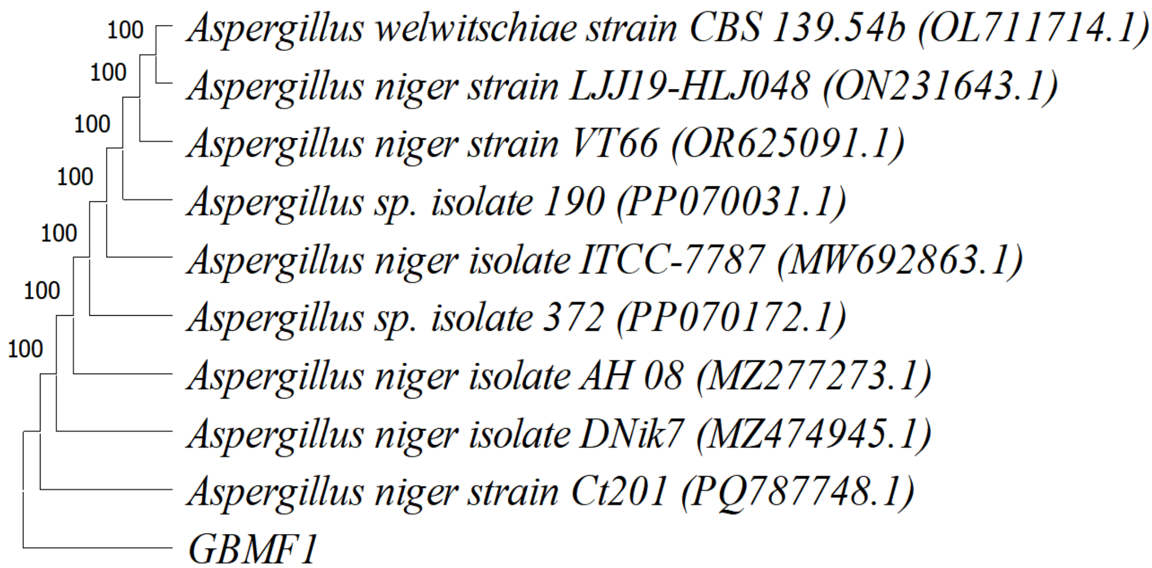


Figure 4. Phylogenetic relationship of isolate GBMF1 with closely related taxa based on ITS. sequences. *Aspergillus niger* is the diminat fungal strain in the GBMF1 isolate. Similarly, *A. welwitschiae* branched off from *A. niger*.

3.8. BLAST Analysis and Phylogenetic Tree Construction for Isolate GBMF4

BLAST analysis of isolate GBMF4 produced weak but notable alignments, with the closest hits to *Knoxdaviesia capensis* showing 85.5% sequence identity, low query coverage (26%), and an E-value of 6×10^{-6}. Additional matches were found with *Emericellopsis* sp. and *Fusarium equiseti*, though these showed even lower identity values (75–88%) and similarly limited coverage. The occurrence of multiple genera among the top hits, coupled with the low coverage and moderate identity, indicates that the sequence may belong to a divergent or underrepresented lineage in the database. While *K. capensis* appears as the closest reference, the limited alignment strength prevents a confident species-level assignment. Thus, GBMF4 is most appropriately described as an unidentified fungal isolate with tentative affinity to *Knoxdaviesia*. The phylogenetic tree (**Figure 5**) was constructed using the Jukes–Cantor model with 500 bootstrap replicates, and bootstrap values are shown at the nodes. The analysis clusters GBMF4 within diverse taxa, including *Knoxdaviesia capensis*, *Emericellopsis* sp. and *Fusarium equiseti*. (See **Appendix B** for the structure of the isolates).

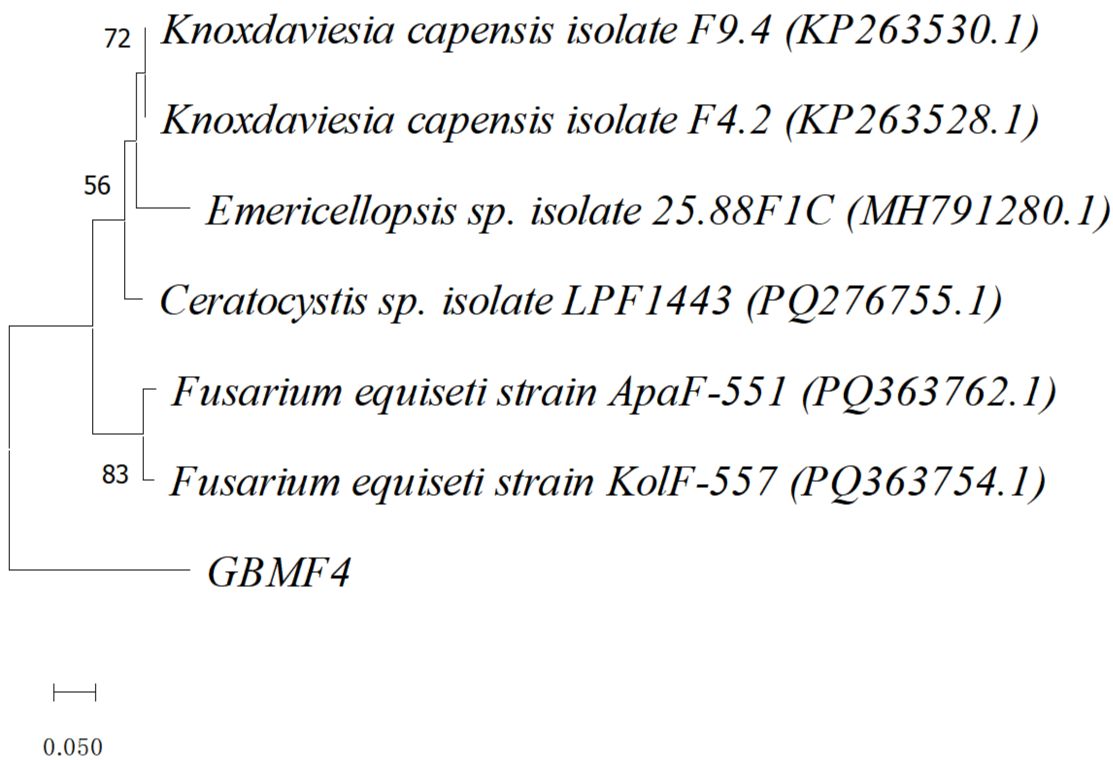


Figure 5. Neighbor-Joining phylogenetic tree based on ITS gene sequences showing the relationship of isolate GBMF4 with reference sequences.

Note: The legend shows a base pair of 0.05. It shows that *K. capensis* and *Ceratocystis spp* isolates originate from the same ancestral group.

Correlation between percent genetic identity and distance between species of bread mold

There was no correlation between the percent genetic identity and distance between bread mold (t = −0.2165, df = 8, p-value > 0.05; cor = −0.3223452). However, the negative correlation sign indicates a tendency for the percent identity to drop with increasing distance between species of bread mold.

4. Discussion

The study on the molecular identification of organisms on bread mold revealed a diverse assemblage of fungal species belonging to the genera *Aspergillus*, *Cladosporium*, *Penicillium*, and *Fusarium*. These fungi are frequently associated with bakery products and are known to cause significant post-production spoilage. The high colony counts recorded at the lower dilution level (10^{-4}) indicate a high degree of fungal contamination in the bread sample. This observation suggests that bread stored under warm and humid conditions provides a favorable environment for fungal growth, particularly when packaging and handling are inadequate. Similar results were reported by Faparusi and Adewole (2019) [14], who isolated *Aspergillus* and *Penicillium* species as the dominant contaminants in bread sold in southwestern Nigeria. Nuhu and Yusuf (2023) [15] also found comparable fungal loads in bread samples from Yobe State, Nigeria, attributing contamination to poor hygiene during production and post-baking exposure.

The morphological and microscopic characterization supported the preliminary identification of these fungi before molecular confirmation. The macroscopic features, such as the dense black mycelia of *Aspergillus*, the grey septate colonies of *Cladosporium*, and the blue-green colonies of *Penicillium*, correspond to descriptions found in classical mycological references. These features are consistent with earlier findings by Ajmal et al. (2022) [16], who observed similar morphological traits in bread-borne molds. The presence of *Fusarium* species in the sample also aligns with the report of Sitoe et al. (2025) [17], which identified *Fusarium oxysporum* and *Fusarium equiseti* as secondary invaders of starchy foods under moist storage conditions. These morphological results illustrate that the bread matrix serves as a rich substrate for diverse fungi capable of surviving environmental stress and nutrient competition.

The DNA extraction results revealed purity ratios (A260/A280) ranging from 1.70 to 1.80, which fall within the acceptable range for high-quality DNA suitable for PCR analysis. These findings align with the results of Langsiri et al. (2025) [18], who reported similar purity values when extracting DNA from filamentous fungi using silica-based and column methods. Adequate purity and concentration are critical for successful molecular analysis because contaminants, such as polysaccharides or proteins, can inhibit enzymatic amplification [19]. The observed DNA concentration values between 24 and 31 ng/µL confirm that sufficient DNA was extracted for molecular work, as supported by Methodology for Extracting High-Molecular-Weight DNA from [20].

The PCR amplification using ITS primers successfully produced distinct DNA bands of approximately 600 base pairs, corresponding to the expected size of the internal transcribed spacer (ITS) region of fungi. The ITS region is widely recognized as the universal DNA barcode for fungal identification due to its variability among species and conservation within genera [20]. The presence of clear, sharp bands in all positive isolates in this study indicates the successful amplification of the fungal ITS region and confirms that the extracted DNA was free from inhibitors.

The sequence analysis revealed that isolate GBMF1 shared 100% similarity with *Aspergillus niger*, a common spoilage mold of bread and baked goods. This result corresponds with findings from Nuhu and Yusuf (2023) and Olumuyiwa et al. (2025) [15,21], who reported *A. niger* as the most prevalent bread spoilage organism in Northern Nigeria. *Aspergillus niger* has been widely implicated in the deterioration of bread because of its ability to produce hydrolytic enzymes such as amylases and proteases that degrade starch and proteins in the product [22]. Moreover, *A. niger* has been reported to produce mycotoxins under favorable conditions, posing potential public health concerns [23]. The molecular confirmation of *A. niger* in this study reinforces its widespread occurrence and dominance in the spoilage of carbohydrate-based food products.

In contrast, isolate GBMF4 showed partial sequence identity (75–88%) with *Knoxdaviesia capensis*, *Emericellopsis sp.*, and *Fusarium equiseti*. The low percentage identity and limited query coverage suggest that this isolate may represent a genetically divergent strain or an underrepresented sequence in current databases. Similar challenges were observed by Geiser et al. (2023) and O'Donnell et al. (2015) [24,25], who noted that ITS alone often lacks resolution for distinguishing closely related or cryptic *Fusarium* lineages. The results of this study support the argument by *Fusarium*-ID v3.0 (2022) that multi-locus sequencing using elongation factor (TEF1-α), RNA polymerase II (RPB1 and RPB2), or β-tubulin genes provides improved phylogenetic resolution within the *Fusarium* complex. Therefore, while GBMF4 shows morphological characteristics of *Fusarium*, its precise taxonomic identity may require further molecular investigation using additional gene targets.

The combined morphological and molecular findings in this study confirm the presence of multiple fungal species contaminating bread samples, with *Aspergillus niger* being the most dominant. These results are consistent with the report of Faparusi and Adewole [14] and Chilaka et al. [26], which identified *Aspergillus* and *Penicillium* species as major contaminants in bread. Similar findings were documented in Brazil by Spoilage Fungi in a Bread Factory (2019), emphasizing that environmental exposure and contaminated ingredients contribute significantly to fungal invasion in bakery products. The identification of *Fusarium*-related isolates in this study also raises the possibility of latent toxigenic contamination, which poses health risks if bread is consumed after visible spoilage. The integration of culture-based and molecular methods provided complementary insights into the fungal diversity of bread molds. This aligns with the conclusion of Langsiri et al. (2021) [18] and Methodology for Fungal DNA Extraction (2021), who advocated that molecular confirmation enhances the reliability of morphological identification. The results, therefore, demonstrate the effectiveness of the ITS-based molecular approach in detecting and identifying fungal contaminants in food matrices. Continuous surveillance and molecular screening of bakery products are essential to prevent economic losses and protect consumers from potential mycotoxin exposure. The genetic distance between both fungal strains (GBMF1 and GBMF4) is not significantly different, which indicates that they both originate from similar ancestral fungal parents. This similarity can evade detection of a unique new strain, which might metamorphose through hybridization. This calls for constant bread monitoring to detect new fungal strains that may pose a public health problem. This study is innovative because there are limited data on bread mold in the region.

It will thus create a database that will serve as a valuable reference point for future sequencing of bread mold. The study aims to expand the sample size in the future by including additional isolates beyond those used in this study (i.e., GBMF1 and GBMF4). A major reason for the low number of samples collected was a lack of funds, but future work will apply for a grant to help collect more samples for more robust results and findings.

5. Conclusions

The study on the Molecular Identification of Organisms on Bread Mold revealed that bread is highly susceptible to fungal contamination, especially when storage and handling conditions are poor. Since bread is the main food eaten by all classes of people, monitoring its production and sale to the public is very important to ensure good public health. Both morphological and molecular analyses confirmed the presence of several fungal species, predominantly belonging to the genera *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*. Among these, *Aspergillus niger* was identified as the most prevalent and dominant contaminant, showing 100% sequence similarity with reference sequences in GenBank. The molecular analysis through ITS amplification and sequencing provided precise identification of fungal isolates beyond what was achievable through morphological observation alone. The successful amplification of the internal transcribed spacer (ITS) region demonstrated the reliability of molecular markers in fungal taxonomy and confirmed their suitability for identifying both culturable and potentially novel fungal species. The presence of *Fusarium*-related isolates with lower sequence similarity (75–88%) also highlighted the existence of genetically diverse or less-characterized fungal strains that may require multi-locus gene sequencing for definitive classification. The findings demonstrate that integrating molecular techniques such as ITS sequencing with traditional culture methods enhances the accuracy and reliability of fungal identification in food spoilage studies. The study underscores the need for continuous monitoring of bakery products to prevent spoilage, economic losses, and potential exposure to mycotoxins associated with toxigenic fungi such as *Aspergillus* and *Fusarium*.

Recommendations

1. Bakeries should maintain strict hygiene during production, packaging, and distribution to prevent contamination from air, surfaces, and handlers.
2. Bread should be stored in dry, cool, and well-ventilated places to reduce moisture and humidity that promote fungal growth.
3. Regular microbial testing should be carried out on bakery environments, equipment, and finished bread to ensure early detection of fungal contaminants.
4. Molecular identification methods such as ITS sequencing should be used routinely in food safety laboratories for accurate identification of spoilage organisms.
5. Consumers should avoid eating visibly moldy bread and be educated on the health risks associated with consuming contaminated bakery products.

Author Contributions

G.M.D. conducted the research and compiled the manuscript; S.C. supervised the work and edited and revised the work; K.J., M.U. and E.B. reviewed and edited the final copy. A.N. coordinated the project. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable because the studies do not involve the use of human and animal subjects.

Data Availability Statement

The data used in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Appendix A

Table A1. DNA Concentration and Purity of Fungal Isolates.

Isolate Code	Duplicate	Purity	DNA Concentration (ng/µL)
GBMF1	1	1.81	24
	2	1.78	26
GBMF4	1	1.68	29
	2	1.72	31

Appendix B. BLAST Analysis of Isolate GBMF1 and GBMF4 Respectively

>GBMF1

CCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAA

>GBMF4

AGAGGAAGTAARMGTCSYGCAGATTCCRAGSGTCGCCWGKRTCCGAAATGACACARAAGAGAAACCTACAAASMAACAATAGTTGTATAGKCAAAACAAACCAACCAAACTTTAASSAATGRCCGKTGAGGGCCCCAAACTCTGRAASGAAGGGAACTTASGAGTTACAGCAAAAAAAGACAAAACTTTCAAAAACGGACTCTTGGTCTGCAACAATGAAGAAACAAGRA

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