Phytology

Application of residual cyanide concentrations level in processed cassava (*Manihot esculenta*) to inhibit the growth of spoilage microorganisms in cooked rice (*Orysa sativa*)

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Abstract

Rice (*Orysa sativa*) is a staple food for human population, providing more than one fifth of the total energy calories consumed worldwide by humans. The shelf life of rice grains when harvested raw milled or unmilled is long. But when it is cooked, it barely lasts for 24hours in storage. The spoilage of rice is due to the presence of different microorganisms such as *Bacillus* spp and Yeast (*Saccharomyces cerevisiae*). Cassava has a high cyanide content and it does not get spoilt easily when cooked. In the processing of cassava, there are several methods of fermentation, sun drying, and frying employed to process it to powdery forms 'Gaari' and 'lafun' and the porridge 'fufu' leading to a great reduction in the cyanide contents. The three processed cassava Gaari, Lafun and fufu were obtained from a local market in Akure, Nigeria. Cyanide concentrations before and after cooking these products from (Day 1 to Day 5) were obtained. The cyanide contents equivalents after cooking were prepared and tested for inhibitory effect on spoilage microorganisms isolated from cooked rice. The objectives of this study are to determine the cyanide concentrations in processed cassava (*Manihot esculenta*) that can inhibit spoilage microorganisms and investigate if the concentration can be added to preserve cooked rice from spoilage. Results obtained showed that Fufu, Gaari and Lafun have residual cyanide contents of 19.63, 10.14 and 7.02mg/kg respectively before cooking and which decreased to 14.94, 4.98 and 4.29mg/kg in day5 after coking. There were no significant correlations between the residual cyanide contents in the cassava products before and after cooking (*Sig* = 0.947 df =14 $p > 0.01$). There was a significant inhibitory effect of cyanide concentrations ($F_{4,20} = 5.307$, *Sig* =0.004 $p = 0 < 0.05$) on the growth of *Saccharomyces cerevisiae*, secondary spoilage microorganisms of cooked rice. Cyanide is effective in controlling spoilage microorganisms in rice.

Keywords: *Manihot esculenta*; *Orysa sativa*; spoilage, bacteria, fungi, residual

Introduction

Rice is a grain of a monocotyledonous plant belonging to the family Gramineae. Rice is a staple food for the world human population. It is probably the most important grain with regards to human nutrition (Boyce *et al*., 1996). Rice could be milled or unmilled. Unmilled rice has the nutritional components retained in it, however, milled rice has relatively low nutritive value as many of the nutrients are lost during processing. The shelf life of rice grains when harvested raw is long. With or without milling, rice can stay for as long as 6 months to 30years when kept free from contaminants, stored in a cool dry and air tight container or in a resealable heavy duty freezer bag. But when cooked, rice does not stay as long at all (Arifa *et al*.,2015). It was reported that after two hours of cooking, bacteria grow rapidly at temperature between 40º and 140ºF. Some of the organisms said to have been isolated from cooked rice products include *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* and although few data available on the occurrence of fungi in ready-to-eat rice, finding show the presence of *Saccharomyces cerevisiae* and *Aspergillus niger* (Boyce *et al*., 2011 and Arifa *et al*., 2012) [2]. Some of these have been reported to have been isolated from cooked rice and *Bacillus cereus* is responsible for food poisoning (Arifa *et al*., 2012) [2]. The microbial quality of ready-to-eat rice is said to be influenced by a number of factors such as cuisine type, cooking, serving methods and management /food handling (FEHD, 1995) [6].

Cassava (*Manihot esculenta*) after processing last longer even at room temperature than rice and any cereal products. For instance, in Nigeria, cassava products include 'Gaari', 'Lafun' and 'Fufu'. They are staple foods of most Nigerians and it can stay for more than 48hours before showing any sign of spoillage. Etonihu *et al*. (2011) [4] claimed cassava from which these products are derived contain antinutritional factors such as cyanogenic glucosides (Linamarine and Linostralin) which offer protection against some herbivores and also makes the plant toxic for human consumption if not well treated or processed. The traditional processing methods used by farmers and other consumers of cassava products lead greatly to a higher reduction in the cyanide content of the plant. Cyanide content in cassava plant ranges from 15mg/kg to about 1000mg/kg of fresh weight sample, depending on the cultivar of the plant (Okafor, 2014 and Orjiekwe *et al*., 2013) [10]. But it has been discovered that the cyanide content reduced greatly by the processing techniques employed since the cyanide is very soluble in water and highly volatile at a temperature of 26ºC (Enidiok *et al*., 2008) [3]. However, no mater the extent cassava root may be processed, it will still contain some doses of Cyanide (Okafor, 2004) [8]. This raises a concern by researchers as to the quantity of cyanide that should be

contained in processed cassava that is safe for consumption, according to FAO/WHO (1991) [5], for fresh cassava intake it is 10mg of HCN/kg of body weight. For products of cassava in Nigeria, gaari and other products contain low level of cyanide 0.14 ± 0.03 to 0.82 ± 0.32 on dry matter basis that is non-toxic (Okafor, 2004) [8]. There is loss of cyanohydrin and linamarine in processed cassava 'gaari' during short-term storage and when it is prepared for consumption (Onabolu *et al*., 2002) [9], the processing reduces the dietary cyanide load for the consumers.

Materials and Methodology

Preparation of Standard Cyanide Solutions

Samples of different concentrations of Potassium cyanide solution 0.01 to 0,1 mol/dm^3 were prepared and 0.5ml of ninhydrin (2,2- dihydroxyindane-1,3-dione) solution was prepared added to each concentration and a deep red colour was obtained. The absorbance of the mixture was measured using the UV5 spectrophotometer (Mettler Toledo) and the values were recorded. Concentrations of the cyanide were plotted against the absorbance reading to obtain a standard cyanide concentration/absorbance curve.

Determination of Cyanide concentration in the cassava products

1g each of the products of cassava were dissolved in about 5ml of distilled water and centrifuged, about 1ml of the supernatants were taken and 0.5ml of ninhydrin (2,2-dihydroxyindane-1,3-dione) solutions were prepared added to each concentration and a deep red colour was obtained. The absorbance of each prepared mixture was taken and an extrapolation was made on the standard cyanide concentration/absorbance curve to obtain the cyanide concentration values in each product.

Assessment of spoilage microorganisms in the preserved cooked rice

The rice was cooked under aseptic condition and stored in a disinfected air-tight container for 24 to 48 hours after microbial examination and enumeration of total bacteria, yeasts and molds were carried out. Serial dilutions up to 10^{-3} were prepared for the determination of Total Viable Count (TVC) and mold count. Pour plate method was employed for the isolation of microorganisms, about 1ml of serially diluted samples (10^{-2} to 10^{-3} dilutions) were poured in each petri plate with 25ml of nutrient agar (NA), Eosine/Methylene blue agar (EMB), Potato Dextrose Agar (PDA), Mac Conkey Agar (MCA) – for coliform bacteria) while MRS agar was poured in differently on each plate for the isolation of bacteria and fungi loads. The petri plates for the Total Viable Count (TVC) and coliform were incubated at 37^{o}C for 24hrs while plates for fungi were incubated for 48hrs. Colonies with diameter greater than 0.5mm were counted and the microbial loads colony forming units (CFU) of the rice sample were calculated per gram of sample. The analyses were accomplished in duplicate trials. All the plates containing the MRS agar were incubated in an anaerobic environment.

Colony characteristics of the representative colonies of bacteria isolated were noted. The bacteria colonies were Gram stained, similarly, the fungi colonies wee stained and identified under the microscope.

Testing the effect of the prepared cyanide concentration on the spoilage microorganisms

The culture media prepared were allowed to gel and using a 20mm diameter cork borer, wells of equal diameters were bored through the gel. 0.2ml of the prepared cyanide concentration replica of what were found in the three different prepared cassava products were introduced into the well. Sterile distilled water was also introduced into some wells to serve as control. On each plate, streaks of the isolated bacteria colonies were made to cover the whole plate and they were incubated at 37^{o}C for about 24 hrs after the plates were observed for zones of inhibition.

Petri plates containing the pure cultures of the fungi colony isolated from the cooked rice samples were prepared, about 2ml of the different concentration of the cyanide residual values of the cassava processed products were pipetted each into different sterile and aseptical petri plates and Potato Dextrose Agar was prepared and allowed to cool and before it jelled, it was poured into each of the plates, swirled together and left for few minutes to jell. After it has gelled, the plates containing the pure fungi cultures were bored with 7mm cork borer at the centre with equal diameters and incubated at 25-27^{o}C. The growth diameter of the fungi cultures was measured after 48, 72, 96 120 and 144hours and the values for each diameter were recorded.

Results

Cyanide concentration and absorbance

Absorbance readings of different concentrations of potassium cyanide prepared against absorbance readings produced a straight-line graph.

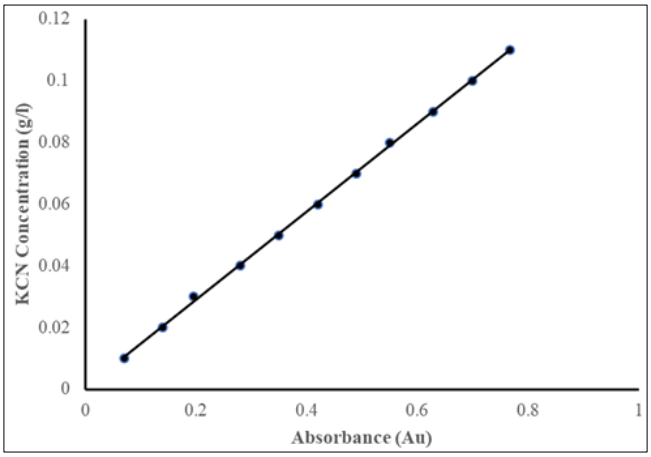

Fig 1

Determination of cyanide content of the processed cassava

The cyanide contents of the products were determined before and after processing the products. The initial cyanide content decreased after these products were processed by cooking. Thereafter, cyanide contents checked over a period of 5days increases, in the processed cassava 'lafun' it increases tremendously up to 48 hours (Table 1) and begin to decrease, the texture became watery, soft and releasing a foul odour; the cyanide contents in the processed cassava gaari, 'eba' increases up to 72 hours (Table 1) and begin to show discolouration but the texture did not change, it was not watery and there was no foul smell. For the cassava processed to 'fufu', the cyanide content was increasing day by day for 5 days (Table 1) before

it began to show any form of discolouration or giving off odour or any sign of spoilage. There were no significant correlations between the residual cyanide contents in the three cassava processed products before and after cooking (*Sig* = 0.947, df =14 and p >.01).

Table 1: Cyanide concentrations of processed cassava products stored for the period of 5 days

Food samples	Mean Cyanide concentrations					
	Befor processing	After processing				
		24hrs	48hrs	72hrs	96hrs	120hrs
Lafun	7.02	2.78 ± 0.12	6.47 ± 1.73	5.74 ± 0.32	5.49 ± 0.43	4.29 ± 0.68
Gaari	10.14	5.61 ± 0.46	7.44 ± 0.76	9.10 ± 0.510	6.53± 0.14	4.98 ± 0.36
Fufu	19.63	11.48 ± 0.23	12.23 ± 0.12	13.35 ± 0.40	14.21 ± 0.32	14.94 ± 0.22

Each value is a mean of triplicate samples ± standard error of mean

Isolation and identification of Microorganisms

Microorganisms were isolated from the cassava products after 24hours to know the microorganisms which may be present as first colonizers, and after 48hours, isolation was done to also know the secondary colonisers. *Bacillus sp.* was found to be in 'gaari' and 'lafun' after 24 and 48hours. It was isolated mostly from 'lafun' in 24hours with up to 144 colony forming units (CFU) at 10^{-2} dilution factor while 'gaari' takes the lowest with 13CFU at 10^{-2} dilution after 48hours.

Some of the fungi species isolated from the products include yeasts (*Saccharomyces cerevisiae*), *Rhizopus* spp, *Aspergillus niger*, *Penicillium* spp. and *Geotrichum* spp (from processed 'gaari' (eba) only) (Table 2). The presence of yeasts was observable in 'lafun' and 'gaari' (eba) in small quantities (up to 9.0 CFU at 10^{-2}) after 48hours but became abundant in 72hours and beyond, while in 'fufu', it was isolated after 72hours in small quantity (at 4.0 CFU at 10^{-2} dilution factor).

Table 2: Microorganisms isolated from the processed cassava products

Microorganisms	Gaari	Lafun	Fufu
Bacillus species	Present but less abundant after 48hours (13 CFU)	Not present	Present but more abundant after 24 hours (144CFU)
Saccharomyces cerevisiae	Isolated after 48 hours (9CFU) but became abundant after 72hours	Isolated after 48 hours in small quantity (9 CFU)	Isolated after 48 hours
Rhizopus spp,	Present after 72 hours	Absent after 72 hours	Present after 48 hours
Aspergillus niger	Isolated after 72 hours	Absent in 120 hours	Present after 48 hours
Penicillium spp	Isolated after 72 hours	Absent in 120 hours	Present after 48 hours
Geotrichum spp	Isolated after 72 hours	Absent	Absent

Microorganisms isolated from the cooked rice samples

Microorganisms were isolated from the cooked rice samples after 24 and 46 hours. Bacteria species were mostly found to be the first colonizers *Bacillus cereus* and *Bacillus subtilis.* Their colony characteristics on plate were almost similar to each other, having a round shaped colony with smooth edge, slightly raised on plate and with an undulated top. They were differentiated after proper identification. Both organisms are gram positive but differ in their morphological characteristics when observed under the microscope. No pathogenic microorganisms were found growing, as there were no growth on the EMB, MRS and MCA. Some fungi species *Saccharomyces cerevisiae* were isolated from the products after 24 and 48 hours (up to 9.0 CFU at 10^{-3} dilution factor).

Effect of the prepared cyanide concentrations on the isolated bacteria and fungi colonies

On bacteria colony, the residual cyanide concentration values of the processed cassava products when prepared and added to the bacteria colonies produced no inhibitory effect after 24 hours of testing.

On fungi colony, the result obtained shows inhibitory effect of *Saccharomyces cerevisiae* in mean growth diameters were significantly different at varying cyanide concentration ($F_{4,20}$ = 5.307, *Sig* =0.004 p = 0 < 0.05) and as the number of days increase ($F_{4,20}$ = 3.557, *Sig* =0.024 p = 0 < 0.05). However, at cyanide concentration values of 0.12 and 0.08g/l inhibitory growth were not very significantly different.

Table 3: Inhibitory effect of cyanide on *Saccharomyces cerevisiae*

Cyanide Concentrations (g/l)	Mean growth diameter				
	Day 1	Day 2	Day 3	Day 4	Day 5
0.16	11.50 ± 1.26	18.83 ±0.92	15.33 ± 0.44	18.33 ± 0.90	19.83± 0.17
0.12	13.33 ± 0.33	16.83 ± 1.01	17.00 ± 0.57	21.92 ± 0.94	23.25 ± 1.01
0.08	13.67 ± 0.93	18.33 ± 1.45	20.62 ± 3.38	24.75 ± 1.75	26.50 ± 2.18
0.04	17.00 ± 4.06	18.66 ± 4.51	23.25 ± 4.99	26.33 ± 5.33	29.58 ± 4.90
Control	17.67 ± 1.15	24.83 ± 6.17	28.42 ± 6.95	32.75 ± 6.31	35.33 ± 5.89

Discussion

The cyanide contents of the cassava products above confirmed that cyanide is lost during processing techniques as the value obtained for each uncooked products was greater than the

values obtained after each product has been processed to gari which is a staple food of the people of Nigeria. Where the product has been obtained, it has a cyanide level which is a little higher than the 10mg/kg recommended by the World Health Organization (WHO, 2004b) [12]. Although Orjiekwe *et al* (2013) [10] reported that gari has the least cyanide contents followed by the unprocessed cassava flour (raw 'lafun') and 'fufu' has the highest cyanide content compared to other cassava products.

Also, considering the result obtained for the products after 24hours, it was noted that the cyanide levels of all the products rose than the initial levels they had after they were processed to values more than the maximum in-take recommended by WHO (2004b) [12]. This may be due to the presence of certain micro-organisms that are capable of producing other forms of cyanide (Knowles, 1976) [7] and this may also be why the cyanide content value of 'fufu' was increasing per day and no preliminary sign of spoilage was noted even after five days and micro-organisms were noted on 'gari' and 'lafun' the day their cyanide content start reducing. Although the cyanide level is significantly lower than the lethal dose of cyanide intoxication of human which has been reported as 200 to 300mg/kg (Akiyama *et al*., 2006) [1] and the oral toxicity standard of 50 to 90mg HCN equivalent /kg body weight (WHO. 2004b) [12] yet over exposure to it has been reported to have significant impact on human health, causing various health challenges such as Tropical Ataxic Neuropathy (TAN), Konzo, Iodine deficiency, dizziness, headache, nausea and vomiting, rapid breathing, restlessness and even weakness (Raji *et al*, 2007, Orjiekwe *et al.,* 2013) [11, 10] and by default we grow up to know that the people in the South Western Nigeria, especially in the villages, consume cassava products which has lasted for more than 24hours, this may be the cause for many of the disease disorder that occur in the villages.

The bacteria isolated from the rice sample, *Bacillus spp* tested negative against being controlled by cyanide, this is in agreement with the report given by Knowles (1976) [7] that certain species of fungi and bacteria have the ability to produce cyanide (i.e they are cyanogenic), some can tolerate cyanide while some can effectively use the cyanide they come in contact with for other metabolic processes. Among the organisms known to effectively covert assimilate and convert low concentrations of cyanide include *Escherichia coli*, *Rhizopus nigricans*, *Fusarium solani* and a species of *Bacillus* known as *B. pyocyaneus* (Knowles, 1976) [7]. Research in this area in the future can look into cyanide toleration and assimilation by other species of *Bacillus* such as *B. cereus* and *B. subtilis* isolated from cooked rice.

Yeast isolated from the same rice sample on the other hand showed large extent sensitive to cyanide at concentration as low as 12mg/kg -16mg/kg. From the result obtained it was noted the yeast could tolerate as low levels of cyanide but more research needs to confirm this assertion, while at high level 16mg/kg, the growth is inhibited. This might be the reason why the organism was occurring in small quantity in the cassava products when the level of cyanide concentration in them is still a little bit high (about 10mg/kg on average) but their number later increased when the cyanide content of the products was reducing as noted.

Conclusion

It is obvious that cyanide is effective in the control of spoilage in food products whose spoilage is initiated by *S. cerevisiae*.

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