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Free and attached cells of *Bacillus subtilis* as starters for production of a soup flavouring ("ogiri egusi")

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ABSTRACT

Aims: This *Bacillus subtilis* has been identified to be the main fermenting bacterium during indigenous production of "ogiri egusi"; a traditional soup flavouring rich in protein. Evaluation of the use of starter and broth cultures of this bacterium in the production of 'ogiri egusi' was therefore undertaken with the view to improve the fermentation process and quality of product.
Methodology and Results: Cowpea granules in association with *Bacillus subtilis* cells were developed as starter cultures for the fermentation. Results obtained showed that the starter cultures resulted in an increase in the amino-nitrogen from 1.67±0.02 to 19.96±0.05 mg N/100 g dry matter in 48 h while the broth cultures increased the amino-nitrogen from 1.63±0.03 to 16.54±0.05 mg N/100 g dry matter in 72 h. There was also a corresponding increase in the protease activity of the fermentation conducted with the starter cultures from 2.69±0.03 to 54.98±0.04 mg N/min in 48 h. The broth cultures produced an increase from 2.65±0.02 to 47.61±0.06 mg N/min in 72 h. Changes in these parameters for the natural process were gradual and reached their peaks at 120 h with values of 9.89±0.13 mg N/100g dry matter and 31.92±0.03 mg N/min respectively. Peroxide values for the fermentation processes increased throughout the period; however the starter cultures produced the lowest value (10.20±0.10 meq/kg) showing that rancidity may not occur in the product fermented by the starter culture.
Conclusion, significance and impact of study: The starter cultures significantly reduced fermentation time from 96 – 120 h in the natural process to 48 h. Thus use of starter cultures optimized the process of fermentation and will eliminate chances of contamination of product with pathogens and spoilage organisms. This ultimately will improve product quality.

Keywords: Amino-nitrogen; *Bacillus subtilis;* ogiri egusi; protease activity; starter cultures

INTRODUCTION

'Ogiri egusi' is an indigenous fermented soup condiment which is used as flavoring agent whose character and organoleptic properties depend on microbial activities (Nwosu and Ojimelukwe, 2000). It has an oily gray pasty consistency with a very strong pungent smell when it is in its raw state. Consumed mainly in southern Nigeria especially by the igbos (Enwere, 1998), it is produced by traditional methods of uncontrolled solid substrate fermentation of melon seeds (*Citrullus vulgaris shrads*) (Achi, 2005). The raw seeds are boiled, the water drained and allowed to ferment naturally for four days in clay pots. The fermented seeds are then mashed into pastes, wrapped in leaves and kept over a fire place to dry. In Nigeria, melon is mostly cultivated in the south-eastern part and is usually inter-planted with yam and cassava where it serves as a cover crop (Ogueke and Nwagwu, 2007). Ogiri egusi is known to contribute to the caloric and protein intake and are generally added to soups as low-cost meat substitute by low income families in parts of Nigeria (Adewusi *et al.*, 2004).

Melon seeds contain good levels of crude protein which are similar to soybean and fluted pumpkin. Crude protein content of melon seeds range from 24.3 to 41.6% dw (Fokou *et al*., 2004) while fat content range from 42.9-57.3% (David and Aderibigbe, 2010; Fokou *et al*., 2004). Oils from melon seeds contain over 60% linoleic acid which is an essential fatty acid, 22% saturated acids (including myristic, palmitic, stearic acids) and 78% unsaturated fatty acids (including oleic, linoleic and linolenic acids) (Ogunsua, 2000). Egusi is a good source of essential amino acids especially tryptophan and methionine. Carbohydrate content of melon seeds range from 4.56-13.3% dw (David and Aderibigbe, 2010; Fokou *et al*., 2004).

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Various workers have identified different microorganisms in fermented melon seeds. These include *Bacillus sp., E.coli, Proteus sp., Pediococcus sp*. and *Alcaligenes sp*. (Barber and Achinewhu, 1992; Barimalaa *et al.*, 1989; Ofuya and Nnajiofor, 1989; Sanni *et al*., 2000). However, *Bacillus subtilis* and *B. licheniformis* have been identified as the main bacteria involved in the fermentation 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 (Barimalaa *et al.*, 1989). *Bacillus* and *Acinetobacter* sp, together with some yeast and fungi (*Rhizopus*) were identified in the fermented product developed from portions of melon (*Citrullus vulgaris*) and groundnut (*Arachis hypogea*) (Nwosu and Ojimelukwe, 2000).

Ogiri egusi fermentation is still carried out using the traditional method. This usually results in variation of quality of products (Ogueke and Nwagwu, 2007). There is also need to optimize the fermentation process with the view to industrialize the production. The use of chance fermentation as practiced traditionally makes the fermentation difficult to control and results in the contamination of products with pathogens or other microorganisms capable of producing toxins and those that cause off flavors (Ogueke and Nwagwu, 2007). These can also introduce undesirable and spoilage organisms which can cause a reduction in the product's shelf-life and increase susceptibility to spoilage. Due to these setbacks, variation in the quality and stability of the products are often observed.

Attempts have been made by a group of workers (Ogueke and Nwagwu, 2007) to produce ogiri egusi using pure cultures of the bacteria isolated from previous ogiri egusi, thus trying to introduce the use of starter cultures. The development of starter cultures for the production will enhance standardization of the product. The work therefore aims at evaluating the effect of *Bacillus subtilis* cells in association with cowpea granules, as starter cultures in 'ogiri egusi' fermentation when compared to the traditional indigenous method.

MATERIALS AND METHODS

Material collection and preparation

Approximately 15 kg of melon seeds (*Citrullus vulgaris*) were purchased from Ihiagwa market in Owerri, Imo State, Nigeria in November, 2011. Seeds obtained were dehulled and sorted to remove bad seeds, hulls and extraneous materials. The seeds were stored at 4 °C until used for the study. Approximately 5.0 kg of cowpea (*Vigna unguiculata*) was purchased from Owerri main market, Imo State, Nigeria and the seeds were properly sorted before use.

Bacillus subtilis used for the study

Bacteria were isolated from various ogiri egusi samples from three states of the consuming region of South Eastern Nigeria. Microbiological analyses were conducted immediately by suspending 10 g of sample in 90 mL sterile maximum recovery diluent(MRD Oxoid CM0733) and homogenized using a Stomacher (A.J.Seward, BA6021) for 1 to 2 min at normal speed. Ten-fold dilutions were then prepared and 0.1 ml of suitable dilution was spread onto Nutrient agar (NA, Oxoid CM0003). Plates were incubated aerobically at 37 °C for 24 - 48 hours. Representative dominant colonies were picked and purified by streaking on same Nutrient agar. Stock cultures were kept in Nutrient broth containing 20% glycerol and stored at -20 °C.

Prior to the genotyping, the Bacillus isolates were phenotyped according to Ouoba *et al.*(2008). Forty bacteria were chosen based on their phenotypic characteristics including colony and cell morphology, cell motility, presence of endospore, Gram and catalase reaction after growth on Nutrient agar (NA; Oxoid CM0003) for 24 h. Fermentation profiles of carbohydrates (API 50CHB galleries; BioMerieux, Basingstoke, UK) were also determined to assist typing. This was done according to the manufacturer's instruction and results were analyzed using the apiweb software.

The identification of the isolates was done using the methods described by Ouoba *et al.* (2008). Thirty three out of the forty isolates were identified to be *Bacillus subtilis*. Thus *B. subtilis* was taken to be the main fermenting bacterium and then used for the fermentation. Before use the identified *Bacillus subtilis* were streaked on Tryptone Soy agar (BIOTEC) and incubated at 35 °C for 18 h. The cultures were then washed by suspending the cells in 10 mL of 0.1 M potassium phosphate buffer (pH 7.0). This was followed by centrifugation at 14000xg for 5 min using an MSE centrifuge (Minor, UK) (Njoku *et al.*, 1990).

Preparation of carrier granules

The method of and Abu (2000) was used with minor modification as shown below. Cowpea (*Vigna unguiculata*) granules were prepared by immersing 500 g of well sorted raw seeds of black-eyed cowpea in 5 L of portable water, autoclaved at 121 °C for 15 min, cooled, drained and air-dried. The boiled seeds were spread evenly on an aluminium foil and placed in a hot-air oven (Weiss-Gallenkamp, UK) at 80 °C for 24-48 h, then cooled. The cooled seeds were dry-milled using a warring blender, sieved into different particle sizes using a laboratory sieve (Endocotts Ltd, UK) and particles sizes of 700 µm was collected. One gram (1 g) each was dispensed into MacCartney bottles and sterilized in a hot-air oven (Weiss-Gallenkamp, UK) at 100 °C for 18-24 h (with the lid loosely closed).

Inoculum preparation and standardization of cultures

Cultures from 18 h Tryptone Soy agar plates (BIOTECH) were examined for purity by microscopic examination of slides prepared from randomly selected colonies. A loopful of the 18 h old culture was inoculated in a flask

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containing 50 mL of Tryptone Soy broth (Oxoid) and was incubated aerobically for 48 h at 35 °C. Cells from the broth culture were harvested and washed three times with 0.1 M potassium phosphate buffer by centrifugation at 14000x*g* for 5 min. The cells were then aseptically re-suspended in 20 mL sterile 0.1M potassium phosphate buffer (pH 7.0). The cell suspension was then standardized using a spectrophometer (Model S2100UV, UNICO, New Jersey, USA) at 550nm wavelength such that 1.0mL of the suspension contained about 3.0 x 10^8 cells.

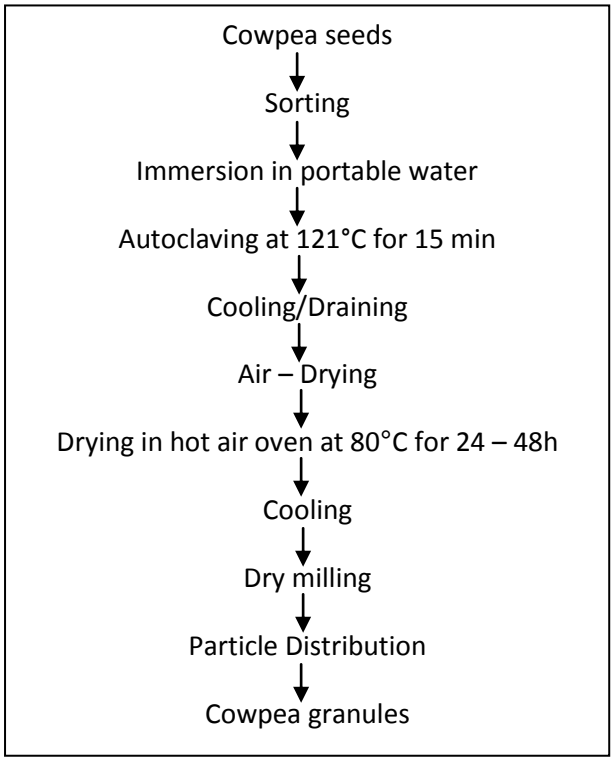

Figure 1: Flow diagram for production of Cowpea granules (carrier).

Transfer of standardized cultures to cowpea granules (carrier)

With a sterile pipette 0.2 mL of the standardized suspension of *B. subtilis* was aseptically transferred into different parts of 1.0 g of the sterile cowpea granules in the Mac Carthney bottles in an inoculation chamber. The contents of the bottles were thoroughly mixed by inversion of the bottles at the rate of 20 times per min for 5 min (Monela *et al.*, 1985). They were then transferred to a rotary shaker to shake them for 5 min (Husmark and Ronner, 1990). The granules with attached Bacillus cells were dried using sterile air from a monopoint dryer (Type 4547, Braun Ag, UK) until a final moisture level of 10% was attained. The attached cells were stored at 4 °C until used for the study. These served as starter cultures.

Standardized broth cultures of *B. subtilis* were also prepared (1.0mL of broth culture contained about 3.0 x 10^8 cells) and used to ferment melon seeds for 'ogiri egusi' production.

Fermentation of melon seeds using starter cultures and standardized broth cultures of *B. subtilis*

Two kilograms of dehulled melon seeds were properly sorted and cleaned, and extraneous materials removed. The seeds were boiled for 2-3 h until the endosperm softened, then drained and cooled to about 30 °C, placed into a sterilized container and inoculated with 10 g of the starter cultures of *B. subtilis*. This was aseptically mixed and allowed to ferment for four days at ambient temperature (30-32 °C).

For the fermentation using standardized broth cultures, 2.0kg of the melon seeds were treated as above except that the boiled and cooled seeds were inoculated with 2.0 mL of the standardized broth cultures.

Natural fermentation of melon seeds for ogiri egusi

This was also prepared as above. However, after boiling and cooling the seeds were allowed to ferment naturally without the addition of the starter cultures or the standardized broth cultures.

The fermentations were monitored for the protease activities, amino nitrogen content, pH, titratable acidity, and peroxide value. All analyses were carried out in triplicate.

Determination of pH

The pH of each fermentation process was measured as follows. Five grams of fermenting seeds was taken from the bulk and mashed in 45 mL of sterile deionized water (Njoku *et al*., 1990). The pH was measured using a digital pH meter (Jenway pH/MV/TEMPERATURE METER, Model 3510, Essex, UK) at ambient temperature (30-32 °C). Before use the pH meter was standardized using buffers (4.0 – 9.0).

Determination of titratable acidity of fermenting seeds

Chilled distilled water (2 mL) was added to 5 g of fermenting mash in a chilled mortar. The sample was crushed with a pestle and then transferred to 100 mL flask. An additional 10 mL of chilled extractant was added and thereafter the flask was stoppered and shaken on a Staurt flask shaker at medium speed for 15 min. The extracts were then centrifuged at 2300x*g* in a bench-top MSE centrifuge at 5 °C. The residue was re-suspended in 8 mL of chilled extractant and subjected to the same procedure. The supernatants were combined and made up to 20 mL with chilled extractant (Njoku and Okemadu, 1989). Ten milliliters of the extract was then titrated with 0.1 M NaOH using phenolphthalein as indicator. A blank was prepared using 10 mL distilled water. The titrable acidity was calculated as mg lactic acid/g of sample.

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Determination of peroxide value

Fifteen grams of fermenting seeds was mashed and 5 g each of the mashed seeds was placed into a 250 mL glass – stoppered Erlenmeyer flask, and 30 mL of solvent mixture (3 parts by volume of Glacial acetic acid + 2 parts by volume of Chloroform) was added. The flask was swirled to dissolve the mashed seeds in the solution. Five hundred microliters of saturated potassium iodide solution was added and the solution was allowed to stand (with occasional shaking) for 1 min, then 30 mL of distilled water was added. The mixture was titrated by gradually adding 0.1 N sodium thiosulphate and constantly shaking vigorously. The titration continued until the yellow color had almost disappeared. Five hundred microliters of starch indicator solution was added. The titration was continued, shaking the flask vigorously at near end point until the iodine from the chloroform layer was liberated. The sodium thiosulphate was then added until the blue color had just disappeared. The tubes were placed in boiling water to boil for about 30 sec. A blank was also prepared at the same time. The peroxide value as milliequivalent of perioxides per 1000 g of sample was calculated using the equation described below:

$$\text{Peroxide value} = \frac{(S - B) \times (N) \times (1000)}{W}$$

B = Titration of blank
S = Titration of sample
W = Weight of sample
N = Normality of sodium thiosulfate solution

Amino nitrogen determination

The formol titration method (Pham and Del-Rosario, 1983) was used. Three grams of fermenting seeds was taken and mashed. Two grams of the mashed seeds was placed in a conical flask, and then 0.5 mL of phenolphthalein (0.5%) and 0.4 mL of neutral saturated potassium oxalate were added. The mixture was kept to stand for few minutes and this was neutralized with 0.1 M NaOH to a standard pink color. Two milliliters of 40% formaldehyde solution was added and allowed to stand for few minutes (until mixture was colorless). This was then titrated with 0.1 M NaOH to pink color. The titer value obtained was designated 'V_1'. A blank was run by titrating a mixture of 2 mL of formaldehyde solution and 10mL distilled water with 0.1 M NaOH to obtain 'V_2'. The amino nitrogen was calculated using the equation: % Amino Nitrogen = 1.7 (V_1 - V_2).

Determination of protease activity

The protease activity of the fermenting materials was determined using the modified casein digestion method as described by Isu and Njoku (1997). Five milliliters of a 10% (w/v) homogenate of the fermenting material was used. Enzyme activity was expressed as amino nitrogen production. One unit of enzyme activity was equivalent to the amount of enzyme required to release 1 µg amino nitrogen under the assay conditions.

Analysis of data

The data obtained from this study were statistically analyzed using Analysis of Variance (ANOVA). The means were separated using Fischer's least significant difference (LSD) at p≤ 0.05 confidence level. Microsoft Excel 2007 was used for the analysis.

RESULTS AND DISCUSSION

Evaluation of pH during fermentation

For all treatments, there was a drop in the pH value within the first 24 h of fermentation (Table 1), but an increase was observed afterwards. The drop in pH could be attributed to the production of organic acids from the utilization of the little amount of carbohydrate present (19). Melon seeds contain 4.56-13.3% dw carbohydrate (David and Aderibigbe, 2010; Fokou *et al.*, 2004). The rise in pH after 24 h could be due to the abundant production of ammonia during fermentation from protein hydrolysis, and deaminase activity following utilization of amino acids as carbon and energy sources by the fermenting bacteria (Njoku and Okemadu, 1989; Ogueke and Nwagwu, 2007). The increase in pH may have encouraged the growth of *Bacillus subtilis*, hence increasing fermentation rate.

Table 1: Mean values of pH change during fermentation of melon seeds

Fermentation Treatment	Fermentation Period (Hour)				
	0	24	48	72	96
Starter cultures	6.46±0.02[a]	5.9±0.10[a]	7.9±0.35[a]	8.0±0.02[a]	8.1±0.40[a]
Broth cultures	6.49±0.03[a]	6.1±0.11[a,b]	7.7±0.41[a]	7.8±0.30[a,b]	8.0±0.29[a]
Natural	6.48±0.04[a]	6.3±0.14[b]	7.4±0.15[b]	7.6±0.28[b]	7.7±0.18[b]

[a b] Mean with different superscripts within each column are significantly different (p ≤ 0.05)

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Bacillus sp have been found to give better fermentation of African Oilbean seeds for ugba at higher pH values (Odunfa and Oyeyiola, 1985). Other workers have also observed an increase in pH during ogiri egusi production (Adewusi et al., 2004; Barber and Achinewhu, 1992; David and Aderibigbe, 2010; Ogunsanwo *et al.*, 1989; Steinkraus, 1995).

However, in this study the pH of the broth cultures and starter cultures were above 7.0 at 48 h fermentation, an indication of period of intense microbial activity, especially the fermentation with the starter cultures. The values obtained for the starter and broth culture fermented mashes were significantly different ($p \leq 0.05$) from that produced by the natural fermentation at 48 h. This has not been observed by other workers whose pH were about 7.0 or less at 48 h fermentation. Thus the use of starter cultures and broth cultures of *B. subtilis* reduced fermentation time. This agrees with the statement of Achi (2005) and observations of Ofuya and Nnajiofor (1989) that the use of starter cultures reduces fermentation time as well as guarantee product quality.

Evaluation of titratable acidity during fermentation

There was an initial increase in the total titratable acidity within 24 h (Table 2) due to production of organic acids (Ogueke and Nwagwu, 2007). These were in agreement with earlier works by David and Aderibigbe (2010). However, after 24 h the titratable acidity decreased. The reduction in titratable acidity afterwards could be attributed to 'alkaline fermentation' (Steinkraus, 1995) and increased protease and deaminase activities (Njoku and Okemadu, 1989).

Evaluation of peroxide value during fermentation

Peroxide value for all treatments showed a remarkable increase as fermentation period increased (Table 3). However, the values did not exceed the threshold value of 30 milliequivalent of active oxygen per kg of oil in oil rich foods (like melon seeds) (Gotoh and Wada, 2006). The fermentation carried out by starter cultures of *Bacillus* cells had the least peroxide value (10.20 Meq/kg) within 96 h while the natural fermentation had the highest value (14.00 Meq/kg). A value of 30 Meq/kg indicate the initial stage of fat and oil deterioration (Gotoh and Wada, 2006). Since the peroxide value is used as a measurement of the extent to which rancidity can occur (Ihekoronye and Ngoddy, 1985) the lower values obtained with the starter cultures showed that the chances of the product going rancid is very low.

Table 2: Mean values of Total Titratable Acidity (% Lactic acid) of fermenting melon seeds

Fermentation Treatment	Fermentation Period (Hour)				
	0	24	48	72	96
Starter cultures	0.28 ± 0.04^{b}	0.79 ± 0.06^{a}	0.21 ± 0.05^{b}	0.19 ± 0.04^{b}	0.18 ± 0.06^{b}
Broth cultures	0.24 ± 0.04^{b}	$0.74\pm0.05^{a,b}$	$0.24\pm0.03^{a,b}$	$0.23\pm0.05^{a,b}$	$0.22\pm0.05^{a,b}$
Natural	0.38 ± 0.04^{a}	0.69 ± 0.05^{b}	0.28 ± 0.04^{a}	0.26 ± 0.04^{a}	0.25 ± 0.05^{a}

$^{a\,b}$ Mean with different superscripts within each column are significantly different ($p \leq 0.05$)

Table 3: Mean values of Peroxide value of fermenting melon seeds (Meq/kg)

Fermentation Treatment	Fermentation Period (Hour)				
	0	24	48	72	96
Starter cultures	7.60 ± 0.17^{a}	7.80 ± 0.03^{c}	8.60 ± 0.03^{c}	9.20 ± 0.07^{c}	10.20 ± 0.10^{c}
Broth cultures	7.60 ± 0.20^{a}	9.00 ± 0.27^{b}	9.80 ± 0.05^{b}	11.00 ± 0.27^{b}	12.80 ± 0.07^{b}
Natural	7.80 ± 0.20^{a}	10.80 ± 0.91^{a}	11.40 ± 0.03^{a}	12.00 ± 0.24^{a}	14.00 ± 0.23^{a}

$^{a\,b\,c}$ Mean with different superscripts within each column are significantly different ($p \leq 0.05$)

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Evaluation of amino-nitrogen during fermentation

Table 4 showed that there was an increase in the amino nitrogen for all the fermentation processes. This implied an increase in the rate of hydrolysis of the protein content by the proteinase enzyme, thus an indication of an increase in fermentation rate. The results also showed that the amino-nitrogen production was highest for the fermentation that employed starter cultures of *Bacillus.* Statistical analysis showed that values obtained with starter cultures were significantly different ($p \leq 0.05$) from the pure culture and naturally fermented mashes from 24 h to the end of fermentation. This also showed that association of the cells with cowpea granules will help increase the activity of the micro-organisms and release of enzymes for optimum fermentation activity. The amino-nitrogen for the fermentation conducted with the starter cultures attained its peak at 48 h, still indicating that the period is the peak of microbial activity and a reduction in fermentation time from 96 – 120 h with the traditional method to 48h with the starter cultures.

Evaluation of protease activity during fermentation

Table 5 shows the protease activities of the cultures during the fermentations. From the results it could be observed that the protease activity of the fermentation conducted with the starter cultures increased rapidly and reached its peak at 48 h (54.96 mg N/min). This was followed by the fermentation carried out by the broth cultures of the bacterium which attained its highest value at 72 h (47.54 mg N/min). These periods for the starter cultures corresponded with periods when the amino-nitrogen and pH values attained their peaks, especially the pH which is the value obtained in a well fermented ogiri egusi after 120 h fermentation through the natural, traditional method (Adewusi *et al*., 2004; Barber and Achinewhu, 1992; Steinkraus, 1995). Statistical analysis showed that the natural fermentation did not produce significant ($p > 0.05$) changes in the protease activity and amino-nitrogen within the period. There was also a significant difference ($p< 0.05$) in these parameters between the starter cultures and broth cultures indicating that the starter cultures have better prospects for use in the production of ogiri egusi, which will also reduce fermentation time to 48 h. Therefore use of starter cultures should be encouraged to guarantee product quality. This will also enhance optimization of the production process. Further studies need to be conducted to determine the effect of fermentation variables (relative humidity, temperature and oxygen transfer) on the overall fermentation process.

Table 4: Mean values of Amino nitrogen of fermenting melon seeds (mg N/100g)

Fermentation Treatment	Fermentation Period (Hour)				
	0	24	48	72	96
Starter cultures	1.67±0.02[a]	10.8 ± 0.03[a]	19.96 ±0.05[a]	17.81±0.04[a]	16.93±0.06[a]
Broth cultures	1.63±0.03[a]	5.79±0.06[b]	11.35±0.03[b]	16.54±0.05[a]	13.70±0.04[b]
Natural	1.66±0.04[a]	3.71±0.04[c]	5.26±0.04[c]	7.47±0.05[b]	9.89±0.13[c]

[a b c] Mean with different superscripts within each column are significantly different ($p \leq 0.05$)

Table 5: Mean values of protease activity during fermentation of melon seeds (mg N/min)

Fermentation Treatment	Fermentation Period (Hour)				
	0	24	48	72	96
Starter cultures	2.69±0.03[a]	29.84 ± 0.02[a]	54.98 ±0.04[a]	50.63±0.02[a]	41.87±0.04[a]
Broth cultures	2.65±0.02[a]	13.99±0.05[b]	37.38±0.02[b]	47.61±0.06[b]	37.68±0.03[b]
Natural	2.68±0.02[a]	3.73±0.02[c]	14.36±0.03[c]	27.53±0.04[c]	31.92±0.03[c]

[a b c] Mean with different superscripts within each column are significantly different ($p \leq 0.05$)

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CONCLUSION

Fermented melon seeds “ogiri egusi” is an indigenous fermented soup condiment that constitutes an important social agro-sustainable business that guarantees an additional income to families. Cowpea granules in association with *B. subtilis* cells were developed as starter cultures for the fermentation. During fermentation higher levels of protease activity and amino nitrogen were observed with the starter culture fermented samples, thus resulting in a significant reduction in fermentation time from 96 – 120 h in natural fermentation to 48 h. Such starters could be explored to improve production and guarantee product quality. This will also create wealth and financial benefits. Further studies need to be conducted to determine the effect of fermentation variables (relative humidity, temperature and oxygen transfer) on the overall fermentation process.

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