Controlled temperature on the microflora of fermented bottle gourd seed.

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Abstract

Effect of controlled temperature on microflora of fermented Bottle Gourd Seeds (BGS) as well as Melon Seeds (MES) was studied. De-hulled seeds were boiled for 6 h and fermented at 28°C, 35°C and 42°C for 96 h. The microbial flora of the fermented BGS and MES were determined with respects to seed type, fermentation time and fermentation temperature. The microorganisms isolated from the fermented samples included; *Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Corynebacterium* sp, and some varieties of fungi. The viable bacterial count for both the BGS and MES were within the range of 5 x 10^8 cfu/g and 36 x 10^8 cfu/g between zero and ninety-six hours. Bacillus subtilis was the major fermenting organism since it was seen at the three temperatures and throughout the fermentation period for all the samples. The absence of other fermenting organisms in some of the samples indicates that these substrates (BGS and MES) differ slightly in their ability to sustain the growth of different species of microorganisms probably due to the differences in their overall composition. The result shows that when the factors that affect the microflora of fermentation are controlled, safe good quality ogiri can be produced from BGS.

Keywords: Bottle gourd seed, Melon seed, Fermentation, Ogiri, Microflora, Controlled temperature.

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Introduction

Fermentation is one of the first methods used by man to produce and preserve food and has continued to play an important role in food processing for thousands of years. It is the breakdown of carbohydrate materials under aerobic or anaerobic conditions by the activities of microorganisms or enzymes elaborated by them (Achi 2005). It provides a way of preservation of food products and also enhances nutritive value of the food [1-4]. It aids in the destruction of undesirable substances making the food safe for consumption [5,6]. Fermentation improves digestibility, palatability and reduces the energy and time required for subsequent cooking [7,8].

Most legumes that are otherwise inedible due to presence of toxicants have been found useful in the production of condiments. These fermented legume-based condiments are generally called iru by the Yorubas of the southwestern Nigeria, dawadawa among the Hausas who inhabit most of the northern part of Nigeria. Ogiriis the name used by the Igbos of the southeastern Nigeria. Owoh on the other hand, is the popular name among the Urhobos and Itsekiris in the Niger Delta region for food condiments. Similarly, okpiye is popular among the Igala and Idoma people of the Middle Belt region [9-14].

The conventional substrates for condiment production are diverse; this implies that condiments can be produced from more than one raw material. Quite often, seeds that are used for fermentation are inedible in their raw unfermented or cooked state. Available literature, reviewed shows that over nine different fermented products in Nigeria are condiments of which ogiri is one of them [15-19]. Ogiri is traditionally prepared by fermenting melon seeds (*Citrullus vulgaris*) [20], fluted pumpkin (*Telferia occidentale*) [21] or castor oil seed (*Ricinus communis)* [1,22,23].

Many indigenous leguminous crops including the bottle gourd *(Lagenaria siceraria)* are under-utilized. This is due to preference to other melon varieties, modernity and ignorance. Traditionally, the gourd is primarily used as a container for water and palm wine while the seeds and the pulp are allowed to rot and are discarded. In today's modern world, the empty gourds are also used for decorative/ornamental purposes. The seeds have not been used for nutritive or culinary purposes though they are high in nutrients [24]. It therefore becomes necessary to discover ways of utilizing this nutritious seed other than using the rind for decoration, musical instrument or bird houses. Also, ogiri is produced traditionally from spontaneous, uncontrolled fermentation of the substrate. The temperature, time, relative humidity and other fermentation variables are not strictly monitored. This results to irreproducible processes and products. As a result, product quality is usually inconsistent. Information in this area is scanty and this has made industrial production difficult. Hence, the objective of this study was to produce ogiri from bottle gourd and melon seeds at 28°C, 35°C, and 42°C respectively, to determine the viable microbial counts of the fermenting samples from 0–96 h of fermentation and to isolate and identify micro-organisms associated with the fermentation of both bottle gourd and melon seeds.

Materials and Methods

Material

Sample collection: The bottle gourd seeds (*Lagenaria siceraria*) were collected from the retail sellers of bottle gourd rinds in Ose market in Onitsha, Anambara state Nigeria who discard the seeds, design and sell the bottles or rind for decorations and musical instruments. The discarded seeds were willingly given to us by the retail sellers since they are only interested in the rinds or bottles. The melon egusi seeds (*Colocynthis citrullus lanatus)* were purchased from Eke-ukwu Owerri market in Imo state, Nigeria.

Equipment: The various equipment used for this study were obtained from Department of Food Science and Technology and Department of Soil and Animal Sciences, Dr. Wesly Braide Laboratory, Nekede, Reliable Research Laboratory services, Umuahia, and Department of Zoology Laboratory, university of Jos, Nigeria.

Chemical reagents: All chemical reagents used in this experiment were of analytical grade and as prescribed by the official methods of analysis.

Methodology

Preparation of 'ogiri' samples

The traditional method of producing ogiri was used with slight modifications. Dry and healthy bottle gourd seeds (*Lagenaria siceraria)* were cracked, de-hulled, washed, and boiled with 3:1 w/w volume of water for six hours until they became soft. The seed (400 g) was then wrapped with tender banana leaves which were briefly passed through flame. The wrap was then boiled for another 30 min drying the boiling water. The sample was milled or mashed with mortar and pestle. The mashed sample was then divided into thirty portions of 10 g each and wrapped with the sterile plantain leaves. Ten wraps each were aseptically transferred into two different incubators preset at 35^{o}C and 42^{o}C respectively while third batch (10 wraps) was covered properly to avoid contamination and kept on the laboratory bench. The samples were properly labeled and fermentation was allowed to progress for ninety-six hours (96 h). The same procedure was followed for melon seed *(Colocynthis citrullus lanatus)*. Samples were collected every 24 h for analysis. The fermented samples were dried and stored in airtight containers for subsequent analysis. The procedure is represented in Figure 1.

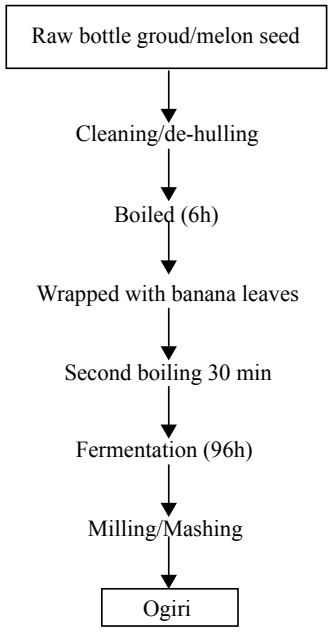

Figure 1. *Production of 'ogiri' using bottle gourd and melon seed.*

Microbial Analysis

Cultivation and isolation of microorganisms

Pour plate method of Jideani and Jideani was adopted [25]. Ten grams (10 g) of each mashed sample was weighed aseptically into 90 ml of distilled water, agitated for 5min and serially diluted up to 10^{-8} and 1 ml of the 10^{-8} dilution was spread on already labelled nutrient agar and potato dextrose agar plates (in duplicates). The nutrient agar plates were incubated at 37^{0}C for 24 h and 48 h while potato dextrose agar plates were incubated at ambient temperature (28^{0}C) for at least 72 h. Un-inoculated plates were also incubated for reference. After the incubation periods the plates were examined and both bacterial and fungal counts were taken from the countable plates. The nutrient agar was used for cultivation of heterotrophic bacteria, and potato dextrose agar for fungi growth.

Observations

The dilution plates for bacteria were examined after 48 h and the number of colonies on each plate counted and recorded. The colony characterisation for the fungal isolates were noted and further identified by microscopic observation of their wet mounts using lactophenol cotton blue. The pH and %TTA (Titratable Acidity) of the fermenting samples were recorded for each sampling day.

Isolation of pure cultures

After incubation, the plates were examined and discrete colonies picked, sub-cultured and put in a slope. Each slope culture was re-streaked on nutrient agar to ensure purity. Each pure culture was then inoculated onto sterile nutrient agar slants in screw capped MacCartney bottles. These were incubated at 37^{0}C for 24 h and preserved in the refrigerator at 4^{0}C as stock cultures for further tests. The pure bacterial cultures were appropriately labelled as A-E. Pure fungal cultures were made by transferring a portion of the growing spores or edge of the colony on a sterile potato dextrose agar plate and incubating at ambient temperature.

Characterisation of bacterial isolates

Purified colonies were identified first by gram staining, then by biochemical reactions and sugar fermentation tests. Colonial morphology and microscopic characteristics were noted and recorded. Further characterisation and identification of bacterial isolates were based on biochemical reactions (catalase, oxidase, coagulase, Indole, citrate tests) and sugar fermentations as described by Buchanan and Gibbson [26].

Results and Discussion

Results

The result in Tables 1-4 shows the viable bacterial counts,

Table 1. *Viable bacterial count of fermenting bottle gourd seed using nutrient agar.*

Viable bacterial count (cfu/g)						
S/No	**Seed type**	**Time (h)**	**28°C**	**35°C**	**42°C**	**Mean**
1	BGS	0	5×10^8	5×10^8	5×10^8	**5×10^8**
2	BGS	24	9×10^8	13×10^9	10×10^8	**10.67×10^8**
3	BGS	48	11×10^8	17×10^8	12×10^8	**13.33×10^8**
4	BGS	72	26×10^8	27×10^8	24×10^8	**25.67×10^8**
5	BGS	96	36×10^8	24×10^8	23×10^8	**27.67×10^8**
Mean			**17.4×10^8**	**17.2×10^8**	**15.8×10^8**	
1	MES	0	nil	nil	nil	
2	MES	24	6×10^8	8×10^8	5×10^8	**6.33×10^8**
3	MES	48	8×10^8	13×10^8	9×10^8	**10×10^8**
4	MES	72	20×10^8	20×10^8	16×10^8	**18.67×10^8**
5	MES	96	27×10^8	21×10^8	16×10^8	**21.33×10^8**
Mean			**13.3×10^8**	**15.5×10^8**	**11.5×10^8**	

Table 2. *Colonial, microscopy and morphological characteristics of bacterial isolates on nutrient agar.*

Isolate	**Morphological characteristics**	**Microscopy**		**Probable Identity of microorganism**
		Spore	**Gram**	
A	Straight rods in short chains with oval central spores, dull and dry irregular flat cream colonies	+	+R	*Bacillus* sp
B.	Cocci in pairs, tetrad or irregular clusters, small round yellow colonies	-	+S	*Micrococcus* sp
C	Straight or slightly curved rods with tapered end circular umbonate cream colonies	-	+R	*Corynebacterium* sp
D	Straight rods in short chains with oval central spores, mucoid and slimy irregular raised colonies	+	+R	*Bacillus* sp
E	Spherical cells (cocci) in clusters, few in pairs or tetrads	-	+S	*Staphylococcus* sp
KEY: S: Spherical; R: Rod; +: Positive; -: Negative.				

Table 3. *Biochemical test and sugar utilization of bacterial isolates.*

Biochemical test										**Sugar Utilization**					**Probable Identity of microorganisms**
Isolate	**Cat**	**Oxi**	**Coag**	**In**	**MR**	**VP**	**Cit**	**NO_3**	**Urease**	**Glu**	**Suc**	**Mal**	**Lac**	**Mann**	
A	+	-	-	-	-	+	+	+	-	+	-	-	+^{s}	+	*Bacillus subtils*
B	+	-	-	-	+	-	+	-	+	-	-	-	-	-	*Micrococcus luteus*
C	+	-	+	-	-	+	+	+	+	+	-	+	-	-	*Corynebacterium* sp
D	+	-	-	-	-	+	+	+	-	+	-	-	+^{s}	-	*Bacillus cereus*
E +	-	+	-	-	-	+	-	+	-	+	+	+	+	+	*Staphylococcus aureus*
KEY: Lac: Lactose; Glu: Glucose; Suc: Sucrose; Mal: Maltose; Fru: Fructose; Cat Catalase; Oxi: Oxidase; Coag: Coagulase; Cit: Citrate; NO_3: Nitrate reduction; In: Indole; VP: Voges Proskaur; MR: Methyl Red; +: Positive; -: Negative; +s: Slow reaction.															

microorganisms identified during BGS and MES fermentation as affected by seed type, fermentation time, and temperature of fermentation.

Discussion

Microorganisms identified during BGS and MES fermentation as affected by seed type: The result in Table 1 showed the Viable Bacterial Counts (VBC) in the fermenting bottle gourd and melon seeds. The Melon Seed (MES) showed no bacterial growth at zero hour while the Bottle Gourd Seeds (BGS) sample had a viable bacterial count (VBC) of 5×10^8 at zero hour. This could suggest that the spores of the inherent microorganisms in BGS survived the heat treatment of boiling. The inherent microorganisms in MES could be mostly vegetative cells which were readily destroyed during boiling. The bacterial count remained generally higher in BGS than MES samples throughout the period of fermentation.

The VBC for both the BGS and MES which were within the range of 5×10^8 cfu/g and 36×10^8 cfu/g between zero and ninety-six hours falls within the range of values reported by most authors. Aminat and Esiobu (2014) reported similar viable bacterial counts (VBC) of 120-163 $\times 10^7$ cfu/g within three days of fermentation of *Parkia biglobosa*. Akinyele and Oloruntoba reported a VBC of 87-91 $\times 10^7$ cfu/g for *Cucumeropsis manni* after 72 h fermentation [27].

Similar microorganisms were found in both fermented BGS and MES. They were gram positive rods and cocci and included *Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Staphylococcus aureus, Corynebacterium* sp. Other organisms identified were the following yeast and moulds: *Saccharomyces* sp*, Rhizopus* sp*, and Penicilium* sp. Results in Table 2 showed the colonial, microscopy, and morphological characteristics of bacterial isolates identified during fermentation of both Bottle Gourd Seeds (BGS) and Melon Seeds (MES) while the biochemical and sugar fermentation tests were shown in Table 3. Previous authors have also reported the prevalence of such microorganisms during production of condiments from legumes. Mbajunwa et al. [28] and Obeta [29] isolated *Staphylococcus saprophyticus, Bacillus pumilus, Bacillus subtilis* and *Bacillus licheniformis* in fermenting ugba samples. Ogunshe et al. [30] observed that *Bacillus* species occurred most consistently and predominated the fermentation of *Albizia saman* into aisa (a condiment), with the production of the highest ammonia-like aroma, characteristic of leguminous-based fermented condiments.

Table 4. *Bacterial isolates from the melon and bottle gourd sample at different temperature of fermentation.*

S.no	Isolate Sample code	Fermentation Time (h)	28°C	35°C	42°C
1	BGS	0	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus subtilis*
2	BGS	24	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus subtilis*
				Staphylococcus aureus	
3	BGS	48	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus subtilis*
			Bacillus subtilis	*Bacillus cereus*	*Staphylococcus aureus*
			Micrococcus luteus	*Staphylococcus aureus*	
4	BGS	72	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus subtilis*
			Bacillus cereus	*Bacillus subtilis*	*Bacillus cereus*
			Micrococcus luteus	*Rhizopus* sp	*Penicillium* sp
5	BGS	96	*Bacillus subtilis*	*Bacillus subtilis*	*Saccharomyces* sp
			Bacillus cereus	*Bacillus subtilis*	
			Rhizopus sp		
6	MES	0	No growth	No growth	No growth
7	MES	24	*Bacillus subitilis*	*Bacillus subitilis*	*Bacillus subitilis*
8	MES	48	*Bacillus subtilis*	*Bacillus subtilis*	*Saccharomyces* sp
			Bacillus cereus	*Bacillus cereus*	*Bacillus subtilis*
			Penicillium sp		
9	MES	72	*Bacillus sutilis*	*Bacillus subtilis*	*Corynebacterium*sp
			Bacillus cereus	*Bacillus cereus*	*Bacillus subtilis*
			Micrococcus luteus	*Rhizopus* sp	
10	MES	96	*Conyrebacberium* sp	*Corynebacterium*	*Bacillus subtilis*
			Bacillus subtilis	*Bacillus subtilis*	*Corynebaterium* sp
			Micrococcus luteus	*Bacillus cereus*	*Saccharomyces* sp

BGS: Bottle gourd seed; MES: Melon seed

However, *Staphylococcus aureus* was found only in the BGS samples but were not present in MES. On the other hand, *Corynebacterium* sp was only found in some of the MES samples. This suggests that the substrates (BGS and MES mash) were slightly different in their ability to sustain the growth of different species of microorganisms probably due to the differences in their overall composition.

Microorganisms identified during fermentation as affected by fermentation time: The results in Table 1 showed that there was a general trend of increase in Viable Bacterial Count (VBC) for all the fermenting samples. The mean value of VBC increased from 5×10^8 cfu/g at 0 h to 27.67×10^8 cfu/g at 96 h for BGS. The VBC increased from 0 cfu/g at 0 h fermentation to a mean value of 21.33×10^8 cfu/g after 96 h for the MES samples. A similar trend had been noted by Aminat and Esiobu [31] who reported similar viable bacterial counts of 120-163 $\times 10^7$ cfu/g within three days of fermentation of *Parkia biglobosa*. Akinyele and Oloruntoba [27] reported 8.7×10^6 cfu/g - 9.1×10^6 cfu/g for fermented *Cucumeropsis mannii*. This can be explained by the fact that the medium is rich in nutrients which enabled the microorganisms to multiply within the fermenting mash.

Bacillus subtilis was identified in all the stages of the fermentation from 24-48 h for BGS and MES samples (Table 4). *Staphylococcus aureus* was observed in some BGS samples after 24 h and 48 h but were absent in the 96 h samples. The reduction in the population of *Staphylococcus aureus* is in agreement with the observation of Babalola and Oluwamodupe [32], that the microbial isolate, *Bacillus* sp, was the major observable fermenting microorganism, and posses' antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus, Proteus* spand *Pseudomonas aeruginosa.*

The fungi *Rhizopus* sp*, penicilium* and *Sacharomyces* sp were identified after 72 h and 96 h of fermentation. These may have contributed to the flavor development.

Some workers have identified different microorganisms in fermented melon seeds. These include *Bacillus* sp, *E. coli, Proteus* sp*, Pediococcus* sp and *Alcaligenes* sp [3,11,33]. However, *Bacillus subtilis* and *B. licheniformis* were identified as the main bacteria involved in the fermentation of melon seed because of their ability to produce the requisite enzymes for the breakdown of proteins and production of the various flavor compounds associated with ogiri egusi [3,34].

Microorganisms identified during fermentation as affected by temperature of fermentation: The viable bacterial count was fewer in both the Bottle Gourd (BGS) and Melon Seed (MES) samples fermented at 42°C. As fermentation temperature increased from 28°C to 42°C, the mean VBC values decreased from 17.4×10^8 cfu/g to 15.8×10^8 cfu/g for BGS and 15.38×10^8 cfu/g to 11.5×10^8 cfu/g for the MES (Table 1). It was also observed that for samples fermented at 35°C and 42°C, the VBC only increased up to 72 h and decreased afterwards. This corresponded with the increase in protease activity which increased within 48-72 h of fermentation and reduced thereafter especially for samples fermented at 35°C and 42°C (Table 1). The decline in microbial population may be attributed to the faster accumulation of toxic compounds and metabolites at higher fermentation temperature. This may be inhibitive to some microorganisms [35,36].

Bacillus subtilis occurred at all the fermentation temperatures, and throughout the period of fermentation. Carpenter [37] reported that *Bacillus subtilis* may grow within the temperature range of 8°C to 55°C with its optimum at 28°C to 40°C. This may account for their growth in all the samples irrespective of the temperature. Another reason could be because *Bacillus* species are spore formers therefore could survive high temperatures.

Micrococcus luteus was only isolated from samples fermented at 28°C and did not occur in samples fermented at 35°C and 42°C. This is in agreement with Carpenter [37] who reported 25°C to 30°C as the optimum temperature for its growth. *Staphylococcus aureus* as well as *Saccharomyces* sp were only isolated from samples fermented at 35°C and 42°C. Optimum temperature for the growth of *Staphylococcus aureus* has also been reported as 35°C to 40°C [37].

Bacillus cereus was identified in some of the samples, although, it was absent in the samples fermented at 42°C for 96 h. The presence of this microorganism poses some health concern since it produces enterotoxins [31]. Aminat and Esiobu reported that the presence of *Bacillus cereus,* which is capable of secreting enterotoxins in these fermented condiment may indeed constitute a health hazard by possibly causing flatulence and diarrhea disease in some individuals [31]. However, a safe practice would be to ferment at a higher temperature (42°C) for a long time (96 h) and to heat the condiment before consumption.

It can also be observed that the first 72 h could be assumed to be the most active period for all the fermentation temperatures (28°C, 35°C and 42°C). It is the period of exponential growth [29-41]. It is therefore possible that fermentation for the rest of the process was sustained more by the enzymes already produced than by the bacterial population [38]. Njoku and Okemadu in their study of ugba fermentation observed that alpha amylase; proteolytic and lipolytic enzymes were detectable at the start of fermentation and attained their maximum levels at 24-36 h fermentation [42]. *Bacillus* species have been reported as producers of enzymes such as amylase, galactanase, galactosidase, and glucosidase which are involved in the degradation of carbohydrates [43,44]. Amylase hydrolyses carbohydrates into sugar. Similarly, galactanase softens the texture of the seeds and liberates sugar for digestion. Most legumes contain large amounts of non-digestible carbohydrate which may include arabinose, starchyose, sucrose and raffinose [38]. These carbohydratess are associated with abdominal distention and flatulence in human [45-47]. But fermentation was found to reduce total flatulent factors (oligosaccharides) from 16.5 to 2.0 mg/g in soy beans [48] and from 0.16 to 0.1 mg/g in groundnut [49].

The significant biochemical changes in most fermented high protein product, protein synthesis and hydrolysis are important factors responsible for changes in textures and flavour [50]. Soluble low molecular weight peptides and amino acids contribute to flavour and are produced through the enzymatic breakdown of proteins [51,52].

Conclusion

The findings from this work have shown that the micro flora as well as the microbial load of fermented BGS and MES are greatly influenced by the seed type, fermentation time and fermentation temperature. It was also noted that the presence of flavour enhancing fungi and enterotoxin secreting bacteria was a function of fermentation time and fermentation temperature respectively. The microflora during the fermentation of fermented food products affect the food quality of the final product as it gives it its peculiar physical properties. Therefore, controlling these factors that affect the microflora of fermented BGS and MES will help in the production of safe, good quality ogiri.

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