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Comparative effect of boiling and solid substrate fermentation using the tempeh fungus (*Rhizopus oligosporus*) on the flatulence potential of African yambean (*Sphenostylis stenocarpa* L.) seeds

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Abstract

The tropical African yambean (AYB, *Sphenostylis stenocarpa* L.) is a protein-rich underutilized African legume. The presence of the flatulence- and diarrhoea-causing raffinose family oligosaccharides (RFO: raffinose, stachyose and verbascose) or α-galactosides has limited the food use of African yambean seeds. To reduce this limitation, non-traditional processing methods are required. Seeds of three varieties were (i) examined for the flatulence- and diarrhoea-causing RFO and (ii) fermented with *Rhizopus oligosporus* for tempeh production. The traditional tempeh production process involved dehulling, soaking in water for 24 h, boiling in water for 30 min, inoculation and fermentation. In addition, the traditional tempeh procedure was modified by using 1% citric acid solution instead of water for soaking and cooking. Comparisons with traditionally cooked beans, which involved boiling in water for 4 h, were made. Boiling seeds for 4 h resulted in 8–30% reduction of total α-galactosides in the three varieties, while the traditional tempeh procedure resulted in an almost complete loss (98%) of the same ($P < 0.05$). The modified procedure resulted in a bacteria-free tempeh but α-galactoside reduction was 22–39%. Both tempeh production processes were clearly more effective than was traditional cooking in reducing the flatulence potential of the AYB seeds.
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1. Introduction

The problem of protein malnutrition persists in Africa, partly because animal protein is unaffordable to the majority of the population. Interest is rising in developing countries in low-cost, protein-rich plant foods, as replacements for, or supplements to, the expensive and frequently scarce animal foods. Legumes have a high potential in this respect because they are rich in protein. Many legumes are, however, underutilized. In this respect, the African yambean (*Sphenostylis stenocarpa*) deserves considerable attention. The African yambean is among the less-known legumes of the humid tropics. It is cultivated in West Africa for its seeds and in East and Central Africa mainly for its tubers. In southern Nigeria, African yambean seeds were preferred to other legumes in the past because they are filling, and for unclear cultural reasons. Cowpea is now the preferred legume (Azeke, Fretzdorff, Buening-Pfaue, Holzapfel, & Betsche, 2005). The chemical composition of African yambean (AYB) has been addressed by several workers (Adeyeye, 1997; Apata & Ologhobo, 1990; Edem, Amugo, & Eka, 1990). A protein content of between 200 and 290 g kg^{-1} has been reported. This is lower than that of soybean (380 g kg^{-1}), but the lysine proportion in the

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protein is reported to be equal to or higher than that in soybean (Agunbiade & Longe, 1999; Apata & Ologhobo, 1990; Evans & Boutler, 1974). The proportion of most essential amino acids corresponds to the WHO/FAO recommendation (Evans & Boutler, 1974). There are, however, at least two major constraints hindering a more extensive use of African yambean in the nutrition plan of Nigeria; these are the presence of the flatulence-causing α-galactosides and the extremely long cooking time required to render the beans palatable. There are only a few reports in the literature about the α-galactoside contents of African yambean (Oboh et al., 2000). Consumers of African yambeans in Edo State, Mid-Western Nigeria, were interviewed about experienced health problems with African yambeans (Azeke et al., 2005). Questionnaires were not applicable under the given conditions. In order of frequency, flatulence, stomach cramp, diarrhea and even dizziness were the common problems experienced by consumers. Although all people interviewed reported the occurrence of several health nuisances, reported frequencies were different: flatulence is general and severe with the black and the brown seeds; stomach cramps are often experienced with all varieties, but the frequency is lower. The interviews confirmed that the common method of processing is cooking in water for as long as 6–8 h, which nonetheless leaves the beans stodgy. Tempeh is a traditional Indonesian fermented food made from soaked and cooked soybean inoculated with a fungus, usually of the genus Rhizopus. After the fermentation, the cooked soybeans are bound together by the mycelium into a compact, white meat-like cake. An important function of the fungus in such food fermentation is the synthesis of enzymes, which hydrolyze soybean constituents and contribute to the development of a desirable texture, aroma and flavour (Paredes-López, Harry, & Gonzalez-Castaneda, 1990). Such enzymatic hydrolysis may also decrease or eliminate antinutritional components and consequently improve the nutritional quality of the fermented product. In the light of the existing problems, three varieties of African yambeans seeds were subjected to solid substrate fermentation in order to effectively reduce α-galactoside contents, thereby improving nutritional quality. Moreover, the application of the processes that we evaluated should be feasible in households and small-scale industries.

2. Materials and methods

2.1. Materials

Seeds of three varieties of African yambean, black, marble and white, were used for this study to ascertain varietal differences, if any, in the response of African yambeans to treatment. The seeds were cultivated and harvested in the same period (January/February 2000) in Edo State in the southern part of Nigeria. *Rhizopus oligosporus* (DSMZ 1964) was obtained from the German Collection of Microorganisms and Cell cultures (DSMZ), Brunswick, Germany.

2.2. Mechanical dehulling

Due to the hardness of seeds of the three AYB varieties, dehulling was done by mechanical abrasion of the seeds by means of a vertical dehulling machine (Type 1, Schule GmbH, Germany). About 1 kg African yambean seeds was placed in the machine for 2–3 min, by which time all the hulls and a part of the endosperm (amounting to 30% of seed weight) were abrased. This was done for the three varieties. The seeds of only the white variety, which are bigger than the other varieties, were further broken down to smaller sizes. This was achieved with the aid of a milling machine, "Prallmühle" (Jehmlich GmbH, D-01683, Nossen, Germany), operating at a frequency of 1.2 Hz. The broken seeds were then passed through a sieve of a pore size of 6.5 mm. The fraction of sample with particle size >6.5 mm (about 55% of the lot) was used for tempeh production. That leaves 45% of unused seeds, which could be processed for the production of animal feed. The larger sizes of seeds of the white variety can hinder tight-packing of seeds during fermentation. Tight-packing contributes to the quality of the final product (Kovac & Raspor, 1997).

2.3. Preparation of spore suspension

R. oligosporus was grown on 12 ml of potato dextrose agar in Petri dishes at 30 °C for 7 days. Spores were harvested by adding 10 ml of sterile water and scraping with a spatula. The suspension was centrifuged at 3000 rpm for 1 min and the sediment containing the spores was resuspended in 5 ml of sterile water, to give suspensions containing about 10^6 spores/ml. The suspension was stored frozen at −20 °C until required (usually within 3 days).

2.4. Tempeh production using R. oligosporus

Tempeh was produced by the traditional procedure employed for soybean (Kovac & Raspor, 1997) as well as by a modified procedure (Fig. 1). The traditional procedure is as follows: dehulled bean seeds were soaked overnight in deionized water; the soak water was drained off and the seeds were cooked for 30 min in boiling deionized water. The seeds were drained and allowed to cool down to room temperature. Portions of 200 g were inoculated with 5 ml of spore suspension, mixed and then packed tightly in perforated polythene bags. The samples were incubated at 30 °C and 75% humidity for 48 h. The tempeh was then dried in an air-oven at 50 °C for about 18 h, milled to a particle size of <0.5 mm and stored at 4 °C pending analysis. Bacterial count was determined by the most probable number method of Oblinger and Koburger (1975). In the modified procedure, 1% of citric acid solution was used for soaking and cooking instead of distilled water. This reduced the pH of seeds from 6.6–6.8 to 3.85. The modified procedure was used because cooling of boiled seeds and subsequent fermentation were done in utensils and

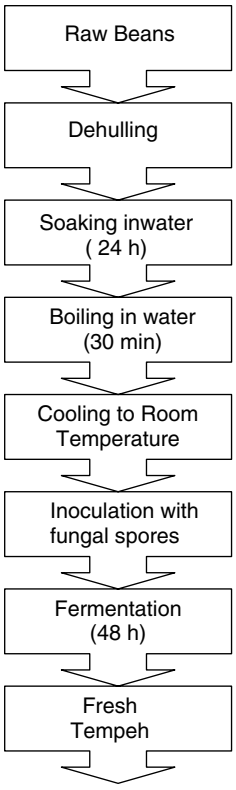

Fig. 1. Flow chart for the production of tempeh. In the modified procedure, 1% citric acid solution is used instead of water.

environments that are not entirely sterile. Consequently, microbial contamination occurs during tempeh production. The absence of bacteria in seeds boiled in citric acid and tempeh cultures produced was confirmed by adding cotyledons to thioglycolate broth (Oxoid, CM173) and incubating at 30 °C for 3 days. Turbidity was not observed in any tube (De Reu, Rombouts, & Nout, 1995; Nout & Rombouts, 1990; van der Riet, Wight, Cilliers, & Datel, 1987).

2.5. Traditional cooking procedure

The dry seeds were cooked in about 4 times their volume of already boiling deionized water for 4 h, by which time all seeds were soft when pressed between the fingers. This was ascertained by several random samplings. After draining, the cooked seeds were dried in an oven at 50 °C for about 18 h, milled to a particle size of <0.5 mm and stored at 4 °C pending analysis.

2.6. α-Galactoside analyses

Soluble sugars were extracted using 80% v/v ethanol (Munzquiz, Rey, & Cuadrado, 1992). In order to optimize extraction, portions of white-seed-meal were extracted once for 30 min, once for 60 min, twice for 30 min and thrice for 30 min. Extracting twice gave a higher yield; the third extraction did not significantly increase the yield ($P < 0.05$). Extraction was therefore done twice: a 2 g sample (<0.5 mm) was extracted twice with 50 ml of 80% ethanol at 60 °C, centrifuged at 3000*g* for 10 min, and the supernatants were vacuum-evaporated. The residues were dissolved in 8 ml of double-distilled water. The effects of three deproteinizing agents, activated charcoal, acetonitrile and Carrez solutions,[1] on sugar yield were compared. All agents produced clear extracts. Activated charcoal and acetonitrile gave lower yields than did Carrez solution. Consequently, Carrez clearing was used for clean-up. The extract was deproteinized with 100 μl each of Carrez solutions I and II, centrifuged and the supernatant filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), which was pre-wetted with 4 ml of methanol and 2 ml of water. A 2.5 ml aliquot was then made up to 10 ml with acetonitrile, centrifuged and the supernatant was used for analysis by HPLC as described by Munzquiz et al. (1992) and Oboh et al. (2000) with a modified mobile phase. A Spherisorb-5-NH_2 column, 250 × 4.6 mm ID (Macherey and Nagel, Düren, Germany), was employed, using acetonitrile–water (60:40 w/w) at a flow rate of 1 ml min^{-1}. Quantification was done on the basis of peak heights. External standards of raffinose, stachyose and verbascose were used for calibration. A linear response was recorded in the range of 0–4 mg ml^{-1} with a correlation coefficient of 0.999 for all standards. Recovery of the α-galactosides tested was 85.6–114%. Coefficients of variation were 2.09% for raffinose, 0.88% for stachyose and 2.95% for verbascose ($n = 10$).

2.7. Statistics

Significance of difference was tested by analysis of variance and Turkey's HSD, using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. α-Galactoside contents of raw AYB seeds

Significant differences in α-galactoside (raffinose, stachyose and verbascose) contents were found among seeds of the three African yambean varieties used in this work ($P < 0.05$). The values ranged from 2.25 to 3.39 g 100 g^{-1} for the three varieties (Table 1). The total α-galactoside contents for AYB reported here were compared with available data. Oboh et al. (2000) reported a similar total α-galactoside content to that in the work presented here. Nwinuka, Abbey, and Ayalogu (1997) reported a higher α-galactoside content (excluding verbascose) for an unspecified AYB variety. The authors employed thin-layer chromatography of the crude sugar extract; therefore, other

[1] Carrez solution I is prepared by dissolving 23.8 g of zinc acetate trihydrate and 3 g of glacial acetic acid in water and diluting to a volume of 100 ml with water. Carrez solution II is prepared by dissolving 10.6 g of potassium ferrocyanide in water and diluting to a volume of 100 ml with distilled water.

Table 1
α-Galactoside contents (g 100 g^{-1}) of raw African yambean seeds

Oligosaccharide	Black	Marble	White
Raffinose	0.60 ± 0.03^{a}	0.82 ± 0.06^{b}	0.29 ± 0.03^{c}
Stachyose	2.17 ± 0.01^{a}	2.46 ± 0.04^{b}	1.81 ± 0.04^{c}
Verbascose	0.15 ± 0.00^{a}	0.11 ± 0.02^{b}	0.15 ± 0.00^{b}
Total α-galactoside	2.92	3.39	2.25

Results are means of triplicate determinations and standard deviation. The values in rows with different letters are significantly different from each other ($P < 0.05$).

substances could have interfered with the assay of oligosaccharide.

3.2. α-Galactoside contents of cooked AYB seeds

The changes in total α-galactoside contents of AYB seeds accompanying traditional cooking are shown in Table 2. There were between 9% and 26% reductions in galactosides following cooking for 4 h. This is, to some extent, in contrast with the reports for other legumes. Ibrahim, Habiba, Shatta, and Embaby (2002) reported 100% reduction in α-galactoside of cowpea seeds after boiling seeds for 45 min, while Abdel-Gawad (1993) reported reductions of 42–47% for faba beans, lentils and common beans after boiling seeds for 60 min. The lower reduction in α-galactosides observed here for AYB seeds may be a result of the very hard nature of their hulls. α-Galactosides may therefore play a major role in the etiology of the health problems, outlined in the introduction, associated with the consumption of cooked AYB seeds. This underscores the need to subject the seeds to other non-traditional methods of processing.

3.3. Traditional tempeh production

At the end of the tempeh fermentation, the seeds were bound together into a compact, white cake by a dense cottony mycelium of the mould, *R. oligosporus*. No sporulation was observed. Fungal sporulation during tempeh production reduces the sensory acceptance of tempeh (Kovac & Raspor, 1997). Fungal growth was inhibited in the white variety. It was initially thought that the grain size of AYB-white seeds made tight-packing impossible, leading to large air spaces, which inhibited fungal growth (Kovac & Raspor, 1997; Paredes-López et al., 1990). Breaking the seeds into smaller pieces enhanced tighter packing, but fungal growth did not improve, indicating that fungal growth may have been inhibited by other factors inherent to the seeds. Literature search showed that there has been only one reported attempt to produce tempeh from AYB (Njoku, Ofuya, & Ogbulie, 1991). The emphasis was on meat pie formulations, sensory evaluation and acceptance but not on the nutritional value of African yambean tempeh. The authors reported sporulation and the loss of pleasant odour at 48 h. This could have been due to the higher inoculum (6×10^7 cells ml^{-1}) and temperature (32.5 °C) used. Inoculum and incubation temperature used in this work were 10^6 cells ml^{-1} and 30 °C, respectively. Nout and Rombouts (1990) reported that inoculation at approximately 10^4 colony forming units ml^{-1} is optimal.

Table 2
α-Galactoside contents (g 100^{-1}) of cooked African yambean

Oligosaccharide	Black	Marble	White
Raffinose	0.22 ± 0.02^{a}	0.46 ± 0.03^{b}	0.13 ± 0.03^{c}
Stachyose	1.95 ± 0.01^{a}	2.20 ± 0.08^{b}	1.43 ± 0.04^{c}
Verbascose	0.15 ± 0.03^{a}	0.13 ± 0.00^{b}	0.10 ± 0.00^{ab}
Total α-galactoside	2.32	2.79	1.66
% Reduction	20.5	8.5	25.9

Results are means of triplicate determinations ± standard deviation. The values in rows with different letters are significantly different from each other ($P < 0.05$).

3.4. The modified tempeh procedure

The bacterial load of AYB-tempeh, produced by the traditional process was high (10^9 most probable number g^{-1}, MPN g^{-1}), while tempeh made by the modified process was bacteria-free and of lighter colour than the other tempeh. The absence of bacteria in seeds boiled in citric acid and tempeh cultures produced was confirmed by adding cotyledons to thioglycolate broth (Oxoid CM173) and incubating at 30 °C for 3 days. Turbidity was not observed in any tube. As mentioned earlier, fungal growth on the AYB-white was inhibited in the traditional procedure. However, in the modified procedure, fungal growth improved as the seeds were bound together into a cake by fungal mycelium after 48 h.

3.5. α-Galactoside contents of AYB tempeh

Because tempeh-type fermentation produced similar effects on the α-galactosides of the three varieties used in this work, the results presented in this section are means for the three varieties. The changes in total α-galactoside contents of AYB seeds accompanying treatments during tempeh production are shown in Table 3. These treatments, as outlined in Section 2, include mechanical dehulling and soaking for about 24 h, followed by 30 min boiling, inoculation with fungal spores (after cooling) and incubation for 48 h at 30 °C. Mechanical dehulling resulted in an average of 5% increase in total α-galactoside contents of AYB seeds. This increase may be due to a concentration effect. Losses due to soaking and cooking of the dehulled seeds were 5.5% and 33.2%, respectively. The subsequent fermentation by *Rhizopus oligosporus* resulted in an almost complete loss (98%) of total α-galactosides (Table 3). This result is similar to the findings of van der Riet et al. (1987) for soybean and Ibrahim et al. (2002) for cowpea. They reported oligosaccharide losses of 91% and 100%, respectively, after tempeh type fermentation. The almost complete loss of α-galactoside may be due to the effects

Table 3
Changes in α-galactoside contents (g 100 g^{-1}) of AYB seeds[a] during tempeh production

AYB	Raffinose	Stachyose	Verbascose	Total RFO	% RFO reduction[b]
Raw	0.57 ± 0.27^{a}	2.15 ± 0.33^{a}	0.14 ± 0.02^{a}	2.85 ± 0.57^{a}	0.0
Dehulled	0.48 ± 0.22^{ab}	2.30 ± 0.40^{a}	0.16 ± 0.02^{a}	2.94 ± 0.59^{a}	+5.0
Soaked (24 h)	0.35 ± 0.17^{ab}	1.88 ± 0.21^{ab}	0.15 ± 0.01^{a}	2.38 ± 0.37^{a}	5.5
Cooked (30 min)	0.26 ± 0.10^{ab}	1.38 ± 0.08^{b}	0.14 ± 0.04^{a}	1.77 ± 0.17^{a}	33.2
Tempeh	0.02 ± 0.02^{b}	0.06 ± 0.06^{c}	0.02 ± 0.01^{b}	0.10 ± 0.08^{b}	98.3

The values in rows with different letters are significantly different from each other ($P < 0.05$).
[a] Results are means for three varieties ± standard deviation.
[b] % Reduction relative to the raw seeds.

Table 4
Effect of acidity on the changes in the contents[a] of α-galactosides (g 100^{-1}) during tempeh-type fermentation

	Raffinose	Stachyose	Verbascose	Total RFO	% RFO reduction[b]
AYB-Marble					
Raw	0.82 ± 0.05^{a}	2.46 ± 0.01^{a}	0.11 ± 0.00^{a}	3.39	0.0
Dehulled	0.70 ± 0.03^{b}	2.63 ± 0.00^{a}	0.14 ± 0.01^{a}	3.47	+2.0
Soaked and cooked	0.39 ± 0.03^{c}	1.67 ± 0.02^{b}	0.14 ± 0.02^{a}	2.20	35.1
Tempeh inoculated (48 h)	0.38 ± 0.00^{c}	1.55 ± 0.01^{b}	0.13 ± 0.01^{a}	2.06	39.2
AYB-White					
Raw	0.29 ± 0.00^{a}	1.81 ± 0.00^{a}	0.15 ± 0.00^{a}	2.25	0.0
Dehulled	0.23 ± 0.00^{b}	1.70 ± 0.07^{a}	0.14 ± 0.01^{a}	2.07	8.0
Soaked and Cooked	0.21 ± 0.00^{b}	1.46 ± 0.03^{b}	0.14 ± 0.02^{a}	1.81	19.6
Tempeh inoculated (48 h)	0.22 ± 0.03^{b}	1.40 ± 0.00^{b}	0.13 ± 0.01^{a}	1.75	22.2

[a] Mean of triplicate determination ± standard deviation.
[b] % Reduction relative to the raw seeds.

of hydrolytic enzymes produced by the accompanying microbial flora and/or by the fungus.

Table 4 shows the changes in the α-galactoside contents of seeds of AYB-marble and AYB-white accompanying treatments for the modified tempeh procedure. Results show that the two varieties responded slightly differently to treatments. Soaking for 24 h and the subsequent 30 min boiling resulted in α-galactoside losses of 35% and 20%, respectively, for the marble and white varieties. This is almost similar to the losses observed for the traditional procedure (33%). Fermentation of the acidified seeds for 48 h resulted in non-significant α-galactoside losses of 4% and 3% for AYB-marble and AYB-white. This implies that the α-galactoside concentrations of fermented seeds are about 60% and 78%, respectively, of the values for the raw seeds. This contrasts sharply with the almost complete loss of α-galactoside in the fermented seeds of the traditional process (non-acidified). These results confirm the report of Ruiz-Terán and Owens (1999) who produced a bacteria-free tempeh from soybean by acidification of the soak medium with 0.11 M lactic acid. The authors reported that the fungus did not contribute to the total α-galactoside losses during fermentation. They attributed the loss of α-galactoside to leaching during the soaking and cooking of seeds (Ruiz-Terán & Owens, 1999). There are, however, two possibilities: either *R. oligosporus* does not produce α-galactosidase needed for oligosaccharide breakdown, or the fungal α-galactosidase is not active at low pH. This means that only the accompanying microflora may have contributed to the reduction in α-galactoside in the traditional procedure. More research is, however, needed in this area.

The implication of these results on the flatulence potential of AYB is discussed below. The raffinose family oligosaccharides (RFOs, or raffinose, stachyose and verbascose) are of physiological importance because of their role in the aetiology of flatulence and diarrhoea. It has been reported that 5 g of raffinose can produce 300 ml of gas after fermentation in the human colon (Calloway & Murphy, 1968; Dibofori, Okoh, & Onigbinde, 1994; Fleming, 1981). In this study totals of 2.92, 3.39 and 2.25 g 100 g^{-1} RFO were found in the raw AYB-black, AYB-marble and AYB-white seeds, respectively. A 200 g portion of raw AYB seeds, therefore, has a theoretical flatulence potential of 250–400 ml of flatus volume (Table 5). After traditional tempeh-type fermentation with *Rhizopus*, the flatus potential of AYB seeds is expected to decrease by more than 97%. The flatus potential of samples cooked for 4 h is expected to remain high. Flatus

Table 5
Flatus potential (ml gas 200 g^{-1}) of AYB seeds as affected by processing

	Black	Marble	White
Raw	350	407	269
Tempeh (48 h)	6.0	9.6	9.6
Modified tempeh (48 h)	nd	247	210
Cooked (4 h)	278	335	199

nd: not determined.

potential of tempeh produced from acidified samples is also expected to remain high (200–250 ml) for the same quantity of sample.

In this work, boiling seeds for 4 h, and solid substrate fermentation using the tempeh fungus *R. oligosporus*, were compared as to their efficiency in reducing the flatulence potential of seeds of the tropical African yambean. The traditional tempeh procedure was clearly more effective than was the modified procedure while both traditional and modified procedures were more effective than was boiling in water. Household application of the traditional procedure has the potential of improving the utilization, not only of the yambean seeds, but also of other underutilized legumes.

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