

Original Research Article

Microbiological Quality of High Quality Cassava Flour Produced from Selected Varieties of Low Postharvest Physiological Deterioration Cassava (*Manihot esculenta Crantz*)

Abstract

The microbial properties of food are important quality characteristics of food materials as it relates directly with the health of the consumer. This study examined the microbiological properties of high quality cassava flours produced from low postharvest physiological deterioration (PPD) cassava. Wholesome four varieties of yellow-fleshed Low PPD cassava and one variety of high PPD cassava were, peeled, washed, grated, pressed, pulverized, flash dried at 120 °C for 8 minutes, milled with cyclone hammer mill fitted with a screen of 250 µm aperture size, cooled and packed into high density polyethylene bag. The high quality cassava flours produced were analyzed for total viable fungal and bacteria count, fungi isolated were further characterized and identified using molecular methods. Data obtained were subjected to one way analysis of variance (ANOVA) using SPSS 25.0 and significant means were separated applying Duncan multiple range test. The mold count ranged from 1.50±0.71 - 2.50±0.71 cfu/ml, with flour produced from IITA-TMS-IBA-011371 and TMEB 419 having the lowest count while the highest was recorded in IITA-TMS-IBA-011368, respectively. The yeast count ranged from 1.00±0.00 - 2.0±0.00 cfu/ml, with flours produced from IITA-TMS-IBA-070593 and IITA-TMS-IBA-011371 having the lowest count while the highest was recorded in flour from IITA-TMS-IBA-011368, respectively. The total viable bacterial and fungal count (microbiological quality) of the flours prepared from IITA-TMS-IBA-011368, IITA-TMS-IBA-070593, IITA-TMS-IBA-011412, IITA-TMS-IBA-011371 and TMEB 419 cassava varieties were within the permissible limit of the microbial load of food allowed for human consumption according to the Standard Organization of Nigeria and CODEX.

Keywords: Low postharvest physiological deterioration, high quality cassava flour, microbiological quality, microbial load, permissible limit

1. Introduction

The quest to add value and fortify a food crop such as cassava roots with essential nutrients such as protein and pro-vitamin A so as to enhance its nutritive value for domestic and industrial application has led to immense research efforts by scientists. Cassava (*Manihot esculenta Crantz*) is a crop that people consume in the tropical regions [1]. Notably, cassava production rose from 132,200,764 tons to exactly 157,271,697 tons in 2010 to 2016, respectively, which was about 18.9% [2]. Also, the production share of cassava by region: Africa (60.7%), Americas (9.9%), Asia (29.3%) and Oceania (0.1%) from 2017 to 2018[3]. The total production of cassava in Africa in 2018 was 169,673,737; Nigeria share in this production was 50,485,047 [3]. Cassava is known to suffer a physiological disorder that takes

effect in about 24-72 hours after the roots have been harvested which impairs its palatability even though it has propensity for increased productivity [4].

Cassava (*Manihot esculenta* Crantz) characteristically has short postharvest life and this is as a result of a phenomenon known as “postharvest physiological deterioration (PPD)”, which consequently reduce its market potential and makes the stakeholders in its value chain loose enthusiasm. This challenge requires that cassava is quickly transported to the point of processing and this has led to the screening of cassava varieties for extended shelf life, improvement in the nutritional composition and yield, thereby overcoming the major challenge confronting cassava value chain.

In countries and regions where wheat grain production is not supported due to unfavorable soil and climatic conditions required for optimum growth of wheat, such countries would largely depend on importation of wheat. In Nigeria, crops such as cassava, cowpea etc. and their flour has been explored and prospected for use in replacing wheat flour up to 30% so as to reduce the over-dependence on wheat importation for use as food and industrial application [5, 6]. Partial substitution of wheat with high quality cassava flour (HQCF) to make composite flour for baking purpose had attracted the attention of the Nigerian Government, which necessitate that Nigerian flour mills should replace wheat flour with cassava flour up to 10%.

Development (screening) of some low PPD cassava varieties aimed at extending the shelf life of cassava root from two days (48hrs) to 5 days (120hrs) and enhancement of nutritional value with vitamin A or β -carotene is very important [7]. Authors found out that a difference in variety goes a long way to affect the quality parameters such as physical, functional and chemical properties of high quality cassava flour [7]. This consequently implies that the flour making properties of such roots and their food uses would also vary [7, 8].

The microbial properties of food are important quality characteristics of food materials as it relates directly with the health of the consumer. This study examined the microbiological properties of high quality cassava flours produced from low postharvest physiological deterioration (PPD) cassava varieties

2. Materials and Methods

2.1 Materials

The materials used for this includes flours from five (5) varieties. Four varieties screened for low postharvest physiological deterioration are: (IITA-TMS-IBA011368, IITA-TMS-IBA070596, IITA-TMS-IBA011412, IITA-TMS-IBA011371) and one variety of high postharvest physiological deterioration (TMEB419) from International Institute of Tropical Agriculture (IITA), Ibadan and refined wheat flour (Nigerian Eagle Flour Mills, Ibadan).

2.2 Preparation of high quality cassava flour (HQCF)

The method reported by [9, 10] was used. Wholesome cassava roots obtained from the International Institute of Tropical Agriculture (IITA) were peeled using stainless steel knives and washed with clean water in a plastic bowl. The washed roots were then grated and

pressed with screw jack press to dewater the mash. The dewatered mash was pulverized and subsequently dried with the aid of flash dryer at 120 °C for 8 min. The flash dried cassava mash was then milled into flour with the aid of cyclone hammer mill fitted with a screen of 250 µm aperture size, cooled and packed into high density polyethylene bag. The sieved cassava flour was allowed to cool and packaged into high density polyethylene bag and subsequently sealed for further analysis.

2.3 Culture Media for Isolation

The isolated microorganisms were cultured using Nutrient Agar (NA) for the isolation of bacteria while Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were used for the isolation of yeasts and moulds. The media were prepared according to the manufacturer's instruction.

2.3.1 Isolation Technique

The isolation of the constituting fungal colonies in the flour samples were carried by doing a 10-fold serial dilution of the sample. 1 gramme of the flour sample was put into a 9ml of peptone water, from this mixture 1ml of the aliquot was then taken and poured into another 9ml of peptone, this process was then repeated for 6 dilution, then 1 ml of the 10^{-1} , 10^{-3} and 10^{-5} were plated on Potatoes Dextrose Agar (PDA) using the pour plate method, the plates were then incubated at 27°C for 3-5 days however, 25µg of chloramphenicol was added to the agar medium before autoclaving. A plate count of emanating moulds and yeasts was carried out after 4 days of incubation, then the isolation of distinct colonies were done by using a flamed inoculating needle to transfer these colonies into freshly prepared agar medium. Then incubation follows at 27°C for 4 days.

2.3.2 Maintenance and preservation of cultures

The pure cultures of isolated yeasts and moulds were maintained on PDA slants which were kept as stock cultures under refrigeration temperature (4°C) for subsequent use and sub-cultured at 3-4 weeks' interval.

2.3.3 Identification of Moulds and yeast

2.3.4 Microscopy

Microscopic examination was carried using the method described by Fawole and Oso [11], by first growing the fungi on Potato Dextrose Agar (PDA), when colonies of the fungi were formed, the mycelia of the fungi was picked with the aid of an inoculating pin which has been flamed red hot and allow to cool, the picked fungal mycelia was placed on a glass slide, 1-2 drops of lactophenol in cotton blue was dropped on to the slide and then the fungi mycelia was teased with the inoculating needle in order to macerate the mycelia for clear view on the microscope, a cover slip was placed gently on the slide to avoid bubble formation. The slide was then viewed under a light microscope at different magnifications, the characteristics observed on the microscope were recorded. The identity of the isolate was partially discovered by comparing their microscopic morphology with the ones found in fungal atlas [11].

2.3.5 Molecular Identification

The five fungal isolates were further identified using molecular methods after microscopic identification [11].

2.3.6 Isolation of Genomic DNA

The genomic DNA of the selected fungi were isolated using NORGEN Fungi DNA Isolation Kit product no 26200 following the manufacturer's instructions.

2.3.7 PCR Amplification of rRNA Genes

PCR amplification was done to confirm the identity of the fungal strain, the small sub-unit rRNA genes (complete ITS) were amplified from the genomic DNA with ITS4: 5-TCCTCCGCTTATTGATATGC-3 and ITS5: 5-GGAAGTAAAAGTCGTAACAAGG-3. The DNA was subjected to the following cocktail mix and condition for the PCR: 10× PCR buffer, 1.0µL; 25mM MgCl₂, 1.0µL; 5 pMol forward primer, 0.5µL; 5pMol reverse primer, 0.5µL; DMSO, 1.0µL; 2.5MmDNTPs, 0.8µL; Taq 5u/ul, 0.1µL; 10ng/µl DNA, 2.0µL; H₂O, 3.1µL using the PCR conditions; 94°c for 5min (Initial denaturation), 94°c for 30sec (Denaturation), 54°c for 35 secs (Annealing temperature), 72°c for 45 sec (Extension), 36 (No. of cycles), 72°c for 7min (Final extension), 10°c for ∞ (Holding temperature). The amplicon from the reaction was loaded on 1.5% agarose gel. The ladder used is 1kb plus ladder from Invitrogen. The expected base pair of the amplicon is around 850bp.

2.3.8 Purification of the PCR Product

The PCR product (540bp) was purified from contaminating products by adding 2vol (20ul) of absolute ethanol to the PCR product followed by incubation at room temperature for 15minutes, It was then spun down at 10000rpm for 15minutes, the supernatant was decanted. Two volume (40ul) of 70% ethanol was added after spinning at 10000rpm for 15minutes and supernatant was decanted, the product was air dried and about 10ul of ultrapure water was added. Finally, amplicon on was checked on 1.5% agarose.

2.3.9 Nucleotide Sequencing

Sequencing pattern – The PCR product was used for another PCR reaction that is now sequencing reaction. The program, process and quantities of everything used was according to BigDye® Terminator v3.1 Cycle Sequencing Kit. After which the sequencing reaction was purified using protocol of BigDye® Terminator v3.1 Cycle Sequencing Kit. The primer used in all sequencing reactions was ITS4: 5-TCCTCCGCTTATTGATATGC-3. Sequencing was then performed using an automated sequencer (ABI genetic analyzer model 3500, Applied Biosystems, USA). The product from the purification process was loaded on the 3130xl genetic analyzer from Applied Biosystems to give the sequences.

2.3.10 Blasting Analysis

Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov/BLASTN/). The nucleotide sequence was entered into the NCBI taxonomy database and the result of similar sequences brought forward and similarities with higher percentage was selected.

2.4 Statistical analysis

The total viable fungal count characteristic data obtained were subjected to one way analysis of variance (ANOVA) using SPSS 25.0 version (SPSS Inc. USA) and significant means were separated applying Duncan's multiple range test.

3.0 Results and Discussion

The results of the total mold count and their frequency of occurrence in the flours produced from different varieties of the low PPD cassava are presented in Table 1. The mold counts were significantly different ($P=.05$) from each other based on varietal differences. The mold count ranged from 1.50 ± 0.71 - 2.50 ± 0.71 cfu/ml, with flour produced from IITA-TMS-IBA-011371 and TMEB 419 having the lowest count while the highest was recorded in IITA-TMS-IBA-011368, respectively. The highest frequency of occurrence (26.32 %) was found in flour prepared from IITA-TMS-IBA-011368 cassava while the lowest (15.79 %) was recorded in IITA-TMS-IBA-011371 and TMEB 418 cassava.

In this study, the presence of *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus spp.* present on cassava root could be due to improper handling during harvesting and processing. Some of these fungi especially *Aspergillus spp.* are able to survive in situations where free water is not available or low moisture condition. The ability of fungi to grow at low moisture levels is of particular importance in their role as plant pathogens i.e. capable of causing diseases to cassava roots [12], and production of spores known as conidia, which are abundantly disseminated into the air [13]. The detection of mold in the flours could be attributed to post production contamination from the environment, temperature, increased water activity and pH. The presence of *Rhizopus stolonifer* has been reported to cause an elevation of pH beyond the safety value of 4.6 and makes environment more conducive for the growth of pathogenic bacteria [14].

Table 1: Total mold count from the low or delayed postharvest physiological deterioration cassava flours

Cassava Variety	Count $\times 10^{-2}$ (cfu/ml)	Frequency of Occurrence (%)
IITA-TMS-IBA-011368	2.50 ± 0.71^a	15.79
IITA-TMS-IBA-070593	2.00 ± 0.00^a	21.05
IITA-TMS-IBA-011412	2.00 ± 0.00^a	21.05
IITA-TMS-IBA-011371	1.50 ± 0.71^a	26.32
TMEB 419	1.50 ± 0.71^a	15.79

Results are expressed as mean \pm standard deviation. Mean values followed by different superscript letter within a column are significantly different ($P=.05$).

Aspergillus and *Rhizopus* species were recorded, but their effects might be non-virulent on cassava root, so they could be secondary pathogens after primary pathogen infection [15]. *Aspergillus niger* can also be used in waste management and bio transformation [15]. According to America Food and Drug Agency, *A. niger* is safe for industrial, medical and agricultural use. It is readily available and has the ability to produce enzymes such as amylases, lipases, celluloses, xylanases and proteases [15].

The total yeast count and their frequency of occurrence in the flours prepared from different varieties of the low PPD cassava are presented in Table 2. The yeast counts were significantly different ($P=0.05$) from each other based on varietal differences. The yeast count ranged from 1.00 ± 0.00 - 2.0 ± 0.00 cfu/ml, with flours produced from IITA-TMS-IBA-070593 and IITA-TMS-IBA-011371 having the lowest count while the highest was recorded in flour from IITA-TMS-IBA-011368, respectively. The highest frequency of occurrence (28.57 %) was found in flour prepared from IITA-TMS-IBA-011368 cassava while the lowest (14.29 %) was recorded in IITA-TMS-IBA-070593 and IITA-TMS-IBA-011371 cassava.

The presence of *Saccharomyces*, *Penicillium* could be due to contamination of the raw materials by air, soil, dust, insects and man and to a less extent on partial fermentation of cassava root, simply because the cassava were processed into flour immediately after harvest in less than five (5) hours of postharvest. In nature, yeasts are found in all habitats where fermentable sugar rich liquids, extracts, and secretion are available for example, in the nectar of flowers and on fruits and leaves [15]. They may also cause tremors, coagulopathy and enteritis [16]. One of the challenges in cassava production is postharvest losses which has been shown to be due to infection by pathogenic fungi of cassava tuber rots like *P. expansum* and *Rhizopus stolonifer* as reported from study conducted in Kogi State [16].

Cassava roots are prone to mechanical damages such as abrasion, bruising, cut etc., during harvest which trigger the generation of reactive oxygen species (ROS). Carotenoids with their antioxidant activity protect cells and tissues from ROS induced oxidative damage. Cassava varieties with elevated β -carotene content had extended shelf-life [17]. PPD depends on the genotypic as well as the environmental conditions and is followed by microbial infection [18]. Flour produced from IITA-TMS-IBA011371 had the highest total carotenoid content of (3.14 μ g/g) whereas flour produced from IITA-TMS-IBA011368 had TC (0.74 \pm 0.01) value which was relatively low. Therefore, the lowest and highest mold count were recorded in IITA-TMS-IBA011371 and IITA-TMS-IBA011368, respectively, and this observation may not be unconnected with the fact that cassava varieties with relatively high β -carotene content exhibits antioxidant activity that protects cells and tissues from ROS induced oxidative damage, reducing microbial activities which consequently would extend the shelf-life of these low PPD cassava flours as noted by Chávez *et al.* [19]. The same phenomenon was observed in the yeast count of the flours.

Table 2: Total yeast count from the low or delayed postharvest physiological deterioration cassava flours

Cassava Variety	Count $\times 10^{-2}$ (cfu/ml)	Frequency of Occurrence (%)
IITA-TMS-IBA-011368	2.0 \pm 0.00 ^a	28.57

IITA-TMS-IBA-070593	1.00±0.00 ^a	14.29
IITA-TMS-IBA-011412	1.5±0.71 ^a	21.43
IITA-TMS-IBA-011371	1.00±0.00 ^a	14.29
TMEB 419	1.50±0.71	21.43

Results are expressed as mean ± standard deviation. Mean values followed by different superscript letter within a column are significantly different ($P=.05$).

The different organisms isolated and identified from flours prepared from different varieties of low PPD cassava are presented in Table 4. *Aspergillus flavus* and *Saccharomyces cerevisiae* were isolated from IITA-TMS-IBA011368 cassava flours while *Rhizopus stolonifer*, and *Saccharomyces cerevisiae* were isolated from IITA-TMS-IBA070593 cassava flour. *Aspergillus flavus* and *Rhizopus microsporium* were isolated from IITA-TMS-IBA011412 cassava flour while *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *Rhizopus*

stolonifer and *Rhizopus microsporum* were isolated from IITA-TMS-IBA011371 cassava flour. *Saccharomyces cerevisiae* and *Aspergillus oryzae* were both isolated from TMEB419 cassava flour which was the only high postharvest physiological deterioration cassava (PPD) cassava among the varieties that were examined. The purified PCR products of yeast samples isolated are presented in Figure 1.

Generally, the microbiological qualities of the flours produced from four (4) low PPD cassava varieties (IITA-TMS-IBA-011368, IITA-TMS-IBA-070593, IITA-TMS-IBA-011412, IITA-TMS-IBA-011371) and one (1) high PPD cassava variety (TMEB 419) were within the permissible limit of the microbial load of food allowed for human consumption which according to the Standard Organization of Nigeria [20] should not exceed 4.00 log cfu g⁻¹ for solid food samples; and within the limit for total viable bacteria count (TVBC) of 5.00 log cfu g⁻¹, total viable fungal count (TVFC) of 3.00 log set by CODEX for cassava flours [21].

Table 3: The total carotenoid content of the selected varieties of low PPD cassava flours

Cassava variety	Total carotenoid (µg/g)
IITA-TMS-IBA-011368	0.74±0.01 ^b
IITA-TMS-IBA-070593	2.82±0.01 ^c
IITA-TMS-IBA-011412	0.75±0.01 ^b
IITA-TMS-IBA-011371	3.14±0.06 ^d
TMEB 419	0.34±0.01 ^a

Results are expressed as mean + standard deviation. Mean values followed by different superscript letter within a column are significantly different ($P=.05$).

Table 4: Results of DNA extraction of organisms isolated from low PPD cassava flours

Sample ID	Nucleic acid ng/μl	A260 (Abs)	A280 (Abs)	260/280 (Abs)	260/230 (Abs)	Sample Type	Factor	Cassava variety	Suspected organism (s)
A	1071.2	21.425	10.28	2.08	2.14	DNA	50	IITA-TMS-IBA011368	<i>Saccharomyces cerevisiae</i>
B	19.5	0.391	0.192	2.04	1.07	DNA	50	IITA-TMS-IBA070593	<i>Aspergillus oryzae</i>
C	5.4	0.108	0.061	1.79	0.58	DNA	50	IITA-TMS-IBA011412	<i>Rhizopus stolonifera</i>
D	67.9	1.358	0.65	2.09	1.68	DNA	50	IITA-TMS-IBA011371	<i>Penicillium sp.</i>
E	11.7	0.235	0.105	2.24	1.12	DNA	50	TMEB419	<i>Aspergillus oryzae</i>
F	587.7	11.754	5.507	2.13	2.29	DNA	50	IITA-TMS-IBA011371	<i>Rhizopus microsporum</i>

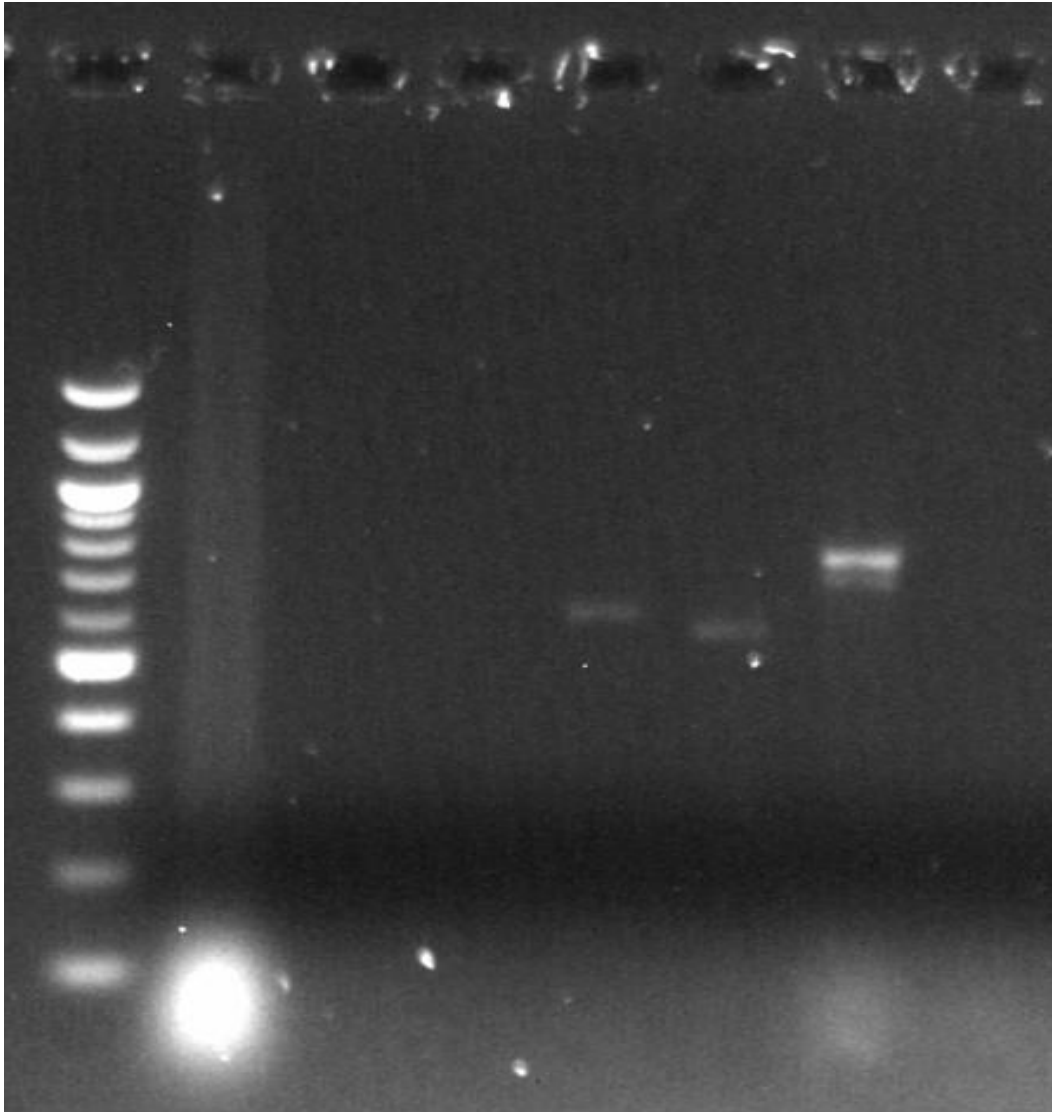


Figure 1: Purified PCR product of yeast samples

4. Conclusion

The microbiological properties of the flours prepared from IITA-TMS-IBA-011368, IITA-TMS-IBA-070593, IITA-TMS-IBA-011412, IITA-TMS-IBA-011371 (low PPD cassava varieties) and TMEB 419 (high PPD cassava variety) were within the permissible limit of the microbial load of food allowed for human consumption which according to the Standard Organization of Nigeria (SON) should not exceed 4.00 log cfu g⁻¹ for solid food samples; and within the limit for total viable bacteria count (TVBC) of 5.00 log cfu g⁻¹, total viable fungal count (TVFC) of 3.00 log set by CODEX for cassava flours.

UNDR PEER REVIEW

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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