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Natural and controlled fermentation of Lima bean (*Phaseolus lunatus*) for *daddawa* production

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ABSTRACT

Aims: *Daddawa* is a traditional fermented condiment produced from legumes in Nigeria. Lima bean is an underutilized legume in Nigeria. Natural fermentation has been the conventional method of producing *daddawa* but the product has been found to be of low quality and consistency. The present study aimed at comparing the microbial and biochemical changes during natural and controlled fermentation of lima bean for production of *daddawa*.
Methodology and results: Lima bean was fermented into *Daddawa* naturally. It was also fermented into *daddawa* using pure starter culture of *Bacillus subtilis* and *Bacillus pumilus* as single starter. The microbial and biochemical changes during both fermentation conditions were evaluated. Lima bean fermented naturally (NF) recorded the highest total viable count at 48 h and 72 h of fermentation respectively. Alpha amylase and protease activities increased with fermentation, and reached their peak at 48 h in both naturally fermented lima bean and pure culture fermented lima bean samples. Lima bean fermented with *B. subtilis* (FBS) recorded the highest total free amino acids at 72 h (54.45 Glycine/g dry wt.).
Conclusion, significance and impact of study: The use of lima bean for *daddawa* production enhanced its utilization. Controlled fermentation of lima bean by *Bacillus* species improved the biochemical properties such as α-amylase and protease activities and free amino acids content of fermenting lima beans into *daddawa*.

Keywords: Daddawa, fermentation, lima bean, microbial, biochemical changes

INTRODUCTION

Daddawa, a Nigerian traditional fermented condiment, is used for taste and flavor enhancement in soup. It is also used as meat substitute in dishes particularly by the low income families. *Daddawa* is nutritious and of health benefit because of the high protein, fibre and vitamins content. *Daddawa* is traditionally produced from African locust beans. Lima bean (*Phaseolus lunatus*), an under-utilized legume, has been found useful as another raw material that could be used in *daddawa* production (Farinde *et al.*, 2011; Adeniran *et al*., 2013; Farinde *et al.*, 2014). Processing of lima bean into *daddawa* involves cleaning of the lima bean seeds, roasting, dehulling, cooking for between 40 to 50 min, draining, placing the beans in clean calabash lined with clean banana leaves and allowing to ferment for 72 h in an incubator at 35 ± 2 °C (Farinde *et al.*, 2011; Adeniran *et al*., 2013). After fermentation, the product becomes soft textured, brownish black in colour with a characteristic ammoniacal smell (Farinde *et al*., 2014).

Traditionally, fermentation of vegetable proteins is usually allowed to proceed naturally, but the use of starter culture has been reported to assist and improve fermentation process (Omafuvbe *et al*., 2002). Microbial starters usually consist of a cultivation media that have been well colonized by the microorganisms used for the fermentation. A pure starter culture is essential for controlled fermentation, and quite a number of *Bacillus* species, *Staphylococcus* and *Streptococcus* have been explored (Abiose *et al*., 1986; Suberu and Akinyanju, 1996; Ouoba *et al*., 2003; Omafuvbe *et al*., 2004). Controlled fermentation of soybeans for producing soy *daddawa* was achieved by using pure single cultures of each of *B. subtilis, B. licheniformis* or their combinations (Sarkar *et al*., 1993; Suberu and Akinyanju, 1996; Omafuvbe *et al*., 2002).

Biochemical changes during fermentation of legumes in production of *daddawa* involve enzymatic activities by microorganisms (Aderibigbe and Odunfa, 1990; Omafuvbe *et al*., 2000). Microbial enzymes including amylases, proteases and lipases have been reported to be associated with the fermentation process (Burhan *et al*., 2003). Proteases and free amino acids have been reported to increase with fermentation time during production of *daddawa* from soybean using *B. subtilis* as

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starter culture (Omafuvbe *et al*., 2000, 2006). The aim of this study was to compare the microbial and biochemical changes during natural and controlled fermentation of lima bean to produce *daddawa*.

MATERIALS AND METHODS

Natural fermentation of lima bean seeds

Matured dried lima bean seeds (*P. lunatus*) were purchased at Ita-Ogbolu, Ondo State, Nigeria. Lima bean fermentation was carried out according to the method used by Farinde *et al*. (2014). Lima bean seeds (2.0 kg) were roasted in an open frying-pan, the roasted beans were allowed to cool down and thereafter manually dehulled by rubbing between palms. The hulls were separated from cotyledon by winnowing. The clean beans (approximately 1.85 kg) were cooked in 2,500 mL of water by boiling for 40 min on gas cooker. The cooked beans were drained and divided into four parts. One portion was left unfermented while the remaining three parts were poured while still warm into three clean calabashes lined with clean banana leaves, covered with more clean banana leaves and with another calabash. The beans were fermented in an incubator at 35 ± 2 °C for 72 h. Fermented samples were taken out in duplicate from each calabash for each fermentation stage into sterile container in an inoculation room at 24 h interval.

Controlled fermentation of lima bean seeds

Controlled fermentation of lima bean seeds was carried out using the method described by Abiose *et al*. (1986) and Omafuvbe *et al*. (2002) with slight modification. Roasted and dehulled lima bean seeds (2 kg) were washed with water and cooked for 30 min on gas cooker. Cooked beans (50 g) were dispensed in 250 mL conical flasks plugged with cotton wool. Contents of the flasks were autoclaved at 121 °C for 15 min at 15 psi pressure to obtain sterile dehulled cooked beans. The sterile cooked beans were cooled to ambient temperature before inoculation.

Preparation of bacterial cell suspension used as starter for controlled fermentation

Culture (18 h old) of bacterial isolate (chosen for use as starter) on tryptone soy agar slant was suspended in 10 mL sterile 0.9% sodium chloride solution by rinsing the agar slant with the sterile saline water. This was standardized by diluting with appropriate amount of sterile 0.9% sodium chloride solution to give a suspension of 0.03 absorbance in a spectrophotometer (Spectrumlab 752×, China) set at 540 nm wavelength (Omafuvbe *et al*., 2002).

Inoculation of sterile lima bean seeds with culture of the isolates

Pure culture of *B. subtilis* and *B. pumilus* isolated and identified as predominant in previously naturally fermenting lima beans seeds (Farinde, 2015) and maintained on tryptone soy agar slant in refrigerator were used as starter singly for controlled fermentation of lima bean seeds to produce *daddawa.* Suspension of 18 h old culture (500 µL) was used to inoculate 50 g sterile lima beans in the conical flasks (this gave approximately 4.0 log CFU/g wet weight of the lima bean seeds). Control was set up with one flask containing 50 g sterile lima bean seeds without inoculum. The flasks and their contents were incubated at 35 °C for 72 h after which samples were taken out in duplicates at interval of 24 h for analysis.

Viable cell count

Total viable count was determined using the method described by Abiose *et al*. (1986) and Omafuvbe *et al*. (2000). Fermenting lima beans (5 g wet weight) was aseptically transferred into sterile 0.9% NaCl solution (45 mL) in sterile stomacher bag and homogenized in a stomacher for 30 sec. Aliquot (1.0 mL) of appropriately diluted sample was plated in triplicates while about 20 mL of nutrient agar (NA) was poured into each plate. The plates were allowed to set and then incubated at 35 ± 2 °C for 24 h. Colonies were counted and expressed as colony forming unit per gram (CFU/g) of the sample.

Determination of pH and moisture content

Hydrogen ion concentration (pH) of the fermenting *daddawa* sample collected at 24 h interval was measured with a standard pH meter (Scholar 425). The pH meter was standardized with buffers of pH 4 and pH 7. One gram of sample was homogenized in 10 mL of distilled water and the pH was read with the pH meter (Ikenebomeh *et al*., 1986; Omafuvbe *et al*., 2002). Moisture content was determined following the method of AOAC (2000).

Determination of enzymatic activities

Amylase activity

Amylase activities were determined following the method described by Adeniran and Abiose (2007). Exactly 5.0 g of the fermenting lima bean samples was suspended in 50 mL of 0.5 M sodium acetate buffer (pH 5.5) in a stomacher bag and homogenized using a stomacher for 30 sec. The homogenate was transferred to conical flask and was mechanically shaken at 150 rpm for 10 min at room temperature on lab-line orbit shaker. The suspension was transferred to a centrifuge tube and centrifuged in a bench centrifuge at 5000 rpm (1118×*g*) for 30 min. The supernatant was collected as crude enzyme and was used for the enzyme assay. To 0.5 mL of 0.5% soluble starch solution, was added 0.3 mL of the crude enzyme and 0.2 mL of 0.1 M sodium acetate buffer (pH 5.5). This mixture was mixed and then incubated at 40 °C for 30 min in water bath. The reaction was

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terminated by adding 2 mL of 3,5-dinitrosalicylic acid reagent (DNSA) and boiling for 5 min in water bath. It was cooled in cold water and diluted with 7 mL distilled water (Miller, 1959). Control was also set for each sample by adding individual enzyme to tubes containing substrate after termination of reaction with DNSA. The tubes and their content were then heated in boiling water bath. The absorbance of the resultant solution was read at 540 nm in a Spectrophotometer (Spectrumlab 752×). Maltose standards were prepared from stock maltose solution (1 mg of maltose in 1 mL of distilled water). The amount of the reducing sugars formed was read from a standard curve prepared with varying concentrations of maltose (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL). One enzyme unit (U) of α-amylase was defined as the amount of enzyme that produced one microgramme (1 µG) of reducing sugar (maltose) under the assay condition (Adeniran and Abiose, 2007).

Protease activity

The method used by Sarkar *et al*. (1993) with slight modification was employed for determination of protease activities. Exactly 3.0 g (wet weight) of the fermented lima bean samples obtained at each fermentation stage was suspended in 5.0 mL of 0.05 M potasium hydrogen phosphate (K_2HPO_4) buffer (pH 7.0) in a stomacher bag and homogenized using a stomacher (Colworth Stomacher 400 model 6021, London) for 20 sec. The homogenate was centrifuged in a bench centrifuge (HARRIER 15/80 MSE Sanyo, Japan) at 10000 rpm (5590×*g*) for 10 min. The supernatant was collected as crude enzyme and used for the enzyme assay. Crude enzyme (0.12 mL) was pipetted into 1.5 mL Eppendorf tube and azocasein (0.25 mL) (2.5 g/L, sigma prepared in 0.05 M potassium hydrogen phosphate buffer, pH 7.0) was added. The reaction mixture in tubes were incubated in water bath set at 37 °C for 1 h and the reaction was terminated by adding 0.75 mL cold 3 M trichloroacetic acid. Control was set for each sample by adding the individual enzyme after terminating reaction with trichloroacetic acid. Undigested protein was allowed to sediment by standing in refrigerator at 4 °C for 1 h. The undigested protein was then removed by centrifugation at 1000 rpm (5590×*g*) for 10 min. The supernatant (0.5 mL) was mixed with 2 mL distilled water and analyzed for free dye by measuring the absorbance at 400 nm in a spectrophotometer (Spectrumlab 752×). One enzyme unit (U) of protease activity was defined as the unit that produced an absorbance increase of 0.01 unit over the blank under the assay conditions (Sarkar *et al*., 1993).

Estimation of free amino acids

Free amino acids content in the fermenting lima bean samples were extracted with 80% ethanol following the method described by Omafuvbe *et al*. (2002). The total free amino acids in the samples were determined using Ninhydrin colorimetric method of Rosen (1957) as described by Omafuvbe (2006). Ethanolic extract of sample was diluted ten-fold. To the aliquot (1.0 mL), was added 0.5 mL of cyanide acetate buffer (pH 5.4) and 0.5 mL of 3% ninhydrin solution in 2-methoxyethanol in a tube. The tubes were heated in a boiling water bath for 15 min after which 10 mL of propan-2-ol water mixture (1:1) was added rapidly and the solution was allowed to cool to ambient temperature. The absorbances of the samples were determined at 570 nm in a spectrophotometer. Glycine standards were prepared from stock glycine solution (10 mg of glycine in 1 mL of distilled water). Free amino acids in the samples were estimated from a standard curve of varying concentrations of glycine (2, 4, 6, 8, 10 mg/mL)

Statistical analysis

Data obtained were subjected to descriptive and inferential statistics (ANOVA) using SPSS (version 17 incorporation, Chicago, Illinois, USA). Means of samples were separated using Duncan Multiple range Test. Significance was accepted at 5% level.

RESULTS AND DISCUSSION

Total viable count of fermenting lima beans

The results of the total viable count of the fermenting lima bean samples are shown in Table 1. The total viable count (TVC) increased rapidly in the first 24 h of fermentation in the three lima bean samples (NF, FBS and FBP). The count then increased steadily, reached peak at 48 h of fermentation after which the count then decreased till the end of the fermentation process.

Table 1: Changes in total viable count (log CFU/g) of microorganisms during natural and controlled fermentation of lima beans to produce *daddawa*.

Fermentation time (h)	NF	FBS	FBP
0	4.65 ± 0.15^{a}	4.15 ± 0.10^{b}	4.12 ± 0.15^{b}
24	7.15 ± 0.09^{b}	7.50 ± 0.11^{a}	7.10 ± 0.10^{c}
48	8.80 ± 0.15^{a}	8.65 ± 0.11^{a}	8.15 ± 0.17^{b}
72	7.40 ± 0.21^{a}	7.25 ± 0.10^{b}	7.16 ± 0.11^{b}

NF = Naturally fermented, FBS = Lima bean fermented with *B. subtilis*, FBP = Lima bean fermented with *B. pumilus*
Values are means of three replicates ± standard error
a, b, c = Means followed by different superscript in the same row are significantly different at $p < 0.05$

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Increase in the total viable count at the early stage of fermentation might be due to high availability of nutrients at this stage which is referred to as logarithmic phase. Drop in total viable count at 72 h of fermentation in both naturally fermented and controlled fermented samples might be as a result of nutrient depletion and accumulation of metabolites at the later stage of fermentation and this is referred to as decline phase of growth of the microorganisms.

There was no significant difference ($p > 0.05$) in total viable count in naturally fermented lima bean (NF) and lima bean fermented with pure starter culture of *Bacillus subtilis* (FBS) at 48 h of fermentation. Lima bean fermented naturally (NF) was significantly higher ($p < 0.05$) in total count at 72 h of fermentation (7.40 log CFU/g) when compared with the controlled samples. This might be as a result of uncontrolled environment which might favor the growth of many other types of microorganisms in the natural environment. Similar pattern of the trend in the TVC was reported by Omafuvbe *et al*. (2002, 2006).

pH and moisture content

The results of the pH and moisture content of the fermenting lima bean samples are shown in Table 2. The pH ranged between 6.18 at 0 h and 6.89 at 72 h during natural fermentation of lima bean (NF). Lima bean fermented with *B. subtilis* and Lima bean fermented with *B. pumillus* recorded an alkaline pH of 8.61 and 8.58 at 72 h respectively). Increase in pH with fermentation time in oil seeds have been reported (Odunfa, 1981; Abiose *et al*., 1986; Omafuvbe *et al*., 2002; Amoo *et al*., 2013). The increase in pH observed during the fermentation of lima bean particularly with controlled fermentation was probably as a result of proteolysis and the release of ammonia following utilization of amino acids by the fermenting microorganisms (Abiose *et al*., 1986; Abiose, 1998; Omafuvbe *et al*., 1999; Omafuvbe *et al*., 2002; Omafuvbe, 2006; Yabaya, 2006; Ojinaka and Ojimelukwe, 2012). Ammonia has been reported to exhibit inhibitory effect on spoilage or pathogenic bacteria in alkaline food fermentations (Leejeerajumnean *et al*., 2000).

Table 2: Changes in pH and moisture content during natural and controlled fermentation of lima beans to produce *daddawa*.

Fermentation time (h)	Sample		
	NF	FBS	FBP
0	6.18 ± 0.02^{a} (46.60 ± 0.90^{a})	6.26 ± 0.06^{a} (43.70 ± 0.55^{b})	6.29 ± 0.01^{a} (46.50 ± 0.24^{a})
24	6.37 ± 0.15^{b} (49.80 ± 0.55^{a})	7.41 ± 0.03^{a} (50.30 ±0.45^{a})	7.26 ± 0.03^{a} (51.60 ± 0.38^{a})
48	6.70 ± 0.15^{c} (50.50 ± 0.45^{b})	8.47 ±0.03^{a} (54.50± 0.81^{a})	7.88 ± 0.04^{b} (52.40 ± 0.60^{a})
72	6.89 ± 0.06^{b} (51.70 ± 0.50^{b})	8.61 ±0.01^{a} (55.40 ± 0.60^{a})	8.58 ± 0.04^{a} (53.50 ± 0.25^{ab})

Moisture content values are shown in parentheses and expressed as percentage (%)
NF = Naturally fermented, FBS = Fermented with *B. subtilis,* FBP = Fermented with *B. pumilus*
Values are means of three replicates ± standard error
a, b, c = Means followed by different superscript in the same row are significantly different at $p < 0.05$

Moisture content increased with fermentation time in both naturally fermented lima bean and lima bean fermented with pure starter culture (controlled fermentation) (Table 2). Moisture content ranged from 43.70% at 0 h to 55.40% at 72 h of fermentation in the three lima bean *daddawa* samples. Increase in moisture content with fermentation time may be due to hydrolytic activity of the microorganisms during fermentation coupled with the fact that the fermenting substrate must have absorbed water during processing such as boiling. This study agrees with the findings of Ogunshe *et al*. (2007).

Biochemical activities associated with fermentation of lima bean into *daddawa*

The result of the changes in the α-amylase activities during fermentation of lima bean into *daddawa* is shown in Figure 1. Alpha amylase activities increased with fermentation time and reached peak at 48 h after which the activity dropped at 72 h of fermentation in NF, FBS and FBP. It increased from 9.00 U at 24 h and reached a peak of 15.91 U at 48 h of fermentation in lima bean fermented naturally. The activities then dropped to 10.96 U at 72 h of fermentation. The same trend was recorded in fermentation of lima bean with pure culture of *B. subtilis* and *B. pumilus.* Lima bean fermented with *B. subtilis* had its α-amylase activity reached its peak at 48 h of fermentation (21.33 U). The activity then dropped to 14.98 U. at 72 h of fermentation. Lima bean fermented with *B. pumilus* also had its α-amylase activity reached peak (19.98 U) at 48 h of fermentation after which the activity dropped to12.66 U at 72 h. The rapid increase in the activity of α- amylase at the early fermentation stage was probably due to increase in the microbial growth (Table 1). The result showed that the logarithmic phase of growth of the microorganisms was optimal at 48 h and subsequently their enzymatic activities were optimal at

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this stage. Lima bean fermented with *B. subtilis* exhibited highest level of amylase activity. Aderibigbe and Odunfa (1990) similarly reported high level of α-amylase production by *B. subtilis* isolated during fermentation of African locust bean. Similar trend were reported by Odunfa and Oyeyiola (1985), Abiose *et al*. (1986) and Omafuvbe *et al*. (2004). Alpha amylases are starch degrading enzymes. They break α-1, 4 glycosidic bonds in polysaccharides to smaller molecules of sugars (Sivaramakrishnan *et al*., 2006).

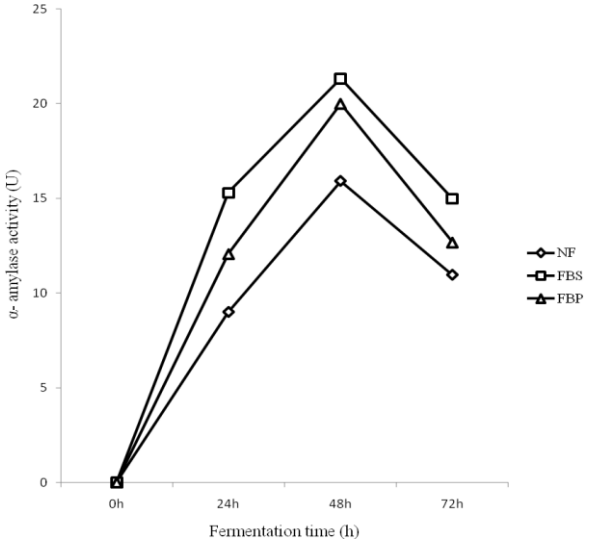

Figure 1: Changes in α-amylase activities during fermentation of lima bean to produce *Daddawa*.

The result of the changes in the protease activities during fermentation of lima bean into *daddawa* is shown in Figure 2. Protease activities increased with fermentation time and reached peak at 48 h of fermentation after which the activities reduce till the end of fermentation in NF, FBS and FBP (Figure 2). It increased from 1.03 U at 24 h to 1.30 U at 48 h of fermentation after which the activity dropped to 1.17 U at 72 h. Similarly, protease activity also increased with fermentation time in lima bean fermented with pure starter cultures and reached their peak at 48 h of fermentation. It increased from 2.15 U at 24 h to 2.75 U at 48 h and then dropped to 2.10 U at 72 h in lima bean fermented with *B. subtilis.* Likewise, protease activity also increased from 1.47 U at 24 h to 2.05 U at 48 h of fermentation in lima bean fermented with *B. pumilus,* the activity then decreased to 1.89 U. at 72 h of fermentation. The trend observed for protease activities is similar to the trend of amylase activities indicating that optimal activities were observed at 48 h of fermentation. Similar trend of increase in proteolytic activity at the initial stage of fermentation and subsequent decrease towards the end of fermentation was also reported by Omafuvbe *et al*. (2000; 2002) during soy *daddawa* production by natural and pure starter fermentations. The result also agreed with the report of Njoku and Okemadu (1989) in which protease activity increased rapidly at early stages of fermentation during *ugba* fermentation. Increase in protease activity corresponds with increase in viable count.

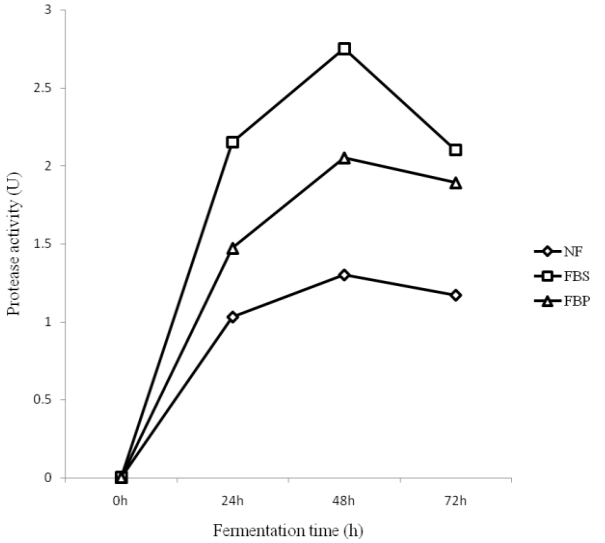

Figure 2: Changes in protease activities during fermentation of lima bean to produce *Daddawa*.

The result showed that *Bacillus* species were involved in the fermentation process of lima bean to produce *daddawa* and that these *Bacillus* species are proteolytic and had their optimal growth at 48 h of fermentation and that the more their count, the more the enzymes being produced and the more their activities to break down protein into smaller molecules of amino acids. Proteolysis has also been reported as the main metabolic activity during fermentation of African locust bean and that it contributes to texture, taste and flavor development of the fermented product (Ouoba *et al*., 2003). *Bacillus subtilis* was significantly higher in protease activity at 48 h of fermentation (2.75 U) when compared with the activity of *B. pumilus* and other microorganisms in the naturally fermented lima bean samples. Omafuvbe *et al*. (2002) in a similar study reported *B. subtilis* as exhibiting highest protease activity at 48 h of fermentation compared to the other *Bacillus* species during pure starter fermentation of soyabean to produce daddawa.

Total free amino acids

The results of the total free amino acids in the fermenting lima bean samples are shown in Table 3. Total free amino acids increased with fermentation time in all the fermented lima bean samples. Lima bean fermented with *B. subtilis* (FBS) recorded the highest value for total free amino acids at 72 h (54.45 mg Glycine/g dry wt.) while the least total free amino acids at 72 h was recorded in naturally fermented lima bean (31.75 mg Glycine/g dry wt.). Free amino acids are amino acids that are not attached by peptide bond to other amino acid to form polypeptides or proteins (David, 2005). They are soluble and are liberated during fermentation of vegetable seeds into condiments. Protease is an important enzyme for

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protein hydrolysis into amino acids and it contributes to texture, taste and flavor development of the fermented product (Ouoba *et al*., 2003). Increase in total free amino acid from 0 h to the end of fermentation was probably due to increase in the protease activity observed during the fermentation of bean seeds (Campbell-Platt, 1980; Odunfa, 1985; Omafuvbe *et al*., 2002).

Table 3: Changes in total free amino acids during natural and controlled fermentation of lima beans to produce *daddawa*.

Fermentation time (h)	Sample/ amino acids (mg Glycine/g dry wt.)		
	NF	FBS	FBP
0	3.70 ± 0.20^{a}	3.75 ± 0.15^{a}	3.75 ± 0.25^{a}
24	21.40 ± 0.20^{ab}	26.95 ± 2.45^{a}	19.80 ± 0.60^{b}
48	27.20 ± 0.08^{b}	37.40 ± 0.50^{a}	30.25 ± 2.65^{b}
72	31.75 ± 1.75^{c}	54.45 ± 1.35^{a}	42.00 ± 1.20^{b}

NF = Lima bean fermented naturally, FBS = Lima bean fermented with *B. subtilis*
FBP = Lima bean fermented with *B. pumilus*
Values are means of three replicates ± standard error
a, b, c = Means in the same row followed by different superscript are significant different at $p < 0.05$

Similar trend of increase in total free amino acids with increase in fermentation time was reported by Omafuvbe *et al*. (1999; 2000) during fermentation of soybean to produce *daddawa*. Ogunshe *et al*. (2007) also reported increase in free amino acids with fermentation time during controlled fermentation of *afiyu*.

CONCLUSION

This study has shown lima bean *daddawa* could be produced by natural and controlled fermentation. Controlled fermentation of lima bean by *Bacillus* species improved the biochemical properties such as α-amylase and protease activities and free amino acids content of fermenting lima beans into *daddawa*. The optimal enzymatic activities were observed at 48 h of fermentation in both the naturally fermented and controlled fermented lima bean samples.

RECOMMENDATION

Further studies should be carried out on effect of fermentation time on the maturity of *daddawa* from lima bean since optimal microbial growth and enzymatic activities were observed at 48 h of fermentation in both natural and controlled environment.

Further studies should also be carried out on effect of combination of two or more species of pure starter culture of *Bacillus* on the quality of *daddawa* produced from lima bean.

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