THE ROLE OF *BACILLUS* SPECIES IN THE CHARACTERISTIC AROMA OF TRADITIONALLY FERMENTED 'SOY- DAWADAWA'.

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ABSTRACT

The traditional production of 'dawadawa' from the fermentation of soybean involves predominantly Bacillus species notably B. subtilis, B. licheniformis, B. pumilus and B. cereus. Although lactic acid bacteria were present in relatively low numbers at the onset of fermentation, they do not appear to play any major role in the fermentation process. The bacterial population, pH and moisture content increased with fermentation. The titratable acidity increased in the first 24 h and then dropped as fermentation progressed. Amylase activity increased rapidly with fermentation attaining a peak at 72h, while the reducing sugar increased in the first 24h and dropped. Protease activity also increased rapidly in the first 72 h and dropped giving higher amounts of free amino acids with the fermentation. The aroma of soydawadawa was predominantly given by a group of alcohols, phenols, acids and pyrazine.

INTRODUCTION

The use of soybean as a high protein human food in Ghana has in recent times received increasing attention. However the effective use of soybean for human consumption could be promoted through the formulation of soybean products acceptable in taste to consumers. Soydawadawa is gradually becoming one of the important fermented products on the Ghanaian market. It is produced from dehulled soybean and has the characteristic ammoniacal smell, taste and stickiness similar to that of African locust bean *(Parkia biglobosa)* dawadawa (Ogbadu and Okagbue, 1988a). It is used in traditional dishes as taste enhancer and low-cost meat substitute.

In Asia, soybean is fermented into products such as natto, misso and tempeh (Carrao *et al.*, 1994). A few like natto (Ohta, 1986) and Kinema (Sarkar *et al.*, 1993) are fermented by *Bacillus* species and appear similar to soydawadawa. Information on the microbiological, biochemical and nutritional changes during the fermentation of African locust bean for dawadawa are well documented (Eka 1980, Odunfa, 1981; 1985; Antai and Ibrahim, 1986; Ogbadu and Okagbue, 1988b; Abiose *et al.*, 1988; Ikenebomeh 1989). Only a few reports on the microbiology (Popoola and Akueshi, 1985; Ogbadu and Okagbue, 1988a), effects of added salt (Omafuvbe, 1994) and the microbiological and biochemical changes in the traditional fermentation of soybean for Soydawadawa (Omafuvbe *et al.*, 2000) has been well documented. There is no information on the characteristic aroma of traditionally fermented soydawadawa. In this paper, the role of *Bacillus* species in the microbiological and biochemical changes including extracellular enzyme activities and aroma compounds released during a 72 h soybean fermentation are reported.

MATERIALS AND METHODS

Preparation of 'Soydawadawa'

Soydawadawa was prepared using approximately 1000g of yellow seeded soybean. The beans were sorted, washed thoroughly and divided into two

portions. One portion was boiled and the other roasted for 30 mins. Both were dehulled, boiled for 1 h and incubated in baskets lined with banana leaves for up to 72h at 37°C ± 1°C.

Isolation and identification of micro-organisms.

Isolation of micro-organisms were made at zero time and 24h intervals. Samples (10g) were taken asceptically from duplicate fermenting mashes and diluted serially in 0.1% peptone water. Aliquots (1.0ml) of appropriate dilutions were plated in duplicate on plate count agar, nutrient agar and de Man, Rogosa and Sharpe agar, for bacteria enumeration and malt extract agar containing 50 μg choloramphenicol ml^{-1} for fungi enumeration. The plates were incubated at 37°C for 48h for bacteria test and 30°C for 4 days for fungi tests,The bacteria colonies were counted and expressed as colony forming units per gram ($cfug^{-1}$) of the sample. Representative colonies from the plate counts were purified by repeated streaking, characterized and identified by using API 50 CHB (Bio Merieux SA)

pH, titratable acidity and moisture content determination

The pH and titratable acidity (expressed as lactic acid) of the fermenting material were determined as previously described (Ikeneboamah, 1989). Moisture content was determined in accordance with AOAC (1990).

Determination of sugar and free amino acids.

The Lane and Eynon's method was used to determine the total sugars. The free amino acid content was determined by the Pico-Tag method and an amino acid analyzer. The amino acid concentration was obtained from peak areas for both the standard and samples and corrected by internal standard. The total sugar was determined by reading from a given table the proportions of the various sugars equivalent to 10 or 25ml of fehlings solution.

Extraction and assay of extracellular enzymes

The extracellular enzymes in the fermenting material (5g) were extracted with an appropriate buffer (50ml) following the method of Young and Wood (1977).

Protease activity

The extracting buffer was 0.1 M sodium hydrogen phosphate buffer, pH6.5. Protease activity was determined following the method of Young and Wood (1977). This involved the hydrolysis of 2% light soluble casein (BDH) (10ml) using 5ml of the enzyme solution at 35°C for 30 min. The reaction was terminated by adding 10ml of 10% trichloroacetic acid (TCA) solution. The mixture was filtered (Whatman No.1) and the optical density of the filtrate was obtained reading the absorbance at 275nm with an SPC 250 spectrophotometer. Enzyme activity was expressed in terms of an arbitrary unit called XS unit and is an enzyme extract which under the stated experimental conditions produced a filtrate with an optical density of 0.500 when measured in a 10mm path length cell, had a strength of 36 XS units per gram (Young and Wood, 1977).

α-amylase activity

The extracting buffer was 1M potassium hydrogen phosphate, pH 6.5. The assay procedure described by Bernfield (1955) was used to determine α-amylase activity. 1ml of 1% starch solution was hydrolysed using 2ml of the enzyme solution at 40°C for 1h. The reaction was stopped by adding 3ml dinitrosalicylic acid reagent (DNS). The mixture was heated in a boiling water bath for 5 minutes, cooled and diluted with 18ml water. The optical density of the solution was obtained reading the absorbance at 550nm with SPC 250 spectrophotometer. The amount of the reducing sugars formed was calculated from a standard curve prepared from known concentrations of maltose (Bernfield,1955).

Determination of Aroma Compounds

A GC-MS Hewlett Packard 6890 GC, 5973 MS, Avondale Pennsylvania was used to separate the soydawadawa extract into individual components

which were readily identified by the mass spectrometer.

Sample (2g) mixed with (10ml) distilled water and (10ml) n-hexane in a100ml separating funnel was separated into aqueous and solvent layers. Solvent was filtered (Whattman no.1) with anhydrous sodium sulphate into a 2ml screw head vial tightly capped for injection into a GC-MS for analysis. Column pressure in Gas Chromatography was 10ml /min and split temperature for the run was 45°C, Ramp and total run time 25 min. All reagents used were of analytical grade (Merck).

RESULT AND DISCUSSION

Bacteria were isolated and were identified as *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus* and *Bacillus cereus* in the three replicate fermentations. The total bacteria count ($cfug^{-1}$) and occurrence of isolates are shown in Table 1. The bacterial population increased with fermentation. The fungi were only isolated at the onset while *Bacillus* species and lactic acid bacteria predominated until the end of the 72h fermentation period. Our results vary from those of Omafuvbe *et al.*(2000) who reported the presence of *Micrcococcus* spp and *Staphylococcus* spp.in their 48h fermentation period for soydawadawa production. However the results are in line with those from Ogbadu and Okagbue (1988a) who also reported the absence of *Micrococcus* spp. and *Staphylocuccus* spp. at the same period of fermentation during soydawadawa production.

Although microbial counts generally increased with time of fermentation, the rate of increase of aerobic mesophiles was higher than that of the lactic acid bacteria due to increased pH in the sample which normally promote the growth of aerobic mesophiles like *Bacillus* species but is not very conducive for growth of lactic acid bacteria.

Aerobic mesophiles – counts on plate count agar including both gram-positive and gram-negative bacteria.

Lactic acid bacterial – enumerated on MRS agar reflecting gram-positive, catalase-negative rods and coccobacilli.

Fungi-determined on malt agar.
B-boiled
R-roasted

While *Bacillus* species have been found responsible for the production of a variety of Asian fermented soybean products like natto (Ohta,1986), kinema (Sarkar *et al.*, 1993) and other African fermented oil seeds other than soybeans (Abiose *et al.*,1988), Barber and Achinewhu (1992) Obeta and Ugwwuanyi (1996) *Staphylococcus* and *Micrococcus* species are hardly associated with the fermentation. Variations were observed in the proportion of the different Bacillus species in the two sets of samples examined.*Bacillus* subtilis however accounted for over 50% of the Bacillus population as shown in Table 2.

The high percentage of Bacillus species in the fermenting soydawadawa highlights the important role played by them during soydawadawa production. This is in line with Parry *et al.*, 1983 findings on the dominant role played by Bacillus species in the fermentation of a traditional staple product.The pretreatment given to the beans during production could be described as a spore activation process favouring selection of *Bacillus* species by virtue of their heat resistant spores.Odunfa (1981), first reported that the predominant fermentation microorganism was a *Bacillus*, possibly *Bacillus subtilis* and other species.Similar findings have been reported elsewhere (Campbell-Platt,1980; Ikenebomeh, 1982).

Moisture content, pH and total free amino acids increased, titratable acidity and reducing sugars increased gradually, while the total soluble sugar level dropped gradually, with fermentation (Table 3).The increase in moisture with fermentation may be due to the moist solid nature of the fermentation

and the hydrolytic decomposition of the fermenting substrate by the implicated micro-organisms. The rise in pH was presumably a result of proteolysis and the release of ammonia following the utilization of amino acids by the fermenting micro-organisms. The release of ammonia is responsible for the ammoniacal odour characteristic of most vegetable protein fermentations (Young and Wood, 1977; Oyeyiola, 1989; Sarkar *et al.* 1993). The observed decrease in titratable acidity after 24h of fermentation with increasing pH have been reported to occur during the fermentation of other protein food (Barber *et al.*, 1988; Oyeyiola, 1989).

However simultaneous increase in pH and titratable acidity have been reported in the fermentation of similar foods (Wagneknecht *et. al.* 1961, Ikenebomeh, 1989). It seems likely that certain changes not yet known could be responsible for the production of acidity in these fermented foods. The total sugar level in fermenting beans decreased gradually after 24h (Table 3), while α -amylase activity in the fermenting beans increased rapidly, attained a peak at 72h and dropped in the final product (Table 3).

Table 1. Total microbial count and occurrence of isolates in fermenting soybean for soydawadawa production

Fermentation Time (h)	Viable count (cfug-1)		Occurrence of isolates
	Boiled soydawadawa	Roasted soydawadawa	
0h			
Aerobic mesophiles	$2.5x10^3$	$9.ox10^3$	+
Lactic acid bacteria	$6.1x10^3$	$6.3x10^4$	+
Fungi	$1.5x10^3$	2.0x10	+
24h			
Aerobic mesophiles	$4.6x10^9$	$1.2x10^9$	+
Lactic acid bacteria	$2.5x10^5$	$5.0x10^5$	+
Fungi	$1.0x10^3$	1.0x10	+
48h			
Aerobic mesophiles	$1.2x10^9$	$9.0X10^9$	+
Lactic acid bacteria	$1.9x10^6$	$7.5x10^6$	+
Fungi	No growth	No growth	-
72h			
Aerobic mesophiles	$1.5x10^{11}$	$1.8x10^9$	+
Lactic acid bacteria	$1.4x10^6$	$5.9x10^5$	+
Fungi	No growth	No growth	–

Table 2: Distribution (percentage) of different *Bacillus* species in soydawadawa during fermentation

Bacillus species	% of Organism	
	B. soydawadawa	R. soydawadawa
B. subtilis	50	48
B. pumilus	20	20
B. licheniformis	6	7
B. cereus	16	16
B. firmus	8	9

** A total of 224 isolates from two samples of soydawadawa taken at a 24h intervals for 3d.*

Table 3: Changes in physical, chemical and enzyme activities during soydawadwa production

Fermentation time (h)	pH		titratable* acidity		reducing** sugars		α-amylase (u/ml)		Protease (u/ml)	
0	6.59	6.41	0.07	0.12	8.0	7.8	0.00	0.10	0.18	0.30
24	6.87	6.57	0.39	0.42	13.0	12.5	0.20	0.25	1.00	1.10
48	8.00	7.90	0.35	0.51	6.5	6.8	0.3	0.32	3.00	2.50
72	8.25	8.15	0.42	0.42	1.5	1.2	0.8	0.82	4.10	3.30

** titratable acidity - (mg lactic acid/g)*
***reducing sugar 0 (mg/g dry weight)*
Note: boiled soydawadawa is represented by the first numbers under each heading and roasted soydawadawa is represented by the second.

Table 4: Major aroma compounds detected by GC-MS dring Production

Aroma component	B. soydawadawa			R.soydawadawa		
	0h	24h	48h	0h	24h	48h
3-Hexanol	+	+	+	+	+	+
9, 12-Octadecanoic ACID	+	+	+	+	+	+
Phenyl ethyl alcohol	-	+	+	-	-	-
2,4-bis(1,1-dimethyl ethyl phenol)	-	-	+	+	-	+
Tetradecanoic acid	-	-	-	+	+	+
1,2-Benzene dicarboxylic acid	-	-	-	-	+	+
2-methoxy-phenol	-	-	-	-	-	+
2.5-dimethyl pyrazine	-	-	-	-	-	+

The rapid increase in the total free amino acids in the early stages of fermentation coincides with the increased protease activity which reached its peak at the 72 hour of fermentation (Table 3). Protease activity has been reported to be abundant in the fermentation of similar protein rich foods (Young and Wood, 1977; Abiose *et al.* 1988; Sarkar *et al.*, 1993).The high level of free amino acids is a reflection of the high protein content of soybean.

Aroma compounds detected in the fermenting beans were basically alcohols, ketones, aromatic aliphatic organic acids and phenols. More aroma compounds were detected in the roasted product and were also at higher levels. Acetophenone and pyrazines detected in the roasted product has been found to be components related to protein degradation during maillard browning in roasted foods. The flavour impressions of many foodstuffs implies a complex mixture of aroma substances and many traditional foodstuffs and beverages are flavoured in situ by the action of micro-organisms example the use of proteases produced by bacteria in the meat industry (Schermers *et al.*, 1976). Many vegetable proteins such as soybeans possess characteristic beany flavours and odour which limit their sale and use by a large number of potential consumers. Non pathogenic bacteria have however been found to proliferate such foods and remove the flavoured and odour from it (Hanson, 1974). Wang *et al.* (1968) found roasting to be necessary in order to produce soy protein products with flavour scores approaching the blandness of wheat flour.

This study has shown that soydawadawa is produced by the fermentation of bacteria notably *Bacillus* species. The *Bacillus* species produced extracellular enzymes which hydrolysed the organic components of the fermenting beans resulting in increased soluble products especially free amino acids. Also it has been established that the method of production affects the aroma of the fermenting beans. It is envisaged that the development of a starter culture and the optimization of the fermentation parameters would help to produce good consistent quality dawadawa.

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