JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

EFFECTS OF SUBSTITUTION OF *ACHA* AND SOYBEAN ON ALPHA-AMYLASE ACTIVITY, SUGARS AND TOTAL FREE AMINO ACID DURING PRODUCTION OF MAIZE *MASA*

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doi: 10.15414/jmbfs.2019/20.9.3.534-538

ARTICLE INFO

Received 16. 8. 2018
Revised 13. 5. 2019
Accepted 30. 5. 2019
Published 1. 12. 2019

Regular article

OPEN ACCESS

ABSTRACT

Food insecurity and malnutrition are the major problems facing developing countries. The aim of this research was to determine alpha-amylase activity, total reducing sugar, individual sugar and total sugar contents of *masa* at six hour interval during fermentation. In this study, different ratios of *acha* was substituted for maize in the production of soybean fortified *masa* to reduce its sugar content and increase the amino acid content. The result showed that α-amylase activity, total reducing sugar, total sugar and total free amino acid were within the range of 4.63 - 9.30 E.U., 13.66 - 37.58 mg glucose/g., 133.25 - 391.56 mg glucose/g and 3.92 - 12.99 mg glycine/g, respectively. Alpha-amylase activity and total reducing sugar increased with increase in maize and were highest in *masa* produced from 100 % maize. Sugars identified during fermentation of *masa* were glucose, galactose, maltose and raffinose. Substitution of maize with *acha* and soybean reduced the sugar contents and α-amylase activity of *masa* while total free amino acid increased with increase in fortification with soybean. *Masa* produced from 60% maize, 20% *acha* and 20% soybean had the highest free amino acid and lowest sugar content.

Keywords: Fortification, α-amylase, sugar, free amino acid, *masa*, *acha*

INTRODUCTION

Masa, a fermented food in Nigeria is made from common cereal such as maize, rice and millet. It is popular in the North and South-West **(Ayo *et al.*, 2007; Igwe *et al.*, 2013; Sanni and Adesulu, 2013)**. Maize (*Zea mays*) is an important cereal grain in the world and it has a diverse form of utilization including human food uses, animal feeds formulation and raw materials for industries **(Sanni and Adesulu, 2013).** Maize can be processed in so many ways depending on the desired product. It can be eaten boiled or roasted, fermented into traditional food products such as *ogi*, *banku*, *kunnu* and *masa*, processed into meal or flour and/ or used as adjunct in breweries **(Oladejo and Adetunji, 2012).** *Acha* (*Digitaria exilis*), an underutilized cereal of West African origin is abundant in the North-Central part of Nigeria **(Philip and Itodo, 2012)** and is considered to be the oldest West African cereal with cultivation dating back to 5000 BC **(Haq and Ogbe, 1995; Lasekan *et al.*, 2001)**. They are perhaps the world's fastest maturing cereal, producing grains six to eight weeks after they are planted **(Ibrahim, 2001)**. *Acha* has the potential of providing enough food for the increasing population of poor people in West Africa and in the world at large **(Kwon-Ndung and Misari, 1999; Ibrahim, 2001; Oyetayo and Agbaje, 2012).** It does not contain any glutenin or gliadine proteins which are the constituents of gluten, making this cereal suitable for people with gluten intolerance **(Jideani, 1999; Ayo *et al.*, 2014).**

Fermentation is one of the oldest methods of food processing. It is one of the important techniques employed to extend the shelf-life of raw food materials **(Isabel *et al.*, 2005).** It is important in improving the rheological properties, acidification, taste and flavour of food **(Hammes *et al.*, 2005).** Improvement in starch digestibility during fermentation can be related to enzymatic properties of fermenting microflora that brings about the breakdown of starch. This enzyme brings about cleavage of amylose and amylopectin to maltose and glucose **(Mugula *et al.*, 2003; Kohajdová and Karovičová, 2007).** Fermentation of cereals by lactic acid bacteria has been reported to increase total free amino acids and their derivatives by proteolysis and/or by metabolic synthesis **(Mugula *et al.*, 2003).**

Fermentation improves absorption of nutrient especially plant product by enzymatic splitting of cellulose, hemicellulose, and related polymers that are not digestible by humans into simpler sugars and sugar derivatives **(Parker, 2018)**. Most of these enzymes are naturally present in cereal grains but at low levels **(Poutanen, 1997)**. Starch hydrolysis by amylases, an enzyme that breaks down starch into sugar, is the basic tool for various industrial processes like preparation of glucose syrups, bread making and brewing. Increase in amylase activity has been reported to increase the level of fermentable and reducing sugars in the flour and dough, thereby promoting yeast fermentation **(Goesaert *et al.*, 2006; Saranraj and Stella, 2013).**

Cereal is generally deficient in tryptophan and lysine which is abundant in legume. Fortification of *masa* with locally available and cheap source of protein such as soybean will increase the amino acid balance of *masa* products. The study evaluated the effect of *acha* substitution and soybean fortification on the sugar and amino acid contents of *masa* during fermentation.

MATERIALS AND METHODS

Materials

Acha was obtained from Sabon Gari market, Zaria, Nigeria and identified at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile - Ife. Quality Protein Maize (Ile-1-OB) and soybeans (TGX 1740 2E) were obtained from Institute of Agricultural Research and Training, Ibadan, Nigeria and chemicals and reagents used were of analytical grade (BDH, England).

Preparation of *masa*

Masa was produced using modified method of **Owusu-Kwarteng and Akabanda (2014)**. Maize and *acha* were cleaned, weighed, washed, steeped in water for 12 h at ambient temperature (27 ± 2 °C), washed and drained. Soybean was cleaned and steeped in water for 2 h at room temperature (27 ± 2 °C), blanched for 20 min in boiling water and dehulled by hand and hull was separated from the cotyledon and drained. Maize, *acha*, and soybean seeds were mixed at ratios: 100:0:0, 0:100:0, 70:20:10, 60:30:10, 60:20:20, 50:40:10, 50:30:20, 40:40:20 and then milled. The batter obtained was divided into three portions. One third of each ground sample was mixed with equal amount of water and then pregelatinized. The pregelatinized portions were mixed with the uncooked two third portions and resulting batter from the mixtures were spontaneously fermented for 24 h at ambient temperature (27 ± 2 °C) and then fried in a stainless steel frying pan containing hot vegetable oil (Bleached Palm Olein) until brown crust is obtained.

Determination of α-amylase activity

Alpha-amylase activity of the fermenting *masa* samples were determined as described by **Adeniran and Abiose (2007)**. Each fermenting *masa* sample (5 g) was weighed at 0, 12, 18 and 24 h of fermentation and homogenised in 50 ml of 0.2 M sodium acetate buffer (pH 4.0). The homogenate was transferred into conical flask and was mechanically shaken at 150 rpm for 10 min at room temperature in a Gallenkamp orbit shaker (3597 C2-2, England). The suspensions were transferred to centrifuge tubes and centrifuged at 5000 rpm for 30 min in centrifuge (Bosch Model No TDL-5, Germany) and the supernatant was collected as crude enzyme for enzyme assay. The substrate for assay was 0.5 ml of 0.5 % soluble starch, buffered with 0.2 ml of 0.2 M sodium acetate (pH 5.6). Crude enzyme extract (0.3 ml) was added to the mixture, mixed and incubated at 40 °C for 30 min in Gallenkomp water bath (HH-S6, England). The reaction was terminated by the addition of 2 ml of 3, 5 Dinitrosalicyclic acid (DNSA) and boiled for 5 min in water bath. The mixture was cooled under running water and 7 ml of distilled water was added. Blank that consisted of 0.3 ml distilled water, 0.5 ml of 0.5 % soluble starch and 0.2 ml of buffer received similar treatment. The optical density of the resultant solution was read at 540 nm in UV Spectrophotometer (Spectrumlab 752S, YM1206PHB2, China). Reducing sugar in the samples was estimated from a standard curve of known concentrations of maltose (0-1000 µg/ml). One unit of α-amylase was defined as the amount of enzyme required to produce 1 microgram of reducing sugar equivalents per minute measured as maltose from soluble starch under the experimental conditions.

$$1 \text{ enzyme unit (E.U.)} = 1 \text{ µg of maltose produced/min}$$

Extraction of sugar from *masa* samples

Dried ground sample (5 g) was weighed into 250 ml conical flask and 50 ml of 80 % ethanol v/v was added. The suspension was mixed properly and 10 ml of petroleum ether was added. The ethanol-petroleum ether suspension was stirred at room temperature for 30 min in Lab line magnetic stirrer (Lab-line, Model No 1580-1, U.S.A.). The mixture was transferred into centrifuge tubes (Bosch Model No TDL-5, Germany) and centrifuged at 5000 rpm for 30 min. The petroleum ether phase was discarded and the clear ethanol phase was used as the sample extract **(Omafuvbe *et al*., 2000**).

Determination of total reducing sugar

Ethanolic extract (1 ml) was measured into each test tube; 2 ml of Dinitrosalicyclic acid reagent was added and boiled for 5 min at 100 ºC in Gallenkamp water bath. After boiling, each tube was thoroughly cooled under running water and 7 ml of distilled water was added. The absorbance was read against reagent blank at 540 nm in a UV Spectrophotometer (Spectrumlab 752S, YM1206PHB2, China) and the amount of reducing sugar in the samples was extrapolated from a standard curve of known concentrations of glucose (0-1000 µg/ml) **(Adepoju *et al*., 2016).**

Identification of sugars

Thin layer chromatography was employed in identification of sugar. Sugar standard was prepared by dissolving 0.5 g of each standard sugar (maltose, glucose, fructose, galactose, xylose, sucrose, and raffinose) in 100 ml distilled water. The solvent system was prepared by mixing ethyl acetate, glacier acetic acid, ethanol and distilled water at ratio 60:15:15:10. Standard sugar solutions (10 µg) and extract (10 µg) were spotted on the plate and dried at room temperature before placing in the chromatograph tank containing the solvent system. The chromatograph plate was positioned upright in the tank and the experiment was run for 1 h. Plate was removed from the tank and allowed to dry at room temperature. Detection agent containing 0.2 ml of concentrated sulphuric acid, 20 mg of naphthol resorcinol and 10 ml of 90 % ethanol was sprayed on the chromatograph plate, and dried in the Gallenkamp hot air oven (Model OV-440) at 100 °C for 30 min **(Adepoju *et al*., 2016).**

Determination of total sugar

Total sugar was determined using the anthrone reagent method of **Morris (1948)**. Stock solution was prepared by dissolving 0.01 g of glucose in 100 ml of distilled water. Standard was prepared by dispensing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml into test tubes and made up to 1ml with distilled water. Ethanolic extract (1 ml) was added to 4 ml of anthrone reagent, heated in boiling water bath (Gallenkomp, HH-S6, England) for 10 min and rapidly cooled. Optical density (O.D) of the solutions was read at 620 nm against a reference blank in spectrophotometer (Spectrumlab 752S, YM1206PHB2, China) and the amount of sugar liberated was obtained from the standard curve based on known concentrations of glucose (10-100mg/l) **(Omafuvbe *et al*., 2000).**

Determination of total free amino acid

Samples (5 g) were weighed into 250 ml conical flask and 50 ml of 80 % ethanol v/v was added. The suspension was mixed properly and 10 ml of petroleum ether was added. The ethanol-petroleum ether suspension was stirred at room temperature for 30 min using a magnetic stirrer (Lab-line, Model No 1580-1, U.S.A). The mixture was transferred to centrifuge and centrifuged at 5000 rpm for 30 min. The petroleum ether phase was discarded and the clear ethanol phase was used as the sample extract **(Omafuvbe *et al*., 2000).** The ninhydrin method described by **Rosen (1957)** was used for determination of free amino acid. The ethanolic extract of the fermenting *masa* (3.9) samples was diluted appropriately. A 0.5 ml of cyanide acetate buffer (pH 5.4) and 0.5 ml of 3.0 % ninhydrin solution (3 g of ninhydrin in 100 ml of 2 methyl ethanol) was added to the extract (1.0 ml) in a tube. The tube was heated in a boiling water bath (Gallenkomp, HH-S6, England) for 15 min after which 10 ml of isopropyl-alcohol (Propan-2-ol) water mixture (1:1) was added rapidly and the solution was allowed to cool to room temperature (27 ± 2 ºC). The optical density of the solutions was read at 570 nm using spectrophotometer (Spectrumlab 752S, YM1206PHB2, China). Total free amino acids in the samples were estimated from a standard curve of known concentrations of glycine (10-100µm/ml) **(Omafuvbe *et al*., 2000).**

Statistical analysis

Data obtained were subjected to Analysis of Variance using SPSS (version, SPSS, Inc., USA). Means of samples were separated using Duncan Multiple range Test **(SAS Institute, 1985).**

RESULTS AND DISCUSSION

Alpha-amylase activity during fermentation of *masa*

Alpha-amylase activity of the fermenting *masa* increased during the period of fermentation as shown in Table 1. It was within the range of 4.63 and 9.30 E.U. during the period of fermentation. It was higher in *masa* produced from 100 % maize (6.00 – 9.30 E. U) than 100 % *acha* (4.63 – 6.07) throughout the period of fermentation and was also higher in *masa* samples fortified with 10 % soybean (5.27- 8.97 E. U) than 20 % soybean (4.97 – 7.70 E. U). It increased with increase in addition of maize and decreased with increase in soybean fortification. This may be due to low carbohydrate content of soybean as reported by **Samuel *et al*. (2015).** Alpha-amylases act by randomly hydrolysing α-1, 4-glucan linkages in the starch polymers: amylose and amylopectin, and then convert all amylose to maltose while glucose, maltose and alpha-limit dextrins are released from the breakdown of amylopectin **(Helland *et al*., 2002).**

Table 1 Alpha-amylase activity of *masa* during fermentation

Masa Sample	Hour of fermentation				
	0	6	12	18	24
100 % M	6.00^{e}±0.03	6.33^{d}±0.04	7.00^{c}±0.05	8.40^{b}±0.03	9.30^{a}±0.03
100 % A	5.07^{c}±0.01	4.67^{d}±0.02	4.63^{d}±0.03	5.33^{b}±0.06	6.07^{a}±0.02
70 % M: 20 % A: 10 % S	5.87^{e}±0.04	7.23^{d} ±0.03	7.50^{c}±0.01	7.93^{b}±0.04	9.13^{a}±0.04
60 % M: 30 % A: 10 % S	5.57^{e}±0.05	6.13^{d}±0.06	6.97^{c}±0.05	7.27^{b}±0.05	8.97^{a}±0.05
60 % M: 20 % A: 20 % S	5.27^{e}±0.03	5.67^{d}±0.03	5.77^{c}±0.01	6.40^{b}±0.01	7.57^{a}±0.03
50 % M: 40 % A: 10 % S	5.23^{e}±0.02	6.00^{d} ±0.05	6.67^{c}±0.02	6.96^{b}±0.05	8.67^{a}±0.06
50 % M: 30 % A: 20 % S	5.13^{e}±0.06	5.86^{d}±0.02	6.00^{c}±0.06	6.83^{b}±0.02	7.70^{a}±0.02
40 % M: 40 % A: 20 % S	4.97^{e}±0.04	5.80^{c}±0.05	5.63^{d}±0.02	6.00^{b}±0.03	6.60^{a}±0.05

M: Maize; A: *Acha*; S: Soybean. Values are means of three replicates ± standard error: Means followed by different superscript in the same row are significantly different at $p < 0.05$

Total reducing sugar of *masa* during fermentation

Total reducing sugar of fermenting *masa* samples is shown in Table 2. It generally increased and was higher in *masa* produced from 100 % maize (29.93 – 36.98 mg/g of glucose) than 100 % *acha* (11.97 – 15.53 mg/g of glucose) throughout the period of fermentation. Addition of *acha* and fortification of *masa* with soybean decreased the total reducing sugar content. *Acha* is reported to relatively evoke low sugar on consumption, an advantage for diabetics (Ayo *et al*., 2007). Samples fortified with 10 % soybean had higher amount of total reducing sugar than samples fortified with 20 % soybean. This decrease could be due to the low carbohydrate content of soybean. Soybean is a legume crop with low carbohydrate (35 %), high protein content (40 %) and high oil (20 %) **(Osho, 2003).** Increase in α-amylase activity could be responsible for increase in the total reducing sugar and utilization of these sugars as carbon source by fermenting microorganisms could be responsible for the decrease. Increase in amylase activity increases the level of fermentable and reducing sugars in flour and dough, thereby promoting yeast fermentation **(Goesaert *et al*., 2006). Adepoju *et al*. (2016)** also reported increase in total reducing sugar in *fura de nunu* during storage.

Table 2 Total reducing sugar during fermentation of *masa*

	Hour of fermentation	
Masa Sample	0	6
100 % M	$29.42^{c}\pm0.12$	$31.46^{bc}\pm0.08$
100 % A	$15.24^{a}\pm0.03$	$14.72^{b}\pm0.09$
70 % M: 20 % A: 10 % S	$30.69^{c}\pm0.07$	$27.47^{d}\pm0.06$
60 % M: 30 % A: 10 % S	$28.68^{c}\pm0.04$	$31.19^{b}\pm0.04$
60 % M: 20 % A: 20 % S	$27.02^{b}\pm0.10$	$25.66^{c}\pm0.07$
50 % M: 40 % A: 10 % S	$29.03^{a}\pm0.05$	$30.51^{a}\pm0.06$
50 % M: 30 % A: 20 % S	$26.94^{d}\pm0.02$	$29.24^{b}\pm0.04$
40 % M: 40 % A: 20 % S	$19.68^{c}\pm0.09$	$27.71^{a}\pm0.05$

M: Maize; A: *Acha*; S: Soybean. Values are means of three replicates ± standard error: Means followed by different superscript in the same row are significantly different at $p < 0.05$

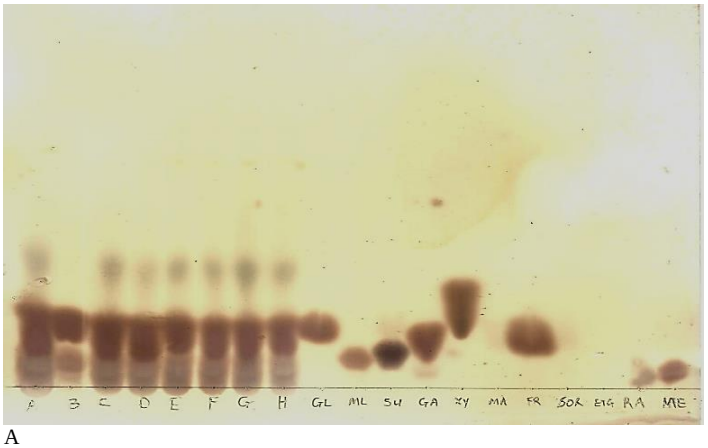
Total sugar of *masa* during fermentation

Total sugar content of fermenting *masa* samples are presented in Table 3. It ranged between 222.02 and 391.56 mg glucose/g during the fermentation period. Total sugar was generally higher in *masa* produced from 100 % maize than 100 % *acha* from 6 h to 24 h of fermentation. Total sugar content was also higher in *masa* samples fortified with 10 % soybean at the beginning of fermentation than in samples fortified with 20 % soybean. It was lowest in *masa* produced from 100 % *acha* from 6 h to 24 h. The fluctuation in total sugar may be as a result of hydrolysis of complex carbohydrate into simple sugars and utilization as carbon source by fermenting organisms may be responsible for the decrease. According to **Oyarekua and Adeyeye (2009),** the fermenting microbes could liberate both alpha and beta amylases; because carbohydrates, starch and **soluble sugar**s are the principal substrates for fermenting organisms.

Table 3 Total sugar of *masa* during fermentation

	Hour of fermentation		
Masa Sample	0	6	12
100 % M	$335.57^{a}\pm0.10$	$242.59^{d}\pm0.04$	323.
100 % A	$342.18^{a}\pm0.05$	$136.50^{c}\pm0.05$	135.
70 % M: 20 % A: 10 % S	$366.73^{b}\pm0.06$	$329.98^{c}\pm0.06$	329.73$^{c}\pm0.11$
60 % M: 30 % A: 10 % S	$299.42^{b}\pm0.09$	$325.40^{a}\pm0.09$	258.
60 % M: 20 % A: 20 % S	$304.29^{b}\pm0.07$	$298.39^{c}\pm0.11$	311.
50 % M: 40 % A: 10 % S	$318.90^{c}\pm0.11$	$341.09^{b}\pm0.05$	391.
50 % M: 30 % A: 20 % S	$318.36^{b}\pm0.05$	$291.71^{c}\pm0.10$	373.
40 % M: 40 % A: 20 % S	$314.57^{b}\pm0.10$	$296.71^{d}\pm0.07$	301.

M: Maize; A: *Acha*; S: Soybean. Values are means of three replicates ± standard error: Means followed by different superscript in the same row are significantly different at $p < 0.05$



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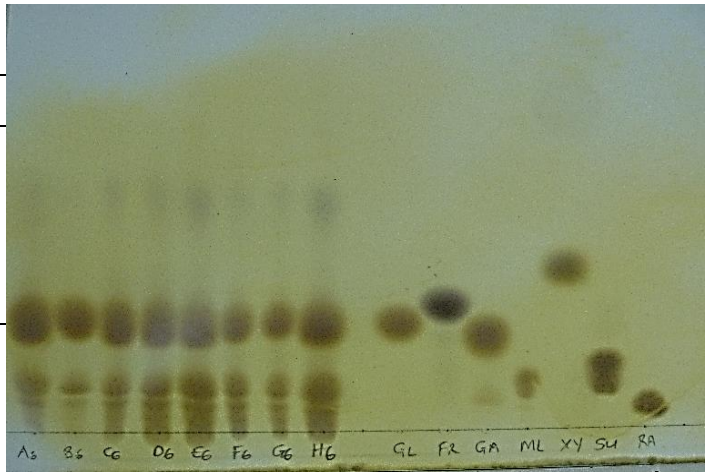

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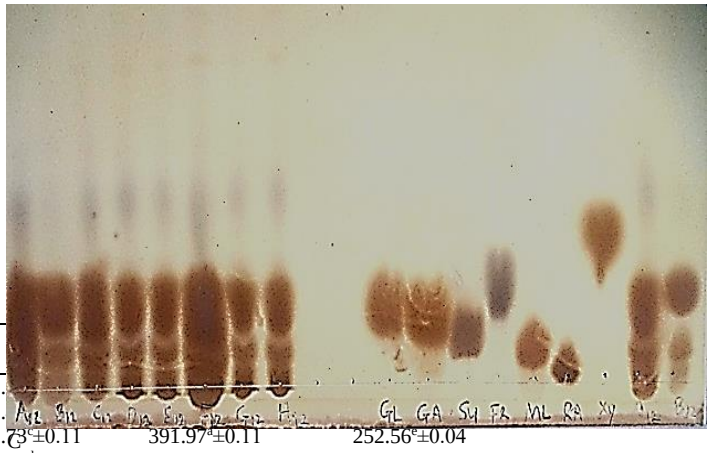

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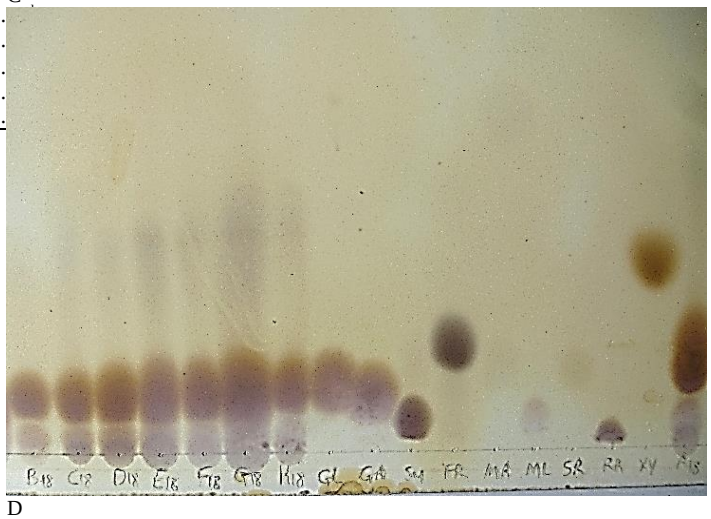

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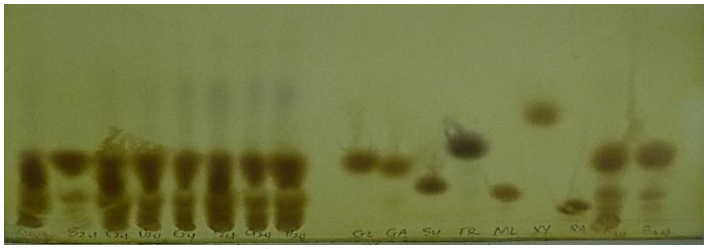

Figure 1 Individual sugars in fermenting *masa*
A: 100% Maize; B: 100% *acha;* C: 70 % Maize: 20 % *Acha*: 10% Soybean; D: 60 % Maize: 30 % *Acha*: 10 % Soybean; E: 60 % Maize: 20 % *Acha*: 20 % Soybean; F: 50 % Maize: 40 % *Acha*: 10 % Soybean; G: 50 % Maize: 30 % *Acha*: 20 % Soybean; H: 40 % Maize: 40 % *Acha*: 20 % Soybean; GL: Glucose; ML: Maltose; SU: Sucrose; GA: Galactose; XY: SOR: Sorbitol; RA: Raffinose; ME: Melibiose; Xylose; MA: Mannitol; FR: Fructose; 6: six hours; 12: twelve hours; 18: eighteen hours; 24: twenty four hours.

Changes in individual sugar composition during fermentation of *masa*

Changes in sugar composition during fermentation of *masa* batter are shown in Figures 1a, 1b, 1c, 1d and 1e. Glucose, maltose and galactose were present in all samples from the beginning to the end of fermentation. At 6 h, glucose, galactose, sucrose and maltose were present in all samples while raffinose was only present in *masa* samples fortified with soybean. The presence of raffinose could be as a result of fortification of *masa* with soybean. **Kumar *et al.* (2010)** also reported the presence of raffinose in soybean. **Adeniran and Abiose (2011)** reported that glucose and maltose were the prominent sugars in starch hydrolysates.

Total free amino acid of *masa* during fermentation

Total free amino acid of *masa* samples (Table 4) ranged between 3.92 anld 12.87 mg glycine/g during the period of fermentation. It was higher in *masa* produced from 100 % maize than 100 % *acha* from 0 to 18 h but was higher in 100 % *acha* at the end of fermentation. Total free amino acid increased with increase in soybean fortification. It was higher in samples fortified with 20 % soybean (10.14 – 11.88 mg glycine/g) than

Table 4 Total Free Amino Acid during Fermentation of *Masa* (mg glycine/g)

	Hour of fermentation				
Masa Sample	0	6	12	18	24
100 % M	10.07^{a}±0.02	9.81^{b}±0.09	8.73^{c}±0.05	6.38^{d}±0.04	5.69^{e}±0.06
100 % A	3.92^{e}±0.08	4.21^{d}±0.05	5.67^{b}±0.04	5.54^{c}±0.07	6.53^{a}±0.12
70 % M: 20 % A: 10 % S	12.96^{b}±0.10	12.07^{a}±0.11	10.90^{c}±0.07	10.89^{c}±0.11	8.74^{d}±0.08
60 % M: 30 % A: 10 % S	11.44^{a}±0.09	10.85^{b}±0.09	8.08^{c}±0.12	7.04^{d}±0.08	7.22^{e}±0.02
60 % M: 20 % A: 20 % S	12.89^{a}±0.04	12.99^{a}±0.11	9.94^{c}±0.08	9.80^{d}±0.03	11.88^{b}±0.08
50 % M: 40 % A: 10 % S	9.37^{a}±0.10	9.06^{b}±0.12	8.69^{c}±0.03	8.22^{d}±0.06	8.92^{b}±0.05
50 % M: 30 % A: 20 % S	9.63^{c}±0.07	10.63^{b}±0.07	8.46^{d}±0.09	10.68^{b}±0.04	12.29^{a}±0.07
40 % M: 40 % A: 20 % S	9.46^{bc}±0.02	9.55^{b}±0.04	8.58^{d}±0.11	9.43^{c}±0.06	10.14^{a}±0.10

M: Maize; A: *Acha*; S: Soybean. Values are means of three replicates ± standard error: Means followed by different superscript in the row are significantly different at $p < 0.05$

10 % soybean (7.22 – 8.92 mg glycine/g) at the end of fermentation period. According to **Ikujenlola (2014)**, legumes contain protein with high amino acid. Increase in total free amino acid has been attributed to hydrolysis of proteins and the activities of proteolytic enzymes during fermentation **(Sripriya *et al.*, 1997)**. Increase in soluble protein improves digestibility of food by increasing the amount of protein that could be readily absorbed in the body **(Ng'ong'ola-Manani *et al*., 2014).**

CONCLUSION

Alpha-amylase activity and total reducing sugar of the fermenting *masa* increased with increase in addition of maize and decreased with increase in soybean fortification. Fortification of *masa* with soybean reduced the sugar content and alpha-amylase activity during fermentation of *masa* but increased the level of total free amino acid content of *masa* thereby increasing the quality of protein. Hydrolysis of starch into simple sugar by the fermenting microorganisms and enzyme α-amylase are important in improving the texture and viscosity of the dough and also absorption and digestibility of food in the gastrointestinal tract. *Acha* and soybean could be incorporated into cereal based food to reduce the glycemic index, the gluten content and improve the protein content thereby advancing the use of *acha* and also reduce malnutrition. The formulation containing 60% maize, 20 % *acha* and 20% soybean had the lowest amount of sugar and highest free amino acid. *Masa* could therefore be produced by fortifying maize and *acha* with soybean since it reduced the sugar contents and increased the free amino acid.

Acknowledgements: Quality protein maize and soybean seeds for this research were obtained from the Institute of Agricultural Research and Training, Ibadan, Nigeria.

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