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Effect of salt and lime on *Bacillus* Fermented Castor Oil Bean Condiment

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The effect of addition of 2% salt and 3% lime on some biochemical changes occurring in the fermentation of castor oil bean into *ogiri* using *B. subtilis* as starter culture were studied. Three different fermented castor oil bean samples were produced viz: B_1(0% NaCl/Lime), B_2 (2% NaCl), B_3 (3% Lime). There were no significant differences (P>0.05) in the pH and temperature values of the fermented castor oil bean samples. Sample B_1 had the highest pH value of 7.15 followed by B_3 and B_2 with mean pH values of 7.01 and 6.74 respectively. Sample B_1 also had the highest mean temperature value of 29.3°C followed by samples B_3 and B_2 with 28.8 °C and 28.5°C.The total viable cell count of *B. subtilis* in the fermented castor oil bean samples increased rapidly 0 – 72h fermentation and less rapidly thereafter. The ammonia content of the three samples increased as the fermentation period progressed. Sample B_2 had the highest value of ammonia content 0.54 µg/ml followed by B_1 and B_3 with 0.20 µg/ml and 0.15 µg/ml respectively. The ricin content of the fermented castor oil bean samples decreased as fermentation period increased. The mean ricin content for the samples were B_1 : 0.060 µg/ml, B_2 :0.072 µg/ml and B_3: 0.084 µg/ml.

***Keywords*:** Castor oil bean*, ogiri, Bacillus subtilis,* NaCl, lime

INTRODUCTION

Ogiri is a food condiment obtained by traditional fermentation of *Ricinus communis* seeds. *Ogiri* is also produced from melon seeds (*Citrullus vulgaris*), iru or dawadawa is produced from locust beans, *Parkia biglobosa*, (Odunfa, 1981, Ogundana,1981) dawadawa from soybeans (Ogbadu and Okagbue, 1988), owoh from cotton seeds (*Gossypium hirsutum*), okpehe from mesquite (*Prosopis africana*). *Ogiri* is predominantly eaten in Eastern Nigeria.

In recent years, the use of fermented food condiments and flavouring agents are becoming popular in the diets of many nations. Apart from the fact that condiments improve sensory properties of foods, they add to the nutritional values providing dietary fibre, energy, minerals and vitamins (Kolapo *et al.,* 2007). Some of them contain antioxidants and nutraceuticals that provide health benefits.

The traditional fermentation of castor oil bean seed into *ogiri* has been reported to be accomplished by mainly bacteria especially Bacillus species notably *B. subtilis, B. pumillus* and *B.licheniformis* (Omafuvbe *et al.,* 2000; Dakwa *et al.,* 2005).

Lime and sodium chloride are additives used in some cultural settings to control the natural fermentation of legume condiments and are known to impact a preservative effect on the end product (Ojimelukwe *et al.,* 2011). Lime (*Citrus aurantifolia*) is known worldwide for its tart, tangy flavor. The values reported for acid content (as citric acid) for lime juices have ranged from 6.10% to 8.32%; about 47 g/L in the juices (Penniston, *et al.,* 2008). Limes contain unique flavonoid compounds that have antioxidant and anti-cancer properties. Of special interest in lime is the flavonoids called flavonol glycosides, including kaemperol-related molecules. While these flavonoids have been shown to stop cell division in many cancer cell lines, they are perhaps most interesting for their antibiotic effect. Lime juice has also been found to have a strong protective effect against cholera. Bioactive compounds from Mexican lime (*Citrus aurantifolia*) juice induce Apoptosis in human pancreatic cells (Jaiprakesh *et al.*, 2009). Many fermented foods require the addition of salt in order to impart a salty taste, improve shelf stability as well as have a selective action on microorganism (Ezeama, 2007).

Addition of 0-3% salt and 0-3% lime in the traditional fermentation of *ogiri* from *Ricinus communis* have been reported to improve the quality of the product (Ojimelukwe *et al.,* 2011). This study seeks to investigate the effect of fermentation period on some biochemical changes during the fermentation of castor oil bean into *ogiri* using *Bacillus subtilis* as starter culture.

MATERIALS AND METHODS

Collection of samples

The castor bean seeds *(Ricinus communis)* used in this research were purchased from a local market in Aba, Abia State, Nigeria. The seeds were sorted to remove contaminants and unwholesome seeds.

Organism

B. subtilis used as starter culture was previously isolated from traditional fermenting castor oil bean, *ogiri* and was maintained on nutrient agar slants in the refrigerator prior to use.

Preparation of 'Ogiri' from *Ricinus communis* using Starter Culture

The laboratory fermentation of castor oil bean into *ogiri* was done using the method of Enujiugha (2009). Approximately 1kg of castor bean seeds were cleaned and sorted to remove defective seeds and contaminants. The cleaned seeds were boiled for eight hours, dehulled, drained and boiled again for two hours. The boiled seeds were soaked for twelve hours, drained and mashed into *ogiri* paste and kept refrigerated for 24h before addition of the starter culture.

One hundred (100)g wet weight portions were put in sterile I L beakers each in three different portions. To one portion was added 2% NaCl, to another 3% lime while to the third portion, no additive was added. The three samples were sterilized in the autoclave at 121^{o}C for 15minutes. The three portions were inoculated with 2ml each of the broth cultures of the *B. subtilis* isolate. The beakers were then covered with aluminum foil and kept in the incubator for 96 hours. Samples were aseptically collected on 24 hour basis to determine the effect of fermentation on the castor bean seeds.

Determination of pH

The pH of the samples were determined by weighing 1g of the fermenting mash, and suspending it in 9ml of distilled water. The pH was measured after shaking with a pH meter (R1 – 02895 HANNA / Italy) every 24 hours for four days.

Determination of Temperature

This was determined by putting a sterile thermometer in the fermenting castor bean seed mash and taking the reading on the thermometer after two minutes every 24 hours for four days.

Determination of the Viable Cell Counts

One (1) g of the sample mash was weighed into 9ml quarter strength Ringers solution. Further dilutions were made in quarter strength Ringers solution and 0.1ml of appropriate dilutions was spread on duplicate plates of sterile Plate Count Agar (Biolab). The plates were incubated at 37^{o}C for 24 hours. The colonies were counted after incubation.

Determination of Ammonia In Fermenting Castor Bean Seed using Technicon Auto-Analyser (TECHNICON AAII)

The digests were run on Technicon auto-analyser (Technicon AAII Australia). This analytical Technique operates on the development of colour intensity of ammonia in the samples and quantizing it as being directly proportional to its concentration.

- Two (2)g of each sample was weighed into a set of 50ml digestion tube.
- 20.0 ml at digestion solution was added and shaken. (Alkaline phenol reagent + Ultra pure water + H_2SO_4) at 20^{o}C. The samples were digested for 1 hour in the fume chamber.
- It was made up 50ml with ultrapure water after cooling to room temperature.
- It was transferred to a set of centrifuge tubes (Thermo Electron Corporation IEC Centra GP8 model, USA) and shaken for 15 minutes and centrifuged for 10 minutes at 5000 rpm.
- The supernatants were sampled in a set of auto-analyzer cups arranged on a tray.

Determining the analyte (Ammonia) and calculation:

- The sample analyte was ran on the Technicon auto-analyzer by first running the standards of the known concentrations (0.0, 2.0, 4.0, 6.0, 8.0 ppm), which the software of the computer system, interphased with the instrument stores. The data was used to calculate the values of the analytes in the samples of unknown concentration.

Calculation: the software was used to carry out the calculation of the analyte values as follows: ppm (in extract) = Dilution factor x Readings or signal intensity

$$\text{ppm (in sample)} = \frac{\text{ppm in extract x volume of samples}}{\text{Wt. of samples}}$$

Analysis of Ricin in Fermenting Castor Bean Seed Using Waters 616/626 HPLC

Procedure used for extraction of the samples:

The analysis of ricin in fermenting castor bean sample was done using Waters 616/626 HPLC. Five (5) gram of castor bean sample was weighed out for blending with 10mlultrapure water. Then 10ml of the homogenously blended sample was measured into sample extraction unit. 35ml of acetone was added and the extraction speed was set at 6000 rpm for 20 minutes.

The supernatant containing Ricin was separated from the precipitate. The sample solution was transferred to a set of centrifuge tubes and covered. The sample was homogenously shaken on a mechanical shaker for 15 minutes. The shaken set of samples was then transferred to a centrifuge set at 3000rpm for 10 minutes. The supernatant was then collected and stored for analysis using HPLC.

Running the Extract on HPLC (Waters 616/6266 Digital/Model)

The column size of 3.26mm by diameter, and 35cm by length was chosen as a stationary phase. Methanol was used as a mobile phase and the detector was a

fluorescence detector. The instrument was programmed according to specifications.

Statistical Analysis

The data for the all the analysis were collected on 24h basis for four days. Statistical analysis was done for each set of data obtained following the procedures of Steel and Torie (1984) for a Factorial Randomized Complete Block Design (Factorial RCBD) while GENSTAT discovery package (2006 edition) was used for the analysis of the data. Comparison of treatment means and significant differences between treatment means separated using Fisher's Least Significant Difference (LSD) as outlined by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Figure 1 shows there was an increase in pH value of the three fermented castor oil bean samples throughout the period of fermentation. The sample with 0% NaCl/Lime (B_1) had the highest pH values followed with samples B_2 (2% NaCl) and B_3 (3% Lime). These results are in agreement with those of Ojimelukwe *et al*., 2011. There was simultaneous increase in the pH of the fermenting castor seeds as fermentation progressed. There was no significant difference ($P>0.05$) in the pH values of the three samples. The pH of the fermenting castor bean samples ranged from 6.24 to 8.01 for B_1, 6.28 to 8.15 for B_2 and 5.84 to 7.60 for B_3 samples respectively. The additives added in the form of 2% NaCl (B_1) and 3% Lime (B_3) did not affect the increase in pH known to be a usual occurrence in alkaline fermentation.

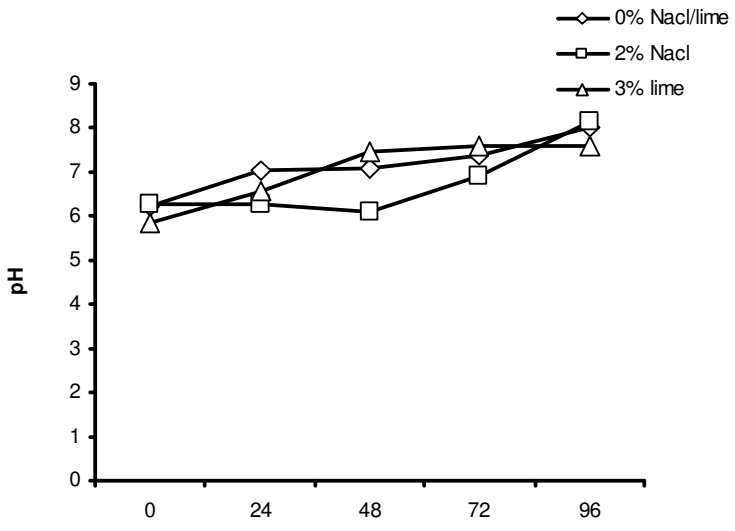


Fig.1 Changes in pH of castor bean mash during fermentation into ogiri $LSD_{0.05}$ = NS

The increase in pH would encourage the growth of *Bacillus sp*. which have been found to grow well at pH 7.0 to 8.0 (Odunfa and Oyeyiola, 1985). It has also been reported that *B.subtilis* use ammonia as nitrogen source in vegetable proteins during fermentation (Odunfa, 1986). However, Njoku and Okemadu (1989) and Ogueke and Aririatu (2004) found that there was pH increase during the fermentation of African oil bean seeds from 5.0 to5.7 at 0hr to 7.9 – 8.7 after 3-5 days of fermentation. The observed pH trend is in accordance with the reports during the fermentation of soybean seeds (Omafuvbe *et al*., 2000). Increasing pH during fermentation has been attributed to proteolytic activities and the release of ammonia by microorganisms involved in the fermentation. The released ammonia is responsible for the pungent smell that usually accompanies most vegetative protein fermentation (Sarkar *et al.,* 1993). The implication of the observed result is that there is likelihood of continued fermentation even in storage (Popoola *et al*., 2007).

There has been a decrease in pH values during tempoyak fermentation (Asian food condiment) from day 0 to 2. This was attributed to the production of metabolites such as lactic acid, acetic acid and carbon dioxide produced from heterofermenters of LAB (Amin *et al*., 2004; Leisner *et al*., 2001). Kpikpi *et al.,* 2009 also reported a decrease in pH in fermented condiment "kantong", though the predominant microorganisms identified at the various stages of fermentation were lactic acid bacteria.

Omafuvbe, (2006) reported that increases in pH values during alkaline fermentations could be as a result of proteolysis and the release of ammonia following the utilization of amino acids by the fermenting *Bacillus* species. The preservative and flavour characteristics of alkaline fermentations are derived in part from the

liberation of ammonia and increased pH (Beaumont, 2002).

The results in Figure 2 shows here was no significant difference (P>0.05) in the temperature of the three fermented castor bean samples. All the three samples had high temperature values ranging from 27°C to 31°C. Sample B_1 with 0% NaCl/Lime increased more steadily compared with those of B_2 (2% NaCl) and B_3 (3%Lime). It could be that the 2% NaCl and 3% lime additives added in samples B_2 and B_3 had liitle effect on the temperature of the fermentation process. Odunfa and Oyewole (1990) reported that the heat generated in fermenting iru beans possibly provided the ideal temperatures for the optimal activity of alpha-galactosidase. This temperature rise agrees with the other documented works on leguminous proteins; Sanni and Ogbonna (1991) in "owoh" and Odunfa and Oyewole (1990) in "iru". Temperature increases have also been reported by Odunfa (1981). Thus fermented castor bean samples is exothermic and this initial increase in temperature has been attributed to the intense metabolic activities of the microorganisms (period of maximum microbial activity) and represents the most active and important period of the fermentation. This is because enzyme studies have revealed that alpha-amylase, proteolytic and lipolytic enzyme activities attained their maximum levels at 24 – 36h of fermentation (Njoku and Okemadu, 1989).

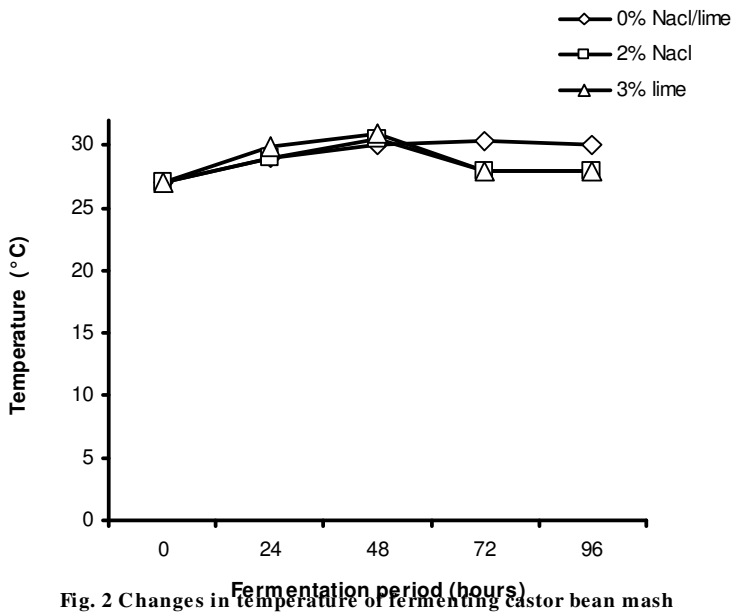


Fig. 2 Changes in temperature of fermenting castor bean mash during fermentation into ogiri $LSD_{0.05}$ = NS

Similar trends have been observed in other plant materials (Achi, 1992; Ogueke and Nwagwu, 2007). The rise in temperature indicates that the fermentation is exothermic with the changes being due to the metabolic activities of the microorganisms (Achi, 1992). The heat generated in the fermenting mash possibly provided the ideal temperature conditions for the optimal activity of the proteolytic enzymes (Odunfa, 1985). An initial increase in temperature followed by a gradual decrease thereafter have been reported during 'ugba' fermentation (Odunfa and Oyeyiola, 1985; Njoku and Okemadu, 1989; Ogueke *et al.,* 2010). During 'iru' production, Odunfa (1981) reported a continuous increase in temperature during fermentation process.

Figure 3 shows that the viable cell counts of bacteria in the fermenting castor bean samples increased rapidly in the 0 - 72h of fermentation and less rapidly thereafter. This trend is not unusual since similar pattern have been reported (Omafuvbe *et al*., 2000; Amin *et al*., 2004). The total viable count of the microbe from the fermenting castor bean samples increased with the length of fermentation period and then started decreasing afterwards. The number of microorganisms increased with the length of fermentation period. However there was no significant difference (P>0.05) in the viable cell counts from the Figure 3 showing the results of interaction in the samples. This shows that the 2% NaCl and 3% lime added differently to fermenting castor mash equally supported the growth of the bacteria. Omafuvbe, (2006) reported that viable cell count was significantly higher in 1% salted daddawa, implying that the increase in viable cell counts in 1% salted daddawa is an indication that low salt concentration creates a favourable medium for the growth of *B.subtilis*. Ojimelukwe *et al*., (2011) reported safe range for lime inclusion as additive to be 0 – 3% in fermented castor seed.

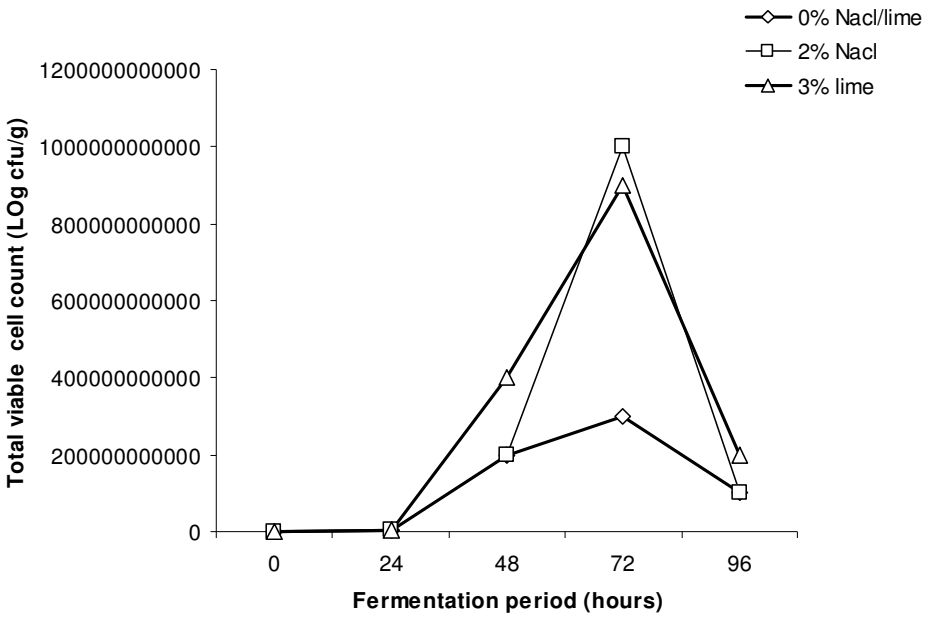


Fig. 3 Changes in total viable counts (log cfu/g) during fermentation of castor bean into *ogiri* .

$LSD_{0.05}$ = NS

From Figure 3, the highest rate was observed within the first 72h then subsequently the decrease in total viable count. This highest period could be assumed to be the most active period in the fermentation process. This initial period increase in population may have been responsible for the heat generated in the first hours of fermentation. This has been documented especially in fermentations involving *Bacillus* species (Obeta, 1983; Odunfa and Oyeyiola, 1985; Sarkar *et al*., 1993). It is therefore possible that fermentation for the rest of the process was sustained more by the enzymes produced than by the increase in bacterial population (Odunfa and Oyeyiola, 1985). Njoku and Okemadu (1989) in their study of ugba fermentation observed that alpha-amylase; proteolytic and lipolytic enzymes were detectable at the start of fermentation and attained their maximum levels at 24-36h fermentation. It could be that the 2% salted *ogiri* and 3% lime added to fermented castor bean mash favoured the growth of *B.subtilis* during fermentation. This has also been reported by Omafuvbe, (2006) in the use of 1% salt daddawa and Yabaya (2006) in fermented *Acacia nilotica* using *B.subtilis*.

The increase in the total viable count can be attributed to the accumulation of compounds such as organic acids and other metabolites (David and Aderibigbe, 2010). Yong and Wood (1979) observed fluctuations in microbial load during the fermentation of soy sauce. Ogunshe *et al*. (2006) observed that *Bacillus species* occurred most consistently and predominated the fermentation of *Albizia saman* into *aisa*, with the production of the highest ammonia-like aroma, characteristic of leguminous-based fermented condiments.

Ikenebomeh (1989) reported that variations in salt (NaCl) content and temperature influenced the microbial development and organoleptic quality of fermenting African locust bean seeds. The influence on microbial growth by different salt contents and temperatures were followed by changes of pH and titratable acidity. And that the predominant microorganism present throughout the fermentation was *Bacillus* species with characteristics similar to *B.subtilis*. He also reported that salt additions above 3% (w/w) and temperatures below 25^{o}C resulted in lower microbial counts, low pH and titratable acid values. Several fermented food condiments rely on the participation of various *Bacillus* species. The microorganisms that produce the changes may be the natural flora on the material to be fermented or may be inocula added as a starter cultures (Pelczar, 1996).

Table 1 shows the result of ammonia production during the 0 – 96h fermentation of castor oil bean seeds into *ogiri*. There exist a significant difference ($P<0.05$) in the samples and fermentation time. The result shows a steady increase in the ammonia content of the samples as fermentation time progressed. The sample containing 2% NaCl, B_2, had the highest value of ammonia content, 0.655 µg/ml followed by sample B_1 (0%NaCl/Lime) with 0.256. This is in agreement with Omafuvbe (2006) who reported that ammonia concentrations in soy-daddawa increased significantly in the first 48h of fermentation after which the value remained stable. The rise in pH coincides with the period of increased production of ammonia in the fermenting seeds (Visessanguan *et al.,* 2005; Omafuvbe, 2006). Sample B_3 had the lowest value of

0.181 µg/ml compared to samples B_1 and B_2. Ammonia formed also makes the substrate unsatisfactory for invasion by microorganisms that might spoil the products (Visessanguan *et al*., 2005).

Omafuvbe (2006) also reported that ammonia concentration was significantly higher in 1% salted daddawa and could 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.* Visessanguan *et al*., (2005) observed similar trend during fermentation of *thua nao,* a traditional Thai fermented soy product. They observed marked changes in ammonia after 12, 18 and 36h of fermentation for the control and those inoculated with *B.subtilis* at 10^2 and 10^4 cfu/g respectively. Inoculation generally accelerated an increase in ammonia nitrogen. The result is in agreement with Van Buren *et al.,* (1972) and Sparringa and Owen (1999) who reported that the increase in pH of tempeh, a rhizopus specie fermented soy product was caused by the liberation of ammonia. Ammonia is a product of the utilization of amino acids by the bacteria as sources of carbon and energy. Stillings and Hackler (1965) observed an increase in free amino acid and ammonia contents as fermentation time increased.

Ammonia production is probably a common feature during fermentation of protein rich plant materials; ammonia is produced during *Bacillus* fermentation of soybeans for kinema (Sarkar *et al*., 1993), during fermentation of African oil bean seeds for ugba (Njoku and Okemadu, 1989) and fermentation of African locust bean seeds for iru (Odunfa, 1981). Accumulation of ammonia in fermented foods may become undesirable at high levels. Thus Njoku and Okemadu (1989) suggested that there should be monitoring of enzyme activity during fermentation for improved quality.

Table 1. Changes in ammonia content (µg/ml) of fermented castor seed, *ogiri*

Fermentation time(hrs)	Samples B_1	B_2	B_3	Mean
0.00	0.1320	0.3060	0.0980	0.1787
24.00	0.1750	0.5320	0.1440	0.2837
48.00	0.2040	0.5980	0.1670	0.3230
72.00	0.2330	0.6130	0.1740	0.3400
96.00	0.2560	0.6550	0.1810	0.3640
Mean	0.2000	0.5408	0.1528	

** B1=0%NaCl/Lime *Ogiri*; B2= 2%NaCl *Ogiri*; B3= 3% Lime *Ogiri*
LSD (0.05%) Ferm_time = 0.01800
LSD (0.05%) Samples = 0.01394
LSD (0.05%) Ferm_time x Samples = 0.03118

The gradual development of unmistakeable ammoniacal odour (Beaumont, 2002) is in agreement with the observed pH changes (alkaline pH ranges) and this agrees with the reported alkaline pH range by Barimala, 1994, Omafuvbe and Oyedapo, 2000 and Omafuvbe *et al.,* 2004 in similar experiment. The preservative and flavour characteristics of alkaline fermentations are derived in part from the liberation of ammonia and increased pH (Beaumont, 2002)., thus the high pH values and ammoniacal odour recorded in the fermented *ogiri* may account for the post-fermentation characteristics. The increase of pH is attributed to the production of ammonia which is characteristic of proteinous food fermentations as a result of proteolytic activity of the fermenting microorganisms. The abundant production of ammonia, which is responsible for the unique aroma sometimes described as ammoniacal or pungent has been reported for other protein food fermentations such as dawadawa, soumbala or netetu (Wang and Fung, 1996; Beaumont, 2002; Yong and Wood, 1977; Sarkar *et al*., 1993; Allegheny *et al*., 1996; Achi *et al.,* 2007; Sarkar and Tamang, 1995).

Leejeerajumnea *et al*., (2000) analysed the concentrations of ammonia in thua nao and Japanese natto by an enzymatic method and found that the ammonia content in fermented thua nao at 72h was 5.0g/kg dry weight, whereas only 1.9g/kg was found in dry thua nao, which suggested that the ammonia was lost during drying. Ammonia has been reported to inhibit spoilage or pathogenic bacteria in alkaline food fermentations (Leejeerajumnean *et al.,* 2000). Some studies have reported total ammonia levels, 1-3mmol/kg wet weight, (Sarkar *et al*., 1993, Allagheny *et al*., 1996) of cooked soybeans. And some have reported maximum total ammonia concentrations of 200 – 300mmol/kg wet weight in the final fermented products (Ohta, 1986; Sarkar *et al*., 1993; Allagheny *et al.,* 1996; Leejeerajumnean, 2000) corresponding to ammonia concentrations at pH 8.0 – 8.5 of 10 – 45mmol/kg wet weight. It is possible that ammonia concentrations are high in the surface layer of microbial growth than these values averaged over the entire bean, but this concentration effect may be counteracted by the near neutral pH value during the phase of maximum microbial growth (Sarkar *et al*., 1993). Thus it appears that ammonia concentrations are unlikely to be

high enough during the fermentation to prevent the growth of many spoilage or pathogenic bacteria.

The result from Figure 4 shows there is no significant difference (P>0.05) in the ricin content of the samples and their fermentation time. It was observed that in all the three samples their ricin content continued to decrease as fermentation time increased. This is in agreement with Anosike and Egwuatu (1981) who reported that the toxicity of ricin is destroyed when a water solution of the ricin is boiled and that given enough time, the toxicity diminishes with an increase in the extent of denaturation. One of the physical effects of thermal denaturation of protein is that its solubility is increased. The oil in the product after boiling and fermenting could coat the protein molecules, preventing the hydrophobic group of the protein from interacting with the water thereby resulting to decreased solubility of the protein (Ihekoronye and Ngoddy, 1985).

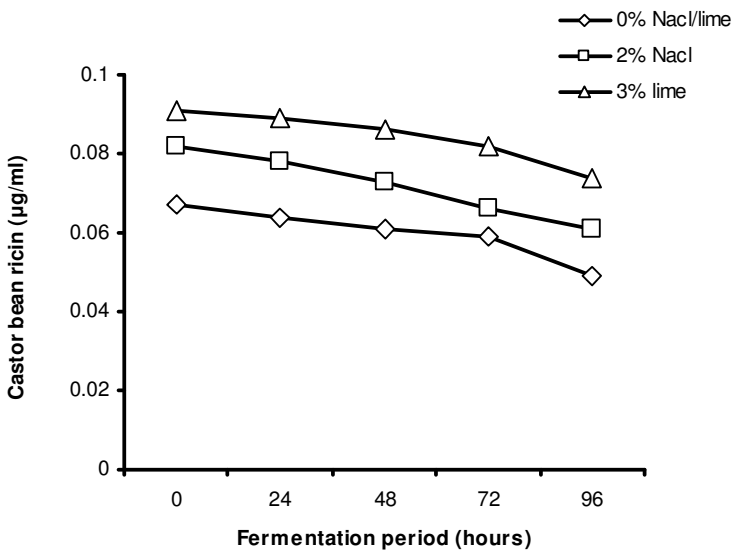


Fig. 4 Changes in the ricin content of fermenting mash during the fermentation of castor oil bean into *ogiri*.

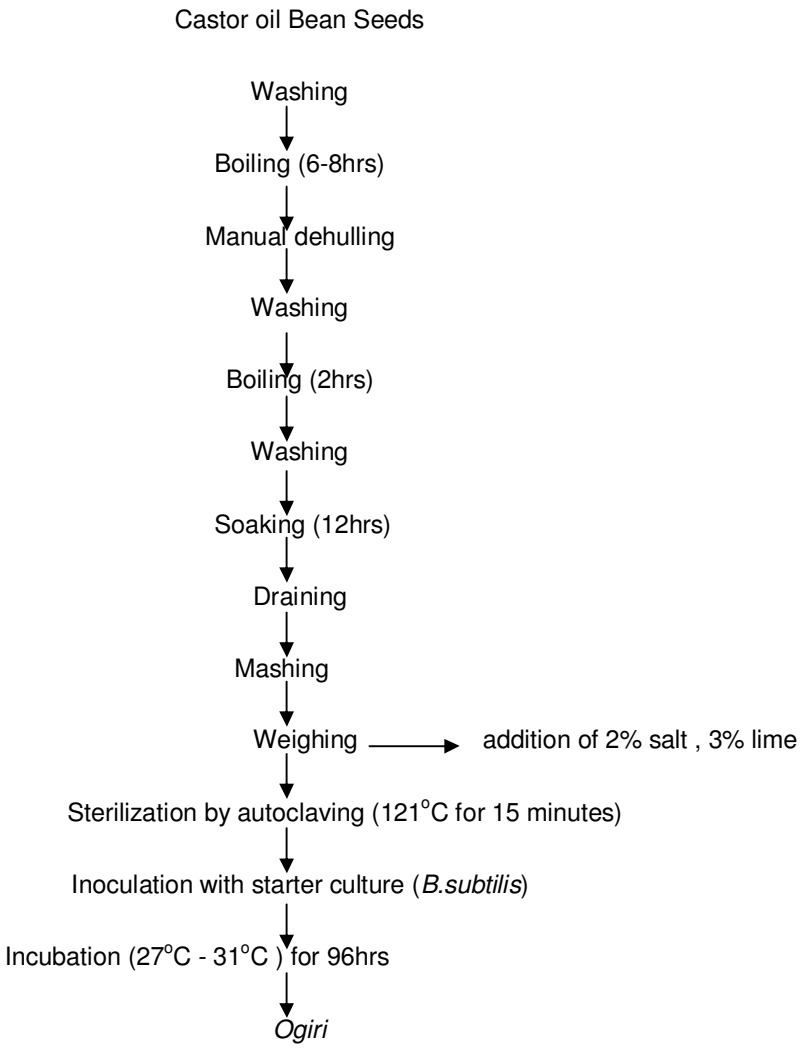
$LSD_{0.05}$ = NS

It was also observed that the ricin content reduced steadily as fermentation time progressed in all the samples thereby making the condiment safe for consumption as seasoning. Ricin being an antinutrient is said to be among the compounds which reduce the nutrient utilization and/or food intake of plants or plant products used as human foods or animal feeds and they play a vital role in determining the use of plants for humans and animals (Soetan and Oyewole, 2009).

Weiss, (1971) and Okagbue, (1993) reported that parts of south eastern Nigeria have long developed a method for treating and detoxifying the unextracted castor seed that is subsequently used as food seasoning. Ricin which occurs in castor beans have been reported to cause poisoning in all classes of human and livestock (Soetan and Oyewole, 2009). Ojimelukwe *et al.,* 1995 reported that soaking fermentation and cooking reduced the hemagglutinating activity of whole seeds of yam bean and soya bean. It has been reported that fermentation is a very reliable method of detoxifying ricin (Odunfa, 1985; Ogunniyi, 2006; Aniche *et al.,* 1993).

Conclusion

Using HPLC to monitor the ricin content of castor oil bean during fermentation for 96h goes to show the in-depth molecular analysis of the changes associated with castor bean fermentation into *ogiri* as well as make appropriate modifications where applicable. The addition of 2% NaCl and 3% lime did not affect the physiochemical properties analyzed. Rather the parameters show that fermentation was alkaline and there could be varieties of samples with respect to product and market differentiation.



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