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Isolation, Multiplication and Preservation of Cassava Fermenting Microorganisms

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Abstract

In Nigeria, Cassava processing and its associated waste have been a source of worry regarding environmental pollution. Uncontrolled disposal of the waste has contributed to a number of environmental concerns including fluxes in microbial population. However, the liquid wastes squeezed out during the processing of cassava have been discovered to be beneficial. Hence, this study is aimed at the isolation, multiplication and preservation of fermenting microflora associated with cassava wastewater for economic value as well as for environmental sustainability. A comparative analysis of microorganisms found on harvested cassava tubers of length 30cm± 0.3m, weight ±378g and microorganisms associated with the fermentation of cassava tubers was carried out. Freshly harvested cassava tuber samples were collected at Idu Industrial District, Port Harcourt, Nigeria at Latitude: 9.05245 N93'8.8254" Longitude: 7.34406 E720'38.61672". The samples were preserved in the refrigerator within 24 hours after collection and transported to the Rivers State University laboratory for microbiological evaluation. Cassava tubers were washed, peeled and cut into 3cm, soaked into 10 liter rubber container containing 1200ml of tap water collected from Mgbuoshimini layout, Port Harcourt, Rivers State. After 5 days of the fermentation process, fermented cassava tubers were mashed and examined using different microbiological parameters. The microorganisms isolated from harvested raw cassava included; bacteria: (*Staphylococcus* spp., *Bacillus* spp, *Lactobacillus* spp.) and fungi: *(Trichosoporon mucoides)* while the microorganisms isolated from fermented cassava included: bacteria*: Bacillus subtilis, Coryrebacterium manihot Bacillus* spp, and fungi: *(Aspergillus niger and Geotrichum candidum)*. Preservation of the cassava fermenting microorganisms was carried out using sterile grinded cow bone (calcium carbonate). This study has revealed that the effluent waste associated with cassava processing can be a veritable resource of fermenting microorganisms that can be sold for economic and environmental sustainability purposes.

Keywords: *Cassava tubers, Environment, Fermentation, Microorganisms, Preservation*
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I. INTRODUCTION

Cassava is scientifically known as *Manihot esculanta* and also referred to as *manioc or mandioca* is a common and popular food for the African population, traditionally processed from fermented fresh cassava tuber plants (Blench, 2014). The cassava tuber plants roots are a rich source of carbohydrates and remains the main food source in Africa and elsewhere. However, the protein content of cassava products can be greatly increased by the addition of protein to the protein-deficient cassava food products such that it does not alter the organoleptic properties of the original food (Arotupin and Akinyosoye, 2005). The use of cassava-derived diets by native Americans can be traced to civilization itself (Blench, 2014). Nowadays, cassava is cultivated in most tropical and developing countries of the world, with Africa leading in most yield, especially for most staple foods and other reasons not limited to industrial production of bioethanol. It has been widely reported that Nigeria is the largest exporter of cassava in the world today (Oboh *et al.*, 2002). Cassava and its produce could serve as a major source of starch and other biopolymers including nutritive and other high-energy products (Oyewole and Odunfa, 2012a). Furthermore, a number of both Governmental and Non-Governmental agencies have funded a number of research in the production of variants of high yielding Cassava species (Guira *et al.*, 2016). These innovations and variants also produced improved resistance to diseases, pest and improved nutrient content (Hesseltine, 2019). The fermentation of cassava tubers have been identified as the most popular approach for the production of consumables. These new varieties of cassava may possess

qualities such as ease of food storage, stability of cassava-produce, improved nutrient, energy and improved taste properties (Flibert *et al.*, 2016). According to Oduro *et al.* (2000), locally available foods from cassava have been staple foods in several regions of Africa. However, there is a dearth of information in most developing countries, on the proper processing, fermentation, preservation and production procedures of cassava foods (Oduro, 2000). A number of authors in Africa have written quite a number of articles on fermented cassava products. This study is targeted at correlating cassava processing and the microbes associated with the processing and shelf stability; as well as, identifying the microflora associated with the cassava handling. Interestingly, cassava plantation can be quite economical, since the starchy roots are capable of growing in poor and acidic soils which are often not suitable for other crops and can also yield a large harvest of cassava tubers even during the period of drought when most other crops have failed due to insufficient availability of water. Usually cassava tubers are processed into different kinds of food products including garri, chips, fufu, flour for baking bread and biscuits (Akingbala *et al.*, 2005; Ijabadeniyi, 2007). Garri has been noted as one of the major cassava products and is consumed by a large population in Nigeria. Over 70% of the processed cassava is consumed in Africa as Garri (Oduro *et al.* 2000; Olaoye *et al*., 2015). Garri is widely a staple food consumed in most parts of Africa due to its ease of preparation and mode of consumption and other reasons that may be due to the immediate energy derived from its consumption. Garri is now produced and consumed in most parts of Africa while it is considered the most vast cassava product. Cassava is rich in cyanogenic glycosides for which the process chain for the production of Garri and other fermented foods (Ross *et al.*, 2002, Adeniran and Ajifolokun, 2015; Zhu *et al*., 2015). This study is aimed at isolating, multiplication and preservation of cassava fermenting flora using locally available resources.

II. MATERIALS AND METHODS

A. Collection and source of samples

Four fresh tubers of cassava were harvested from agricultural farmland located at Idu Industrial District 900106 Port Harcourt, Nigeria at: Latitude: 9.05245 N93’8.8254 and Longitude: 7.34406 E $7^0$20’38.61672”, packed in a sterile ten-litre rubber bucket containing ice packs and transported within 24 hours after collection to the Microbiology laboratory of Rivers State University for microbiological analyses. Thereafter, the fresh cassava tubers were manually selected and prepared (Camargo *et al.*, 2018). The selected cassava tubers were weighed accordingly and then peeled manually using a clean laboratory knife as presented in Figure 1. The peeled cassava tubers were thoroughly washed with clean running water and cut into 3cm $\pm$ 0.03m long pieces, after which it was soaked in 10-liter rubber buckets containing 1,200 ml of tap water from Mgbuoshimini Port Harcourt, Rivers State. After 5 days, the soft cassava tubers were then squeezed to obtain the cassava wastewater. The samples were transported using an ice-cold chest under standard conditions and preserved in ice bags for microbiological analyses.

Figure 1. Freshly harvested cassava tubers

B. Sterilization of laboratory equipment and media

All glassware used were properly washed with detergent, rinsed under clean running water and finally re-rinsed with deionized water. The glass wares were sterilized via a hot air oven at 170^0C for 2 hours. The required media used were autoclaved at 121^0C temperature under 15 psi for 15 minutes (Holt *et al.*, 2012).

C. Enumeration and isolation of microorganisms

Samples from freshly harvested cassava and fermented cassava wastewater were serially diluted. Aliquots (1ml) of the appropriate dilution were inoculated onto freshly prepared nutrient agar plates using a Pasteur pipette and spread evenly while the cultured plates were incubated at 37^0C for 24 hours. After incubation, the plates were observed for microbial growth. The colonies that developed on the culture plates were characterized, recorded and sub-cultured onto freshly prepared nutrient agar plates and distinct colonies were isolated and stored in nutrient agar slants at 4^0C for further analyses (Holt *et al.*, 2012).

D. Characterization and identification of bacterial isolates

Identification of the microbial isolates was carried out based on their colonial morphological features, cellular morphology and biochemical reactions (Tables 1-3). The results were interpreted using the dichotomous keys as documented in microbiological repositories. Morphological indices were observed from the cultured plate after 24 hours of incubation (Cheesbrough, 2006; Oluwole *et al.*, 2007;

Holt *et al.*, 2012).

Table 1. Colonial morphological characteristics of bacterial isolated from freshly harvested and fermented cassava water samples

Isolate	Margin	Elevation	Color	Opacity	Surface	Texture	Size	Shape	Suspected microorganism
HCBA	Entire	Raised	Yellow	Opaque	Smooth	Wet-Dry	3.0mm	Round	*Staphylococcus* sp.
HCBB	Entire	Flat	Creamy	Opaque	Rough	Wet- Dry	7.0mm	Irregular	*Bacillus* sp.
HCBC	Entire	Raised	Creamy	Opaque	Smooth	Dry	5.0mm	Round	*Lactobacillus*
HCBD	Entire	Raised	Creamy	Opaque	Smooth	Wet-Dry	0.5mm	Round	*Bacillus* sp.
HCBE	Entire	Flat	Creamy	Opaque	Smooth	Wet	5.0mm	Round	*Staphylococcus* sp.
FCBF	Entire	Raised	Milk	Opaque	Smooth	Wet	6.0mm	Round	*Bacillus subtilis*
FCBG	Entire	Raised	Creamy	Opaque	Smooth	Wet	3.0mm	Irregular	*Coryrebacterium manihot*
FCBH	Entire	Raised	Creamy	Opaque	Smooth	Wet	4.0mm	Round	*Bacillus* sp.
FCBI	Entire	flat	Creamy	Opaque	Rough	Dry	5.0mm	Irregular	*Bacillus* sp.

Key; HCBA; Harvested Cassava Bacteria Isolate A, B, C, D, E and FCBF; Fermented Cassava Bacteria Isolate F, G, H, I

Table 2. Biochemical Characterization of bacterial isolates from freshly harvested and fermented cassava water samples

Sample type	Gram stain	Cat	Cit	Ind	Mot	Mr	Vp	Suc	Glu	Lac	Man	Starch Hyd	Suspected microorganism
HCBA	+ve cocci	+	+	+	+	+	_	+A	+A	_	+A	+	*Staphylococcus* sp.
HCBB	+ve Rods	+	+	-	+	+	_	+A	+A	_	+A &G	+	*Bacillus* sp.
HCBC	+ve Rods	_	+	+	+	+	_	+A	+A,G	_	+A	_	*Lactobacillus*
HCBD	+ve Rods	+	+	_	+	+	_	+A	+A	_	+A	+	*Bacillus* sp.
HCBE	+ve cocci	+	+	+	+	+	_	+A	+A		+A	_	*Staphylococcus* sp.
FCBF	+ve Rods	+	+	+	+	+	_	+A	+A	+	+A	+	*Bacillus subtilis*
FCBG	+ve Rods	+	+	-	+	+	_	+A	+A	_	_	+	*Coryrebacterium manihot*
FCBH	+ve Rods	+	+	_	_	_	+	+A	+A,G	_	_	+	*Bacillus* sp.
FCBI	+ve Rods	+	+	+	_	+	+	+A	+A	_	_	+	*Bacillus* sp.

Key; HCBA; Harvested Cassava Bacteria Isolate A, B, C, D, E and FCBF; Fermented Cassava Bacteria Isolate F, G, H, I
Cat: Catalase, Cit: Citrate, Glu: Glucose, Hyd: hydrolysis, Ind: Indole , Lac: Lactose, Man: Manitol, Mot: Motility, MR: Methyl red, Suc: Sucrose, VP: Voges – Proskauer.

Table 3. Colonial morphological characterization of fungi isolates from fresh harvested cassava and fermented cassava water samples

Sample type	Morphological appearance on SDA plate	Wet-mount	Suspected microorganisms
HCF1	Raised and waxy appearance	Appearance of arthrocondia and blastocondidia	*Trichosoporon mucoides*
FCF2	Creamy mucoid flat growth with the yellow reverse side	Barrel shaped arthroconidia in chains and dischotomous branching hyphae	*Geotrichum candidum*
FCF3	White Lawny growth with the yellow reverse side	Smooth and Colorless Conidiophores and spores, globose and dark brown conidial heads	*Aspergillus niger*

Key: HCF1: Harvested Cassava Fungal Isolate 1, FCF2:Fermented Cassava Fungal Isolate 2 FCF3: Fermented Cassava Fungal Isolate 3

E. Multiplication of fermenting microorganisms of cassava tubers

1- Multiplication of fermenting bacterial isolate

The nutrient broth was prepared accordingly using the manufacturer's directions as follows: The conical flasks containing the freshly prepared nutrient broth were inoculated with the bacterial isolates accordingly and properly labelled. The inoculated conical flasks were observed for the multiplication of the fermenting microorganisms after seven days of incubation in an incubator at 37^0C (Adewoye and Sawyeer, 2016; Omotioma *et al.*, 2016).

2-Multiplication of fermenting fungal isolate

Potato dextrose broth was freshly prepared using fresh raw Irish potato as mentioned elsewhere, the fungal isolates from the fermented cassava water were then, inoculated into the potato dextrose broth and observed for 7 days for the multiplication of the fungal isolates (Adewoye and Sawyeer, 2016; Omotioma *et al.*, 2016).

F. Preservation of the cassava fermenting microorganisms

Fresh waste Cow bone was used for the preservation of the cassava fermenting microorganisms. This was obtained from mile 3 market Port Harcourt, Rivers State (Figure 2). The fresh bones were dried in a hot air oven for 4 days. The dried bones were then grinded using a laboratory grinder into powder with the use of a clean laboratory ceramic mortar. The grinded powdered bone served as a source of calcium carbonate and was poured into a 500 ml conical flask, thereafter, sterilized in a hot air oven. Calcium carbonate (15g) was added into different beakers and labelled accordingly for the preservation process. Thereafter, 5ml of each broth culture was added and thoroughly mixed. The mixture was then, transferred into small-sized waterproof nylon, sachet forms and sealed with a sealing machine (Omotioma *et al.*, 2016; Okoronkwo *et al.*, 2017).

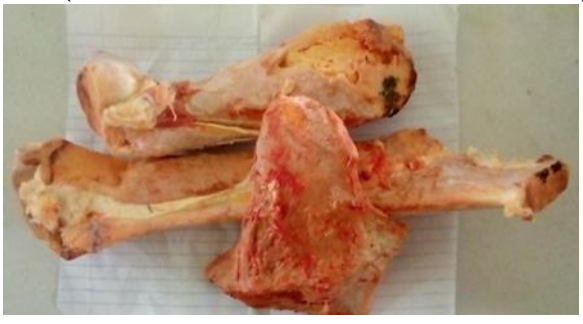

Figure 2. Cow bone (calcium carbonate) served as a base for the preservation of cassava fermenting microorganisms

G. Evaluation of preserved fermenting microorganism

Preserved fermenting microorganisms were evaluated. This was done to ascertain the reappearance of the various isolated fermenting microorganisms on their respective culture media. The procedure was carried out after five days. (Figure 3). One gram each of calcium carbonate-containing the preserved cassava fermenting microorganisms was serially diluted using 10-fold serial dilutions. The bacterial isolates were sub-cultured on freshly prepared nutrient agar media plates, while the fungal isolates were cultured on freshly prepared sabouraud dextrose agar plates. Inoculation of the various isolates was carried out on the different culture media plates accordingly and spread evenly. The inoculated bacterial culture plates were incubated at 37^0C for 24 hours, while the fungal culture plates were incubated for 72 hours at 28^0C (Ehiri *et al.*, 2001). After incubation, the initial fermenting flora isolated from cassava wastewater reappeared on the cultured plates accordingly following morphological characterization of various sub-cultured preserved fermenting microorganisms (Okoronkwo *et al.*, 2017).

Figure 3. Preservation of the Cassava Fermenting Microorganisms in Sachets after 5 days (Cow bone used as a base for preservation). Bacterial isolate. A: *Bacillus Subtilis*, B: *Corynebacterium manihot*. Fungal isolate C: *Geotrichum candidum*, D: *Aspergillus niger*

III. RESULTS

The total microbial counts of microorganisms isolated from the freshly harvested cassava and fermented cassava water are presented in Table 4. The freshly harvested cassava recorded higher counts of bacterial population (2.90 X 10^9 CFU/ml) than the fermented cassava water (1.06 X 10^7 CFU/ml). On the other hand, the fermented cassava water had a higher number of fungal counts (2.0 X 10^4 CFU/ml) than the freshly harvested cassava (1.0 X 10^3 CFU/ml).

Table 4. Total counts of microbial isolates from freshly harvested and fermented cassava water

Sample type	Total heterotrophic bacteria count (cfu/ml)	Total fungi count (cfu/ml)
Freshly Harvested Cassava	2.90 X 109	1.0 X 103
Fermented Cassava Water	1.06 X 107	2.0 X 104

Microbial isolates of freshly harvested cassava (FHC) included three bacteria and one fungus. The bacterial isolates were as follows: *Bacillus* spp., *Lactobacillus* spp. and *Staphylococcus* sp. while the fungal isolate was *Trichosoporon mucoides.* On the other hand, microbial isolates of fermented cassava water (FCW) included four bacteria and two fungi. The bacterial isolates were as follows: *Bacillus* spp. (1), *Bacillus* spp (2) *Bacillus subtilis* and *Corynebacterium manihot.* While the fungal isolate were: *Aspergillus* sp. and *Geotrichum candidum*.

Bacillus spp was the most dominating microorganism during the fermentation process. Also, after preservation of cassava fermenting microorganisms with sterile grinded cow bone (calcium carbonate), and subcultured, it was observed that the bacteria *Bacillus* spp. had the highest bacterial counts of $2.50X10^7$ CFU/g while the fungi *Geotrichum candiudum* had the highest fungal counts of $8.0X10^4$ CFU/g.

Table 5. Microorganisms and Microbial Counts (CFU/g) in preserved bone meal medium

Sample type	Microorganisms (Bacteria/Fungi)	Total heterotrophic bacteria / Total fungi count (CFU/g)
FCWBF	*Bacillus subtilis*	1.18×10^7
FCWBG	*Coryrebacterium manihot*	1.06×10^7
FCWBH	*Bacillus* spp.	2.50×10^7
FCWBI	*Bacillus* spp.	1.80×10^7
FCWF2	*Geotrichum candidum.*	8.0×10^4
FCWF3	*Aspergillus niger*	2.0×10^4

Key: FCWBF: Fermented Cassava Water Bacteria Isolate F, G, H, I, FCWF2: Fermented Cassava Water Fungi Isolate 2, 3

IV. DISCUSSION

Freshly harvested cassava exhibited a higher bacterial population than fermented cassava water. In contrast, fungal counts were greater in fermented cassava water than in freshly harvested cassava. It was observed that the increase in viable counts as the fermentation period progressed could be as a result of the presence of the substrates and the prevailing conducive environment for the microorganisms to metabolize the available substrates (Akinyosoye *et al*., 2003). Furthermore, the observation of the higher bacterial counts of the harvested cassava may be due to a lack of efficient control measures taken during the discharge of the wastewater into the environment. Furthermore, Uzochukwu *et al*. (2001) identified the role of cassava effluent and the remote pollution effect it has on the environment. Furthermore, Uzochukwu *et al*. (2001) identified that the residues of cassava production also contain a huge amount of nutrients which could serve as a source for a number of economic benefits. Furthermore, Ume *et al*., (2020) observed and documented that cassava wastewater (effluent) contained minerals such as Ca, Fe, Mg, K, Na, and Zn ions. The mineral content of cassava waste may be a contributing factor to the positive correlation to microbial counts. Nevertheless, the lower fungal counts of the cassava wastewater as observed may be due to the regular sanitation, disinfection and fumigation of the environment practiced as confirmed by the factory workers.

FHC yielded three bacterial isolates (*Bacillus spp*., *Lactobacillus spp*., and Staphylococcus sp.) and one fungus (*Trichosporon mucoides*). In contrast, FCW had four bacterial isolates (Bacillus spp. (1), Bacillus spp. (2), Bacillus subtilis, and *Corynebacterium manihot*) and two fungi (Aspergillus sp. and *Geotrichum candidum*). Isolates such as *Bacillus* species have been implicated in both fresh harvested cassava samples and the cassava waste water samples. Therefore, the isolate, *Bacillus* species can be regarded as transient microorganisms surviving in both environmental conditions (Oranusi *et al*., 2013; Olowoyo *et al*., 2021). However, more varieties of these bacterial species were isolated from fresh harvested cassava than from fermented cassava water. They may exist as normal flora of the soil and may have crossed from the critical control points associated with the cassava processing. The presence of *Staphylococcus* spp. and *Trichosporon mucoide* as observed in the harvested cassava samples could be due to contamination on the raw cassava surface and from the soil where the cassava tubers were harvested. Several researchers including; Olowoyo *et al*., (2021); Okafor, (2018); Oboh (2005); Akinyosoye *et al*., (2003), have reported similar microbial species isolated from cassava wastewater samples. The presence, increased number and the type of fungi isolated after the fermentation process may be a result of processing contamination of the cassava tuber. From our study, the bacteria; *Corynebacterium manihot* was isolated in only the fermented cassava water sample. This reveals that *Corynebacterium manihot* is a major fermenting bacteria. Previous studies have shown that *Corynebacterium* species of bacteria are known to be early colonizers that are associated with cassava fermentation and possess capabilities of converting starch into lactic acid and formic acid, thus lowering the pH of the substrate medium (Akingbala *et al*., 2005). Also, Akinyosoye *et al*. (2003) stated clearly that, the bacteria; *Lactobacillus* species have been implicated in the production of organic acids. Furthermore, the fungi; *Geotrichum candidum* group of microorganisms are known to contribute to the unique flavor and taste of the finished products during the processing of cassava tubers into food products like garri (Adewoye and Sawyeer, 2016).

Bacillus spp. emerged as the predominant microorganism in the fermentation of cassava. The type of microorganisms isolated in the present study is in accordance with work done by Oyewole and Odunfa, (2012b) on the

characterization and distribution of bacteria during cassava fermentation. Furthermore, researchers isolated similar species of microorganisms in their studies on cassava fermenting microorganisms (Schwan *et al*., 2014; Okafor *et al*., 2018; Sanni *et al*. 2018).

V. CONCLUSION

To conclude, The fermentation process is a very vital aspect of cassava processing that helps in the removal of cyanide substances that are harmful to humans by microorganisms. From the present study, it was observed that cassava wastewater contained microorganisms which are beneficial in the fermentation processing of cassava into other finished food products. Bacteria; *Bacillus* spp. was the most dominating microorganism during the fermentation process while the fungi isolate; *Geotrichum candiudum* had the highest microbial load. The fermenting microorganisms have revealed potential for preservation for further purposes. Therefore, it is recommended that after the processing of cassava into finished products, the fermented cassava wastewater generated could be explored and converted into beneficial valuables of economic importance. This would help in creating other job opportunities for the teeming population as well as maintaining a safe and friendly environment in the regions where cassava tubers are constantly processed in Nigeria.

Proper care should be taken during the processing and fermentation of cassava to avoid contamination of cassava food products (garri and fufu). Cassava should be allowed to ferment at least 5-6 days to enable the removal of harmful cyanide substance. Grinded bone (calcium carbonate) should be used for preservation of cassava fermenting microorganism and other microorganisms that are of industrial and economic importance to humans.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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