SCREENING FOR CYANIDE DEGRADING CAPABILITY OF SOME MICROBIAL CASSAVA FERMENTERS

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DECLARATION

I declare that this research titled**'Screening for cyanide degrading capability of some microbial cassava fermenters'**was carried out by me in the Department of Microbiology under the supervision of Prof. J. B. Ameh and Prof. C. M. Z. Whong.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any institution.

Name of Student	Signature	Date

CERTIFICATION

This thesis entitled SCREENING FOR CYANIDEDEGRADING CAPABILITY OF SOMEMICROBIAL CASSAVA FERMENTERS by Onoja ODA meets the regulations governing the award of the degree of a Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Cyanide is a chemical compound that occurs in biomolecules such as cassava as cyanogenic glycoside. Cassava and cassava products are therefore unsafe for consumption if not properly processed. A very efficient method of processing cassava is fermentation. Seven microbial cassava fermenters were isolated and characterized as *Weissellaconfusa*, *Lactobacillus brevis1*, *Lactobacillusfermentum 1*, *Lactobacillusplantarum 1*, *Aspergillusniger*, *Fusarium*sp and *Trichoderma*sp. The lactic acid bacteria were isolated on deMann, Rogosa and Sharpe (MRS) agar while the moulds were isolated on malt extract agar (MEA). The bacteria were characterized with analytical profile index (API) 50 CH and API 50 CHL medium and the moulds were characterized using microscopy and fungal atlas. McFarland turbidity standards and haemocytometre were used for the determination of the inoculum size of bacteria and mould respectively. Result of cyanide screening showed that reduction of cyanogenic glycoside was significant on the third and fourth day of fermentation as indicated by the result of the analysis of variance (ANOVA), $P<0.05$, with *Lactobacillus plantarum 1* as the organism with the highest cyanide degrading potential, with residual cyanide level of0.10 ± 0.10^{d} mgHCN/100 g cassava wet weight.*Lactobacillus plantarum 1* having the most efficient cyanide degrading capabilitywas therefore used as starter culture for gari preparation.Another sample of gari was produced without the starter culture and sensory evaluation of the two gari samples was carried out by seven panelsof judges according to the Hedonic Scale, on five attributes namely appearance, aroma, taste, flavour and general acceptability. A t–test analysis was done to compare the two samples of gari and the result showed a significant difference ($P< 0.05$), with gari produced with starter culture as being more preferable. The result of screening for cyanide degrading capability which showed that all organisms isolated degrade cyanide in varying degrees is consistent with

the observation of Amoa-Awua*et al*.,(1996) who reported that all bacteria, yeasts and moulds identified in traditional cassava dough inocular exhibited linamarase activity and were therefore capable of degrading cyanogenic glycoside. It was concluded that *Lactobacillus plantarum 1*, the organism with the highest cyanide degrading potential, combined with the very good sensory attributes it produced in gari makes it a good starter culture for gari production.

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ABBREVIATIONS

ANOVA	-	Analysis of variance
HCN	-	Hydrogen cyanide
ICMI	-	International Cyanide Management Institute
ISO	-	International Standardization Organisation
FAO	-	Food and Agricultural Organisation
IITA	-	International Institute for Tropical Agriculture
MRS	-	de Mann, Rogosa and Sharpe
MEA	-	Malt Extract Agar
CFU	-	Colony Forming Unit
ASTM	-	Association of Standard Testing Materials

CHAPTER ONE

1.0 INTRODUCTION

It is widely known that consumption of certain cassava varieties in their raw or even boiled state cause serious poisoning and subsequently death. Upon research, it was discovered that the poisoning is caused by a biomolecule called cyanogenic glycoside commonly known as cyanide(ICMI, 2012).

Cyanide is a chemical that contains the cyano group, -C≡N, which consists of a carbon atom triple bonded to a nitrogen atom. It is a potentially deadly chemical that can exist as a colourless gas, such as hydrogen cyanide (HCN) or cyanogen chloride (CNCl), or a crystal form such as sodium cyanide (NaCN) or potassium cyanide (KCN) and biomolecules as cyanogenicglycoside(ICMI, 2012).

Cyanide is found in food stuffs such as cassava, cabbage, spinach and almonds, and as amygdalin in apple pips, peach, plum, cherry and almonds kernel (Montgomery *et al*., 2010). In Kernels, amygdalin seems to be completely harmless as long as it is relatively dry (Montgomery *et al.,* 2010). Natural oil of bitter almonds contains 4 % (w/v HCN), American white lima beans contains 10 mg cyanide\100 g bean, the dried root of cassava may contain 245 mg cyanide/100 g root, (Montgomery *et al.,* 2010).

Local farmers process their cassava by soaking them in water for a few days, a process known as fermentation. Most of these farmers are only aware that the product gets better after soaking it this way without having the knowledge of the mechanism behind it. Researching on cassava fermentation to improve on it and educating local farmers would help in food security and safety.

Cassava (*Manihotesculentacrantz*) leaves are wide and palmated with five to seven lobes. They are carried by a long and thin petiole. Bunch of mature roots from 30 to120 cm long and from 4 to 15 cm in diameter appear in the ground. The root is composed of two parts: the external part constitutes the skin and the internal part the pulp (Kobawila*et al.,* 2005).

Cassava roots are quantitatively the third most important food in the tropics, after rice and corn. It is an important source of calories because it covers 60 % of the daily calorific needs of the populations in tropical Africa and in Central America (Nartey, 2010). Cassava roots are however rich in cyanide in the form of cyanogenic glycoside, which is poisonous (Dunstan *et al.,* 1996 and Montgomery*et al.,* 2010). The recognition of cyanide in cassava goes back to antiquity (Meredith and Jacobsen, 2010). The hydrolysis of cyanide detoxifies cassava, releasing cyanhydric acid (hydrogen cyanide), which is toxic. Unhydrolysed cyanide remaining in cassava root after fermentation can constitute a health problem for the consumers (Cooke,1978; Ikediobi*et al*., 1980; Gomez and Valdiviesco, 1985;Nartley, 2010). Traditional technologies have been developed in Africa to eliminate cyanide in cassava, such that, they are safe for man and animal consumption. These technologies have fermentation as basis for operation. The use of pure cultures of microorganisms such as *Saccharomyces* sp and *Lactobacillus* sp or combinations of these had been reported to cause a substantial decrease in cyanogenic glycoside content (Oboh and Akindahunsi, 2003; Oboh, 2006).

1.1 Statement of research problem

The chronic exposure to cyanide due to the consumption of non-detoxified cassava products is associated with certain number of diseases such as goitre, dwarfism and the tropical ataxic neuropathy in man and impaired thyroid function and growth, neonatal deaths and lower

birth rates in animals(Meredith and Jacobsen, 2010). It is particularly a problem in regions where cassava is the major source of calories (Oke, 1980; Tewe, 1984; Umoh, 1985; Balagopalan*et al.*, 1988).

Chronic low-dose neurotoxicity has been suggested by epidemiological studies of populations ingesting naturally occurring plant glycosides (Blanc *etal*., 1993). These glycosides are present in a wide variety of plant species, most notably the cassava plant, one of the major tropical food stuffs (Conn, 1973; Cooke and Coursey, 1981). Cyanohydric acid is lethal at a consumption dose of 0.5 to 3.5mg per kilogram body weight (Kalyanaraman*et al*.,2010). Cassava roots and leaves contain cyanide (Naughton, 2010).

Excess cyanide residue from improper preparation of cassava root is known to cause acute cyanide intoxication (Peters *et al*., 2010). Symptoms of acute cyanide intoxication appear four or more hours after ingesting raw or poorly processed cassava (Peters *et al*., 2010). In some cases, death may result within one or two hours (Peters *et al*., 2010).

1.2 Justification of the study

Due to inadequate medical facilities in our own part of the world, Africa in general, and Nigeria in particular, cyanide poisoning may not be readily diagnosed and hence appropriate medication may not be administered. It is therefore imperative to develop technologies that would prevent the poisoning from occurring.

Sun drying is a common method used in the treatment of cassava for consumption, but this is only partially effective in reducing cyanogenic glycoside content (Tewe, 1993). However, fermentation has been described as a highly effective method in the detoxification of cyanide in cassava. Cyanophilic microorganisms have been shown to possess the enzymes linamarase,

hydroxynitrilelyase and cyanide hydratase that catalyses the sequential degradation of cyanogenic glycosides into hydrogen cyanide which is subsequently converted into formamide which they use as both nitrogen and carbon source (Adamafio,2008).

It would be however better, to identify the organisms that are most effective in the fermentation process so that starter cultures for *gari* and other cassava products can be developed.

1.3 Aim of study

To determine and compare the degrading capability of cyanide by some microbial fermenters of cassava.

1.4 Specific objectives

(1) To isolate and characterize some microbial cassava fermenters.

(2)To determine which of the microbial fermenters of cassava degrade cyanide.

(3)To prepare starter cultures for further *gari*fermentations.

(4) To prepare *gari* in the laboratory using the isolates.

(5) To carry out sensory evaluation of the prepared laboratory *gari*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical review of cyanide poisoning

The recognition of cyanide as a poison in bitter almonds, cherry laurel leaves and cassava goes back to antiquity. Dioscoride in the first century A.D. was aware of the poisonous properties of bitter almonds (Sykes, 1981).

The first description of cyanide poisoning was by Wepter in 1679 and dealt with the effects of the administration of extract of bitter almonds (Sykes, 1981). Two fatal cases of poisoning in Ireland caused by cherry laurel water used as a flavouring agent in cooking and to dilute brandy led to the experiment of Madden in 1731 (Meredithand Jacobsen, 2010). He showed that cherry laurel water contained a poison; given orally, into the rectum or by injection, it rapidly killed dogs (Meredith and Jacobsen, 2010). The first isolation of pure hydrogen cyanide (HCN) was done in 1786 (Scheele, 1981).

2.2Forms of cyanide

2.2.1 Free Cyanide (CN_F)

Only hydrogen cyanide and the cyanide ion (CN-) in solution can be classified as ‘free cyanide’. The proportions of HCN and CN- in solution is a function oftheir equilibrium equation which is influenced by the solution pH (**International Cyanide Management Institute ICMI, 2012).**

Methods used to detect free cyanide should not alter the stability of weaker cyanide complexes, as they may otherwise be included in the free cyanide result. Methods used to detect

free cyanide should be clear of interferences due to the presence of high concentrations of more stable cyanide complexes or other cyanide forms. If not, the interference must be quantified and allowed for in the result (ICMI, 2012).

2.2.2Weak Acid Dissociable Cyanide (CN_{WAD})

Unlike the definition of "free cyanide" which identifies the specific cyanide species being measured, WAD cyanide refers to those cyanide species measured by specific analytical techniques. WAD cyanide include those cyanide species liberated at moderate pH of 4.5 such as aqueous hydrogen cyanide(**ICMI,2012).**

Methods used to measure WAD should be free from interferences due to the presence of high concentrations ofmore stable cyanide complexes or other cyanide forms. If not, the interference must be quantified and allowed for in the result (ICMI, 2012).

2.2.3Total Cyanide (CN_T)

This measurement of cyanide includes all free cyanide, all dissociable cyanide complexes and all strong metal cyanide including ferro-cyanide $Fe(CN)_6^{-4}$, ferri-cyanide $Fe(CN)_6^{-3}$ and portions of hexacyanocobaltate $Co(CN)_6^{-3}$ and those of gold and platinum. Only the related or derived compounds cyanate (CNO^-) and thiocyanate (SCN^-) are excluded from the definition of total cyanide (ICMI, 2012).

Methods used to determine total cyanide must be shown to be capable of quantitatively determining all stable complexes of cyanide, including the cobalt cyanide complex. If

thesemethodsdetermine otheranalytes as well, including (SCN-), those analytes need to be determined separately and allowed for in the total result (ICMI, 2012).

2.3 The mechanism of cyanide toxicity

Cyanide has specific affinity for the ferric ions that occur in the cytochrome oxidase, the terminal oxidative respiratory enzyme in the mitochondria. This enzyme is an essential catalyst for tissue utilization of oxygen. When cytochrome oxidase is inhibited by cyanide, histotoxic anoxia occurs as anaerobic metabolism becomes inhibited. In massive cyanide poisoning, the mechanism of toxicity is more complex. It is possible that autonomic shock from the release of biogenic amines plays a role by causing cardiac failure (Burrows and Way, 1993). Cyanide could cause both pulmonary arteriolar and or coronary arterial vasoconstriction, which would result either directly or indirectly in pump failure and a decrease in cardiac output (Vick and Froelich, 1993). This theory is supported by the sharp increase in central venous pressure that was observed by Vick and Froelich at the time when the arterial blood pressure fell after the administration of sodium cyanide to dogs (Meredith and Jacobsen, 2010). Inhalation of amyl nitrate, a potent arteriolar vasodilating agent, resulted in the survival of dogs in these experimental circumstances. This could have been due to reversal of early cyanide-induced vasoconstriction with restoration of normal cardiac function (Vick and Froelich, 1993).

2.4 Detoxification of cyanide

2.4.1 Biological detoxification of cyanide

The major pathway of endogenous detoxification is conversion, by means of thiosulfate to thiocyanate. Major routes of elimination are excretion of hydrogen cyanide through the lungs and binding to cysteine or hydroxycobalamine (Meredith and Jacobsen, 2010).

2.4.2 Metabolite detoxification of cyanide

The detoxification of cyanide occurs slowly at the rate of 0.017mg/kg per minute (McNamara, 1976).

A sulphurtransferase enzyme is needed to catalyse the transfer of a sulphur atom from the donor thiosulfate to cyanide. The classical theory indicating that the mitochondrial thiosulphatesulphurtransferase is the most important enzyme in this reaction is now in doubt because thiosulfate penetrates lipid membranes slowly and would therefore not be readily available as a source of sulphur in cyanide poisoning. The modern concept assumes a greater role for the serum albumin-sulfate complex, which is the primary cyanide detoxification buffer operating in normal metabolism (Sylvester *et al*., 1983).

2.5Antidote to cyanide poisoning

2.5.1 Oxygen

It is difficult to understand how oxygen has a favourable effect in cyanide poisoning, because inhibition of cytochrome oxidase is non-competitive. However, oxygen has always been regarded as an important first aid measure in cyanide poisoning and there is now experimental evidence that oxygen has specific antidotal activity. Oxygen accelerates the reactivation of cytochrome oxidase and protects against cytochrome oxidase inhibition by cyanide (Takano *et al*., 1993).

Hyperbaric oxygen is recommended for smoke inhalation victims suffering from combined carbon monoxide and cyanide poisoning, since these two agents are synergistically toxic (Meredithand Jacobsen, 2010).

2.5.2 Sodium thiosulfate

The major route of cyanide detoxification in the body is conversion to thiocyanate by rhodanese,although other sulfurtransferases, such as beta-mercaptopyruvatesulfurtrasferase, may also be involved (Meredith and Jacobsen, 2010). This reaction requires a source of sulphur, but endogenous supply of this substance is limited. Cyanide poisoning is an intra-mitochondrial process and an intravenous supply of sulphur will only penetrate the mitochondria slowly (Meredith and Jacobsen, 2010). While sodium thiosulfate may be sufficient alone in mild to moderate cases, it should be administered with other antidotes in cases of severe poisoning. It is also the antidote of choice when the diagnosis of cyanide intoxication is not certain, for example, in cases of smoke inhalation (Meredith and Jacobsen, 2010).

2.5.3 Hydroxycobalamine (Vitamin B_{12})

This antidote binds strongly to form cyanocobalamine and compared to nitrite and 4-DMAP therapy, it has great advantage of not interfering with tissue oxygenation (Meredith and Jacobsen, 2010). The disadvantage of cyanocobalamine as a cyanide antidote is the large dose required for it to be effective. Detoxification of 1mol cyanide (corresponding to 65mg KCN) needs 1406mg hydroxycobalamine. In most countries, it is only commercially available in formulations of 1-2mg per ampoule (Meredith and Jacobsen, 2010). Some authors have reported a reduced antidotal effect as a result of mixing hydroxycobalamine with sodium thiosulfate in the same solution (Evans, 1964; Friedberg and Shukla, 1975). Histological changes in the liver, myocardium and kidney apparently induced by hydroxycobalamine have been reported in animal experiments (Hoebel*et al*., 1980), but their relevance to man has not yet been established (Meredith and Jacobsen, 2010).

2.5.4 Dicobaltedetate

This agent has been shown to be effective in the treatment of cyanide poisoning in man, and in the United Kingdom, it is the current treatment of choice provided that cyanide toxicity is definitely present (Meredith and Jacobsen, 2010). This is a strict criterion, because as a result of the manufacturing process, some free cobalt ions are always present in solutions of dicobaltedetate. Cobalt ions are toxic and the use of dicobaltedetate in the absence of cyanide, will lead to serious cobalt toxicity (Meredith and Jacobsen, 2010). There is evidence from animal experiments that glucose protects against cobalt toxicity and it is recommended that this be given at the same time as dicobaltedetate. Serious adverse effects recorded include vomiting, urticaria, anaphylactic shock, hypotension and ventriculararrhymiasis (Hilmann*et al*., 1974; Naughton, 1993).

2.6 Supportive treatment

Although effective antidotes are available, general supportive measures should not be ignored and may be life saving (Jacobs, 1984).According to Jacobs (1984), who reported his personal experience of 104 industrial poisoning cases, the use of specific antidotes was indicated only in severe intoxication with deep coma, wide non-reactive pupils and respiratory insufficiency in combination with circulatory insufficiency. In patients with moderately severe poisoning, who had suffered only a brief period of unconsciousness, convulsions, vomiting and cyanosis, therapy consisted of intensive care and intravenous sodium thiosulfate. In cases of mild intoxication with dizziness, nausea and drowsiness, rest and oxygen alone were used (Oslein, 1999).

Peden*et al*. (1996) described patients poisoned by hydrogen cyanide released by a leak from a valve. Three of them were briefly unconscious but recovered rapidly after being moved from the area where they had been working. The arterial whole-blood cyanide concentrations on admission were 3.5, 3.1 and 2.8mg/l respectively. The cyanide concentrations in the other cases ranged between 2.6 and 0.93mg/l. All recovered with supportive therapy alone (Meredith andJacobsen, 2010).

Between 1970 and 1984, three other men were treated similarly, two were transiently unconscious and in these cases, the cyanide concentrations after 30 minutes exposure were 7.7 and 4.7mg/l. The concentration in the other patient was 1.6mg/l. All three patients recovered without the use of cyanide antidotes (Meredith and Jacobsen,2010). Small numbers of comatose patients with potentially lethal blood concentrations on admission and who recovered withoutcyanide antidotes have been reported (Graham *et al*., 1993).

2.7Sampling for cyanide analysis

The results of analysis can be no better than the sample on which it was performed.While the taking of either aqueous or solid samples may appear easy, the collection of correct samples, both in terms of location and with respect to the analytes to be monitored, is fraught with difficulties. Any sampling must have as its aim the collection of a representative portion of the substance to be analysed. When this portion is presented for analysis, the parameters to be determined must be present in the same concentration and chemical or biological form as found in the original environment from which the portion was removed(ICMI, 2012).Samples representative of a site, or of a portion of a site, provide information that is often extrapolated to include the whole area under investigation. This is true whether the entity being sampled is a

contaminated section of land, surface water, an industrial outfall, or a drum containing waste material. Therefore, samples must be representative of the specific entity being sampled, but not necessarily representative of the entire area of which it is part(ICMI, 2012).

The overall objectives of a sampling program must be considered in the development of the sampling plan. Sampling may be performed for one of several purposes:Maximum, minimum and average values for a near steady state stream with the aim of monitoring compliance versus set specifications (process control, environmental criteria). Such data can illustrate the likelihood and magnitude of occurring non-compliance provided enough data points have been analyzed from samples. Process, residue, and effluent stream analysis could have this type of objective. Even aquifer sampling (bore-holes) would fit this description. Often the relative mass-flows have to be known for proper data integration(ICMI, 2012).

Maximum, minimum and average values derived from the analysis of "batch streams" such as treated backfill portions or detoxified waste batches usually require a minimum of one data point per batch to ensure a representative sample. The major objective remains one of compliance and/or verification of effective management procedures for the batch streams involved(ICMI, 2012).

2.8Analytical methods for cyanide assay

2.8.1 Qualitative methods

2.8.1.1 Detection in blood with a detector tube (bedside test)

The time required, assuming that instrumentation and chemicals are readily available, is 2-3 minutes (Meredith and Jacobsen,2010). Hydrocyanic acid is liberated from blood by acidification and is then passed through a detector tube using gas detector pump. If hydrocyanic

acid is present, the reactive zone of the tube changes its colour from yellow to red as a result of the following reaction:

i. HCN $\xrightarrow{HgCl_2}$ HCl

ii. HCl + Methyl red $\longrightarrow$ Red reaction product (Meredith and Jacobsen, 2010).

A semi-quantitative determination of the hydrogen cyanide concentration can be made by means of a scale on the tube (Clark and Lambertsen, 1993).

2.8.1.2 Spot test

The time required, assuming that instrumentation and reagents are readily available is 2-3 minutes (Meredith and Jacbsen, 2010).Hydrogen cyanide is liberated from biological fluids by acidification. The evolved HCN is passed through a filter paper impregnated with alkaline and solution of pallidiumdimethylglyoxime in which pallidium is a constituent of an inner-sphere complex anion. Cyanide ions lead to a demasking of dimethylglyoxime, which reacts with nickel (II) to produce red nickel-dimethylglyoxime (Jacobs, 1984).

2.8.2Quantitative methods

2.8.2.1Silver nitrate titration method

The preferred method for the analytical determination of free cyanide is silver nitrate titration. Silver ions are added to the solution to complex the free cyanide ions. When all free cyanide is consumed as silver cyanide complex, the excess silver ions indicate the end-point of the titration. The analytical equipment used for the titration is rather simple. To accurately determine the cyanide concentration, a normalized silver nitrate solution is dosed with a manual

or automatic burette, which should be capable of measuring volumes to an accuracy of better than 0.005 ml (ICMI, 2012).

Several techniques can be used for the end-point determination. The easiest possibility is to use an indicator such as potassium iodide or rhodamine that changes colour upon appearance of free silver ions. It is important that the first colour change is used as end-point indication because the silver ions tend to liberate cyanide ions from other complexes, which leads to the disappearance of the colour. The potentiometric endpoint detection is a more accurate way to determine the endpoint as a more easily identified peak signal is produced (ICMI, 2012).

2.8.2.2 Distillation method

The preferred analytical method to determine weak acid dissociable cyanide and total cyanide is the distillation method according to International Standardization Organization(ISO). This method creates a chemical condition which allows the CN_{WAD} to be liberated as dissolved hydrogen cyanide gas which is then carried in an air stream to caustic soda absorption where the CN_{WAD} appears as CN_F (ICMI, 2012). As the hydrogen cyanide is adsorbed in a much smaller volume than the original sample solution, the CN_F concentration to be analyzed is typically at least 10 times higher than the original CN_{WAD} concentration in the sample solution. The CN_F concentration in the distillation product sample is then determined using silver nitrate titration as described above. The method is similar to that of ASTM. However, the results from ISO method are more accurate than those from the ASTM method for samples containing a high concentration of copper cyanide (ICMI, 2012).

2.9 History of cassava

Wild populations of *Manihotesculenta*, subspecies *flabillifolia* shown to be the progenitor of domesticated cassava are centred in West-Central Brazil, where it was first likely domesticated more than 10,000 years (Oslen and Schaal, 1999). By 6,600 BC, manioc pollen appears in the Gulf of Mexico lowlands, at the San Adre's archaeological site (Pope *et al*., 1999). The oldest direct evidence of cassava cultivation comes from 91,400-year old Maya site in East Salvador, but the species *Manihotesculenta* likely originated further south in Brazil and Paraguay. With its high food potential, it had become a staple food of the native populations of Northern South America, Southern Meso-America and the Caribbean by the time of the Spanish conquest and its cultivation was continued by the colonial Portuguese and Spanish (Pope *et al.*, 1999). Forms of the modern domesticated species can be found growing in the wild in the South of Brazil. While several *Manihot*speciesare wild, all varieties of *Manihotesculenta* are cultigens (Pope *et al*., 1999).

Cassava was a staple food for pre-Columbian people in the Americas and was often portrayed in indigenous art. The Moche people often depicted *yucca* in their ceramics (Berrin, 1997).

Cassava is one of the most important staple food crops grown in tropical Africa. It plays a major role in efforts to alleviate the African food crises because of its efficient production of food energy, year round availability and suitability to present farming and food systems in Africa (Hahn *et al*., 2012).

Since being introduced by Portuguese traders from Brazil in the 16th century, cassava and maize have replaced traditional food crops as the continents' most important staple food crops

(FAO, 1990). Cassava is sometimes described as the bread of the tropics (Adams *et al*., 2009), but should not be confused with the Tropical and Equatorial bread tree.

2.10 Description of cassava roots

The cassava root is long and tapered, with a firm, homogenous flesh encased in a detachable rind, about 1mm thick, rough and brown on the outside (Ravindran, 1992). Commercial varieties can be 5 to 10cm in diameter at the top and around 15cm to 30cm long. A woody cordon runs along the root axis, the flesh can be chalk-white or yellowish. Cassava roots are very rich in starch, and contain significant amount of calcium (50mg/100g), phosphorus (40mg/100g) and vitamin C (25mg/100g). However, they are poor in protein and other nutrients. In contrast cassava leaves are good sources of protein (rich in lysine), but deficient in the amino acid methionine and possibly tryptophan (Ravindran, 1992).

2.11 Economic importance of cassava

World production of cassava root was estimated to be 184million tonnes in 2002, rising to 230 million tonnes in 2008 (FAO, 2012). The majority of production in 2002 was in Africa, where 99.1 million tonnes were grown in Asia and 33.2 million tonnes in Latin America and the Caribbean. Nigeria is the world largest producer of cassava(FAO, 1990). However, based on the statistics from the Food and Agricultural Organisation of the United Nations, Thailand is the largest exporting country of dried cassava, with a total of 77% of world export in 2005. The second largest exporting country is Vietnam, with 13.6%, followed by Indonesia (5.8%) and Costa Rica (2.1 %) (Fredrick and Hog, 2008).

Cassava, yams and sweet potatoes are important food sources in the tropics. Cassava plant gives the highest yield of carbohydrates per cultivated area among crop plants, except for

sugarcane and beets (FAO, 2012). Cassava plays a particularly important role in agriculture in developing countries, especially in sub-Saharan Africa, because it does well on poor soils and with low rainfall and because it is a perennial that can be harvested as required. Its wide harvesting window allows it to act as famine reserve and is invaluable in managing labour schedules. It also offers flexibility to resource-poor farmers because it serves as either subsistence or cash crop (Stone, 2002).

No continent depends as much on root and tuber crops in feeding its population as does Africa. In the humid and sub-humid areas of tropical Africa, it is either a primary staple food or a secondary co-staple. In Ghana, for example, cassava and yams occupy an important position in the agricultural economy and contribute about 46% of the agricultural gross domestic product (FAO, 1990). Cassava accounts for a daily calorific intake of 30% in Ghana and is grown by nearly every farming family (Hahn*et al*., 2012). The importance of cassava in many African countries is epitomised in the *Ewe* (a language spoken in Ghana, Togo and Benin) which name the plant*agbeli*, meaning there is life (Hahn *et al.*, 2012). The price of cassava has risen significantly in the last half decade, and lower income people have turned to other carbohydrate-rich foods such as rice (Hahn*etal.*,2012).

2.12 Uses of cassava

2.12.1 Culinary use

Cassava based diets are widely consumed wherever the plant is cultivated; some have regional, national or ethnic importance. Cassava must be cooked properly to detoxify it before it is eaten (Fredrick and Hog, 2008).

Cassava can be cooked in various ways. The soft boiled root has a delicate flavour and can replace boiled potatoes in many uses: as an accompaniment for meat dishes or made into purees, dumpling, soups, stews, gravies, etc. (Fredrick and Hog, 2008). This plant is used in cholent in some households as well, deep fried (after boiling or steaming)it can replace fried potatoes, with a distinctive flavour (Fredrick and Hog, 2008). In Brazil, detoxified manioc is ground and cooked to a dry often hard crunchy meal which is used as a condiment, toasted in butter, or eaten alone as a side dish (Fredrick and Hog, 2008).

2.12.2 Fufu

Fufu is made in Africa by first pounding cassava in a mortar to make flour, which is then sifted before being put in hot water to become fufu (Fredrick and Hog, 2008).Fufu is made from the starchy cassava root flour. Tapioca or fecula, essentially a flavourless, starchy ingredient produced from treated and dried cassava (*manioc*)root, is used in cooking (Fredrick and Hog, 2008).

2.12.3Gari

Gari is a creamy-white, granules flour with a slightly sour fermented flavour from fermented, gelatinized fresh cassava tubers (Fredrick and Hog, 2008). Gari soaking is a delicacy in Ghana that cost less than US $1 (Fredrick and Hog, 2008). One can simply soak gari in cold water, add a bit of sugar and roasted groundnut (peanut) to taste and add whatever quantity of evaporated milk one desires (Fredrick and Hog, 2008). Gari soaking with coconut water tastes better.

2.12.4 Palaver

The leaves can be pounded into fine chaff and cooked as palaver sauce as is done in Liberia and Sierra Leone, usually with palm oil, but other vegetables can also be used (Fredrick and Hog, 2008). Palaver sauces contain meat and fish, as well. The leaf chaff must be washed several times to remove the bitterness.

2.12.5 Cassareep

The juice of bitter cassava, boiled to the consistency of thick soup and flavoured with spices is called cassareep. It is used as a basis for various sauces and as a culinary flavouring, principally in tropical countries (Ikpi*et al*., 1986). It is exported chiefly from Guyana, where it is started as a traditional recipe with its origin in Amerindian practices (Ikpi*et al*., 1986).

2.12.6 Beverages

Cassava was also used to make alcoholic beverages. The English explorer and naturalist, CharlesWaterton in1836 reported in Wandering, South America that the natives of Guyana used cassava to make liquor which they abandoned when *rum* became available (Charles, 2009).Hamilton Rice in 1913 also remarked on liquor being made from cassava in the Brazilian rain forest (Charles, 2009). The native tribes from all over Brazil, used made alcoholic beverages made from this native root (Charles, 2009). These beverages were known by many different names, being most well known as *Kasiri* and *Cauim*(Charles, 2009). In the 16th century, Jean de Lery published a book titled voyage to the land of Brazil, in which it has an account on how the Tupynambas used to make the beverage (Charles, 2009).

The Tiros and Erwarhoyunas, Indian tribes from Northern Brazil and Surinam, make a beverage called Sakura with the sweet manioc variety of cassava, *yucca* (Olumide, 2004). The same beverage is made by the Jivaro in Ecuador and Peru (the Shuara, Achuara, Aguaruna and Mayna people); they call it *Nijimanche* (Olumide, 2004).

The sweet manioc beer *Nijimanche* is prepared by first peeling and washing the tubers in the stream near the garden. Then the water and the *manioc* are brought to the house, where the tubers are cut up and put in a pot to boil. The *manioc* is then mashed and stirred to a soft consistency with the aid of a special wooden paddle (Olumide, 2004). While the woman stirs the mash, she chews handfuls of it and spits them back into the pot, a process that may take half an hour or longer (Olumide, 2004). After the mash has been prepared, it is transferred to a beer storage jar and left to ferment (Olumide, 2004). The resultant liquid taste somewhat like a pleasing alcoholic butter-milk and is most refreshing (Olumide, 2004).

2.12.7 Biofuel

Ethanol also called ethyl alcohol; can be used as fuel alcohol, drinking alcohol, and grain alcohol. The common type of ethanol is the one found in alcoholic beverages. It is also used as fuel for cars and often called alcohol or spirit (Agbogun, 2011).

Cassava tuber as a case study has in the past been an efficient source of ethanol in Western Nigeria, where foreign and local investors have the opportunity to invest in the production of ethanol even in large amount. Cassava, an edible starchy tuberous root, is grown in large quantities and well suited for the production of ethanol (Agbogun, 2011).

Cassava ethanol in Nigeria has been in existence since independence and apart from food and pharmaceutical uses, cassava ethanol plays a major role in renewable energy as in ethanol or

biodiesel. Cassava produces a better quality alcohol, and the distilling technique employed ensures a high quality product, free from all type of odors and not harmful to the environment(Agbogun, 2011).

Nigerian biofuel plant would bring in fresh cassava, wash and peel, grate, cook in a jet cooker, ferment, distil and bottled. In addition, a steam boiler, generating set, effluent treatment plant, and power source are required. The actual amount of cassava needed is dependent upon the starch content. As a guide, a tonne of cassava at 25 % starch will produce 230 litres of alcohol. A typical plant will produce approximately10,000litres/day at 40% (Agbogun, 2011). This plant will also produce around 2-3 tonnes of effluent. The effluent has to be disposed of properly and is normally used as animal feed. This alcohol can be collected by batch fermentation and can process approximately 4-6 batches/day. Producing 520 litres of alcohol/ batch (Agbogun, 2011).

Cassava has contributed immensely to the economy of the West African Nations, with the Nigerian state being a major beneficiary. Apart from acquisition of staple food for both man and animal, cassava also serves as a major raw material in ethanol production. Several hundreds oflitres of ethanol are produced on a daily basis in Western Nigeria, Oyo State (IITA, 2005).

2.13 Cassava processing

2.13.1 Need for cassava processing

Fresh cassava roots cannot be stored for long because they rot within 3-4 days of harvest (Hahn*et al*., 2012). They are bulky with about 70% moisture content and therefore transportation of the tubers to urban market is difficult and expensive. The roots and leaves contain varying amount of cyanide which is toxic to humans and animals, while the raw cassava roots and uncooked leaves are not palatable (Hahn*et al.,* 2012). Therefore cassava must be processed into

various forms in order to increase the shelf life of the products, facilitate transportation and marketing, reduce cyanide content and improve palatability (Hahn*et al.*, 2012). The nutritional status of cassava can also be improved through fortification with other protein rich crops (Hahn*et al.*, 2012). Processing reduces food losses and stabilizes seasonal fluctuations in the supply of the crop (Hahn*et al.*, 2012).

2.13.2 Processing techniques and reduction of cyanide in cassava

Cassava processing procedures vary, depending on products, from sample processing (peel, boil and eat) to complicated procedures for processing into *gari*, for example, which involves more steps namely, peeling, grating, pressing, fermenting, sifting and roasting. Some of these steps reduce cyanide more effectively than others (Hahn*et al*., 2012).

Processing techniques and procedures differ with countries and localities within a country according to food cultures, environmental factors such as availability of water and fuel-wood, the cassava variety used and the types of processing equipment and technologies available (Hahn*et al.*, 2012).

2.13.2.1 Fermentation

Fermentation consists of two distinct methods: aerobic and anaerobic fermentation (Hahn*et al.*, 2012). For aerobic fermentation, the peeled and sliced cassava roots are first surface-dried for 1-2 hours and then heaped together, covered with straw or leaves and left to ferment in air for 3 to 4 days until the pieces become mouldy. The fermented mouldy pieces are sundried after the mould has been scraped off. The processed and dried pieces (called *Mokopa*in Uganda) are then milled into flour, which is prepared for meal as *kowan* in Uganda (Hahn*et al.*, 2012).

The growth of mould on the root pieces increases the protein content of the final product three to eight times (FAO, 1990).

In anaerobic fermentation, grated cassava for processing into*gari* is placed in sacks and pressed with stones or a jack between wooden platforms (Odunfa, 1985). Whole roots or pieces or roots for processing into fufu are placed in water for3-5 days (Odunfa, 1985). During the first stage of *gari*production, bacteria attack the starch of the roots, leading to the production of various organic acids (such as lactic and formic acid) and the lowering of substrate pH (Odunfa, 1985). In the second stage, the acid condition stimulate the growth of a mould, which proliferate rapidly, causing further acidification and production of a series of aldehydes in*gari* (Odunfa, 1985). The optimum temperature for fermentation for *gari* processing is 35^0C, increasing up to 45^0C (Odunfa, 1985).

For *lafun* production in Nigeria, peeled or unpeeled cassava tubers are immersed in stationary water (near a stream) or in an earthen ware vessel and fermented until the roots become soft (Odunfa, 1985). The peeled and central fibres of the fermented roots are manually removed and the recovered pulp is hand washed or pounded (Odunfa, 1985).

2.13.2.2 Dewatering the fermented cassava

During or after fermentation, the grated pulp is put is sacks (jute or polypropylene) on which stones are placed or jacked-wood platforms are set to drain or press off the excess liquid from the pulp (Hahn *et al*., 2012). In Zaire, the cassava pulp is heaped up on the racks in the sun for further fermentation and draining of the excess moisture. In this way, much of the cyanide is effectively lost with the liquid (Hahn*et al.*, 2012).

2.13.2.3 Tissue disintegration

Tissue disintegration in the presence of excess moisture during grating or fermentation in water permits the rapid hydrolysis of glycosides, effectively reducing both free and residual cyanide in the products. Fermentation in water appears a more efficient method for reducing the cyanide content of roots. For example, this process reduces cyanide by 70-95 percent of the original level after the roots were soaked in water for 3 days (Hahn *et al*., 2012). Gari obtained through the processing procedures involving grating and or fermentation showed 80-90 percent reduction in total cyanide content relative to freshly peeled roots (Mahungu*et al*., 1987). Oke (1968) reported HCN content of 1.9mg/100g for *gari*, 2.5mg/100g for *fufu*(Nigeria) and 1.0mg/100g forlafun (Nigeria). HCN concentration in 200*gari* samples collected across cassava growing areas of Nigeria had 0.0-3.2mg/100g with a mean of 0.6mg/100g (Oke, 1968). Akinrele*et al*. (2009) stated that 0.3mgHCN/100 g is an acceptable level in *gari*. When *gari* is prepared into *eba*, HCN is further reduced to even safer levels (Mahungu*et al*., 1987).

2.13.2.3 Drying

Drying is the simplest method of processing cassava. Drying reduces moisture, volume and cyanide content of roots, thereby prolonging product shelf life. The processing is practiced primarily in areas with less supply of water (Hahn*et al*., 2012).

Total cyanide content of cassava chips could be decreased by only 10-30 percent through fast air drying. Slow sun-drying however, produces greater loss of cyanide. Sun-drying the peeled cut pieces of roots gave a HCN concentration lower than 10mg/100g and was more effective than oven drying (Mahungu*et al*., 1987). Drying may be in the sun or over a fire. The

former is more common because it is simple and does not require fuel-wood (Mahungu*et al*., 1987).

2.13.2.4 Boiling

Boiling the peeled roots did not effectively remove HCN. Pounding the boiled roots into pounded *fufu* decreased HCN concentration by only 10 percent. Therefore, only cultivars containing low cyanide are recommended for this method of preparation (Mahungu*et al*., 1987).

2.13.2.5Milling

The dried root pieces and fermented dried pulp are milled into flour by pounding in mortar or using hammer mills. Milling with hammer mills, done at village level, may also reduce cyanide (Mahungu*et al.,* 1987). The dried cassava roots (both fermented and unfermented) are often mixed in a ratio of 2-3 parts cassava with 1 part of sorghum, millet and or maize and milled into composite flour. Mixing cassava with cereals increases food protein and enhances palatability by improving consistency (Mahungu*et al*., 1987).

2.14 Microbial cassava fermenters

The bacterial population of fermenting cassava is essentially constituted of lactic acid bacteria, notably *Lactobacillus* (73.3%), including *Lactobacilluscoprophillus*(53.3%), *Lactobacillus fermentum*(6.7%), *Lactobacillusdelbrueckii* (13 %). The remainder is made up mainly of cocci (26.7 %), of which *Lactococcuslactis*(6.7%), *Leuconostocmesenteroides* (13.3%) and *Leuconostoclactis* (6.7%) are prominent (Kobawila*et al*., 2005).

Some of the lactic acid bacteria such as *L. coprophilus,L. delbrueckii,L. fermentum, Lactobacillus plantarum, L. mesenteroides* and *L. lactis* have the capacity to resist strong concentration of free cyanide from 200 to 800ppm (Kobawila*et al*., 2005).

Microorganisms associated with *gari,* produced in Cameroun, Africa, by the traditional method and that produced in the laboratory, had been isolated, identified and screened for their abilities to produce *gari*flavor. *Gari* production, under the controlled conditions was attempted utilizing these isolates and the dairy starter cultures. Some of the volatiles responsible for the *gari*flavour were isolated by gas chromatography employing the head-space analysis technique and tentatively identified by mass spectral analysis (Ngabaand Lee,2011).

Cassava fermentation involves mainly the lactic acid fermentation. *Lactobacillus* spp, and to a less extent, *Streptococcus* spp were responsible for the acid production and gariflavor development during this fermentation. A starter culture that consisted of *Lactobacillus plantarum* or a combination of *L. plantarum* and *Streptococcus* sp were suitable for use in gari production. *L. acidophilus* 3532, a widely used dairy starter culture, was as effective as *L. plantarum* in producing acid but it contributed a foreign (dairy) flavour to the gari. Yeasts were not found to play a major role in gari production and the moulds were detrimental (Ngaba and Lee, 2011).

2.14.1 Lactic acid bacteria (order *Lactobacillales*)

Many members of the order *Lactobacillales* produce lactic acid as their major or sole fermentation product and are sometimes collectively called lactic acid bacteria, *LAB* (Prescott *et al*., 2008). Lactic acid bacteria are diverse bacterial group consisting of 11 genera. These bacteria are Gram-positive, non-spore forming cocci or rods which produce lactic acid as their main metabolic product (Prescott*et al*., 2008). *Streptococcus,Enterococcus, Lactococcus,*

Lactobacillus and *Leuconostoc* are all members of the group (Prescott *et al*., 2008). They normally depend on sugar fermentation for energy. They lack cytochromes and obtain energy by substrate-level-phosphorylation rather than by electron transport and oxidative phosphorylation. Nutritionally, they are fastidious and many vitamins, amino acids, purines and pyrimidines must be supplied because of their limited biosynthetic capabilities (Prescott *et al*., 2008).

Lactic acid bacteria usually are categorized as facultative anaerobes, but some are classified asaerotolerant anaerobes (Prescott *et al*., 2008).

2.14.1.1*Lactobacillus*

The largest genus in the order *Lactobacillales* is *Lactobacillus* with around 100species. *Lactobacillus* contains non-sporing rods and sometimes cocco-bacilli that lack catalase and cytochromes, are usually facultative anaerobes or microaerophilic, produce lactic acid as their main or sole fermentation product and have complex nutritional requirement (Prescott *et al*., 2008). *Lactobacilli* carry out either a homolactic fermentation using the Embden-Meyerhof pathway orheterolactic fermentation with the pentose phosphate pathway. They grow optimally under slightly acidic condition, when the pH is between 4.5 - 6.4. The genus is found on plant surfaces and in dairy products, meats, water, sewage, beer, fruits and many other materials. *Lactobacilli* also are part of normal flora of the human body, in the mouth, intestinal tract and vagina. They are usually not pathogenic (Prescott *et al*., 2008).

2.14.1.2*Leuconostoc*

Family Leuconostocaceae, contains facultative Gram positive cocci, which may be elongated or elliptical and arranged in pairs or chains (Prescott *et al*., 2008). *Leuconostocs* lack catalase and cytochromes and carry out heterolactic fermentation by converting glucose to D-

lactate and ethanol or acetic acid by means of the phosphoketolase pathway (Prescott *et al*., 2008). They can be isolated from plants, sillage and milk. *Leuconostocmesenteroides*synthesizes dextrans from sucrose and is important in industrial dextran production. *Leuconostoc* species are involved in food spoilage and tolerates high sugar concentration so well that they grow in syrup and are major problems in sugar refineries (Prescott *et al*., 2008).

2.14.1.3 Role of lactic acid bacteria in food industry

Lactic acid bacteria are important in food and dairy industries because the lactic acid and other organic acids produced by these bacteria act as natural preservatives as well as flavour enhancers (Harrigan and MacCence, 1976). Lactic acid bacteria find increasing acceptance as probiotics which aid a stimulating immune responses, preventing infections by enteropathogenic bacteria and treating and preventing diarrhoea. Lactic acid bacteria have a long history as GRAS (generally regarded as safe) organisms and especially members of the genus *Lactobacillus, Lactococcus*and *Streptococcus* are widely used in fermentation industry (Harrigan and MacCence, 1976).

Lactobacillus is indispensable to the food and dairy industry. *Lactobacilli* are used in the production of fermented vegetable foods (sauerkraut, pickles, silage), beverages (beer, yoghurt, sausage (Prescott *et al*., 2008).

2.14.2*Corynebacteriummanihot*

A species of *Corynebacterium* not described in the 7th edition of *Bergey's Manual* has been isolated from grated cassava (*Manihotutilissima*) root allowed to ferment for some days during the preparation of gari, a farinaceous food eaten in Nigeria. The organism ferments starch, dextrose, maltose, sucrose, salicin, xylose and arabinose with the production of acid only, and

produces bright yellow colonies of a characteristic form on litmus-lactose agar. It has been suggested that this organism, if accorded specific status, should be named *Corynebacteriummanihot (Collard, 2008).*

2.14.3 Yeasts and moulds

Aspergillusniger*, A. tamari, Geotricumcandidum*and*Penicilliumexpansum* had been isolated and identified from cassava waste water while standard analytical methods had been used to determine the ability of the isolates to produce linamarase and the **proximate composition**, pH and titrable acidity of the fermenting mash(Ihaotu*et al,* 2011).

2.15 Food fermentation

Together with drying and salting, fermentation is one of the oldest methods of food preservation. Its importance in modern-day life is underlined by the wide spectrum of fermented foods marketed both in developing and industrialised countries, not only for the benefit of preservation and safety, but also for their highly appreciated sensory attributes. Fermented foods are treasured as major dietary constituents in numerous developing countries primarily because of their keeping quality under ambient conditions, and also for their safety and traditional acceptability (Holzapfel, 1997).

As a technology, food fermentation dates back at least 6000 years and probably originated from microbial interactions of an acceptable nature. Fermentation has enabled our ancestors in temperate and cooler regions to survive winter season and those in the tropics to survive drought periods, by improving the shelf life and safety of foods (Holzapfel, 1997).

Through the ages, fermentation has had a major impact on nutritional habits and traditions, on culture and on the commercial distribution and storage of food. Traditional fermentation process

still serves as a substitute where refrigeration or other means are not available for the safe keeping of food. Fermented foods can, in general, be described as palatable and wholesome foods prepared from raw or heated raw materials. They are generally appreciated for attributes such as pleasant flavour, aroma, texture and improved cooking and processing properties. Microorganisms, by virtue of their metabolic activities, contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life and safety. Enzymes indigenous to the raw materials may play a role in enhancing these characteristics (Hammes, 1990). Through trial and error, traditional skills have been developed for controlling technical parameters during fermentation processes.

Experience has also shown that back sloping, or the inoculation of raw materials with a residue from a previous batch, accelerates the initial phase of fermentation and results in the promotion of desirable changes during the fermentation process (Hammes, 1990).

2.15.1 Concept of starter culture

A starter culture may be defined as a preparation or material containing large numbers of variable microorganisms, which may be added to accelerate a fermentation process. Being adapted to the substrate, a typical starter facilitates improved control of a fermentation process and predictability of its products (Holzapfel, 1997). In addition, starter cultures facilitate control over the initial phase of a fermentation process (Holzapfel, 1997).

2.15.2 Traditional approaches: spontaneous fermentation and back-sloping

Modern starter cultures are selected either as single or multiple strains, specifically for their adaptation to a substrate or raw material (Holzapfel, 1997). Spontaneous fermentations i.e. processes initiated without the use of a starter inoculum have been applied in food preservation for millennia and were elucidated through trial and error, perhaps over thousands of years

(Holzapfel, 1997). The majority of small-scale fermentations in developing countries and even some industrial processes such as sauerkraut fermentations are still conducted as spontaneous processes. Various types of starter cultures and even back-sloping are widely used in fermentation processes,even in industrialised countries.

Spontaneous fermentations typically result from the competitive activities of a variety of contaminating microorganisms (Holzapfel, 1997). Those best adapted to the food substrate and to technical control parameters, eventually dominate the process. The production of metabolites (e.g. organic acids) inhibitory to other contaminating microbes (e.g. Enterobacteriaceae) may provide an additional advantage during fermentation (Holzapfel,1997).

Bacteria typically dominate the early stages of fermentation processes, owing to their relatively high growth rate, followed by yeasts, in substrates that are rich in fermentable sugars (Holzapfel, 1997). In numerous traditional processes, material from a previous successful batch is added to facilitate the initiation of a new process. Through this practice of back-sloping, the initial phase of the fermentation process is shortened and the risk of fermentation failure reduced. Repeated use of back-slopping results in selection of the best-adapted strains, some of which may possess features that are desirable for use as starter cultures (Holzapfel,1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample collection

Fresh leaf was collectedfrom the cassava plant whose root was used for the researchand taken for identification in the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria and was identified as*Manihotesculentacrantz* with the variety number, 2741.

3.2 Preparation of substrate for isolation of microbial cassava fermenters

One kilogram of cassava root was obtained, peeled, washed, sliced into fragments and fermented in sterile distilled water in an air-tight plastic container for two days to get the inoculum.

3.3 Isolation of Lactic acid bacteria

Serial dilution of the order, 10^{-1}-10^{-3} was made from the inoculum and 0.1 ml of each of the dilutions was seeded in MRS(de Mann Rogosa and Sharpe) agar and incubated at 30^{o}c for 48 hours. Distinct colonies were sub-cultured on individual plates of MRS agar and subsequently preserved on slants of the same medium for identification.

3.3.1 Biochemical tests for confirmation of Lactic acid bacteria

3.3.1.1 Catalase test

A drop of 6 % hydrogen peroxide solution was placed on the glass slide and a loop full of the bacterial growth was placed in the solution. The solution was stirred using a sterilewire loop and observed for reaction.

3.3.1.2 Gram's stain

A thin smear of the bacterial growth was made on glass slide and heat fixed. It was stained with crystal violet and then washed with water without blotting and covered with Gram's iodine. It was decolorized for 10-30 seconds with gentle agitation in 30 % acetone solution, washed with water and stained with safranin for 10-30 seconds. It was washed and allowed to dry.

3.3.1.3 Biochemical characterization of lactic acid bacteria

The bacteria were characterized to species level using the API 50 CH kits following the manufacturer's instruction.The test is a standardized system associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The API 50 CHL medium rehydrates the substrates. During incubation, fermentation is being revealed by a colour change from purple to yellow in the tube by anaerobic production of acid indicated by pH indicator in the tube (Appendix III). The first tube does not contain any active ingredient and is used as negative control.

Pure isolates of lactic acid bacteria were grown on MRS (de Mann, Rogosa and Sharpe) agar and harvested after 48 hours. The bacterial cells were prepared in API 50CHL medium and

used to inoculate the test strips immediately. The strips were incubated anaerobically in their incubation trays at 30^{oc} and read after 48 hours. The biochemical profile was referred to the api*web*^{TM}identification software with data base (V5.1) for strains identification.

3.4 Isolation and identification of moulds

Serial dilution of the inoculum was prepared and 10^{-1}-10^{-3} diluentwas inoculated on separate plates of Malt Extract Agar (MEA) for isolation of moulds. Isolates were purified and stored on slants of same medium for use.

3.4.1 Microscopic characterization of moulds

Moulds were characterized using 40 x objective of the light microscope using the fungal atlas of Burnet (1972). During microscopy, key features such as conidiophores, conidia, phialides and hyphae were looked out for and compared with those of standard strains on fungi atlas.

3.5 Fermentation of cassava pulp using pure culture of microbial isolates

Cassava roots were peeled, sliced and washed then50 g of the sliced cassava pulp was weighed and placed ineach of nine volumetric flasks of 250 ml volume and 100 ml of sterile distilled water was added into each flask to submerge the substrateto screen for cyanide degrading capability for each of the seven strains of microbial isolates including the consortium and the control. The consortium was made to consist of microorganisms from the seven isolates while the control experiment had no isolate inoculated into the substrate.Five millilitre (5ml) of inoculum size of 6.0 x 10^8colony forming unit(CFU) /ml for the bacteria strains and 6.0 x 10^8spores per millitrefor the moulds strains was introduced into each fermenter accordingly with

each coveredwith cotton wool immediately inoculation was made andallowed to ferment for 4 days.Each day, 5 g of the fermenting substrate (cassava pulp) was taken and analysed for cyanide level.McFarland turbidity standard and haemocytometer were used for the determination of inoculum size of bacteria and mould respectivelyas follows:

3.5.1 Preparation of bacterial inoculum size for fermentation

The bacterial inoculum size for fermentation of cassava pulp was prepared using McFarland turbidity standards, McFarland scale number two (Appendix V)

3.5.1.1 Preparation of McFarland turbidity standards

The McFarland turbidity standards were prepared using the method ofMicrobioLab(2010).This method involves preparing 1 % solution of anhydrous $BaCl_2$ and 1 % solution of H_2SO_4, mixed in proportion (Appendix V),tightly sealed in sets of tubes and stored at room temperature in the dark.

3.5.2 Preparation of mould inoculum size for fermentation

The inoculum size of mould was prepared using the spore count method with the haemocytometer.

3.5.2.1 Spore count using haemocytometer

Mould spore count was carried out using the method of Abcam (2012) as follows:

Preparation of normal saline buffer: The preparation of normal saline buffer involved preparation of 2.5 % tween 80 of the physiological saline. This was obtained by making a

solution of 5 ml of tween 80 in 195 ml of normal saline and shaken to mix. It was dispensed in bottles and sterilized by autoclaving.

Preparation of haemocytometer: The haemocytometer was cleaned using 70 % ethanol, the shoulders were moistened, and cover slip was affixed using gentle pressure and small circular motions. The cell (spore) suspension to be counted was well mixed by gentle agitation of the bottles containing the cells (spores).Onemillitre(1ml) of cell suspension was taken using a serological pipette and placed in an Eppendorf tube. One hundredmicrolitre (100µl)pipette was used to mix the cells in the tube and shaken vigorously to ensure spore suspension.

Gilson pipette was used to draw up some spore suspension carefully and used to fill the haemocytometer by gently resting the end of the Gilson tip at the edge of the chambers. Care was taken not to overfill the chamber. The sample was allowed to be drawn out of the pipette by capillary action, as the fluid ran to the edges of the grooves only. The pipette was re-loaded and used to fill the second chamber of thehaemocytometer. The grid lines of the haemocytometer was focused using the 10 x objective of the microscope, focusing on one set of the four 16 corner square as indicated in the circle (Appendix IV).

The counting was done according to the method of Abcam (2012). The spores in a set of the16 corner squares were counted. Cells that were counted included those within the square and any position on the right hand or bottom boundary line. This was repeated for the other three set of squares.

The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 / ml (Abcam, 2012).To obtain the count, the

total count from 4 sets of 16 corner squares = (cells / ml $x10^4$) x 4 squares from one haemocytometer grid (MicrobioLab,2010).

3.6. Determination of cyanogenic glycoside (HCN)

The determination of cyanogenic glycoside was carried out using alkaline picrate method of Onwuka (2005) as follows:

3.6.1 Cyanide extraction

Five grams each of ground cassava pulp sample was weighed and dissolved in 50 ml of sterile distilled water in corkedErlenmeyerflask. It was allowed to stay overnight for cyanide extraction and then filtered with grade 1Whatman filter paper.

3.6.2 Preparation of cyanide standard curve and cyanide analysis

Different standard concentrations of potassium cyanide (KCN) solution of 0.1 to 1.0 mg/ml were prepared.

To 1 ml of the sample filtrate and standard cyanide solutions in test tubes, 4 ml of alkaline picrate solution (1 g of picrate and 5 g Na_2CO_3 in warm 200 ml distilled water) was added and incubated in a water bath for 15 minutes. After colour development, the absorbance was taken at 490 nm against a blank containing only 1 ml distilled water and 4 ml alkaline picrate solution.

The cyanide content was extrapolated from the cyanide standard curve using the formula:

Cyanogenic glycoside (mg/100g) = C (mg) / (weight of sample) × 100; Where (C) is concentration of cyanide from standard curve.The percentage cyanide degrading efficiency of the

isolates (microbial cassava fermenters) was determined using the formula: Percentage cyanide degrading efficiency = (Initial cyanide content – Residual cyanide)/ Initial cyanide content x 100.

3.7 Cassava processing into gari using starterculture (pure isolate of *L. Plantarum1*)

Gari was prepared using the method of IITA (2005), with some modifications. Mature cassava roots were harvested and those without rots were selected and peeled by hands. The woody tips were removed and the pulps were washed to remove pieces of peels and sand. They were grated using cassava hand grater and5Kg of grated cassava pulp was weighed in two parts.Isolate with the highest cyanide degrading capability*Lactobacillus plantarum 1*,was inoculated into one of the two 5Kg of grated cassava pulp as starter culture while the other was left without inoculation.They were poured into separate jute bags and labelled as sample O and sample W, with sample O with starter culture and sample W without starter culture respectively. A heavy stone was placed on each of them to press and they were both allowed to ferment for four days under the same conditions. They were sieved and roasted in a shallow cast-iron over fire, with constant stirring with a piece of broken calabash for 20-30 minutes. They were thereafter cooled to room temperature and packaged in nylon bags for sensory evaluation.

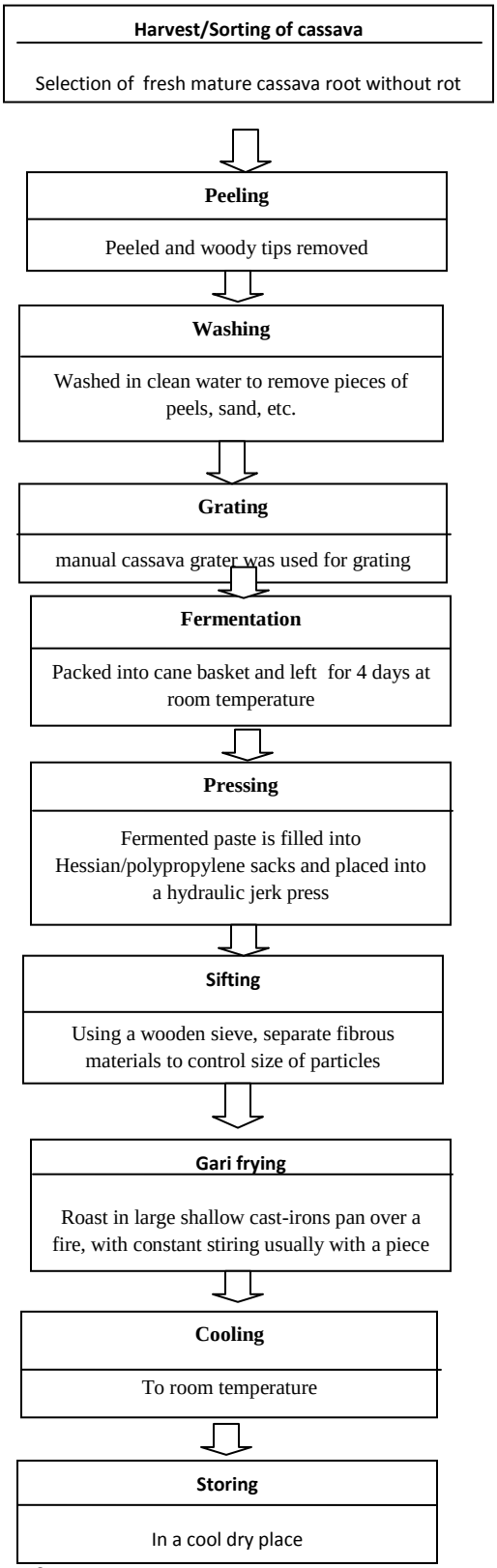


Figure 3.1: Flow Chart for Gari Production (IITA, 2005).

3.8 Sensory evaluation of laboratory gari

Sensory evaluation of the twogari samples was carried out by the method ofOnyesom and Okpokunu(2008) which involved sevenpanelsof judges according toHedonic scaleusing appropriate questionnaires (Appendix VI). They were assessed and rated on five attributes namely: appearance, aroma, taste, flavour and general acceptability, using a five point scale from a score of 5.0 very desirable to 1.0.

3.9 Statistical analysis

Statistical Programme for Social Science, version 17.0 (SPSS V 17.0) for windows (SPSS Inc., Chicago, IL, USA) was used for the statistiscal analysis of the data. One-way analysis of variance (ANOVA) was used to compare the relative degrading capability of the seven isolatesincluding the consortium and the control at 95 % confidence interval while student's t – test was used to analyse the result of the sensory evaluation at P = 0.05.

CHAPTER FOUR

4.0 RESULTS

Lactic acid bacteria namely*Weissellaconfusa, Lactobacillus brevis1*, *Lactobacillusfermentum 1*, *Lactobacillusplantarum 1* and mould namely*Aspergillusniger*, *Fusarium*sp and *Trichoderma*sp were isolated from fermenting cassava pulp and screened forcyanide degrading capability.

Table 4.1shows the morphological and biochemical properties of bacteria isolates. It shows that all the isolates were Gram positive, catalase negative and formed no spores.The result indicated that although they did not have same morphological features but had same biochemical features, suggesting a close relation among the them.Tables4.2 to table 4.5 show the result of the biochemical characterization of the four bacteria using API kit. The API result showed that all isolates are lactic acid bacteria.

Table4.6 shows the basic features used for the identification of the mould isolates. All the mouldswere maintained on Malt Extract Agar (MEA) slant on which presumptive identification was made. Identity of isolates was confirmed by microscopy using lactophenol cotton blue stain. During microscopy, key features such as conidiophores, conidia, phialides and hyphae were looked out for and compared with those of standard strains on fungi atlas.

Plates I to IIIshow pictures of the mould isolates indicating their macroscopic features.They are *A. niger, Trichoderma*sp *and Fusarium*sp respectively.*A. niger*was Woolly at first, then developed white to yellow and turned black on the third day of inoculation, *Trichoderma*sp had white fluff which covered agar at first and green patches eventually formed on the agar and *Fusarium*sp was white and cottony and then developed a pink to violet

centre.Pictures were taken on their various period of maturity, third day, fifth day and fourth day after inoculation for *A. niger*, *Trichoderma*sp *and Fusarium*sp respectively.

Table 4.1:Morphological and Biochemical Properties of Bacterial Isolates from the Inoculum

Code	Colonial Morphology	Vegetative Morphology	Gram Stain	Catalase Test	Spore Formation
B01	entire, white and transluscent	cocci	+	-	-
B02	entire, milky and transluscent	Rods	+	-	-
B03	entire, milky and transluscent	Rods	+	-	-
B04	entire, milky and transluscent	Rods	+	-	-

B01, B02, B03 and B04 are codes of the bacteria isolates before characterization

Table 4.2: Biochemical Profile of *Weissellaconfusa* Using API Kit

TM	O	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
24h	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-
48h	-		-	-	-	+	+	-	-	-	+	+	+	+	-	-	-

	0	GLY	ERY	DARA	LARA	RIB	DYYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL

TM	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
24h	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-
48h	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-

	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU

TM	34	35	36	37	38	39	40	41	42	43	44	45	46
24h	-	-	-	-	-	+	-	-	-	-	-	-	-
48h	-	-	-	-	-	+	-	-	-	-	-	-	-

	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL

GLY=Glycerol, ERY=Erythritol, DARA=D-Arabinose, LARA=L-Arabinose, RIB=D-Ribose, DXLY=D-Xylose, LXLY=L-XyLose, ADO=D-Adonitol, MDX=Methyl-BD-Xylopyranoside, GAL=D-Galactose, Glu=D-Glucose, FRU=D-Fructose, MNE=D-Mannose, SBE=Sorbose, RHA=L-Rhamnose, DUL=Dulcitol, INO=Inositol, MAN=D-Mannitol, SOR=D-Sorbitol, MDM=Methyl-αD-Mannopyranoside, MDG=Methyl-αD-Glucopyrnoside, NAG=N-AcetylGlucoseamine, AMY=Amygdaline, ARB=Arbutine, ESC=Esculine, SAL=Salicine, MAL=D-Maltose, LAC=D-Lactose, MEL=D-Melibiose, SAC=Saccharose, TRE=D-Trehalose, INU=Inuline, MLZ=D-Melezitose, RAF=Raffinose, AMD=Amidon, GLYG=Glycogene, XLT=Xylitol, GEN=Gentiobiose, TUR=D-Turanose, LXY=D-Lyxose, TAG=Tagatose, DFUC=D-Fucose, LFUC=L-Fucose, DARL=D-ArabitoL, LARL=L-ArabitoL, GNT=Potassium GlucoNate, 2KG=Potassium 2-cetogluconate, 5KG=Potassium 5-cetogluconate.

Table 4.3: Biochemical Profile of *Lactobacillus brevis* 1 Using API Kit

TM	O	1	2	3	4	5	6	7	8	9	10	11	12	1
24h	-	-	-	-	-	+	+	-	-	-	+	+	+	+
48h	-	-	-	-	-	+	+	-	-	-	+	+	+	+
		GLY	ERY	DARA	LARA	RIB	DYYL	LXYL	ADO	MDX	GAL	GLU	FRU	

TM	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
24h	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	-
48h	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	-
	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU

TM	34	35	36	37	38	39	40	41	42	43	44	45	46
24h	-	-	-	-	-	+	-	-	+	-	-	-	-
48h	-	-	-	-	-	+	-	-	+	-	-	-	-
	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL

GLY=Glycerol, ERY=Erythritol, DARA=D-Arabinose, LARA=L-Arabinose, RIB=D-Ribose, DXLY=D-Xylose, LXLY=L-XyLose, ADO=D-Adonitol, MDX=Methyl-BD-Xylopyranoside, GAL=D-Galactose, Glu=D-Glucose, FRU=D-Fructose, MNE=D-Mannose, SBE=Sorbose, RHA=L-Rhamnose, DUL=Dulcitol, INO=Inositol, MAN=D-Mannitol, SOR=D-Sorbitol, MDM=Methyl-αD-Mannopyranoside, MDG=Methyl-αD-Glucopyrnoside, NAG=N-AcetylGlucoseamine, AMY=Amygdaline, ARB=Arbutine, ESC=Esculine, SAL=Salicine, MAL=D-Maltose, LAC=D-Lactose, MEL=D-Melibiose, SAC=Saccharose, TRE=D-

Trehalose, INU=Inuline, MLZ=D-Melezitose, RAF=Raffinose, AMD=Amidon, GLYG=Glycogene, XLT=Xylitol, GEN=Gentiobiose, TUR=D-Turanose, LXY=D-Lyxose, TAG=Tagatose, DFUC=D-Fucose, LFUC=L-Fucose, DARL=D-ArabitoL, LARL=L-ArabitoL, GNT=Potassium GlucoNate, 2KG=Potassium 2-cetogluconate, 5KG=Potassium 5-cetogluconate.

Table 4.4: Biochemical Profile of *Lactobacillus fermentum 1* UsingAPI Kit

TM	O	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
24h	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-
48h	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-

	GLY	ERY	DARA	LARA	RIB	DYYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL

TM	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-

	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU

TM	34	35	36	37	38	39	40	41	42	43	44	45	46
	-	+	-	-	-	-	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-	-	-	-	-

	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL				

GLY=Glycerol, ERY=Erythritol, DARA=D-Arabinose, LARA=L-Arabinose, RIB=D-Ribose, DXLY=D-Xylose, LXLY=L-XyLose, ADO=D-Adonitol, MDX=Methyl-BD-Xylopyranoside, GAL=D-Galactose, Glu=D-Glucose, FRU=D-Fructose, MNE=D-Mannose, SBE=Sorbose, RHA=L-Rhamnose, DUL=Dulcitol, INO=Inositol, MAN=D-Mannitol, SOR=D-Sorbitol, MDM=Methyl-αD-Mannopyranoside, MDG=Methyl-αD-Glucopyrnoside, NAG=N-AcetylGlucoseamine, AMY=Amygdaline, ARB=Arbutine, ESC=Esculine, SAL=Salicine, MAL=D-Maltose, LAC=D-Lactose, MEL=D-Melibiose, SAC=Saccharose, TRE=D-Trehalose, INU=Inuline, MLZ=D-Melezitose, RAF=Raffinose, AMD=Amidon, GLYG=Glycogene, XLT=Xylitol, GEN=Gentiobiose, TUR=D-Turanose, LXY=D-Lyxose, TAG=Tagatose, DFUC=D-Fucose, LFUC=L-Fucose, DARL=D-ArabitoL, LARL=L-ArabitoL, GNT=Potassium GlucoNate, 2KG=Potassium 2-cetogluconate, 5KG=Potassium 5-cetogluconate.

Table 4.5: Biochemical Profile of *Lactobacillus plantarum 1* Using API Kit

TM	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
24h	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-
48	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-

	0	GLY	ERY	DARA	LARA	RIB	DYYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL
TM	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-
	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-

	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU		
TM	34		35		36		37		38		39		40		41		42		43		44		45		46
	+		-		-		-		-		+		+		-		-		-		-		-		-
	+		-		-		-		-		+		+		-		-		-		-		-		-
	MLZ		RAF		AMD		GLYG		XLT		GEN		TUR		LYX		TAG		DFUC		LFUC		DARL		LARL

GLY=Glycerol, ERY=Erythritol, DARA=D-Arabinose, LARA=L-Arabinose, RIB=D-Ribose, DXLY=D-Xylose, LXLY=L-XyLose, ADO=D-Adonitol, MDX=Methyl-BD-Xylopyranoside, GAL=D-Galactose, Glu=D-Glucose, FRU=D-Fructose, MNE=D-Mannose, SBE=Sorbose, RHA=L-Rhamnose, DUL=Dulcitol, INO=Inositol, MAN=D-Mannitol, SOR=D-Sorbitol, MDM=Methyl-αD-Mannopyranoside, MDG=Methyl-αD-Glucopyrnoside, NAG=N-AcetylGlucoseamine, AMY=Amygdaline, ARB=Arbutine, ESC=Esculine, SAL=Salicine, MAL=D-Maltose, LAC=D-Lactose, MEL=D-Melibiose, SAC=Saccharose, TRE=D-Trehalose, INU=Inuline, MLZ=D-Melezitose, RAF=Raffinose, AMD=Amidon, GLYG=Glycogene, XLT=Xylitol, GEN=Gentiobiose, TUR=D-Turanose, LXY=D-Lyxose, TAG=Tagatose, DFUC=D-Fucose, LFUC=L-Fucose, DARL=D-ArabitoL, LARL=L-ArabitoL, GNT=Potassium GlucoNate, 2KG=Potassium 2-cetogluconate, 5KG=Potassium 5-cetogluconate.

Table 4.6: Identification of Mould Isolates from Fermenting Cassava Pulp Inoculum

Code	Period of maturity	Macroscopic morphology	Microscopic morpholoy				identification
			Conidiophores	Philiades	hyphae	Conidia	
M01	Day 3	Woolly, at first white to yellow, then turned black	Long and smooth	Biseriate, covered entire vesicle with radiate head	septate	Large, dark and globuse head	A. niger
M02	Day 5	White fluff covered agar, green patches eventually formed	Short and branched	Flask shape	septate	Round or oval singly or in clusters	Trichodermasp
M03	Day 4	At first white and cottony, developed a pink or violet centre	Branched or unbranched, long or short	Canoe or sickle shape	septate	One or two celled conidia	Fusariumsp

M01 = *A. niger*,M02 = *Trichoderma*sp, M03 = *Fusarium*sp

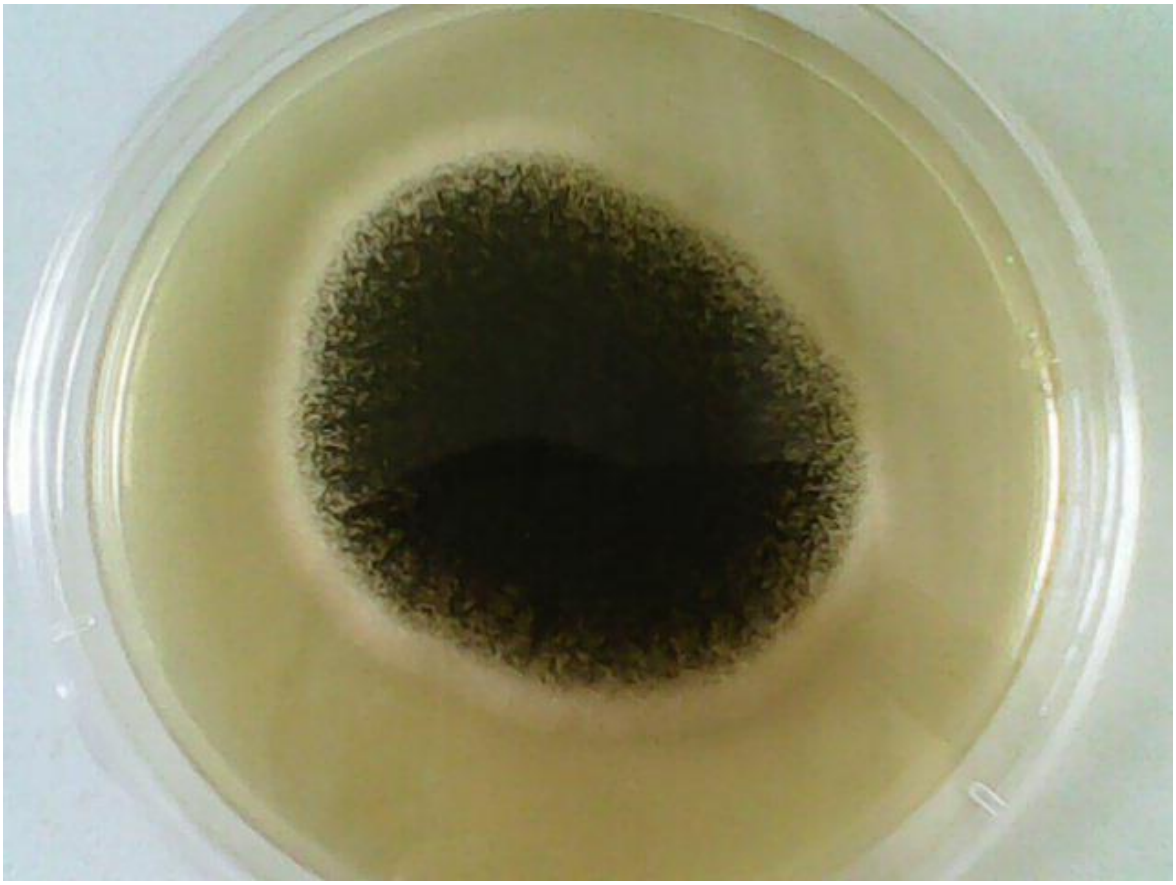


Plate I: Macroscopic Morphology of*A. niger*on MEA

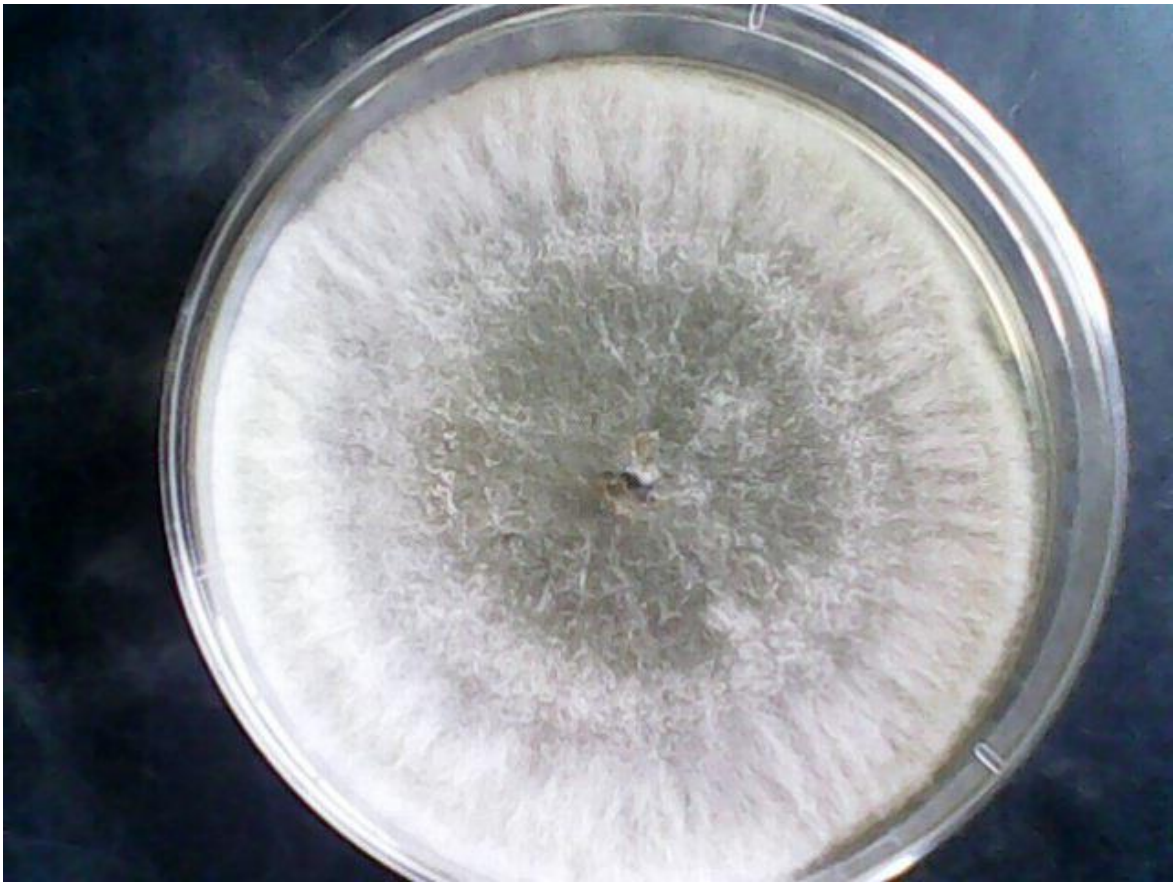


Plate II: Macroscopic Morphology of *Trichoderma*spon MEA



Plate III: Macroscopic Morphology of *Fusarium* sp on MEA

Table 4.7 shows changes in cyanide content of cassava samples using pure isolates of microbial cassava fermenters in mg HCN/100 g cassava wet weight. Summary of ANOVA result (Appendix I) shows a significant difference in cyanide reduction on day 3 and day 4 of fermentation,$P < 0.05$ (Appendix I); hence the ranking of the residual cyanide of the two days.The ranking is indicated by the superscript on the residual cyanide of the two days and showedthat*L. plantarum 1*had the lowest residual cyanide 0.10 ± 0.10^{d} mg HCN/100 g, which suggests that it had the highest cyanide degrading capabilitywhile the control experiment had the highest residual cyanide 2.60 ± 0.20^{a} mg HCN/100 g, which suggests that its cyanide degrading capability was the lowest.

Figure 4.1 was derived from Table 4.7 to show the percentage cyanide degrading capability of the test organisms including the consortium and control using the formular:Percentage cyanide degrading capability = (Initial cyanide content – Residual cyanide)/ Initial cyanide content x 100.

The chart reveals *L. plantarum 1*to have the highest percentage cyanide degrading capability of the test organisms with the value, (98.00 %), followed closely by *L. fermentum 1* with percentage cyanide degrading capability of 95.42 %, with*W. confusa* having the least percentage cyanide degrading capability of (78.48 %). The control experiment with no test organism had 43.43 % cyanide degrading capability.

Table 4.8 presents the mean scores of the sensory evaluation of the two *gari*samples; sample O and sample W, as rated by seven panels of judges. Result of t-test analysis(Table 4.8) shows that sample O, *gari*prepared with starter culture was more preferred to sample W, *gari*prepared without starter culture, ($P < 0.05$); indicating a significant difference between the two *gari* samples with sample O,*gari* prepared with

stater culture, *Lactobacillus plantarum 1* being more preferable to sample W, *gari* prepared without starter culture.

Table 4.7: Changes in Cyanide Content of Cassava Sample Using Pure Isolates of Microbial Cassava Fermenters (mg HCN/100 g cassava wet weight)

Duration of fermentation (Days)	*W. confusa*	*L. brevis*	*L.fermentum 1*	*L.plantarum 1*	*A. Niger*	*Fusarium*sp	*Trichoderma*sp	Control	Consortium
1	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40	4.60±0.40
2	3.20±0.40	2.80±0.60	2.60±0.20	2.01±0.21	2.40±0.20	3.20±0.40	2.90±0.30	3.60±0.40	2.90±0.30
3	1.20 ± 0.40^{bc}	1.80 ± 0.40^{bc}	2.00 ± 0.20^{ab}	1.20 ± 0.20^{bc}	0.80 ± 0.00^{c}	2.00 ± 0.20^{ab}	2.10 ± 0.30^{ab}	3.00 ± 0.40^{a}	1.70 ± 0.20^{bc}
4	1.00 ± 0.20^{b}	$0.40\pm0.20b^{cd}$	0.21 ± 0.01^{cd}	0.10 ± 0.10^{d}	0.60 ± 0.00^{bcd}	0.30 ± 0.10^{bcd}	0.90 ± 0.10^{bc}	2.60 ± 0.20^{a}	0.54 ± 0.46^{bcd}

Values are expressed as mean ± SEM from two determinations

SEM = Standard error of mean

Control = fermentation done without microbial isolate

Consortium = fermentation done with combination of the seven isolates of microorganisms

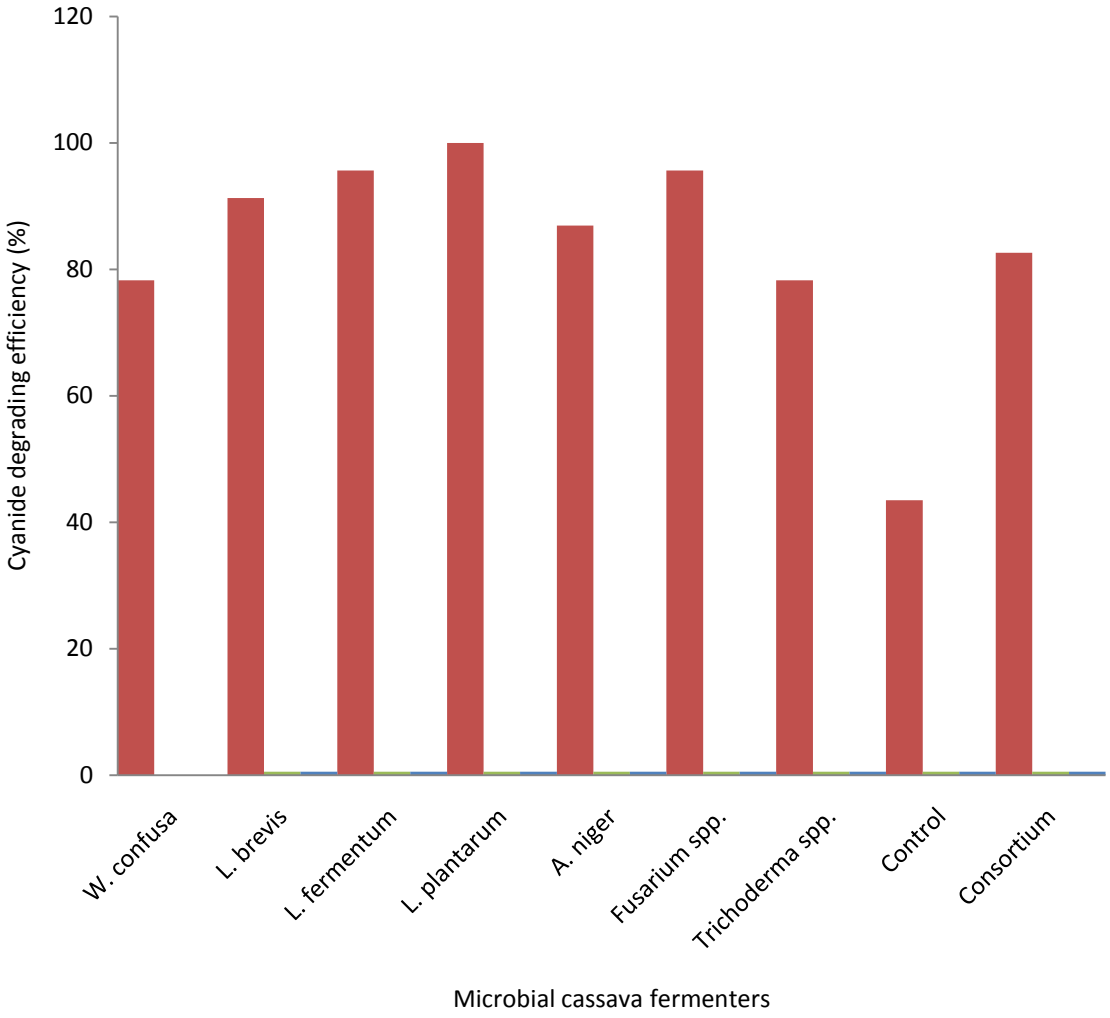


Figure 4.1: Percentage Cyanide Degrading Capability of Microbial Cassava Fermenters

Table 4.8: Result of Sensory Evaluation of the Two Gari Samples using Hedonic Scale

	Mean ± SEM score of attributes	
Attributes	Sample O	Sample W
Appearance	4.40 ± 0.17	3.20 ± 0.21
Aroma	3.40 ± 0.30	2.60 ± 0.25
Taste	4.40 ± 0.20	2.60 ± 0.22
Flavour	4.00 ± 0.27	3.20 ± 0.12
General acceptability	4.00 ± 0.15	3.20 ± 0.24

SEM = Standard error of mean. $P < 0.05$ is significant

Sample O = gari prepared with starter culture, *L. plantarum 1*

Sample W = gari prepared without starter culture

CHAPTER FIVE

5.0 DISCUSSION

The microbial cassava fermenters isolated included both bacteria and moulds. This is consistent with the work of Kobawila*et al*. (2005) and Ihaotu*et al*. (2005).

Lactic acid bacteria could not be identified to species level on presumptive basis. This could be because apart from the large number of species in the group, they possess very similar colonial morphology and biochemical characteristics (Table 4.1). This may be responsible for the many fermentable carbohydrates and carbohydrate derivative (Tables 2 - 5) used to characterize them to species level using the API 50 CH test. Biochemical characterization showed that all four strains of lactic acid bacteria tested negative to glycerol, erithritol and D-arabinose fermentation while testing positive to esculin. It is possible that fermentation of these carbohydrates is common to all or most lactic acid bacteria. If this is found to be repeated in more members of the group, the test could be suggested as confirmatory tests for lactic acid bacteria like the others such as Gram positive, catalase negative and non-spore forming as preliminary tests for identification of lactic acid bacteria.

Isolation of moulds from cassava was made less difficult due to foreknowledge of the types of moulds associated with fermenting cassava dough. With this knowledge, it was easier identifying the isolates by comparing their macroscopic and microscopic features with pictures of standard strain on fungal atlas.

The result ofscreening for cyanide degrading capability (Table 4.7) shows that all isolates showed cyanide degrading capability in varying rates. This is consistent with the observation of Amoa-Awua*et al.* (1997) that all bacteria, yeasts and moulds identified in

traditional dough inocula exhibited linamarase activity and were therefore capable of degrading cyanogenic glycosides.

Table 4.7 shows the residual cyanide content of the control, (2.60±0.20 mg HCN/100 g cassava wet weight) on day 4 of fermentation which is more than acceptably safe cyanide level according to Akinlere *et al.* (2009) who stated 0.3 mg HCN/100 g to be the maximum acceptable safe level of cyanide content of cassava and cassava products for consumption. However, upon the introduction of microorganisms, cyanide level was reduced to safe level in same fermentation period (4 days fermentation). This showed the importance of the involvement of microorganisms in cassava processing. In addition, (Table 4.7) also shows that only three of the isolates, *L. plantarum 1*, *L.fermentum* and *Fusarium*sp reduced cyanide content of cassava to safe levels on the fourth day of fermentation and according to Onyesom and Okpokunu (2008), sensory attributes of cassava products are best in four days fermentation period. This suggests the necessity of the use of starter culture in cassava processing as all microorganisms do not degrade cyanide at the same rates and also, it suggests that any of the three organisms could be used for the preparation of starter culture for cassava processing.

Expectations were that the consortium would have the highest cyanide degrading capability however, it came fourth by ranking. This means that the synergy anticipated was not obtained. Possible reason for this could be inter-species competition among the microbial isolates. Figure 4.1 shows that *Lactobacillus plantarum 1* had 98 % cyanide degrading efficiency with residual cyanide of 0.10±0.10 mg HCN/100 g cassava wet weight (Table 4.7), being the isolate with the lowest residual cyanide. This suggests that it had the highest cyanide degrading capability among the seven isolates including the consortium. This is excellent as compared to drying, which, according to Mahungu*et*

al.,(1987), removes only 30 % of total cyanide, and boiling and steaming which reduce cyanide level by approximately 16 % and 47 % respectively. Fermentation therefore, is about the best method of cyanide detoxification, especially in products for consumption.

The control experiment with no microbial isolates still had its cyanide level reduced from 4.60±0.40 mg HCN/100 g to 2.60±0.20 mg HCN/100 g and rated at 43.43 % cyanide degrading efficiency as shown on Figure 4.1. This could be due to the fact that the enzymes,linamarase and hydroxynitrilelyase which catalyse the degradation of cyanogenic glycoside to release cyanide (HCN), are sequestered in different tissues of the cassava and released when the tissue is disrupted (Kimaryo*et al*., 2000).

In this study, the variety of cassava used was *Manihotesculentacrantz* with the variety number, 2741 as identified in the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University Zaria. Upon laboratory analysis of its cyanogenic glycoside content, was found to contain a mean concentration of 4.60±0.40 mg HCN/100 g cassava wet weight of cyanogenic glycoside (Table 4.7). This means that this variety belong to the non-toxic variety according to Kobawila (2005) classification of cassava on the basis cyanogenic glycoside content. Consumption of this variety of cassava may not have acute effect on the consumer but does have chronic effect, that is, a deleterious cumulative effect according to the findings of Gomez (1985). Consumption of raw and or non-detoxified cassava is therefore completely discouraged, whether its a toxic or a non-toxic variety.

The result of one-way analysis of variance (ANOVA) shows that there was significant difference in cyanide reduction only on the third and fourth day of fermentation, $P< 0.05$ (Appendix I). This shows that the length of fermentation is very important and attention should be paid to it. T-test analysis of the two samples of *gari* (

Table4.8) shows that sample O (*gari* produced with starter culture, *L. plantarum 1*) was significantly more preferred to sample W (gari produced without starter cultures), $P < 0.05$. This suggests that apart from cyanide degradation, pure culture of appropriate microorganisms improve the sensory characteristics (colour, texture, aroma, taste) of *gari.* This would therefore increase acceptability by consumers and hence increasing its market value. This finding is consistent with the work of Dunican(1990), who reported that sensory attributes of *gari* depends on the types of microorganisms involved in the fermentation, the nature of cassava roots and the fermentation conditions.

CHAPTER SIX

6.0 CONCLUSION AND SUMMARY

6.1 SUMMARY

Weissellaconfusa, Lactobacillusbrevis 1, Lactobacillusfermentum 1, Lactobacillusplantarum 1,Aspergillusniger, *Trichoderma*sp and *Fusarium*sp were isolated from fermentingcassava pulp and screened for cyanide degrading capability and were all found toshow cyanide degrading capability in varying degrees with *Lactobacillusplantarum 1*,having the highest cyanide degrading capability. Having found *Lactobacillusplantarum 1*to have a high cyanide degrading capability, it was used as starter culture for *gar*i preparation. Another sample of *gari* was prepared but without the starter culture to serve as a control. The two samples of *gari* were assessed on the basis of sensory attributes and the results were compared using student's t – test. There was a significant difference between the two *gari* samples($P < 0.05$), with *gari* prepared with starter culture being more preferable.

6.2 CONCLUSIONS

On the basis of the results obtained, it was concluded that *Weissellaconfusa, Lactobacillusbrevis 1, Lactobacillusfermentum 1, Lactobacillusplantarum 1,Aspergillusniger*, *Trichoderma*spand *Fusarium*spare indigenous to fermenting cassava dough and degrade cyanide and therefore are cyanophilic microorganisms. *Lactobacillus plantarum 1* had the highest cyanide degrading capability and combined with very good sensory attributes it produced in *gari*, makes it a good starter culture for *gari*preparation. Cyanide level in cassava reduces appreciably from the third day of fermentation.

6.3 RECOMMENDATIONS

In view of the health threat posed by cumulative residual cyanide consumption, the following recommendations are made:

1. Starter cultures made of *L. plantarum 1*or any of the organisms with very high cyanide degrading capabilityshould be used for improved *gari* preparation, however preference should be given to the lactic acid bacteria because apart from their high cyanide degrading capability, according to Chantaraporn (2006),produce metabolic compounds (diacetyl and bacteriocins) with antimicrobial property against pathogens such as *B. cereus* and *S. aureus*.

2. Cassava pulp for *gari* preparation or for other cassava products should not be fermented for less than 3 days fermentation period to eliminate or reduce cyanide concentration to a safe level.

3. Cassava should never be eaten raw no matter how small the cyanide content is because residual cyanide has a cumulativedeleterious effect.

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APPENDIXI

One way analysis of variance (ANOVA) comparing reduction in cyanide among days

Day		Sum of squares	Df	Mean of squares	F	P
1	Between groups	0.000	8	0.000	0.000	1.000
	Within group	2.880	9	0.320		
	Total	2.880	17			
2	Between groups	3.571	8	0.446	1.756	0.209
	Within group	2.288	9	0.254		
	Total	5.859	17			
3	Between groups	6.644	8	0.831	4.792	0.015
	Within group	1.560	9	0.173		
	Total	8.204	17			
4	Between groups	9.224	8	1.153	14.345	0.000
	Within group	0.723	9	0.080		
	Total	9.948	17			

*= Significant mean difference at the 0.05 level.

APPENDIX II

Composition of API 50 CH

Tube	Test	Active ingredients	QTY (mg/cup.)
0		CONTROL	-
1	GLY	GLYcerol	1.64
2	ERY	ERYthritol	1.44
3	DARA	D-ARAbinose	1.4
4	LARA	L-ARAbinose	1.4
5	RIB	D-RIBose	1.4
6	DXYL	D-XYLose	1.4
7	LXYL	L-XYLose	1.4
8	ADO	D-ADOnitol	1.36
9	MDX	Methyl-ßD-Xylopyranoside	1.28

Tube	Test	Active ingredients	QTY (mg/cup.)
10	GAL	D-GALactose	1.4
11	GLU	D-GLUcose	1.56
12	FRU	D-FRUctose	1.4
13	MNE	D-MaNosE	1.4
14	SBE	L-SorBosE	1.4
15	RHA	L-RHAmnose	1.36

16	DUL	DULcitol	1.36
17	INO	INOsitol	1.4
18	MAN	D-MANnitol	1.36
19	SOR	D-SORbitol	1.36

Tube	Test	Active ingredients	QTY (mg/cup.)
20	MDM	Methyl-αD-Mannopyranoside	1.28
21	MDG	Methyl-αD-Glucopyranoside	1.28
22	NAG	N-Acetylglucosamine	1.28
23	AMY	AMYgdalin	1.08
24	ARB	ARButin	1.08
25	ESC	ESCulin ferric citrate	1.16 0.152
26	SAL	SALicin	1.04
27	CEL	D-CELIobiose	1.32
28	MAL	D-MALtose	1.4
29	LAC	D-LACtose (bovine origin)	1.4

Tube	Test	Active components	QTY (mg/cup.)

30	MEL	D-MELibiose	1.32
31	SAC	D-SACcharose (sucrose)	1.32
32	TRE	D-TREhalose	1.32
33	INU	INUlin	1.28
34	MLZ	D-Melezitose	1.32
35	RAF	D-RAFfinose	1.56
36	AMD	AmiDon (starch)	1.28
37	GLYG	GLYcoGen	1.28
38	XLT	XyLitol	1.4
39	GEN	GENtibiose	0.5

Tube	Test	Active components	QTY (mg/cup.)
40	TUR	D-TURanose	1.32
41	LXY	D-LYXose	1.4
42	TAG	D-TAGatose	1.4
43	DFUC	D-FUCose	1.28
44	LFUC	L-FUCose	1.28
45	DARL	D-ARabitoL	1.4
46	LARL	L-ARabitol	1.4
47	GNT	Potassium GlucoNaTe	1.84

48	2KG	Potassium 2-KetoGluconate	2.12
49	5KG	Potassium 5-Ketogluconate	1.8

APPENDIX III

API 50 CH before fermentation



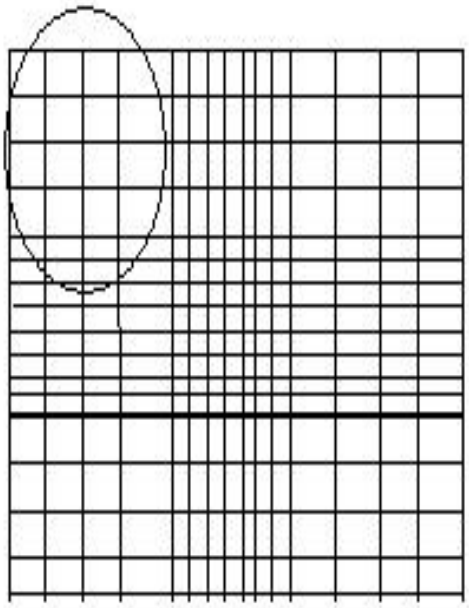
APPENDIX III CONT'D

API 50 CH after 48 hour fermentation



APPENDIX IV

Haemocytometre grid line (Abcam, 2012)



APPENDIX V

McFarland standard scale

McFarland scale No	Amt of 1% $BaCl_2$(ml)	Amt of 1% H_2SO_4(ml)	Bacteria in million/ml
0.5	0.05	9.95	100
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1200
5	0.5	9.5	1500
6	0.6	9.4	1800
7	0.7	9.3	2100
8	0.8	9.2	2400
9	0.9	9.1	2700
10	1.0	9.0	3000

APPENDIX VI

Questionnaire to compare qualities of two gari samples

The two gari samples are products of MSc. research work in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria. They were processed under highly hygienic conditions. Please kindly assess them based on the attributes below by your personal candid judgement.

Thank you.

Please underline accordingly.

Sample O

1. Appearance A B C D E
2. Aroma A B C D E
3. Taste A B C D E
4. Flavour A B C D E
5. Acceptability A B C D E

Sample W

1. Appearance A B C D E
2. Aroma A B C D E
3. Taste A B C D E
4. Flavour A B C D E
5. Acceptability A B C D E

Key: A = very desirable, B = desirable, C = fairly desirable, D = undesirable, E = very undesirable.

APPENDIX VII

Cyanidestandardcurve

