The Microbial Changes of Fermenting Cowpea Seeds into a Novel Food Condiment (*Agwaure*) at Varying Temperatures and pH

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ABSTRACT

This work is aimed at studying the microbiological changes in Cowpea seeds fermentation at varying temperatures and pH. Cowpea seeds used for this study were randomly purchased from Ekeukwu market in Owerri, Imo State, Nigeria. Using standard microbiological techniques and media these changes were monitored. The bacteria isolated from the fermented agwaure were Bacillus spp, Lactabacillus spp, and Pediococcus spp. In the heterotrophic counts an increase in counts were observed as the fermentation days increased. An initial viable heterotrophic count of 10.2 x 10^4 and 8.4 x 10^4 was observed at 25°C and 35°C respectively after the first day of fermentation. Eventually the counts increased across the fermentation period till the 5th day with counts of 24.5 x 10^4 and 18.9 x 10^4 respectively. Thus, the study established that changes occur across the days of fermentation at different pH, temperature and the proximate analysis. Thus during fermentation the appropriate temperature and pH should be maintained to optimize the fermentation process.

Keywords: Food Condiment, Fermentation, pH, Temperature

INTRODUCTION

Cowpea seeds (*Vigna unguiculata*) is one of the major legumes produced in Nigeria, particularly the northern states. Nigeria is responsible for 70% of the world total dry cowpea seeds production (Emmanuel *et. al*., 2004). The seed is rich in protein, carbohydrates, Vitamin B complex and minerals. The protein content ranges between 18.3 to 30.3%. As a pulse its carbohydrate is high (36-68%), the crude fibre content ranges from 2.7 to 5.8%, total ash from 2.5 to 4.2% and fat 0.9 to 2.4%. Cowpea seeds contain iron (6.2mg/100g) and calcium (110mg/100g). The seeds are rich in phosphorus, potassium, magnesium and sodium. Cowpea seeds also contain a wide range of toxin compounds and anti-nutrients polyphenols, phytate, trypsin inhibitor, saponins lectins and oligosacchrides). These affects digestibility and food value (Achi, 2005).

A food condiment is obtained from fermented seeds of the cowpea seeds (*Vigna unguiculata*).The plant is a legume that is known as 'kunde' in swahili and 'black eyed peas' in America, it is known as 'Agwa' among the Igbos in Nigeria and originates in the centre of Africa and is one of the continent's oldest known crop, first discovered as a small creeping vine in the Sahara desert. Cowpea seeds is extremely drought resistant and adapted to poor soil, making it a useful staple crop for farmers in areas that face increasingly water scarcity and hot temperature due to climate change, perhaps for this reason alone, it is understandable that cowpea is the second most widely grown legume in Africa only the peanut originating in America (Afoakwa*et. al*., 2004).

Cowpea seeds can be eaten at different stages throughout its development and forms the basis of a wide variety of meals. The leaves and young pods are eaten like vegetables and the seeds

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are consumed as a side dish or made into sauce or dry grains. The seeds are also ground into flour that can be pressed into deep fried cakes called "Akara balls" or steamed cakes' known as 'moi-moi', the dry seeds can all be ground and fermented into a food condiment (Emmanuel *et. al.,* 2004: Akinyele and Akinlosotu, 1991).

The people who cultivate and eat cowpea seeds are beneficial to the soil which it grows. The plants deep tap root, the part that makes it so tolerant to dry growing conditions and helps to stabilize the soil, while its shade and dense cover help preserve moisture, like all legumes, cowpea seeds fix nitrogen in the soil, making the location where it grows more hospitable to other vegetable and staple crops.

The aim of the work is to study the nutritional changes and micro-organisms associated with fermenting Cowpea seeds into a food condiment at varying temperature and pH. The objectives of this study include:

1. To isolate the micro-organisms involved in fermenting Cowpea seeds.
2. To study the microbial load at varying temperatures and pH.
3. To study the nutritional changes in fermenting Cowpea seeds.

Fermentation is one of the most important technological processes in the food industry. It converts the less to the more nutritious forms of the seed by the use of oxidizing agents such as bacteria. The process enhances the nutritional value of processed foods, adds a variety of flavors and extends the shelf life. In Nigeria, the traditional fermented foods are derived from roots, tubers, cereals, legumes and dairy products such as milk. Alcoholic beverages are also prepared from fermented cereal juice fruits or liquids from trees (Achi, 2005).

Fermented vegetable proteins have potential uses as protein supplements and functional ingredients in fabricated foods. The local food flavoring condiments are normally prepared by traditional methods which are uncontrolled resulting in extensive hydrolysis of the protein and carbohydrate components. Apart from increasing the shelf life and reduction in the anti-nutritional factors, fermentation improves the digestibility, nutritive value and flavours of the raw seed (Antai and Ibrahim, 2009).

In the household or cottage industry preparation of dawadawa, the techniques employed are simple and non-sterile materials are used. This fermentation relies on natural inoculate under uncontrolled fermentation conditions. This uncontrolled fermentation can lead to inconsistent products and shorter shelf life. However, in modern industrial processing, processes are more closely monitored and controlled with direct inoculation with isolated and purified microorganisms. In this case longer shelf life is obtained for the commercial product.

The studies of Antai and Ibrahim (2009) and Odunfa (2005) found several microorganisms associated with dawadawa but most abundant and the major agent of fermentation after 72hours was *Bacillus subtilis*. Other microorganisms that was present after the fermentation include *Leuconostoc mesenteriodes* and *Staphylococcus* sp.

Fermenting to produce the condiment was found to be exothermic and the pH also increased during the period. During fermentation soluble amino acid, namely glutamic acid is liberated the salt of the amino acid monosodium glutamate is used widely as an additive to enhance flavour (Odunfa, 2005). Dawadawa is also an important source of vitamin B in the form of riboflavin which is generally deficient in most African diets. Dawadawa contains the highest riboflavin content when compared to 33 common plant foods, about 0.80mg per 100g.

MATERIALS AND METHODS

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The Study Area

This study was conducted in Science Technology Laboratory, Federal Polytechnic Nekede Owerri, Imo State, Nigeria.

Source of Sampling

Cowpea seeds were carefully and randomly purchased from Ekeukwu Owerri main market.

Sterilization of Materials

All glassware, diluents and media with the exception of the pipettes, was sterilized at 121°C for 15 minutes at 15 psi using autoclave as described by Ogbulie *et al* (2001). The pipettes were sterilized in a hot air oven at 160^{0}C for 1hour 30 minutes. Wire loop was sterilized by direct flaming to red hot using Bunsen burner. Glass spreader was sterilized by first dipping into ethanol and passing through a Bunsen burner flame.

Sample Processing

The sample was processed using the method of Singh *et al* (2003). Whole cowpea seeds were soaked in water for 4 minutes to loosen the seed coats. The seed was drained and dehulled using a heavy duty blender.

The hull was separated by floatation in water. One portion was dried in an oven temperature of 65°C for a period of 20-24 hours and the remaining portion steamed for 4minutes. Subsequently, the steamed seed was oven dried at 65°C and milled into flour using a heavy duty blender.

Estimation of pH

Ten grams of dried flour was mixed with 100ml distilled water. The mixture was allowed to stand for 15 minutes, shaken at 5 minutes intervals and centrifuged at 3000 rpm for 15 minutes using a centrifuge. The supernatant was decanted and its pH was determined using a pH meter (Hana, Italy). It was noted and maintained at 6, 7 and 8. Acid and base was used to maintain stability.

Fermentation Process

Fermentation was achieved by the addition of weighed portions of dehulled and steamed cowpea seeds flour kneaded into 50% moisture dough, wrapped with banana leaves and allowed to ferment at room temperature for a period for 5days. The bioload and the proximate analysis on the various samples was carried out at 24hrs interval for the for 5day period.

Media Used: MacConkey and Nutrient agar (Oxoid, England) were used for the cultivation of the bacterial organisms.

Microbiological Analysis

Serial Dilution and Inoculation of Samples

To estimate the microbial load, the fermenting samples were first serially diluted using 10-fold serial dilution method as described by Dozie and Uwaezuoke, (2003). This was aimed at obtaining a clearer aliquot for inoculation and effectively isolating the inherent microorganisms in the samples. Based on the serial dilution, spread plate technique was employed after the transfer of the inoculums into the Petri dish-medium to adequately separate viable cells from each other. In this technique, the propagules of microorganisms are spread over solidified agar medium with the help of an L-shaped (bent) glass rod called glass spreader. The inoculated Petri dishes were then incubated at 37^{0}C for 24 hours.

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Plate Count

Duplicate plates of each dilution were used to achieve a plate count of between 30 and 300 Colony Forming Unit (CFU) as described by Dozie and Uwaezuoke (2003). This quantitative determination of bacterial and fungal populations was carried out by standard or viable plate count method using a Colony counter.

Purification and Preservation of Isolates

After the various Colony counts, bacterial isolates was pick with a wire loop based on their morphological appearance. The picked colonies were sub- cultured unto freshly prepared nutrient agar plates for purification. They were further incubated for 24hrs at 37^0C. After incubation pure cultures were stored in agar slant in a refrigerator.

Preparation of Standard Inocula

The methods of Dozie and Uwaezuoke, (2003) was adopted for the preparation of the standard inoculums of bacteria. Isolates from the stock cultures were revived by picking two balls each from the agar slant and sub-culture into sterile prepared Nutrient agar plates for another 24hours, at 37^0C. The fresh cultures of bacteria were used as standard inoculums for the different morphological and biochemical tests.

Characterization and Identification of Microbial Isolates

Gram Staining

The Gram staining method of Cruickshank *et al*., (1982) was adopted for the determination of the Gram straining reactions for all bacterial isolates. Using sterile wire loop, isolates was smeared onto clean microscope slide, air-dried and fixed. Crystal violet solution was poured on the smear and after 30 seconds, washed in a gentle running tap water for 5 seconds, covered with Lugols was iodine solution and washed again. The stained smear was thereafter decolorized using 95% ethanol, washed with water and finally counter stained with 3% safranin solution for 15 seconds. The smear was finally washed in clean tap water, air-dried and observed first under x 60 magnification and finally under oil immersion objective lens (x100).

Motility Test

The semi-solid method for motility test as reported by Cruickshank et al., (1982) was adopted in preference to the medium for the motility test contain g/L tryptone soy broth, 25g and agar-agar, 5g. It was dispensed in 5ml volume into a set test tube and sterilized. After cooling, the tube was separately stab-inoculated with the inoculums or test bacterial using sterile wire loop. The inoculated tube was incubated at 37^oC for 24 hours. Diffused, brush-like growth indicates a positive result for motility.

Biochemical Tests

Catalase Test

For catalase test, the method of Okereke and Kanu, 2004 was adopted.

Indole Test

The method of Kovacs (1928) as reported by Cruickshank *et al*., (1982) was adopted for the determination of indole production among bacterial isolates.

Citrate Utilization Test

This test determines the ability of an isolate to utilize citrate as a sole source of carbon for metabolism. It is therefore a useful test in the identification of organisms in the Enterobacteriaceae and other genera. Simon's citrate agar medium was used. This test of utilization of citrate by isolates was carried out using the method described by Cruickshank *et al*., (1982).

Test for Fermentation of Sugars

Bacteria isolates from stock cultures were used for this test. They were tested for their ability to ferment sugars like maltose, lactose, glucose, sucrose and mannitol (Cruickshank *et al*., 1982).

Urease Production Test and Hydrogen Sulphide Production Tests

The method of Cruickshank *et al*., (1982) was adopted for these tests.

Methyl Red (MR) and Vogues Proskaur (V.P) Test

The tests are employed in the identification of members of the Enterobacteriaceae and were adopted from the method of Ogbulie *et al.,* (2001).

Coagulase Test

This test was carried out as described by Ogbulie *et al.* (2001).

Oxidase Test

The Kovac's method as described by Ogbulie *et. al.,* (2001) was used. Few drops of tetramethyl phenylenedramine hydrochloride (Kovac's reagent) was impregnated into a filter paper in a Petridish. With sterile glass rod, a smear of bacterial growth was made into the impregnated filter paper, a purple coloration indicates oxidase positive while a delayed reaction was recorded negative.

Spore Staining

The smear of the bacterial isolates were made on the clean glass slide and air-dried. The smear were flooded with 5% Malachite green and steamed for 1 minute. The stain was washed under running water. The slides were counterstained with safranin solution for 15 minutes and were then rinsed with water and dried. The slide was viewed under the microscope using oil immersion objective lens. Bacterial cells stained red while spores stained green.

Identification of Lactobacillus Species

The gram positive *Lactobacillussp*. was identified by growth at 15^0C and at 45^0C in MRS broth (Oxoid, England).

Total Heterotrophic Bacterial Count

This was determined with the nutrient agar using the spread plate technique as described by Okereke & Kanu (2004). One milliliter of each serially diluted sample was inoculated onto different sterile nutrient agar plates in duplicates. The plates were incubated for 24hours at 37^0C.

Total Coliform Count

The method of Okereke & Kanu (2004) was adopted. One milliliter of each serially diluted sample was inoculated onto different sterile MacConkey agar plates in duplicates, the

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inoculums were then spread evenly on the surface of the media using a sterile spreader. This was followed by incubation at 37^oC for 24 hours, after which the colonies was counted and the mean total coliform count expressed as Cfu/ml. These counts were carried out on the fermenting sample at various temperatures and pH. All these were performed on triplicate samples.

Determination of Moisture Content

The moisture content was determined according to AOAC methods (AOAC, 1995). The dried and fermenting cowpea seeds sample was weight the residue was converted to percent total solids (dry matter) and the moisture content was calculated as the difference. However, the moisture contents of the dried vegetables was determined by drying approximately 1g samples using an infrared balance-cum-drier to constant weight and calculated as percent loss in weight.

Determination of Total Ash Content

Total ash was determined by AOAC methods (AOAC, 1995). Approximately 2g of the cowpea seeds samples was weighed into porcelain ashing dishes previously dried in a hot air oven at 98^0 – 100^0C, cooled and tarred. The dish was held in a muffle furnace at approximately 600^0C for 4 hrs. They were then cooled to room temperature in desiccators and weighed. The weight of residue represented the total ash.

Determination of Crude Fibre

Crude fibre content was determined following AOAC methods (AOAC, 1995). Approximately 2g of dry ground sample was used to determine the crude fibre as loss on ignition of dried residue remaining after boiling of the sample with 2.04N sulphuric acid and 1.78N sodium hydroxide solutions under specific conditions.

Determination of Crude Protein

Crude protein was determined as total nitrogen using the semi-micro Kjeldahl method (AOAC, 1995).

Determination of Crude Lipid Content and determination of Carbohydrate Content

The AOAC (1995) method was used for these properties.

Statistical Analysis

The mean, standard deviation and ANOVA (analysis of Variance) was calculated using statistical package for social sciences (SPSS) for windows version 15. Means was separated using Duncan multiple range test and significance was judged at $P<0.05$.

RESULTS AND DISCUSSION

The mean result of the microbiological and chemical (proximate) analysis of the fermenting cowpea seeds samples examined is as shown in the tables and figures below. The variation of the different microbiological and chemical parameters at the different sampling hours were determined by subjecting the mean result to Analysis of variance (ANOVA) and Duncan multiple range test (T-Test). Tables 1 and 2 show the Total Heterotropic Bacterial Count based on Temperature and pH, respectively. The result after statistical analysis is as shown in figures 1 to 4.The morphological and biochemical characteristics of the isolated bacterial are as shown in Tables, 1 and 2, respectively. Part of the proximate analysis result is shown in

figures 5 to 6 based on pH and on temperature, respectively. The result was based on the two temperature of 25^{0} and 37^{0}C and pH of 6, 7 and 8, respectively.

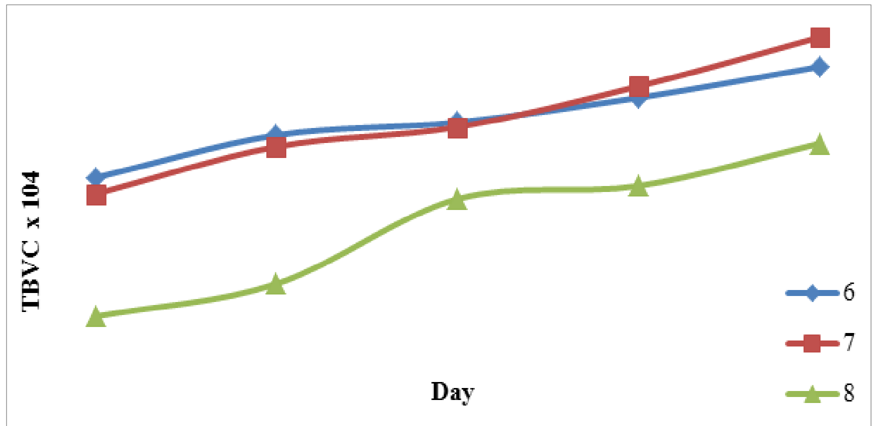

Figure 1. Total Viable Bacterial Count of Fermenting Cowpea Seeds Based On pH

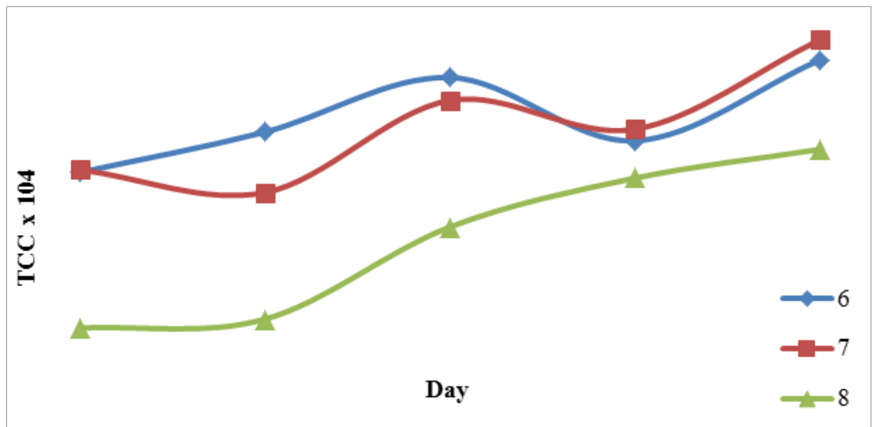

Figure 2. Total Coliform Count of Fermenting Cowpea Seeds Based On pH

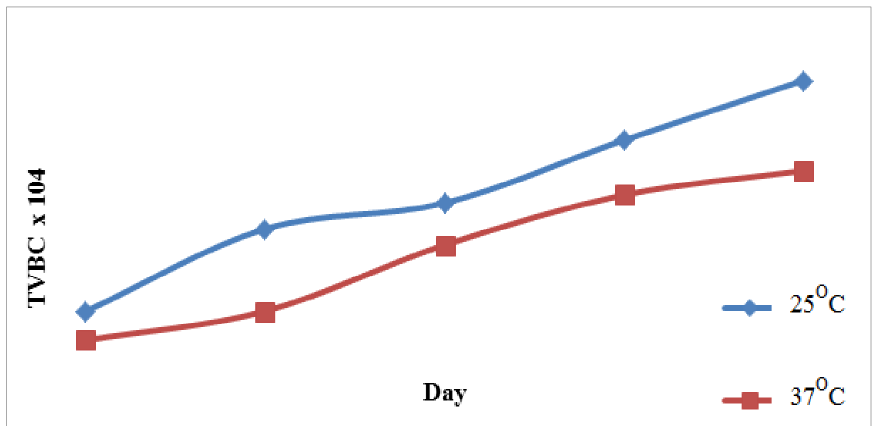

Figure 3. Total Viable Bacterial Count of Fermenting Cowpea Seeds Based On Temperature

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25°
37°

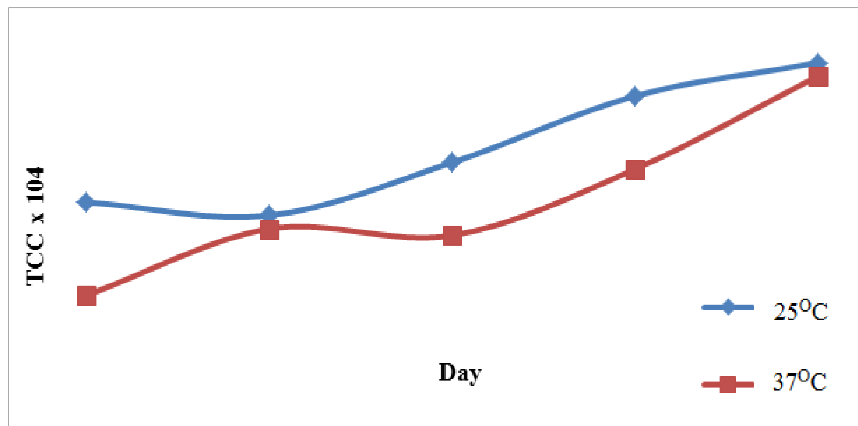

Figure 4. Total Coliform Count of Fermenting Cowpea Seeds Based On Temperature

Table 1. Morphological Characterizations of Bacterial Isolates

	Morphology	*Shape*	*Grams Reaction*	*Motility*	*Motility*	*Probable Identity*
1.	Large round, thick, opaque colonies with dull irregular edges	Rod	G+ ve	-	-	*Bacillus sp*
2.	Moderate round, beige, smooth colonies with glistering irregular edges	cocci	G+ ve	-	-	*Lactobacillus sp*
3.	Large round, thick, opaque colonies with dull irregular edges	Tetrad cocci	G+ ve	-	-	*Pediococcus sp*

Table 2. Biochemical Characterization of Bacterial Isolates

	Coagulse	*Citrate*	*Oxidase*	*Urease*	*Indole*	*Methyl Red*	*Voges Proskauer*	*Hydrogen Souphide Production*	*Carbohydrates Metabolizing*	*Catalase*	*Maltose*	*Glucose*	*Lactose*	*Sucrose*	*Mannitol*	*Probable Identity*
1.	-	+	+	+	-	+	-	+	O.F	+	A	A / G	A	A / G	A / G	Bacillus sp
2.	-	+	+	+	-	+	-	+	F	-	A	A	A	A	A	Lactobacillus sp
3.	-	+	+	+	-	+	-	+	F	-	A	A	A	-	A	Pediococcus sp

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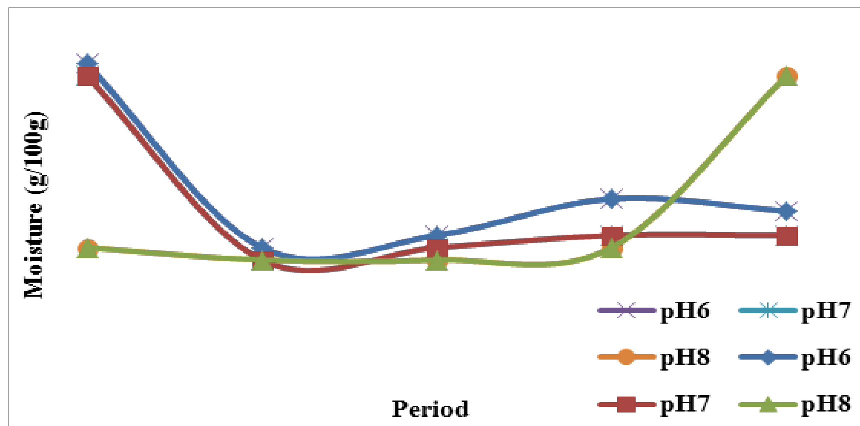

Figure 5. Crude Moisture Content of Fermenting Cowpea Seeds Based on pH

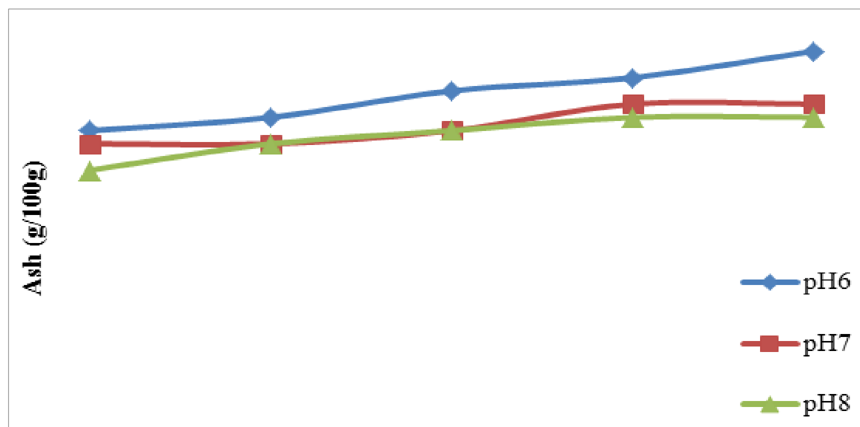

Figure 6. Ash Content of Fermenting Cowpea Seeds Based On pH

This study x-rays the microbiological and nutritional changes in Cowpea seeds fermentation at varying temperature and pH. The bacteria isolated from the fermented dawadawa as shown in Table 1 were *Bacillus spp, Lactabacillus spp, and Pediococcus spp.* Previous studies have also reported some of these organisms to be associated with fermenting food of plant origin and are not pathogenic (Sanni, 1993).

These organisms especially *Lactobacillus* and *Pediococcus* are known as lactic acid bacteria species and have been reported to possess the enzyme lactase that convert sugar (lactose) during fermentation to glucose and galactose. The development of lactic acid bacteria is also stimulated by the presence of yeasts which provide soluble nitrogen compounds and co-factors like B-vitamin. Lactic acid fermentation involves production of lactic acid and carbon dioxide among other by products. The small number units of *Enterobacteriaceae* and other gram- negative bacteria showed that their growth might have also been inhibited due to the presence of lactobacilli which produced lactic acid.

Nonetheless; in the heterotrophic counts an increase in counts were observed as the fermentation days increased. An initial viable heterotrophic count of 10.2 x 10^4 and 8.4 x 10^4 was observed at 25°C and 35°C respectively after the first day of fermentation. Eventually the counts increased across the fermentation period till the 5th day with counts of 24.5 x 10^4 and 18.9 x 10^4 respective. This suggests that growth of microbes were more favored during the earlier temperature of as against the later of 35°C. These high bacteria counts recorded at 25°C in the beginning of the fermentation could be attributed to the presence of excess carbohydrate glucose content. According to Abegaz (2002) substrate (glucose fructose and sucrose) are consumed fast within the first 1-4 days of fermentation hence the maximum

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population in bacteria counts.

Alternatively, a fluctuating reduction in viable bacteria counts were observed at the different pH of 6, 7 and 8 during the 5 day fermentation period. The result of this study revealed that microbial life was more prominent at the least pH (pH6). Thus, it may be imperative to state that a pH near neutrality (6) or slightly alkaline enhances the growth, proliferation and metabolic activities of the fermenting organism: hence the prevalence of lactic acid producing Bacteria. Also changes in the external pi I also might affect the ionization of nutrient molecules and thus reduce their availability to the organism. Microorganisms frequently change the pH of their own habitat by releasing acidic or basic metabolic waste product. Acidophilic microbes have their growth optimum between pH 1.0 and 5.5, neutrophiles between pH5.5 and 8.0 and alkalophiles prefer pH range of 8.5 to 11.5. Thus it has been stated that generally different microbial group have characteristic pH preferences (Abegaz et al., 2002). Similar observations were also recorded in the coliform counts respectively.

Similarly, on the total coliform count, observations were also in line with the heterotrophic viable bacteria counts across the pH and temperatures respectively. The mixture of total plate counts showed both homo- fermentative and hetero-fermentative bacteria.

CONCLUSION

The result of the study has revealed the microbiological changes in Cowpea Seeds fermentation; thus, the study established that changes occur across the days of fermentation at different pH, temperature and the proximate analysis. Thus during fermentation the appropriate temperature and pH should be maintained to optimize the fermentation process.

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