International Journal of Frontline Research in
Multidisciplinary Studies
Journal homepage: https://frontlinejournals.com/ijfrms/
ISSN: 2945-4875 (Online)

(RESEARCH ARTICLE)

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Isolation and identification of microorganisms associated with fermented cassava (*Manihot esculenta Crantz*) for the production of Akpu

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International Journal of Frontline Research in Multidisciplinary Studies, 2022, 01(01), 008–015

Publication history: Received on 03 April 2022; revised on 04 June 2022; accepted on 06 June 2022

Article DOI: https://doi.org/10.56355/ijfrms.2022.1.1.0026

Abstract

Akpu, an acid-fermented cassava product traditionally produced and consumed in Nigeria and other West African countries and hence it is necessary that microbiological study is varied out to evaluate its consumption safety and quality the isolated organisms can play an important role in industrial processes. This study looks into the possibility of utilizing cassava waste water as a source of industrial isolate for further purpose; it involves isolating and identifying the organisms present during the akpu production so as to make and ensure a safe product. The cassava tubers were harvested, peeled and soaked in the water for 4 days. The retting was closely monitored for the 4day period after which the mash was cultured on appropriate media to determine the organisms present. The methods used involve traditional retting method to ret the tubers in the laboratory. Isolation and identification of microorganism associated with fermented cassava for the production of akpu was examined. During the 4th day of retting process a total of seven microorganisms were isolated from the retting water. Three bacteria *Bacillus subtilis*, *Staphylococcus epidermidis Staphylococcus aureus,* while fungi and yeasts isolated were *Saccharomyces cerevisiae, Candida tropicalis, Aspergillusspp and Rhizopusstolonifer*. The presence, number and the kind of microorganisms during the fermentation to Akpu could be as a result processing contamination and microbial flora present in the cassava tuber. The consumption of akpu is very high in Nigeria and hence safety in the production process should be considered.

Keywords: Fermentation; Retting; Akpu; Isolation; Identification

1. Introduction

Akpu is an acid-fermented cassava product produced through submerged fermentation of peeled cassava roots in water and consumed in Nigeria, West African countries and other parts of the world [1]. The cassava (*Manihotesculenta Crantz*) is cultivated mainly in the tropic and sub-tropic regions of the world, over a wide range of environmental and soil conditions. It is tolerant of insect pests and diseases, and is very tolerant of drought and heat stress. The cassava is not a labour intensive crop and produces well on marginal soils. In many of the cassava growing regions of the world, however, the cassava does not achieve its yield potential, due primarily to disease and limited inputs such as fertiliser and irrigation [2]. Estimates of the Food and Agriculture Organisation of the United Nations put world production of cassava at more than 230 million metric tonnes annually [3]. Major producers of cassava include Nigeria (37.5 million

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tonnes per annum), Brazil (24.5 million tonnes) and Thailand (22.0 million tonnes). For the Caribbean region, annual production is in excess of 1.2 million tonnes, with the Bahamas contributing about 240 tonnes to this figure.

Cassava is grown in Nigeria and many other regions (countries) of the tropics, where it serves as one of the basic food source for about 200-300 million people [4]. The main cassava food products of considerable domestic importance in Nigeria are garri in the south, lafun in the west and fufu in the east [5]. The principal parts of the mature cassava plant expressed as a percentage of the whole plant are 6% leaves; 44% stems and 50% storage roots. The roots and leaves of the cassava plant are the two nutritionally valuable parts, which serve as food. The cassava root is composed of 60-65% moisture, 20-31% carbohydrate, 0.2-0.6% ether extracts, 1-2% crude protein and a comparatively low content of vitamins and minerals. The root carbohydrate is made up of 64-72% starch. However, the roots are rich in calcium and vitamin C and contain a nutritionally significant quantity of thiamine, riboflavin and nicotinic acid that are lost during processing [6].

The cassava is an important component in the diets of more than 800 million people around the world [7] and is the third largest carbohydrate food source within the tropical regions, after rice and corn [8]. It is used mainly as a fresh food item, but is also processed into various food and non-food products, such as starch, flour, beverages, animal feeds, biofuels and textiles. There is much variation in the nutrient quality of the cassava root [9]. In the tropical regions, cassava is the most important root crop and, as a source of energy, the calorific value of cassava is high, compared to most starchy crops [10]. The starch content of the fresh cassava root is about 30%, and gives the highest yield of starch per unit area of any crop known [11]. The protein content is extremely low, however, and ranges between 1-3% [12,13]. The cassava root contains a number of mineral elements, in appreciable amounts, that are useful in the human diet. The root contains significant amounts of iron, phosphorus and calcium, and is relatively rich in vitamin C [14].

There are several thousand varieties of cassava and about 100 related wild species [15] with hydrogen cyanide (HCN) contents of their roots ranging from 1-1550 parts per million (ppm) [16]. Cassava plants are generally categorized as bitter or sweet, depending upon their cyanide content. The low-HCN, or sweet cassava, has less than 50 ppm of cyanogenic equivalents, while the high-HCN or bitter cassava has more than 100 ppm. [17]. According to Adepoju *et al*., [18], the food value of cassava is greatly compromised by its toxic hydrogen cyanide content [19]. The sweet cassava can be cooked and eaten as they are, while the bitter cassava needs to be processed before being consumed. A large amount of variation exists among the cassava leaf, stem and root characteristics. These characteristics, which include leaf morphology, stem colour, branching habit and storage root shape and colour, may influence cassava yield [20]. Other, not so obvious, characteristics include resistance to insect pests and diseases. A proper understanding of these variations in plant characteristics would assist the selection of cassava types with the desired traits. This, in turn, will contribute to improved crop establishment and increased yields. The fermentation process is initiated as a result of chance inoculation by microorganisms from the environment. Although, convenient there are concerns about its reliability the control of which is the basis of all technological measures that are used to obtain product at a defined quality. The presence of unspecified microorganisms complicates the control of the fermentation process and lead to the production of objectionable odours. Such problems have led to the development of several other processing techniques suitable for odourless fufu [21]. During fermentation of fufu, lactic acid bacteria, yeast and other bacteria contribute significantly to starch breakdown, acidification, detoxification and flavour development [22]. Among the fermented products of cassava, Akpu is one of the favorite consumed in many parts of West Africa countries [23]. This is produced by steeping in water peeled cassava cubes for 24-72 hours. The fermented cassava is sieved to remove the fibers and allow to sediment. After sedimentation, the water is decanted and the sediment is dried [24]. Akpu is produced, sold and eaten in Nigeria and other African countries without any formal regulations or certification and this necessitate microbiological studies in other to ascertain its consumption and quality. The objective of this present study is to isolate and identify the number of organism isolate responsible for the fermentation of cassava tuber and the time at which these organisms will take to ferment the cassava (*Manihotesculentacrantz*) tubers to produce Akpu, so as to make a product safer for consumption.

2. Material and methods

2.1 Source of materials

Cassava tubers One year old cassava tubers of the specie TMS 30555 were harvested from the farm at the Nnamdi Azikiwe University Awka premises and immediately transported to the laboratory for processing within 24 hours. Culture media, chemicals and reagents used was obtained from the Applied Microbiology and Brewing Laboratory of the institution and were of analytical grade.

2.2 Methods of akpu production

The method of wet akpu mash production by Oyewole and Ogundele [25]. was used to produce wet akpu mash in the laboratory. The tubers after harvest were peeled, cut into cylindrical portions (4-7 cm long) and washed with tap water. Three kg (3 kg) of the peeled cut tubers were soaked in 5liters of water for 4 days using plastic buckets with lid. The retting waters and tubers were monitored daily for retting ability and the presence of microbial flora.

After retting the tubers were washed, mashed in clean water and sieved remove the fibers and the vascular bundles. The mixture was allowedto settle and excess water decanted. The wet akpu mash was transferred into a clean jute bag and the remaining water pressed out [25].

2.3 Method of analysis

Determination of the retting ability of the tubers. The retting ability of the tubers will be determined manually using the method of Umeh and Odibo [26].

2.4 Isolation of microorganism

The pour plate method was used to determine the microbial counts in the retting water. Characterization and identification of the bacterial isolates was carried out as stipulated by Krieg and Holt, [27].

2.5 Microbiological analysis

Sample included fermentation of water and the cassava mixture at an interval during the 96hr fermentation period. For all sample, 0,1ml of the appropriate serial dilutions were spread plated on duplicate plates. The following media were used: nutrient agar (NA) for general bacterial. Saboruad dextrose agar (SDA) for general molds. Incubation was at room temperature for 24-48hrs.

2.6 Gram staining

Carefully cleaned slides were properly labeled. A loopful of a 24hr culture of the organism was collected and smeared on the slide. It was then fixed by passing the smeared slide quickly through the flame so as not to scorch. The heat-fixed film of the organism in question was then immersed with crystal violet reagent for 60seconds after which it was washed with a gentle stream 0f tap water. it was then immersed in iodine mordent for 60seconds and again washed with gentle stream of tap water and allowed to dry.95%(v/v) ethanol was then used to decolorize the crystal violet for 30seconds after which the film was counter stained with safranine red for 10seconds and washed with water until no color appeared on the effluent. The slide was allowed to dry and then viewed under a microscope using oil immersion. Gram positive bacteria appeared purple while gram negative appeared pink.

2.7 Catalase

A loopful of the organisms from the nutrient agar plate was made on a clean slide and a few drops of hydrogen peroxide [H_20_2] were placed on it. The presence or absence of effervescence indicated a positive [¬+] or a negative [-] result respectively.

2.8 H2s production

A tube of sterile peptone water was inoculated with the organism. A strip of lead acetate paper was placed at the top of the tube and held in place with a cotton- wool plug. The tubes were incubated for 48hrs and examined for blackening of paper. Blackening indicates positive otherwise negative.

2.9 Motility

A ring of Vaseline was made on a glass slide. A drop of the suspension of the organism was then placed at the center of a clean cover-slip using a Pasteur pipette. The slide was then inverted over the cover-slip, which adhere to the slide and both were quickly re-inverted top produce a hanging drop of the culture on the cover slip. Motility was then examined under low power objectives X40 motile and non-motile isolates were noted.

2.10 Methyl red

This test as updated by Aryal, 2018., uses organisms taken from 18-24 hours culture and lightly inoculate into the methylred VOGES-PROSKAUER broth (buffered peptone=7g/l, glucose=5g/l, dipotassium phosphate=5g/l) and incubate aerobically for 24hrs. 1ml of the broth is added to a clean testube and the remaining is reincubated for

additional 24 hrs. 2-3 drops of methylred indicator is added to the aliquot in the testube. A red colour shows a positive result.

2.11 Voges-proskauer test

This test as updated by Aryal, 2018., uses organisms taken from 18-24 hours culture and lightly inoculate into the methyl red voges-proskauer broth (buffered peptone=7g/l, glucose=5g/l, dipotassium phosphate=5g/l) and incubate aerobically for 24hrs. after 24hrs incubation,2mls of the broth is added to a testube and the rest is reincubated for additional 24hrs. 6 drops of 5% alpha-naphthol was added and mixed well to aerate. 2 drops of 40% potassium hydroxide is added and mixedwell to aerate. The presence of a pink colour at the surface after 30 mins shows a positive result.

2.12 INDOLE

To a 4day peptone water culture of the organism is added about 0.5ml of xylene. The mixture was thoroughly shaken and allowed to stand for 30 minutes, a few drops of kovac's reagent was then added. The development of a rose pink colour showed a positive result while a yellow colour indicated a negative result while a yellow colour indicated a negative result.

2.13 Lactophenol cotton blue mount (yeasts)

A small drop of lactophenol cotton blue was placed in the center of a clean slide. A fragment of the yeast colony was removed with a probe or an inoculating needle and placed into the drop of the lactophenol cotton blue. A coverslip was then lowered gently on the preparation to mount it. The organism was then viewed under low power objective (x40) to observe the morphology of the yeast isolates.

2.14 Sugar fermentation test

Durham tube was placed in an inverted position in a bijou bottle containing 1% solution of the differential sugar (namely glucose, maltose, mannitol, fructose e.tc) in 1% peptone water with bromothymol blue (0.01g/l) as indicator before autoclaving at 1150 c for 10minutes. After autoclaving, the medium was allowed to cool to 40°c for organisms were inoculated. The bottles were then incubated at 370c for 48hrs.At the end of the incubation period, the tubes were observed for gas formation and acid production. The presence of air space in the durham tubes indicate was formation otherwise, no gas formation. On the other hand, change in colour of the medium from green to yellow indicates acid production otherwise negative (no acid production).

3. Results

Table 1,2, 3 show the different isolates of bacteria, fungi and yeast from the fermented cassava tubers (*Manihotesculentacrantz*) and biochemical test. Table 4 shows the microbial count form the first to the last day of the fermentation.

Table 1 Characteristics and Morphology of the Bacterial Isolate and Its Biochemical Test

Colony morphology	Gram stain	Spore	Motility	Urease	Catalase	Citrate	M.R	V.P	Indole	H_2S	Coagulase	Glucose	Lactose	Maltose	Sucrose	Manitol	Probable Organisms
Cream, rough, opaque and circular yellow	+ve, long rod in chains	+	+	_	+	+	+	_	+	_	_	AG	_	_	_	_	*Bacillus subtilis*
Smooth, raised and circular	+ve, cocci in clusters	_	_	_	+	+	_	_	_	_	_	AG	A	_	+	_	*Staphylococcus epidermidis*
Cream, smooth, raised and circular	+ve, circular cocci	_	_	_	+	+	_	_	_	_	_	A	_	_	_	_	*Staphylococcus aureus*

Key: _ absent; + present

Table 2 Characteristics and Morphology of the Yeast Isolate and Its Biochemical Test Sugar Fermentation

Cell morphology	Glucose	Maltose	Lactose	Galactose	Sucrose	Manitol	Urease	Probable organisms
Budding cell and pseudohyphea	+	+	_	_	+	_	+	*Candida tropicalis*
Budding cells	+	+	_	_	+	_	_	*Saccharomyces cerevisiae*

Key: _ absent; + present

Table 3 Morphological Characteristics of the fungi isolates

Young culture morphology	Old culture morphology	Microscopy	Texture	Days	Probable organisms
Whitish with yellow reverse	Blue-green or dark-green	Double branching, septate hyphae and stuartcondiophores.	Powdery and velvety.	3-4	*Aspergillusniger*
Dense grayish	Green to brown to black filling plate	Oval non septate hyphae with sporamgiospores.	Fluffy and cottony.	2-3	*Rhizopusstolonifer*

Table 4 Daily changes in the microbial counts of retting water

Days	Heterotrophic Bacterial count ×10⁶cfu/ml	Yeast count ×10⁶cfu/ml	Mould count ×10⁶cfu/ml	Lactic acid ×10⁶cfu/ml
0	27.0	nd	nd	nd
1	40.0	30.5	28.5	29.0
2	51.0	40.0	46.0	30.5
3	55.5	46.5	53.0	39.5
4	65.0	50.0	56.0	48.0

Key: nd = not determinable

4. Discussion

The fermentation of cassava roots, allows softening for further processing and reduction of potentially toxic cyanogenic glucosides present in the roots. Clearly, it is evident that production of cassava fufu is initially mediated by a diverse microflora, which eventually is dominated by the lactic acid bacteria. This pattern of microbial succession is a general feature of fermenting plant materials [28, 29, 30]. During the 4th day of retting process a total of seven microorganisms were isolated from the retting water. From the Table 1, three bacteria is identified as the probable organisms found and they include *Bacillus subtilis, Staphylococcus epidemidis, Staphylococcus aureus*. This agrees with the work of Inetianbor *et al.* [1]. The presence of *Staphylococcus aureus* could be due to contamination from the skin, mouth, or nose of the handlers during the processing since it is a member of the normal flora of the skin. it does not portray a serious concern since the temperature of most of the Akpu is raised before final consumption [1]. According to the work of Ezemba *et al*., [31]. several microorganisms including *Bacillus sp*., lactic acid bacteria (*Lactobacilli, Leuconostoc*, *Streptococci*)and yeasts, (*Aspergillus*, *Fusarium, Penicillium* and *Trichoderma)* strains are known for their detoxification activities during cassava fermentation which is in agreement with the result in this work as their presence is being recorded. *Bacillus sp* was also found in the work of *Inetianbor et al*., [1], it is an opportunistic pathogen of humans and also a frequent inhabitant of soil, leaf surfaces and wrapping materials. Its presence in the Akpu may results from the soil and materials used in wrapping, covering the fermentation tank or drum [32]. Table 2 showed the yeasts present in the fermentation. *Candida tropicals, Saccharomyces cerevisiae were* isolated. This result agrees with the work of Inetianbor *et al*., [1]. Table 3 showed presence of moulds and they include *Aspergillus sp,Rhizopus stolonifer. Aspergillus sp* and *Rhizopus stolonifer*was also reported to be isolated in the work of Obadina *et al*., [33].. Three bacteria isolates as shown in table 1, in table 2 yeast isolates, in table 3,mould isolates. Isolates are presented respectively. This result in line with the work of Umeh and Odibo [34], who isolated eleven bacteria isolates, two yeast and two mould isolates. Many microbes had been reported to be responsible for cassava fermentation [34; 35, 36].Table 4 showed the microbial count at different days of retting. On the first day, the bacteria count is 27x10⁶cfu/ml and on the 4th day the count was, 65 x10⁶cfu/ml showing the increase in microbial count as the days increased. This result was also in line with the report of Ezemba *et al*., [37], who also recorded an increase in microbial with increase in fermentation period. There was no increase in the microbial count for mould and yeast but on the 4thaday, the yeast count is 50 x10⁶cfu/ml and the mould count is 56 x10⁶cfu/ml which shows increased microbial count of yeast and mould as the days increases. Microbial count of these organisms in the retting water increased daily with increase in the retting days (table 4). This is in agreement with the findings of Fagbemi and Ijah [35]. The increase in counts may be as a result of favorable conditions which enable them to multiply [35]. The multiplication of coliforms, especially in the early and intermediate days of fermentation is a characteristic of mixed acid fermentations. It was also observed that the fungal and lactic acid bacterial counts were very small on the zero days and is considered as not determinable (nd) .The proliferation of coliforms, especially in the early and intermediate days is a characteristic of mixed acid fermentations [35]. Some of these organisms, as suggested by Fagbemi and Ijah [35], may be originated from the water used for fermentation, surrounding air or the bowels used for the retting of the tubers, or due to prevailing favorable aerobic conditions [35]. The increase in the counts of the yeast and lactic acid bacteria in the retting water which favored their growth. This was in line with the finding of Fagbemi and Ijah [35]. The report that yeast and different bacterial species were involved in cassava fermentation agrees with the report of Gaffa and Gaffa [38], that yeast and other lactic acid bacteria are very common in a variety of traditional food and beverage fermentations.

5. Conclusion

It was observed that the organisms isolated from the fermentation system of cassava tubers are active in retting it. The *Bacillus subtilis, Staphylococcus epidemidis, Saccharomyces cerevisiae, Candida tropicalis* were able to ret the tubers and yield fermentation process. The *Bacillus* present tends to hydrolyse the starch and help in the retting process to get the final product. Therefore if fresh cassava tubers were peeled, washed, cut and rewashed with 70% ethanol and rinsed with sterile water, any of these organisms can be inoculated aseptically and the resultant wet Akpu. More safety precautions can be taken to get a safer Akpu. This study has therefore given us a better insight ion the microorganisms involved in the fermentation of cassava tubers for the production of Akpu.

Compliance with ethical standards

Acknowledgments

This is to acknowledge the supervisors, and all the authors that participated in this work.

Disclosure of conflict of interest

There is no conflict of interest among the authors in this manuscript.

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