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Biocatalysis of *H. sabdariffa* during 'dawadawan botso' production and biogeneration of volatile compounds

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ABSTRACT

The analysis of free amino acid and volatile compounds were conducted to understand how the fermenting organism's biocatalyse *H. sabdariffa* seeds and its role in the biogeneration of free amino acids and flavour compounds in "dawadawan botso". Fermentation increased the quantity of all essential amino acids except of threonine which decrease by 1.23 g/100 g protein. Arginine had the highest increase with a value of 3.06 g/100 g protein while proline had the least value of 0.22 g/100 g protein. Fermentation increased the total free amino acid from 68.32% to 76.79%. The values of bitter, sweet and MSG-like free amino acids in the unfermented seeds and "dawadawan botso" were different. A total of 22 compounds were identified from the fresh "dawadawan botso" and locally produced dried "dawadawan botso". Predominant among them are Methyl (9Z) – 12- hydroxyl -9 –octadecenoate (40.66%) in fresh, Methyl (14E) – 14, 17- Octadecadienoate (33.97%) in dried and Cis -9- Hexedecenal (19.96%, 15.13%) in both samples. The compounds include alcohols, acids, esters, aldehydes, and alkanes. In this study, fermentation increased the bioavailability of free amino acids and volatile compounds in "dawadawan botso".

Keywords: "Dawadawan botso", Fermentation, Free amino acids, GC-MS analysis, *H. sabdariffa*, Volatile flavor compounds.

INTRODUCTION

Plant and animal products that are fermented by microorganisms for human consumption contain a great variety of organic compounds and microorganisms that ferment them or grow on them cause many biochemical changes which result into a variety of products. These changes mostly affect the major constituents of foods, improves the digestibility, nutritive value and flavour (Achi, 2005). High amount of ammonia-like flavor in local condiment is a displeasing factor to some consumers. Free amino acids (FAA) following proteolysis have been reported to undergo further transformation to yield aroma compounds (Tavaria et al., 2002).

'Dawadawan botso' is a condiment produced by a traditional uncontrolled fermentation of the seeds of *Hibiscus sabdariffa* by the rural dwellers of Zuru who call it "Chwande". This condiment is also produced by other northern states such as Plateau (Schippers, 2000), where the Tarok

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people ferment Roselle seeds to make a cake to be used as “sorrel meat” or Iyu (Schippers, 2000) and Borno where the Babur/ Bura ethnic groups, call it “Nwanza Ntuza” (Ayodele and Musa, 2008) and African Countries such as Burkina Faso where it is called ‘Bikalga’, Mali where it is called ‘Datou’, Cameroon where it is called ‘Mbuja’, Sudan where it is called ‘Furundu’ and Niger where it is called “Dawadawan botso” (Parkouda et al., 2008; Bengaly et al., 2006a). Naturally occurring food proteins do not contribute chemically to flavor formation in foods but peptides and amino acids do have a flavour. Amino acids taste either bitter or sweet, with the exception of aspartic and glutamic acid which have a sour taste (Shallenberger, 1994). Traditionally, these hydrolyzed products, available as flavour enhancers for soups and stock cubes, have been produced by acid hydrolysis of proteins during heat treatment. Flavours produced in this way are widely used in the food industry. Increasing concern about the less healthy compounds that develop during acid hydrolysis has led to alternative flavor production processes based on enzymatic hydrolysis of protein (Soe, 2007).

Proteolysis and lipolysis are the most principal and complex biochemical event occurring during the preparation of most fermented food condiments (Parkouda et al., 2009; Azokpota et al., 2006; Beaumont, 2002; Aderibigbe and Odunfa, 1990). The degradation products not only have a considerable influence on the nutritional values, but also contribute directly to the taste characteristics, or serve as a precursor of aromatic products (Han et al., 2004; Kiers et al., 2000). It is well established that the water-soluble fraction contains the majority of taste compounds such as salt and amino acids produced during proteolysis (Kim and Lee, 2003). Consequently, quality indexes such as free amino acids of many soy-fermented foods have been reported (Han et al., 2004; Kim and Lee, 2003; Omafuvbe et al., 2000). Studies on the volatile flavour compounds in traditional soy sauce made in several regions such as Japan, Korea, including Indonesia have been reported (Kobayashi and Sugawara, 1999; Kim et al., 1996; Seo et al., 1996). However, little or no reports are available about the amino acid profile and sensory properties such as taste and aroma of “Dawadawan botso”. This study was undertaken to investigate the amino acid profile, and to identify the profiles of odorants responsible for its unique flavours using GC-MS analysis of its chloroform extracts.

MATERIALS AND METHODS

The seeds of *Hibiscus sabdariffa* used in this study were purchased from Zuru in Kebbi State. The procedure for “Dawadawan botso” production among Dakarkari ethnic group of Zuru, Kebbi State, Nigeria is presented in Figure 1.

Determination of amino acid profile

The amino acid profile was determined using methods described by Spackman et al. (1958). The samples were dried to constant weight, defatted, hydrolysed and analysed using sequential Multi-sample Amino Acid Analyzer (TSM). The samples were defatted by weighing a known weight of the dried sample into extraction thimble and the fat was extracted with chloroform/methanol (2:1 mixture) using soxhlet extraction apparatus as described by AOAC (2006). Nitrogen was determined using MicroKjedhal methods (AOAC 2006). The samples were hydrolyzed by weighing the defatted sample into glass ampoule and seven millilitres (7 ml) of 6N HCl was added and oxygen was expelled by passing nitrogen into the ampoule. The glass ampoule was sealed with Bunsen burner flame and put in an oven preset at 105 ^{0}C for 22 hours and the content was filtered to remove the humins. The filtrate was evaporated to dryness at 40 ^{0}C under vacuum in a rotary evaporator and the residue was dissolved with 5 ml of acetate buffer (pH 2.0) and stored in plastic bottles and kept in freezer. The Hydrolysate was loaded into the TSM

Hibiscus sabdariffa raw seeds
↓
Dry cleaning (Pounding, winnowing and removal of stones)
↓
Dry cleaned seeds
↓
Water washing
↓
Cleaned and selected seeds
↓
Cooking (8-10h.)
↓
Cooked seeds
↓
First fermentation (2 days)
↓
Deep pounding
↓
Mixing with ash leacheate
↓
Second fermentation (1day)
↓
Drying under sun for 2-3 days according to sunshine intensity
↓
“Dawadawan botso”

Figure 1: Flow chart for “Dawadawan botso” production in Zuru.

analyzer by loading 5 to 10 microlitres (5 for acidic/ neutral amino acid and 10 for basic amino acids). This was dispensed into the cartridge of the analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of an analysis lasted for 76 minutes.

Grouping of free amino acid

This was done in accordance to the taste characteristics described by Tseng et al. (2005), amino acids were grouped as MSG-like (monosodium glutamate-like) (Asp+Glu), sweet (Ala+Gly+Ser+Thr), bitter (Arg+His+Ile+Leu+Met+Phe+Trp+Try+Val), and tasteless (Cys+Lys+Pro).

Extraction of volatile compounds

Extraction of volatile compounds was done by direct solvent extraction method. Two gram of spoilt tomatoes was weighed into a bottle and saturated with 20 ml of chloroform. It was allowed to stand at room temperature for 24 hours, filtered using Whatman filter paper and the filtrate was collected in a sterile bottle, closed tightly before the GC-MS analysis.

Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis was performed using GC-MS-QP2010 plus (Shimadzu, Japan) equipped with flame ionization detector (FID). The injection was conducted in split less mode at 250 ^{0}C for 3 min by using an inlet of 0.75 mm i.d to minimize peak broadening. Chromatography separations were performed by using DB-WAX analytical column 30 m 0.25 mm, 0.25 mm (J&W scientific, Folsom C.A) with helium as carrier gas at a constant flow rate of 0.8 ml/min. The oven temperature was programmed at 60 ^{o}C for 5 min, followed by an increase (held for 5 min), and finally at 10 ^{0}C/min to 280 ^{o}C (held for 10 min). The temperature of the FID was set to 250 ^{o}C. MS operating conditions (electron impact ionization mode) were an ion source temperature of 200 ^{o}C, ionization voltage of 70 eV and mass scan range of m/z 23- 450 at 2.76 scans/s.

Identification and quantification of volatile compounds

The chromatographic peak identification was carried out by comparing their mass spectra with those of the bibliography data of unknown compounds from the NIST library mass spectra database on the basis of the criterion similarity (SI)>800 (the highest value being 1,000). According to the method of (Wanakhachornkrai and Lertsiri, 2003) approximate quantification of volatile compounds was estimated by the integration of peaks on the total ion chromatogram using Xcalibur software (Vienna, VA). The results are presented as the peak area normalized (%).

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RESULTS

The content of individual free amino acid and total free amino acid in fresh and dried “dawadawan botso” are shown in Table 1 and Figure 2. Fermentation increased the quantity of all essential amino acids except threonine which decrease by 1.23 g/100 g protein. Arginine had the highest increase with a value of 3.06 g/ 100 g protein, while proline recorded the least value of 0.22 g/100 g protein. The free amino acids groupings based on their taste characteristics as described by Tseng et al. (2005) are presented in Figure 3. A decrease of 1.4% and 5.54% was observed after fermentation in the content of two free amino acid classes, MSG-like and sweet free amino acid. However, for tasteless free amino acid class, a slight increase of 0.21% was observed, and an increase of 6.73% was also observed in the content of bitter free amino acid.

A total of 22 compounds were identified in the freshly produced “dawadawan botso” and locally produced dried “dawadawan botso”. The compounds include alcohols, acids, esters, aldehydes, ketones, and alkanes. Volatile compounds were also analyzed to understand the unique flavor of “dawadawan botso”. As observed from Table 2. A total of 11 and 17 kinds of compounds were identified in the freshly fermented and the locally fermented dried samples, respectively. In total, 22 kinds of volatile compounds were identified, including alcohols (3), acids (8), esters (7), aldehydes (3), and alkanes (1). The major compound in the fresh sample was methyl (9Z) – 12-hydroxyl -9 –octadecenoate with 40.66% and methyl (14E) – 14, 17- octadecadienoate was the dominated compound in the dried sample with 33.97%.

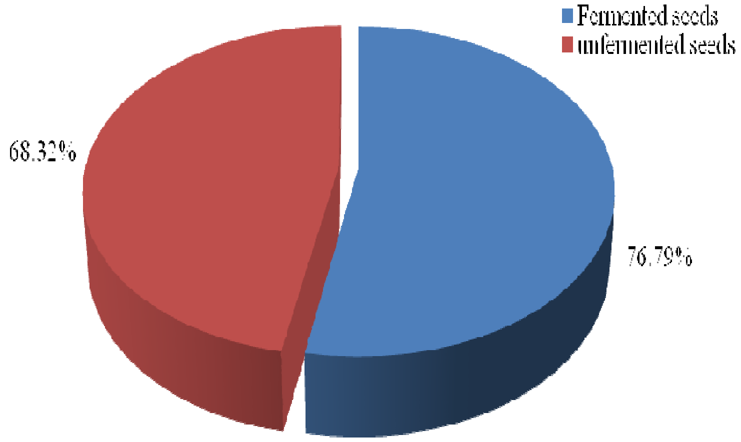

Figure 2: Total free amino acid content (%) of unfermented seeds and fermented seeds of *H. sabdariffa.*

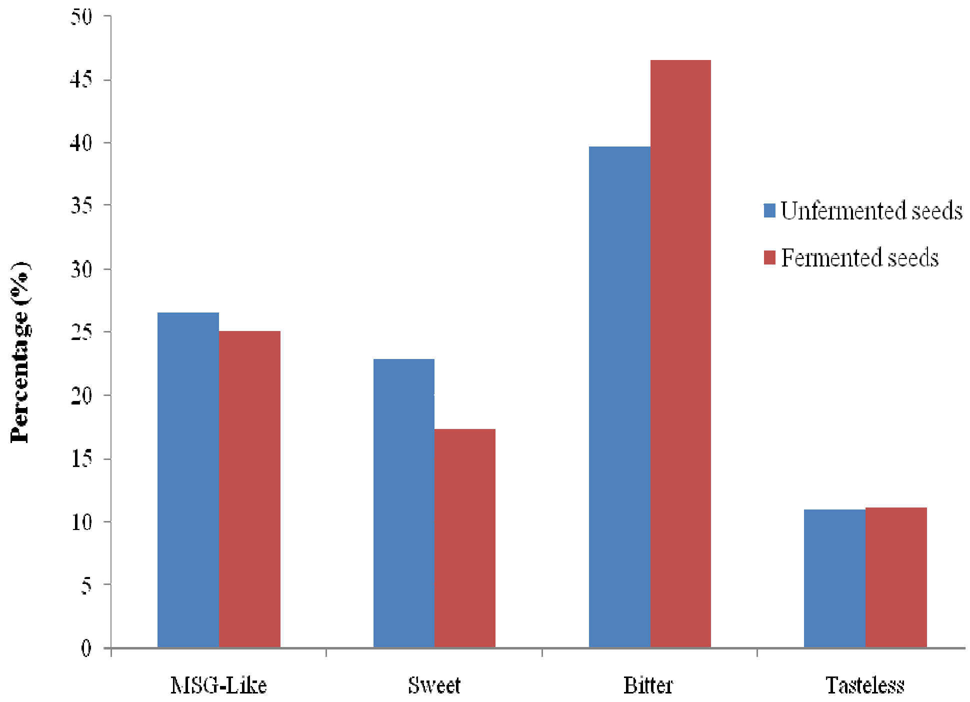

Figure 3: Free amino acid classes (%) imparted the different taste in unfermented seeds and fermented seeds of *H. sabdariffa.*

Table 1: Amino acid profile of unfermented seeds of *H. sabdariffa* and "dawadawan botso" (g/100 g protein).

Amino acids	Unfermented	'dawadawan botso'
Essential amino acids		
Lysine	2.79	3.92 (1.13)
Histidine	2.26	3.07 (0.81)
Threonine	3.39	2.16 (-1.23)
Valine	3.95	4.30 (0.35)
Methionine	1.02	1.25 (0.23)
Isoleucine	3.39	4.27 (0.88)
Leucine	5.38	6.92 (1.54)
Phenylalanine	4.57	5.58 (1.01)
Non-essential amino acids		
Arginine	4.94	8.00 (3.06)
Aspartic acid	8.35	9.10 (0.75)
Serine	2.64	2.37 (-0.27)
Glutamic acid	9.77	10.19 (0.42)
Proline	2.97	3.19 (0.22)
Glycine	4.96	4.52 (-0.44)
Alanine	4.63	4.25 (-0.38)
Cystine	1.70	1.44 (-0.26)
Tyrosine	1.61	2.26 (0.65)

Values in parentheses are the differences between the unfermented seeds and "dawadawan botso"

DISCUSSION

Amino acid profiles of *H. sabdariffa* seeds and "dawadawan botso" was evaluated. The results suggest a slight difference in amino acid content between *H. sabdariffa* seed, however no significant difference at $P<0.05$ was observed. The major amino acids are glutamic acid, aspartic acid and arginine. Similar observations were reported on Egyptian roselle (El-Adawy and Khalil, 1994) and Burkina Faso roselle (Bengaly et al., 2006a). The increased in the quantity of some essential amino acids and total free amino acid after fermentation may be due to the proteolytic activities of the bacteria during fermentation.

The free amino acids groupings based on their taste characteristics showed a decrease of 1.4% and 5.54% after fermentation in the content of MSG-like and sweet free amino acid. The decrease in the content of MSG like and sweet free amino acid class could also be explained by the fact that the fermenting organisms might have utilized for their metabolism and further biotransformed them to eventually yield volatile aroma compounds during fermentation. Similar result has been observed in cheese (Tavaria et al., 2002). The taste of the "dawadawan botso" was MSG-like, sweet and slight saltiness. The slight saltiness may be directly related to the ash leachate added during the production process and MSG-like may be due to high concentration of aspartic and glutamic acid. Similar result has been reported for Chinese solid fermented soy sauce (Tseng et al., 2005). Despite an increase of 6.73% observed in the content of bitter free amino acid, the overall taste of the "dawadawan botso" was not affected. This may be attributed to the fact that the other classes of free amino acid class have a kind of masking effect on the bitter amino acid class. Similar result has been observed in soybean paste (Kim and Lee, 2003).

Table 2: Volatile Compounds of the chloroform extract in fresh and dried "dawadawan botso".

RT[1] (Min)	Volatile compounds	Peak area normalized (%)	
		Fresh	**Dried**
6.63	Ethylmethylacetic acid (2-methyl-butanoic acid)	-	0.80
9.88	4-methyl-pentanioc acid (Isobutylacetic acid)	-	1.09
13.80	1 –Methyl-4-ispropenylcyclohexan-3-ol	-	0.83
18.13	3,7 – Dimethyldecane	-	0.13
18.43	4–Methyl–1-hexanol	-	0.06
22.81	1-methylcyclopentanethiol	-	0.13
26.11	Methyl 15 – Methylhexadecanoate	-	0.17
26.56	Undecanoic acid	-	0.23
27. 68	Methyl hexadecanoate	-	3.22
27. 69	Hexadecanoic acid, methyl ester (Metholene)	-	12.87
28.01	Hexadecanoic acid	-	7.38
28.06	n-Hexadecanoic acid (Palmitic acid)	-	10.71
28.88	Methyl Cis – 6 – octadecenoate	-	9.59
28.88	Methyl (14E) – 14, 17- Octadecadienoate	-	33.97
29.01	Octadecanoic acid, methyl ester (Kemester 9718)	-	6.38
29.23	Cis -9- Hexedecenal	19.96	15.13
29.33	Octadecanoic acid	6.61	-
30.10	Methyl (9Z) – 12- hydroxyl -9 –octadecenoate	40.66	-
30.58	Oleic acid amide (Adogen 73)	2.26	3.24
30.83	Cis, Cis – 7, 10 – Hexadecadienal	2.71	-
30.83	Z – 7 – Tetradecenal	-	6.06
31.13	Linoleic acid chloride	5.55	5.88

[1] Retention time (RT) on DB-WBXcolumn in GC-MS.

Ethylmethylacetic acid and isobutylacetic acid are the main compounds in these acidic compounds which contribute to the old cheesy and cheesy flavor of "dawadawan botso" respectively. Undecanoic acid and hexadecanoic acid are known to give the products their characteristic waxy aroma. Hexadecanoic acid, methyl ester is also known to impart the sweet aroma that is perceived in this product. Ethylmethylacetic acid and isobutylacetic acid have being identified in Chinese solid fermented soy sauce (Yanfang and Wenyi, 2009).

The carbonyl compounds included 3 aldehydes, as Cis-9-hexedecenal, Cis, Cis–7, 10–hexadecadienal, and Z–7–tetradecenal in the product. The evolution of these compounds may be explained by the fact that aldehydes are intermediate unstable compounds being easily reduced to alcohols (Estrella et al., 2004). The ester compounds included methyl- hexadecanoate, methyl cis-6-octadecenoate, methyl (9Z)-12-hydroxyl-9-octadecenoate, methyl 15-methylhexadecanoate, methyl (14E)-14, 17-octadecadienoate, octadecanoic acid, methyl ester (methyl n-octadecanoate), and oleic acid amide (9-octadecenamide). The importance of these esters which contribute to food aroma has been emphasized particularly with the fact that esters with low carbon atoms are highly volatile at ambient temperatures and the perception thresholds are 10 times lower than their alcohol precursors (Izco and Torre, 2000;

Nogueira et al., 2005). In addition to imparting a fruity floral character, esters can diminish or mask the sharpness of unpleasant free amino acid-derived notes. These esters might have been formed by esterification between the short-chain acids and the alcohols. Similar result has been observed in Chinese solid fermented soy sauce (Yanfang and Wenyi, 2009). Methyl 15-methylhexadecanoate could confirm the assertion by its consumers that, its consumption makes them look young and healthy even at old age; this is because it is used as skin conditioning agent and emollients. Oleic acid amide (9-octadecenamide) has been described to possess germicidal and insecticidal properties explaining the reasons why pathogens and insects are not found in this product especially during drying when it is exposed under the sun.

Alcohols constitute some percentage of the total volatiles detected in "dawadawan botso" among which 1-methyl-4-ispropenylcyclohexan-3-ol, 4–Methyl–1-hexanol, and 1-methyl-cyclopentanethiol were detected in the product. One (1)-methyl-cyclopentanethiol might be responsible for the powerful meaty flavour in "dawadawan botso". This compound has been detected and reported to contribute to meaty and soy sauce-like flavour in Chinese solid fermented soy sauce (Chung, 1999).

Conclusion

In this study, fermentation increased the quantity of all essential amino acids except threonine which decrease by 1.23 g/100 g protein. Arginine had the highest increase with a value of 3.06 g/ 100 g protein, while proline recorded the least value of 0.22 g/100 g protein. A decrease of 1.4% and 5.54% was observed after fermentation in the content of two free amino acid classes, MSG-like and sweet free amino acid. However, for tasteless free amino acid class, a slight increase of 0.21% was observed, and an increase of 6.73% was also observed in the content of bitter free amino acid. A total of 11 and 17 kinds of compounds were identified in the freshly fermented and the locally fermented dried samples, respectively. In total, 22 kinds of volatile compounds were identified, including alcohols (3), acids (8), esters (7), aldehydes (3), and alkanes (1). The major compound in the fresh sample was methyl (9Z) – 12- hydroxyl -9 –octadecenoate with 40.66% and methyl (14E) – 14, 17-octadecadienoate was the dominated compound in the dried sample with 33.97%. In this study, fermentation increased the bioavailability of free amino acids and volatile compounds in "dawadawan botso". However, there is need to validate this fact by conducting further studies on the interaction of these free amino acids groupings based on their taste characteristics and the source of the increase in bitter amino acid class.

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