Influence of Solid State Fermentation Technology on Bush Mango (*Irvingia gabonensis*) Seed Cotyledons; Proximate and Microbial Quality

OO Olusola-Makinde[1] and YD Oluwafemi[2]*

[1]Department of Microbiology, School of Sciences, Federal University of Technology, Akure, Ondo State, Nigeria
[2]Department of Biological Sciences, Faculty of Science, University of Medical Sciences, Ondo, Ondo State, Nigeria

*Corresponding Author: YD Oluwafemi, Department of Biological Sciences, Faculty of Science, University of Medical Sciences, Ondo, Ondo State, Nigeria.

Abstract

Fermentation is well-known to advance the dietary worth of foods. This study was piloted to evaluate the impact of fermentation on bush mango with the aim of mitigating environmental devaluation. The seed cotyledons of bush mango were subjected to solid state fermentation for 96h at room temperature. Isolation and presumptive identification of bacteria and fungi associated with the fermentation process were carried out using standard colonial, morphological and biochemical microbiological techniques. Comparative analyses were determined between unfermented seeds and the fermented products by proximate constituent, nutrient component and selected anti-nutrient compositions. Bacteria identified from fermented bush mango seeds were *Bacillus* spp., *Staphylococcus* spp. and *Lactobacillus fermentum*. The fungi isolated were while fungi identified in this study were *Penicillium chrysogenum, Aspergillus flavus, A. niger, Saccharomyces cerevisiae* and *Trichoderma viride*. The total bacterial count of the untreated and autoclaved sample ranged from 5.2×10^4 cfu/ml and 0.0 to 9.8×10^5 cfu/ml respectively. The total fungal count of the untreated and autoclaved sample ranged from 0.0 to 1.5×10^4 sfu/ml and 0.0 to 8.0×10^3 sfu/ml respectively. The fermented product had a decreased fat content of (58.33%) unlike the unfermented seeds (60.32%) while the crude protein and moisture content increased from 7.98% to 8.99% and 3.14% to 3.51% respectively. This indicates that fermented seed cotyledons of bush mango can be employed as a low-cost, easily accessible high protein and low fat food for man and animals.

Keywords: Bush Mango Seed Cotyledons; Protein; Minerals; Low Fat; Solid State Fermentation

Introduction

Irvingia gabonensis is a non-timber forest produce, comprising of fruits, leaves, stem and roots. The fruit is made up of a fleshy part and the nut has a hard shell and the seed. Its seeds have an external brown hull and two white cotyledons [1]. The seeds of bush mango have water-soluble dietary fiber that defers stomach emptying and permits for a more steady absorption of dietary sugar. The high fiber content of bush mango is responsible for its valuable metabolic effects. Researches on bush mango for its probable role in curing diabetes, metabolic syndrome, and obesity are on-going [2]. The bush mango is claimed to have antibacterial, astringent, analgesic, anti-inflammatory and anti-oxidative properties. Traditionally, bush mango has been employed to manage a variety of ailments, including diarrhea, wounds, dysentery, yellow fever and tympanic membrane infections. It could also be beneficial in dropping cholesterol and blood sugar levels. In addition, bush mango is used for weight loss because it may decrease appetite and stimulate the body to burn fat stores for energy [2,3].

Irvingia gabonensis literally known as bush mango is valued communally, especially for their fat and protein-rich seed (Atangana., *et al.* 2002). The faintly aromatic seeds are typically air dried and sold whole or powdered. They could be grind to a paste. The high mucilage content enables seeds to be used as thickening agents for dishes [1]. The seed could also be pressed for vegetable oil. It have some medicinal value in which the high soluble fiber content of *I. gabonensis* seed can melt away belly fat and trim waste line.

Solid-state fermentation has arisen as a probable technology for animal feed, energy, food, chemicals and therapeutic products. It is involves solids in absence (or almost absence) of free water, though, it is necessary that the substrate possess adequate moisture to sustain growth and development of microbes [4]. Microbial growth on naturally humid solid is stimulated by solid-state fermentation. Solid substrates generally offer a habitable environment to the microbial flora. Here, filamentous fungi are most studied for SSF because of their

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hyphae, which have the ability to develop on the particles' surface of the substrate, and its capacity to penetrate through the surfaces. Several agricultural crops such as rice bran, cassava bagasse, coconut oil cake, soybean cake, among others are frequently used as substrates for SSF processes [4]. For this process, hydrolytic exo-enzymes are produced by the associated microorganisms and released out of the cells, which produce and support in retrieving carbon source and nutrients through the cells. This, on the other hand, encourages biosynthesis and microbial actions (Bhavsar., *et al.* 2010, Díaz., *et al.* 2007).

Aim of the Study

This work was aimed at the study of microorganisms associated during the fermentation process of bush mango seed and evaluation of the effect of fermentation on the proximate constituents of bush mango seeds.

Figure: Bush mango seed cotyledons.

Materials and Methods

Collection and preparation of *I. gabonensis* seed sample

I. gabonensis seeds were collected from Akure main market and transported to the Microbiology laboratory, Federal University of Technology, Akure. The seeds were cleaned and screened to eliminate broken and cracked seeds, dust, stones and other foreign materials. The surface of the seeds was dipped in 80% ethyl alcohol for 5 minutes and washed with sterile distilled water. The sterile seeds were grounded aseptically with a clean electric blender (Binatone, British Hongkong BLG 699).

Solid state fermentation of *I. gabonensis* seed sample

Two treatments of powdered seeds were prepared; sample A was sterilized in an autoclave at 121°C for 15 mins while sample U was not sterilised. Seventy milliliter of sterile distilled water was added to the powdered seeds and allowed to ferment naturally for 96 hours at room temperature.

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Determination of titratable value

Total titratable acidity (TTA) was determined on 5 ml aliquot of the sample against 0.01M NaOH using phenolphthalein as an indicator to AOAC [5].

Determination of pH of bush mango seed sample

pH meter was used to measure the pH of fermented samples. This was done by first standardizing the pH meter (Hanna multi-parameter-H1-9828) fitted with glass electrodes with buffer solutions and then the pH reading of the sample taken.

This was done at 0h, 24h, 48h, 72h and 96h. The initial pH of the sample was taken as soon as the sample was brought to the laboratory (Ademoroti, 1996).

Isolation and presumptive identification of bacteria and fungi associated with bush mango seed fermentation

From the fermented samples of *I. gabonensis*, 1g was aseptically taken with the aid of sterile spatula and dispensed into 9 ml of distilled water where it was serially diluted to 10^{-2}. One millilitre was cultured on nutrient agar (Oxoid), De Man, Rogosa and Sharpe (MRS) agar (Oxoid) and potato dextrose agar (Oxoid). The nutrient agar, MRS and potato dextrose agar was incubated for bacteria and fungi respectively. Plates containing the nutrient agar were incubated in an inverted position at 37°C for 24 hours. Plates containing potato dextrose agar were incubated at 25°C for 3 - 5 days. Plates containing MRS agar was incubated anaerobically for 24h. Bacterial isolates were identified and characterized based on their morphological and biochemical features as described by Fawole and Oso [6] while fungal isolates were identified using cultural and morphological features according to Barnett and Hunter (1972).

Comparative proximate and mineral analyses of unfermented and fermented with bush mango seed

Moisture content was determined by drying to constant weight at 105°C in an oven, ash by ignition at 55°C in a muffle furnace, oil content by soxhlet extraction with hexane, protein by micro Kjedahl method and crude fibre by Acid/Alkali digestion methods (AOAC, 1990). The mineral were analyzed by dry ashing the sample at 55°C to constants weight and dissolving the ash in volumetric flask using distilled, de-ionized water with 10 ml of 10% hydrochloric acid solution. Sodium and Potassium were determined by using a flame photometer (model 405, corning UK) using NaCl and KCl salts to prepare the standards. All other metals were determined by Atomic absorption spectrophotometer (Perkin-Elmer model 403, Norwalk CT, USA). All chemicals used were of analytical grade. Earlier, the detection limit of the metal had been determined according to Techtron (1975). The optimum analytical grade was 0.1 to 0.5 absorbance unit with a coefficient of variance of variance of (0.87 - 2.20%) the mineral reported as mg/100g. Phytate and tannin was determined using AOAC (1990) methods while oxalate content was done by the titrimetric method as modified by Akindahunsi and Salawu (2005).

Proximate analysis of fermented and unfermented bush mango seed sample

Moisture Determination

Moisture was determined by the loss in weight that occurs when a sample is dried to a constant weight in an oven. About 2g of a feed sample is weighed into a silica dish previously dried and weighed. The sample is then dried in an oven for 65°C for 36 hours, cool in a desiccator and weigh. The drying and weighing continues until a constant weight is achieved:

%Moisture = wt of sample + dish before drying- wt of sample+ dish after drying x100

Weight of sample taken

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Since the water content of feed varied very widely, ingredients and feed are usually compared for their nutrient content on moisture free or dry matter (DM) basis:

%DM = 100 - %Moisture [7].

Crude fibre analysis

The organic residue left after sequential extraction of feed with ether was used to determine the crude fibre. The fat-free material is then transferred into a flask and 200 ml of pre-heated 1.25% H_2SO_4 is added and the solution is gently boiled for about 30 minutes, maintaining constant volume of acid by the addition of hot water.

The buckner flask funnel fitted with Whatman filter was pre-heated by pouring hot water into the funnel. The boiled acid sample mixture is then filtered hot through the funnel under sufficient suction. The residue was then washed several times with boiling water (until the residue is neutral to litmus paper) and transferred back into the beaker. Then 200 ml of pre-heated 1.25% Na_2SO_4 is added and boiled for another 30 minutes. It was filtered under suction and washed thoroughly with hot water and twice with ethanol. The residue was dried at 65°C for 24 h and weighed. The residue was transferred into a crucible and placed in muffle furnace (400 - 600°C) and ash for 4h, then cooled in desiccator and weighed:

%Crude fibre = <u>Dry weight of residue before ashing - wt of residue after ashing x100</u>

Weight of sample

Crude protein analysis

Crude protein was determined by measuring the nitrogen content of the feed and multiplying it by a factor of 6.25. This factor is based on the fact that most protein contains 16% nitrogen. Crude protein is determined by kjeldahl method. The method involved: digestion, distillation and titration. Digestion was done by weighing 2g of the sample into kjeldahl flask with the addition of 25 ml of concentrated sulphuric acid, 0.5g of copper sulphate, 5g of sodium sulphate and a speck of selenium tablet. Heat was applied in a fume cupboard slowly at first to prevent undue frothing, continue to digest for 45 minutes until the digested substance become clear pale green. Leave until completely cool and rapidly add 100 ml of distilled water. Rinse the digestion flask 2 - 3 times and add the rinsing to the bulk.

Distillation was done using Markham distillation apparatus. We steamed up the distillation apparatus and added 10 ml of the digest into the apparatus through a funnel and allowed to boil. Then, 10 ml of sodium hydroxide from the measuring cylinder was added so that ammonia is not lost. Distil into 50 ml of 2% boric acid containing screened methyl red indicator.

Titration was done when the alkaline ammonium borate formed is titrated directly with 0.1N HCl. The titre value which is the volume of acid used is recorded. The volume of acid used is fitted into the formula which becomes

%N = <u>(14 x V_A x 0.1) w x10</u>

1000x100

V_A = Volume of acid used; w= Weight of sample

%crude protein = %N x 6.25 [7].

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Determination of fat content

The ether extract of a feed represents the fat and oil in the sample. For the determination of ether extract using soxhlet apparatus, 150 ml of an anhydrous diethyl ether (petroleum ether) of boiling point of 40 - 60°C was placed in the flask. 2 - 5g of the sample is weighed into a thimble and the thimble is plugged with cotton wool. The thimble with content is placed into the extractor; the ether in the flask is then heated. As the ether vapour reaches the condenser through the side arm of the extractor, it condenses to liquid form and drop back into the sample in the thimble, the ether soluble substances are dissolved and are carried into solution through the siphon tube back into the flask. The extraction continues for at least 4h. The thimble is removed and most of the solvent is distilled from the flask into the extractor. The flask was then disconnected and placed in an oven at 65°C for 4h, cool in desiccator and weighed.

%Ether extract = Weight of flask + extract - tare weight of flask x100 [7].

Weight of sample

Ash content analysis

Ash is the inorganic residue obtained by burning off the organic matter of feedstuff at 400 - 600°C in muffle furnace for 4hrs. 2g of the sample is weighed into a pre-heated crucible. The crucible is placed into muffle furnace at 400 - 600°C for 4hrs or until whitish-grey ash is obtained. The crucible is then placed in the desiccator and weighed

%Ash = Weight of crucible+ash - weight of crucible [7].

Weight of sample

Results and Discussion

In this study a total of eight (8) microorganisms were isolated. Three of the above microorganisms were bacteria while the remaining five were fungi. The bacteria isolated were *Bacillus* spp., *Staphylococcus* spp. and *Lactobacillus fermentum* as illustrated in table 1. The fungi isolated were *Penicillium chrysogenum, Aspergillus flavus, Aspergillus niger, Saccharomyces cerevisiae* and *Trichoderma viride* as shown in table 2. *Staphylococcus* spp. and *Bacillus* spp. that were isolated in bush mango seed cotyledons were found in the fermentation of *Parkia biglobosa* (locust beans seed) in the production of "Iru" a food condiment [8]. Fungi isolated from the seed cotyledons are similar to those isolated by Aboloma and Ogunbusola [9]. The work of Ojokoh [10] showed the presence of *Aspergillus* spp. in the fermenting mango peel as also isolated from the bush mango seed cotyledon. The isolated fungi were predominantly filamentous. This agrees with the work of Manpreet., *et al.* [11] that filamentous fungi are the major group of microorganisms which predominate in the SSF process. Filamentous fungi that are usually found associated with SSF process include many species of *Aspergillus, Rhizopus, Alternaria, Fusarium, Monilia, Mucor, Trichoderma* and some species of *Penicillium*. The microbes associated with fermentation of the ogbono seed powder were similar with few differences from those in untreated samples.

Gram reaction	H$_2$S formation	Fructose	Lactose	Maltose	Mannitol	Glucose	Sucrose	Starch hydrolysis	Indole production	Motility	Suspected isolate
+	-	AG	AG	AG	AG	AG	AG	-	-	-	*Lactobacillus fermentum*
+	+	+	+	+	-	+	+	ND	ND	ND	*Staphylococcus* spp.
+	+	+	-	+	ND	+	-	-	-	+	*Bacillus* spp.

Table 1: Morphology and biochemical characteristics of bacterial isolates associated with fermented bush mango seeds
Key: +: Positive; -: Negative; AG: Gas Production; ND: Not Detected.

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Colony description	Morphological characteristics	Probable fungi
Black colonies	Colonial heads were typically radiate, later split to form loose columns, biserate but having some heads with phialides borne directly on the vesicle. Conidiophores were hyaline coarsely roughened, noticeably near the visicle. Conodia are globos, pale-green and conspicuously echinulate.	*Aspergillus flavus*
Black colonies	Large globose, dark-brown conidial heads radiate with ability to split into several loose columns. Smooth-walled conidiophores, turning dark towards the vesicle. Conidial heads were biserate with phialides borne on brown and septate metulae	*Aspergillus niger*
Greenish colonies	Conidiophores arising from the mycelium, synnemata, branched near the apex, penicillate, ending in a group of phialides; conidia (phialospores) brightly coloured in mass, 1-celled ovoid in dry basipetal chains	*Penicillium* species
White colonies	Smooth glaborous and yeast-like in appearance. Microscopic morphology shows large, ellipsoidal budding yeast-like cell	*Saccharomyces cerevisiae*
Greenish yellow colonies	Slightly granular with green conidia distributed throughout. An irregular yellow zone without conidia. White pustules were also found growing on the green mat of conidia. Conidia were also globose and slender	*Trichoderma viride*

Table 2: *Morphological characteristics of fungal isolates associated with fermented bush mango seeds.*

The result of proximate composition of bush mango seed displayed in table 3 showed that there was reduction in ash content of the untreated samples (the fermented and the unfermented) from 1.73 to 1.53%, fat from 60.31 to 58.33%, and crude fibre from 13.34 to 12.50% while there was an increase in (moisture content) from 60.13 to 59.93%, crude protein from 8.43 to 8.98%. The result of proximate composition of bush mango seeds showed that there was reduction in ash content of the autoclaved samples (the fermented and the unfermented) from 1.71 to 1.40%, fat from 62.85 to 55.55%, and crude fibre from 13.04 to 12.69% while there was an increase in (moisture content) from 49.51 to 49.51%, crude protein from 7.98 to 8.87%. Generally, there was reduction in ash, fat and crude fibre content while there was increase in protein. The increase in the moisture content could be attributed to the addition of water to the sample prior to fermentation as also observed by Ojokoh., *et al* [12]. Reduction in fat content is advantageous to prevent excess fat in the body. Ekpe., *et al.* [1] also observed a decrease in the crude fat of *I. gabonensis* as a result of processing. Since the ash content is a measure of the total amount of minerals present within a food, a reduction in its level during microbial fermentation could be as a result of the minerals being used up by the fermenting organisms as a mineral source during their metabolism [13]. The increase in protein content of the seed is in line with the work of Fadare and Ajaiyeoba [14]. The high protein contents could be attributed to the ability of microorganisms to secret some extra cellular enzymes capable of degrading cellulolytic materials during fermentation [15]. The increase in protein level could also be attributed to increased microbial nitrogen during fermentation as a result of increased production of single cell proteins [1] during the fermentation of bush mango seed cotyledons which is a testament to the ubiquity of microorganisms.

Unfermented	Moisture	Ash	Fat	Fibre	Protein
Untreated	59.935	1.734	60.317	13.347	8.432
Autoclaved	49.516	1.714	62.857	13.040	7.988
Fermented	**Moisture**	**Ash**	**Fat**	**Fibre**	**Protein**
Untreated	60.129	1.563	58.333	12.500	8.988
Autoclaved	60.813	1.408	55.556	12.690	8.875

Table 3: *Proximate composition of bush mango seeds.*

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At 0h, the untreated samples after incubation on nutrient and Potato dextrose agar plates, showed the growth of *S. cerevisiae, Penicillium* and *Bacillus* sp. There was no growth at 0h in the autoclaved samples. Killing of the most resistant spores due to exposure to moist heat at 121°C for 10 to 30 minutes must have been responsible for no growth and few isolates present in the autoclaved samples [16].

Due to the low water activity, the system created an unfavourable condition for bacterial growth. So, bacterial colonization was minimal in the fermentation process. Bacteria usually require at least 0.91 water activity while fungi require lesser water activity, 0.7. However, *Bacillus* sp, survived for 48h before the rise of *Lactobacillus*. The survival of Bacillus probably was due to their ability to form spores and withstand unfavourable circumstances [10].

The eventual disappearance of *Bacillus* spp. may not be unconnected with the increase in the acidity of the medium as a result of the fermentative activity of the *Lactobacillus*. After 96h, *S. cerevisiae, L. fermentatis, A. niger, A. flavus* and *Trichoderma* spp. were the organisms isolated from the system. There was no growth at 0h in the autoclaved samples. Killing of the most resistant spores due to exposure to moist heat at 121°C for 10 to 30 minutes must have been responsible for no growth and few isolates present in the autoclaved samples [16].

At 24h, *S. cerevisiae, Penicillium* spp. *and Aspergillus* spp. were found to colonize the samples with *Penicillium* spp. disappearing at 48h. *S. cerevisiae* and *Aspergillus* spp. dominated the rest of the fermentation period. The isolation from the untreated samples revealed the presence of *Staphylococcus* spp. and *Aspergillus* spp. In addition to the isolates isolated from other samples. The presence of *Staphylococcus* sp. could have been as a result of contamination during handling and processing. *Aspergillus* sp. is known to be associated with grains where they cause toxification by the release of aflatoxin. These microbes were absent in the autoclaved samples the first day probably due to the heat treatment the samples underwent.

Antibiotic sensitivity pattern was carried out to know the particular antibiotics capable of inhibiting the growth of these organisms. Ciprofloxacin is potent antibiotic that was capable of inhibiting the growth of almost all the organisms in which its highest inhibitory power was on *Staphylococcus* spp. (14 mm).

Time (hours)	Bacterial count (cfu/ml)		Fungal count (sfu/ml)	
	Untreated	Treated	Untreated	Treated
0	70×10^3	0	0	0×10^3
24	156×10^3	98×10^3	4×10^3	5×10^3
48	102×10^3	96×10^3	7×10^3	7×10^3
72	75×10^3	65×10^3	11×10^3	7×10^3
96	52×10^3	40×10^3	15×10^3	8×10^3

Table 4: *Microbial count of untreated and autoclaved fermented bush mango seeds against time.*

Organisms	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E
Bacillus species	4	2	-	-	-	6	5	1	-	-
Staphylococcus species	-	18	-	-	10	-	14	3	-	21

Table 5: *Antibiotic sensitivity pattern of bacterial organisms isolated from Irvingia gabonensis.*
Key: PEF: Perfloxacin (10 μg); CN: Gentamycin (10 μg); APX: Ampiclox (30 μg); Z: Zinnacef (20 μg); AM: Amoxicillin (30 μg);
R: Rocephin (25 μg); CPX: Ciprofloxacin (10 μg); S: Streptomycin (30 μg); SXT: Septrin (30 μg); ERY: Erythromycin (5 μg).

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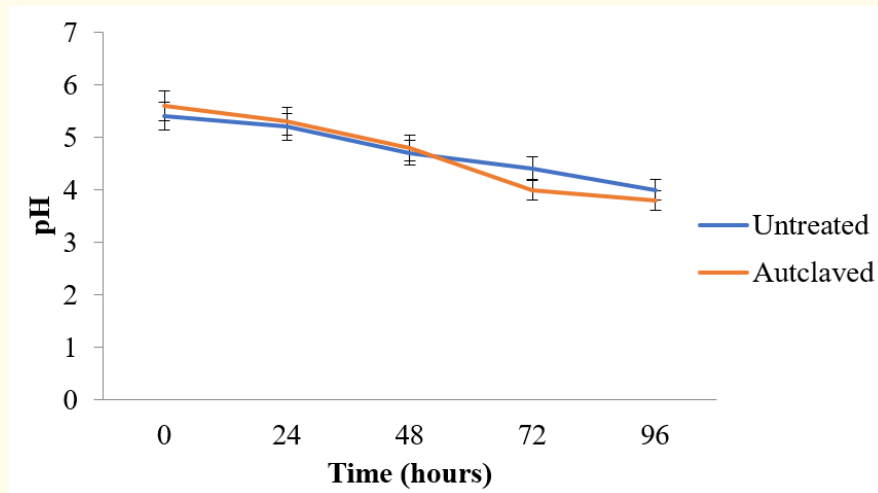
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Figure 1: *pH of untreated and autoclaved fermented bush mango seeds against time.*

Conclusion

Overall findings from this study indicates that fermented seed cotyledons of *Irvingia gabonensis* can be employed as a low-cost, easily accessible high protein and low fat food for man and animals due to its sophisticated high mineral and proximate contents and appreciable antimicrobial quality. It was also deduced that the effect of solid state fermentation on *Irvingia gabonensis* seeds has douse environmental depreciation of bush mango seeds and has been recommended for its great economic importance and marketability.

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