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Molecular Characterization and Potential of Fungal Species Associated with Cassava Waste

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Authors' contributions

This work was carried out in collaboration between both authors. Author AIE designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors AIE and NUA managed the literature searches and performed the analyses. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Efficient utilization of cassava waste for value addition depends largely on proper understanding of its true microbial diversity. The aim of this study was to characterize using molecular methods, fungal species associated with cassava waste and to highlight their industrial potential.
Study Design: Cassava peel (CP) waste from CP waste dumpsites and cassava waste water from cassava wastewater discharge outlets were collected from major cassava processing centres in Abeokuta, Ogun State, Nigeria, for the study.
Place and Duration of Study: Biotechnology Centre, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria; between June 2011 and March 2012.
Methodology: Two molecular methods namely, total fungal community DNA and isolates DNA sequence analysis were employed to characterize and identify the fungal species. Total fungal community DNA was extracted directly from CP waste and cassava wastewater, using the Soil DNA isolation kit (Norgen, Canada), while total genomic DNA was extracted from fungal isolates,

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using the same kit. The fungal ITS2 (Internal transcribed spacer) gene sequence of total fungal community and genomic DNA was amplified by Polymerase Chain Reaction (PCR) using ITS2 primers. Total fungal community DNA amplicons were spliced into PCR-TRAP Cloning Vector, used to transform competent cells of *Escherichia coli* and sequenced. Sequences were identified by aligning with sequences in the GenBank.

Results: Results showed that 17 fungal species including *Eurotiomycetes – Eurotiales* (6 species), *Mucormycotina – Mucorales* (1 species), *Sordariomycetes - Hypocreales* (1 species), *Saccharomycetes Saccharomycetales* (8 species), and unidentified fungi (1 species) were present in cassava peel (CP). The dominant species was *Aspergillus niger* (15.2%). However, cassava wastewater had 27 fungal species including *Eurotiomycetes – Eurotiales* (2 species), *Saccharomycetes Saccharomycetales* (24 species) *Tremellomycetes-Tremellales* (1 species); the dominant species being *Saccharomyces cerevisiae* and *Candida krusei* each with 8.7% relative abundance.

Conclusion: This study shows that cassava waste, on account of its rich fungal diversity, is an important microbial resource.

Keywords: Cassava waste; ITS2 gene; molecular characterization; fungal diversity; total community DNA; Aspergillus niger; yeast.

1. INTRODUCTION

Huge amount of waste such as peels, fibrous core and carbohydrate rich pressing slurry, is generated during cassava processing and utilization. It is estimated that about 0.3 - 0.4 tonne of cassava peel (CP) is generated when 1 tonne of starch is produced [1]. In Nigeria, about 450,000 tonnes of CP is generated annually, with an increasing trend [2]. On the average, 2.62 m^3 $tonnes^{-1}$ of residues from washing and 3.68 m^3 $tonnes^{-1}$ from the water residues resulting from cassava flour production have been reported [3,4]. Presently, these wastes generated pose serious environmental challenges, especially when dispose of indiscriminately. With the projected total world cassava utilization of 275 million tonnes by 2020 [5,6], resulting from ongoing effort at stimulating cassava production and utilization globally, a more challenging environmental concern is undoubtedly anticipated.

Over the years, researchers have made tremendous impact on the use of cassava waste for value added products. The production of animal feed from CP has been well studied [7-9]. A major improvement in this regard, has been in nutrient enrichment of CP by fermentation using microorganisms such as yeast and lactic acid bacteria [10], *Aspergillus and Trichoderma* species [11], for enhanced animal nutrition. CP has also been used for the production of functional food [12], ethanol [13], biofertilizer [14], and as substrate for mushroom cultivation [15]. Similarly, cassava wastewater has been used for the production of butanol [16], organic acids [17], biosurfactant [18], volatile aromatic compounds [19], and has even been considered for the production of a probiotic beverage [20]. Besides, even much more could be achieved if the true microbial diversity and dynamics of cassava waste is clearly understood.

Cassava waste is a known veritable source of important microorganisms [21,14,22], with industrial importance. Yet information on the microbial diversity of cassava waste is scanty. The natural decomposition of cassava waste is a complex process involving a community of fungi and bacteria. A recent study revealed a rich bacterial diversity in cassava waste [23], indicating its potential for industrial applications. A similar study on fungal diversity in cassava waste, especially employing molecular procedures, would complement this finding. This would enhance better utilization of the waste. Novel fungal species of industrial importance may possibly be identified. In addition, fungal species already identified from cassava waste by cultural/biochemical methods could be authenticated and fully exploited if characterized using molecular methods. The aim of this study was therefore to characterize using molecular methods, fungal species associated with cassava waste in order to reveal its true diversity. Industrial potential of the species are also highlighted.

2. MATERIALS AND METHODS

2.1 Sample Collection and Pre-treatment

Cassava peel (CP) waste from CP waste dumpsites and cassava wastewater from

cassava wastewater discharge outlets were collected from major cassava processing centres in Abeokuta, Ogun State, Nigeria. Prior to DNA extraction, samples were pre-treated as follows: 5 g of CP waste was added to 10 ml of sterile physiological solution (0.9%, w/v NaCl) in a beaker containing glass beads and shaken with a vortex mixer (Vortex genie 2, Scientific Industries, USA) at room temperature (30±2°C) for 15 min to dislodge microorganisms from the peels. The resulting mixture designated as pre-treated CP sample, was sieved using cheese cloth to remove debris. The filtrate from CP waste and the cassava wastewater, respectively, were centrifuged for 5 min at 4000 x g using a microcentrifuge (Spectrafuge 24D, Labnet International, USA). In each case, the pellet was discarded while the supernatant was centrifuged for 10 min at 10,000 x g to pellet cells. This was repeated until enough amount of cell was obtained. The pellet was washed in Tris-EDTA (TE) buffer, centrifuged for 10 min at 6000 x g and the supernatant was discarded. This step was repeated for 3 times. The resulting cell pellet was used for fungal DNA extraction.

2.2 Isolation of Fungal Species

For fungal isolation, pre-treated CP waste was used while cassava wastewater was used directly. The pre-treated sample was allowed to settle and the supernatant was kept for use while the sediment was discarded. The supernatant as well as the cassava wastewater were diluted serially to 10^{-8} and 10^{-5} respectively. One milliliter (1 ml) of each sample was pour plated on Sabouraud dextrose agar (SDA) to which chloramphenicol (0.25 mg/l) was added, to suppress bacteria growth, and incubated at 28°C for 48 h for isolation of fungi. Discrete representative colonies were picked from the plates and streaked out on SDA to obtain pure cultures which were transferred to slant and stored at 4°C.

2.3 Characterization and Identification of Fungal Species

Two molecular methods namely, total fungal community DNA and isolates DNA sequence analysis were employed to characterize and identify the fungal species, to ensure that no species was left undetected. The former method is a culture-independent method while the later is a culture-dependent method.

2.4 Total Fungal Community DNA Extraction

Total fungal community DNA was extracted directly from CP waste and cassava wastewater respectively, using the Soil DNA isolation kit (Norgen, Canada), with slight modification of the procedure provided by the manufacturer. Cell pellet already washed in TE buffer was resuspended in 750 µl of lysis solution and transferred to a bead tube using a micropipette. Lysis additive (100 µl) was added and vortexed briefly. The tube was secured horizontally on a flat-bed vortex pad with tape, vortexed for 5 min at maximum speed and then centrifuged for 1 min at 14,000 x g. The supernatant (450 µl) was transferred to a DNAase-free microcentrifuge tube. Binding solution (100 µl) was added, mixed by inverting the tube 5 times, incubated for 5 min on ice and then spun for 1 min at 14,000 x g to pellet any protein and soil particles. The supernatant (450 µl) was transferred into a DNAase-free microcentrifuge tube. An equal volume supernatant (450 µl) of 70% ethanol was added to the lysate collected above, and mixed by vortexing. The clarified lysate and 96 -100% ethanol (600 µl) was applied onto the spin column and centrifuged for 1 min at 14,000 x g. The flow-through was discarded and the spin column reassembled with the collection tube. Wash solution I (500 µl) was added to the column and centrifuged for 1 min at 14,000 x g. The flow-through was also discarded and the spin column reassembled with the collection tube. Wash solution II (500 µl) was added to the column and centrifuged for 1 min at 14,000 x g. This was repeated twice. The column was spun for 2 min in order to thoroughly dry the resin. The collection tube was discarded while the column was placed in a fresh 1.7 ml elution tube. The elution buffer (50 µl) was added to the column, centrifuged for 2 min at 200 x g, followed by a 1 min spin at 14,000 x g to obtain the DNA solution. The purified genomic DNA was stored at -20°C for use.

2.5 Fungal Isolate Genomic DNA Extraction

Total genomic DNA was extracted from fungal isolates using the Soil DNA isolation kit (Norgen, Canada), with slight modification of the procedure provided by the manufacturer. Pure isolates were inoculated in potato dextrose broth (PDB) and incubated at 28°C for 48 h. Cells were harvested in a microcentrifuge tube by

centrifuging for 10 min at 5000 x g and the supernatant discarded. The pellet was subsequently used for lysate preparation, binding of DNA to column, column wash and DNA elution following the protocol reported above for fungal community DNA extraction

2.6 Amplification of the ITS2 Gene

The fungal ITS2 gene sequence from total fungal community and genomic DNA was amplified using the ITS2 primers (ITS86F – GTGAATCATCGAATCTTTGAA and ITS4 – TCCTCCGCTTATTGATATGC). PCR amplification was carried out in a Techne TC-412 Thermal Cycler (Model FTC41H2D, Bibby Scientific Ltd, UK) in a 50 µl reactions containing 25 µl of 2 X PCR Master Mix (Norgen Biotek, Canada), 1.5 µl of template DNA (0.5 µg), 1 µl of both forward and reverse primers (2.5 µM of each) and 21.5 µl of nuclease free water in a PCR tube added in that order. PCR was carried out at an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, 45°C for 30 sec and 72°C for 2 min, and a final extension step at 72°C for 5 min. PCR products (amplicons) were separated by electrophoresis on a 1% agarose TAE gel containing ethidium bromide and visualized by UV transillumination (Foto/UV 15, Model 3-3017, Fotodyne, USA).

2.7 Cloning

Cloning was carried out using the PCR TRAP Cloning System (GenHunter Corporation, USA), following the manufacturer's protocol. Amplicons from total fungal community DNA were spliced into the PCR TRAP Cloning Vector using the T4 DNA ligase. Competent cells were transformed with the recombinant DNA and inoculated in Luria-Bertani (LB)-Tet agar (containing 20 µg/ml of tertacycline). Plasmids containing the gene insert were verified by colony-PCR method using the Lgh and Rgh primers that flank the cloning site of the PCR-TRAP vector. Tetracycline resistant colony was picked with a clean pipette tip and transferred into 50 µl of the colony lysis buffer in a microcentrifuge tube. The tube was incubated in boiling water for 10 min, spun (10,000 x g) at room temperature (30±2°C) for 2 min to pellet the cell debris, and the supernatant (cell lysate) transferred into a clean microcentrifuge tube. The lysate was used immediately for PCR analysis in a PCR reaction containing 20 µl reaction mixture: 10 µl of 2 X PCR Master Mix (Norgen Biotek, Canada), 2.0 µl of colony lysate, 2.0 µl of both Lgh and Rgh primers (2.0 µM of each) and 4.0 µl of nuclease free water in a PCR tube. The PCR mixture was mixed thoroughly and spun down before placing the PCR tube in the Thermocycler. The PCR was carried out using an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR product after colony-PCR was verified to be of correct length (120 bp longer than the original PCR insert before cloning due to the flanking vector sequence being amplified) after electrophoresis on 1% agarose.

2.8 DNA Sequencing and Analysis

PCR products from the total community and genomic DNAs were sequenced with 518F and 800R primers using ABI PRISM Big Dye Terminator cycle sequencer (Macrogen, USA). The gene sequences obtained were compared by aligning the result with sequences in the GenBank using the Basic Local Alignment Search Tool (BLAST) search program at the National Center for Biotechnology Information (NCBI).

2.9 Phylogenetic Analysis

Evolutionary analysis was conducted in Molecular Evolutionary Genetics Analysis (MEGA)5 [24]. The evolutionary history was inferred using the Neighbor-Joining method [25]. The evolutionary distances were computed using the Kimura 2-parameter method [26], and were in the units of the number of transversional substitutions per site.

3. RESULTS AND DISCUSSION

This study reports on molecular characterization and identification of fungal species associated with cassava waste and showcases their industrial potential. Gel electrophoresis profile of amplified ITS2 sequence of total fungal community and fungal isolates DNA from cassava peel and cassava wastewater are presented in plate 1, 2 and 3. PCR amplification of the ITS2 sequences yielded amplicons of about 350 bp confirming that the ITS2 sequence was successfully amplified.

3.1 Fungal Species Associated with Cassava Peel

The distribution of fungi identified in the ITS2 clone library of CP fungal community DNA is presented in Table 1. The result showed that 46 fungal clones belonging to 5 major fungal

domains were detected in the ITS2 clone library of CP. These included *Eurotiomycetes* – *Eurotiales* (24 clones), *Mucormycotina* – *Mucorales* (4 clones), *Sordariomycetes* - *Hypocreales* (2 clones), *Saccharomycetes Saccharomycetales* (14 clones) and unidentified fungi (2 clones). However, 18 fungal isolates including *Eurotiomycetes* – *Eurotiales* (12 isolates), *Mucormycotina* – *Mucorales* (2 isolates), *Sordariomycetes* - *Hypocreales* (1 isolate), and *Saccharomycetes Saccharomycetales* (3 isolates), were detected from the ITS2 gene sequence analysis of fungal isolates DNA from CP (Table 2).

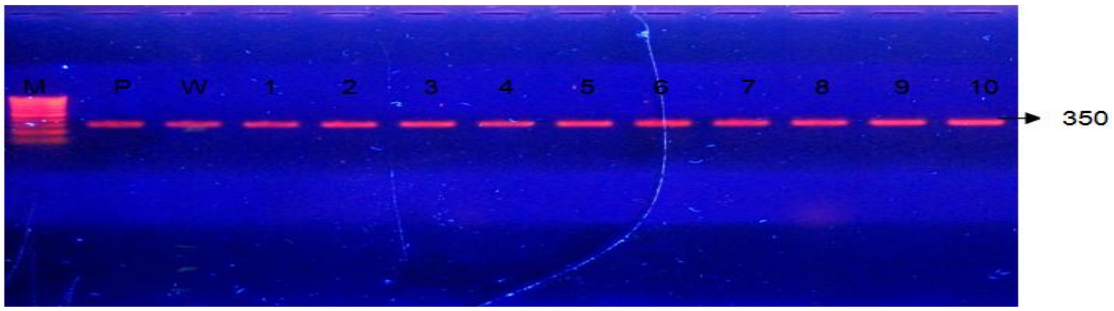

Plate 1. Gel electrophoresis profile of amplified ITS2 sequence of total fungal community DNA and fungal isolates DNA from cassava peel and cassava wastewater. lane M, 1,000 bp DNA ladder; lane P, total fungal community DNA from cassava peel; lane W, total fungal community DNA from cassava wastewater; lane 1 – 10, cassava peel fungal isolates' DNA

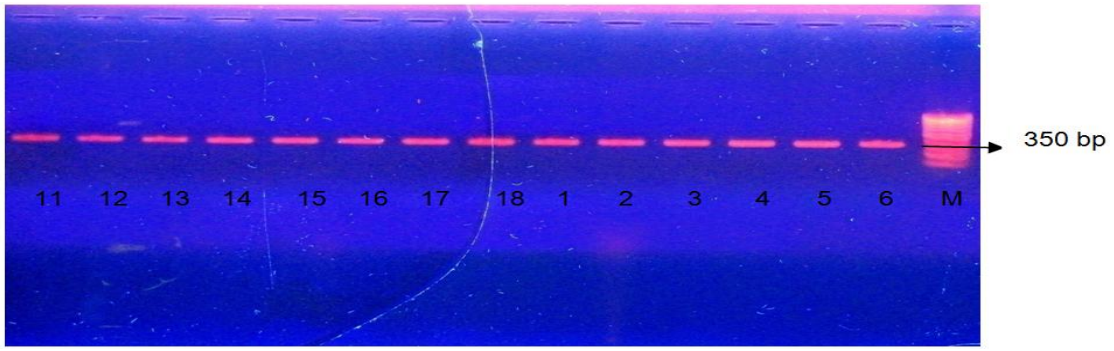

Plate 2. Gel electrophoresis profile of amplified ITS2 sequence of fungal isolates DNA from cassava peel and cassava wastewater. Lane 11 - 18, cassava peel fungal Isolates' DNA; Lane 1 – 6, cassava wastewater fungal isolates' DNA; lane M, 1,000 bp DNA ladder

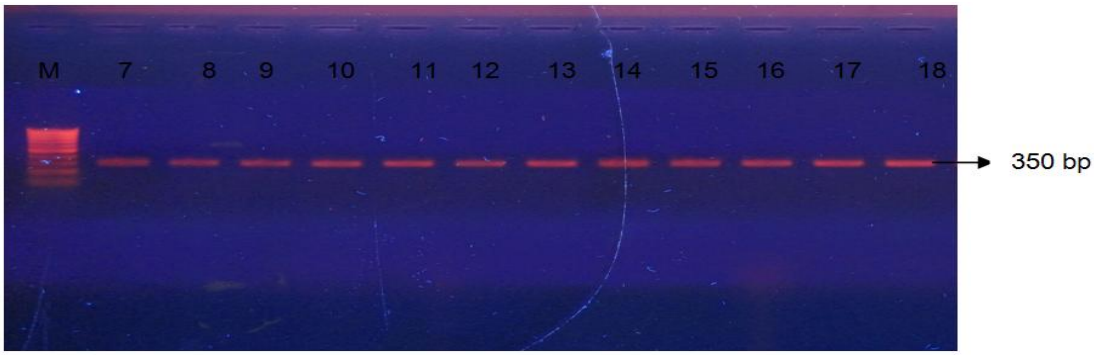

Plate 3. Gel electrophoresis profile of amplified ITS2 sequence of fungal isolates DNA from cassava wastewater. lane 7 - 18, cassava wastewater fungal isolates' DNA lane M, 1,000 bp DNA ladder

In all, a total of 17 fungal species were detected in CP including *Eurotiomycetes – Eurotiales* (6 species), *Mucormycotina – Mucorales* (1 species), *Sordariomycetes - Hypocreales* (1 species), *Saccharomycetes Saccharomycetales* (8 species), and unidentified fungi (1 species). The dominant species was *Aspergillus niger* (15.2%).

A comparison of fungal species identified by total fungal community DNA and isolates DNA sequence analyses revealed as expected that all fungal species detected by isolates' ITS2 DNA sequence analysis were also detected by total fungal community ITS2 DNA sequence analysis. However, *Saccharomyces exigus*, *Zygosaccharomyces bailli*, *Z. florentinus*, *Candida krusei*, *C. gulliermondii* and an unidentified fungus clone B5F31 were detected only by total fungal community ITS2 DNA sequence analysis. It is generally believed that uncultured organisms comprise the vast majority of the microbial world [27].

Most of these species detected in CP have been reported to be associated with cassava fermentation for various food products. Other researchers [11,14] have also reported the dominance of *Aspergillus* species in decaying cassava peels. *Aspergillus niger* has been used to convert cassava wastes by semi-solid fermentation technique to phosphate biofertilizer [14]. *Aspergillus niger* is a filamentous ascomycete fungus that is ubiquitous in the environment particularly the soil. Although it has been implicated in opportunistic infections of humans [28], *A. niger* has industrial relevance. It produces a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. The hydrolytic enzymes are responsible for polysaccharide degradation while the oxidative and extracellular ligninolytic enzymes degrade lignin and open phenyl rings [29]. *Aspergillus niger* is also an important model organism for several important research areas including the study of eukaryotic protein secretion in general, the effects of various environmental factors on suppressing or triggering the export of various biomass degrading enzymes, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology [30]. In addition, it has been reported that microorganisms isolated from fermented pulp juice including *Aspergillus niger* and *Aspergillus flavus* were capable of reducing the levels of cyanogenic glycosides in cassava peels to non-toxic levels and also improving the nutritional value of the peels by increasing the protein content of the peels appreciably [31].

Penicillium expansum, Trichoderma viride and an unidentified fungus similar (98%) to an uncultured fungus clone B5F31 identified in this study were reported for the first time in cassava peel. These new strains were clustered together in the phylogenetic tree (Fig. 1), suggesting that they are closely related to one another.

Penicillium expansum is a known producer of important enzymes such as pectinase, protopectinase, amylase, cellulases, invertase, phospholipase and lipase. However, it is best known as the cause of distinct brown rot of apples in storage and also to lesser extent, implicated in a number of soft fruits [32], where it produces a mycotoxin – patulin.. Almost 100% of *P. expansum* isolates are patulin producers [33,34]. Human exposure by ingestion to patulin-contaminated products and can lead to serious health problems. Animals fed with *P. expansum* contaminated cassava peel can therefore be exposed to serious health hazard.

The use of biological control agents, particularly the ones also isolated from cassava peel in this study, is promising for the treatment of cassava peel for animal feed, following reports that *Panteoa agglomerans* [34], *Pichia anomala* [35] and *Rhodotorula glutinis* [36] are effective in the control of *Penicillium expansum.*

Independent phylogenetic tree was constructed for each genus of fungi identified in this study in order to establish the relatedness between species of fungi identified in this study and closely related species from the Genebank. Results showed that except for *Aspergillus flavus* (Fig. 2), other fungal species including *Aspergillus niger* (Fig. 3), *Penicillium expansum* (Fig. 4) and *Rhizopus oryzae* (Fig. 5) were closely related to sequences of species from the Genebank.

Fig. 3 showed that the *Aspergillus niger* isolated from cassava peel was closely related to *Aspergillus niger* strain AG68 isolated from a sugar factory in Thailand [37], while the *Penicillium expansum* isolated from cassava peel was closely related to *Penicillium expansum* strain isolated from decayed kiwifruit (*Actinidia arguta*) in China [38]. On the other hand, the *Rhizopus oryzae* isolated from cassava peel was closely related to *Rhizopus oryzae* strain 2013-028 isolated from sugar beet root rot in Germany.

Table 1. Distribution of fungi identified in the ITS2 clone library from cassava waste fungal community

Clone identity/fungal domain	Accession number	% identity	No of clones (relative abundance)	
			P	W
Eurotiomycetes – Eurotiales				
Aspergillus niger	JN862797.1	100	7 (15.2%)	2 (3.5%)
Aspergillus flavus	JX231008.1	96	2 (4.3%)	-
Aspergillus fumigates	JX231009.1	100	5 (10.8%)	-
Aspergillus oryzae	DQ335983.1	99	3 (6.5%)	-
Aspergillus tamari	JX110981.1	98	4 (8.6%)	-
Penicillium expansum	HQ873319.1	100	3 (6.5%)	1 (1.7%)
Mucormycotina – Mucorales				
Rhizopus oryzae	AB181305.1	100	4 (8.6%)	-
Sordariomycetes –Hypocreales				
Trichoderma viride	AJ223773.1	98	2 (4.3%)	-
Saccharomycetes-Saccharomycetales				
Geotrichum candidum	FN376415.1	97	-	3 (5.2%)
Geotrichum fragrans	AB499021.1	95	-	2 (3.5%))
Saccharomyces cerevisiae	AM900397.1	100	4 (8.6%)	5 (8.7%)
Saccharomyces exigus	AJ229063.1	99	2 (4.3%)	1 (1.7%)
Zygosaccharomyces bailli	X84641.1	99	1 (2.1%)	1 (1.7%)
Zygosaccharomyces florentinus	Z48363.1	98	1 (2.1%)	1 (1.7%)
Candida tropicalis	FJ011533.1	100	2 (4.3%)	4 (7.0%)
Candida krusei	AB054034.1	100	2 (4.3%)	5 (8.7%)
Candida rugopelliculosa	FR819719.1	92	-	1 (1.7%)
Candida inconspicua	EU315758.1	100	-	3(5.2%)
Candida maris	D43812.1	99	-	1 (1.7%)
Candida glabrata	U70498.1	97	-	1 (1.7%)
Candida humilus	HE572527.1	100	-	2 (3.5%)
Candida ethanolica	FJ662418.1	98	-	1 (1.7%)
Candida valida	AB053233.1	100	-	1 (1.7%)
Candida holmii	Z75740.1	93	-	2 (3.5%)
Candida utilus	U70504.1	100	1 (2.1%)	3 (5.2%)
Pichia rhodanensis	D83149.1	100	-	2 (3.5%)
Pichia anomala	FJ605112.1	98	-	2 (3.5%)
Pichia gulliermondii	DQ680842.1	96	1 (2.1%)	1 (1.7%)
Pichia kudriavzevii	JX174414.1	95	-	2 (3.5%)
Galactomyces geotrichum	JN974290.1	100	-	4 (7.0%)
Issatchenkia scutulata	D89349.1	100	-	4 (7.0%)
Hanseniaspora guilliermondii	AJ512433.1	97	-	1 (1.7%)
Tremellomycetes-Tremellales				
Trichosporon asahii	JQ863250.1	100	-	1 (1.7%)
Uncultured fungus				
Uncultured fungus clone B5F31	GU073031.1	98	2 (4.3%)	-

CP = Cassava peel, W = Cassava wastewater

Table 2. Distribution of fungi identified in the ITS2 fragment analysis of cassava waste fungal isolates' DNA

Isolate identity/ Bacteria domain	Accession number	% identity	No of isolates (relative abundance)	
			P	W
Eurotiomycetes – Eurotiales				
Aspergillus niger	JN862797.1	100	4 (22.2%)	-
Aspergillus flavus	JX231008.1	96	1 (5.5%)	-
Aspergillus fumigates	JX231009.1	100	3 (16.6%)	-
Aspergillus oryzae	DQ335983.1	99	1 (5.5%)	-
Aspergillus tamari	JX110981.1	98	1 (5.5%)	-
Penicillium expansum	HQ873319.1	100	2 (11.1%)	-
Mucormycotina – Mucorales				
Rhizopus oryzae	JX120698.1	98	2 (11.1%)	-
Sordariomycetes – Hypocreales				
Trichoderma viride	AJ230673.1	100	1 (5.5%)	-
Saccharomycetes Saccharomycetales				
Geotrichum candidum	FN376415.1	91	-	1 (5.5%)
Geotrichum fragrans	GQ222355.1	94	-	1 (5.5%)
Saccharomyces cerevisiae	AM900397.1	100	2 (11.1%)	4 (22.2%)
Saccharomyces exigus	AJ229063.1	100	-	1 (5.5%)
Candida tropicalis	FJ011533.1	99	1 (5.5%)	2 (11.1%)
Candida krusei	AB054034.1	98	-	3 (16.6%)
Candida inconspicua	EU315758.1	97	-	1 (5.5%)
Candida utilus	U70504.1	100	-	2 (11.1%)
Issatchenkia scutulata	AJ512433.1	97	-	2 (11.1%)
Galactomyces geotrichum	JN974290.1	100	-	1 (5.5%)

CP = cassava peel, W = cassava wastewater

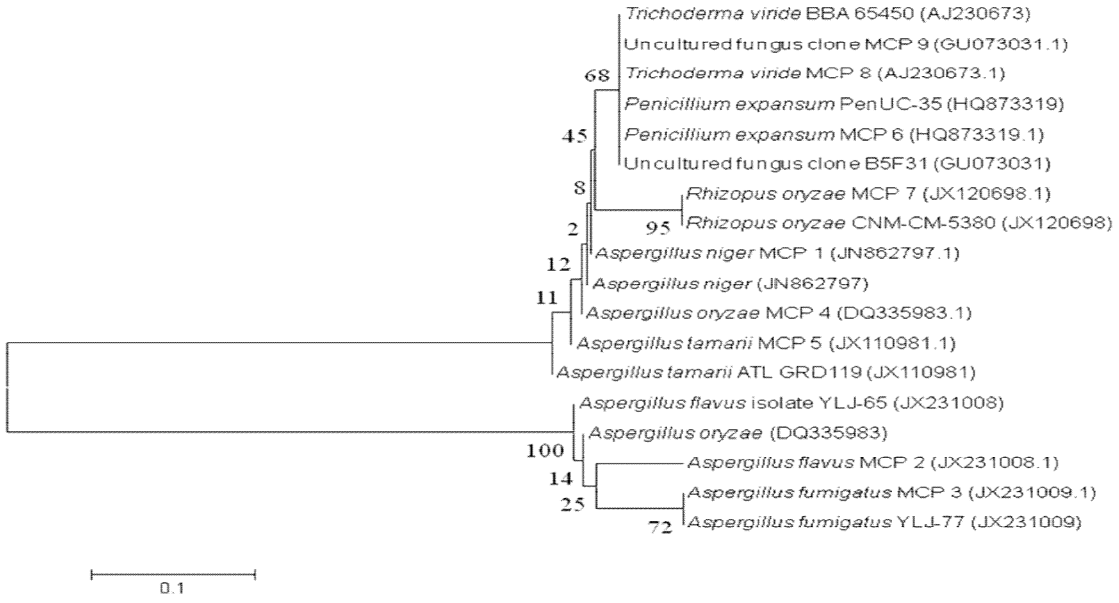

Fig. 1. Phylogenetic tree showing grouping patterns of ITS2 sequences from mould isolated from cassava peel. MCP, mould isolated from cassava peel in this study; the rest are close relatives from database. See materials and methods for details of phylogenetic reconstruction

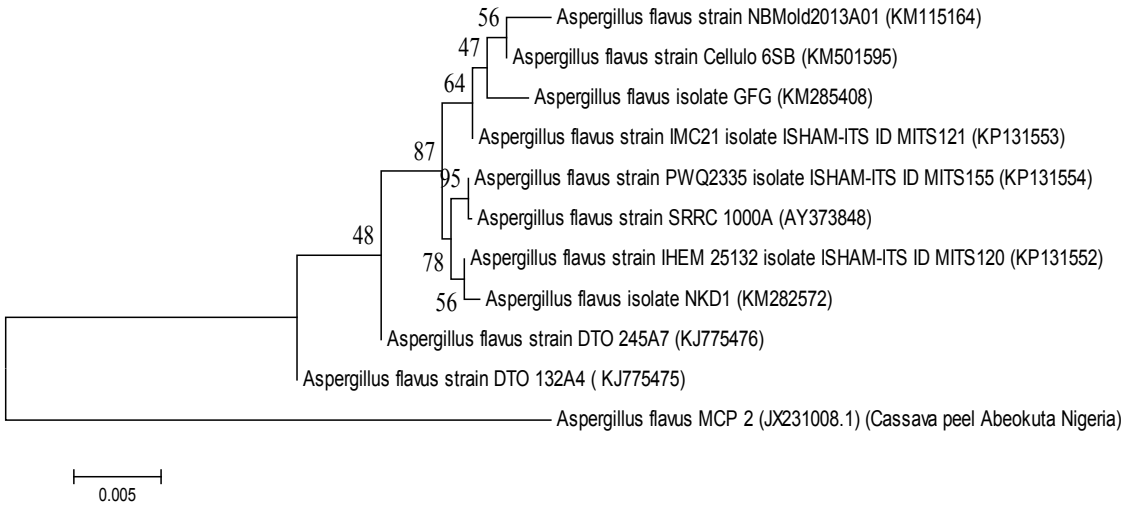

Fig. 2. Phylogenetic tree showing grouping patterns of ITS2 sequences from *Aspergillus flavus* isolated from cassava peel and sequences of closely related species from the genebank. MCP, *Aspergillus flavus* isolated from cassava peel in this study

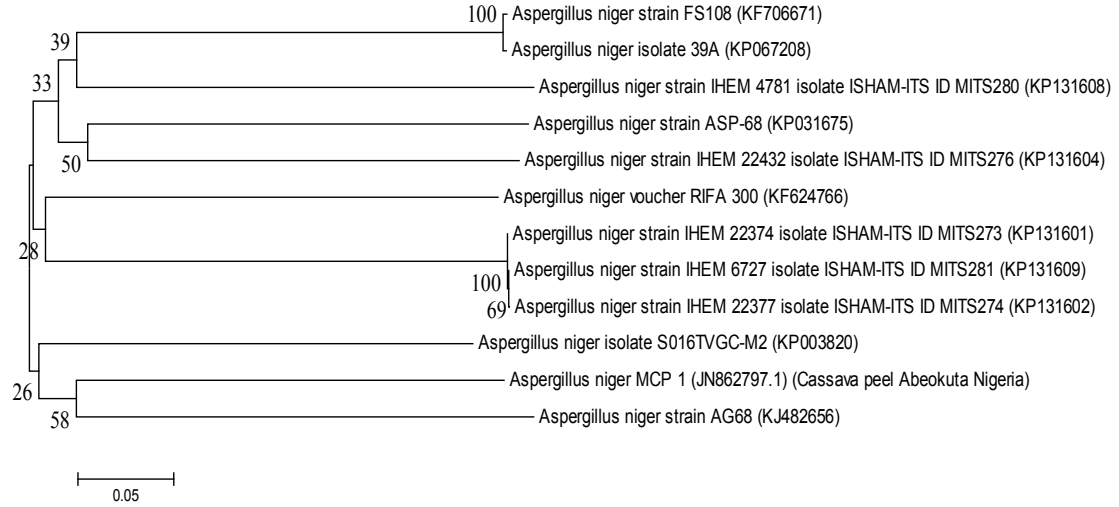

Fig. 3. Phylogenetic tree showing grouping patterns of ITS2 sequences from *Aspergillus niger* isolated from cassava peel and sequences of closely related species from the genebank. MCP, *Aspergillus niger* isolated from cassava peel in this study

3.2 Microorganisms Associated with Cassava Wastewater

The distribution of fungi identified from the ITS2 clone library of cassava wastewater fungal community DNA is also presented in Table 1. The result showed that 57 fungal clones were detected in cassava wastewater. The clones belonged to 3 major fungal domains namely *Eurotiomycetes – Eurotiales* (3 clones), *Saccharomycetes Saccharomycetales* (53 clones) and *Tremellomycetes-Tremellales* (1 clone). However, only 18 fungal isolates belonging to *Saccharomycetes-Saccharomycetales* domain were obtained from cassava wastewater (Table 2). In all, a total of 27 fungal species were detected in cassava wastewater belonging to *Eurotiomycetes – Eurotiales* (2 species), *Saccharomycetes Saccharomycetales* (24 species) *Tremellomycetes-Tremellales* (1 species). The dominant species were *Saccharomyces cerevisiae* (8.7%) and *Candida krusei* (8.7%).

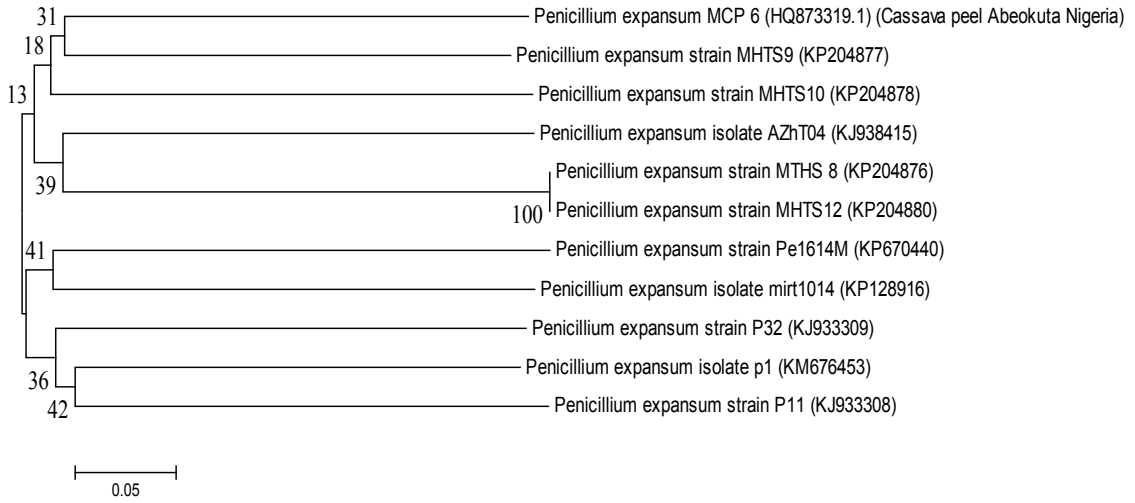

Fig. 4. Phylogenetic tree showing grouping patterns of ITS2 sequences from *Penicillium expansum* isolated from cassava peel and sequences of closely related species from the genebank. MCP, *Penicillium expansum* isolated from cassava peel in this study

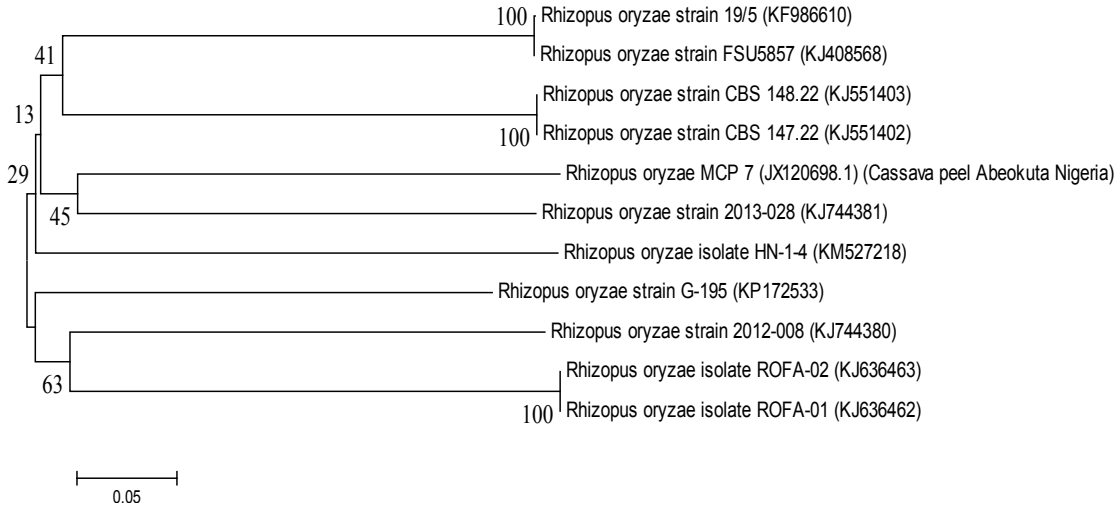

Fig. 5. Phylogenetic tree showing grouping patterns of ITS2 sequences from *Rhizopus oryzae* isolated from cassava peel and sequences of closely related species from the genebank. MCP, *Rhizopus* oryzae isolated from cassava peel in this study

A comparison of fungal species identified by total fungal community DNA and fungal isolates DNA analyses showed that all organisms detected by isolate DNA analysis were also detected by total fungal community DNA analysis. Nevertheless, *Aspergillus niger*, *Penicillium expansum*, *Zygosaccharomyces bailli*, *Z. florentinus*, *Candida rugopelliculosa*, *C. gulliermondi, C. maris, C. glabrata, C. humilus, C. ethanolica, C. holmii*, *Pichia anomala, P. rhodanensis*, *P. kudriavzevii, Issatchenkia scutulata, Hanseniaspora guilliermondii* and *Trichosporon asahii* were detected only by total fungal community DNA analysis.

In principle, all species detected by isolates' DNA analysis should have been found in the clone library. However, this was not the case as few isolates detected by isolates' DNA analysis were not found in the clone library, demonstrating that cultivation-dependent and independent methods are complementary. As expected, more organisms were detected by total microbial community DNA analysis than by isolates DNA

analysis. This is also in line with the generally accepted view that plate culturing techniques reveal only a little portion of the true microbial population in a natural ecosystem.

Majority (47%) of the fungal species found in cassava wastewater were yeasts. Previous studies have reported the presence of different species of yeasts in cassava wastewater [39,40]. Likewise, different authors have reported the dominance of different species of yeast in cassava fermentation. Earlier studies reported *Geotrichum, Saccharomyces* and *Brettanomyces* as the predominant genera of yeasts in cassava fermentations [41,42]. However, it was later reported that *Galactomyces geothricum* and *Issatchenkia* spp. were the prevalent yeasts [43]. In "lafun" fermentation, *Saccharomyces cerevisiae* was reported as the predominant yeast species [44] while *Candida tropicalis* was the predominant species in "Attieke" [45]. The inconsistencies in the dominance of species could result from the fact that the cassava wastewater used in the different studies was obtained from different food products.

Trichosporon asahii identified in this study has been reported, previously, but at low incidence (<5%) during cassava fermentation for "lafun" production [46]. Phylogenetic analysis (result not presented) showed that *Saccharomyces cerevisiae* is not closely related to *Saccharomyces exiguus* in both cassava peel and cassava wastewater.

Generally, yeasts have been identified as the second predominant microorganisms involved in cassava fermentation after lactic acid bacteria [47,48]. It has been has shown that different strains of *Candida* species participated in the traditional fermentation of cassava [49]. *Candida* species are important, especially, for their co-metabolism ability with lactic acid bacteria, a parameter reported as desirable for adequate fermentation of cassava [50]. According to another researcher [51], yeast seems to have an important role in the survival and in the activity of lactic acid bacteria during the cassava fermentation process, as they are involved in the cassava starch hydrolysis into simple sugars, which are converted into organic acids by the lactic acid bacteria.

This study has revealed the presence of fungal species such as *Zygosaccharomyces bailli*, *Geotrichum fragrans*, *Z. florentinus, Candida. maris, C. humilus, C. ethanolica, C. holmii*, *Pichia anomala* and *Issatchenkia scutulata* in cassava wastewater, which were not reported in previous studies. Some of these species possess many interesting technological properties which hold promises for industrial applications. It has been reported that under cassava wastewater cultivation, *Goetrichum fragrans* produced fruit aroma volatile compounds such as ester, alcohol and acid-like fruit aromas: 1-butanol, 3-methyl 1-butanol (isoamylic alcohol), 2-methyl 1-butanol, 1-3 butanodiol and phenylethanol; ethyl acetate, ethyl propionate, 2-methyl ethyl propionate and 2-methyl propanoic [19]. This aerobic microorganism isolated from cassava wastewater also had cyanide resistant respiration which enhanced its ability to reduce the COD value of the cassava wastewater by 40% [19].

Similarly, *Geotrichum candidum* (anamorph; *Galactomyces geotrichum*) has demonstrated potential in food applications, particularly in the dairy industry, where it plays functional roles in cheese making. On some cheeses, like St. Marcellin, it is responsible for the appearance of the cheese, imparting a uniform, white, velvety coat to the surface [52]. On soft cheeses, such as Camembert, and semihard cheeses, such as St. Nectaire and Reblochon, the biochemical attributes of *G. candidum* impact the course of cheese ripening [53]. In addition, *G. candidum* reduces bitterness in industrial Camemberts [54]. Metabolites produced by *G. candidum* can also inhibit *Listeria monocytogenes* [55], which can cause food poisoning. Thus, the *G. candidum* identified in this study could be investigated as a possible source of cheese ripening enzymes.

Also, Pichia anomala has been reported as a good flavouring agent, and has applications in producing food bioemulsifiers [56]. It has also been reported to produce biosurfactant. A thermotolerant strain which was isolated from "Khao Mhak", a Thai fermented food, displayed surface lowering capacities and has attracted much interest since it is regarded as a source of protein and vitamin [57]. In addition, *P. anomala* has biotechnological significance as it is amenable to genetic analysis because of its heterothallic nature with known strains of stable opposite mating types [58].

Different strains of *Pichia guilliermondii* have been successfully applied to control postharvest pathogens on a number of fruits and vegetables, such as *Penicillium digitatum* on grapefruits, *Penicillum expansum* on apples and *Rhizopus nigricans* on tomato fruit [59,60] and

Colletotrichum capsici on chillifruit [61]. Generally, competition for nutrients and space is considered the main mode of action of yeast biocontrol agents [62].

4. CONCLUSION

This study has shown that cassava waste, on account of its rich fungal diversity, is an important microbial resource. Most of the species identified have great industrial potential. The results indicated *Aspergillus niger* as the dominant fungal species in CP waste while *Saccharomyces cerevisiae* and *Candida krusei* are the dominant species in cassava wastewater. The use of molecular identification methods has further revealed novel fungal species hitherto not reported in cassava waste, thus demonstrating the importance and the need to adopt this new approach in studying microbial diversity in food and related samples.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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