Towards the development of a starter culture for gari production

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Declaration

I declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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Candidate

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Abstract

Cassava is a food crop planted in many countries in Africa. Its tubers are a major source of food and are processed to produce a variety of food products, one of which is the fermented product called gari. This research report aimed to evaluate the performance of three lactic acid bacteria for several properties with regard to the fermentation of cassava to produce gari. Three organisms were used for the evaluation, namely *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Leuconostoc mesenteroides.* The organisms were evaluated for viability, biomass formation and glucose utilisation in static flasks, biomass formation and glucose utilisation in 2 L fermenters, cell viability after dehydration processes and pH and cyanide reduction in cassava substrate. In static flasks, the organisms were found to retain above 80% cell viability after cryopreservation. Maximum biomass of 10^8 cells/ml was formed within the first 12 hours by all the organisms. While *L. fermentum*, depleted glucose within 24 hours, *L. plantarum* formed the highest biomass of 4 x 10^8 cells/ml. In 2 L Braunstat B fermenters, a cell count of 10^9 cells/ml was obtained by *L. fermentum* and *Leuconostoc mesenteroides* within 12-15 hours. Biomass formation for *L. plantarum* during the same period was 10^{10} cells/ml. Glucose was depleted within 12 - 15 hours. The viability of cells between the dehydration processes of centrifugation, glycerol and maltodextrin addition and lyophilisation, was above 80% for all the organisms. However, this high cell viability was influenced by concentration of cells during the centrifugation step. In cassava substrate, *L. fermentum*, though heterofermentative, was found to be particularly acid tolerant and reduced pH to 3.98. All the organisms were able to retain good viability after lyophilisation. However, the results of cyanide reduction were inconclusive. These results show that while cultures show promise for pilot scale studies of starter culture development, further cyanide experiments need to be conducted, and synergy between the organisms investigated.

Dedication

In memory of my grandmother

Louise Mbapeua Haakuria

1922 – 1991

To my daughter, *Vesora Kuzeue Haakuria*, who had to put up with my prolonged absence while I engaged in my pursuit of knowledge as the noblest of all human endeavours.

Acknowledgements

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I am forever indebted to the Roman Catholic Church and in particular Fathers Edward Kangootui and Anton Biesel for putting me through school.

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List of Symbols

α – alpha

β - beta

g – gram

mg- milligram

ml - milliliter

X_{max} – Maximum Biomass

μ – micro

CFU – Colony Forming Units

Nomenclature

ADP – Adenosine diphosphate

ATP – adenosine triphosphate

CSIR – Council for Scientific and Industrial Research

EMP – Embden-Meyerhof-Parnas Pathway

FAO - Food and Agricultural Organisation

IITA – International Institute of Tropical Agriculture

LAB – lactic acid bacteria

Lyophilisation - freezedrying

NAD – Nicotine-Adenine-Dinucleotide

SANAS – South African National Accreditation System

Phosphoketolase pathway - a mixed acid fermentation pathway used by *Lactobacillus* and *Leuconostoc* spp. to generate energy

Introduction

1.1. General introduction

The production of fermented foods is one of the oldest food processing technologies known to man (Fitzgerald and Caplice, 1999). Since the dawn of civilisation, man has used these technologies to preserve and prolong the shelf-life of foods such as milk, meat, vegetables and cereals. While the main focus of such fermentations was to preserve foodstuffs, it was also used to improve the taste, flavour, aroma and texture for enhanced digestibility. Microorganisms, by virtue of their metabolic activities contribute to the development of attributes such as flavour, aroma, texture and improved cooking and processing properties. The earliest production of fermented foods was based on spontaneous fermentation due to microorganisms that occur naturally on the raw material. However, fermentation times were long due to the lag phase of the organisms and it was difficult to produce an end product of consistent quality. In time, this spontaneous fermentation was optimised through inoculation of the raw material with a small quantity from a previous successful fermentation (De Vuyst and Leroy, 2004). This process is called backslopping. This was a way to use a selected culture to shorten fermentation process and to produce an end product of improved and consistent quality. This was necessary since the quality of the end product and the fermentation time are dependent on the microbial load. To date, backslopping is still the preferred process to produce foodstuffs such as sauerkraut and sourdough (Harris, 1998).

Today, the production of fermented foods and beverages through spontaneous fermentation and backslopping is used as a cheap and reliable method of preserving foodstuffs in less developed countries. In industrialised countries, large-scale production of fermented foods is an important branch of the food industry. Production of beer and wine and organic acids such as lactic and citric acids are multibillion dollar industries annually. The breakthrough in the processing of fermented foods was made possible by the use of starter cultures (De Vuyst and Leroy, 2004). The ability to isolate strains of microorganisms with desirable physiological and metabolic characteristics from natural fermentations for use as inocula in industrial

fermmetations, resulted in a high degree of control over the fermentation process. With these starter cultures, end products with particular characteristics could be produced in a specified time. Figure 1 gives a general outline of a food fermentation process using a starter culture.

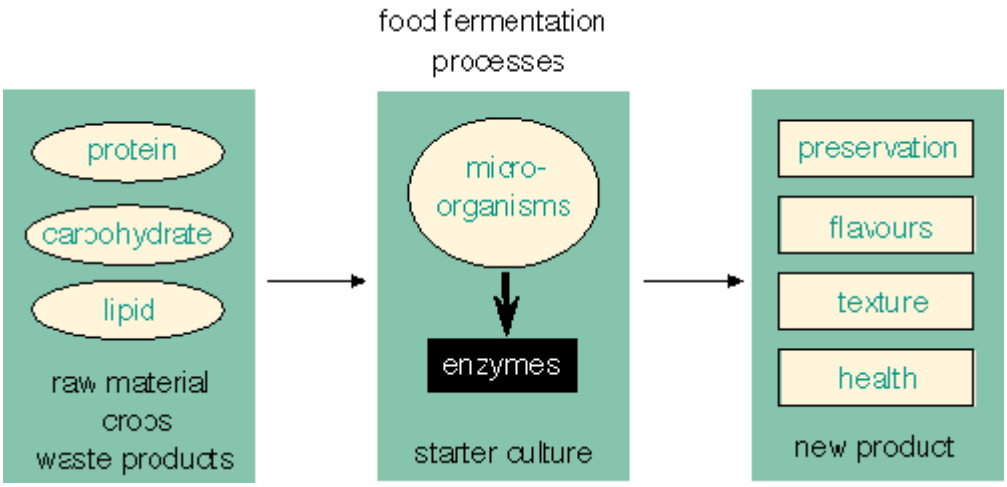

Figure 1: A food fermentation process typically consists of raw materials, fermenting microorganisms and the products (http://www.fooddoc.com/NFS430/fermentation.htm).

Lactic acid bacteria play a central role in fermentation process and have a long history of application and consumption in the production of fermented foods and beverages (Table 1). Apart from the good sensory qualities they impart to food, lactic acid bacteria are appreciated for their reported health benefits. They are, therefore, widely used as probiotics, which are live micoorganisms administered in adequate amounts to confer health benefits to the host.

Fermentation substrates range from milk (cheeses; butter; yoghurt etc), meat (pickles), vegetables (sauerkraut etc.) and a variety of other crops such as wheat (beer and spirits), grapes (wines) and potatoes (whiskies). One of the most important and abundant food crops in Africa from which a variety of fermented products are produced, is cassava/manioc/tapioca. It serves as a substrate for many fermentation products at subsistence level.

Table 1: Some fermented foods and beverages and their associated LAB (De Vuyst and Leroy, 2004)

Hard cheeses without eyes	*L. lactic* subsp. *lactis*
Cheeses with small eyes	*L. lactic* subsp. *lactis*, *Leuconostoc mesent*eroides
Yoghurt	*L. delbrueckii* subsp *bulgaricus, S. thermophilus*
Fermented probiotic milk	*L. casei, L. acidophilus, B. bifidum*
Sauerkraut	*Leuconostoc mesenteroides, L. plantarum*
Pickles	*Leuconostoc mesenteroides., L. brevis*
Fermented cereals	*L. fermentum, L. brevis*
Sourdough	*L. plantarum, L. reuteri*
Wine (malolactic fermentation)	*O. oeni*
Rice wine	*L. sakei*
kefir	*L. brevis*

1.2 Cassava

Cassava, *Manihot esculenta Crantz*, is a perennial woody shrub with an edible root growing in tropical and subtropical areas of the world. It is cultivated for its starchy tubers in areas where the temperature exceeds 20°C (see Figure 2) and has many properties that make it an ideal crop in many areas of the world. One of them is its ability to grow in nutrient poor soils where other crops will not grow easily. This ability makes it an ideal crop in many poor soil areas around the world, especially in Third World countries where agriculture is the mainstay of the economies. Cassava is perennial and can be cultivated throughout the year without regard to the seasons. It generally has high yield per unit acre and grows with limited water which makes it drought tolerant. As such, cassava has all the attributes to serve as a food security crop in many parts of the developing world.

Figure 2: Cassava plant showing the starchy tubers (IITA, 2004).

1.2.1 Cassava utilisation

In Africa, cassava is used mainly as a food source for local consumption, though some is exported to Europe. It plays a role as a food security crop in areas prone to drought, famine and civil strife. Cassava provides a stable food base because of its flexibility in terms of planting and harvesting strategies and relative tolerance to nutrient poor soils and drought resistance. It is a major source of energy for low-income consumers in many parts of tropical Africa, including major urban areas (Nweke, 1994). It is estimated that more than 500 million people in Africa rely on cassava as a major energy source (Nweke,1994). As such, cassava is regarded as the most important food crop in many villages in African countries. Because of the high starch content cassava roots have great potential for industrial applications.

1.2.2 Nutritional contribution to the diet

Cassvava roots are rich in carbohydrate with most of it being present as starch (31% of fresh weight, Table 2). However, cassava roots are deficient in protein (less than 1.5% of fresh weight) fat (0.17%, Table 2). Though deficient in protein, cassava is generally considered to have high content of dietary fibre, magnesium, sodium,

riboflavin, nicotinic acid, thiamine and citrate (Bradbury and Holloway, 1988). Iron and vitamin A levels, however, are considered to be low, though some yellow varieties contain significant concentrations of ß-carotene up to 1 mg/100g on a dry weight basis (McDowell and Onduro, 1983).

Table 2: Composition of cassava roots in the Pacific Islands (Scott *et al*., 2000)

Component	**Roots**	**Leaves**
Moistre (%)	62.8	74.8
Energy (KJ/100 g)	580	-
Protein (%)	0.53	5.1
Fat (%)	0.17	2.0
Starch (%)	31.0	-
Sugar (%)	0.83	-
Dietary fibre (%)	1.48	5.1
Ash (%)	0.84	2.7
Ca (mg/100 g)	20	320
K (mg/100 g)	302	56
P (mg/100 g)	46	-
Mg (mg/100 g)	30	-
Fe (mg/100 g)	0.23	-

1.2.3 Cassava deficiencies

As can be seen from Table 2 above, cassava has major deficiencies that limit its use as a food source. It contains levels of antinutritive cyanogenic glucosides, notably linamarin (2-[β-D-glucopyranosyloxyl-isobutyronitrile]) (Figure 4) and to a lesser extent, lotaustralin , its derivative.

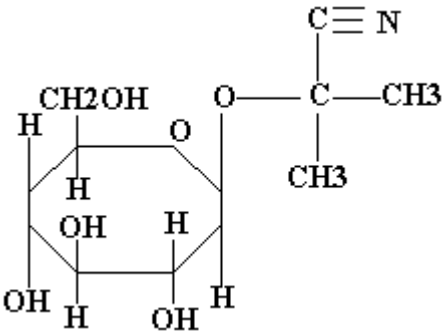

Figure 3: The molecular structure of linamarin.

Linamarin occurs in all parts of the plant but is concentrated in the roots and leaves and accounts for more than 80% of the cassava cyanogenic glucosides (Cereda and Mattos, 1996). Linamarin is a β-glucoside of acetone cyanohydrin and ethyl-methyl-ketone-cyanohydrin. The β-linkage of linamarin can only be broken down under high pressure, temperature and use of mineral acids. However, the cassava plant has endogenous linamarase (β-glucosidase), an enzyme which can easily break down linamarin. The enzymatic reaction occurs under optimum conditions at 25°C and pH 5.5 – 6.0 (Cereda and Mattos, 1996). When the enzyme and linamarin come in contact, linamarase breaks down linamarin into acetone cyanohydrin and glucose (Figure 3). The acetone cyanohydrin is then further degraded into acetone and hydrocyanic acid by another enzyme hydroxynitrile lyase (Figure 5). Hydroxynitrile lyase only occurs in the leaves, however, the conversion of acetone cyanohydrin to hydrocyanic acid and acetone can occur spontaneously at pH > 5 (Holzapfel, 2000). The HCN and acetone may either evaporate or is removed during successive stages of preparation.

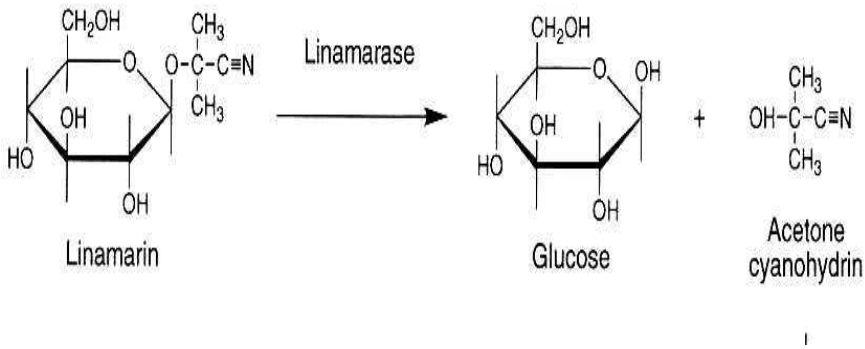

Figure 4: β-glucosidase breaks down linamarin into glucose and acetone cyanohydrin

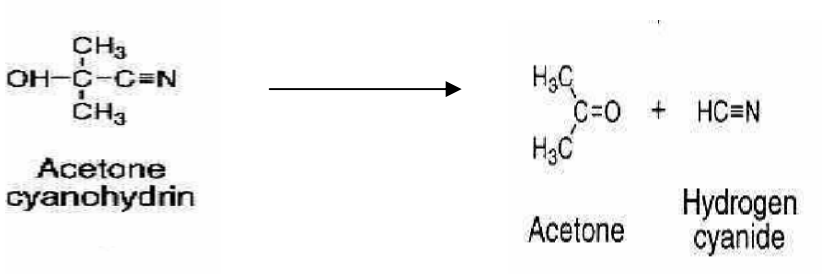

Figure 5: The breakdown of acetone cyanohydrin into acetone and HCN. Cyanide released can evaporate or dissolve in water.

Hydrocyanic acid is toxic to higher animals because cyanide forms a very strong bond with the heme-group by displacing Fe^{2+} to form the cyanohemoglobin. This results in a paralytic disease called Konzo. Exposure of humans to cyanide also causes acute toxic effects, iodine deficiency disorder, tropical ataxic neuropathy and destruction of cells in the body (Tylleskar *et al.,* 1992). Inhaled from atmospheric air or absorbed through the skin during processing, hydrocyanic acid passes into the bloodstream and undergoes various reactions in the liver and kidneys. In microorganisms, cyanide interferes with the oxidative phosphorylation pathway by combining with cytochrome-oxidate and inhibiting electronic transportation, and consequently, ATP formation. However, some microorganisms can grow in cyanide-containing substrates due to their anaerobic metabolism, their alternative metabolism regarding the respiratory chain and their capacity to detoxify cyanide by splitting the CN^- radical into carbon and nitrogen (Jensen and Abdel-Ghaffar, 1979). The average lethal dose of cyanide in higher animals was experimentally determined to be 1 mg/kg of live weight (Cereda and Mattos, 1996). Though hydrocyanic concentrations of 15 – 400 mg/kg of fresh weight in cassava varieties are reported in the literature, more frequent values fall within the interval o f 15 – 150 mg/kg (Cereda and Mattos, 1996)). There are, however, cassava varieties which contain concentrations above 1000 mg/kg of CN^- (Cereda and Mattos, 1996). While successive preparation processes reduce cyanide concentrations, residual cyanide has cumulative toxic effects in consumers of cassava-based products. Since cassava is almost completely devoid of protein and deficient in vitamins and minerals (Table 2), malnourishment is often a problem among lactating women, infants and pre-school children when it is consumed as a sole staple food. To improve its nutritional quality, cassava is often mixed with soybean for protein and palm oil (west Africa) or coconut milk (east Africa) for vitamin A.

Another problem with cassava is its short shelf-life, owing to ease of spoilage by microorganisms. Once out of the soil, post-harvest deterioration sets in, in about 24 – 48 hours (Wenham, 1995). This involves discoloration and microbial spoilage. Another serious threat is posed by the growth of filamentous fungi on stored cassava roots at a relative moistre content of about 17 – 40 percent (Sanni, 1996). These fungi can produce mycotoxins, extracellular zootoxic exotoxins. Some mycotoxins, such as aflatoxins produced by *Aspergillus flavus*, are implicated in oesophagal cancer. Other effects of mycotoxins include allergic reactions, reproductive failure, loss of appetite and suppression of immune system.

To reduce toxicity due to cyanogenic glucosides and to prolong shelf-life, cassava is processed into a variety of food products. Cassava products can be classified into nine groups as follows according to Ugbwu and Ay (1992): cooked fresh roots, granular roasted cassava (gari), granular cooked cassava (attieke, kwosai), fermented pasties, sedimented starches, drinks with cassava components, leaves cooked as vegetables and medicines. Fermented foods are popular as fermentation not only has preservation effect but also gives these foods their unique aroma, flavour and texture which are much appreciated by consumers. The conditions generated by the fermentation are essential in ensuring shelf-life and microbiological safety.

1.2.4 Cassava as a fermentation substrate

As pointed out earlier, cassava forms a substrate for a variety of food products in Africa. The most popular cassava processing method is fermentation. Making use of naturally occurring microorganisms, cassava is allowed to ferment in solid state to produce food products with desirable taste, texture and aroma. These include gari, attieke, kivunde and kapok pogari. Of these, gari is the most important foods in Africa.

1.2.5 Gari

Gari is the most important staple produced from cassava (Sanni, 1996). It is a coarse granular gelatinised flour that is primarily consumed in the form of a meal called eba. Its preparation follows the scheme as illustrated in Figure 6.

Root preparation: The roots are prepared within 48 hours after harvesting. Processing involves removing the ends and chopping the remainder into short pieces of about 15 – 20 cm long (Steinkraus *et al*., 1977). The roots are then fed into the peeler or peeled by hand.

Peeling: The cassava peeler is a rotating concrete mixer-like eccentric drum with an abrasive lining. Peeling is accomplished within 3 minutes through the combined action of the abrasive lining and the cassava roots rubbing one another as the rotating drum revolves at 40 rpm. Water washes the peels away from the roots. Loss of material can be as high as 40 % if the process is unduly prolonged.

Grating: The peeled roots are fed into a grating machine with revolving blades of 2.5 cm impact cross section. The grating brings the previously compartmentalised cyanogenic glucosides in contact with linamarin, where it is broken down to acetone cyanohydrin and glucose. The mash resulting from grating is dewatered to about 50 % moistre content while retaining at least 70 % of its weight. The dewatering facilitates the subsequent solid state fermentation.

Fermentation: The fermentation of cassava is the most important step in gari production. The cassava mash is inoculated with cassava liquor from a three-day-old fermented mash (backslopping) at a rate of 1 litre of liquor to 45 kg of pulp (Steinkraus *et al.,* 1977). This reduces the fermentation time from 4 days to 24 hours. The grated pulp is transferred to a jute sack and left to ferment in solid state.
Spontaneous fermentation is also used where the process is initiated without the use of an inoculum. However, due to the lag phase that microorganisms have to go through, fermentation times are longer and the risk of fermentation failure is high. The 3-day old cassava juice used for seeding contains microorganisms in their early stationary phase. Such microorganisms will have synthesised the necessary enzymes to be able to utilise nutrients in the substrate, hence the absence of the lag phase.

Thus, through repeated use of inoculums from a previous successful fermentation, the best-adapted strains are selected. Many organisms have been isolated from cassava fermentations. The predominant lactic acid bacteria isolated from fermenting cassava products including gari are *Lactobacilli, Leuconostoc* and *Streptococci* (Okafor and Uzuegbu, 1987). Some fungi have also been isolated from cassava fermentations, notably *Geotrichum candidum* and *Candida tropicalis*. Some of these fungi are thought to be responsible for starch hydrolysis into simpler carbohydrates which are then utilised by bacteria. However, lactic acid bacteria are thought to be responsible for the sensory qualities and texture of gari.

Dewatering: The fermented pulp is placed in a nylon mesh bags and dewatered in a hydraulic press to form a cake of 47-50 % moistre content.

Roasting (garification): The filtered cake is disintegrated and then roasted in a rotary kiln. When the core temperature in the kiln has reached 260 to 280°C, the cassava mash becomes partially gelatinised with a moistre content of 40 %. The gelatinisation process requires high heat and low mass transfer. The roasting stage is critical for the swelling of the gari.

Drying: The drying of the gelatinised mash cassava requires low heat and high mass transfer. The final product has a moistre content of about 8 % when it has cooled down.

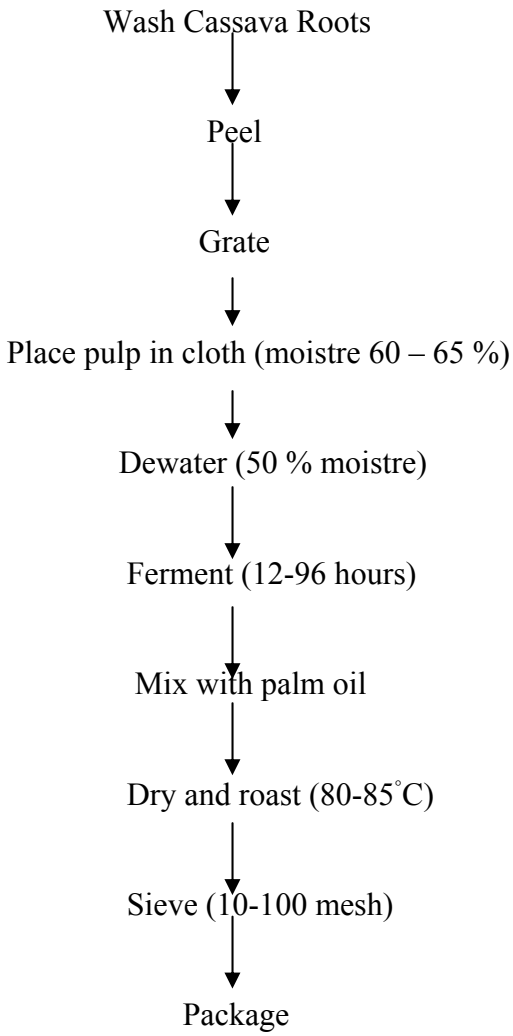

Figure 6: Flow sheet: Traditional household scale production of Nigerian gari (Okafor, 1977).

1.2.6 Control of processes

Fermentation and roasting are the most important stages in gari production and are responsible for product quality. To obtain an acceptable product, these stages must be carefully controlled. Rapid acid production is necessary and the process proceeds best at a temperature of about 35°C. Sunlight and frequent mixing of the pulp accelerates fermentation (Steinkraus *et al*., 1977). Most of the cyanide (HCN) and cyanogenic glucosides are removed during fermentation (Ofuya and Nnajiofor, 1988). This makes control and optimisation of fermentation a crucial stage in producing a quality product.

Gari having a total acidity of 0.53 to 1.2 % as lactic acid has satisfactory flavour (Steinkrauss *et al*., 1977). The sour taste is produced by organic acids produced during fermentation. Acetic, lactic, propionic, succinic and pyruvic acids have been identified as components of flavour and aroma in gari (Steinkraus *et al*., 1977). Aldehydes and esters also contribute to the characteristic aroma in gari. The fine granular, creamy texture of gelatinised gari which replaces the fibrous texture of cassava roots, makes it more digestible.

1.2.7 Microbiology of the fermentation

Starch is a complex carbohydrate that can be degraded only by organisms that produce α-amylase to produce simple sugars that can then be readily metabolised by many microorganisms. Stable co-metabolism between bacteria and yeasts is common in many food fermentations, enabling the utilisation of substances that are otherwise non-fermentable, such as starch (Gobbetti and Corsetti, 1997). It appears that the early stages of this fermentation are dominated by *Lactobacillus* and *Leuconostoc* spp. (Ofuya and Nnajiofor, 1988). Lactic acid bacteria lack a fully functional electron transport chain and TCA cycle for energy generation and make use of substrate level phosphorylation to generate ATP. During this process, pyruvate is used as both an electron donor and acceptor, resulting in the formation of lactic acid as an end-product. The acidic environment created by lactic acid bacteria, favours the proliferation of yeasts while the growth of bacteria is stimulated by vitamins and soluble nitrogen compounds produced by the yeasts (Nout, 1991). Thus the association of lactic acid bacteria during fermentation also contribute metabolites which impart taste and flavour to gari (Hansen and Hansen, 1996).

As pointed out earlier, *Geotrichum candidum* and *Candida tropicalis* are the main yeasts isolated from cassava fermentations. The main lactic acid bacteria isolated from cassava fermentations include *Lactobacillus plantarum, L. fermentum, L. brevis, L. salivarius, L. delbruckii and L. coryneformis.* Others bacteria are *Leuconostoc mesenteroides* subspecies *acremonis* and *Alcaligenes*. However, it is possible that other microorganisms are also present, but are masked by the rapid growing lactic acid bacteria.

The flavour, aroma and texture of gari is evidently a combined result of the interaction of all these microorganisms. It is believed that for efficient fermentation, rapid production of acid is useful. The acidic medium so created would discourage and inhibit non-fermenting microorganisms. It is also believed that hydrolysis of linamarin is enhanced at low pH (Ofuya and Nnajiofor, 1988).

However, both traditional methods of gari production, spontaneous fermentation and backslopping are inadequate. Initiation of a spontaneous fermentation takes long (24-48 hrs) with high risk for failure (Holzapfel *et al*, 2000). During the long lag phase, contaminating organisms from the environment slowly increase in number and compete for nutrients in order to produce metabolites. In addition, spontaneous fermentation leads to product inconsistencies.

While backslopping improves the fermentation time considerably, residual cyanide in the gari remains at levels that may cause cumulative toxicity. Though the maximum value recommended by Codex/FAO for cassava flour is 10 mg/kg, concentrations lower than this can accumulate to cause toxicity over prolonged periods (Holzapfel *et al.* 2000).

1.2.8 Starter cultures

Traditional methods of gari production are too time consuming and labour intensive. It takes, for example, 96 hours to obtain a good quality gari from cassava. Most of this time is for fermentation. In addition, traditional fermentation methods leave high residual cyanide in the gari, because the endogenous linamarase does not permit the complete breakdown of linamarin (Giraud *et al.,* 1993). Therefore, an improved fermentation method that will not compromise the quality and safety of the product, is necessary.

One way suggested to reduce cyanide in the final product was the use of exogenous linamarase (Ofuya and Nnajiofor, 1988). However, even if this was practical, it will raise the overall cost of the product and the technology may not be within the reach of local producers, because enzymes are quite expensive and their use would require some expertise.

One possibility that has not been adequately explored, is the use of starter cultures. A starter culture is a preparation containing numbers of variable microorganisms to

accelerate the fermentation process. The use of a pure inoculum will provide a means of standardising the production process resulting in a product of uniform quality and contribute to reducing the processing time. However, the main criteria for such organisms would be their ability to reduce cyanide to below toxic levels while retaining the desirable organoleptic qualities of gari. Such a culture should have no lag phase and be able to grow rapidly to significantly shorten fermentation time. It is also desirable for the culture to rapidly drop the pH and increase the acidity, as acidic conditions are said to promote the degradation of linamarin and may inhibit the growth of and toxin production by enteropathogens (Mugula *et al*., 2002).

In experiments done by Ofuya and Nnajiofor (1988), the growth rate of starter cultures consisting of *Leuconostoc*, *Streptococcus, Lactobacillus* and a *Saccharomyces* spp., was similar to that of the natural inoculum. However, the starter cultures grew faster with specific growth rate (μ) of 0.07/h after fermentation of 24 hours. Whereas natural inoculum had a steady growth rate throughout the period of fermentation, the starter culture grew exponentially. Thus, fermentation time can be reduced by as much as 50 % using an appropriate starter culture (Okafor *et al*.,1997).

Since cassava is rich in starch, a starter that rapidly produces more reducing sugars would be a prerequisite for rapid pH drop and increase in titratable acidity. Such a starter culture would consist of microorganisms that are able to produce α-amylase. ά - Amylase hydrolyses starch to produce glucose and amylopectin.

Glucose is metabolised by fermenting bacteria to a variety of organic acids such as citric acid, acetic acid, propionic acid, butyric acid and lactic acid, which are responsible for the drop in pH and increase in titratable acidity. For cassava fermentation, a high lactic acid concentration (% w/w) is desired within the shortest possible time (Mugula *et al*., 2002). It appears that a desirable pH is quickly reached when a combined starter culture is used and this is necessary in order to obtain good sensory properties (Mugula *et al*., 2002).

1.2.9 Lactic acid bacteria

Lactic acid bacteria are generally mesophilic microorganisms but can grow at temperatures ranging from 5°C to 45 °C. They produce lactic acid from hexoses since they lack functional heme-linked electron transport chains and a functional

Tricarboxilic Acid Cycle (Krebs cycle). They obtain energy via substrate-level phosphorylation. There are two types of lactic acid bacteria, depending on the glycolytic pathway they use for energy generation. The homofermentative bacteria make use of the Embden-Meyerhof-Parnas pathway in which pyruvate is converted by lactate-dehydrogenase into lactic acid (Figure 7). During the process, pyruvate is used as both electron acceptor and electron donor and NAD^+ is regenerated. This is the same pathway used by yeasts, such as *Saccharomyces cerevisiae*, to produce ethanol. Heterofermentative lactic acid bacteria, on the other hand, produce a combination of lactic and acetic acid and ethanol. In addition to lactate-dehydrogenase, two other enzymes are present. Pyruvatedecarboxylase converts pyruvate to acetaldehyde, which is then converted to ethanol by alcoholdehydrogenase (Figure 8). These mixed-acid fermentations make lactic acid bacteria very useful in the food industry as the fermentation products are organic acids that impart aroma, flavour and texture and other organoleptic properties to food. The acids produced during fermentation also improve the shelf-life of food products because acids inhibit the growth of unwanted microorganisms.

Lactic acid, acetic acid and traces of butyric and propionic acids and ethanol are produced simultaneously in natural fermentations. However, lactic acid increases with time suggesting the more acid tolerant homofermentative lactic acid bacteria (eg. *L. plantarum*) dominate later stages of fermentation (Oyewole and Odunfa, 1990). Homofermentative lactic acid bacteria produce lactic acid as the sole end-product of glucose fermentation (Fitzgerald and Caplice, 1999). It appears the acid-sensitive *Leuconostoc mesenteroides* initiate fermentation and are later supplanted by the acid-tolerant *L. plantarum*. A number of lactic acid bacteria isolated from cassava fermentations have been found to produce linamarase (Okafor and Ejiofor, 1990).

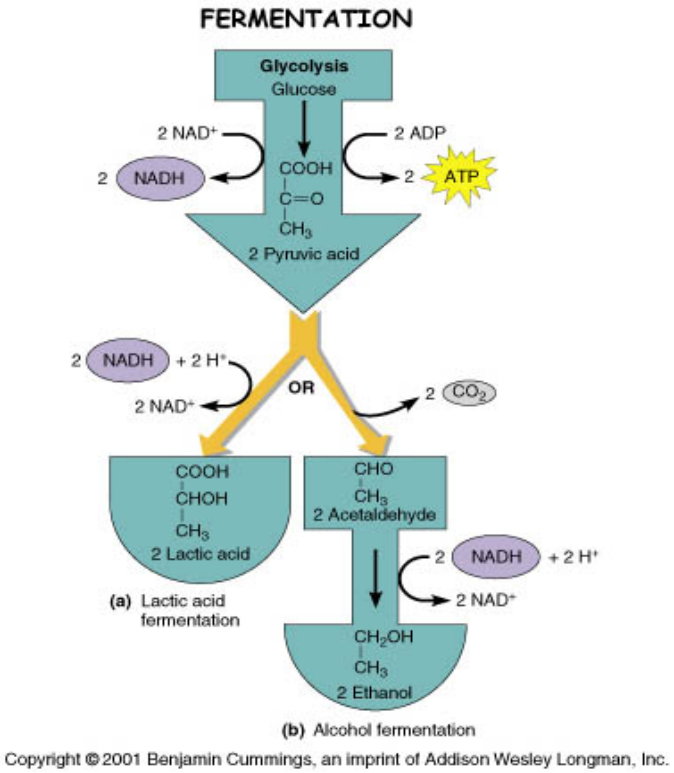

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Figure 7: The Emden Meyerhof pathway to lactic acid in lactic acid bacteria. This pathway is used by homolactic acid bacteria (*lactobacillus* sp.). The EMP can lead to a wide array of endproducts depending on the pathway taken in the reductive steps taken after the formation of pyruvate. In homolactic fermentation, lactic acid is the sole endproduct (http://www.utc.edu/Faculty/Becky-Bell/P-210-fermentation.jpg).

While some yeasts notably *Geotrichum candidum* and *Candida tropicalis* have been isolated from cassava fermentations, *Lactobacillus* spp. are the predominant fermentative microflora: *Lactobacillus plantarum, L. brevis, L. fermentum, L. salivarius and Leuconostoc mesenteroides* subsp. *cremoris* are the dominant organisms isolated from cassava fermentations (Amoa-Awua *et al.*, 2004). *Lactobacillus plantarum* and *Leuconostoc mesenteroides* subsp. *cremoris* are able to produce β-glucosidase in culture (Holzapfel *et al*., 2000). Various other lactic acid bacteria have the ability to hydrolyse linamarin (Giraud *et al*., 1992). In addition, there are *Lactobacillus* spp. with both extracellular amylolytic and intracellular linamarase activities. *Lactobacillus plantarum* strain A6, is able to simultaneously produce linamarase and amylase in significant amounts (Giraud *et al*., 1993), though synthesis of these enzymes is reported to be repressed by glucose.

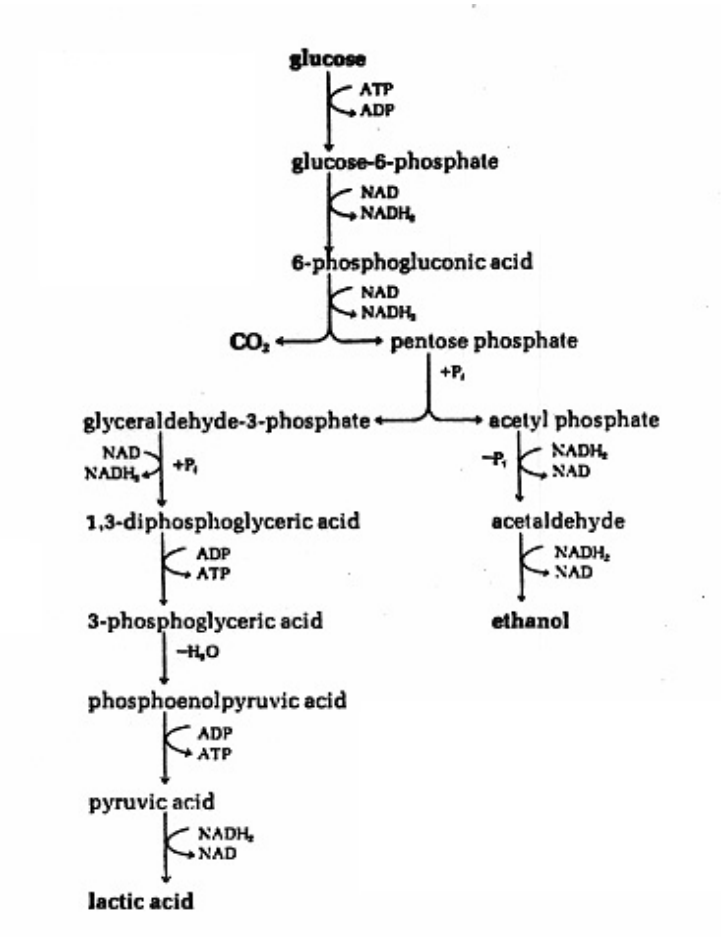

Figure 8: Heterolactic (Phosphoketolase) Pathway of fermentation. This pathway is used by *Lactobacillus* spp. and *Leuconostoc.* (http://www.bact.wisc.edu/Bact303/bact303metabolism).

Such organisms would make good starter organisms due to the ability to hydrolyse starch and degrade linamarin. A mixed culture of microorganisms that are able to produce α-amylase and linamarase simultaneously, should be able to rapidly produce acidic conditions for detoxification of gari. However, since microorganisms play a synergistic role, a starter culture can be constituted of microorganisms that are α-amylase negative and linamarase positive. Glucose released by α-amylase positive organisms could be utilised by the others for growth. Other organisms that produce neither α-amylase nor β-glucosidase (linamarase), might be responsible for the sensory qualities of the final product. A starter culture can therefore consist of a variety of synergistic organisms some of which would produce α-amylase and others only contributing to other qualities of the end product.

1.3 Aims

This study was aimed at evaluating selected lactic acid bacteria for use in a commercial dry powder starter culture formulation for gari production. The project was EU-funded and involved six partners: Germany (isolation and chracterisation of the microorganisms), CSIR (starter culture development and production), Belgium (pilot plant production), Benin (stability studies) and Kenya (market studies and impact assessment). The phases of the project are illustrated in Figure 9. The evaluation was based on the microorganisms' growth (maximum biomass formation), ability to shorten fermentation time to below 18 hours, drop the pH within 12 hours and reduce cyanide to below 10 mg/kg. The viability of cells after cryopreservation, biomass production in 2 litre fermenters, dehydration studies and solid state fermentation was also evaluated. It was important to determine cell viability after the long cryopreservation process. At present, no formulated starter culture for cassava fermentation is commercially available.

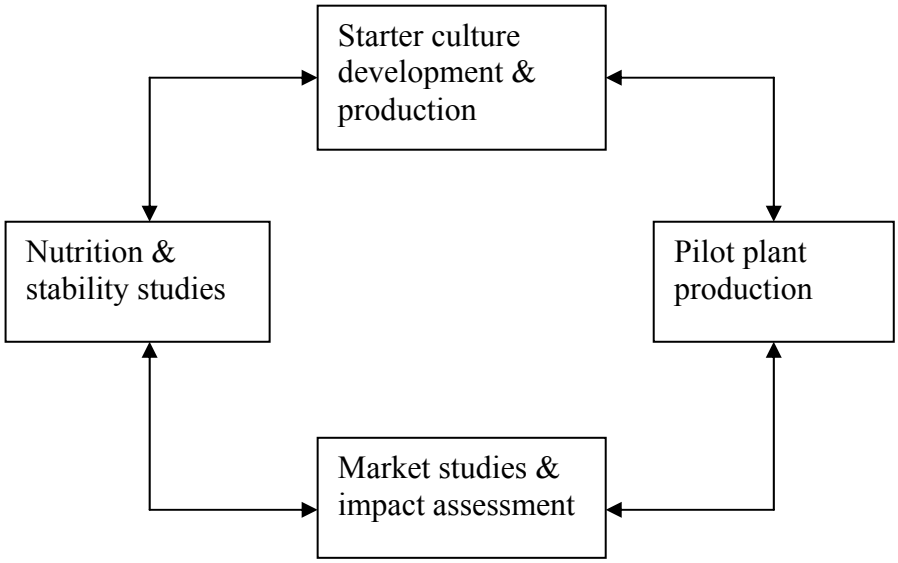

Figure 9: An illustration of the project phases.

2. Materials and Methods

2.1 Isolation of bacteria

Lactic acid bacteria have already been isolated from cassava fermentations in Benin and screened for both ß-glucosidase and α-amylase activities in Germany. The organisms were selected to represent the variety of organisms naturally found on the cassava roots, some of which produce both α-amylase and β-glucosidase, one of these enzymes or none. These isolates were cyopreserved at -78˚C at CSIR Bio/Chemtek (Food, Biological and Chemical Technologies) in Modderfontein.

Table 3: Characteristics of the isolated bacteria.

	L. plantarum	*L. plantarum*	*L. fermentum*	*Leuconostoc mesenteroides*
α -amylase	-	-	-	-
B-glucosidase	+	+	-	+
Fermentation products	Lactic acid	Lactic acid	Lactic acid ethanol	Lactic acid ethanol

2.2 Static flask studies (Growth Profiles)

Monocultures of the respective organisms were cryopreserved as follows: 750 µl of the culture was added to 750 µl taken from a 50:50 solution of glycerol and water. The cultures were stored at -78˚C. The cryovials were removed from the freezer and hand-thawed. A small drop was placed on a slide and studied under a microscope to see if every culture was monoculture. From previous work, it was found that *Lactobacillus* grows best in MRS medium. Hence this medium was used for static flask studies to determine growth profiles. An MRS broth medium (100 ml) of pH 6.0 was prepared in a 250 ml conical flask and sterilised for 15 minutes at 121°C. The flask volume-to-medium volume ratio was therefore 2.5:1. The medium was inoculated with whole content (1.5 ml) of a cryovial (monoculture) and incubated at 30°C for 48 hours. A sample was taken immediately for plate counts and

determination of glucose, optical density and pH. This sample time was taken as t = 0. Subsequent sampling was done every 3 hours for the first 12 hours and then at 24 and 48 hours. A total of 2 ml was taken for sampling from each flask at every given time. To reduce the risk of contamination, the sample for plate counts was sampled in a different McCartney bottle. The other sample (1 ml) was used for the analysis of glucose, optical density (OD_{660}) and pH. All samples were collected in autoclaved sample bottles. Glucose was determined using the Accutrend® *alpha* (Boehringer Mannheim), pH using pH Meter 3310 Jenway and the optical density using the Spectronic® 20 Genesys™ at 660 nm. Sterile water was used as a blank. The glucose reading was converted from mmol/l to g/l.

Dilutions were made by serially transferring 1 ml of sample to 9 ml of sterile water in a 100 ml volumetric flask. For plate counts, 2 µL was plated in duplicate onto MRS Agar and incubated at 30°C for 24 hours. Throughout the experiments, viable cell counts method (mean values) was used to estimate biomass as it is the most sensitive method of biomass estimation and is suitable for unicellular microorganisms (Rhodes and Stanbury,1997). The number of colonies that formed as a function of time where enumerated and expressed as colony-forming units/millimetre (CFU/ml). Other methods of biomass estimation (microbiological and physical methods) were explored (data not included). Of these, viable cell counts method was selected on the basis of its ease of use, accuracy (sensitivity) and limited hardware required. The physical methods of dry weight and turbidimetry are inaccurate when the fermentation medium contains undissolved particles. As a result, dry weight method was only used for correlation with viable cell count method.

2.3 Biomass Production (Submerged fermentation)

Biomass was produced in 2 L Biostat B (B. Braun Biotech International) fermentors. A working volume of 1.4 L was used, consisting of 1.3 L growth medium and 100 ml inoculum. The production medium for *Lactobacillus plantarum* was constituted as follows: 0.033 g/L $MgSO_4$, 0.033 g/L $MnSO_4$, 0.033 g/L $FeSO_4$, 13 g/L Corn Steep Liquor (Roquette), 26 g/L Yeast Extract, 1.3 g/L Malt Extract and 50 g/L glucose monohydrate. The medium for the other *L. plantarum* did not contain Meat Extract, but was otherwise the same as for the other *L. plantarum* strain. However, the

production medium for the heterolactic cultures (*L. fermentum* and *Leuconostoc mesenteroides*) consisted of an MRS medium with glucose adjusted to 20g/l. The medium was sterilised at 121°C for 15 minutes. Glucose monohydrate was sterilised separately. All chemicals were supplied by Merck and were anhydrous. The set point pH was 5.6, temperature 30˚C, stirrer speed 1000 rpm and airflow 0.3 slpm (standard liters per minute). The pH was controlled with 1N HCl and NaOH. The fermentation was run over 48 hours until glucose was depleted. Samples (2 ml) were taken every 3 hours for pH, glucose, OD_{660} (optical density) and viable cell counts. Biomass was estimated using the dry weight method. However, due to interference of the production medium, viable cell counts method was instead used to estimate biomass formation and the dry weight method was only used for correlation.

2.4 Centrifugation

The biomass produced from each organism was collected and centrifuged at 10 000 rpm in a Beckman ® Model J2 – 21, Rotor JA 14 for 10 minutes. After centrifugation the pellet was collected and sampled for plate counts. The supernatant was also sampled for plate counts. Cryoprotectants, 2 % and 5 % glycerol and maltodextrin, respectively, were added to each pellet. The amount of glycerol and maltodexrin added were calculated as a percentage of the mass of the cell biomass to which they were added. A sample was taken for plate counts to evaluate cell viability. At each stage, the pellets were weighed to keep track of mass balances. The final pellet was freeze-dried. For plate counts, 0.1g (equivalent to 100µL) of pellet was added to 0.9 ml (900µL) of sterile water to make up 1 ml. Then 9 ml of sterile water was added to the 1 ml of pellet and serial dilutions subsequently made.

2.5 Lyophilisation

The pellets were lyophilised as illustrated in Figure 10 below and stored indefinitely at -20˚C. To test for cell viability 0.1g of freeze-dried biomass was added to 900µL of sterile and the contents subsequently added to 9 ml of sterile water. Serial dilutions were then made and 2µl plated onto MRS Agar for plate counts.

Lyophilisation procedure

The vacuum set point for all steps was 80 mT (milliTor).

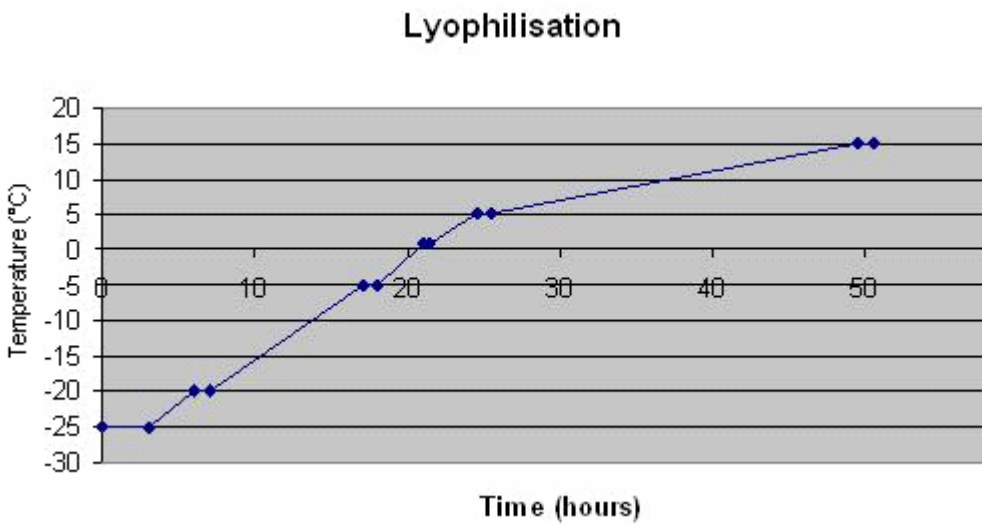

Figure 10: Lyophilisation of the concentrated bacterial culture. Note that actual drying takes place at 50.5 hours.

2.6 Solid state fermentation

2.6.1 Preparation of fermentation substrate

Cassava tubers were obtained from Mozambique and Mpumalanga and stored at -20°C. Batches were hand-peeled, washed and milled in a ball mill. The batches were vacuum-sealed and stored at 4.5°C. The masses of the cassava were recorded at each stage of processing to keep track of the mass balances. Soybean was soaked in water for 30 minutes and then coarse-milled. Following drying at 50°C for 12 hours, it was fine-milled and stored at 4.5°C. Palm oil was stored at 4.5°C and thawed in a water bath before use.

2.6.2 Inoculation with freeze-dried biomass

Cassava substrate was prepared as in the previous section. Cassava (500g) was inoculated with 0.1g of dry biomass dissolved in 1ml of 1% saline and mixed well. The buckets were incubated at 30°C for 48 hours. Samples were taken every 4 hours for pH and cyanide measurements and plate counts. For plate counts, 1g of solid

sample was added to 900 µl to constitute a 1 ml sample. This was then transferred to a 100 ml volumetric flask with 9 ml sterile water and serially diluted. Five replicate samples (10 samples) were prepared for each organism in each case.

2.6.3 Cyanide determination

Cyanide was analysed as Total Cyanide by AMPATH, a SANAS accredited laboratory to ISO/IEC 17025: Analytical science - M0066. The Conway-Dish method of Cyanide analysis was used where the sample was UV-digested in a closed environment to release all residual cyanide followed by colorimetric (spectrophotometric) analysis of the released cyanide. Cyanide remaining in the cassava substrate was expressed as Total Cyanide in microgram/gram (µg/g) of sample.

3. Results and Discussion

3.1 Static Flask studies

As can be seen from Figures 11 (a), (b), (c) and (d), all organisms formed maximum biomass of 10^8 CFU/ml within the first 12 hours. *Lactobacillus plantarum* (G4/18) formed the highest biomass of 4.5 x 10^8 CFU/ml (Figure 11(a)). The same strain produced 10^8 CFU/ml within 6 hours. It appears that the homofermentative organisms performed better in terms of biomass formation. Both *L. plantarum* strains formed a biomass of 4.5 x 10^8 and 2.5 x 10^8 CFU/ml within 12 hours respectively (Figure 11 (a) and (c)). This could possibly be attributed to acid tolerance of the organisms. A rapid pH reduction, particularly by the acid tolerant *L. plantarum* strains could be observed during the same time. *Leuconostoc mesenteroides,* being heterofermentative, is sensitive to low pH and would grow slower at these pHs. *Lactobacillus fermentum,* though heterofermentative, is reported to be particularly acid tolerant (Guyot, 2003). This can be seen from the 1.8 x10^8 CFU/ml that was formed at pH 4 at 12 hours compared to 1.5 x 10^8 CFU/ml at pH 5 for *Leuconostoc mesenteroides* (Figures 11(b) and (d)). From Figures 11 (a), (b), (c) and (d), it can be observed that OD_{660} correlated well with biomass as estimated by viable cell counts. *L. fermentum* utilised glucose quicker than the others: 17 g/L in 24 hours (Figure 11(b). However, this glucose utilisation was not translated into biomass production as 1.8 x 10^8 cells/ml were formed in 15 hours. In contrast, G4/18 utilised only about 12 g/l glucose and formed the highest biomass of 4.5 x 10^8 cells/ml in 24 hours (Figure 11(a). From this it appears like growth rate is independent of glucose utilisation. Within 24 and 48 hours, all the cells for *L. fermentum* and *Leuconostoc mesenteroides* had died off, while *L. plantarum* (G4/18), had a cell count of 3 x 10^8 CFU/ml at 48 hours (Figure 11 (a) - (d)). *L. fermentum* appeared to be the most sensitive to glucose concentration. From Figure 11(b), it can be seen that there was complete glucose depletion and die-off of cells at 24 hours. Viability of the cultures after cryopreservation was good since cell concentration of 10^8 CFU/ml was obtained within 12 hours. The apparent high growth of *L. plantarum* (G4/18) is comparable to that of the other three organisms since this high biomass was formed after a longer time period (24 hours). Thus, in terms of biomass formation in static flasks, none of the organisms performed better than the others. G4/18, however, formed the highest

biomass in 24 hours. The other three organisms formed less biomass in about 15 hours.

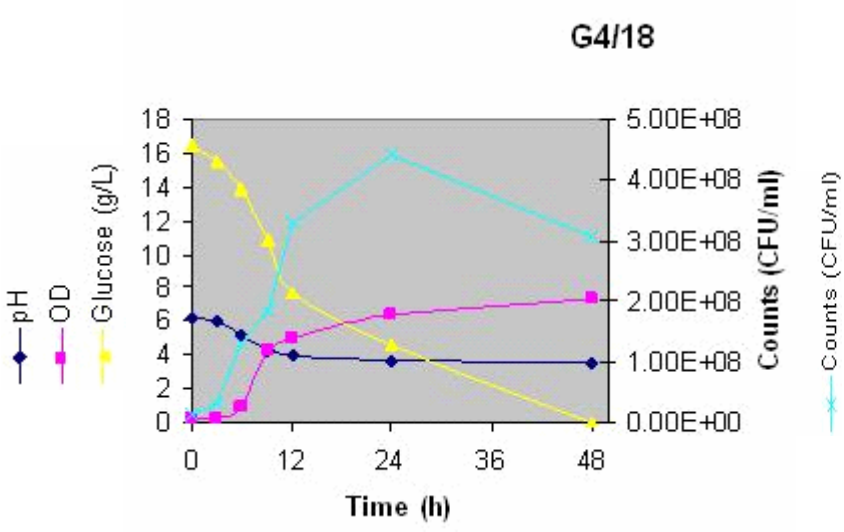

Figure 11 (a): Profiles of growth, pH, OD_{660} and glucose of *L. plantarum* in MRS medium.

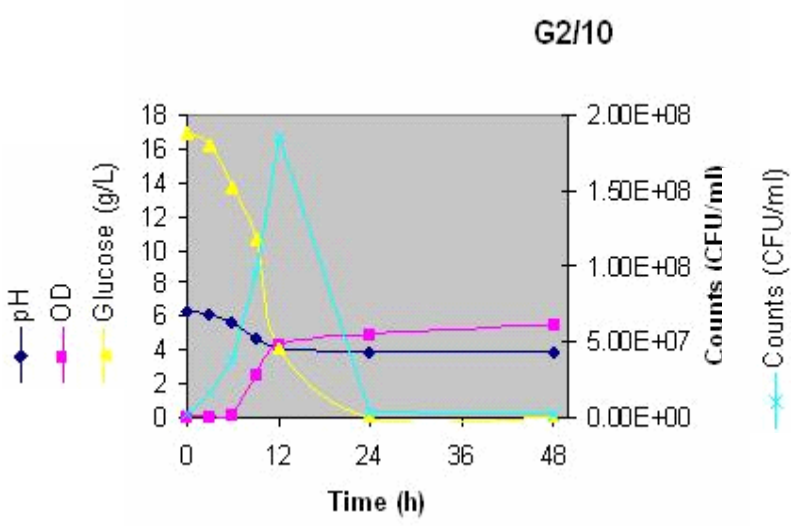

Figure 11(b): Profiles of growth, pH, OD_{660} and glucose of *L. fermentum* in MRS medium.

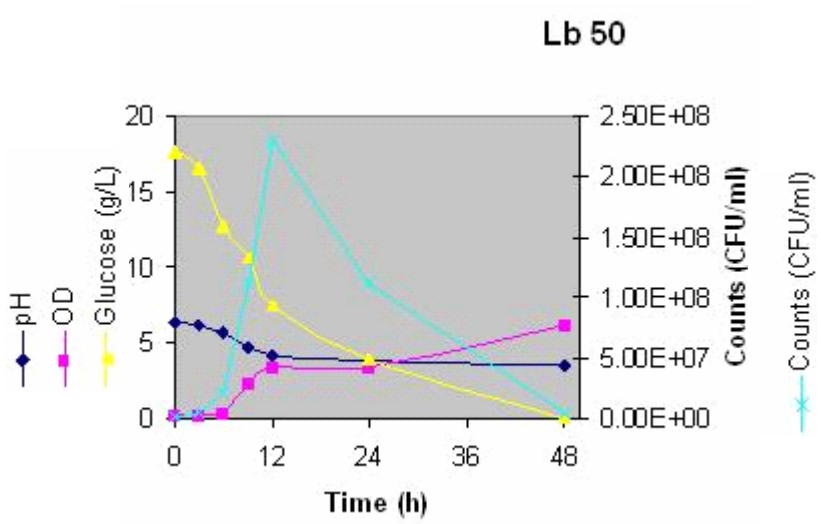

Figure 11(c): Profiles of growth, pH, OD_{660} and glucose of *L. plantarum* in MRS medium.

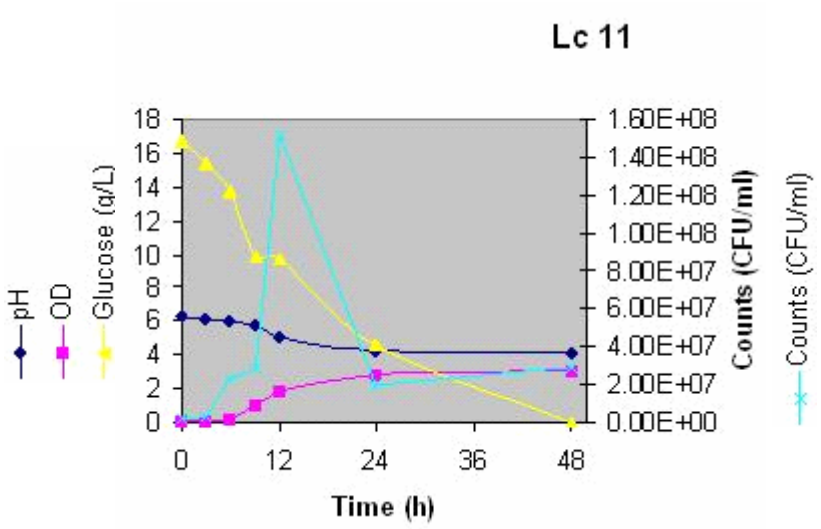

Figure 11(d): Profiles of growth, pH, OD_{660} and glucose of *Leuconostoc mesenteroides* in MRS medium.

3.2 Biomass Production (2L Braun Biostat fermenter)

The production media used appear to have promoted rapid growth and biomass formation. Peak biomass of 10^9 CFU/ml was formed within the first 6 hours for *L. fermentum, L. plantarum* (LB50) and *Leuconostoc mesenteroides*, as can be observed in Figures 12 (b), (c) and (d). Again, *L. plantarum* (G4/18) formed 10^{10} CFU/ml during the same period of time (Figure 12 (a). This could indicate that Meat Extract promotes growth as LB 50 and G4/18 were both *L. plantarum* strains and the production medium for G4/18 contained Meat Extract. However, since no such experiments were done with the two media, this can not be concluded. The acid tolerance of *L. fermentum* is again shown by its peak cell concentration at pH 5.6 (Figure 12 9b)). From Figures 12 (a), (b), (c) and (d), it can be seen that glucose was depleted in roughly half the time compared to that in MRS medium used for static flask studies. This possibly means that the production media were ideal in promoting optimum growth. It suggests that what limited growth for the heteroferemetative organisms, *L .fermentum* and *Leuconostoc*. *mesenteroides*, was the glucose concentration since the production medium contained 20 g/L of glucose compared to the 17 g/L of regular MRS medium used for static flask studies. Figures 12 (a), (b), (c) and (d) show good correlation between biomass, as estimated by plate counts, and $OD_{660.}$ However, dry weight is not a good measure of biomass for the Meat Extract-containing medium. The particles in the production medium may have contributed to the high biomass initially (Figure 12 (a). As the fermentation proceeded, these Meat Extract particles dissolved and the biomass subsequently declined. It is, therefore, important to pay careful attention to the method to be used for estimating biomass formation. However, for the other *L. plantarum* strain (LB50) grown on Meat Extract-deficient medium, dry weight correlated with viable cell counts (Figure 12 (b)). From Figures 12 (b), (c) and (d), an increase in biomass, even when glucose dropped to zero can be observed. It is not clear why this was the case. A series of experiments are required to investigate this phenomenon. The main objective of maximum biomass (X_{max}) production and glucose depletion within 12 hours was achieved. The production media used at bench-scale, should be evaluated in the pilot plant

production. At the given specifications, these cultures can form good amounts of biomass.

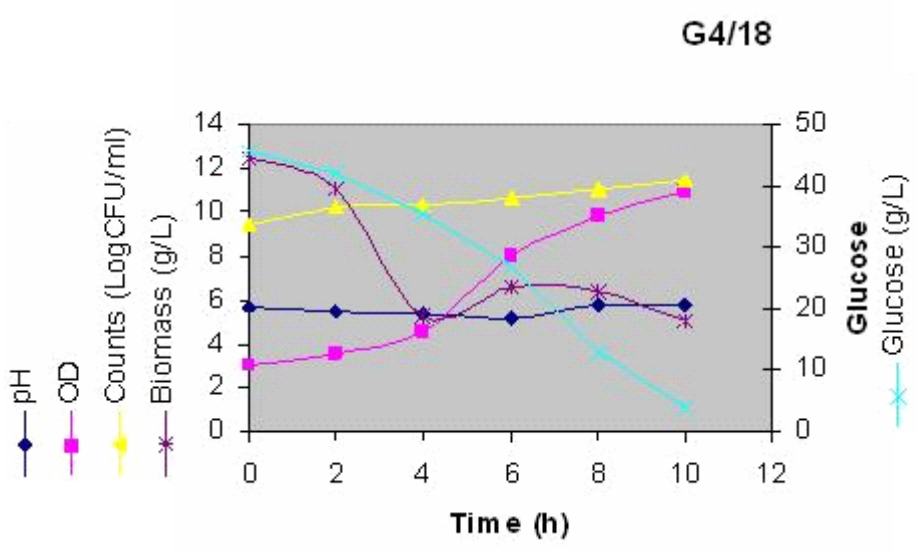

Figure 12 (a): Profiles of *L. plantarum* in 2 L Braun B Biostat fermenter.

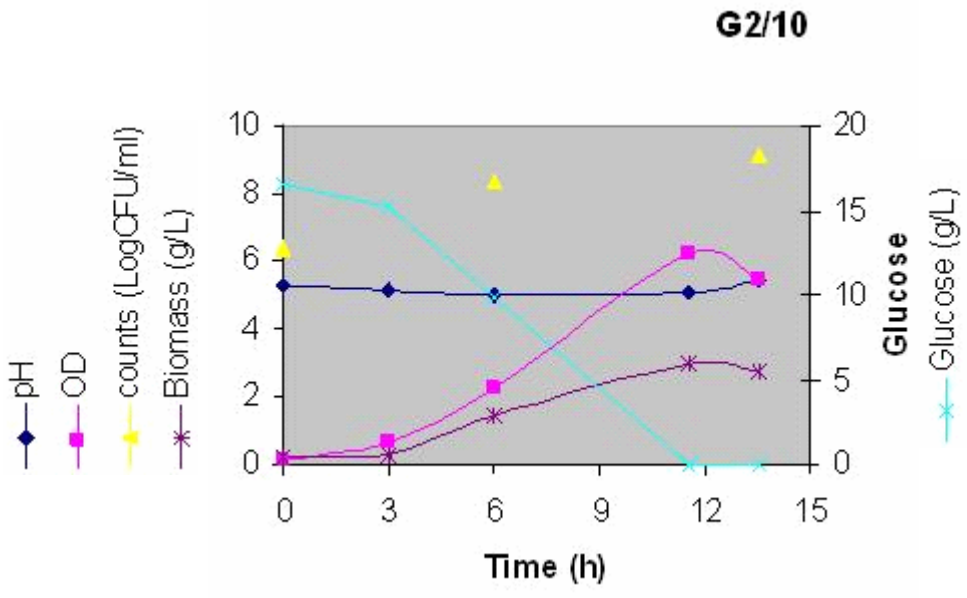

Figure 12 (b): Profiles of *L. fermentum* in 2 L Braun B Biostat fermenter.

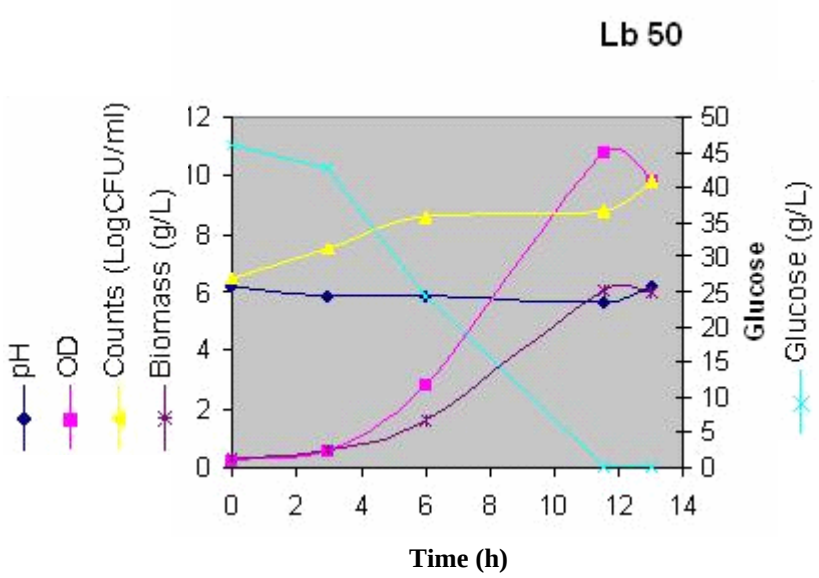

Figure 12 (c): Profiles of *L. plantarum* in 2 L Braun B Biostat fermenter.

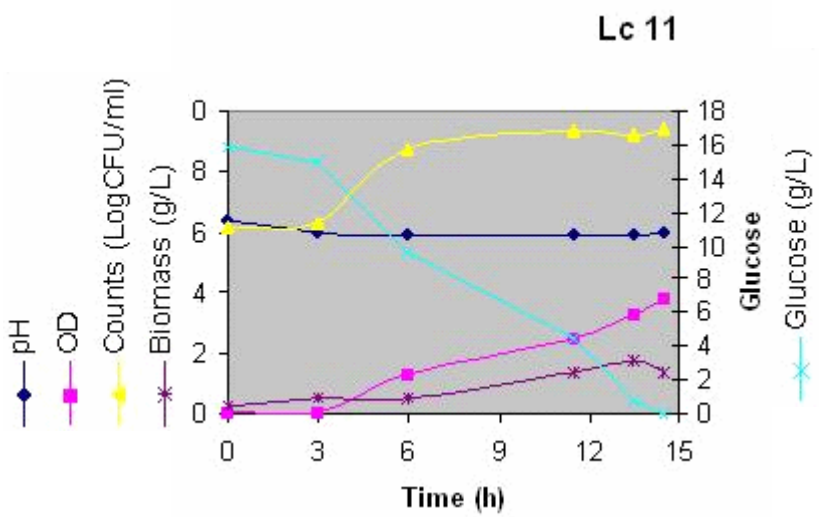

Figure 12 (d): Profiles of *Leuconostoc mesenteroides* in 2 L Braun B Biostat fermenter.

3.3 Centrifugation and lyophilisation

It can be observed from Table 4 that there was a hundred fold increase in cell concentration for *L. fermentum* and *Leuconostoc mesenteroides*. The increase in cell numbers could be due to the concentration of cells by the centrifugation step. There was a tenfold decrease for *Lactobacillus plantarum* (G4/18) and an increase for the other *Lactobacillus plantarum* species (LB50) in cell numbers/ml. Though it appears the organisms retained good viability when centrifuged and lyophilised at the stated specifications, the increase in viability at the centrifugation step could be due to the concentration of cells by this operation. Therefore, it can not be concluded with certainty that centrifugation operation gave good cell viability. For *L. plantarum* (G4/18), there was a loss of 10^2 cells for every 10^{10} cells/ml which still resulted in a viability of 80%. It is not known if different concentrations of the cryoprotectants (2% glycerol and 5% maltodextrin) or different cryoprotectants altogether would have improved the viability. Such experiments were not conducted and viability before and after addition of cryoprotectants was not determined. Therefore, it can not be concluded if the loss in viability was due to centrifugation, cryoprotectants or lyophilisation. Future viability experiments will have to be planned differently to bring into consideration the concentration of cell numbers by the centrifugation step.

Table 4: Cell viability between fermentation and lyophilisation (dehydration studies)

Organism	Fermentation (Biomass production) cells/ml	After lyophilisation (cells/ml)
L. plantarum (G4/18)	10^{10}	10^{8}
L. plantarum (LB50)	10^{9}	10^{11}
L. fermentum (G2/10)	10^{9}	10^{10}
Leuconostoc mesenteroides	10^{9}	10^{11}

3.4 Solid state fermentation (Cassava substrate)

3.4.1 Growth (cell biomass)

As can be observed from Figures 13 (a), (b), (c), (d) and (e), maximum cell numbers/ml were obtained in the first 12-15 hours of fermentation. *L. fermentum* and *Leuconostoc mesenteroides* produced the highest growth of 2 x 10^{11} and 5 x10^{10} cells/ml respectively (Figure13 (b), (d) and 14). Growth in cassava substrate can be compared as the initial concentration was taken. The growth of the organisms in cassava substrate, in general, compared favourably with the findings of Ofuya and Nnajiofor (1988) for cassava fermentation by lactic acid bacteria over 48 hours: 6.9 x 10^8 cells/ml. The control sample (not inoculated) had an initial lag phase of about 5 hours. Perhaps it is worth pointing out that the cassava substrate was stored at -20˚C for 3 months. Thus, the apparent lag phase in the control could be due to the low viability of the natural organisms caused by the long storage. The apparent absence of a lag phase in the inoculated samples seems to confirm this.

Though lactic acid bacteria are known for high growth rates on fermentable sugars, (Brauman *et al*., 1996), the phenomenal growth of the inoculated organisms was not expected, considering they were all α-amylase negative. There should not have been any glucose and hence, no carbon source for them to grow. However, since the substrate contained natural organisms, some of which could have been α-amylase positive, it is likely that glucose was provided by these organisms. It appears that the control organisms grew the slowest. Its peak cell concentration (X_{max}) was slightly less than 10^{10} cells/ml, as observed in Figure 13 (e) and 14.This could be due to the three months storage of cassava at -20°C. The peak cell concentrations (X_{max}) of the inoculated organisms were above 10^{10} cells/ml. From Figure 14, a general decline in growth (cell numbers/ml) after 12 - 15 hours can be observed which could be explained by glucose limitation. Glucose produced by natural organisms would have been quickly depleted due to the high cell numbers in the inoculum. The natural organisms would have been far outnumbered by the inoculum organisms, leading to rapid depletion of glucose. However it is unclear as to why there was a sudden increase in growth after 25 hours for *L. plantarum* (LB50) (Fig.12 and 13).

3.4.2 pH

From Figure 15, a pronounced reduction in pH between the control and the inoculated samples could be observed. At all intervals, the control pH was expectedly higher than in the inoculated samples (Fig.15). The lowest pH for the control sample was 4.5 compared to an average value of 3.98 for the inoculated samples (Figure 15). A notable difference was the sudden drop in pH from 6.4 to 4.5 (1.9 log units) for the inoculated samples compared to a more gradual drop of only 0.4 log units (from 6.4 to 6) for the control sample for the first 8 hours (Figure 14). This suggests a lag phase of 8 hours for the control before a critical mass of cells (cell concentration) were generated to rapidly drop the pH. This is confirmed by the growth of cells as observed in Figures 14 (a) – (e). A similar trend could be observed for *L. plantarum* (G4/18), which had a gradual pH reduction {Figure 13 (a) and 15}. In general, the organisms were able to drop the pH by two log units (from 6.5 to below 4.5) within 12 - 15 hours {Figures 13 (a) – (e)}. From Figures 13 (a), (b), (c) and 15, it can be observed that the acid-tolerant organisms were able to drop the pH to 3.98. This strain of *L. fermentum*'s acid tolerance, though being heterofermentative, was demonstrated here by its growth at pH 4.5 (Figure 13 (b) and 15)). For *L. fermentum* and the *L. plantarum* strains, this rapid pH drop correlated with X_{max} production. This is expected as more cells would produce more lactic acid. For *Leuconostoc mesenteroides*, the low pH probably inhibited its growth.

3.4.3 Cyanide reduction

There was a correlation between the cell numbers and cyanide concentration. The decline in cell numbers after 15 hours correlated with an increase in cyanide concentration over the same period of time (Figures.14 and 16). From Figures 14 and 15, it can be observed that when the cell numbers dropped from 10^{11} to just below 10^{10} cells/ml for *L. fermentum,* there was a corresponding increase in cyanide concentration from 3 - 8 μg/g during the same period of time (Figure 16). It appears like the log phase of the organisms correlate to the drop in cyanide concentration. This suggests that the enzyme production is related to biomass formation. This is not surprising as enzymes are growth related. The breakdown of cyanide is therefore linked to the linear growth of the organisms. It is safe to conclude that β-glucosidase was produced, leading to the breakdown of the cyanide complex in the substrate. This

assertion is further strengthened by the correlation between increased growth of *L. plantarum* (LB50) at 24 hours and decreased cyanide levels in the sample over the same period (Figures 14 and 16). As stated earlier, it is worth noting that maximum cell numbers (biomass) at 15 hours corresponded to lowest cyanide concentrations (Figures 14 and 16). However, it is not clear why such a high cyanide amount was released from the sample inoculated with G4/18 at 24 hours. The concentrations of the other samples at 24 hours, could be explained by the corresponding decline in cell numbers. However, this appeared to be contradicted by the fact that the control and *L. fermentum,* (G2/10) which was β-glucosidase negative, were able to reduce cyanide levels. It must be noted that the control and the sample inoculated with *L. fermentum* would have natural organisms, some of which could produce β-glucosidase. These organisms, since their growth compared well with the inoculums, must have been able to produce reasonable enzyme levels to degrade the cyanide complex. However, as no enzyme activity tests were done, this is only speculation. Equally, how the 3 months storage of cassava substrate's matrix was affected, needs to be investigated. Further experiments with fresh cassava should provide information in this regard. What is known is that the prolonged storage lead to the reduction of cyanide as the milling process brings the endogenous linamarase into contact with the substrate. This is possibly why the cyanide values in the cassava substrate were below 10 μg/g at t = 0. To exclude any influence due to this process, further experiments need to be conducted with fresh cassava.

There was no significant difference between the natural organisms in the control and *L. fermentum* samples on the one hand and the other samples that had high concentrations of β-glucosidase-producing organisms in terms of their ability to reduce cyanide. Therefore, it could not be concluded from these results if β-glucosidase (linamarase) was responsible for cyanide breakdown. Since cyanide diffuses out of the substrate over time, there is therefore a progressive decline in its concentration. This may explain the low concentration at 48 hours for all samples. It is possible that any reduction in cyanide concentration was due to endogenous linamarase rather than the linamarase from the organisms. In terms of cyanide reduction, a definite difference between the organisms that have this enzyme and those that don't was expected. Thus, the absence of this difference points to the role of

endogenous linamarase. The dependence of cyanide degradation on the pH was confirmed (Figures 15 and 16).

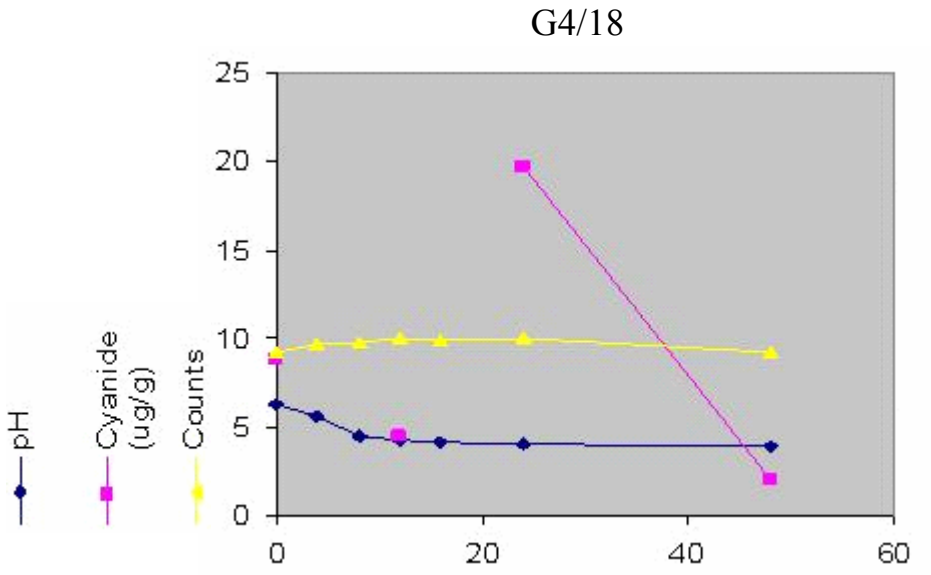

Figure 13 (a): Profiles of *L. plantarum* in cassava substrate.

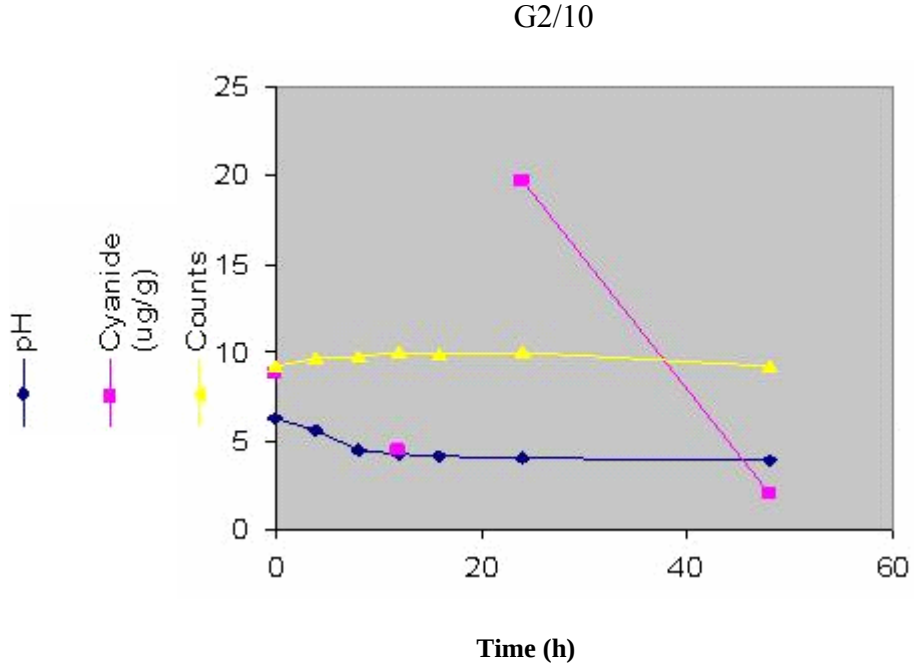

Figure 13 (b): Profiles of *L. fermentum* in cassava substrate.

LB50

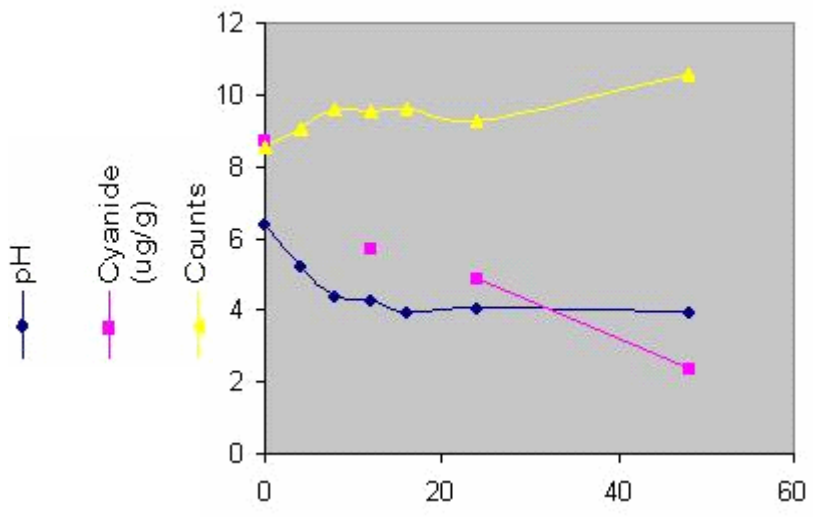

Figure 13 (c): Profiles of *L. plantarum* in cassava substrate.

LC11

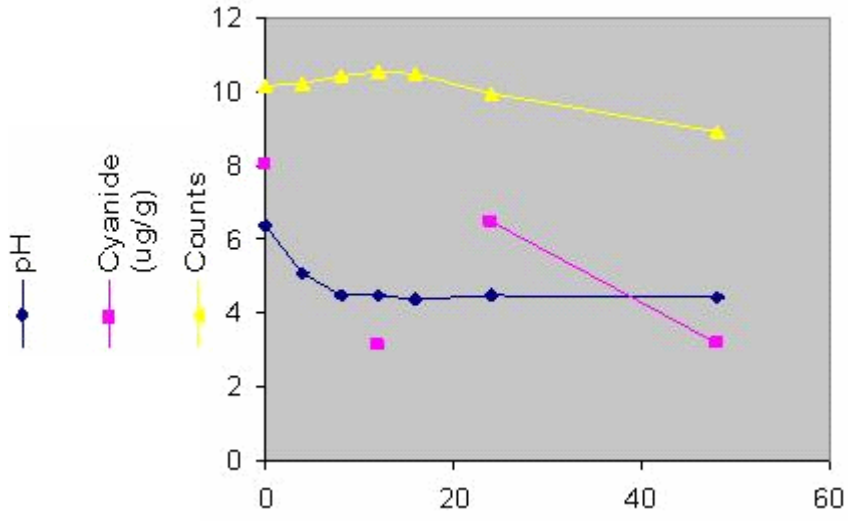

Figure 13 (d): Profiles of *Leuconostoc mesenteroides* in cassava substrate.

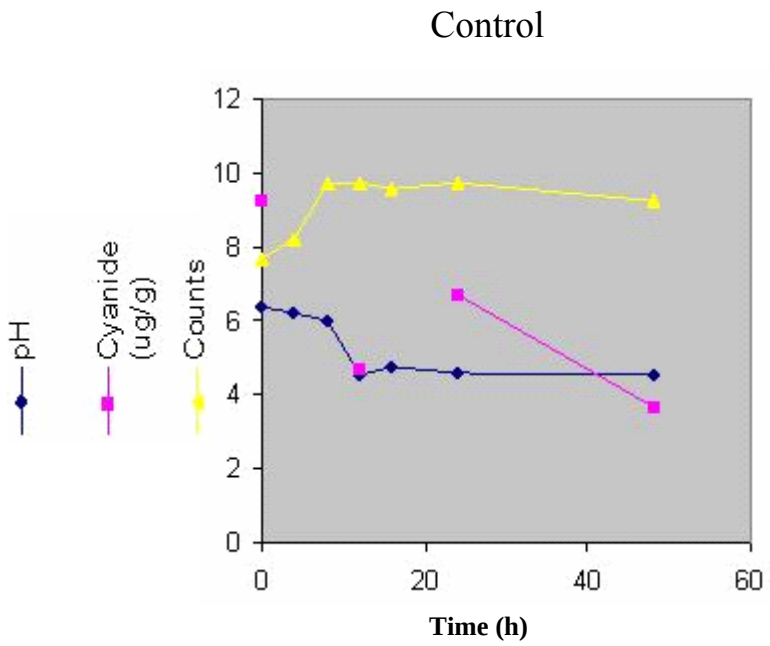

Figure 13 (e): Profiles of natural organisms (control) in cassava substrate.

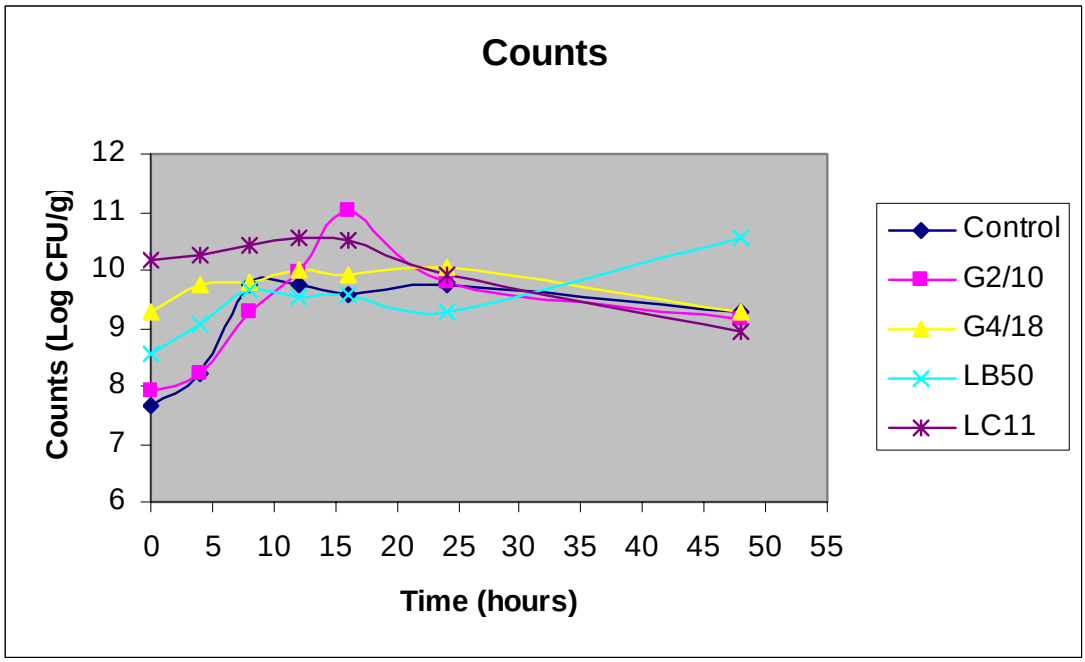

Figure 14: Comparison of the growth (biomass estimation) of the organisms in cassava substrate: G4/18 - *L. plantarum*, G2/10 - *L. fermentum*, LB50 - *L. plantarum*, LC11 - *Leuconostoc mesenteroides*, Control - natural organisms.

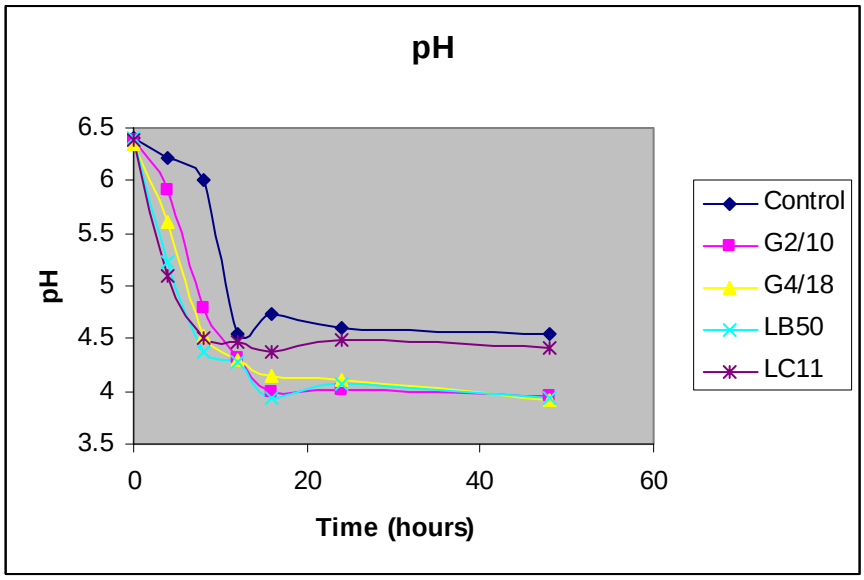

Figure 15: Comparison of pH reduction by the organisms in cassava substrate: G4/18 - *L. plantarum*, G2/10 - *L. fermentum*, LB50 - *L. plantarum*, LC11-*Leuconostoc mesenteroides*, Control - natural organisms.

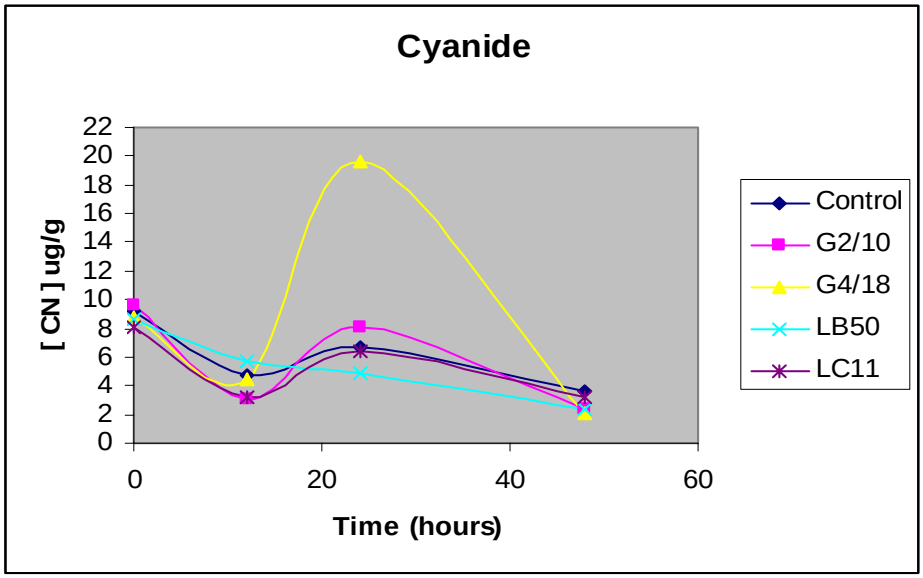

Figure 16: Comparison of cyanide reduction by the organisms in cassava substrate: G4/18 - *L. plantarum*, G2/10 - *L. fermentum*, LB50 - *L. plantarum*, LC11 - *Leuconostoc mesenteroides*, Control-natural organisms.

4. Conclusions

This work revealed that cultures of *L. plantarum, L .fermentum and Leuconostoc mesenteroides* could retain their viability after a prolonged period of cryopreservation at -78˚C. It could also be concluded that these organisms grow well in 2 L Braun Biostat fermenters on the production media used for each as evidenced by the rapid biomass formation and glucose utilisation. Viability retention for bench-scale dehydration processes of centrifugation and lyophilisation was inconclusive due to the concentration effect of the centrifugation step. Future experiments should be planned differently to cater for the influence of centrifugation on the concentration of the cells. Equally, further experiments need to be done to determine the contribution of each step (centrifugation, addition and concentration of cryoprotectants and lyophilisation) to the loss in viability. This could involve determination of viable cell counts after each dehydration step. However, it is not known if such high viability retention would be achieved if these processes were scaled up. In this regard, additional experiments involving viable cell counts of samples taken from scaled up processes should provide valuable information.

There seemed to be a correlation between growth of the organisms (biomass formation) and cyanide reduction. It appeared like to reduce cyanide, the cultures should be kept in the log phase. It is recommended that organisms that produce α-amylase be selected for a starter culture production so that there is no glucose limitation during fermentation. It could not be concluded if the organisms used had any measurable advantage over the natural ones in terms of cyanide reduction. Further cyanide experiments need to be conducted with fresh cassava to exclude the possible impact of the long cold storage on the fermentation outcome. This includes running the fermentation with fresh cassava and determining the residual cyanide. Since cyanide reduction in cassava was the major aim of this research, it could not be concluded if these organisms are suitable for use in pilot-scale studies of starter culture development. Future experiments should evaluate combinations of the microorganisms as microorganisms often behave synergistically.

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