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Changes in the Protein and Cyanide Contents of Pupuru as Affected by Duration of Fermentation of Cassava with Species of *Rhizopus*

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Abstract

Fermentation with micro-fungi has been identified as an inexpensive tool for increasing the protein level of substrates in a solid media fermentation technique. This study investigated influence of cassava fermentation using selected species of *Rhizopus* (*R. oryzae, R. oligosporous and R. nigricans*) in an attempt to enhance its nutritional value. Three cassava varieties namely 'odongbo', 'oko-iyawo' and 'arubielu' used were peeled and grated and subsequently inoculated with single culture and combinations from the three species of *Rhizopus* at different fermentation days (2, 4 and 6 days). The fermented mash samples were subsequently processed into pupuru. All the samples were analysed for their protein and cyanide contents. With single species of *Rhizopus* fermentation, protein content of 'pupuru' ranged from 10.20-11.28%. Protein content of 7.94-11.24% was obtained with combination of the organisms. There was a significant reduction in the cyanide level which ranged from 0.42-0.80 mgHCN/kg.

Keywords

Cassava, *Rhizopus*, Fermentation, Pupuru, Protein, Cyanoglucosides

Introduction

Cassava (*Manihot* esculenta Crantz), serves as an important food crop due to its efficient production of food energy, tolerances to extreme stress conditions, availability suitable for present farming and food system in Africa. It is a starchy root crop and a major source of food security in Africa because of its ability to grow in low-quality soil, its resistance to drought and disease, and its flexible cultivation cycle [1-3]. Nigeria is the world's leading cassava producer, with about 21 percent share in the global market [3, 4].

Cassava is the most widely consumed food staple in Nigeria [3, 5] and the main traditional cassava food products in Nigeria are gari, lafun, fufu and pupuru. A cassava staple is consumed by more than 130 million people in Nigeria [6], however it has high levels of carbohydrate but lacks essential nutrients such as protein, fat, vitamins and minerals needed in adequate supply by the body.

The two important methods of increasing the protein content of fermented cassava products have been identified to consist of adding protein to the deficient food from external sources in such a way as not to alter significantly the organoleptic qualities of the food. Consequently, several studies have examined the fortification of cassava meal with vegetable protein like soybean flour, groundnut flour and sesame flour in varying proportions in which for instance the biological value of gari has been found to be raised from 47 to 68 with this blend [7, 8]. The second method is through controlled fermentation where the micro-

flora could be made in large numbers in the mash by solid state fermentation with the use of filamentous microfungi [9].

Fungal fermentation has been identified as an inexpensive tool for increasing the protein level of substrates in a solid media fermentation technique [10]. Solid-state fermentation (SSF) involves the growth of microorganisms on predominantly insoluble substrates, with low moisture content (no free liquid) [11]. SSF is a low energy technique that has played vital role in the use of microorganisms to produce beneficial and commercially viable products. It has been successfully used in the transformation of agricultural wastes such as yam peels [12] and has been exploited for the production of feed from carbohydrate substrates (wastes), which has great potential as animal and livestock feed [13]. The possibility of using fungal strains to upgrade the protein content of cassava products by solid state fermentation has been investigated by several authors [9, 10, 14, 15]. This study is therefore aimed at evaluating the effect of fermentation of cassava variety with selected *Rhizopus* species at different fermentation time on the protein and cyanide contents of pupuru.

Materials and Method

Materials

Three varieties of cassava namely Oko iyawo, Odongbo and Arubielu variety were collected from the Research and Teaching farm of Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria while the *Rhizopus* species (*R. oryzae, R. oligosporous* and *R. nigricans*) that were used were obtained from the Microbiology Unit of Department Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso. The chemicals that were used were of analytical grade manufactured by May and Baker Ltd.

Sub culturing of the species of *Rhizopus*

Each species of the *Rhizopus* was sub-cultured by transferring a bit of each typical colony from its edge onto a sterile Potato dextrose agar (PDA) and incubated for 3-5 days [16]. Following incubation, plates of the organisms were kept for subsequent inoculation into the cassava mash.

Preparation of cassava mash for inoculation with species of *Rhizopus*

The method of Oboh and Elusiyan [9] was adopted with little modifications. Each of the varieties of cassava was peeled, washed, grated and pressed after which 1 kg of the mash was spread on a tray. About 0.5 g freshly sub-cultured pure strains of *R. oryzae, R. oligosporous or R. nigricans* each, their combinations and the consortium of the microorganisms in 730 ml nutrient solution [containing urea (80.0 g), $MgSO_4.2H_2O$ (70.0 g), KH_2PO_4 (13 g) and citric acid (20 g)] was carefully added to the solid matrix and thoroughly mixed. The mash was allowed to ferment for two, four and six days. The control experiment was also carried out on the cassava mash fermented without the *Rhizopus* species. After the fermentation, the mash samples were processed into pupuru as indicated below (Figure 1).

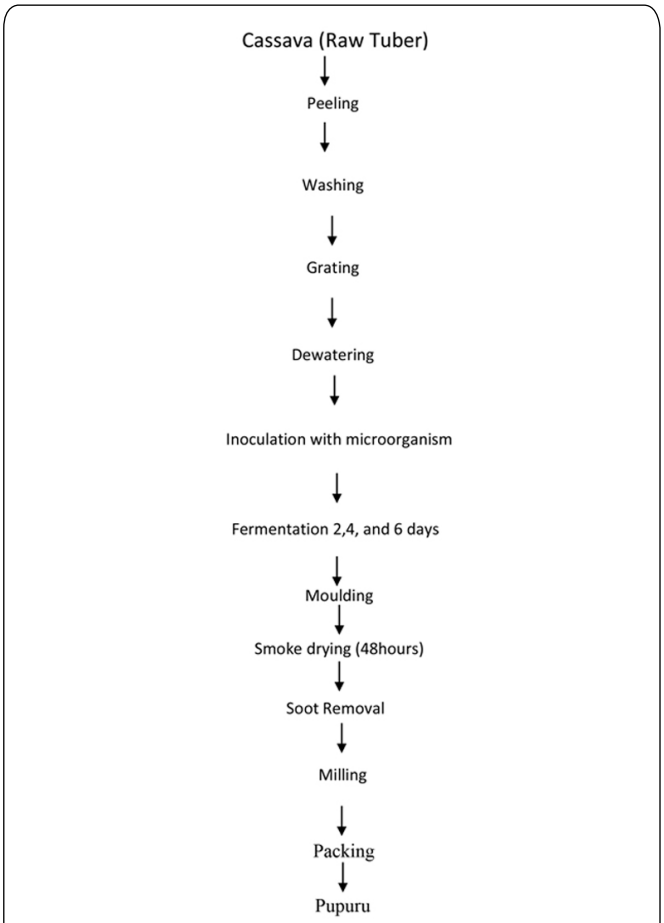

Figure 1: Flow chart illustrates the production of Pupuru.

Pupuru processing

The fermented mash was made into a ball shape and smoked until a light dark-brownish colour was obtained. The smoked ball was then pulverized and sieved followed by toasting and milling into flour [17]. The resultant product known as pupuru was packaged and kept for further analyses.

Chemical analyses

Determination of protein content

This was determined according to the procedure of Official Methods of Analysis of AOAC International, 18th edition [18].

Determination of cyanide content

The method of Nwokoro et al. [19] as modified by Orjiekwe et al. [20] was adopted. Residual cyanogenic glucosides were extracted from the pupuru samples by mixing about 10g of the sample with 50 ml of water in corked conical flask and was allowed to stand for 24 hrs. The mixture was later filtered to obtain the extract cyanogenic glucosides. This was followed by determination of the free cyanide which involves preparation of varying concentrations of standard KCN solution from stock solution. The KCN solution was sealed with Picrate paper after being acidified with 20% HCl and kept at 80 ^{0}C for 10 mins. The picrate paper was removed and kept at room temperature for 24 hr which later turned red. The picrate paper was then rinsed in 50% ethanol solution after which the absorbance was read using spectrometer and was

plotted against the graph after which the amount of cyanide was calculated.

Results and Discussions

Protein content of pupuru as affected by fermentation with species of *Rhizopus*

The results of protein content of pupuru flour samples are presented in Tables 1-3. The protein content of pupuru flour samples obtained from fermentation with single species of *Rhizopus* ranged between 7.94 and 10.32% (Table 1) with Odogbo variety. Fermentation with *R. oryzae* for four days gave the highest value of protein content (10.32%). With Oko-iyawo, the protein content obtained ranged between 8.41 and 10.42% with highest value obtained from fermentation with *R. nigricans* for six days. The lowest value (8.41%) was obtained with fermentation with *R. oligosporous* for six days. With fermentation using single species of *Rhizopus*, significant changes were observed in the protein content of pupuru flour samples across the three varieties of cassava. As fermentation days progressed there was increase in protein content. In Odongbo cassava variety, fermentation with *R. oryzae* in the 4th day of fermentation produced pupuru flour samples with highest protein content of 10.32% but later decreased to 10.21% in the 6th day of the fermentation. Pupuru flour samples obtained from Oko-iyawo cassava variety produced protein content of 10.42% with fermentation using *R. nigricans* with 6 days fermentation which is the highest value among the samples. Lowest value of protein (8.41%) was obtained with fermentation using *R. oligosporous* with 6 days fermentation. Samples of pupuru flour prepared from Arubielu cassava variety indicated the highest protein content of 10.24% with fermentation using *R. oryzae* for 6 days. Moreover, among the samples from the Arubielu cassava variety, protein content was found to increase significantly as fermentation days increased though noticeable decrease was observed at 6 days fermentation with *R. nigricans*.

Table 1: Protein content (%) of pupuru as influenced by length of fermentation with single of species of *Rhizopus*.

	Cassava Varieties		
Pupuru Samples	**Odongbo**	**Oko-iyawo**	**Arubielu**
SP1	10.06^{b}	10.21^{b}	7.24^{g}
SP2	10.32^{a}	9.68^{c}	9.16^{b}
SP3	10.21^{a}	9.54^{f}	10.24^{a}
SP4	8.24^{e}	9.82^{d}	8.24^{e}
SP5	7.94^{d}	9.52^{f}	8.52^{c}
SP6	10.14^{a}	8.41^{g}	8.93^{c}
SP7	10.24^{a}	9.54^{f}	7.54^{e}
SP8	9.25^{b}	9.62^{e}	7.36^{f}
SP9	9.36^{b}	10.42^{a}	7.25^{h}
NP	3.33^{e}	3.41^{h}	3.25^{i}

Values are means of triplicate determinations.
Means in the same column bearing different superscript are significantly different ($p < 0.05$).
SP1- Fermentation with *R. oryzae* for 2 days.
SP2 - Fermentation with *R. oryzae* for 4 days.
SP3 - Fermentation with *R. oryzae* for 6 days.
SP4 - Fermentation with *R. oligosporous* for 2 days.
SP5 - Fermentation with *R. oligosporous* for 4 days.
SP6 - Fermentation with *R. oligosporous* for 6 days.
SP7 - Fermentation with *R. nigricans* for 2 days.
SP8 - Fermentation with *R. nigricans* for 4 days.
SP9 - Fermentation with *R. nigricans* for 6 days.
NP - Control, Natural Fermentation (without inoculation fermented for three days).

Using combination of species of *Rhizopus* as indicated in Table 2 for fermentation, pupuru flour samples indicated higher amount of protein content particularly with samples from Oko-iyawo cassava variety (10.24-11.94%). The values were higher than the ones obtained in samples obtained from fermentation with single species of *Rhizopus*. Similarly significant changes were observed among the samples with combination of the species of *Rhizopus* for fermentation.

Table 2: Protein content (%) of pupuru as influenced by length of fermentation with combination of species of *Rhizopus*.

	Cassava Varieties		
Pupuru Samples	**Odongbo**	**Oko-iyawo**	**Arubielu**
MP1	10.92^{a}	11.92^{a}	8.24^{g}
MP2	10.44^{e}	11.94^{a}	8.42^{h}
MP3	9.44^{g}	10.94^{d}	9.20^{f}
MP4	10.24^{f}	10.94^{d}	9.00^{g}
MP5	10.64^{d}	10.84e	9.35^{e}
MP6	9.84^{g}	11.00^{d}	9.45^{d}
MP7	10.74^{c}	11.24^{b}	10.42^{c}
MP8	10.82^{b}	11.15^{c}	10.52^{b}
MP9	10.84^{b}	10.24^{e}	10.62^{a}
NP	3.30^{h}	3.41^{f}	3.24^{j}

Vales are means of triplicate determinations.
Means in the same column bearing different superscript are significantly different ($p < 0.05$).
MP1- Fermentation with combination of *R. oryzae* and *R. oligosporous* for 2 days.
MP2 - Fermentation with combination of *R. oryzae* and *R. oligosporous* for 4 days.
MP3 - Fermentation with combination of *R. oryzae* and *R. oligosporous* for 6 days.
MP4 - Fermentation with combination of *R. oryzae* and *R. nigricans* for 2 days.
MP5 - Fermentation with combination of *R. oryzae* and *R. nigricans* for 4 days.
MP6 - Fermentation with combination of *R. oryzae* and *R. nigricans* for 6 days.
MP7- Fermentation with combination of *R. oligosporous* and *R. nigricans* for 2 days.
MP8 - Fermentation with combination of combination of *R. oligosporous* and *R. nigricans* for 4 days.
MP9 - Fermentation with combination of *R. oligosporous* and *R. nigricans* for 6 days.
NP - Control, Natural Fermentation (without inoculation fermented for three days).

Using the consortium of the three species of *Rhizopus* for fermentation (Table 3) produced pupuru flour sample with highest protein content of 11.05% with fermentation using *R. oryzae* fermented for 2 days in Arubielu cassava variety. Similarly significant changes were recorded in the protein

Table 3: Protein content (%) of pupuru as in influenced by length of fermentation with consortium of species of *Rhizopus*.

	Cassava Varieties		
Pupuru Samples	**Odongbo**	**Oko-iyawo**	**Arubielu**
CP1	11.02^{a}	10.24^{a}	11.05^{a}
CP2	10.42^{b}	9.14^{b}	10.50^{b}
CP3	10.33^{b}	10.24^{a}	9.42^{c}
NP	3.33^{c}	3.41^{c}	3.25^{d}

Values are means of triplicate determinations.
Means in the same column bearing different superscript are significantly different ($p < 0.05$).
CP1: Fermentation with consortium of the three species for 2 days.
CP2: Fermentation with consortium of the three species for 4 days.
CP3: Fermentation with consortium of the three species 6 days.
NP: Control Sample by natural fermentation.

content across the cassava varieties as fermentation days progressed. Likewise control samples from all the treatments were lower in protein content than the samples obtained from fermentation with species of *Rhizopus*.

According to Akindahunsi et al. [21] and Okafor N. [22], the increase in protein may be due to metabolic activities of the microfungi as a result of extracellular enzymes during the fermentation of cassava mash which according to Reed G. [23] has been enhanced by the presence of ammonia as a nitrogenous base of the medium. This has resulted to the multiplication of the fungi in form of single cell protein. This may also be as a result of the urea added as the basal medium for the organisms.

Changes in protein quality and quantity during different fermentation techniques have also been studied. Crude protein was enhanced to 2.56% during fufu production and to 3.68% in *Pukuru* (Pupuru) but was reduced to 1.43% in gari and 1.14% in Kpokpo gari compared with 2.04% in cassava chips [24]. Although there was reduction in protein content in the latter products, there was general improvement in feed intake and growth rate of rats fed on diets containing fermented products. During fufu production, a 20% reduction in protein content was observed. At the end of 72 h, value dropped from 1.5% to 0.9%, subsequently increasing to 1.2%, which might be attributed to increased biomass production [25].

Cyanide content of pupuru as affected by fermentation with species of *Rhizopus*

The results of cyanide content of pupuru as influenced by fermentation with species of *Rhizopus* are presented in Figures 2-4. With fermentation using single species of *Rhizopus*, significant changes were observed as fermentation days progressed across the three varieties of cassava with cyanide content ranged from 0.52 -0.75 mgHCN/kg (Odongbo cassava variety); 0.32-0.73 mgHCN/kg (Oko-iyawo cassava variety) and 0.25-0.72 mgHCN/kg (Arubielu cassava variety).

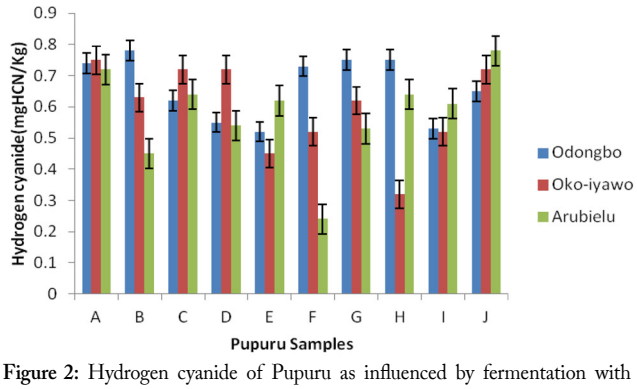

Figure 2: Hydrogen cyanide of Pupuru as influenced by fermentation with single species of *Rhizopus* with different cassava varieties.
A = Control
B = Fermentation with *R. oryzae* for 2 days
C = Fermentation with *R. oryzae* and for 4 days
D = Fermentation with *R. oryzae* for 6 days
E = Fermentation with *R. oligosporous* for 2 days
F = Fermentation with *R. oligosporous* for 4 days
G = Fermentation with *R. oligosporous* for 6 days
H = Fermentation with *R. nigricans* for 2 days
I = Fermentation with *R. nigricans* for 4 days
J = Fermentation with *R. nigricans* for 6 days

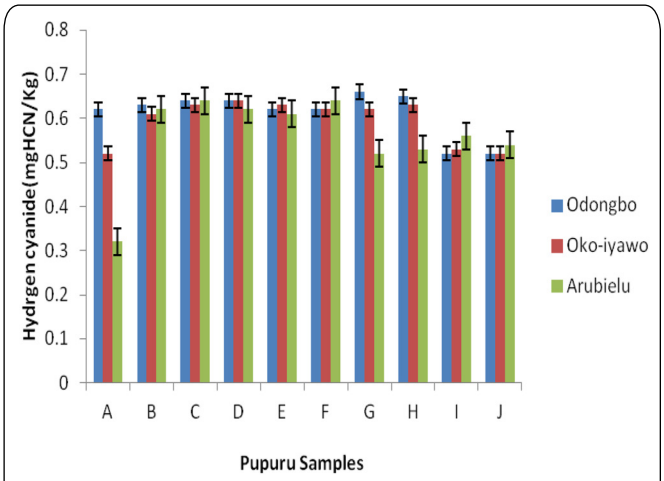

Figure 3: Hydrogen cyanide of Pupuru as influenced by fermentation with combination of species of *Rhizopus* with different cassava varieties.
A -Sample from fermentation with combination of *R. oryzae* and *R. oligosporous* for 2 days
B -Sample from fermentation with combination of *R. oryzae* and *R. oligosporous* for 4 days
C -Sample from fermentation with combination of *R. oryzae* and *R. oligosporous* for 6 days
D -Sample from fermentation with combination of *R. oryzae* and *R. nigricans* for 2 days
E - Sample from fermentation with combination of *R. oryzae* and *R. nigricans* for 4 days
F - Sample from fermentation with combination of *R. oryzae* and *R. nigricans* for 6 days
G - Sample from fermentation with combination of *R. oligosporous* and *R. nigricans* for 2 days
H - Sample from fermentation with combination of *R. oligosporous* and *R. nigricans* for 4 days
I -Sample from fermentation with combination of *R. oligosporous* and *R. nigricans* for 6 days
J - Control, Natural Fermentation

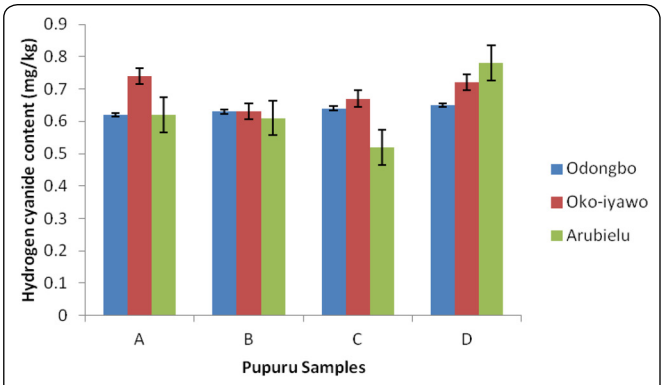

Figure 4: Hydrogen cyanide of gari as influenced by fermentation with consortium of three species of *Rhizopus* with different cassava varieties.
A - Fermentation with consortium of the three species for 2 days.
B - Fermentation with consortium of the three species for 4 days.
C - Fermentation with consortium of the three species 6 days.
D - Control Sample by natural fermentation.

Pupuru samples obtained from fermentation using combination of species of *Rhizopus* indicated the value of cyanide to range between 0.56-0.64 mgHCN/kg (Odongbo); 0.52-0.62 mgHCN/kg (Oko-iyawo cassava variety) and 0.25-0.72 mgHCN/kg (Arubielu cassava variety). Pupuru samples obtained from fermentation with consortium of species of *Rhizopus* indicated the values of cyanide to range between 0.61- 0.62 mgHCN/kg (Odongbo cassava variety); 0.60-

0.72 mgHCN/kg (Oko-iyawo cassava variety) and 0.53-0.60 mgHCN/kg (Arubielu).

Fermentation with species of *Rhizopus* reduced the cyanide content to a safe level in all the samples of Pupuru. A value of 2-3 mg/100g has been regarded as acceptable level of cyanide in gari [26]. The cyanide levels of the pupuru sample falls within the recommended acceptable and safe levels, however studies have shown that long exposure to small doses of cyanide could be fatal and result in the increased blood cyanide levels with symptoms like headache, dizziness, nausea, vomiting, paralysis, nerve lesions and miscarriages [20, 27, 28].

Conclusion

Protein content of pupuru samples obtained from fermentation of cassava with combinations of the species of *Rhizopus* was observed to be higher than the samples obtained from fermentation with single species of the organisms. The protein content of the control sample obtained by natural fermentation was much lower than the samples obtained from fermentation with the species of *Rhizopus*. The cyanogenic glucosides levels in the pupuru samples ranged from 0.42 to 0.80 mgHCN/Kg which is relatively very safe and within the acceptable limit of 10 mg HCN equivalent/Kg body weight recommended by FAO.

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