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Full Length Research Paper

Effect of salt on the fermentation of soybean (*Glycine max*) into daddawa using *Bacillus subtilis* as starter culture

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Previous studies showed that 1% salt improved the organoleptic attributes of traditional fermented daddawa. Also, *Bacillus subtilis* as a monoculture starter produced daddawa of same quality with traditional daddawa. The aim of this study was to investigate the effect of 1% salt on some biochemical changes occurring in the fermentation of soybean into daddawa using *Bacillus subtilis* as starter. Viable cell count of *Bacillus subtilis* increased with fermentation and was significantly (P<0.05) higher in 1% salted daddawa. The pH value increased with fermentation with no significant difference between the salt free and 1% salted daddawa after 24 h of fermentation. Ammonia concentration increased in both fermentations with higher values in 1% salted daddawa. Proteolytic activity which increased rapidly in the first 48 h and dropped giving higher amounts of free amino acids with fermentation was also higher in 1% salted daddawa. The total soluble sugars decreased in both fermentations with significantly lower levels in 1% salted daddawa after 24 h of fermentation. Although there was no significant difference in the organoleptic properties of salt free and 1% salted daddawa, the panelist showed preference for 1% salted daddawa.

Key words: *Bacillus subtilis*, biochemical changes, fermentation, organoleptic quality, salt, soy-daddawa.

INTRODUCTION

Soy-daddawa is a product of alkaline fermentation of soybean (*Glycine max* (L) Merrill) used as a food condiment in Nigeria. Soy-daddawa is similar in sensory attributes to African locust bean (*Parkia biglobosa*) daddawa (Ogbadu and Okagbue, 1988; Omafuvbe et al., 2002a).

To make traditional soy-daddawa, soybeans are cleaned, soaked overnight in tap water, dehulled manually and cooked by boiling for two hours (Omafuvbe et al., 2000). The dehulled cooked beans are then placed in calabashes or bamboo baskets lined with banana or plantain leaves and left to ferment spontaneously in a warm place for up to 72 h. The traditional fermentation is accomplished with no conscious inoculation of microorganisms, resulting in products of variable quality.

In order to develop controlled fermentation and improve on the quality of soy-daddawa, the science of the traditional process must be well understood. The traditional fermentation has been reported to be accomplished by mainly bacteria especially *Bacillus* species, notably *B. subtilis, B. pumilus*, and *B. licheniformis* (Ogbadu and Okagbue, 1988; Omafuvbe et al., 2000; Dakwa et al., 2005). Of these *Bacillus* species, only *B. subtilis* as a monoculture starter produced soy-daddawa of the same sensory attributes with the traditional fermented soy-daddawa (Omafuvbe et al., 2002b). Biochemical changes associated with the traditional production of soy-daddawa have also been examined (Popoola and Akueshi, 1986, Omafuvbe et al., 2000; Dakwa et al., 2005). Addition of 1% salt (NaCl) in the traditional production of soy-daddawa has been reported to improve the organoleptic quality of the product (Omafuvbe, 1994). There are no reports on the biochemical changes associated with the enhancement

of the organoleptic quality of soy-daddawa following the addition of 1% salt to the fermenting substrate. The aim of this study was to investigate the effect of 1% salt on the biochemical changes occurring during the fermentation of soybean into daddawa using *Bacillus subtilis* SDA3 as starter culture.

MATERIALS AND METHODS

Seeds

Soybean seeds used for this study were purchased from a local market in Ile – Ife, Osun State, Nigeria.

Organism

Bacillus subtilis SDA3 used as starter culture was previously isolated from natural fermenting soy-daddawa (Omafuvbe et al., 2000). The organism was maintained on nutrient agar (Oxoid CM3) slope in the refrigerator.

Preparation of soy-daddawa by starter culture

The traditional method of boiling for 2 h (Omafuvbe et al., 2000) was replaced with autoclaving at 121°C for 20 minutes to obtain sterile cooked beans (Omafuvbe et al., 2002b). Essentially, approximately 1.2 kg of soybeans were sorted, washed thoroughly in tap water and then soaked in 3 liter of tap water at room temperature for 12 h. The soaked beans were dehulled manually and divided into 2 equal lots. To one lot was added 1% salt (NaCl, May & Baker (w/w)) and mixed thoroughly in a stomacher bag (Seward Ltd). Each lot was distributed in approximately 50 g (wet wt.) amounts into 250 ml conical flasks plugged with cotton wool. The contents of the flask were steamed at 121°C for 20 min to obtain sterile cooked beans.

B. subtilis SDA3, grown on nutrient agar (Oxoid CM 3) at 35°C for 16 h, was suspended in 10 ml of sterile maximum recovery diluent (MRD, Oxoid CM 733) and diluted with the diluent to give a suspension with absorbance of 0.03 in a spectrophotometer (CE 202 Ultraviolet Spectrophotometer, Cecil Instrument) set at 540 nm wavelength (10 mm light path length). About 500 µl of the suspension of predominantly vegetative cells was inoculated into each of the sterile cooked beans held in 250 ml conical flasks (this gave approximately 10^5 cells/ g wet wt.). The inoculated beans were incubated at 35°C for 72 h. Triplicate flasks were removed for examination at selected times from each of the 2 sets of fermentation.

Viable cell counts

Fermenting beans (5.0 g wet wt.) were homogenized with 45 ml of sterile MRD (Oxoid CM 733) by stomaching for 2 min (Colworth Stomacher 400). Further dilutions were made in MRD and 1.0 ml of appropriate dilutions was plated in duplicate plate count agar (Oxoid CM 325). Inoculated plates were incubated at 35°C for 48 h after which the bacteria colonies were counted and expressed as colony forming units per gram (c.f.u g^{-1}) of the sample.

pH determination

Approximately 2 g fermenting beans (wet wt.) were homogenized with 8 ml of distilled water and the pH of the slurry was measured with a pH meter (Hanna Instruments, 8520).

Determination of soluble sugar and free amino acids

The soluble sugar and free amino acid content of the fermenting beans were extracted with 80% ethanol (v/v) as previously described (Omafuvbe et al., 2000). The ethanolic extract was appropriately diluted for the various determinations.

The total free amino acid content was determined by the ninhydrin colorimetric method (Rosen, 1957). The amino acid concentration was calculated from standard curve of known concentrations of glycine. The total soluble sugar was determined by the anthrone reagent method of Morris (1948) while the reducing sugar was estimated by the colorimetric method (Somogyi, 1945) using glucose as standard solution.

Proteolytic activity

The assay for proteolytic activity was based on the method described by Sarkar et al. (1993). Fermenting beans (3.0 g wet wt.) were homogenized with 10 ml of cold 0.05 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged (Harrier 15/80 centrifuge) at 13,000 rpm for 2 min and the supernatant used in the assay. The assay mixture comprised 250 µl of azocasein (2.5 g/l; sigma A2765) in 0.05 M potassium phosphate buffer and 120 µl of the supernatant, contained in 1.5 ml Eppendorf tube. The tube was incubated at 37°C for 1 h and adding 750 µl of cold 3 M trichloroacetic acid terminated the reaction. Undigested protein was allowed to sediment by standing at 4°C for 1 h and then removed by centrifugation at 13,000 rpm for 10 min. The resulting supernatant (500 µl) was mixed with 2 ml of freshly distilled water and analysed for free dye by measuring the absorbance at 400 nm in a spectrophotometer (Pharmacia Biotech, Novaspec II). One unit of proteolytic activity is defined as the amount that produced an absorbance increase of 0.01 units under the assay conditions.

Estimation of ammonia

Fermenting beans (2.0 g wet wt.) were homogenized with 10 ml 1 M perchloric acid. The homogenate was diluted with 10 ml freshly distilled water and the pH adjusted to 7.0 first with 5 M KOH and then exactly with 2 M KOH solutions. The mixture was transferred quantitatively into 50 ml volumetric flask and the volume made to 50 ml mark with distilled water. The mixture was cooled in a refrigerator for 20 min (to precipitate protein) and centrifuged at 13,000 rpm for 10 min. The clear supernatant was used for ammonia determination. The extracted ammonia was assayed enzymatically (Boehringer kit 11 112 732 035; Boehringer-Mannheim/R- Biopharm, Germany).

Sensory analysis

Soy-daddawa samples (salt free and 1% salted daddawa) produced with monoculture starter of *B. subtilis* SDA3 were organoleptically evaluated as previously described (Omafuvbe et al., 2002b). The samples were evaluated by a panel of 10 regular consumers of soy-daddawa using a score range of 1 (dislike extremely) to 5 (like extremely). The data obtained were subjected to statistical analysis.

Statistical analysis

The data obtained in this study were subjected to analysis of

variance followed by Student - Newman - Keuls post hoc test (Primer for Biostatistics software package version 3.01 by Glantz (1992)). Statistical significance was accepted at P value equal to or less than 0.05.

RESULTS AND DISCUSSION

The viable cell counts of *B. subtilis* SDA3 in the fermenting salt free and 1% salted soy-daddawa increased rapidly in the 1st 24 h of fermentation and less rapidly in the later stages (48 – 72 h) of fermentation (Table 1). The increase in cell count from the onset of fermentation to the end of fermentation was significant ($P<0.05$) with the two treatments except for 1% salted daddawa where the difference in cell count was not significantly different between the 48th and 72nd hour of fermentation. This trend is not unusual since similar pattern have been reported (Omafuvbe et al., 2000; Amin et al., 2004). In general, the viable cell count was significantly higher in 1% salted daddawa. The increase in viable cell counts in 1% salted daddawa is an indication that low salt concentration creates a favorable medium for the growth of *B. subtilis* SDA3.

The proteolytic activity of salt free and 1% salted daddawa increased significantly up to the 48th hour of fermentation and dropped thereafter (Table 2) The total free amino acids increased significantly throughout the fermentation process but most rapidly in the first 48 h (Table 2). The rapid increase in total free amino acids in the early stages of fermentation is an indication of rapid protein hydrolysis and this coincides with the period of increased proteolytic activity in the fermenting beans. This trend in the fermenting beans has been reported to indicate a two 'stage process'. The first stage is linked to the growth and protease production while the 2nd stage of less activity is linked to product ripening (Omafuvbe et al., 2002b). The high proteolytic activity is not a surprise since *B. subtilis* SDA3 is proteolytic (Omafuvbe et al., 2002b). Salt may have enhanced the production and activity of protease enzyme since the level of protease activity in 1% salted daddawa was significantly higher than values obtained for salt free daddawa. 1% and 0.4 % NaCl have been reported to give maximum protease activity and enhanced protease production respectively in *Bacillus clausii* (Joo and Chang, 2005). Also, NaCl have been reported to influence the hydrolysis of protein in sufu (fermented soybean curd) to a large extent resulting in increased level of free amino acids (Han et al., 2003).

The pH increased significantly from 6.52 to 8.35 and 6.68 to 8.38 in 1 % salted and salt free daddawa respectively with fermentation (Table 1). The pH was significantly higher in salt free daddawa in the first 24 h of fermentation and thereafter the values became similar in the later stages of fermentation. The rise in pH is a result of proteolysis and the release of ammonia following the utilization of amino acids by the fermenting *Bacillus* sp. The release of ammonia or other basic end products have been reported to be common features in the fermentation of vegetable proteins (Hesseltine, 1965). Ammonia concentrations in soy-daddawa increased significantly in the first 48 h of fermentation after which the value remained stable (Table 2). The rise in pH coincides with the period of increased production of ammonia in the fermenting seeds. Ammonia concentration which was significantly higher in 1% salted daddawa may be a reflection of the enhanced proteolytic activity and release of ammonia following the utilization of amino acids by the increased population of *B. subtilis* SDA3.

The total soluble sugar decreased significantly while the reducing sugar increased in the first 12 h and decreased significantly thereafter in soy-daddawa with fermentation (Table 3). The utilization of the soluble sugars by the increasing population of *B. subtilis* SDA3 (Table 1) may have been responsible for the significant drop in the total soluble and reducing sugar levels. Similar trend have been reported in other fermented vegetable protein (Sanni and Ogbonna, 1991). The soluble sugar levels were significantly lower in 1% salted daddawa in the later stages of fermentation. The metabolic activities of *Bacillus* population that coincidentally was higher in the later stage of 1% salted daddawa fermentation (Table 1) may have been responsible for this observation.

There was no significant difference ($P<0.05$) in the organoleptic attributes scored for salt free and 1% salted daddawa (Table 4). However, the panelist consisting of regular soy-daddawa consumers showed preference for 1% salted daddawa. The difference in the level of biochemical constituents brought about by salt, most especially the enhanced increased levels of proteolytic activity and free amino acids may have improved the flavor of 1% salted daddawa.

The results of this study suggest that the addition of 1% salt improved the organoleptic quality of soy-daddawa produced by monoculture of *B. subtilis* SDA3 starter. The salt created a favorable medium for the growth of *B. subtilis* SDA3, resulting in increased populations, enhanced proteolytic activity and increased free amino acids which are considered to be important flavor – enhancing compounds in many fermented foods. Since volatile compounds formed from amino acid by transformation contributes to flavor (Chung, 1999), it is suggested that further studies on the amino acid profile and the volatile aroma compounds are necessary to clarify the contribution of salt in the flavor enhancement of soy-daddawa.

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Table 1. Changes in viable cell count and pH during fermentation of soybean with *Bacillus subtilis* SDA3 to produce soy-daddawa.

Fermentation time (h)	Viable count (log_{10} c.f.u.g^{-1}wet wt.)		pH	
	Salt free daddawa	1% salted daddawa	Salt free daddawa	1% Salted daddawa
0	5.13 ± 0.02^{a}	5.13 ± 0.02^{a}	6.68 ± 0.02^{j}	6.52 ± 0.00^{k}
12	9.01 ± 0.04^{b}	9.29 ± 0.08^{f}	6.96 ± 0.01^{m}	6.90 ± 0.01^{r}
24	9.77 ± 0.08^{c}	9.98 ± 0.06^{g}	7.20 ± 0.03^{n}	7.11 ± 0.01^{s}
48	10.04 ± 0.02^{d}	10.23 ± 0.04^{h}	8.16 ± 0.02^{p}	8.15 ± 0.01^{p}
72	10.23 ± 0.02^{e}	10.33 ± 0.05^{eh}	8.38 ± 0.00^{q}	8.35 ± 0.00^{q}

Values are means ± SE of three replicate fermentations.
Means having the same superscripts within the same column or row do not differ significantly (P< 0.05).

Table 2. Changes in free amino acids proteolytic activity and ammonia during fermentation of soybean with *Bacillus subtilis* SDA3 to produce soy-daddawa.

Fermantation time (h)	Free amino acid (mg glycine g^{-1}dry wt.)		Relative proteolytic activity*		NH_3 concentration (mg/100g wet wt.)	
	Salt free daddawa	1% salted daddawa	Salt free daddawa	1% salted daddawa	Salt free daddawa	1% salted daddawa
0	4.37 ± 0.06^{a}	4.47 ± 0.09^{a}	0.00 ± 0.00^{k}	0.00 ± 0.00^{k}	1.30 ± 0.01^{d}	1.30 ± 0.02^{d}
12	6.66 ± 0.05^{b}	7.28 ± 0.08^{f}	7.40 ± 0.02^{m}	7.90 ± 0.03^{r}	2.30 ± 0.02^{c}	2.80 ± 0.03^{e}
24	18.72 ± 0.10^{c}	19.79 ± 0.06^{g}	12.60 ± 0.06^{n}	14.30 ± 0.04^{s}	6.90 ± 0.04^{b}	7.20 ± 0.08^{f}
48	55.50 ± 0.06^{d}	$59.77\ 0.08^{h}$	18.50 ± 0.03^{p}	21.60 ± 0.10^{t}	33.00 ± 0.06^{n}	33.30 ± 0.10^{g}
72	59.87 ± 0.07^{e}	62.71 ± 0.10^{j}	16.70 ± 0.07^{q}	17.70 ± 0.05^{v}	33.00 ± 0.07^{ng}	33.20 ± 0.10^{g}

Values are means ± SE of three replicate fermentations.
*Measured as activity on azocasein.
Means having the same superscripts within the same column or row do not differ significantly (P<0.05).

Table 3. Changes in soluble sugar level of soy-daddawa during fermentation with *Bacillus subtilis* SDA3.

Fermentation time (h)	Total soluble sugar (mg glucose g^{-1} dry wt.)		Reducing sugar (mg glucose g^{-1} dry wt.)	
	Salt free daddawa	1% salted daddawa	Salt free daddawa	1% salted daddawa
0	28.14 ± 0.11^{a}	28.32 ± 0.06^{a}	2.27 ± 0.03^{j}	2.29 ± 0.04^{j}
12	24.36 ± 0.06^{b}	24.10 ± 0.13^{b}	9.14 ± 0.09^{k}	9.48 ± 0.05^{p}
24	18.13 ± 0.12^{c}	15.42 ± 0.10^{f}	7.77 ± 0.10^{i}	7.14 ± 0.09^{r}
48	15.95 ± 0.09^{d}	14.82 ± 0.06^{g}	7.30 ± 0.09^{m}	6.77 ± 0.03^{s}
72	15.39 ± 0.07^{e}	14.54 ± 0.05^{h}	7.02 ± 0.05^{n}	6.53 ± 0.05^{t}

Values are means ± SE of three replicate fermentations.
Means having the same superscripts within the same column or row do not differ significantly (P< 0.05).

Table 4. Sensory evaluation of soy-daddawa produced by starter culture of *Bacillus subtilis* SDA3.

Sample	Organoleptic attributes scored			
	Color	Aroma	Taste	General acceptability
Salt free daddawa	2.90 ± 0.23	3.50 ± 0.27	3.60 ± 0.22	3.90 ± 0.18
1% salted daddawa	3.10 ± 0.28	3.80 ± 0.25	4.20 ± 0.13	4.30 ± 0.21

Values are mean scores ± SE (n= 10).
The mean score for each attribute do not differ significantly (P< 0.05).

Manneheiem/R-Biopharm, Germany) and azocasein (Sigma A 2565)

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