SELECTION, PRODUCT CHARACTERISTICS AND METABOLITE SPECTRUM OF A COMMON STARTER CULTURE FOR *FUFU* AND *USI* PRODUCTION

BY

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ABSTRACT

Fermented cassava products like *fufu* and *usi* (edible starch) are important staple foods in many African homes. Natural fermentation time is usually long resulting in slower acidification process and inconsistent nutritional composition of products which could be overcome with the use of starter culture. However, most available starter cultures are used for single food fermentation and are uneconomical. This necessitates the development of a starter culture for multiple related food products to reduce cost. Hence this study was designed to produce a common starter culture for the production of *fufu* and *usi.*

Cassava varieties TME 30572, TME 4(2)1425 and TME 50395 were obtained from the International Institute of Tropical Agriculture, Ibadan and landraces from Bodija market. Fresh, peeled, chipped and grated cassava tubers were spontaneously fermented in the laboratory. Lactic Acid Bacteria (LAB) were isolated from the fermenting mash and identified phenotypically. Genotypically identified starters were selected based on screening for starch hydrolysis, linamarase and pectinase enzyme production, antimicrobial compound production and rate of acidification using standard methods. The starters were utilised singly and randomly combined to initiate fermentation for production of *fufu* and *usi*. Un-inoculated fermentation mash served as control. Rate of production of organic acids, various sugars, metabolic enzyme assays, nutritional and anti-nutritional content of the resulting mashes were monitored using standard procedures. Best starter was applied in the final production of *fufu* and *usi*. Shelf-life of the products were evaluated and compared with the control. Data were subjected to descriptive statistics and ANOVA technique at p=0.05.

Ninety-eight LABs were identified as *Lactobacillus plantarum* (50.0%), *L. acidilactici* (12.2%), *L. brevis* (11.3%), *L. fermentum* (10.3%), *L. delbrueckii* (8.2%), *L. mesenteroides* (6.0%), and *L. lactis* (2.0%). Screened isolates did not hydrolyse starch but produced pectinase, linamarase alongside hydrogen peroxide, diacetyl and lactate with a rapid decrease in medium pH (6.5 - 3.6). Selected potential starters were genotypically identified as *L. pentosus* F2A (A), *L. plantarum* subsp. *argentolarensis* F2B (B), *L. plantarum* F2C (C), *L. plantarum* U2A (G) and *L. paraplantarum* U2C (I). The best starter combination CGI gave significant reduction in fermentation pH (7.1 - 3.7) and lactic acid ranged between 0.04mg/ mL and 6.9mg/mL. Sugars produced include xylose

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(3.2µg/mL), arabinose (1.4µg/mL), fructose (26.2µg/mL), glucose (30.3µg/mL) and sucrose (99.7g/mL). Enzyme assay revealed peak amylase (10.1U/mL) and pectinase (4.4U/mL) activities at 24 hours as well as linamarase (0.8U/mL) at 48 hours in *fufu*, whereas, in *usi*, highest linamarase (0.7U/mL) and pectinase (1.0U/mL) activities were recorded at 72hours with no amylase activity. The CGI-produced *fufu* and *usi* had significant reduction in phytate (0.3-0.1mg/g and 0.3-0.27mg/g), tannin (35.4-34.0mg/g and 35.4-32.3mg/g), cyanide (0.1-0.05mg/g and 0.1-0.0mg/g), and moisture (7.3%-5.1% and 7.3%-5.4%) content while total protein content increased (1.0-1.3% and 1.0-1.8%) respectively. Starter fermented *fufu* and *usi* had shelf-life of five days while control had three days.

The selected starter was able to ferment both *fufu* and *usi* to yield products with improved nutritional content, better shelf-life and reduced anti-nutritional composition. This could be employed in the production of indigenous fermented foods.

Keywords: Lactic acid bacteria, Starter culture, *Fufu* and *Usi*, Shelf-life, Fermented food
Word count: 495

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DEDICATION

This thesis is dedicated to the memory of my late father, Murtala Olayinka OGUNMOLA.

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I am grateful to Almighty Allah, without whom this thesis would not have manifested, and for His continued guidance. I am especially thankful for the gift of life. To him alone be the glory and adoration forever.

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Your names will remain evergreen in my mind.

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CERTIFICATION

I certify that this work was carried out by **Kubrat Abiola, OYINLOLA** under my supervision in the Department of Microbiology, University of Ibadan, Ibadan. Nigeria.

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TABLE OF CONTENTS

	Page
ABSTRACT	**ii**
DEDICATION	**iv**
ACKNOWLEDGEMENT	**v**
CERTIFICATION	**vi**
TABLE OF CONTENTS	**vii**
LIST OF TABLES	**xiv**
LIST OF PLATES	**xvi**
LIST OF FIGURES	**xvii**
LIST OF ABBREVIATIONS	**xix**

CHAPTER ONE

INTRODUCTION

1.1	Background	1
1.2	Statement of Problem	3
1.3	Justification	3
1.4	Scope of the research	3
1.5	Aim and Objectives	4

CHAPTER TWO

LITERATURE REVIEW

2.1	Cassava	5
2.1.1	Origin of cassava	5
2.1.2	Importance and consumption	6
2.1.3	Shelf life	7
2.1.4	Cassava varieties	7
2.1.5	Nutritional composition	8

UNIVERSITY OF IBADAN

Content		Page

2.1.6 Anti-nutritional factors of cassava 11
2.2 Fermentation 13
2.2.1 Definition 13
2.2.2 Advantages of fermentation 13
2.2.3 Types of fermentation 15
2.2.3.1 Homolactic acid fermentation 16
2.2.3.2 Heterolactic acid fermentation 17
2.2.4 Fermented foods 17
2.3 Cassava fermentation 20
2.3.1 Submerged fermentation 20
2.3.2 Microbiology of cassava fermentation 21
2.3.3 Cassava products 22
2.3.3.1 *Fufu* 23
2.3.3.2 Usi 24
2.4 Starter culture 24
2.4.1 Definition 24
2.4.2 Properties of starter culture 28
2.4.3 Effect of starter on cassava fermentation 28
2.4.4 Metabolite spectrum of cassava fermentation 30

CHAPTER THREE
MATERIALS AND METHODS
3.1 Sample collection 33
3.1.1 Cassava samples 33
3.1.2 Organic acids and sugar standards 33
3.1.3 Test pathogenic isolates 33
3.2 Media and sample preparation 33
3.2.1 Media preparation 33
3.2.2 Sample Preparation and fermentation 34

UNIVERSITY OF IBADAN

Content		Page
3.3	Total titratable acidity of fermenting mash	34
3.4	pH of fermenting mash	34
3.5	Isolation and identification of organisms from fermenting cassava tubers	34
3.5.1	Isolation technique	
3.5.2	Characterization of selected isolates	34
3.5.2.1	Morphological Characterization	35
	i Macroscopic	35
	ii Microscopic	35
3.5.2.2	Biochemical Characterization	35
	i Catalase test	35
	ii Motility test	36
	iii Voges-Proskauer test	36
	iv Oxidase test	36
	v Indole test	37
	vi Methyl red test	37
	vii Starch Hydrolysis	37
	viii Production of ammonium from arginine	37
	ix Sugar fermentation test	37
3.5.3	Identification of organisms	38
3.6	Molecular characterization	38
3.6.1	DNA extraction	38
3.6.2	DNA amplification	38
3.6.3	Profile sequencing	39
3.7	Screening for potential starters	39
3.7.1	Starch hydrolysis	39
3.7.2	Production of linamarase	39
3.7.3	Production of pectinase	40
3.7.4	Acidification of growth medium by selected isolates	40
3.7.5	Production of Lactic acid	40
3.7.6	Production of Hydrogen peroxide	40
3.7.7	Production of Diacetyl	41
3.7.8	Antimicrobial activity against pathogenic organisms	41

UNIVERSITY OF IBADAN

Content	Page
3.7.8.1 Preparation of Cell-Free Filtrate	41
3.7.8.2 Agar Well Diffusion Test	41
3.8 Fermentation studies with identified isolates	42
3.8.1 Preparation of cassava for controlled fermentation	42
3.8.2 Determination of inoculum size	42
3.9 Performance studies on fermenting mash	43
3.9.1 pH of fermenting mash	43
3.9.2 Total titratable acidity of fermenting mash	43
3.9.3 Nutritional Analysis of fermenting mash	43
3.9.3.1 Determination of moisture content	43
3.9.3.2 Determination of crude protein	44
3.9.3.3 Determination of crude fat	44
3.9.3.4 Determination of ash content	45
3.9.3.5 Determination of fibre content	45
3.9.3.6 Determination of total carbohydrate	45
3.9.4 Anti-nutritional factors of the fermenting cassava mash	45
3.9.4.1Determination of cyanide content	45
3.9.4.2 Estimation of phytic acid	46
3.9.4.3 Estimation of tannin content	46
3.10 Estimation of enzyme activities during fermentation	47
3.10.1 Enzyme crude extracts	47
3.10.2 Amylase activity	47
3.10.3 Linamarase assay	47
3.10.4 Pectinase assay	48
3.11 Analysis of metabolites in fermenting mash	48
3.12 Selection of starter	49
3.13 Optimization studies on selected starter	49
3.13.1 Determination of inoculum size	49
3.13.2 Determination of optimal pH	49
3.13.3 Determination of optimal incubation temperature	49
3.13.4 Determination of optimal salt concentration	50

UNIVERSITY OF IBADAN

Content		Page

3.13.5 Effect of agitation on growth 50

3.13.6 Effect of carbon sources on growth 50

3.13.7 Effect of nitrogen sources on growth 50

3.13.8 Effect of different incubation time on growth 51

3.14 Application of starter in traditional processing of *fufu* and *usi* under optimized growth conditions and performance study on the products 51

3.14.1 Inoculum preparation 51

3.14.2 Preparation of *Fufu* and *Usi* 51

3.14.3 Nutritional analysis of mash fermented with starter grown under optimised condition 52

3.14.4 Sensory evaluation of products 52

3.14.5 Microbiological assessment and physical evaluation of products during storage 53

3.15 Statistical Analysis 53

CHAPTER FOUR

RESULTS

4.1 Physico-chemical analysis of spontaneously-fermenting cassava for *fufu* and *usi* 54

4.2 Microbial load during spontaneous *fufu* and *usi* fermentation 54

4.3 Isolation of Lactic Acid Bacteria from fermenting cassava mash 58

4.4 Screening for potential starters among selected Lactic Acid Bacteria 61

4.5 Molecular identification of screened potential starters 68

4.6 Fermentation of cassava with identified potential starters 71

4.6.1 pH of starter-fermented cassava mash 71

4.6.2 Total titratable acidity (%) of starter-fermented cassava mash 71

UNIVERSITY OF IBADAN

Content		Page

4.6.3 Proximate analysis of starter-fermented cassava for *fufu* and *usi* production 77

4.6.4 Anti-nutritional factors of starter-fermented cassava for *fufu* and *usi* production 80

4.6.5 Selection of a common starter for both products 82

4.6.6 Enzyme activities during starter fermentation for *fufu* and *usi* production 85

4.6.7 Analysis of organic acid and sugars during *fufu* and *usi* fermentation 89

4.7 Optimization of growth conditions for starter CGI 95

4.7.1 Effect of different pH values on the growth of selected starter 95

4.7.2 Effect of different incubation temperature on the growth of selected starter 95

4.7.3 Effect of different salt concentrations on the growth of selected starter 98

4.7.4 Effect of different agitation speed on the growth of selected starter 98

4.7.5 Effect of different carbon sources on the growth of selected starter 98

4.7.6 Effect of different nitrogen sources on the growth of selected starter 98

4.7.7 Effect of different incubation time on the growth of selected starter 103

4.8 Effect of optimization of starter growth conditions on proximate and anti-nutritional factors (%) of fermented *fufu* and *usi* mashes 103

4.9 Sensory evaluation of starter-fermented *fufu* and *usi* 107

4.10 Microbial load during fufu and usi storage at room temperature 107

UNIVERSITY OF IBADAN

CHAPTER FIVE

5.0 DISCUSSION, SUMMARY AND CONCLUSION 115

CONTRIBUTION OF THE STUDY TO KNOWLEDGE 136
REFERENCES 137
APPENDIX 185

UNIVERSITY OF IBADAN

LIST OF TABLES

Table	Content	Page
2.1	Nutrient Composition of fresh cassava roots per 100g of edible portion	10
2.2	Different classification of fermented foods	19
4.1	Microbial count (10^8 CFU/mL) of spontaneously-fermenting cassava for *fufu* and *usi*	57
4.2	Colonial morphology of randomly selected Lactic Acid Bacteria	59
4.3	Biochemical characteristics of selected LAB.	60
4.4	Frequency of occurrence (%) of selected Lactic Acid Bacteria during spontaneous cassava fermentation.	62
4.5	Starch hydrolysis, linamarase and pectinase enzyme production by selected isolates	63
4.6	Acidification (pH) of growth medium by selected isolates	64
4.7	Quantity of lactic acid produced in g/L by selected strains of Lactic Acid Bacteria	65
4.8	Quantity of hydrogen peroxide produced in µg/L by selected strains of Lactic Acid Bacteria	66
4.9	Quantity of diacetyl produced in g/L by selected strains of Lactic Acid Bacteria	67
4.10	Antagonistic effect of selected isolates against test pathogenic organisms.	69
4.11	Molecular identification of selected potential starters	70
4.12	Single and randomly combined LAB isolates used as potential starters for *fufu* and *usi* production.	73
4.13	pH during starter fermentation of cassava for *fufu* and *usi* production	75
4.14	Total titratable acidity of mash during starter fermentation of cassava for *fufu* and *usi* production	76
4.15	Proximate composition (%) of starter fermented cassava for *fufu* production after 72-hour fermentation	78

UNIVERSITY OF IBADAN

Table	Content	Page
4.16	Proximate composition (%) of starter fermented cassava for *usi* production after 72hours fermentation	79
4.17	Anti-nutritional factors of fresh and starter-fermented cassava for *fufu* and *usi* production	81
4.18	Selection of a common starter using pH and anti-nutritional factors	83
4.19	Selection of a common starter using proximate composition	84
4.20	Effect of optimization of starter growth conditions on nutritional contents (%) of fermented *fufu* and *usi* mashes	105
4.21	Effect of optimization of starter growth conditions on anti-nutritional contents (mg/g) of fermented *fufu* and *usi* mashes	106
4.22a	Sensory evaluation of starter-fermented and spontaneously fermented *usi*	109
4.22b	Sensory evaluation of starter-fermented and spontaneously fermented *fufu*	110
4.23a	Microbial load during *usi* storage at room temperature	111
4.23b	Microbial load during *fufu* storage at room temperature	112

UNIVERSITY OF IBADAN

LIST OF PLATES

Plate		Page
4.1	Cassava fermentation with single and combined starter cultures using plastic bioreactors	74
4.2	Laboratory prepared starter-fermented *usi* and *fufu*	108
4.3	Spoilage symptoms on *usi* and *fufu* on the 7th storage day	114

UNIVERSITY OF IBADAN

LIST OF FIGURES

Figure	Content	Page
2.1	Flow chart for traditional *fufu* and *usi* production	26
4.1	pH of spontaneously-fermenting cassava for *fufu* and *usi*	55
4.2	Titratable acidity (%) of spontaneously-fermenting cassava for *fufu* and *usi*.	56
4.3	Phylogenetic relationship between identified starters and closely related organisms in the GenBank.	72
4.4	Amylase activities in starter fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation	86
4.5	Pectinase activities in starter fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation	87
4.6	Linamarase activities in starter fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation	88
4.7	Lactic acid quantities at different time intervals during spontaneous and starter (CGI) fermentation for *fufu*	90
4.8	Lactic acid quantities at different time intervals during spontaneous and starter (CGI) fermentation for *usi*.	91
4.9a	Sugar quantities at different time intervals during starter (CGI) fermentation for *usi*.	93
4.9b	Sugar quantities at different time intervals during starter (CGI) fermentation for *usi*.	93
4.10a	Sugar quantities at different time intervals during starter (CGI) fermentation for *fufu*.	94
4.10b	Sugar quantities at different time intervals during starter (CGI) fermentation for *fufu*.	94
4.11	Effect of different pH values on the growth of selected starter (CGI)	96
4.12	Effect of different incubation temperature on the growth of selected starter (CGI).	97
4.13	Effect of different NaCl concentrations on the growth of selected starter (CGI)	99

UNIVERSITY OF IBADAN

Figure	Content	Page

4.14 Effect of different agitation speed on growth of selected starter (CGI). 100
4.15 Effect of different carbon sources on the growth of selected starter (CGI). 101
4.16 Effect of different nitrogen sources on the growth of selected starter (CGI) 102
4.17 Effect of different incubation time on the growth of selected starter (CGI) 104

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LIST OF ABBREVIATIONS

%	Percentage
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
Bp	Boiling point
cal	Calorie
CDM	Chemically defined medium
CFU	Colony forming unit
cm	Centimetre
DM	Dry matter
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalysilic acid
FAO	Food and Agricultural Organization of United Nations
FW	Fresh weight
g	Gram
g/L	Gram per litre
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrogen chloride
HCN	Hydrogen cyanide
kcal	Kilocalorie
kg	Kilogramme
KH_2PO_4	Potassium dihydrogen phosphate
$KMnO_4$	Potassium permanganate
L	Litre
LAB	Lactic acid bacteria
m	Meter
M	Molar
MEA	Malt Extract Agar
MEGA	Molecular Evolution Genetics Analysis
mg	Milligramme
mg/g	Milligram per gram
mL	Millilitre

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mm	Millimetre
MRS	Mann Rogosa Sharpe
NAD+	Nicotinamide Adenine dinucleotide
NADH	Nicotinamide Adenine dinucleotide hydrogenase
NaOH	Sodium hydroxide
nm	Nanometer
^{O}C	Degree Celcius
OD	Optical density
PCA	Plate Count Agar
pKa	Acid dissociation constant
PNPG	Para-nitrophenyl-B-D-glucopyranoside
ppm	Parts per million
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SON	Standards Organization of Nigeria
Sp.	Species (singular)
spp.	Species (plural)
SSF	Solid state fermentation
TRIP	Tuber and Root Improvement Programme
USA	United States of America
UV	Ultraviolet
v/v	Volume per volume
VRBGA	Violet Red Bile Glucose Agar
w/v	Weight per volume
β	Beta
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

In Africa, cassava is very important to the people because fermented cassava products are known to constitute a major part of the daily diets in many homes. It is cultivated widely as a food crop, ranked as the world′s sixth most important (Soccol, 1996) and fourth on the list of major food crops in developing countries after rice, wheat and maize (Mingli *et al*., 1992). De Bruijin and Fresco (1989) reported a progressive increase in demand of fermented cassava products yearly as a result of the high energy content due to the fact that it provides averagely more than 50% of daily energy intake.

However, cyanogenic glucosides inherent in cassava usually restrict its use as a food crop (Koch *et al.,* 1992; Peifan *et al.,* 2004) even though there is an endogenous linamarase (β-glucosidase), an enzyme which can easily hydrolyse linamarin, situated in the cell wall (Mkpong *et a*l., 1990). It was reported that the endogenous linamarase could not completely breakdown the linamarin (Ikediobi and Onyike, 1982; Mkpong *et al*., 1989) thus, bringing about the addition of an exogenous linamarase during fermentation, which is by far the most important and widely used means of processing cassava (Oyewole, 1992; Nweke *et al*., 2002) to reduce cyanogenic toxicity (Ikediobi and Onyike, 1982).

Fermentation, the oldest method of food processing, started over 6000 years ago (Holzapfel, 2002) in which the traditional methods and outdated techniques of producing fermented foods were based on spontaneous fermentation due to naturally occurring microorganisms in the environment and on the raw materials. However, fermentation durations were long due to the lag phase of the organisms, thus, yielding a longer acidification process and making it difficult to produce an end product of consistent quality. Developing countries cannot continue to be dependent on the historic methods for food processing because of factors such as increasing populations,

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drought and other natural disasters, inadequate food production as well as other associated problems such as long fermentation time, inconsistencies in final products and the presence of pathogenic organisms, all because it depended on chance inoculation from the environment.

Common research approaches have included isolation and characterization of microorganisms that could be used as starter culture with modifications to fermentation regimes. To date, little of this research has been put to use. Part of technology considerations suggested by Baseline Consultancy Report for Cassava in 2010 included the use of isolated starter cultures in maintaining product quality. Therefore, an improved fermentation method that will not compromise the quality and safety of the product would be through the use of starter cultures which are preparations or materials containing large number of viable microorganisms which may be added to facilitate improved and controlled fermentation process (Holzapfel, 1997, 2002).

Fufu and *Usi* (edible starch) are among the products of cassava fermentation in Africa (Etejere and Bhat, 1985). *Fufu* is an important basic commodity, ranked next to gari as a native food of most Nigerians (Sanni *et al.*, 1998) and widely eaten in many parts of West Africa and the Tropics (Sanni, 1989). It is a sticky cassava mash which is cooked in boiling water and consumed with soup. It is eaten mostly in the Eastern and Western parts of Southern Nigeria as well as some other areas of West and Central Africa; and unlike other fermented cassava products, it has very intense odour (Lancaster *et al.*, 1982). *Usi* is an indigenous food of the Itsekiri and Urhobo in Southern Nigeria, who also refer to it as edible starch. It is a very pasty, light yellow food eaten with any oil or pepper soup. The starch is precipitated out of the solution pressed out of the grated cassava during the preparation of gari and sometimes, obtained from grated cassava, soaked directly in water (Etejere and Bhat, 1985). Both cassava products undergo lactic acid fermentation by several microorganisms, thus yielding various metabolites which confer positive effects such as preservation, flavour development, cyanide reduction and changes in functional properties on the final product (Akindahunsi *et al.*, 1999).

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The use of starters will provide a means of standardising the production process resulting in products of uniform quality and contributes to reduction in processing time. Furthermore, such starter will have the ability to detoxify, while retaining the desirable organoleptic qualities of the product, grow rapidly to significantly shorten fermentation time, rapidly drop the pH and increase the acidity, as acidic conditions inhibit the growth of and toxin production by pathogens (Mugula *et al*., 2002).

1.2 Statement of Problem

Constantly, there is an increase in demand for fermented cassava products because they are high energy yielding foods but indigenous spontaneous fermentation have been characterized with longer acidification time, inconsistencies in the nutritional composition and quality of the final products. Use of starter culture has brought some improvement on fermented cassava products, but most available starter cultures are used for single food fermentation and are uneconomical.

1.3 Justification

The ability to isolate strains of microorganisms with desirable physiological and metabolic characteristics for use as starter culture will result in a high degree of control over the fermentation process, thus, maintaining consistency. Furthermore, the possibility of developing a common starter culture for multiple related food products will reduce cost and be of economic importance.

1.4 Scope of the research

In order to establish the selection process, this study was approached in four phases, namely; isolation, characterization, identification and screening for potential starters; utilization of potential starters both singly and in combination for controlled fermentation; physiological studies and optimisation of growth conditions of selected starter(s); utilisation of the starter(s) in *fufu* and *usi* fermentation and final product assessment.

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1.5 Aim and objectives

The overall aim of this study is to select a common starter culture for the production of *fufu* and *usi*. Specifically, this study was designed to:

- Isolate, characterize and identify lactic acid bacteria involved in the fermentation of cassava to produce *fufu* and *usi*.
- Screen for, and genotypically identify potential starters.
- Utilise selected isolates solely and in combination to ferment cassava in *fufu* and *usi* production, monitoring microbiological, nutritional and technological properties as well as derived metabolites during the fermentation processes, thus, select isolate(s) of best fit.
- Carry out optimization of growth studies on the potential starter(s) selected
- Apply the selected starter(s) in the production of the two products and analyse the end products.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Cassava

Cassava (*Manihot esculenta Crantz*), a 1-2 m high woody shrub with an edible root is a perennial crop. It is made up of an ariel part (2-4 m) and an underground part (edible root) clustered around the lowermost part of the plant and extends about 60 cm on all sides (Pandey *et al*., 2000). A single root may weigh as much as four kilogram under convinient conditions. The number of roots per plant at harvest varies from 2 to 7, each averaging 27.7 to 43.3 cm long and from 4.5 to 7.4 cm in diameter. A central vascular core, the cortex (flesh) and the phelloderm (peels) makes up a mature root. Cassava peels account for 10–12% of the total dry matter of the root and are 1–4 mm thick (Nartey, 1979).

Cassava, one of the most useful tropical crops widely exploited as a cheap energy source in Africa, Asia, South America and India (de Bruijin and Fresco, 1989) was however castigated as an inferior food crop (Kwatia, 1986), poor people′s crop (Hahn and Keyser, 1985) and a dangerous crop (Cheok, 1978) because of major limitations like low protein content, short postharvest shelf life and the presence of toxic cyanogenic glucosides.

2.1.1 Origin of cassava

In tropical Africa cassava has assumed the status of a security and industrial crop, alleviating the food crisis in many war-torn and drought ravaged parts of Africa because it can be cultivated throughout the year without regard to the seasons. It produces high yield and grows with limited water (Hahn and Keysar, 1985; Oyewole, 2002).

Although, it was reported to have originated in Venezuela, South America during 2700B.C. (Soccol, 1996), Philips (1982) established its being introduced to Nigeria and other parts of West Africa by the Portuguese. It was estimated by

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FAO that 37% of the 13.4 million ton world production was produced by African countries. The main cassava producing countries of Africa include Nigeria, Benin, Kenya, Zambia, Tanzania, Uganda, Ghana, Zimbabwe, Democratic Republic of Congo and Mozambique (Okigbo, 1980; Nweke *et al*., 2002). In 1996, Soccol reported Africa as the largest producer with about 53% of the world′s production. Although, it was cultivated in about 88 countries, only 5 countries accounted for 67% of the production and these include Nigeria, Brazil, Thailand, Zaire and Indonesia. Purseglove (1968) reported that cassava was known around the North of the River Niger in 1914 but it has now become one of the most important staple food crops of the inhabitants in Nigeria and known by different names among the ethnic groups of the country. The Yoruba call it *gbaguda* or *ege*, the Hausa, *rogo, karaza* or *doyar kudu,* the Ibo, *akpu, abacha* or *jigbo,* the Benin, *igari*, the Efik, *iwa unene* while the Urhobo refer to it as *imidaka* (Etejere and Bhat, 1985)

2.1.2 Importance and Consumption of cassava

Cassava plays a major role in efforts to alleviate the African food crisis because of its efficient production of food energy, year-round availability, tolerance to extreme stress conditions, and suitability to present farming and food systems in Africa (Hahn and Keyser 1985, Hahn *et al*., 1987). Much recognition has been dwelled on the importance of cassava and among reported ones are its use as source of income and raw material in the manufacturing of processed food, animal feed as well as industrial products (Beléia *et al*., 2004). Wider utilization of cassava products can be a catalyst for rural industrial development and raise the income for producers, processors and traders, contribute to the food security status of its producing and consuming households as well as its high efficiency in converting solar energy to starch (Dufour *et al*., 1996).

Total world cassava use was projected to increase from 172.7 million ton to 275 million ton over a period of 27 years (1993-2020), using the International Food Policy Research Institute baseline data whereas, a higher prediction of demand and production growth puts the 2020 production at 291 million tons (Scott *et al*., 2000). In both projections, cassava use in Africa is equivalent to 62% of total world production with an average of about 102 kg/person/year or 220 kcal/person/day (Giraud, 1993) and Nigeria is the current leading producing country (FAO, 2008). Almost all the

UNIVERSITY OF IBADAN

cassava produced globally is used for human consumption, either in natural form as flour or in fermented forms and other products with only 5% being used industrially (Bokanga and Otoo, 1991; FAO, 2002; Ajao & Adegun, 2009).

There is an increase in demand yearly as it was reported by Seigler and Pereira in 1981 to have been consumed by 300 million people. However, Cock (1982, 1985), FAO (2000) and Mroso (2003) reported it to be consumed by more than 500 million people worldwide, including 80 million from Nigeria alone (Okafor and Ejiofor, 1990).

2.1.3 Shelf life of cassava

Other tuber crops such as yam and sweet potato are not as perishable as cassava roots (Poulter, 1995). Physiological deterioration (primary or secondary) occurs 2-3 days after harvesting, followed by microbial deterioration 3-5 days thereafter (Rickard and Coursey, 1981; Rickard, 1985; Akingbala *et al*., 2005). The primary deterioration is an endogenous physiological process called vascular streaking brought about by damage to the roots during postharvest handling which results in a fine blue-black or brown discoloration. This usually occurs when phenolic compounds present in the tubers are converted to coloured compounds called quinines and the process is catalyzed by the enzyme, polyphenol oxidase which acts on the phenolic compounds in the presence of oxygen (Rickard and Coursey, 1981; Rickard, 1985; Sakai *et al*., 1986). Furthermore, dehydration caused by the physical damage to the tubers worsens the conditions thus, making the tissues become portals of entry for pathogens leading to the secondary deterioration by microorganisms. The microbial spoilage involves rotting, softening or fermentation of the tissue by microorganisms (Rickard and Coursey, 1981; Uritani *et al.,* 1984).

2.1.4 Cassava varieties

There are many natural cassava varieties (cultivars) and are classified according to morphological traits as well as taste, cyanide content, average yield, disease performance and pubescence (MIC, 2007; Gbadegesin *et al*., 2013). However, in recent times, a number of regional programs have been initiated to breed improved varieties of cassava to increase yield and resistance to diseases. Various studies have shown that the physicochemical, functional and other quality characteristics of cassava

UNIVERSITY OF IBADAN

products are significantly affected by varietal chemical composition differences such as dry matter, starch content and quality (Safo-Kantanka and Owusu-Nipah, 1992). Even though more than 5,000 cassava cultivars are recognized globally (Best, 1993; Gade, 2003; IFAD/FAO, 2005), several improved cassava varieties have been recommended and released in Nigeria (IITA, 2004). Among the most commonly grown are TME 30572, 4(2)1425, 92/0326 and NR8082. The major genetic factor that determines quality of roots is dry matter content and more recently, 42 new improved genotypes have been made available to farmers (IITA, 2004) with qualities like multiple resistance/tolerance to cassava mosaic disease and other major problems of cassava, bacterial blight disease, anthracnose, green mite, and mealybug. They are high yielding varieties, suitable for use as food and livestock feed as well as a raw material in industry.

2.1.5 Nutritional composition of cassava

Cassava roots, though deficient in protein (less than 1.5% of fresh weight), are rich in carbohydrates (31% of fresh weight) with most of it being present as starch hence, its utilization by most people in tropics as sources of carbohydrate (Blagbrough *et al*., 2010). The edible starchy flesh comprises some 80- 90% total weight of the root as water, forming the major components (Wheatley *et al*., 1993; Harris and Koomson, 2011) and it is between the range of 60.3 - 87.1% (Padonou *et al*., 2005; Zvinavashe *et al*., 2011). Water is an important parameter in the storage of cassava with low levels giving desirable and relatively longer shelf life (Padonou *et al*., 2010; Harris and Koomson, 2011).

Cassava contains about 1-2% protein which makes it a predominantly starchy food (Charles *et al*., 2005). The protein content is low (1% - 3%) on a dry matter basis (Buitrago, 1990) and between 0.4 and 1.5% per 100 g fresh weight (Bradbury and Holloway, 1988). However, about 50% of the crude protein in the roots consists of whole protein and the other 50% in the form of free amino acids (primarily glutamic and aspartic acids) and non-protein components such as nitrite, nitrate and cyanogenic compounds (Zvinavashe *et al*., 2011). The root also has high content of dietary fibre, magnesium, sodium, riboflavin, nicotinic acid, thiamine and citrate (Bradbury and Holloway, 1988) but was reported to be low in iron and vitamin A (McDowell and Onduro, 1983).

UNIVERSITY OF IBADAN

Even though, cassava tuber has been criticized for its low and poor protein content, it produces more weight of carbohydrate per unit area than other staple food crops under comparable agro-climatic conditions thus, being an energy-dense food and therefore ranked high for its energy value of 250 x 10^3 cal/ha/day as compared to 176 x 10^3 for rice, 110 x 10^3 for wheat, 200 x 10^3 for maize, and 114 x 10^3 for sorghum (Okigbo, 1980; Jisha *et al*., 2010). Montagnac (2009) and Zvinavashe *et al*. (2011) reported that the root is a physiological energy reserve with high carbohydrate content, which ranged from 32% to 35% on a fresh weight (FW) basis, and from 80% to 90% on a dry matter (DM) basis. Raw cassava root however, has more carbohydrate than potatoes and less carbohydrate than wheat, rice, yellow corn, and sorghum on a 100 g basis (Montagnac, 2009).

The lipid content in cassava roots ranges from 0.1% to 0.3% on a fresh weight basis with values ranging from 0.1% to 0.4% (Charles *et al*., 2005) and 0.65% (Padonou *et al*., 2005) on a dry weight basis. This content is relatively low compared to maize and sorghum, but higher than potato and comparable to rice. The lipids are either non-polar (45%) or glycolipids (52%) according to Hudson and Ogunsua (1974), mainly galactose-diglyceride (Gil and Buitrago, 2002). Predominant fatty acids include palmitate and oleate (Hudson and Ogunsua, 1974).

UNIVERSITY OF IBADAN

Table 2.1: Nutrient Composition of fresh cassava root per 100g of edible portion

Nutrients	Unit	Cassava root
Food energy	Kjoules	0.61
Water	G	62.5
Carbohydrate	G	34.7
Protein	G	1.20
Fat	G	0.3
Calcium	Mg	33
Iron	Mg	0.7
Vitamin A	I.U	Trace
Thiamine	Mg	0.06
Riboflavin	Mg	0.03
Niacin	Mg	0.06
Vitamin C	Mg	36

Source: FAO Food Composition (Nweke *et al*., 2002)

UNIVERSITY OF IBADAN

2.1.6 Anti-nutritional factors of cassava

Cassava has significant deficiencies that restrict its usefulness as a food source, one of which is the precence of cyanogenic glucosides; linamarin (2-β-Dglucopyranosyloxy-2-methylpropanenitrile) and lotaustralin [(2R)-2- β -Dglucopyranosyloxy- 2-methylbutyronitrile] derived from valine and isoleucine, respectively (Peifan *et al.,* 2004; Koch *et al.,* 1992). The ratio of linamarin to lotuastralin in leaves and roots was reported to be about 93:7 by Nartey (1978) and Liangcheng *et al.* (1995) while less than 83% linamarin was recounted by other researchers (Cereda and Mattos, 1996; Kimaryo *et al*., 2000).

Linamarin is a β-glucoside of acetone cyanohydrin and ethyl-methylketone-cyanohydrins which is stored in the vacuoles of the cassava cells (McMahon *et al*., 1995) and whose β-linkage can only be broken down under high pressure, temperature and use of mineral acids. Linamarin is bitter thereby making high-cyanide cassava (>100ppm) bitter, thus called bitter cassava.

Cyanogens are present in variable concentrations ranging from 300 to 500 ppm (El Tinay *et al*., 1984) depending on the type of cassava. Bitter varieties, which contain higher amounts of cyanogenic glucosides therefore needs processing to remove the toxic compounds before consumption, whereas, sweet varieties (<100 ppm) have low levels and can be eaten fresh (Rosling, 1990). Despite this, populations which use cassava as main staple food, mainly grow the bitter varieties due to their higher yields (Mozambique Ministry of Health, 1984), resistance to insects, pests and therefore rely on processing methods for detoxification. Cardoso *et al.* (2005) puts the range of total cyanide content to be from 1 to 1550 ppm. Although hydrocyanic concentrations of 15 to 400 mg/kg of fresh weight in cassava varieties are reported, more frequent values fall within the interval of 15 – 150 mg/kg (Cereda and Mattos, 1996) even though the minimum tolerant level recommended by Standard Organisation of Nigeria is 50 mg/kg (SON, 1985). There are, however, cassava varieties which contain concentrations above 1000 mg/kg (Cereda and Mattos, 1996). These variations were reported to be due to different rate of biosynthesis, degradation or transport (Elias *et al.,* 1997). Environmental factors, the cultivar and growth conditions have also been documented (Cooke, 1978; Bradbury *et al.,* 1991).

Cassava plant has endogenous linamarase (β-glucosidase), an enzyme which can easily hydrolyse linamarin, situated in the cell wall (Mkpong *et al*., 1990). The

UNIVERSITY OF IBADAN

grating or mincing of the roots permits, through the cell structure damage, the release of endogenous linamarase able to hydrolyse linamarin into glucose and acetone cyanohydrin (Conn, 1969), under optimum conditions at 25°C and pH range 5.5 – 6.0 (Cereda and Mattos, 1996).

As reported by Mkpong *et al*. (1989) as well as Ikediobi and Onyike (1982), the endogenous linamarase content could not permit the complete breakdown of linamarin. However, it was demonstrated (Ikediobi and Onyike 1982) that it is possible to lower the cyanogen toxicity by the introduction of an exogenous linamarase during fermentation. Many authors (Ikediobi and Onyike, 1982; Padmaja and Balagopal, 1985; Okafor and Ejiofor, 1990) have suggested the inoculation of fermenting cassava with a linamarase-producing microorganism. This reduces the cyanogen content because the microorganisms produce linamarase which break down the linamarin (Guyot *et al*., 1998). Fermentation of cassava with water is the simplest method to reduce the cyanide content (Cumbana *et al*., 2007; Bradbury and Denton, 2010) as the water will facilitate swelling of the cells and allow linamarase to come into contact with linamarin leading to hydrolysis (Bradbury, 2006). Uyoh *et al*. (2009) also observed that using unchanged water during fermentation will reduce cyanide content significantly. Reduction in the cyanide content of fermenting cassava as reported by Westby and Choo (1994) ranged from 65 to 110 mgHCN/kg for a period of 12 to 96 hours while Onyesom *et al*. (2008) puts the range between 7.02 and 2.70 mgHCN/100g cassava wet weight for a period of 24-96 hours.

Although, much of these toxic components were removed during processing of cassava, a quantity still remains, depending on the process used according to Nartey (1981) as well as Nambisan and Sundaresan (1985). Consumption of cassava which still contains residual levels of cyanogenic compound can result in chronic diseases such as goitre, cretinism, topical ataxic neuropathy, iodine deficiency, destructions of cells and tropical diabetes (Cock 1982; Tylleskar *et al.,* 1992). Since cassava is completely devoid of protein and highly deficient in vitamins and minerals, it causes malnutrition as well, when consumed solely.

UNIVERSITY OF IBADAN

2.2 Fermentation

2.2.1 Definition

Adams (1990) describes fermentation as a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is partially oxidised, and an organic carbohydrate acts as the electron acceptor. But according to Campbell-Platt (1987), Fermentation is the subjecting of food to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification to the food. It was also defined as the process by which alcohol or lactic acid was produced by living cells in solutions that contain sugars (Ribéreau-Gayon *et al*., 2000). William and Dennis (2011) as well as Wikipedia (2012) described fermentation as the conversion of carbohydrates to alcohol and carbondioxide or organic acids using yeast and/or bacteria, under anaerobic conditions.

Fermentation of food has however been termed as one of the oldest method of food preparation and preservation (Pederson 1971; Steinkraus *et al*., 1983; Campbell-Platt, 1994) used as far back as 6000 BC in the Middle East, although then, it was without any knowledge of the roles of the microorganisms involved (Caplice and Fitzgerald, 1999).

2.2.2 Advantages of fermentation

Fermentation processes are used for production of a vast number of valuable products using various fermentation media (substrates) and microorganisms (Mshandete, 2011). It is widely used to transform and preserve food because of its low technology, energy requirements and the unequaled organoleptic qualities it confer on the final product (Daeschel *et al*., 1987). Some of the importance of fermentation includes flavour enhancement, improved nutritional quality, preservation, detoxification, inhibitory metabolite production, food edibility and income generation.

Fermentation tend to make food more palatable by enhancing its aroma and flavour thereby, making the organoleptic properties of fermented food more popular than the unfermented one in terms of consumer acceptance (Blandino *et al*., 2003; Osungbaro, 2009). The microorganisms and metabolites responsible for these changes have been described (Ramaite and Cloete, 2006). However, the specific mechanisms by which flavour is generated are still subject to investigation. Fermentation is unique in that it modifies the unfermented food in diverse ways, resulting in new sensory properties in the fermented product (Leroy and De Vuyst, 2004).

UNIVERSITY OF IBADAN

A number of foods especially cereals are poor in nutritional value, and they constitute the main staple diet of the low income populations. Fermentation has however, been shown to improve the nutritional value and digestibility of these foods (Obiri-Danso *et al*., 1997; Nout, 2009). It was reported that the activities of microbial enzymes which include amylases, proteases, phytases and lipases were enhanced by the acidic nature of the fermentation products at a temperature range of 22-25ºC (Mokoena *et al*., 2005), thus, modifying the primary food products through hydrolysis of polysaccharides, proteins, phytates and lipids respectively. Anti-nutrients such as phytic acid and tannins in foods were also reduced through lactic fermentation leading to increased bioavailability of minerals such as iron, protein and simple sugars (Sripriya *et al*., 1997; Chelule *et al*., 2010)

By lowering pH below 4.0 through acid production, lactic fermentation hinders the growth of pathogenic microorganisms which cause food spoilage, food poisoning and diseases (Ananou *et al*., 2007). The production of lactic and acetic acids resulting in pH decrease and increase in titratable acidity as reported by Abdel and Dardir (2009) and Olukoya *et al.* (2011) accounted for the overgrowth of desirable bacteria in food than other non-desirable food spoilage bacteria, thus, prolonging the shelf life of fermented food. Detoxification of toxins (mycotoxin) in food through fermentation has also been documented over the years (Mokoena *et al*., 2005, 2006; Schnurer and Magnusson, 2005; Chelule *et al*., 2010; Dalie *et al*., 2010). Fermentation has been reported to be more advantageous than using alkaline ammonia treatment because of its mild nature which preserves the nutritive value and flavour of decontaminated food (Bata and Lasztity, 1999). Furthermore, the mycotoxins are irreversibly degraded without leaving any toxic residues and this was believed to be through the toxin-binding effect or an enzymatic interaction (Zinedine *et al*., 2005). Endogenous linamarase production or the use of a linamarase-producing microorganism as starter culture during fermentation has also been reported to detoxify cassava (Ravi and Padjama, 1997; Sweeney and Dobson, 1998).

Some of the inhibitory compounds produced against unwanted bacteria include hydrogen peroxide, carbon dioxide, diacetyl, broad spectrum antimicrobials such as reuterin, organic acids and bacteriocins (De Vuyst and Vandamme, 1994; Oyewole, 1997), which act as deterrents for pathogenic enteric bacteria and non-acid tolerant bacteria (Olukoya, 2011). This antagonistic effect by the organic acids was believed to have resulted from their action on the bacterial cytoplasmic membrane which

UNIVERSITY OF IBADAN

interferes with the maintenance of membrane potentials and inhibits active transport (Sheu *et al*., 1972; Eklund, 1989; De Vuyst and Vandamme, 1994a). Hydrogen peroxide inhibitory mechanism was said to be mediated through the strong oxidizing effect on membrane lipids and cell proteins (Lindgren and Dobrogosz, 1990). Carbon dioxide can directly confer an anaerobic environment which is toxic to aerobic food microorganisms through its action on cell membrane and its ability to lower internal and external pH (De Vuyst and Vandamme, 1994a). (De Vuyst and Vandamme, (1994a) and Motlagh *et al*. (1991) ascertained that gram negative bacteria, yeasts and moulds are more sensitive to diacetyl and its mode of action was believed to be due to its interference with utilization of arginine. Bacteriocins, regarded as extracellularly released primary or modified products of bacterial ribosomal synthesis by Jack *et al*. (1995) have a relatively narrow spectrum of bactericidal activity.

African locust bean, oil bean and cassava, for example are inedible in their unfermented state but according to Odunfa (1983), during fermentation, they become edible as a result of hydrolysis of the indigestible components which are removed by the action of microorganisms. Fermented food production has also been found to provide a source of income to a lot of people around the world. Anon (1995) reported in the FAO of nation that there was value added through processing and marketing of raw products. About 60% of workforces in sub-Sahara Africa are employed in small scale food processing sector and between ⅓ to ⅔ in manufacturing of agricultural raw materials (Anon, 1989; Conroy *et al.,* 1995). It was also observed that rural-urban migration and the associated social problems were reduced by the generation of employment opportunities in the rural areas and small scale food industries (Aworh, 2008).

UNIVERSITY OF IBADAN

2.2.3 Types of fermentation

Soni and Sandhu (1990) described four main fermentation processes namely, alcoholic, lactic acid, acetic acid and alkali fermentations. Alcoholic fermentation brings about the production of ethanol, and yeasts are the predominant organisms. Products of such fermentation include wine and beer. Lactic acid fermentation produces foods such as fermented milks and cereals, mainly carried out by Lactic Acid Bacteria. A second group of bacteria of importance in food fermentations are the acetic acid producers from the *Acetobacter* species which converts alcohol to acetic acid in the presence of excess oxygen. Alkali fermentation often takes place during the

fermentation of fish and seeds, popularly known as condiment (McKay and Baldwin, 1990). Chisti (1999) however, classified most commercially useful fermentations as solid state or submerged, each possessing particular advantages over the other and have been reviewed (Pandey *et al*., 2000; Pérez-Guerra *et al*., 2003; Cauto and Sanromán, 2006). Although different, the processes are influenced by numerous factors, including temperature, pH, nature and composition of the medium, dissolved oxygen, dissolved CO_2, operational system (batch, fed batch, continuous), mixing and most especially, fermentation microorganisms. Variations in these factors could affect the rate of fermentation, product spectrum and yield, organoleptic properties of the products (taste, texture, smell and appearance), nutritional qualities and other physico chemical properties (Chisti, 1999).

During lactic fermentation, all lactic acid bacteria produce lactic acid from hexoses and since they lack functional heme-linked electron transport chains and a functional Kreb's cycle, they obtain energy via substrate level phosphorylation. The pathways by which hexoses are metabolised thereby, divide Lactic Acid Bacteria into two groups: homofermentative and heterofermentative (Kockova *et al*., 2011)

2.2.3.1 Homofermentative lactic fermentation

Homofermentative bacteria transform nearly all the sugars they utilise, especially glucose into lactic acid thus, the major or sole end product. Homofermenters use the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose and produce approximately, twice the energy per mole of glucose as heterofermenters (Kockova *et al*., 2011). The pathway includes a first phase involving the conversion of hexose to pyruvate (glycolysis). The terminal electron acceptor is pyruvate which is further reduced to lactic acid (Khalid, 2011). Lactate dehydrogenase catalyzes reduction of the keto group in pyruvate to a hydroxyl, converting both molecules of pyruvate to lactate as NADH is oxidized to NAD+ (Wikipedia, 2012). Examples of homolactic acid fermentation involve the production of *gari, fufu, lafun* etc.

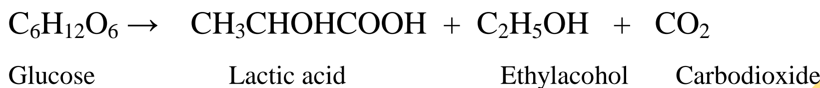
$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$

Glucose Lactate

UNIVERSITY OF IBADAN

2.2.3.2 Heterofermentative lactic fermentation

Heterofermentative bacteria use the pentose phosphate pathway in which NADH is converted to NAD+ in the reaction catalyzed by pyruvate and alcohol dehydrogenase. One molecule of pyruvate is converted to lactate while the other is converted to ethanol and carbon dioxide or acetate (acetic acid) through the enzyme acetate kinase (Wikipedia, 2012). The final products of this pathway are equimolar amount of lactic acid and ethanol in a slightly aerated environment as well as lactate and acetate in an aerated environment (Khalid, 2011). An example of heterolactic acid fermentation is production of burukutu.



$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$$

Glucose Lactic acid Ethylacohol Carbodioxide

2.2.4 Fermented foods

A great majority of the foods we eat are fermented; from bread and cheese to yogurt, beer, wine, coffee, vinegar, pickled vegetables, sausages, to mention only a few. Harlender (1992) defined fermented foods as those products which have been subjected to the effect of microorganisms or enzymes to cause desirable biochemical changes. Holzapfel (2000) described them as palatable and wholesome foods prepared from raw or heated raw materials. However, Eisenbrand (2005), in the DFG Commission on Food Safety referred to fermented foods as consumable products, generated from either thermally treated or untreated raw materials of plant/animal origin which have characteristic sensory and nutritional value as well as properties determining shelf life and hygiene, conferred on them by microorganisms and/or enzymes from the raw materials. The history of fermented foods has early records in Southeast Asia, where China was regarded as the cradle of mold-fermented foods, and in Africa where the Egyptians developed the concept of the combined brewery bakery. The early Egyptian beers were probably quite similar to some of the traditional opaque sorghum, maize, or millet beers found in various African countries today (Hesseltine, 1981).

When consumed by humans, it was reported that fermented foods often introduce microflora that inhabit the human body (Ross *et al.,* 2002; Reid *et al.*, 2003; Picard *et al*., 2005). This probiotic effect and the reduced level of pathogenic bacteria observed in them are especially important when it comes to developing countries

UNIVERSITY OF IBADAN

where fermented foods have been reported to reduce the severity, duration and morbidity of diarrhoea (Mensah, 1997; Kimmons *et al.*, 1999). Nutritionally, they also have reduced antinutritional composition (Paredes-López and Harry, 1988), increased concentrations of vitamins, minerals and protein when measured on a dry weight basis (Adams, 1990).

They are generally safe and wholesome because the dominant microorganisms involved in their fermentations do not appear to be associated with any health risks. These beneficial microorganisms serve to some extent in safeguarding against pathogens and spoilage organisms. Acidification to pH values of less than 4.2 constituted a major safety concern because a number of metabolites, such as acetic acid, hydrogen peroxide and bacteriocins, produced during the fermentation process, exhibit antimicrobial properties which may contribute to the safety of lactic fermented foods in particular (Holzapfel, 2002).

Fermented foods have been classified in many different ways according to the views of different authors. It could be according to the kind of microorganism involved (Yokotsuka, 1982) or based on commodity (Campbell-Platt, 1987; Odunfa, 1988; Kuboye, 1985). Nigeria has a variety of people and culture, thereby, difficulty in choosing one national dish. Abdel and Dardir (2009) and Adebayo *et al.* (2010) stated that each area has its own regional favourite food and such is dependent on custom, tradition and religion. The fermentation processes for such native food constitute a vital body of indigenous knowledge used for food preservation, acquired by observations or experience, and passed on from generation to generation (Aworh, 2008; Chelule *et al.*, 2010).

UNIVERSITY OF IBADAN

Table 2.2: Different classification of fermented foods

Yokotsuka (1982)	Campell-Platt (1987)	Odunfa (1988)	Sudanese (Dirar 1993)
1. Alcoholic beverages (yeast) 2. Vinegars (Acetobacter) 3. Milk products (*Lactobacilli*) 4. Pickles (*Lactobacilli*) 5. Fish or meat (Enzymes and *Lactobacilli*) 6. Plant protein (moulds with or without *Lactobacilli* and yeasts)	1. Beverages 2. Cereal products 3. Dairy products 4. Fish products 5. Fruit and Vegetable products 6. Legumes 7. Meat products 8. Starch crop products 9. Miscellenous products.	1. Starchy roots 2. Cereals 3.Alcoholic Beverages 4. Vegetable protein 5 Animal protein	1. Kisser – staples 2. Millet – sauces and relishes for the staples. 3. Marayiss – beers and other alcoholic drinks 4. Akil-munasabat – food for special occassions

Source: (Dirar, 1993)

UNIVERSITY OF IBADAN

2.3 Cassava fermentation

Fermentation of cassava was reported to be the most important and widely used means of processing cassava (Oyewole, 1992; Nweke *et al*., 2002). As of date, the ancient traditional processing of cassava is still being used and this practice is however, afflicted with so many problems because it depended on chance inoculation from the environment (Oyewole, 1990, 1995). Thus, the fermentation period is rather slower, with inconsistent quality of the products from one processor to the other as well as from one production batch to the other even by the same processor, and from one season to the other (Oyewole and Sanni, 1995). Improvements in cassava processing, which have been employed over the years helped to reduce the duration of processing to economically viable limits, maximise the detoxification process and improve the physical and nutritional qualities of the products.

While the methods of fermentation vary from one locality to another (grated root fermentation, underwater/soaking fermentation, and mold fermentation), cassava fermentation in sub-Saharan Africa has been categorized either as solid state or submerged (Oyewole, 1992).

2.3.1 Submerged Fermentation

Submerged fermentation is the cultivation of microorganisms in liquid nutrient medium (Renge *et al*., 2012) in which the bioactive compounds are secreted into the fermentation medium (Subramaniyam and Vimala, 2012).

Submerged fermentation of cassava involves the soaking of cassava roots under water for 3 -5 days, causing the root to soften and swell, thus having a combined effect of enabling linamarase and linamarin to mix as well as leaching of cyanogens (Westby and Choo, 1994). The softened roots can be easily broken into pieces by hand, passed through a sieve to remove the fibre, leaving a smooth paste. Initially, a mixed microflora was reported to be involved, but it was later dominated by lactic acid bacteria (Oyewole and Odunfa, 1988; Achi and Akubor, 2000; Obilie *et al*., 2004; Kostinek *et al*, 2005). The size to which the roots were cut prior to soaking was also found to affect the rate of fermentation and the quality of product (Oyewole and Odunfa, 1992). Different groups of lactic acid bacteria isolated from submerged fermenting cassava includes *Lactobacillus cellobiosus, E. bulgaricus, L. brevis, L. coprophilus, L. plantarum* and *Leuconostoc mesenteroides* with *Lactobacillus plantarum* being predominant during the last 36 hours.

UNIVERSITY OF IBADAN

2.3.2 Microbiology of cassava fermentation

Starch is a complex carbohydrate that can be degraded either by microorganisms that produce α-amylase or an inducible/constitutive amylase to produce simple sugars which can then be readily metabolised by many microorganisms. The indigenous natural fermentation has been reported to involve mixed colony of microorganisms such as molds, bacteria and yeasts (Anthony and Chandra, 1997; Kobawila *et al*., 2005; Ekundayo and Okoroafor, 2012). Even though, microbial size in food is usually small, their influence on the nature of the food, especially in terms of flavour, and other organoleptic properties, is profound (Okafor, 2009). Thus, fermentation products are based on the microorganisms involved in the fermentation and the type of bacterial flora developed in each fermented food varies based on water activity, pH, salt concentration, temperature and substrate composition (Blandino *et al*., 2003). These microorganisms are harmless to the consumer and produces enzymes such as proteases, amylases and lipases that hydrolyze food complexes into simple non-toxic products with desirable texture and aroma that makes them palatable for consumption (Steinkraus, 1997).

Numerous authors have linked a wide spectrum of microorganisms to cassava fermentation and these includes *Bacillus, Leuconostoc, Klebsiella, Corynebacterium, Lactobacillus, Aspergillus, Candida*, *Geotrichum, Streptococcus, Enterococcus, Aerococcus* and *Pediococcus* species (Oyewole and Odunfa, 1988; Anthony and Chandra, 1997; Hirayama and Rafter, 1999; Holzapfel, 2002; Blandino *et al*., 2003). Furthermore, yeasts and molds such as *Saccharomyces, Candida, Kluyeromyces, Aspergillus, Rhizopus, Mucor, Penicillium* and *Debaryomyces* were also reported (Wouters *et al*., 2002; Omemu *et al*., 2007).

However, rapid growing Lactic Acid Bacteria (LAB) are the most common prominent microorganisms for fermentation and preservation of foods. Their importance was known to be associated mainly with their safe metabolic activity while growing in foods, utilising available sugar for the production of organic acids and other metabolites. Their common occurrence in foods, coupled with their long-lived use contributed to their natural acceptance as GRAS (Generally Recognised as Safe) for human consumption (Aguirre & Collins, 1993). *Lactobacillus plantarum* has been shown to be the predominant LAB specie in sour cassava starch (Ngaba and Lee, 1979; Amoa-Awua *et al*., 1996; Ben Omar *et al*., 2000; Lacerda *et al*., 2005; Kostinek *et al*., 2007), even though, most species were found not to produce α-amylase and this

UNIVERSITY OF IBADAN

was quite surprising because cassava has about 84% of the carbohydrates in the form of starch (Ketiku and Oyenuga, 1972). Sanni *et al*. (2002) stated that only a few amylolytic LABs have been isolated from starchy fermented foods in Africa but more studies over time, had led to the discovery of more (Diaz-Ruiz *et al*., 2003; Putri *et al*., 2011a, 2011b; Mukisa *et al*., 2012) . In other cases, there are many kinds of fermented foods in which the dominating processes and end products are dependent on a mixture of endogenous enzymes and mixed microbial cultures which were earlier reported to have originated from the native microflora of the raw materials utilised in most of the traditional food fermentation processes according to Anthony and Chandra (1997).
Some of the inhibitory compounds formed during fermentation include organic acids (e.g. palmitic, pyruvic, lactic, acetic, propionic and butyric acids), alcohols (mainly ethanol) aldehydes and ketones (acetaldehyde, acetoin, 2-methyl butanol) (Campbell-Platt, 1994). These varieties of metabolites are antagonistic in action to competing bacteria (Breidt and Fleming, 1997). The inhibition has been attributed to the protonated form of the acids, which are uncharged and may therefore cross biological membranes, thus inhibiting growth due to lowered pH of the cytoplasm and/or accumulation of anions inside the cell (Adams, 1990; Russel, 1992; Breidt & Fleming, 1997). In other words, they interfere with the maintenance of cell membrane potential, inhibiting active transport and a variety of metabolic functions as well as reducing intracellular pH (Ross *et al*., 2002).

2.3.3 Cassava products

As earlier stated, cassava roots are bulky with about 70% moisture content, and therefore transportation of the tubers to urban markets is difficult and expensive. Moreso, the raw roots and uncooked leaves are not palatable and they contain varying amounts of cyanide which is toxic to humans and animals. Therefore, cassava must be processed into various forms in order to increase the shelf life, facilitate transportation and marketing, reduce cyanide content and improve palatability. Traditionally, cassava roots are processed by various methods into numerous products and utilised in various ways according to local customs and preferences. In some countries, the leaves are consumed as vegetables, and many traditional foods are processed from cassava roots and leaves. The nutritional status of cassava can also be improved through fortification with other protein-rich crops. Processing reduces food losses and stabilizes seasonal fluctuations in the supply of the crop. Various traditional processing methods are

UNIVERSITY OF IBADAN

known which include boiling, smoking, drying and fermentation while some of the products of these processes in Nigeria include *gari, abacha, lafun, usi, fufu, tapioca cakes* etc.

2.3.3.1 *Fufu*

Fufu is traditionally produced and marketed as a fermented wet, pasty food product, which is also made into porridge in boiling water before consumption. Mostly consumed in the Eastern and Western parts of Nigeria as well as some other parts of West and Central Africa, it is known as *chikwuangue* or *chikwange* in Zaire; *fufu* or *foo-foo* in Southern Nigeria and *akpu* in some parts of Eastern Nigeria (Okafor *et al*., 1984; Longe, 1990).

Traditional *fufu* fermentation involves peeling and washing cassava roots that are manually cut into different sizes by using a hand knife and soaking in earthen pots or drums of water for 3 to 5 days to undergo lactic acid fermentation. Reports indicated that during soaking, the pH value decreases, the root softens and this facilitates the reduction in potentially toxic cyanogenic compounds (Oyewole and Odunfa, 1992; Westby and Choo, 1994; Oyewole *et al*., 2001; Aworh, 2008; Uyoh, *et al*., 2009). The soft roots were broken with clean hands and the fibres removed by sieving which is done by adding water to the retted mass on a sieve. The starch suspension is allowed to sediment for about 24 hours after which the water is decanted. Fine, clean starch is further dewatered by putting in raffia or cotton bags and pressed with heavy stones overnight (Oyewole and Odunfa, 1989). To prepare for consumption, a quantity of the slurry containing about 25% of *fufu* paste in water was boiled in an open pan. After continuous stirring using a wooden rod, strong dough was formed (Kwatia, 1986; Ayankunbi *et al*., 1991; Anon, 1994). This method is commonly reported among the Yoruba tribe. The Igbo and Efik tribes of Eastern Nigeria who refer to it as *nni akpu* and *udep utim* respectively, rolls the starch into large balls, wrap it with wilted plantain leaves and partially steam cook. The balls are removed and pounded in a wooden mortar to give a fine, smooth and soft mash. It is further rolled into small balls, wrapped in leaves and thoroughly steamed, after which the balls are finally pound together (Etejere and Bhat, 1985). The cooked *fufu* is usually eaten warm with fish, meat, vegetable stew or soup. However, unlike other fermented cassava products, it has a very strong odour (Lancaster *et al*., 1982; Okafor *et al.,* 1998) and considered by consumers to be of good quality when it has a smooth

UNIVERSITY OF IBADAN

texture, characteristic aroma and creamy white, grey or yellow colour (Akingbala *et al.*, 1991; Oyewole and Odunfa, 1992; Blanshard, 1994).

Variability in the quality of *fufu* could be as a result of chance inoculation involving varieties of microorganisms (Oyewole and Sanni, 1995), little or no control over the process (Oyewole, 1997), roots cut size (Okafor *et al.*, 1984), difference in dry matter content (Hahn, 1989), root quality and fermentation water.

2.3.3.2 *Usi*

Usi is one of the major native foods of the Itsekiri and Urhobo in Southern Nigeria, who also refer to it as edible starch (Etejere and Bhat, 1985). At household level, different techniques are used to obtain the fermented starch. It may be precipitated from the solution pressed out of the grated cassava roots or from grated cassava that is soaked directly in water. The starch is produced either in a wet form or more commonly, dried. The cassava roots were peeled, washed and grated. The grated pulp is steeped for 2-3 days in a large quantity of water. The mixture is stirred and filtered through a piece of cloth sieve. The filtrate stands overnight and the supernatant is then decanted. The fine starch paste is collected and put in a wide metal pan that is already smeared with red palm oil. Water is added and then stirred with the hand to dissolve completely. The pot is put on fire and the solution constantly stirred with a wooden rod until it is converted to a very sticky, light yellow mass. This is eaten with any oil or soup (Etejere and Bhat, 1985).

2.4 Starter culture

2.4.1 Definition

Spontaneous fermentation has been used for the production of verieties of fermented foods based on the microflora present in the fermentation environment as well as the raw material (Vogel *et al.*, 2002) and it has been reported that the initiation of a natural fermentation takes long with high risk for failure (Holzapfel *et al.*, 2000). During the long lag phase which was characterised with microorganisms physicochemically equilibrating with their environment, contaminating organisms from the environment slowly increase in number and compete for nutrients in order to produce metabolites. In addition, spontaneous fermentation has been reported by Oyewole and Sanni (1995) to lead to product inconsistencies since the end-product was dependent on the types and number of microorganism in the raw material. Thus,

UNIVERSITY OF IBADAN

the use of a preparation containing a large number of viable microorganisms was recommended, as this would lead to rapid acidification of fermentation process, the inhibition of spoilage and pathogenic organisms (Holzapfel, 1997, 2002), as well as a product with consistent quality.

A starter culture may therefore be defined as a preparation or material containing large numbers of viable 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). Leroy and De Vuyst (2004) further define it as a microbial preparation of large numbers of cells containing at least one microorganism which is added to a raw material to produce a fermented food by accelerating its fermentation process. However, according to the DFG Senate Commission on Food Safety, "starter cultures are preparations of live microorganisms or their resting forms, whose metabolic activity has desired effects in the fermentation substrate, the food".

The early technologies in starter usage include the transfer of an old batch of fermented products to a new batch (back-slopping) and the indigenously-derived cultures (Westby *et al.*, 1997). It was customary in the beginning when cereals were fermented by their natural flora, to put aside pieces called 'sours' or 'starters' for fermenting subsequent batches.

UNIVERSITY OF IBADAN

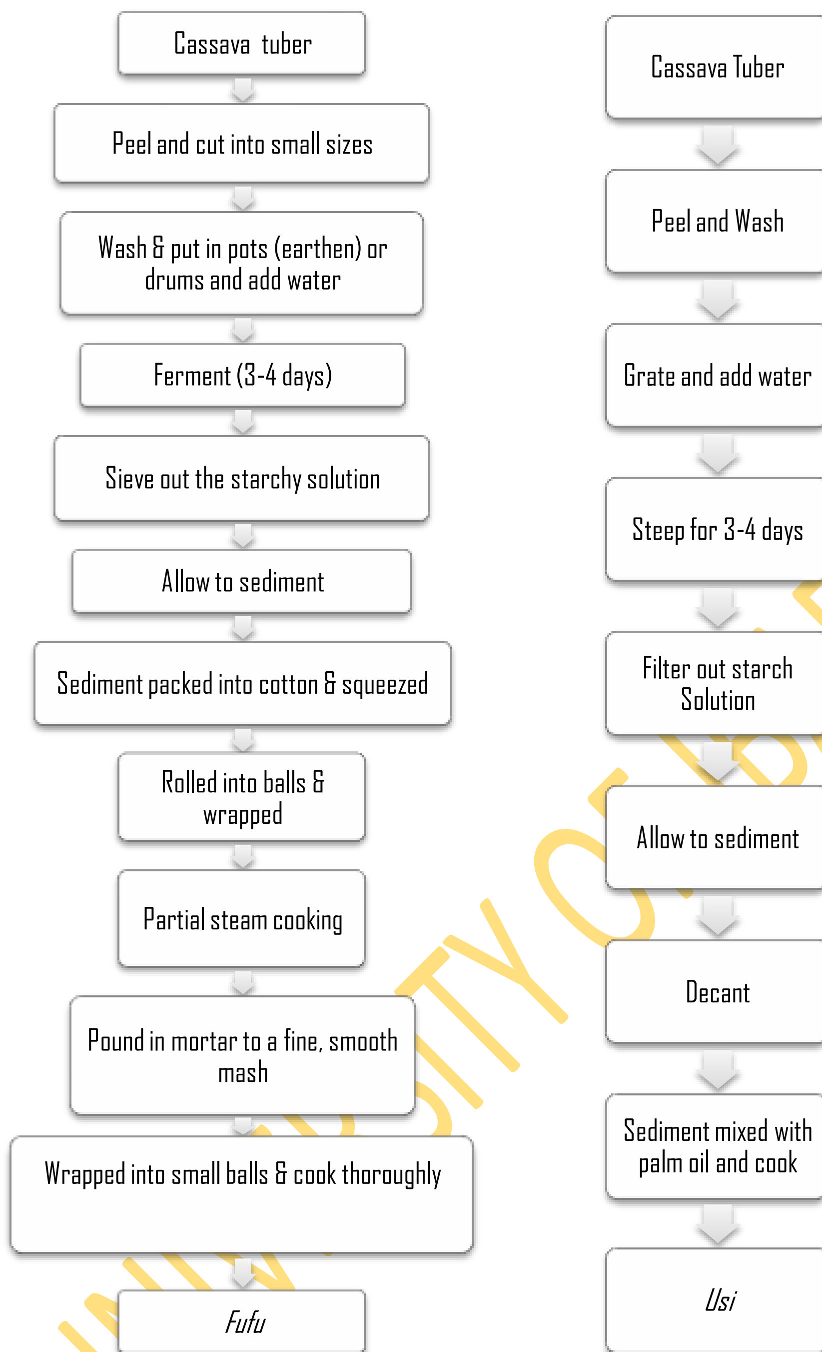


Figure 2.1: Flow chart for traditional *fufu* (Oyewole and Odunfa, 1989) and *usi* (Etejere and Bhat, 1985) production

This back-slopping process often resulted in irregularities and unpredictability that led to the development and use of defined starter cultures (Holzapfel, 2002). Commercial starter cultures generally originated from food substrates or from the processes in which they were applied (Holzapfel, 2002), even though there has been a trend to isolate wild-type strains from traditional products (Beukes *et al.*, 2001; De Vuyst *et al.*, 2002; Leroy and De Vuyst, 2004). Edward *et al.* (2012) envisaged that the microorganisms isolated from the samples taken would be ideal for the development of a starter culture, as they would be typical of the fermentation and well adapted to the ecological conditions.

Since the majority of starter cultures are natural isolates of the desirable microorganisms found normally in the substrates (Holzapfel, 2002; De Vuyst and Vancanneyt, 2007), a wide variety of species of organisms have been used in the food industry and many have been investigated for their potential use as starter cultures (De Vuyst and Neysens, 2005; Gaggiano *et al.*, 2007). As at 2009, it was reported that relatively few LABs, out of millions of specie, have been isolated from starchy fermented foods in Africa and used as starter cultures (Yao *et al.*, 2009), even though some reports indicated that *Lactobacillus brevis, L. fermentum, L. plantarum, L. reuteri, Pediococcus pentosaceus* and *P. acidilactici* exhibited superior performance in lactic fermented cereal, root crops and vegetable products (Steinkraus, 1996, 1997; Holzapfel, 1997; Lee, 1997; Oyewole, 1997).

Some of the earlier studies that reported the use of defined starter cultures include Guiraud *et al.* (1998), Guiraud and Raimbault (1993) and Kimaryo *et al.* (2000) whom mentioned the use of the amylolytic *L. plantarum* as a starter culture during cassava fermentation for *gari* and *kivunde* respectively. Okafor *et al.* (1998) inoculated *L. coryneformis* and *Saccharomyces* sp. as a starter culture in cassava mash for *gari* production. Oyewole (1990) and Okolie *et al.*, (1992) have both reported successfully producing acceptable *fufu* by the use of isolated pure starter cultures and the survival of these microorganisms was studied when placed alone or mixed in different carriers (Okafor *et al.*, 1999). Various other reports have shown the utilization of starters during cassava fermentation (Kimaryo *et al.*, 2000; Egounlety *et al.*, 2007; Asmahan and Muna, 2009; Yao *et al.*, 2009; Padonou *et al.*, 2010).

Starter cultures could be acquired in a ready-to-use, highly concentrated frozen /freeze dried form, or being propagated (Høier *et al.*, 1999; Buckenhuskes, 1993; Hansen, 2002), ensuring the principles of hazard analysis critical control point (HACCP).

UNIVERSITY OF IBADAN

However, continuous propagation of starters from the original mixed microflora of traditional foods over time has led to the loss of certain beneficial strains as a result of natural ecosystem alterations. Moreso, certain metabolic characteristics of some starters, encoded in the plasmid are sometimes lost during propagation and these factors have been reported to reduce the biodiversity of starter cultures (Asmahan, 2010). This has prompted more studies towards the isolation of more strains even though typical commercial starters remain highly expensive which make people to still use small batches from a previous fermentation.

2.4.2 Properties of a starter culture

Spontaneous food fermentations are neither predictable nor controllable. Pure cultures isolated from mixed populations of traditional fermented foods exhibit a diversity of metabolic activities, which vary even among strains. These include variability in growth rate, adaptation to a particular substrate, ability to degrade antinutritive compounds, antimicrobial properties, flavour and quality attributes and competitive growth behaviour in mixed cultures (Holzapfel, 1997). Therefore, the selection of suitable starter must be based on its ability to compete for survival, antagonise pathogens and spoilage microorganisms, produce acid or alcohol rapidly, confer desirable organoleptic changes, produce primary metabolites, degrade antinutritive and improve nutritive factors, detoxify and have probiotic features (Holzapfel, 1997).

2.4.3 Effects of starter on cassava fermentation

Even though Hansen (2002) stated that the primary activity of the culture in a food fermentation is to convert carbohydrates to desired metabolites as alcohol, acetic acid, lactic acid or CO_2, the introduction of starter culture to initiate a fermentation process is expected to bring about certain physical, chemical, organoleptic and nutritional changes to the products and such have been reported by numerous authors (Hernandez-Jover *et al.*, 1997; Johanningsmier *et al.*, 2007; Ogunbanwo *et al.*, 2008; Ojokoh, 2009; Henshaw and Ikpor, 2010; Adetunde *et al.*, 2011; Ahaotu *et al.*, 2011; Hati *et al.*, 2013).

.The potential starters for cassava fermentation are pre-selected on the basis of suitable technological characteristics such as enzyme (amylase, pectinase, glucosidase, phytatse etc) and antimicrobial compound (bacteriocin, lactic acid, diacetyl, hydrogen

UNIVERSITY OF IBADAN

peroxide) production, survival rate, hydrolysis of complex carbohydrates, and rapid acidification of the fermentation process (Kostinek *et al*., 2007).

Reduction of anti-nutritional compounds has been an important factor to be considered during the analysis of fermented products. Although lactic fermentation has been shown in certain studies to have reduced the phytate content, which is an antinutritive component of some cereals (Lopez *et al*., 1983; Mahajan and Chauhan, 1987; Svanberg and Sandberg, 1988; Khetarpaul and Chauhan, 1989; Svanberg *et al*., 1993), phytic acid-degrading ability is relatively rare among pure LAB cultures. Some *L. plantarum* strains have however, been reported to be capable of degrading phytic acid on incubation at 37°C for 120 hours (Holzapfel, 1997). Phytase activity was not detectable in *Bacillus sp.* associated with the fermentation of the African locust bean *Parkia biglobosa* which was used in the preparation of iru or dawadawa (Aderibigbe and Odunfa, 1990).

Detoxification during the fermentation of cassava was reported by Westby and Choo (1994) to be dependent primarily on microbial activity, even though, endogenous linamarinase present in cassava play a significant role in the process. Different starter cultures such as *Lactobacillus* sp, *Bacillus* sp, yeasts and molds play important roles during cassava processing (Ejiofor and Okafor, 1981; Hahn, 1989; Essers *et al*., 1995; Amoa-Awua and Jakobsen, 1995; Amoa-Awua *et al*., 1996; Olasupo *et al*., 1997) but an experiment comparing the effects of spontaneous fermentation, back-slopping and the use of starter cultures for the reduction of cyanogenic glucosides in cassava (Kimaryo *et al*., 2000) revealed that all three types of fermentations contributed significantly to the detoxification of cassava, with the starter-fermented *(L. plantarum* strains) cassava, having the best results.

Rapid acidification rate is an important factor in cassava fermentation as this creates an unsuitable environment for spoilage and pathogenic microorganisms as well as hastening the entire process. It was reported by Henshaw and Ikpoh (2010) that after 96-hour controlled fermentation of cassava for *fufu* production, *L. plantarum, Bacillus subtilis, Klebsilla sp* and *L. mesenteroides* reduced the initial pH of fermenting mash from 6.2 to 3.68, 4.90, 4.88 and 4.68 respectively, with *L. plantarum* showing the highest acid-producing ability and this had earlier been reported by Oyewole (1990) who associated *L. plantarum* strain with high acid production during cassava fermentation for the production of *fufu.* Also, initial pH reduction from 5.62 to 3.05,

UNIVERSITY OF IBADAN

3.37 and 3.65 by *L. plantarum, L. mesenteroides* and *S cerevisiae* respectively, was reported during starter selection (Edema and Sanni, 2008).

The production of acid, diacetyl, hydrogen peroxide and some other inhibitory compounds, in the fermenting meals were significantly higher in substrates fermented with starter cultures than in the spontaneously fermented ones as shown in the studies conducted by Edema and Sanni (2008) as well as Ogueke (2008).

Nutritional, organoleptic and sensory qualities of starter fermented foods are expected to improve after the fermentation process. Cook (1994) and Steinkraus (1996) reported that the use of selected starter cultures for fermentation during processing of foods has been found to improve shelf life, nutritional status and to promote safety. Furthermore, Opere *et al*. (2012) observed increased nutritive value, acceptable organoleptic characteristics, and acceptable flavour properties in the fermentation of cereal gruel with starter culture. Ogunbanwo *et al*. (2013) also reported improved organoleptic property in burukutu while using mixed starter culture. Other numerous studies have shown the effect of starters on the nutritive and general sensory qualities of fermented foods (Kristek *et al*., 2004; Singh *et al*., 2012; Kabuo *et al*., 2013; Hasan *et al*., 2014). In general, the prospect of applying starter cultures will be achieved only if benefits, such as reduction of costs (e.g. energy), reduced fermentation duration, reduced risk of spoilage (increased shelf-life), improved process control, sensory quality (taste, aroma, visual appearance, texture, consistency), safety attributes (e.g. lower risk of diarrhoea, detoxification of cassava) and reduced preparation procedures for the final product, were perceived (Holzapfel, 2002).

2.4.4 Metabolite spectrum of cassava fermentation

Through fermentation, microorganisms growing on inexpensive carbon sources can produce valuable products such as simple sugars, amino acids, nucleotides, vitamins and organic acids which had been proven to enhance flavour or increase nutritive values (Demain 1980). Similarly, microorganisms also produce a range of metabolites during food fermentation processes that can suppress the growth and survival of undesirable microflora in foods (Ross *et al.,* 2002). Caplice and Fitzgerald (1999) reported that these metabolites not only increase the shelf life and microbiological safety of a food, but also make foods more digestible and reduce toxicity of the substrate.

UNIVERSITY OF IBADAN

Organic acids have been reported to be naturally present in foods or they are synthesized either during biochemical metabolic processes or bacteria metabolism (Akalin *et al*., 2002; Soyer *et al*., 2003; Karadeniz, 2004). They have important roles in food because they affect the organoleptic properties, stability, nutrition, acceptability and maintaining quality (Santalad *et al*., 2007). These acids have, not only antimicrobial effects but the organic acid-producing organisms are also considered as antimicrobial agents due to acid and antibodies production which lowers the pH of food/substrate to suppress the growth of other microorganisms. It has been reported that acetic and propionic acids produced by LAB strains may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al*., 1986). Earnshaw (1992) stated that both are usually more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percentage of undissociated acids.

Acetic acid has been shown to have more inhibitory property than lactic acid bacteria towards *Listeria monocytogenes* (Ahmad and Marth, 1989; Richards *et al*., 1995), and toward the growth and germination of *Bacillus cereus* (Wong and Chen, 1988). It also acted synergistically with lactic acid, decreasing the pH of the medium, thereby increasing the toxicity (Adams and Hall, 1988).

These organic acids are used as flavouring agents and the acceptability of food products depends largely on the flavour profiles, which are complex and are type-specific. Akalin *et al.* (2002) reported that those flavour profiles are influenced by many substances, e.g organic acids, sulphur compounds, lactones, methyl ketones and alcohols, as well as phenolic substances (Seitz 1990; Urbach, 1993). The flavour substances are formed as a result of the hydrolysis of fatty acids, normal bovine metabolic processes, and bacterial growth during food processing (Adda *et al.,* 1982). Quantitative determination of organic acids is important to flavour studies for nutritional reasons and as an indicator of bacterial activity (Pham and Nakai 1983). With the use of High Performance Liquid Chromatography, number of authors have attempted to use organic acid content as an indicator of microbial metabolism and classification parameter of various food products (Marsili, 1985; Panari, 1986; Bevilacqua and Califano, 1989; Lombardi *et al.,* 1994; Lues and Botha, 1998; Califano and Bevilacqua, 1999; Aka *et al*., 2008; Nour *et al*., 2010; Yuwono *et al*., 2011; Miguel *et al*., 2014). Mugula *et al.* (2003) reported the production of lactate during togwa fermentation whereas lactate, butyrate, acetate and formate were reported during

UNIVERSITY OF IBADAN

fermentation of ogi and maize dough by Banigo and Muller (1972) as well as Mensah *et al.* (1991), respectively. Sanni *et al.* (1999), during the analysis of cereal based beverages in Nigeria, detected the presence on lactic, acetic, malic, succinic and formic acids. In general, the organic acids are not only recommended as food additives by FAO for taste, aroma and texture but are also food preservatives. Among the organic acid metabolites are citrate, acetate, propionate, fumarate and most importantly, lactate which was the first organic acid to be produced on an industrial scale by fermentation in 1880 (Zadow, 1992) through controlled fermentation from the hexose sugars, molasses, corn, or milk.

UNIVERSITY OF IBADAN

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

3.1.1 Cassava samples

Healthy cassava varieties TME 30572, TME 4(2)1425 and TME 50395 were obtained from the Tuber and Root Improvement Programme (TRIP), International Institute of Tropical Agriculture, Ibadan as well as local variety from a retailer at Bodija market, Ibadan and transported to the Microbial Physiology and Biotechnology laboratory, Department of Microbiology, University of Ibadan.

3.1.2 Organic acid and sugar standards

The linamarin analogue (4-nitrophenyl-B-D-glucopyranoside), acetonitrile, KH_2PO_4 (HPLC grade), organic acids and sugar standards were obtained from Sigma (Sigma- Aldrich, Germany)

3.1.3 Test Isolates

Test pathogenic organisms isolated from food samples were obtained from Food Microbiology and Biotechnology Unit of the Department of Microbiology, University of Ibadan.

3.2 Media and Sample preparation

3.2.1 Media preparation

De Mann Rogosa and Sharpe (MRS) Agar, Violet Red Bile Glucose Agar (VRBGA), Malt Extract Agar and Plate Count Agar were used for Lactic Acid Bacteria enumeration, coliforms, yeast and total bacterial count respectively. They were prepared according to manufacturer's instruction (Appendix I, II, III and IV), sterilized at 121°C and 15 psi for 15 minutes.

UNIVERSITY OF IBADAN

3.2.2 Sample Preparation and fermentation

The method of Oyewole and Odunfa (1989) was used in which the cassava tubers were sorted by visual assessment, peeled, washed with clean tap water, and cut into small sizes (3-5 cm). Two hundred gram (200 g) of the cassava was submerged in 2 L tap water in plastic fermenters of 10 L capacity for the 72-hour fermentation at room temperature. The method of Etejere and Bhat (1985) was used for the fermentation of *usi*. Two hundred gram (200 g) cassava roots were grated and the pulp steeped in 2 litres tap water in a 10 L capacity plastic fermenter for 3 days. Samples of fermenting mash were taken every 24 hours and analyzed for microbiological and physicochemical parameters.

3.3 Total titratable acidity of fermenting cassava mash

Titratable acidity was determined using the standard titration procedure for total titratable acidity (TTA) described by Lonner *et al*. (1986). Ten gram (10 g) of the fermenting mash was mixed with 90 mL sterile distilled water and homogenized. The mixture was filtered through Whatman Filter paper (No.1) and the filtrate titrated against 1M NaOH using 1% phenolphthalein as indicator. Acid equivalent is the amount of NaOH consumed in mL and each mL of 1M NaOH is equivalent to 90.08 mg of lactic acid.

3.4 pH of the fermenting cassava mash

Ten gram (10 g) of fermenting cassava was weighed, mixed with 90 mL of sterile distilled water, homogenized and filtered through Whatman Filter paper (No.1). The pH of the resulting filtrate was then measured using a pH meter (H19107, Hanna), after standardizing with phosphate buffer solution at pH 4.0 and 7.0.

3.5 Isolation and identification of organisms from fermenting cassava tubers

3.5.1 Isolation technique

Ten gram (10 g) of each sample was aseptically added to 90 mL of sterile peptone water and homogenized for 2 minutes. Samples were further diluted in a tenfold serial dilution up to 10^{-9}. One millilitre (1 mL) from dilutions 10^{-5}, 10^{-7} and

UNIVERSITY OF IBADAN

10^{-9} was introduced into sterile petri dishes and sterile PCA, VRBGA, MEA and MRS agar were added and allowed to solidify (Harrigan and MacCance, 1976). MRS agar culture plates were incubated at 30°C for 48 hours and the others at 30°C for 24 hours after which microbial counts were carried out. Representative LAB colonies were picked randomly from the plates and purified by repeated sub-culturing on fresh agar plates. Pure cultures were grown on MRS agar slants and kept at 4°C for further use. The stock cultures were stored at -4°C in glycerol broth for subsequent use.

3.5.2 Characterization of selected isolates

Isolates were characterized and identified on the basis of their morphological (microscopic, macroscopic) and biochemical properties.

3.5.2.1 Morphological Characterization

i **Macroscopic:** They were observed based on shape, colour, elevation, size, edge and surface on respective agar plate.

ii **Microscopic:** This was carried out using the simple staining technique described by Norris and Ribbond (1971). A thin smear of the isolate was made on a clean glass slide and heat-fixed. Two drops of Crystal violet were applied onto the smear for 60 seconds. It was washed with water and stained with Grams iodine solution for 1 minute. The stain was decolourized by flooding the slides with ethanol until no more violet coloration was observed. Two drops of counter stain Safranin reagent were added for a minute, rinsed with water and blotted dry using filter paper. Microscopic observation was carried out under the oil immersion objective (Fisher Scientific, USA). Gram positive organisms were characterized by purple colouration after counter staining while Gram negative cells were pink in colour. Their shapes were also observed.

3.5.2.2 Biochemical Characterization

i **Catalase test**

Twenty four (24) hour old culture was obtained by sub-culturing on MRS agar plates and incubated at 30°C for 24 hours. A drop of freshly prepared 3% hydrogen peroxide solution was added to a clean glass slide. With the aid of a sterile wire loop, the culture was aseptically picked onto the slide (Seeley and Van Demark, 1972).

UNIVERSITY OF IBADAN

Evolution of gas as white froth indicated catalase positive reaction. Catalase negative isolates were selected for further tests.

ii Motility test

In semi-solid agar medium, motile organisms swarm and gave a diffuse spreading growth that was easily visually recognized. Agar agar (0.2% w/v) was dissolved in 100 mL nutrient broth and 10 mL of the medium was dispensed into test tubes. The medium was sterilized at 121°C, 15 psi for 15 minutes and allowed to set in a vertical position. Inoculation was done with a sterile needle by making a single stab down the centre of the tube to about half the depth of the medium and incubated at 37°C for 48 hours. Non-motile organisms gave growth that were confined to the stab line, had sharply defined margins and left the surrounding medium clearly transparent. Motile organisms gave diffuse hazy growth that spread throughout the medium (Olutiola *et al*., 2000)

iii Voges-Proskauer test

The selected isolates were each inoculated into sterile glucose phosphate broth (Glucose 0.5 g, KH_2PO_4 0.5 g, Peptone 0.5 g and distilled water 100 mL) and incubated at 37°C for 2-5days. Aliquot (1 mL) of 66% alpha-naphtol solution and 1 mL of 10% NaOH was added and the culture thoroughly shaken. Appearance of a pale pink colouration within 5 minutes was noted as positive reaction. The solution was left for a while to check for slow reaction in case of false negative result (Reuter, 1970).

iv Oxidase test

Whatman No. 1 filter paper was soaked in oxidase reagent (1% aqueous tetramethyl-p-phenylenediamine dihydrochloride). Sterile wire loop was used to touch colony of the test isolates, then transferred onto the reagent spot on the filter paper. Formation of a very deep purple coloration within 10 seconds indicated a positive reaction, while absence of a deep coloration indicated negative reaction (Seeley and Van Demark, 1972).

UNIVERSITY OF IBADAN

v Indole test

Each isolate was inoculated into sterile peptone water medium (Peptone 9 g, distilled water 100 mL) and incubated at 37°C for 48 hours. Five to six drops of Kovac's reagent were then added to the culture. Development of rose pink coloration on the surface of the culture indicated positive reaction of indole production. No colour change was recorded as negative (Kovac, 1956).

vi Methyl red test

Glucose phosphate peptone broth (Glucose 0.5 g, KH_2PO_4 0.5 g, Peptone 0.5 g and distilled water 100 mL) was prepared as described by Harrigan and McCance (1966). Ten millilitres (10 mL) of the broth was dispensed into screw cap tubes and sterilized at 121°C and 15 psi for 15 minutes. Inoculation with 24 hour old culture was aseptically done and incubated at 37°C for 2-5 days. After incubation, a few drops of methyl red indicator were added to the culture and a resultant definite red colouration was considered positive result.

vii Production of ammonium from arginine

This test was done to detect the ability of the organisms to produce deaminase enzyme which enables them to break down proteins to release ammonia. Modified MRS broth (10 mL) containing 0.3% arginine (Appendix V) was dispensed into screw cap tubes and sterilized at 121°C and 15 psi for 15 minutes. Loopful of cultures were inoculated aseptically and incubated at 37°C for 5-7 days. Uninoculated tube served as control. A loopful of Nesslers reagent was added to a loopful of the culture on a clean glass slide and observed. Presence of ammonia is indicated by the formation of orange to brown colour while pale yellow or no colour change indicated no reaction (Olutiola *et al*., 2000)

viii Sugar fermentation test

This test was for investigating the ability of the bacteria isolates to utilize different sugars. 10 g of different sugars (Glucose, maltose, mannitol, sucrose, melibiose, galactose, fructose, sorbitol, raffinose, lactose, xylose, arabinose, inositol and sorbose) were dissolved in 100 mL distilled water and each was sterilized at 121°C, 15 psi for 10 minutes. 1 mL of each sugar solution was added to 9 mL of sterile

UNIVERSITY OF IBADAN

modified MRS broth in test tubes, as the sole carbon source with methyl red indicator to give a final concentration of 1% (v/v). Each tube contained an inverted Durham tube. The tubes were inoculated with 24 hour old LAB cultures and incubated at 30°C for 5 days. Un-inoculated tubes served as control. Positive reaction with acid production was indicated by a colour change from red to yellow and/or gas production which was collected in the Durham tubes (Olutiola *et al*., 2000)

3.5.3 Identification of organisms

Selected isolates were identified based on comparison of the results obtained from the macroscopic, microscopic and biochemical characterization with the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3.6 Molecular Identification

3.6.1 DNA extraction

The DNA was extracted according to the procedure of Pitcher *et al*. (1989) with some modifications. Bacteria culture grown overnight was centrifuged at 13000 g for 2 minutes. Cell pellet was suspended in lysis buffer (pH 8.0) containing 25 mM Tris-HCl (Sigma), 10 mM EDTA, 50 mM sucrose, 10 mg/mL lysozyme and incubated at 37 ^{o}C for 30 minutes. 0.5 mL aliquots of the mixture of 5M guanidine thiocyanate, 0.1N EDTA and 0.5% N- lauroyl sarcosine sodium salt (Sigma, England) was added and incubated at 30 ^{o}C for 15 minutes. Precipitation was done using chloroform: isoamylalcohol (24:1) and centrifugation at 13000 g for 10 minutes. Upper protein precipitate was removed, added to isopropanol and centrifuged for 5 minutes. Resultant pellets were then washed in 70% ethanol. Purification was done by dissolving in 1X TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH=8.0) containing 10 mg/mL of RNase and incubated at 37 °C for 30 minutes.

3.6.2 DNA Amplification

The bacterial universal primers 27F (5'-AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') previously shown to be useful for identification of Lactic Acid Bacteria (Lane, 1991) were used to amplify approximately 600 base pairs of the genomic 16S conserved region.

Amplification method of Tajabadi *et al.* (2013) was used. The PCR reaction was carried out in a total volume of 25 µL with a reaction mixture with the following: 1×

UNIVERSITY OF IBADAN

Taq Master Mix (Promega, UK), deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.25 mM forward primer, 0.25 mM reverse primer and 0.4 mg of genomic DNA. The reaction mixture in micro-centrifuge tube was amplified in a thermocycler PCR system (Techne-Progene, UK) in which an initial heating of 95°C for 3 minutes was followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 55 seconds, extension at 72°C for 1 minute, and terminating with a 10-minute final incubation at 72°C.

3.6.3 Profile sequencing

The successful PCR amplification products were sequenced using an ABI Bigdye 3.1 sequencing kit (Applied Biosystems, California USA) on ABI 3730XL automated sequencing analyzer at Laragen Inc., Culver City, California.
Sequences (Appendix VI) obtained were compared with sequences listed in GenBank database using the NCBI Basic Local Alignment Search Tools (BLAST) and identified based on the closest relative (Altschul *et al.*, 1997). The 16S rRNA gene clone sequences that had 99-100% similarity to sequences deposited in GenBank were designated as belonging to the corresponding species and strains. Phylogenetic analysis was conducted utilising computer software plan and neighbour-joining methods with MEGA (6) package. All bacterial 16S rRNA gene sequences were entrusted in GenBank using their accession numbers.

3.7 Screening for potential starters

3.7.1 Starch hydrolysis

Selected LAB isolates were streaked on sterile modified MRS agar containing 0.4% (w/v) soluble starch as the sole carbon source. The cultures were incubated at 37°C for 24 hours, after which they were flooded with Gram's iodine. Degradation of starch was evident by zone of inhibition around the streak, indicating starch utilisation (Edward *et al.*, 2012).

3.7.2 Production of linamarase

A medium for testing linamarase (in the form of β –glucosidase) production was prepared by adding 0.1 g of 4-nitrophenyl-B-D-glucopyranoside (Sigma Aldrich,

UNIVERSITY OF IBADAN

Germany) to 100 mL 0.666 M NaH_2PO_4 (pH 6). The mixture was dissolved and filter-sterilized. The test culture was grown on MRS agar for 24 hours at 30°C. Colonies were picked from the plates using a sterile loop and were emulsified in physiologic saline to McFarland Turbidity Standard No. 3. Thereafter, 0.75 mL of culture was added to 0.25 mL of the test medium. It was incubated at 30°C overnight. Positive isolates that produced β -glucosidase degraded the linamarin analogue and changed the colour of the mixture from colourless to distinct yellow (Edward *et al*., 2012)

3.7.3 Production of pectinase

Chemically defined medium (CDM) containing pectin (Appendix VII) for the screening for pectinase enzyme was used. The test culture was grown on MRS agar for 18 hours at 30°C. Colonies were picked from the plates using a sterile loop and streaked on the sterile CDM plate, then incubated for 24 hours. Colonies showing zone of inhibition upon flooding with 1% cetyltrimethyl ammonium bromide were confirmed as pectinase producers (Altan, 2004).

3.7.4 Acidification of growth medium by selected isolates

MRS broth was prepared from a single batch with initial pH 6.50. Aliqouts (10 mL) were dispensed into screw cap tubes and autoclaved at 121°C and 15 psi for 15 minutes. The selected isolates were inoculated into each tube and incubated at 30°C. Acid production was determined by measuring the pH of the culture medium at 24, 48 and 72 hours respectively (Kostinek *et al.*, 2005).

3.7.5 Production of Lactic acid

The amount of lactic acid produced in the fermented cassava was determined by the standard titration procedure for total titratable acidity (Lonner *et al.,* 1986). Twenty five millilitres (25 mL) of broth culture was titrated with 1M NaOH using phenolphthalein as indicator. Acid equivalent is the amount of NaOH (mL) consumed. Each mL of 1M NaOH is equivalent to 90.08 mg of lactic acid.

3.7.6 Production of Hydrogen peroxide

Hydrogen peroxide production was determined by measuring 25mL of broth culture into a 100mL flask. To this was added 25 mL of dilute H_2SO_4 (10%). The preparation was then titrated with 0.1M potassium permanganate ($KMnO_4$). The end

UNIVERSITY OF IBADAN

point was the point at which the pale pink colour persisted for 15 seconds before de-colourization. Each mL of 0.1M $KMnO_4$ is equivalent to 1.701 mg of H_2O_2 (Sanni *et al*., 1995). The volume of H_2O_2 produced was then calculated as follows:

$$H_2O_2\,\text{concentration} = \frac{KMnO_4(mL) \times KMnO_4(M) \times M.E. \times 100}{H_2SO_4(mL) \times sample(mL)}$$

3.7.7 Production of Diacetyl

To 25 mL each of the broth culture, 7.5 mL of hydroxylamine solution (1M) was added in two flasks (one flask was for residual titration). Both flasks were titrated with 0.1M HCl to a greenish yellow end point using bromophenol blue as indicator (Sanni *et al*., 1995). The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated as follows:

$$Ak = \frac{(R - S)(100E)}{W}$$

where Ak (mg) is the percentage of diacetyl, R the volume of 0.1M HCl used in residual titration, S the volume of 0.1M HCl used in titration of sample, E the equivalence factor and W is the volume of sample.

3.7.8 Antimicrobial activity against pathogenic organisms

3.7.8.1 Preparation of Cell-Free Filtrate

MRS broth (10 mL) was inoculated with selected isolates and incubated at 37°C for 48 hours. A cell-free solution was obtained by centrifuging the bacterial culture at 6000 rpm for 15 minutes followed by filtration of the supernatant through 0.2 mm pore size filter, thus, obtaining cell free filtrate (Kalalou *et al*., 2004).

3.7.8.2 Agar Well Diffusion Test

The antimicrobial activity of the isolated LAB (cell free filtrate) against *Escherichia coli, Bacillus cereus*, *Proteus* species, *Salmonella* sp., *Corynebacterium* sp. and *Shigella* sp. was performed by the agar well diffusion assay. Loopful of the test pathogenic bacteria were incubated in brain heart Infusion (BHI) broth at 30°C for 24 hours. Petri dishes containing 20 mL of sterile Muller Hinton agar were streaked with 0.1 mL of 24 hour-old broth culture of each pathogenic bacterium. Wells were cut on

UNIVERSITY OF IBADAN

the agar using sterile 5 mm corkborer and filled with 100 µL of cell-free filtrate. The plates were stored for 2 hours in the middle compartment of the refrigerator and then incubated at 37°C for 24 hours. The antimicrobial activity was determined by measuring the diameter (mm) of inhibition zone around the wells (Schillinger and Lucke, 1989).

3.8 Fermentation studies with identified isolates

This was carried out by inoculating the isolates singly and in-combination into a 10 L capacity bioreactor containing cassava in cut pieces and grates, soaked in 2 L sterile distilled water (pH 7.1) for *fufu* and *usi* production respectively.

3.8.1 Preparation of cassava for controlled fermentation

Cassava tubers for *fufu* were cut into small pieces of about 3 – 5 cm long while those for usi were grated. Cut/grated cassava tubers (200 g) were sterilized using 0.1% HgCl in 70% ethanol followed by rinses with sterile distilled water (Adetunde and Onilude, 2010).

3.8.2 Determination of Inoculum size

The selected lactic acid bacteria were inoculated into sterile MRS broth and incubated at 30°C for 48 hours. Aliquot (1 mL) of broth culture was introduced into a fresh sterile broth and incubated for 24 hours. At the end of incubation period, the broth was centrifuged (Himac CR21GII, Japan) at 5000 rpm for 10 minutes. The supernatant was decanted while the pellet was washed with sterile distilled water and re-centrifuged before being suspended in sterile normal saline.

The inoculum size was determined using McFarland standard. Dilutions were made with sterile normal saline to McFarland standard (No 4) using a spectrophotometer (Cecil CE 1011, Cambridge, England) to give 0.669 optical density at 600 nm, resulting in an approximate cell density of 1.2×10^9 CFU/mL (McFarland, 1907). Aliquots (5 mL) of each resultant diluent was used as inoculum in all cases either singly or in-combination to inoculate 200 g of the cassava soaked in 2 litres sterile distilled water and fermentation was allowed for 72 hours at room temperature.

UNIVERSITY OF IBADAN

3.9 Performance studies on fermenting mash

3.9.1 pH of fermenting mash

Ten gram (10 g) of fermenting cassava samples was mixed with 90 mL of sterile distilled water, homogenized and filtered through a Whatman's Filter paper (No. 1). The pH was then measured at 24 hours interval using a pH meter (H19107 Hanna), after standardizing with phosphate buffer solution of pH 4.0 and 7.0.

3.9.2 Total titratable acidity of fermenting mash

Titratable acidity was determined using the standard titration procedure for total titratable acidity (TTA) as described by Lonner *et al*. (1986). Ten gram of the fermenting mash was mixed with 90 mL sterile distilled water and homogenized. The mixture was filtered through Whatman filter paper (No. 1) and the filtrate titrated against 1M NaOH using 1% phenolphthalein as indicator. Acid equivalent is the amount of NaOH consumed in mL. Each mL of 1M NaOH is equivalent to 90.08 mg of lactic acid.

3.9.3 Nutritional Analysis of fermenting mash

Methods described by AOAC (2005) were used to estimate moisture content, crude protein, fat, fibre, ash and total carbohydrate.

3.9.3.1 Determination of moisture content

A metallic dish was dried in an oven (Fisher scientific, model 655F, USA) at 110°C for 10 minutes to a constant weight. It was cooled in a desiccator for 30 minutes and weighed. Five gram of the sample was placed in the dish and weighed. The dish with the sample was then dried in an oven at 105°C for 4 hours to achieve a constant weight and was quickly transferred to the desiccator to cool. It was re-weighed immediately after cooling with minimum exposure to the atmosphere. The loss in weight of the sample during drying was the moisture content.

UNIVERSITY OF IBADAN

3.9.3.2 Determination of crude protein

The crude protein content of fermenting cassava mash was determined by the micro Kjeldahl method using a protein conversion factor of 5.80. Sample (0.5 g) was weighed and placed on filter paper, then folded and dropped into a Kjeldahl digestion tube. Three gram (3 g) digesting mixed catalyst ($CuSO_4$ + Na_2SO_4) and 25 mL of concentrated Na_2SO_4 were added to the sample in the digestion tube. The mixture was transferred to the Kjeldahl digestion apparatus with the heater regulated at a temperature below the boiling point of the acid until frothing ceased. The mixture was allowed to boil vigorously as the temperature increased, until a clear (light) green colour was obtained. The digest was allowed to cool, then transferred into 100 mL volumetric flask and diluted with distilled water to make up 100 mL. Ten millilitres (10 mL) aliquot of the digest was introduced into the distillation jacket of the micro steam distillation apparatus that was connected to the main, as the water in the distiller flask boils. 20 mL of 40% (w/v) NaOH was added to the digest in the distillation jacket. 50 mL of 40% boric acid was measured into 250 mL conical flasks and four (4) drops of methyl red indicator were added each. The conical flask containing the mixture was placed onto the distillation apparatus with the outlet tube inserted into the conical flask and NH_3 was collected through the condenser. The distillation continued until 25 mL of the distillate was trapped into the boric acid solution and colour changes from red to yellow. The distillate was then titrated against 0.02 M HCl and the titre values recorded. Percentage crude protein was calculated (AOAC, 2005).

3.9.3.3 Determination of crude fat

The fat content was determined by direct Sohxlet extraction using petroleum ether (bp 40–60^oC) as solvent (AOAC, 2005). Sample (0.5 g) contained in a filter paper was placed in the sohxlet extraction apparatus (Osk, Japan) and the set-up was placed on a heating mantle. The heat source was adjusted so that the solvent boiled gently and refluxed several times for 6 hours until the ether had siphoned over and the barrel of the extractor was empty. On removal, the filter paper containing the sample was dried in an oven at 50^oC to a constant weight and percentage lipid (fat) was then calculated (AOAC, 2005).

UNIVERSITY OF IBADAN

3.7.3.4 Determination of ash content

The ash content of the fermented cassava was determined by placing a pre-weighed crucible with 5 g of the sample on a burning flame for 15 minutes until smoke ceased. It was then transferred into a muffle furnace (Gallenkamp, England), and heated at 550°C for 4 hours, leaving a white ash. The crucible was removed, immediately covered and placed in a desiccator to cool. The weight was measured and the percentage ash was calculated (AOAC, 2005).

3.9.3.5 Determination of fibre content

Cassava sample (5 g) was weighed into a round bottom flask. Sulphuric acid 100 mL, 0.25 M) was added and the mixture boiled for 30 minutes. The hot solution was quickly filtered under suction. The residue was thoroughly washed with hot water until acid free. This was further transferred into labeled flask and 100 mL of hot 0.3 M sodium hydroxide solution was added. The mixture was allowed to boil again under reflux for 30 minutes and filtered quickly under suction. Insoluble residue was washed with hot water until it was base free, dried to a constant weight in an oven at 100ºC for 2 hours, cooled in desiccator and weighed. The weighed samples were then incinerated, and reweighed. Percentage crube fibre content was calculated (AOAC, 2005).

3.9.3.6 Determination of total carbohydrate

The total carbohydrate content was calculated by difference in which the sum of the percentage moisture, ash, crude lipid, crude protein and crude fibre was subtracted from 100%.

3.9.4 Anti-nutritional factors of the fermenting cassava mash

3.9.4.1 Determination of cyanide content

The cyanide content of the fresh and fermenting cassava was determined using the method of Ojimelukwe (1997). Twenty gram (20 g) of the crushed cassava was homogenized in 200 mL distilled water for 10 minutes. The homogenate was incubated for 18 hours at room temperature after which 100 mL of 5% $NaHCO_3$ was added to it before distillation. After distillation, the filtrate was collected and titrated

UNIVERSITY OF IBADAN

against 0.2% iodine solution using 1% starch as indicator. Cyanide content was calculated using the titre value.

3.7.3.7 Estimation of phytic acid

The phytic acid was determined using the procedure described by Markkar *et al*. (1993). Two gram (2g) of sample was weighed into 250 mL conical flask. A hundred millilitre concentrated HCl acid (2% v/v) was used to soak the sample in the conical flask for 3 hours and then filtered through a double layer of hardened filter papers. The filtrate (50 mL) was placed in 250 mL beaker and 100 mL of distilled water was added to give proper acidity. Ten millilitres (10 mL) of 0.3% (w/v) ammonium thiocyanate solution was added to the solution as indicator. The solution was titrated with standard iron chloride solution, which contained 0.00195 g iron per millilitre. The end point colour was slightly brownish-yellow which persisted for 5 minutes. The percentage phytic acid was calculated using the titre value.

3.7.3.8 Estimation of tannin content

The method described by Markkar *et al*. (1993) was adopted. Four hundred milligram (400 mg) of sample was placed in 500 mL conical flasks and 40 mL diethyl ether containing 1% acetic acid (v/v) was added, then the mixture was properly mixed to remove the pigment materials. The supernatant was carefully discarded after 5 minutes and 20 mL of 70% aqueous acetone was added and the flasks were sealed with cotton plug covered with aluminum foil, then kept in shaker for 2 hours for extraction. The content of each flask was filtered through Whatman filter paper (No. 1) and samples (filtrates) were used for analysis. Aliquot (50 mL) of tannin extract from the sample was introduced into test tubes and the volume was made up to 100 mL with distilled water. Folin-Ciocalteua reagent (0.5mL) was added to each and mixed properly after which 2.5 mL of 20% (w/v) sodium carbonate solution was added and further mixed. The mixture was kept for 40 minutes at room temperature, and absorbance was read at 725 nm using spectrophotometer (Cecil CE 1011, Cambridge, England). Tannin concentration was estimated from the tannic acid standard curve.

UNIVERSITY OF IBADAN

3.10 Estimation of enzyme activities during fermentation

3.10.1 Enzyme crude extract

An 80 mL sample of 0.1 M citrate buffer, pH 6.5, was added to 40 g of cassava mash, homogenized and kept overnight at 4°C. The mixture was centrifuged (Himac CR21GII Hitachi, Japan) at 12,000 rpm for 30 minutes. The resulting filtrate was used for enzyme assay (Ampe and Brauman, 1995).

3.10.2 Amylase activity

This was determined by DNS method (Miller, 1959). One millilitre (1 mL) of sample was added to 1 mL of standard starch solution (containing 1% soluble starch in 0.1 M phosphate buffer pH 7) and incubated at room temperature for 30 minutes. Reducing sugars were estimated by stopping the reaction with the addition of 1 mL Di-nitrosalicylic acid (DNS) reagent, boiled for 5 minutes in a water bath (Clifton, England) and cooled under running tap water. Thereafter, 2 mL of sterile distilled water was added to dilute the solution. The absorbance of the resulting solution was determined at 540 nm with spectrophotometer (Cecil CE 1011, Cambridge, England) against a reagent blank. A standard graph was generated using standard glucose solution (Appendix VIII). One unit of amylase activity was taken as the amount of enzyme in 1 mL of crude amylase that produced 1 mg of reducing sugars under the standard assay conditions (Panduranga *et al*., 2010).

3.10.3 Linamarase assay

Quantification of linamarase was based on the degradation of linamarin analogue, para-nitrophenolgalactosidase (PNPG) and determination of the released p-nitrophenol (Ikediobi *et al*., 1980). The assay medium included 0.5 mL of enzyme extract and 1 mL of PNPG (5 mM) in 0.01 M phosphate buffer (pH 6.8). The mixture was incubated for 15 minutes at 65°C, and the reaction was terminated by the addition of 2 mL of 0.2 M borate buffer (pH 9.8). Colour of the released p-nitrophenol was measured at 425 nm against enzyme blank using spectrophotometer (Cecil CE 1011, Cambridge, England). A standard graph was generated using standard PNPG solution (Appendix IX). One unit of linamarase activity was expressed as the amount that caused a change in absorbance of 0.01 unit against an enzyme blank under the defined assay conditions (Ugwuanyi *et al*., 2007).

UNIVERSITY OF IBADAN

3.10.4 Pectinase assay

Pectinase enzyme was assayed using the colorimetric method as outlined by Miller (1959). Five milliliters (5 mL) of cell free supernatant was incubated with 2% pectin in 0.1 M acetate buffer (pH 6.0) and the reaction mixture was incubated at 40°C for 10 minutes. After adding 1.0 mL of DNSA reagent (without sodium potassium tartarate), the mixture was boiled for 5 minutes at 90°C. The reaction was stopped by adding 1 mL of Rochelle's salt (sodium potassium tartarate - Sigma, USA). The mixture was further diluted by adding 2 mL of distilled water after which the absorbance was read at 595 nm using spectrophotometer (Cecil CE 1011, Cambridge, England) to estimate the reducing sugar released. A standard graph was generated using standard glucose solution. One unit of pectinase activity was defined as the amount of enzyme which liberated 1 µm glucose/minute (Karthik *et al*., 2011).

3.11 Analysis of metabolites in the fermenting mash

Metabolites were analyzed by the high performance liquid chromatography (HPLC) according to the method of Andersson *et al.* (2007), with slight modification to the flow rate. The organic acid concentration was measured using HPLC (CECIL CE4200, Cambridge England) equipped with a C18 column (Ultra Aqueous, 5μm, 150mm x 4.6 mm, Restek). The mobile phase was 50 mM KH_2PO_4 buffer with 5% acetonitrile (pH 2.5 adjusted by 37% HCl) at a flow rate of 1.0 mL/minute. Samples (20μL) were injected manually for analysis with ultra violet detection at 210 nm. Samples for acid analysis were taken at 24 hour intervals and centrifuged (Himac CR21GII Hitachi, Japan) at 10 000 rpm for 10 minutes at 4°C. The supernatant was diluted with the mobile phase and 37% hydrochloric acid in the ratio 0.1:0.8:0.1 mL respectively and further filtered through a 0.2 μm syringe filter.

Sugar concentration was determined using the same HPLC system as above but equipped with a Series 200 refractive index (RI) detector (Perkin-Elmer), guard column, and an ion exchange column (Aminex HPX87-P, BioRad). The column was kept at 85°C in a column oven for optimal performance. Prior to analysis, the samples were centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatant was diluted with water and filtered through a 0.2 μm syringe filter. Samples (20 μL) were injected manually and water, at a flow rate of 1.0 mL min^{-1} was used as the mobile phase.

The analysis was externally calibrated using standard solutions as prepared for the samples. Peak area from the chromatograms were evaluated by comparison to standard

UNIVERSITY OF IBADAN

curves generated (Sigma Aldrich, Germany) with known concentrations of organic acids (lactic acid, acetic acid, propionic acid and butyric acid) as well as sugar standards (sucrose, glucose, fructose, xylose, maltose, ribose, arabinose, lactose and rhamnose).

3.12 Selection of starter

The selection of a common starter for the two products was based on improved nutritional contents, reduced anti-nutritional contents and rapid acidification. Results were statistically analysed using SPSS 20.0 software package and the most common to both food products was selected for further use.

3.13 Optimization studies on selected starter

3.13.1 Determination of inoculum size

The inoculum size was determined using McFarland standard. Dilutions were made using 18-hour-old culture of the selected starter with sterile normal saline to McFarland standard (No 4) using a spectrophotometer (Cecil CE 1011, Cambridge, England) to give 0.669 optical density at 600nm, resulting in an approximate cell density of 1.2×10^9 CFU/mL (McFarland, 1907).

3.13.2 Determination of optimal pH

Effect of different pH values on starter growth was determined by inoculating 0.1 mL of 18-hour-old culture (10^7) onto sterile MRS broth that was adjusted to different pH values (3.5, 4.5, 5.5, 6.5, and 7.5). The broth culture was incubated for 24 hours and the optical density (OD) determined at 600 nm using spectrophotometer (Cecil CE 1011, Cambridge, England) (Tofan *et al*., 2002).

3.13.3 Determination of optimal incubation temperature

Effect of different incubation temperatures on starter growth was determined by inoculating 0.1 mL of 18-hour-old culture (10^7) onto sterile MRS broth and incubated at different temperatures (25°C, 30°C, 37°C, 40°C and 45°C) for 24 hours. The OD at 600 nm was determined (Tofan *et al*., 2002).

UNIVERSITY OF IBADAN

3.13.4 Determination of optimal salt concentration

Optimal salt concentration for starter growth was determined by inoculating 0.1 mL of 18-hour-old culture (10^7) onto modified MRS broth containing different NaCl concentrations (2%, 4%, 6%, 8% and 10%). They were incubated at 30°C for 24 hours and OD at 600 nm was determined (Tofan *et al*., 2002)

3.13.5 Effect of agitation on growth

Agitation intensity had effect on the mixing and oxygen transfer rates in a culture medium and thus influences cell growth and product formation (Wijbenga *et al*., 1991). Broth cultures were subjected to different agitation speed using an incubator shaker (Thermoscientific, USA). Aliquot (0.1 mL) of 18-hour old culture (10^7) was inoculated into sterile MRS broth and subjected to agitation at 50 rpm, 100 rpm, 150 rpm, 200 rpm and 250 rpm respectively. OD at 600 nm was determined after 24 hours using a spectrophotometer (Cecil CE 1011, Cambridge, England).

3.13.6 Effect of carbon sources on growth

The effect of different carbon sources (starch, glucose, galactose, maltose and lactose) on growth of selected starter was determined according to the method of Enitan *et al*. (2011) with modification to the optical density used. Overnight culture (0.1mL) was inoculated into modified MRS broth containing 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.4H_2O$, 5 g sodium acetate, 1.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 10 g peptone, 5 g yeast extract, 1 mL of Tween 80 per litre of distilled water and each sugar at 2% w/v was introduced as the sole carbon source. Incubation was done at 30°C and optical density at 500 nm determined using a spectrophotometer (Cecil CE 1011, Cambridge, England) after 24-hour incubation.

3.13.7 Effect of nitrogen sources on growth

The effect of different nitrogen sources (peptone, yeast extract, casein, urea and $[NH_4]_2SO_4$) on growth of the selected starter was determined by inoculating 0.1mL of overnight culture into modified MRS broth containing 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4$. $4H_2O$, 5 g sodium acetate, 1.5 g KH_2PO_4, 6% w/v glucose, 1.0 mL vitamin solution (0.2 g vitamins B6, 0.1 g riboflavin and 0.1 g folic acid in 100 mL of 20% ethanol) per litre of distilled water and each listed substrate at 2% w/v as its sole nitrogen source. Incubation was done at 30°C for 24 hours and the optical density was

UNIVERSITY OF IBADAN

determined at 500 nm using a spectrophotometer (Cecil CE 1011, Cambridge, England) (Enitan *et al*., 2011).

3.13.8 Effect of different incubation time on growth

The growth rate was determined by inoculating 0.1 mL of 18-hour old culture into sterile MRS broth. It was incubated at 30°C and samples withdrawn at 24-hour intervals for three days. The optical density at 620 nm was determined using a spectrophotometer (Cecil CE 1011, Cambridge, England) (Manca De Nadra *et al*., 2003).

3.14 Application of starter in traditional processing of *fufu/usi* under optimized conditions and performance studies on the products

3.14.1 Inoculum preparation

The selected starter(s) were cultivated on sterile MRS agar. The culture plates were incubated at 30°C for 48 hours. Pure colonies were introduced onto a fresh broth (30 mL) using the determined optimal growth conditions and incubated at 30°C for 24 hours. At the end of the incubation period, the broth was centrifuged (Himac CR21GII Hitachi, Japan) at 5000 rpm for 10 minutes at 4°C. The supernatants were decanted while the pellets were washed with sterile distilled water and re-centrifuged before being re-suspended in sterile distilled water. Dilutions were made with sterile normal saline to McFarland standard (No 4) using a spectrophotometer (Cecil CE 1011, Cambridge, England) to give 0.669 optical density at 600 nm, resulting in an approximate cell density of 1.2×10^{9} CFU/mL. Aliqout (5 mL) of each diluent was used singly and in-combination as inoculum for the fermentation process. An un-inoculated experiment served as the control (McFarland, 1907).

3.14.2 Preparation of *Fufu* and *Usi*

Fufu: The fermented roots were taken out after 72-hour fermentation, broken with clean hands and the fibres removed by sieving through a muslin cloth after the addition of water. The starch suspension was allowed to sediment in a large container for about 24 hours after which the water was decanted. The fine, clean starch was thereafter dewatered by putting in cotton bags and pressed with heavy stones overnight (Oyewole and Odunfa, 1989). A quantity of the slurry containing about 25% of *fufu*

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paste in water was boiled in an open pan. After continuous stirring using a wooden rod, strong dough (ready-to-eat *fufu*) was formed (Kwatia, 1986; Ayankunbi *et al*., 1991; Anon, 1994).

Usi: The fermented grated root mixture was stirred and filtered through a piece of cloth (sieve). The filtrate stands overnight and the supernatant was decanted. The fine starch paste was collected and put in a wide metal pan already smeared with red palm oil. Water was added and then stirred with the hand to dissolve completely. The pot was put on fire and the solution constantly stirred with a wooden rod until being converted to a very sticky, light yellow mass (Etejere and Bhat, 1985).

3.12.3 Nutritional analysis of cassava fermented with starter cultivated under optimized growth condition

Methods described by AOAC (2005) were used to estimate crude protein, fat, carbohydrate, fibre, ash and moisture contents as earlier described. Anti-nutritional content (tannin, phytic acid and cyanide) were also estimated as earlier described. The results were compared to un-inoculated samples (control) as well as the samples obtained from the starter that was not subjected to optimized growth conditions.

3.12.4 Sensory evaluation of products

The acceptability of the cassava products was determined through sensory evaluation using Hedonic scale test. The products were presented in small plates and coded, to avoid bias from panelists. Modified method of (Iwe, 2002) was used in which a 20-man trained panel each that was familiar with each food product was set up to determine their general acceptability. Panellists were provided with drinking water to rinse their mouth after tasting each sample. A 7-point Hedonic rating on the degrees of acceptability was conducted with score 7 having like extremely, 6- like very much, 5- like moderately, 4- indifferent, 3- dislike moderately, 2- dislike very much and 1 having dislike extremely for the following attributes (texture, colour, taste, odour and overall acceptability). Each product was considered against a control (spontaneously fermented) product.

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3.12.5 Microbiological assessment and physical evaluation of products during storage

Samples of stored *fufu* and *usi* were taken at 24 hours interval for microbial enumeration over a 7-day period. One gram (1 g) each of starter-fermented and the spontaneously-fermented *fufu* and *usi* were homogenized in 9 mL of sterile peptone water. Ten-fold serial dilution was done aseptically and 0.1 mL of 10^{-9} dilution was introduced into sterile petri dishes. Sterile Plate Count Agar, MRS agar, Violet Red Bile Glucose Agar, Malt Extract Agar, Potato Dextrose Agar and Manitol Salt Agar for the enumeration of total bacteria, LAB, coliforms, yeast, mold and *Staphylococcus* respectively, were poured into the plates, allowed to set and incubated. Microbial count was done thereafter on each agar medium.

Furthermore, spoilage symptoms (appearance of mold and slime, change in colour, texture odour and firmness) were also observed for a period of 7 days (Aderiye *et al.*, 2006). Each product was considered against a control (spontaneously-fermented) product.

3.15 Statistical Analysis

Data obtained were subjected to statistical analysis. Duncan's Multiple Range Test was used to separate mean values and Analysis of variance (ANOVA) at 5% level of significance was used to determine differences. Data in tables were presented as mean ± standard deviation while figures were presented as mean ± error bars.

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CHAPTER FOUR

RESULTS

4.1 Physico-chemical analysis of spontaneously-fermenting cassava for *fufu* and *usi* production

A rapid decrease in pH was first observed within the first 24 hours, after which the reduction was gradual till the end of the fermentation during the spontenous fermentations for both *fufu* and *usi* (Figure 4.1). The mean pH of the spontaneously fermented cassava showed decrease in pH values with increase in fermentation time for both products. The initial pH for *fufu* and *usi* mashes at zero hour was 7.1 ± 0.31. The values decreased, reaching its minimum 4.43 ± 0.31 and 4.27 ± 0.21, respectively at 72 hours. The pH values for fermenting *usi* mashes were slightly reduced than those for *fufu.*

A progressive increase in the percentage titratable acidity was observed during *fufu* fermentation while a decrease from the beginning of fermentation up till the 48th hour occurred during *usi* fermentation with a slight increase at 72 hours. The mean titratable acidity (%) of fermenting *fufu* ranged from 0.027 ± 0.01 to 0.393 ± 0.12 while those for *usi* was between 0.039 ± 0.01 and 0.084 ± 0.03 as illustrated in Figure 4.2.

4.2 Microbial load during spontaneous *fufu* and *usi* fermentation

The mean microbial count on Plate Count Agar, De Mann Rogosa Sharpe agar, Violet Red Bile Glucose Agar and Malt Extract Agar for total bacteria, Lactic Acid Bacteria, coliforms and yeasts, respectively, were shown in Table 4.1. During *usi* fermentation, the highest total count (7.50×10^8 CFU/mL) was observed at 24 hours while a sharp decrease (1.76×10^8 CFU/mL and 0.13×10^8 CFU/mL) was at 48 and 72 hours, respectively. There was an increase in LAB count at the beginning of fermentation up till 24 hours from 2.9×10^8 CFU/mL to $5.5.7 \times 10^8$ CFU/mL, but decreased at 48 hours to 0.91×10^8 CFU/mL and further 0.07×10^8 CFU/mL at 72hours.

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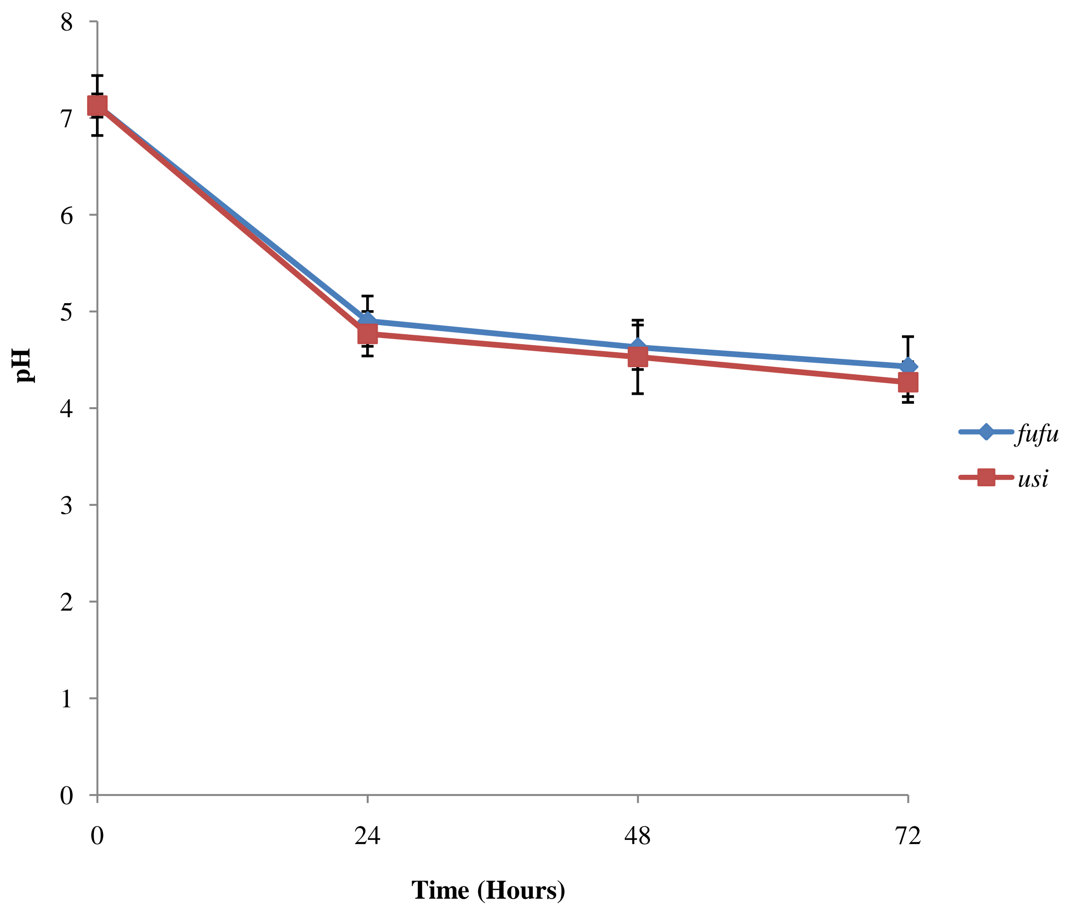

Figure 4.1: pH of spontaneously-fermenting cassava for *fufu* and *usi*

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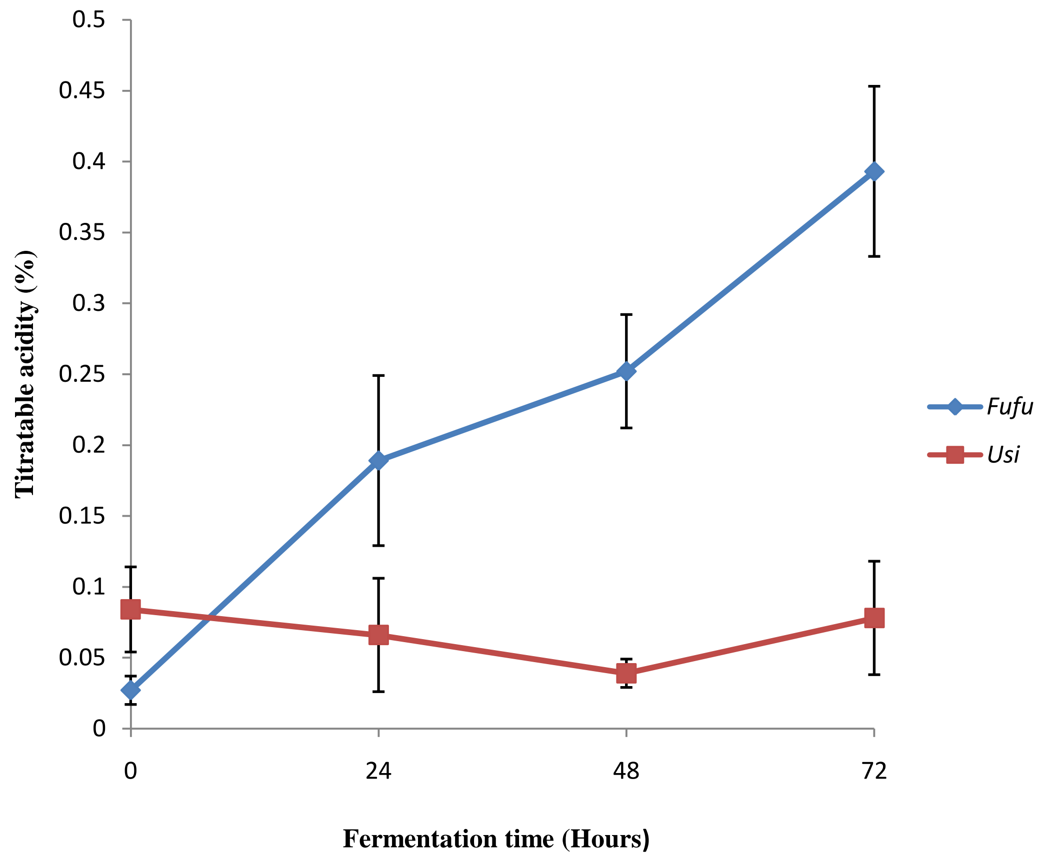

Figure 4.2: Titratable acidity (%) of spontaneously-fermenting cassava for *fufu* and *usi*

Table 4.1: Microbial count (10^8 CFU/mL) during spontaneous fermentation of cassava for *fufu* and *usi* production

	Time (Hours)/Products/ Microbial counts (10^8CFU/mL)							
	Fresh cassava		24		48		72	
Sample	F	U	F	U	F	U	F	U
TBC	6.17±5.28	4.10±1.57	4.43±2.47	7.50±1.04	5.29±4.69	1.76±2.81	3.44±4.37	0.13±0.04
LC	2.03±1.86	2.90±0.85	2.67±1.36	5.70±0.3	3.43±1.50	0.91±1.38	4.03±1.69	0.07±0.04
CC	1.56±1.29	1.22±0.33	1.37±0.48	1.19±0.53	1.09±0.98	0.39±0.61	0.16±1.18	0.06±0.02
YC	2.10±0.54	1.90±1.02	2.12±0.71	1.50±1.21	0.91±0.88	0.72±1.10	0.82±1.21	0.56±0.09

Values are mean ± SD, n=3; F (*Fufu*), U (*Usi*), TBC (Total bateria count), LC (LAB count), CC (Coliform count), YC (Yeast count).

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Coliform count also decreased from the peak value (1.22×10^8 CFU/mL) at the beginning of fermentation to 1.19×10^8 CFU/mL, 0.39×10^8 CFU/mL and 0.06×10^8 CFU/mL at 24, 48 and 72 hours respectively. Total bacteria count was maximal at the beginning of fermentation (6.17×10^8 CFU/mL) and the least (3.44×10^8 CFU/mL) was at 72 hours during *fufu* fermentation. There was gradual increase in LAB count as fermentation progressed with count ranging between 2.03×10^8 CFU/mL and 4.03×10^8 CFU/mL. Decrease in coliform count (1.56×10^8 CFU/mL to 0.16×10^8 CFU/mL) was however observed with increase in fermentation time. In both fermentations, yeast count decreased till the end of the fermentation ranging between 1.9×10^8 CFU/mL and 0.56×10^8 CFU/mL in *usi* while it was between 2.10×10^8 CFU/mL and 0.82×10^8 CFU/mL in *fufu.*

In general, the total bacterial count in *usi* increased within the first 24 hours, but decreased at 48 hours and further at 72 hours, whereas, a lower value at the beginning of fermentation up till 24 hours which later increased at the 48th hour was observed in *fufu*. LAB count increased with increase in fermentation time in *fufu* while it increased up till 24 hours and then decreased gradually in *usi*. Coliform and yeast counts decreased till the 72nd hour in both fermentations.

4.3 Isolation of Lactic Acid Bacteria from fermenting cassava mash

Ninety-eight (98) Lactic Acid Bacteria isolates were obtained from the fermenting cassava mashes for both *fufu* and *usi*. The isolates were characterized and identified based their cultural, morphological, physiological and biochemical properties.

The colonial morphology of the randomly selected isolates varied from small, medium to big colonies, shiny, creamy and whitish in colour. Most were concave while some were either flat or embedded on the agar mdium (Table 4.2). Biochemical characterization showed the isolates to be Gram positive colonies of medium, short and long rods while some are cocci in shape, catalase negative, non motile and do not hydrolyze starch and gelatin. Some produced ammonia from arginine and grew at 6.5% NaCl. Hydrogen sulphide was produced by all isolates, but they were negative to methyl red test. Varied sugar utilization pattern was observed by the organisms where glucose, sucrose, fructose, lactose and maltose were fermented by all isolates. Sorbose, inositol and starch were not utilized as shown in Table 4.3.

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Table 4.2: Colonial morphology of randomly selected Lactic Acid Bacteria

Isolate code	Elevation	Colour	Surface	Edge	Opacity	Shape	Size	Number of randomly selected isolates
I	Convex	White	Shiny	Entire	Opaque	Circular	Small	17
II	Convex	White	Shiny	Entire	Opaque	Circular	Big	8
III	Flat	Cream	Shiny	Irregular	Opaque	Star like	Medium	10
IV	Embedded	Cream	Shiny	Irregular	Opaque	Spindle	Small	6
V	Convex	Cream	Shiny	Irregular	Opaque	Spindle	Small	6
VI	Embedded	Cream	Shiny	Entire	Opaque	Circular	Big	8
VII	Convex	Cream	Shiny	Entire	Opaque	Circular	Medium	27
VIII	Flat	Cream	Shiny	Irregular	Opaque	Irregular	Medium	10
IX	Flat	Cream	Shiny	Entire	Opaque	Circular	Small	6

Key

I - F1E, F1F, FIG, F1J, F1K, LFA, F2A, F2B, F2C, F2E, F2F, F3I, F3J, F3N, U1A, U1B, U1D

II - LFF, U1C, U2I, LFK, U3A, U3G, U3J, U3P

III - F3M, U1N, LFJ, U3C, U3H, LFB, F2J, F3B, F3L, U1M

IV - U3P, LFE, F2K, U1P, U2J, U2P

V - U2C, U2D, U2F, F1I, U1K, U2L

VI – F1H, F1L, F2N, F3D, LFH, LFG, LFI, U1F

VII - U2H, U2K, U2L, U3D, U3E, U3F, U3I, U3K, F1A, F1B, F1D, F2G, F2I, F3A, F3C, F3E, F3F, F3G, F3H, U1E, U1G, U1H, U1I, U1J, U1L, U2A, U2B

VIII - LFC, F2D, F3K, U3O, U3B, U2E, U1O, U2M, U3N, U2L

IX - F1C, F1J, LFD, F2H, F2L, F2M

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Table 4.3: Biochemical characteristics of selected LAB isolates

Isolate code	Grams rxn	Shape	Catalase	NH3 from Arginine	4% NaCl	6.5%NaCl	8% NaCl	Motility	Starch hydr	MR	VP	Gelatin	H2S pdtn	Glucose	Maltose	Mannitol	Sucrose	Melibiose	Galactose	Fructose	Sorbitol	Raffinose	Lactose	Xylose	Starch	Arabinose	Inositol	Sorbose	No. of occurence	Probable id
I	+	rod	-	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	49	*L. plantarum*
II	+	cocci	-	-	+	-	-	-	-	+	-	-	+	+	-	-	-		-	+	-	+	+	-	-	-	-	-	6	*Leuconostoc. mesenteroides*
III	+	rod	-	-	+	+	-	-	-	+	-	-	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	12	*L. acidilactici*
IV	+	rod	-	+	+	+	-	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	10	*L fermentum*
V	+	rod	-	-	+	+	-	-	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	8	*L. delbruekii*
VI	+	rod	-	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	11	*L. brevis*
VII	+	cocci	-	+	+	-	-	-	-	+	-	-	+	+	+	-	+	+	+	+	-	+	+	-	-	+	-	-	2	*L. lactis*

Key: + positive, - negative

I - F1A, F1B, F1D,F1E, F1F, FIG,F1K, LFA, F2A, F2B, F2C, F2E, F2F, F2G, F2I, F3A, F3C, F3E, F3F, F3G, F3H,F3I, F3J, F3N, U1A, U1B, U1D, U1E, U1G, U1H, U1I, U1J, U1L, U2A, U2B, U2C, U2D, U2F, U2G, U2H, U2K, U2L,U2Q U3D, U3E, U3F, U3I, U3K, U3M.

II – F1C, F1J, LFD, F2H, F2L, F2M

III – F1H, F1L, F2N, F3D, LFH, F3M, U1N, LFJ, U3C, U3H, U3N,U3L

IV – F1I, LFC, F2D, F3K, U3O, U3B, U2E, U1O, U2M, U1K

V – LFB, F2J, F3B, F3L, U1M, U1P, U2J, U2P

VI – LFE, F2K, LFF, U1C, U2I, LFK, U3A, U3G, U3J, U3P, U1F

VII – LFG, LFI

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The selected Lactic Acid Bacteria were identified as *Lactobacillus plantarum* (50.0%, 50.0%), *Lactobacillus fermentum* (8.0%, 12.5%), *Lactobacillus brevis* (6.0%, 16.7%), *Leuconostoc mesenteroides* (12.0%, 0.0%), *Lactobacillus delbruekii* (8.0%, 8.3%), *Lactobacillus acidilactici* (12.0%, 12.5%) and *Lactobacillus lactis* (4.0%, 0.0%) for *fufu* and *usi*, respectively (Table 4.4). *L. plantarum* had the highest percentage of occurrence in both fermentations hence selected for further screening.

4.4 Screening for potential starters among selected Lactic Acid Bacteria

All the forty-eight (48) selected *Lactobacillus plantarum* strains did not hydrolyze starch, when grown on modified MRS agar but produced pectinase as well as linamarase in the form of β –glucosidase, a linamarin analogue when grown in a medium containing 4-nitrophenyl-B-D-glucopyranoside (Table 4.5). Rate of acid production in growth medium as monitored showed decrease in values with increasing incubation time, ranging from a starting pH of 6.50 to 3.58 after 72- hour incubation. The least pH at 24 hours was 4.62 by isolate F2B, 4.05 at 48 hours by U2C and 3.58 at 72 hours by isolate F2B (Table 4.6). Twenty (20) isolates that produced the lowest pH values after 72 hours incubation were selected for further screening (production of antimicrobial compounds).

Production of lactic acid, hydrogen peroxide and diacetyl by the selected isolates was shown in Tables 4.7- 4.9 respectively. Lactic acid concentration produced ranged from 1.10 g/L by isolate U3K to 1.78 g/L by isolate U2C at 24 hours, 1.22 g/L by isolate F1B to 2.45 g/L by isolate F2A at 48 hours and 0.57 g/L and 2.55g/L by isolates F1F, U3K, respectively.

The highest hydrogen peroxide concentration produced was 0.629 µg/L by isolate F2A at 24 hours while the least was 0.136 µg/L by isolate U1D at 72 hours. Least concentration (1.08 g/L) of diacetyl was the produced by both F2A and U1H at 24 hours while the highest was 2.86 g/L by F1B at 48 hours.

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Table 4.4: Frequency of occurrence (%) of selected lactic acid bacteria during spontaneous cassava fermentation

Probable identity	*Fufu*		*Usi*	
	Freq. of occurrence	% occurence	Freq. of occurrence	% occurence
L. plantarum	25	50.0	24	50.0
L. fermentum	4	8.0	6	12.5
L. brevis	3	6.0	8	16.7
Leuc. mesenteroides	6	12.0	-	-
L. delbruekii	4	8.0	4	8.3
L. acidilactici	6	12.0	6	12.5
L. lactis	2	4.0	-	-
Total	50	100	48	100

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Table 4.5: Starch hydrolysis, linamarase and pectinase enzyme production by selected isolates

Isolates	Starch hrdrolysis	Linamarase	Pectinase
F1A	-	+	+
F1B	-	+	+
F1D	-	+	+
F1E	-	+	+
F1F	-	+	+
F1G	-	+	+
F1K	-	+	+
LFA	-	+	+
U1A	-	+	+
U1B	-	+	+
U1D	-	+	+
U1E	-	+	+
U1G	-	+	+
U1H	-	+	+
U1I	-	+	+
U1J	-	+	+
U1L	-	+	+
F2A	-	+	+
F2B	-	+	+
F2C	-	+	+
F2E	-	+	+
F2F	-	+	+
F2G	-	+	+
F2I	-	+	+
F2J	-	+	+
U2A	-	+	+
U2B	-	+	+
U2C	-	+	+
U2D	-	+	+
U2F	-	+	+
U2G	-	+	+
U2H	-	+	+
U2K	-	+	+
U2L	-	+	+
F3A	-	+	+
F3C	-	+	+
F3E	-	+	+
F3F	-	+	+
F3G	-	+	+
F3H	-	+	+
F3I	-	+	+
F3J	-	+	+
F3N	-	+	+
U3D	-	+	+
U3E	-	+	+
U3F	-	+	+
U3I	-	+	+
U3K	-	+	+
U3M	-	+	+

Key: - negative, + positive

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Table 4.6: Acidification (pH) of growth medium by selected isolates

Isolates	Time (Hours)/ pH		
	24	**48**	**72**
F1A	5.33^{f*}	4.86^{k}	4.52^{n}
F1B	**5.26^{m}**	**4.53^{y}**	**4.43^{q}**
F1D	**5.27^{l}**	**4.58^{x}**	**4.50^{p}**
F1E	**5.06^{q}**	**4.33^{zb}**	**4.25^{w}**
F1F	**5.04^{s}**	**4.68^{s}**	**4.51^{o}**
F1G	5.35^{e}	5.06^{f}	4.64^{i}
F1K	5.32^{g}	4.98^{g}	4.71^{g}
LFA	5.29^{j}	4.76^{o}	4.41^{r}
U1A	5.27^{l}	4.79^{n}	4.73^{f}
U1B	5.31^{h}	4.86^{k}	4.52^{n}
U1D	**5.15^{n}**	**4.22^{zf}**	**4.17^{za}**
U1E	5.30^{i}	5.10^{d}	4.82^{c}
U1G	5.36^{d}	4.90^{i}	4.62^{k}
U1H	**4.94^{x}**	**4.29^{zc}**	**4.25^{w}**
U1I	5.32^{g}	4.98^{g}	4.71^{g}
U1J	5.28^{k}	4.68^{s}	4.61^{l}
U1L	5.41^{c}	5.18^{b}	4.63^{j}
F2A	**4.75^{zc}**	**4.06^{zh}**	**4.01^{zd}**
F2B	**4.62^{zd}**	**3.66^{zj}**	**3.58^{ze}**
F2C	**4.89^{z}**	**4.29^{zc}**	**4.18^{z}**
F2E	**4.93^{y}**	**4.33^{zb}**	**4.27^{v}**
F2F	5.36^{d}	5.09e	4.74^{e}
F2G	5.30^{i}	5.1^{d}	4.82^{c}
F2I	5.31^{h}	4.87^{j}	4.68^{h}
F2J	5.28^{k}	4.68^{s}	4.61^{l}
U2A	**4.82^{za}**	**4.13^{zg}**	**4.05^{zc}**
U2B	5.30^{i}	5.10^{d}	4.82^{c}
U2C	**4.76^{zb}**	**4.05^{zi}**	**4.01^{zd}**
U2D	5.27^{l}	4.73^{q}	4.61^{l}
U2F	**4.99^{u}**	**4.26^{ze}**	**4.26^{v}**
U2G	5.31^{h}	4.85^{l}	4.76^{d}
U2H	5.28^{k}	4.68^{s}	4.61^{l}
U2K	5.32^{g}	4.92^{h}	4.61^{l}
U2L	5.29^{j}	4.60^{v}	4.39^{s}
F3A	**4.95^{w}**	**4.33^{zb}**	**4.22^{y}**
F3C	**4.97^{v}**	**4.22^{zf}**	**4.18^{z}**
F3E	**5.06^{q}**	**4.64^{u}**	**4.52^{n}**
F3F	5.30^{i}	4.72^{r}	4.68^{h}
F3G	**5.15^{n}**	**4.28^{zd}**	**4.23^{x}**
F3H	5.27^{l}	4.74^{p}	4.31^{t}
F3I	5.32	4.82^{m}	4.59^{m}
F3J	5.44^{a}	5.17^{c}	4.93^{b}
F3N	5.00^{t}	4.59^{w}	4.31^{t}
U3D	**5.12^{o}**	**4.26^{ze}**	**4.09^{zb}**
U3E	5.29^{j}	4.65^{t}	4.59^{m}
U3F	**5.05^{r}**	**4.42^{z}**	**4.26^{v}**
U3I	5.42^{b}	5.31^{a}	5.10^{a}
U3K	**5.07^{p}**	**4.35^{za}**	**4.23^{x}**
U3M	5.30i	4.82^{m}	4.71^{g}

*The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$). **Bold values**: Least pH values selected for further screening

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Table 4.7: Quantity of lactic acid produced in g/L by selected strains of Lactic Acid Bacteria

Isolates	Time (Hours)		
	Lactic acid (g/L)		
	24	48	72
F2A	1.24^{m}*	2.45^{a}	0.74^{m}
F2B	1.48^{hij}	1.69^{i}	1.13^{g}
F2C	1.71^{b}	1.62^{j}	1.03^{h}
F2E	1.57^{e}	1.81^{h}	0.98^{i}
U2A	1.50^{ghi}	2.34^{b}	1.23^{f}
U2C	1.78^{a}	2.18^{c}	0.78^{l}
U2F	1.64^{c}	1.64^{j}	0.83^{k}
F1B	1.57^{e}	1.22^{n}	0.94^{j}
F1D	1.54^{efg}	1.47^{k}	0.74^{m}
F1E	1.33^{l}	2.10^{d}	0.79^{l}
F1F	1.30^{l}	1.93^{f}	0.57^{o}
U1D	1.59^{d}	1.47^{k}	0.72^{m}
U1H	1.45^{jk}	1.48^{k}	1.15^{g}
F3C	1.46^{ij}	1.86^{g}	2.11^{e}
F3A	1.18^{n}	1.26^{m}	0.69^{n}
F3E	1.41^{j}	1.37^{l}	2.39^{c}
U3D	1.58^{de}	1.88^{g}	2.46^{b}
U3F	1.10^{o}	1.99^{e}	2.25^{d}
U3J	1.51^{fgh}	1.86^{g}	2.55a
F3G	1.55^{def}	2.07^{d}	1.14^{g}

*The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$)

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Table 4.8: Quantity of hydrogen peroxide produced in µg/L by selected strains of Lactic Acid Bacteria

Isolates	Time (Hours) Hydrogen peroxide (µg/L)		
	24	48	72
F2A	0.629^{a}*	0.340^{a}	0.204^{c}
F2B	0.442^{b}	0.272^{e}	0.221^{b}
F2C	0.238^{j}	0.323^{b}	0.221^{b}
F2E	0.187^{l}	0.204^{i}	0.170^{e}
U2A	0.272^{i}	0.255^{f}	0.187^{d}
U2C	0.323^{g}	0.306^{c}	0.187^{d}
U2F	0.306^{h}	0.221^{h}	0.204^{c}
F1B	0.340^{f}	0.289^{d}	0.204^{c}
F1D	0.357^{e}	0.238^{g}	0.221^{b}
F1E	0.391^{c}	0.204^{i}	0.204^{c}
F1F	0.306^{h}	0.204^{i}	0.374^{a}
U1D	0.323^{g}	0.238^{g}	0.136^{g}
U1H	0.374^{d}	0.204^{i}	0.187^{d}
F3C	0.221^{k}	0.323^{b}	0.153^{f}
F3A	0.391^{c}	0.255^{f}	0.204^{c}
F3E	0.238^{j}	0.204^{i}	0.204^{c}
U3D	0.374^{d}	0.204^{i}	0.204^{c}
U3F	0.357^{e}	0.187^{j}	0.221^{b}
U3J	0.374^{d}	0.204^{i}	0.187^{d}
F3G	0.323^{g}	0.289^{d}	0.204^{c}

*The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$)

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Table 4.9: Quantity of diacetyl produced in g/L by selected strains of Lactic Acid Bacteria

Isolates	Time (Hours)		
	Diacetyl (g/L)		
	24	48	72
F2A	1.08^{j}*	1.91^{i}	1.72^{i}
F2B	1.72^{f}	2.35^{d}	2.18^{cd}
F2C	1.91^{je}	2.51^{c}	2.31^{b}
F2E	2.00^{d}	2.82^{a}	2.51^{a}
U2A	2.11^{c}	2.25^{ef}	2.10^{e}
U2C	1.51^{i}	2.10^{g}	1.98^{fgh}
U2F	1.09^{j}	1.97^{hi}	1.77^{i}
F1B	2.33^{a}	2.86^{a}	2.52^{a}
F1D	1.98^{d}	2.10^{g}	1.90^{h}
F1E	1.56^{h}	2.00^{h}	1.72^{i}
F1F	1.91^{e}	2.32^{de}	2.13^{de}
U1D	2.10^{c}	2.61^{b}	2.23^{c}
U1H	1.08^{j}	2.22^{f}	1.96^{fg}
F3C	1.62^{g}	1.98^{hi}	1.72^{i}
F3A	1.52^{ki}	1.98^{hi}	1.77^{i}
F3E	2.21^{b}	2.51^{c}	2.32^{b}
U3D	1.99^{d}	2.13^{g}	2.01^{f}
U3F	2.33^{a}	2.64^{b}	2.38^{b}
U3J	1.56^{h}	1.99^{h}	1.70^{i}
F3G	1.73^{f}	2.13^{g}	1.93^{gh}

*The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$)

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Most of the isolates inhibited the growth of test pathogens by showing zones of inhibition around the colonies. Only a few showed no inhibition zones at all, most especially against *Corynebaterium* specie (Table 4.10). All the isolates inhibited the growth of *E. coli* with zones ranging from 5 to 7 mm. All except F3E and U3K inhibited the growth of *Salmonella* with inhibition zones between 4 and 9 mm. The growth of *Bacillus cereus* was not inhibited by F1D and while only F1F showed no inhibition against *Shigella* sp.

The best five (5) overall producers after being analyzed statistically, using Duncan Multiple Range Test at 0.05% level of probability, across the three compounds were isolates F2A, F2B, F2C, U2A and U2C. They were thus selected as the potential starters and identified genotypically.

4.5 Molecular identification of screened potential starters.

The result of the amplified nucleotide sequences (Appendix IX) of the five (5) selected isolates was shown in Table 4.11. On the basis of the database information available on National Centre for Biotechnology Information (NCBI) site using the Basic Local Alignment Search Tool (BLAST), the isolates were classified and identified using the highest percentage similarity with organism of the nearest homology. All the isolates belong to the family *Lactobacillaceae* and genus *Lactobacillus.*

Isolate F2A was identified as *Lactobacillus pentosus* F2A with accerssion number KJ778115, having showed 99% homology alignment with *Lactobacillus pentosus* strain 405 in the GenBank. F2B was *L. plantarum subsp. argentolarensis* F2B (KJ778116) with 99% nucleotide homology with *L. plantarum subsp. argentolarensis* strain Ni1031 while F2C was *L. plantarum* F2C (KJ77117) showing 99% homology with *L. plantarum* strain 097. 100% similarity was observed between isolate U2A, which was *L. plantarum* U2A (KJ78118) and *L. plantarum* P2 whereas U2C was 99% homologous with *L. paraplantarum* DSM 10667 nucleotide sequence in the NCBI Genbank.

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Table 4.10: Antagonistic effect of selected isolates against test pathogenic organisms.

Isolates	Test pathogenic organisms — Zone of inhibition (mm)					
	E. coli	*Proteus* sp.	*Salmonella* sp.	*B. cereus*	*Shigella* sp.	*Corynebacterium* sp.
F2A	6	2	6	4	12	NI
F2B	6	2	8	4	9	NI
F2C	6	3	7	3	11	NI
F2E	7	5	9	3	10	NI
U2A	9	NI	7	6	10	NI
U2C	6	2	7	2	9	7
U2F	6	NI	8	6	8	3
F1B	6	NI	7	3	4	6
F1D	6	2	4	NI	9	4
F1E	7	2	9	4	10	8
F1F	5	2	5	4	NI	NI
U1D	6	3	9	3	10	3
U1H	6	2	8	5	11	3
F3C	5	6	8	3	10	NI
F3A	7	2	7	6	7	NI
F3E	6	NI	NI	5	9	NI
U3D	5	8	8	6	10	3
U3F	5	5	5	8	9	NI
U3J	6	NI	NI	3	10	3
F3G	7	3	5	2	9	2

NI - No Inhibition.

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Table 4.11: Molecular identification of selected potential starters

Isolate code	Closely related species/GenBank Accerssion number	Percentage similarity (%)	Base pair analyzed	Identification	Accerssion number
F2A	*Lactobacillus pentosus* 405 (AB775188.1)	99	517	*Lactobacillus pentosus*	KJ778115
F2B	*L. plantarum subsp. argentolarensis* Ni1031(AB598953.1)	99	496	*L. plantarum subsp. argentolarensis*	KJ778116
F2C	*Lactobacillus plantarum* 097 (JN560914.1)	99	521	*Lactobacillus plantarum*	KJ778117
U2A	*Lactobacillus plantarum* P2 (EU167523.1)	100	500	*Lactobacillus plantarum*	KJ778118
U2C	*Lactobacillus paraplantarum* DSM10667 (NR117813.1)	99	520	*Lactobacillus paraplantarum*	KJ778119

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Phylogenetic tree constructed using the Molecular Evolution Genetics Analysis (MEGA) version 6 established the relationship among the organisms and their nearest homologies (Figure 4.3). The neighbour joining method revealed the clustering of the identified isolates into four clusters of closely related strains.

4.6 Fermentation of cassava with identified potential starters

The genotypically identified potential starters were utilised singly and in-combination (Table 4.12) to ferment cassava for *fufu* and *usi* production (Plate 4.1). Inoculum size of approximately 1.2×10^9 CFU/mL as earlier described was used for each organism and 5 mL of single and randomly combined starters were used as the final inoculum.

4.6.1 pH of starter-fermented cassava mash

Decrease in pH values with increasing fermentation time was observed in both *fufu* and *usi* fermentations with values ranging from initial pH of 7.10 to 3.68 in *fufu* and 3.53 in *usi*. The least pH value after 72-hour fermentation in *fufu* was 3.68 by the combined starter CGI (*Lactobacillus plantarum* F2C/*L. plantarum* U2A/*L. plantarum* U2C) while *usi* had 3.53 by the same starter combination. The un-inoculated control however had the highest pH values throughout the fermentation processes with a minimum of 4.12 (*fufu*) and 4.05 (*usi*) at 72 hours (Table 4.13).

4.6.2 Total titratable acidity (%) of starter-fermented cassava mash

The highest percentage total titratable acidity in the form of lactic acid produced at 24 hours during *fufu* fermentation was 0.77% by the combined starter AB (*L. pentosus* F2A/*L. plantarum subsp. argentolarensis* F2B). Reduction (0.41%) in the maximal quantity was observed at 48 hours while a slight increase (0.45%) in value was produced at 72 hours by *L. plantarum* F2C. During *usi* fermentation, the maximal total titratable acidity reduced with increase in fermentation time. Combined starter CG (*L. plantarum* F2C/ *L. plantarum* U2A) produced the highest titratable acidity (0.279%) at 24 hours whereas, the value decreased to 0.025% at 48 hours with a much lower quantity (0.002%) at 72 hours by the same starter. It was however noted that the observed highest values in both fermentations were produced by different starter cultures (Table 4.14).

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Lactobacillus pentosus 405
Lactobacillus plantarum E7304
Lactobacillus plantarum P2
Lactobacillus pentosus 124-2
Lactobacillus paraplantarum DSM 10667
Lactobacillus plantarum TW57-4
Lactobacillus plantarum KLDS 1.0725
***Lactobacillus plantarum subsp argentolarensis* F2B (KJ778116)**
***Lactobacillus plantarum* F2C (KJ778117)**
***Lactobacillus paraplantarum* U2C (KJ778119)**
***Lactobacillus pentosus* F2A (KJ778115)**
***Lactobacillus plantarum* U2A (KJ778118)**
Lactobacillus plantarum 097
Lactobacillus plantarum subsp. argentoratensis Ni1031
Lactobacillus plantarum PM411
Lactobacillus plantarum C21-41
Lactobacillus plantarum AF1

Figure 4.3: Phylogenetic relationship between identified starters and closely related organisms in the GenBank.

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Table 4.12: Single and randomly combined LAB isolates used as potential starters for *fufu* and *usi* production.

Codes	Isolates combinations
A	*Lactobacillus pentosus* F2A
B	*Lactobacillus plantarum susp. argentolarensis* F2B
C	*Lactobacillus plantarum* F2C
G	*Lactobacillus plantarum* U2A
I	*Lactobacilluc paraplantarum* U2C
AB	*L. pentosus*F2A + *L. plantarum subsp.argentolarensis* F2B
ABC	*L. pentosus* F2A + *L. plantarum subsp.argentolarensis* F2B +*L. plantarum* F2C
ABCG	*L. pentosus* F2A + *L. plantarum subsp.argentolarensis* F2B +*L. plantarum* F2C +*L. plantarum* U2A
ABCGI	*L. pentosus* F2A + *L. plantarum subsp.argentolarensis* F2B +*L. plantarum* F2C +*L. plantarum* U2A +*L. paraplantarum* U2C
BC	*Lactobacillus plantarum susp. argentolarensis* F2B + *L. plantarum* F2C
BCG	*L. plantarum subsp.argentolarensis* F2B +*L. plantarum* F2C +*L. plantarum* U2A
BCGI	*L. plantarum subsp.argentolarensis* F2B +*L. plantarum* F2C +*L. plantarum* U2A+ *L. paraplantarum* U2C
CG	*L. plantarum* F2C +*L. plantarum* U2A
CGI	*L. plantarum* F2C +*L. plantarum* U2A +*L. paraplantarum* U2C
GI	*L. plantarum* U2A+*L. paraplantarum* U2C
Control	Un-inoculated

Plate 4.1: Cassava fermentation with single and combined starter cultures using plastic bioreactors

X – Main improvised bioreactor

Y – Cassava samples

Z – Fermenting liquor collection outlet

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Table 4.13: pH during starter fermentation of cassava for *fufu* and *usi* production

Potential starter	Time (Hour)/Cassava product/ pH					
	24		**48**		**72**	
	F	**U**	**F**	**U**	**F**	**U**
A	4.23^{c}*	4.22^{d}	3.78^{m}	3.76^{i}	3.68^{k}	3.75^{j}
B	4.17^{g}	4.14^{e}	3.95^{e}	3.9^{e}	3.81^{d}	3.88^{e}
C	4.18^{c}	3.95^{k}	3.93^{f}	3.76^{i}	3.77^{g}	3.75^{j}
G	4.21^{d}	4.28^{c}	3.95^{e}	4.04^{c}	3.79^{e}	3.99^{c}
I	4.11^{k}	3.95^{k}	3.96^{d}	3.74^{j}	3.95^{b}	3.73^{k}
AB	4.20^{e}	4.05^{h}	3.93^{f}	3.77^{h}	3.78^{f}	3.76i
ABC	4.28^{b}	4.08^{g}	4.05^{b}	3.66^{l}	3.82^{c}	3.64^{n}
ABCG	4.17^{g}	5.16^{a}	3.82^{l}	4.33^{a}	3.81^{d}	4.03^{b}
ABCGI	4.17^{g}	3.99^{j}	3.87^{j}	3.78^{g}	3.78^{f}	3.78^{h}
BC	4.01^{m}	4.02^{i}	3.91^{g}	3.74^{j}	3.77^{g}	3.72^{l}
BCG	4.12^{j}	4.14^{e}	3.89^{h}	3.9^{e}	3.74^{i}	3.86^{f}
BCGI	4.15^{h}	4.08^{g}	3.96^{d}	3.53^{m}	3.77^{g}	3.53^{o}
CG	4.10^{l}	4.1^{f}	3.83^{k}	3.94^{d}	3.74^{i}	3.91^{d}
CGI	4.11^{k}	4.14^{e}	3.88^{i}	3.72^{k}	3.71^{j}	3.7^{m}
GI	4.13^{i}	4.1^{f}	3.98^{c}	3.87^{f}	3.75^{h}	3.81^{g}
Un-inoculated	4.75^{a}	4.93^{b}	4.25^{a}	4.2^{b}	4.12^{a}	4.05^{a}

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp. argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** – *L. plantarum U2A*, **I** – *L. paraplantarum*
F- *Fufu*, U- *Usi.**The means reported with the same superscript in each column indicated no significant difference (p≤0.05).

Table 4.14: Total titratable acidity (%) during starter fermentation of cassava for *fufu* and usi production

Potential starter	Time (Hour)/Cassava product/Total titratable acidity					
	24		**48**		**72**	
	F	**U**	**F**	**U**	**F**	**U**
A	0.549^{c*}	0.252^{d}	0.360^{b}	0.023^{d}	0.252^{f}	0.0020^{d}
B	0.342^{g}	0.198^{f}	0.198^{c}	0.018^{f}	0.252^{f}	0.0016^{f}
C	0.594^{b}	0.189^{g}	0.108^{i}	0.017^{g}	0.459^{a}	0.0015^{g}
G	0.270^{j}	0.180^{h}	0.135^{g}	0.016^{h}	0.144^{j}	0.00145^{h}
I	0.306^{i}	0.225^{e}	0.117^{h}	0.020^{e}	0.117^{l}	0.00182^{e}
AB	0.774^{a}	0.153^{i}	0.054^{n}	0.014^{j}	0.144^{j}	0.00123^{i}
ABC	0.522^{d}	0.144^{j}	0.099j	0.013^{k}	0.243^{g}	0.00117^{j}
ABCG	0.504^{e}	0.270^{b}	0.135^{g}	0.024^{b}	0.126^{k}	0.00219^{b}
ABCGI	0.315^{h}	0.153^{i}	0.090^{k}	0.014^{j}	0.180^{i}	0.00124^{i}
BC	0.342^{g}	0.198^{f}	0.153^{e}	0.018^{f}	0.324^{c}	0.0016^{f}
BCG	0.216^{l}	0.180^{h}	0.072^{l}	0.016^{i}	0.252^{f}	0.00145^{h}
BCGI	0.189^{m}	0.135^{k}	0.072^{l}	0.012^{l}	0.261^{e}	0.0011^{k}
CG	0.153^{n}	0.279^{a}	0.063^{m}	0.025^{a}	0.342^{b}	0.0023^{a}
CGI	0.252^{k}	0.189^{g}	0.144^{f}	0.017^{g}	0.315^{d}	0.0015^{g}
GI	0.549^{c}	0.189^{g}	0.414^{a}	0.017^{g}	0.108^{l}	0.0015^{g}
Un-inoculated	0.387^{f}	0.261^{c}	0.162^{d}	0.023^{c}	0.189^{h}	0.0021^{c}

A- *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp. argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** – *L. plantarum U2A*, **I** – *L. paraplantarum*

F- *Fufu*, U- *Usi*. *The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$).

4.6.3 Proximate analysis of starter-fermented cassava for *fufu* and *usi* production

The proximate analysis of the fresh cassava indicated moisture content to be 7.28%, crude protein 1.02%, crude fat 0.48%, crude fibre 1.75%, ash 1.57% and the total carbohydrate 89.65%.

Table 4.15 showed the proximate analysis of *fufu* after 72-hour fermentation. The moisture content ranged between 5.10% by the combined starter CGI (*L. plantarum* F2C/*L. plantarum* U2A/*L. paraplantarum* U2C) and 8.61% by starter C (*L. plantarum* F2C). Only two starters (*L. plantarum* F2C and *L. paraplantarum* U2C) showed higher moisture content (8.61% and 7.72%) than the un-inoculated experiment (control) which had 7.28%. Protein content ranged between 0.73% and 1.34%. The combined starter CGI had the highest protein content of 1.34% even though combined starter ABC and ABCGI also had higher protein content (1.24% and 1.16%) than the fresh cassava (1.02%). *L. plantarun* U2A had the least crude fat content (0.24%) from an initial 0.48%, although, some starters showed higher fat content (0.52 - 0.91%) than the fresh cassava after fermentation. *L. paraplantarum* U2C recorded the highest crude fibre (3.28%) and ash content (2.85%) while the total carbohydrate was maximal at 91.33% by the combined starter GI (*L. plantarum* U2A/*L. paraplantarum* U2C).

As shown in Table 4.16, the proximate composition of *usi* after fermentation indicated that moisture content was between 5.34% and 9.38% by combined starter GI (*L. plantarum* U2A/ *L. paraplantarum* U2C) and CG (*L. plantarum* F2C/*L. plantarum* U2A) respectively, even though, starters *Lactobacillus plantarum* F2A, combined starter CGI (*Lactobacillus plantarum* F2C/*L. plantarum* U2A/*L. paraplantarum* U2C) and the un-inoculated batch had values that were not significantly different from the lowest moisture content (5.34%). Increased protein contents ranging from 1.14% to 1.82% was observed in most samples after fermentation. The highest (1.82%) however, was by the combined starter CGI (*Lactobacillus plantarum* F2C/*L. plantarum* U2A/*L. paraplantarum* U2C). Meanwhile, reduction in protein content was also observed in the un-inoculated sample as well as when combined starter GI (*L. plantarum* U2A/*L. paraplantarum* U2C) and ABCGI were used. All the starter-fermented samples had reduced fat content except *L. paraplantarum* U2C which had 2.06% and the least (0.04%) observed when combined starter BC was used.

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Table 4.15: Proximate composition (%) of starter fermented cassava for *fufu* production after 72 hour fermentation

	Proximate composition (%)					
Potential starters	**Moisture content**	**Crude protein**	**Crude fat**	**Crude fibre**	**Ash**	**Total carbohydrate†**
A	5.51±0.06^{i}*	0.91±0.05^{f}	0.83±0.04^{b}	1.73±0.04^{i}	1.19±0.01^{j}	89.83±0.06^{c}
B	7.23±0.04^{c}	0.89±0.02^{g}	0.52±0.02^{e}	1.58±0.03^{j}	1.68±0.03^{c}	88.10±0.08^{l}
C	8.61±0.05^{a}	1.02±0.12^{d}	0.45±0.02^{h}	1.83±0.03^{fgh}	1.57±0.04^{f}	86.52±0.07^{m}
G	5.63±0.06^{g}	0.98±0.11^{e}	0.24±0.02^{k}	2.00±0.02^{d}	1.29±0.01^{h}	89.86±0.14^{b}
I	7.72±0.04^{b}	0.89±0.02^{g}	0.29±0.02^{j}	3.28±0.03^{a}	2.85±0.07^{a}	84.97±0.09^{n}
AB	5.38±0.04^{k}	1.02±0.12^{d}	0.42±0.02^{gh}	2.15±0.01^{c}	1.19±0.02^{j}	89.84±0.10^{c}
ABC	6.18±0.03^{f}	1.24±0.13^{b}	0.39±0.01^{d}	1.86±0.06^{efg}	1.29±0.02^{h}	89.04±0.14^{g}
ABCG	6.70±0.04^{d}	0.91±0.05^{f}	0.58±0.02^{e}	2.18±0.03^{c}	1.48±0.02^{g}	88.15±0.06^{l}
ABCGI	5.16±0.02^{l}	1.16±0.13^{c}	0.69±0.02^{c}	2.41±0.03^{b}	1.79±0.03^{b}	88.79±0.13^{i}
BC	6.58±0.06^{e}	1.02±0.12^{d}	0.69±0.03^{c}	1.69±0.03^{i}	1.48±0.03^{g}	88.54±0.15^{j}
BCG	6.70±0.03^{d}	1.02±0.12^{d}	0.54±0.05^{ef}	1.70±0.02^{hi}	1.24±0.06^{i}	88.80±0.21^{h}
BCGI	5.62±0.05^{g}	0.89±0.02^{g}	0.55±0.05^{ef}	1.40±0.03^{k}	1.14±0.03^{k}	89.10±0.07^{f}
CG	5.6±0.044^{h}	0.81±0.13^{h}	0.91±0.04^{a}	1.70±0.02^{hi}	1.61±0.03^{e}	89.37±0.11^{e}
CGI	5.10±0.09^{n}	1.34±0.05^{a}	0.33±0.06^{i}	1.91±0.04^{def}	1.67±0.02^{d}	89.65±0.15^{d}
GI	5.11±0.01^{m}	0.73±0.13^{i}	0.28±0.01^{jk}	1.77±0.03^{ghi}	0.78±0.06^{l}	91.33±0.14^{a}
Uninoculated	5.48±0.04^{j}	0.91±0.05^{f}	0.90±0.04^{a}	1.90±0.02^{de}	1.78±0.02^{b}	89.03±0.12^{f}
Fresh cassava	7.28±0.03	1.02±0.12	0.48±0.01	1.75±0.03	1.57±0.04	87.90±0.11

Values are means ±SD, n=3

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp.argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** – *L. plantarum U2A*, **I** – *L. paraplantarum U2C.*

* The means reported with the same superscript in each column indicated no significant difference ($p \leq 0.05$).

† By difference

Table 4.16: Proximate composition (%) of starter fermented cassava for *usi* production after 72-hour fermentation

	Proximate composition (%)					
Potential starters	**Moisture content**	**Crude protein**	**Crude fat**	**Crude fibre**	**Ash**	**Total carbohydrate†**
A	6.18 ± 2.25^{c}*	1.02 ± 0.12^{h}	0.31 ± 0.02^{c}	1.93 ± 0.05^{bcdef}	0.93 ± 0.02^{bc}	89.63 ± 0.18^{d}
B	7.32 ± 0.06^{abc}	1.02 ± 0.12^{h}	0.20 ± 0.01^{d}	1.94 ± 0.05^{bcdef}	0.89 ± 0.02^{c}	88.63 ± 0.16^{g}
C	7.35 ± 0.02^{abc}	1.24 ± 0.13^{f}	0.29 ± 0.02^{d}	1.96 ± 0.02^{bcdef}	0.78 ± 0.03^{def}	88.37 ± 0.13^{i}
G	7.36 ± 0.10^{abc}	1.68 ± 0.13^{b}	0.42 ± 0.02^{b}	1.78 ± 0.02^{ef}	0.77 ± 0.03^{ef}	87.99 ± 0.13^{j}
I	6.56 ± 0.06^{bc}	1.38 ± 0.13^{c}	2.06 ± 0.03^{a}	1.81 ± 0.02^{def}	0.81 ± 0.02^{de}	87.38 ± 0.09^{k}
AB	6.29 ± 0.02^{bc}	1.38 ± 0.13^{c}	0.16 ± 0.01^{de}	1.58 ± 0.06^{f}	0.79 ± 0.03^{de}	88.80 ± 0.16^{f}
ABC	6.79 ± 0.03^{bc}	1.24 ± 0.13^{f}	0.39 ± 0.02^{bc}	2.24 ± 0.02^{abc}	1.03 ± 0.01^{a}	88.31 ± 1.28^{i}
ABCG	6.64 ± 0.06^{bc}	1.34 ± 0.05^{d}	0.29 ± 0.02^{d}	2.35 ± 0.04^{a}	0.93 ± 0.03^{bc}	88.45 ± 0.06^{h}
ABCGI	6.45 ± 0.03^{bc}	0.95 ± 0.12^{i}	0.06 ± 0.01^{f}	2.14 ± 0.02^{abcde}	0.74 ± 0.05^{f}	89.66 ± 0.1^{c}
BC	6.34 ± 0.55^{bc}	1.68 ± 0.13^{b}	0.04 ± 0.01^{f}	2.31 ± 0.03^{ab}	0.74 ± 0.05^{f}	88.89 ± 0.08^{f}
BCG	5.39 ± 0.03^{d}	1.28 ± 0.05^{e}	0.14 ± 0.01^{e}	2.14 ± 0.02^{abcde}	0.90 ± 0.04^{b}	89.55 ± 0.04^{e}
BCGI	8.41 ± 0.06^{ab}	1.24 ± 0.13^{f}	0.38 ± 0.03^{bc}	1.89 ± 0.02^{cdef}	0.82 ± 0.02^{d}	87.26 ± 0.15^{l}
CG	9.38 ± 0.03^{a}	1.14 ± 0.08^{g}	0.27 ± 0.03^{d}	2.21 ± 0.02^{abc}	1.01 ± 0.01^{a}	85.99 ± 0.05^{m}
CGI	5.36 ± 0.04^{c}	1.82 ± 0.13^{a}	0.16 ± 0.03^{de}	2.35 ± 0.05^{a}	0.69 ± 0.02^{g}	89.62 ± 0.08^{d}
GI	5.37 ± 0.07^{c}	0.85 ± 0.05^{j}	0.28 ± 0.03^{d}	2.17 ± 0.03^{abcd}	0.93 ± 0.06^{bc}	90.40 ± 0.15^{b}
Uninoculated	5.68 ± 0.42^{c}	0.95 ± 0.12^{i}	0.29 ± 0.02^{d}	1.91 ± 0.03^{cdef}	0.57 ± 0.03^{h}	90.60 ± 0.52^{a}
Fresh cassava	7.28±0.03	1.02±0.12	0.48±0.01	1.75±0.03	1.57±0.04	87.90±0.11

Values are means ±SD, n=3

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp.argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** - *L. plantarum U2A*, **I** - *L. paraplantarum U2C.*

* The means reported with the same superscript in each column indicated no significant difference (p≤0.05).

† By difference

Fibre content increased after fermentation ranging between 1.78% and 2.35% from an initial 1.75%, except for the combined starter AB (*L. pentosus* F2A/*L.plantarum subsp. argentolarensis* F2B) which had reduced fibre content (1.58%). All the starters yielded samples with reduced ash content after fermentation when compared to the fresh cassava with values ranging between 0.51% and 1.03%.

Nonetheless, the highest ash content (1.03%) was observed with the combined starter ABC (*L. pentosus* F2A/*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C). Total carbohydrate ranged from 85.99% to 90.6%. The highest value (90.6%) after fermentation was observed in the spontaneously fermented sample while the least (85.99%) was by the combined starter CG (*L. plantarum* F2C/*L. plantarum* U2A).

4.6.4 Anti-nutritional factors of starter-fermented cassava for *fufu* and *usi*

The anti-nutritional factors of the fresh and starter-fermented cassava for *fufu* and *usi* were shown in Table 4.17. The fresh cassava tuber had 0.30 mg of phytic acid, 35.40 mg tannin and 0.10 mg cyanide per gram of the tuber. After 72-hour *fufu* fermentation, the phytic acid ranged between 0.1 mg/g to 0.5 mg/g with a minimum value of 0.1 mg/g by numerous starter combinations (A, AB, ABCG, ABCGI, CGI and CG). It was however, observed that starter combination ABC (*L. pentosus* F2A/*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C) and G (*L. plantarum* U2A) had higher phytic acid values (0.4 mg/g and 0.5 mg/g) than the fresh cassava. Tannin ranged between 34 mg/g and 62.7 mg/g with the least value of 34 mg/g by the combined starter CGI (*L. plantarum* F2C/*L. plantarum* U2A/*L. paraplantarum* U2C) while all other starter-fermented samples yielded tannin content that was higher than that of the fresh cassava. Cyanide content was not detected in samples fermented with starters B (*L. plantarum subsp. argentolarensis* F2B), BC (*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C), CG (*L. plantarum* F2C/*L. plantarum* U2A) and BCGI.

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Table 4.17: Anti-nutritional factors (mg/g) of fresh and starter (CGI)-fermented cassava for *fufu* and *usi* production

Potential starters	Cassava Product/ Anti-nutritional factors (mg/g)					
	Phytate		Tannin		Cyanide	
	Fufu	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*
A	0.1±0.00^{defg}*	0.32±0.003^{a}	45.7±0.35^{g}	46.4±0.04^{b}	0.08±0.004^{b}	0.2±0.01^{c}
B	0.2±0.01^{cd}	0.32±0.003^{a}	45.9±0.19^{f}	35.5±0.03^{e}	0.00^{c}	0.1±0.00^{d}
C	0.2±0.01^{defg}	0.28±0.00^{bc}	42.1±0.02^{i}	64.0±0.02^{a}	0.1±0.00^{a}	0.6±0.03^{b}
G	0.5±0.01^{a}	0.28±0.00^{bc}	37.5±0.13^{k}	40.6±0.02^{c}	0.08±0.004^{b}	0.8±0.02^{a}
I	0.2±0.00^{defg}	0.277±0.0004^{c}	37.3±0.13^{l}	33.8±0.02^{i}	0.05±0.00^{bc}	0.1±0.01^{d}
AB	0.1±0.00^{fg}	0.28±0.001^{bc}	43.2±0.13^{h}	33.0±0.01^{k}	0.1±0.00^{a}	0.00^{e}
ABC	0.4±0.02^{ab}	0.277±0.0004^{c}	61.8±0.06^{b}	34.6±0.02^{g}	0.05±0.00^{bc}	0.00^{e}
ABCG	0.1±0.00^{efg}	0.28±0.00^{bc}	46.3±0.16^{e}	32.3±0.01^{l}	0.05±0.00^{bc}	0.1±0.01^{d}
ABCGI	0.1±0.00^{efg}	0.28±0.00^{bc}	55.5±0.06^{c}	35.5±0.03^{e}	0.08±0.004^{b}	0.00^{e}
BC	0.2±0.01^{def}	0.275±0.00^{c}	62.7±0.07^{a}	33.7±0.01^{i}	0.00^{c}	0.00^{e}
BCG	0.2±0.01^{de}	0.275±0.00^{c}	43.7±0.39^{d}	37.5±0.02^{e}	0.08±0.004^{b}	0.00^{e}
BCGI	0.2±0.01^{cd}	0.275±0.00^{c}	36.9±0.09^{m}	35.3±0.02^{f}	0.00^{c}	0.00^{e}
CG	0.1±0.00^{fg}	0.289±0.0002^{b}	35.9±0.26^{n}	39.0±0.02^{d}	0.00^{c}	0.00^{e}
CGI	0.1±0.00^{g}	0.277±0.0004^{c}	34.0±0.28^{o}	32.3±0.01^{l}	0.05±0.00^{bc}	0.00^{e}
GI	0.2±0.01^{defg}	0.277±0.0004^{c}	39.9±0.17^{j}	34.4±0.03^{h}	0.05±0.00^{bc}	0.00^{e}
Un-inoculated	0.3±0.01^{bc}	0.29±0.001^{b}	45.7±0.01^{g}	33.4±0.01^{j}	0.05±0.00^{bc}	0.00^{e}
Fresh cassava	0.30±0.004		35.40±0.03		0.10±0.01	

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum* subsp. *argentolarensis F2B*, **C**- *L. plantarum F2C*, **G** – *L. plantarum U2A*, **I** – *L. paraplantarum U2C*. Values are means ±SD, n=3; * Means reported with the same superscript in each column indicated no significant difference (p≤0.05).

In *usi*, cyanide was not detected in most of the samples fermented with starters where as, phytic acid value was within the range of 0.275 mg/g and 0.32 mg/g with starters BC (*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C), BCG (*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C/*L. plantarum* U2A) and BCGI (*L. plantarum subsp. argentolarensis* F2B/*L.plantarum* F2C/*L. plantarum* U2A/*L. paraplantarum* U2C), having the least phytic acid values. The tannin content after fermentation ranged between 32.3 mg/g and 64 mg/g. The combined starter CGI had the least (32.3 mg/g) tannin.

4.6.5 Selection of a common starter culture for both products

The starter combination that had the most reduced anti-nutritional factor, improved proximate composition and a faster acidification was considered for further study. Five starters with the best results for the above mentioned parameters were considered, after which the most common was selected. For *usi*, starters BC, BCG and BCGI had the least phytate (0.275 mg/g), followed closely by I, ABC, CGI and GI having 0.277 mg/g. It was however observed that the values were not significantly different at 5% level of probability. The CGI and ABCG starter fermented samples had the least tannin (32.30 mg/g). Absence of cyanide compound was observed in majority of the starter fermented samples. For *fufu*, the least phytate (0.10 mg/g) and tannin (34.0 mg/g) was produced by the combined starter CGI while cyanide compound was not detected in starters C, BC, BCGI and CG. The combined starter CGI (*Lactobacillus plantarum* F2C/*Lactobacillus plantarum* U2A/*Lactobacillus paraplantarum* U2C) was the most common starter to reduce phytate, tannin and cyanide in both *fufu* and *usi* even though there are some other starters that reduced the anti-nutritional factors, but were not common among the three factors (Table 4.18).

Lactobacillus petosus F2A and the combined starter BCGI had the least pH values of 3.68 and 3.53 for *fufu* and *usi*, respectively after the fermentation process. Since the least pH values were produced by different starters, the next reduced values (3.71 and 3.7) were considered and these were from samples fermented with starter CGI.

Increased protein content and lower moisture content were crucial factors in fermented foods. The least moisture content (5.10% and 5.36%) and highest protein (1.34% and 1.88%) recorded during starter fermentation of *fufu* and *usi* respectively were observed when the combined starter CGI was used (Table 4.19).

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Table 4.18: Selection a common starter among the most reduced pH values and anti-nutritional factors after the 72-hour cassava fermentation for *fufu* and *usi* production

pH		Phytate		Tannin		Cyanide	
Fufu	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*
A	BCGI	**CGI**	BC	**CGI**	**CGI**	B	BCG
CGI*	ABC	CG	BCG	-	ABCG	BC	BCGI
CG	**CGI**	AB	BCGI	-	AB	BCGI	CG
BCG	BC	ABCG	**CGI**	-	Control	CG	**CGI**
GI	I	A	CG	-	BC	**CGI**	GI

*Starters in bold are the most common among the parameters analysed and thus, selected for further work.

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp.argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** - *L. plantarum U2A*, **I** - *L. paraplantarum U2C.*

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Table 4.19: Selection of a common starter among the most improved proximate composition after the 72-hour cassava fermentation for *fufu* and *usi* production

Moisture content		Crude protein		Crude fat		Ash content		Carbohydrate	
Fufu	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*	*Fufu*	*Usi*
CGI*	**CGI**	**CGI**	**CGI**	G	ABCGI	I	CG	GI	Control
GI	GI	ABC	G	GI	BC	ABCGI	BCG	G	GI
ABCGI	Control	G	BC	I	BCG	B	GI	A	ABCGI
AB	A	ABCGI	I	**CGI**	**CGI**	**CGI**	A	AB	**CGI**
Control	BCG	A	AB	ABC	AB	CG	B	**CGI**	A

*Starters in bold are the most common among the parameters analysed and thus, selected for further work.

A - *Lactobacillus pentosus F2A*, **B** – *L. plantarum subsp.argentolarensis F2B*, **C** - *L. plantarum F2C*, **G** - *L. plantarum U2A*, **I** - *L. paraplantarum U2C.*

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In general, starter combination of CGI, comprising of *Lactobacillus plantarum* F2C, *Lactobacillus plantarum* U2A and *Lactobacillus paraplantarum* U2C was the most frequent and common to both product and thus, selected for further experiment.

4.6.6 Enzyme activities during starter fermentation for *fufu* and *usi* production

The assay for amylase, pectinase and linamarase during the fermentation indicated their production and activities. The selected starter combination (*Lactobacillus plantarum* F2C/*Lactobacillus plantarum* U2A/*Lactobacillus paraplantarum* U2C) showed decrease in amylase activity with increase in fermentation time in both fermentations. Starter-fermented *fufu* mash had highest activity (10.1 U/mL) at 24 hours, 5.18 U/mL and 3.93 U/mL at 48 and 72 respectively, whereas, activity in *usi* ranged between 4.37 U/mL and 2.17 U/mL. In *fufu*, the un-inoculated experiment (control) had reduced activities than the starter-fermented batch, except at 72 hours, while amylase activities (1.04 U/mL, 0.76U/mL) were detected at 48 and 72 hours, respectively in *usi* (Figure 4.4).

Pectinase activity during *fufu* fermentation decreased with increase in fermentation time in both the starter and un-inoculated fermentations. The activities ranged from 4.44 U/mL at 24 hours, to 3.81 U/mL at 48 hours and 1.74 U/mL at 72 hours. The control experiment had a higher activity (6.32 U/mL) than the starter-fermented batch at 24 hours after which the activities were lower than the latter throughout the fermentation. In *usi*, activity was detected at 24 hours (0.88 U/mL) and 72 hours (1.01 U/mL), but not at 48 hours (Figure 4.5).

Starter-fermented experiment had higher linamarase activities than the control in both products. *Fufu* fermentation had 0.17 U/mL linamarase activity at 24 hours, increased gradually to 0.83 U/mL at 48 hours and had a sharp decrease to 0.39 U/mL at 72 hours while *usi* had a slight decrease from 0.38 U/mL at 24 hours to 0.34 U/mL at 48 hours and subsequently increased to 0.71 U/mL at 72 hours (Figure 4.6).

However, it was generally observed that the un-inoculated (control) fermentation for *usi* had none of the enzyme activities at the 24th hour and most of the enzyme activities in the starter fermented experiments were higher than the in the un-inoculated experiments.

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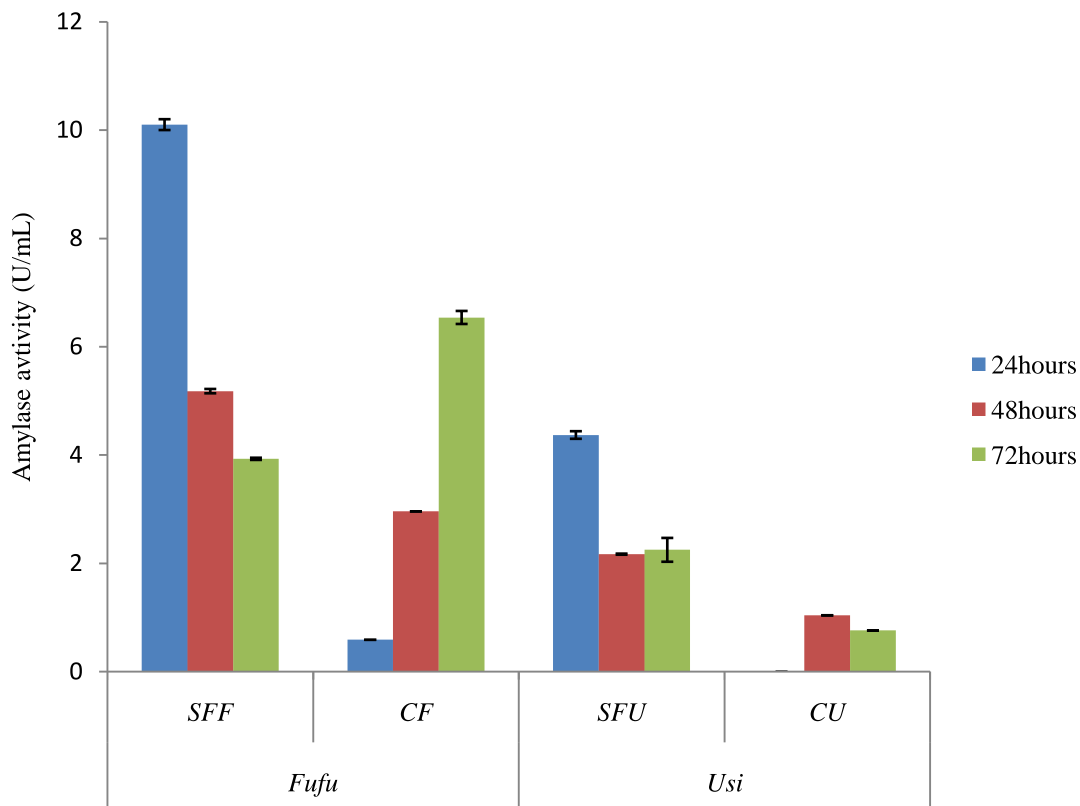

Figure 4.4: Amylase activities in starter (CGI)-fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation

Key:

SFF- Starter-fermented *fufu;* CF- Spontaneously-fermented *fufu* (control)*;* SFU- Starter-fermented *usi;* CU- Spontaneously-fermented *usi* (control)

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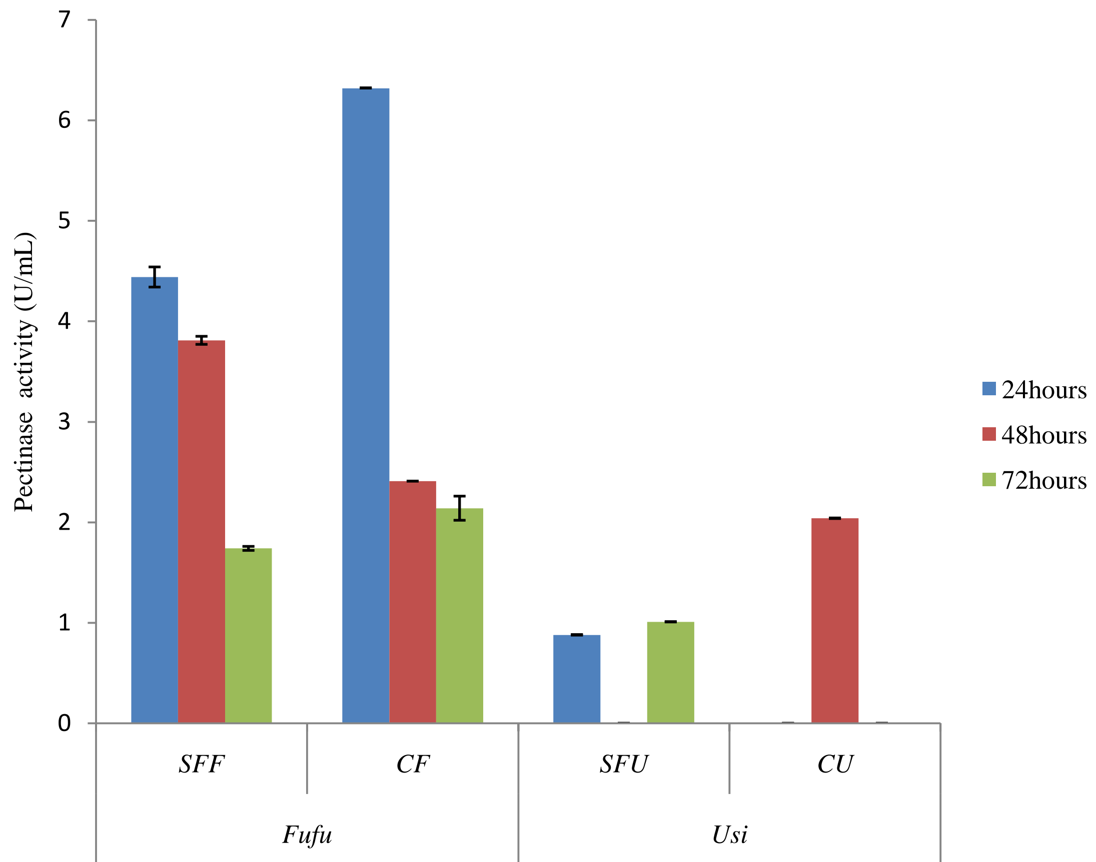

Figure 4.5: Pectinase activities in starter (CGI)-fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation

Key:

SFF- Starter-fermented *fufu;* CF- Spontaneously-fermented *fufu* (control)*;* SFU- Starter-fermented *usi;* CU- Spontaneously-fermented *usi* (control)

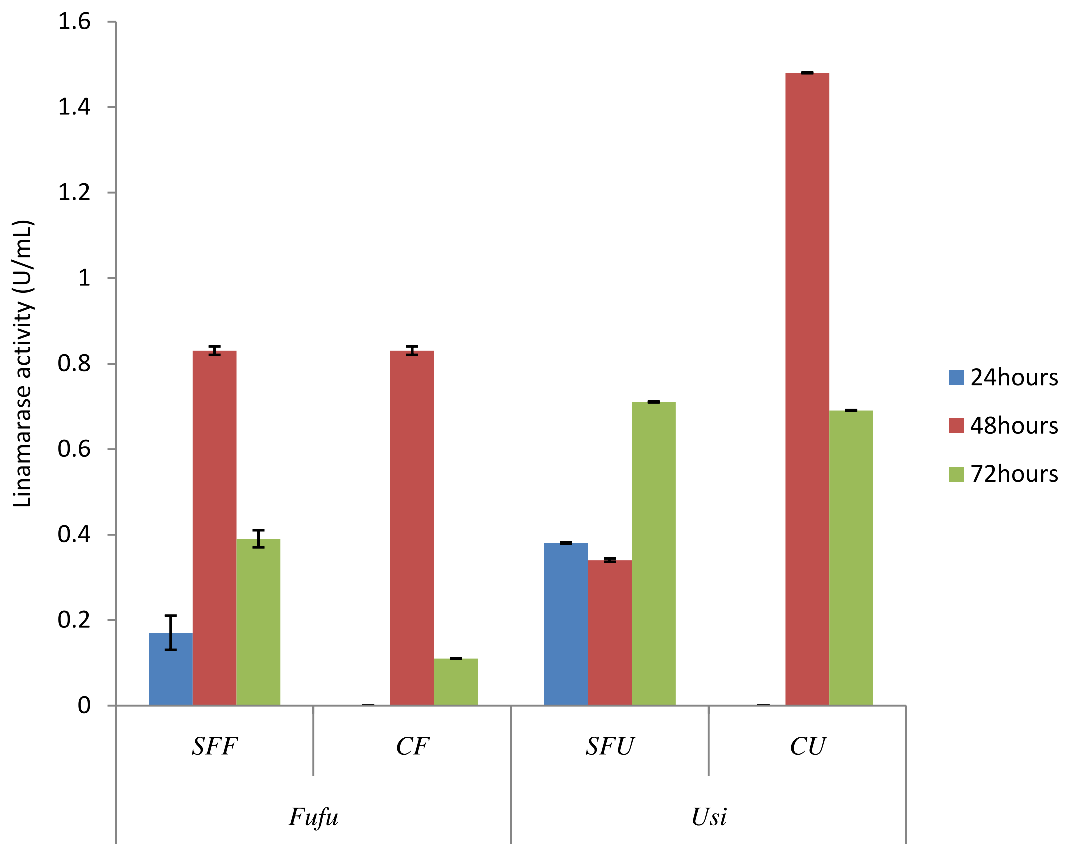

Figure 4.6: Linamarase activities in starter (CGI)-fermented and un-inoculated cassava mash during *fufu* and *usi* fermentation

Key:

SFF- Starter-fermented *fufu;* CF- Spontaneously-fermented *fufu* (control)*;* SFU- Starter-fermented *usi;* CU- Spontaneously-fermented *usi* (control)

4.6.7 Analysis of organic acids and sugars during *fufu* and *usi* fermentation

Since flavour enhancement, increase in nutritive values, suppression of growth of undesirable microflora, thus, increasing shelf life and general acceptability of fermented products have been attributed to the production of fermentation metabolites, the organic acids analyzed during fermentation process involving the utilization of the selected starter combination comprising *L. plantarum* F2C, *L. plantarum* U2A and *L. paraplantarum* U2C indicated that lactic acid was the major organic acid produced throughout the fermentation process with little traces of acetic acid. It has a retention time of 2 minutes 6 seconds (02mins:06secs). Production of other acids (butyric and propionic) was not detected.

During *fufu* fermentation, lactic acid was not detected at zero hour, 0.51 mg/mL was produced at 24 hours after which a subsequent decrease (0.04 mg/mL) was observed at 48 hours and a much higher increased value of 1.23 mg/mL at 72 hours whereas, in the un-inoculated control, the acid was not produced at the zero hour but after which its production was decreasing with increase in fermentation time. It was also observed that higher quantities were produced in the starter fermented experiment than in the uninoculated batch throughout the fermentation process (Figure 4.7).

Production of lactic acid was detected at zero hour, during *usi* fermentation. A slight decrease was observed from zero to 24 hours, after which there was an increase in quantity till the end of fermentation, with the highest value (6.91 mg/mL) at 72 hours. 2.71 mg/mL, 2.68 mg/mL and 4.38 mg/mL were produced at 0, 24 and 48 hours, respectively. In the un-inoculated experiment, there was increase in quantity produced with increasing time. Gradual increase (0.22 mg/mL, 0.97 mg/mL and 1.38 mg/mL) was observed from zero to 48 hours, after which production was more than doubled (3.98 mg/mL) at 72 hours. It was also noted that higher lactic acid was produced in the starter fermented batch than the un-inoculated batch (Figure 4.8). However, acetic acid was detected at the beginning of the fermentation (0 hour) both in the starter-fermented *fufu* (0.14 mg/mL) and *usi* (0.15 mg/mL).

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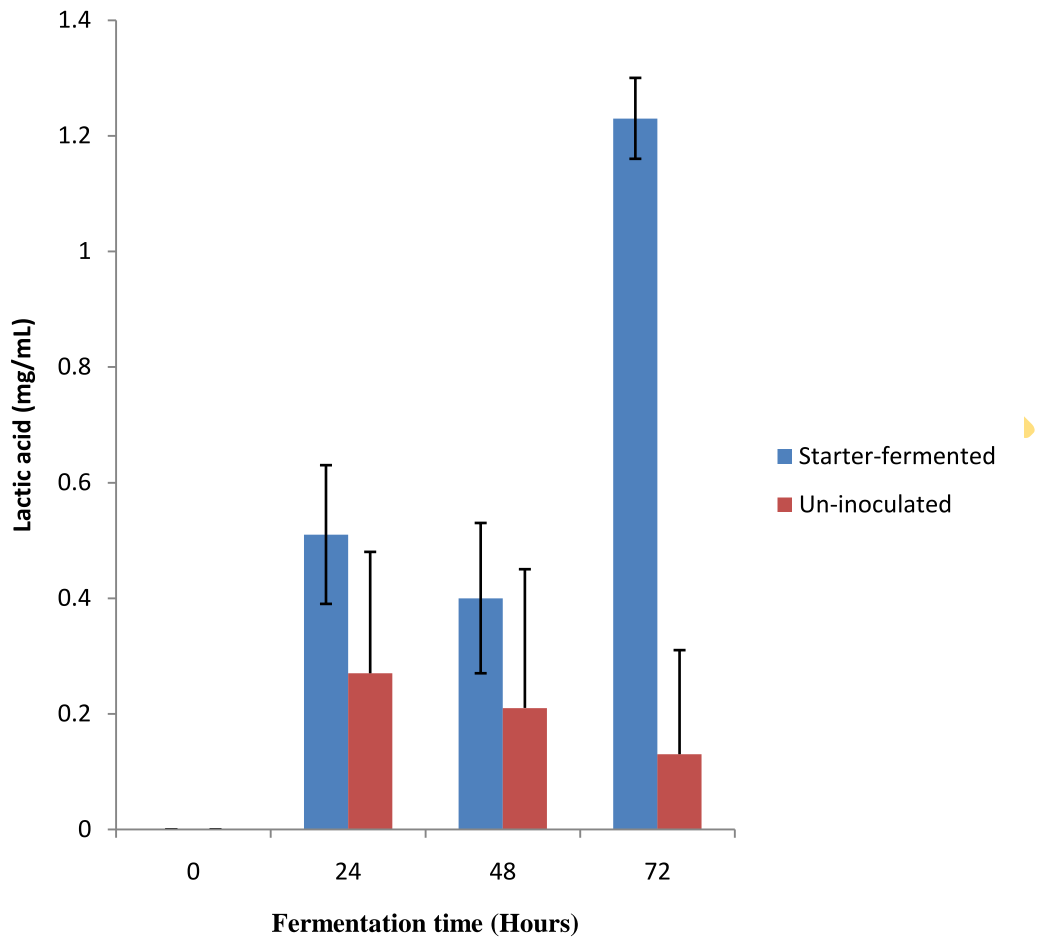

Figure 4.7: Lactic acid quantities at different time intervals during the spontaneous and starter (CGI) fermentation for *fufu*.

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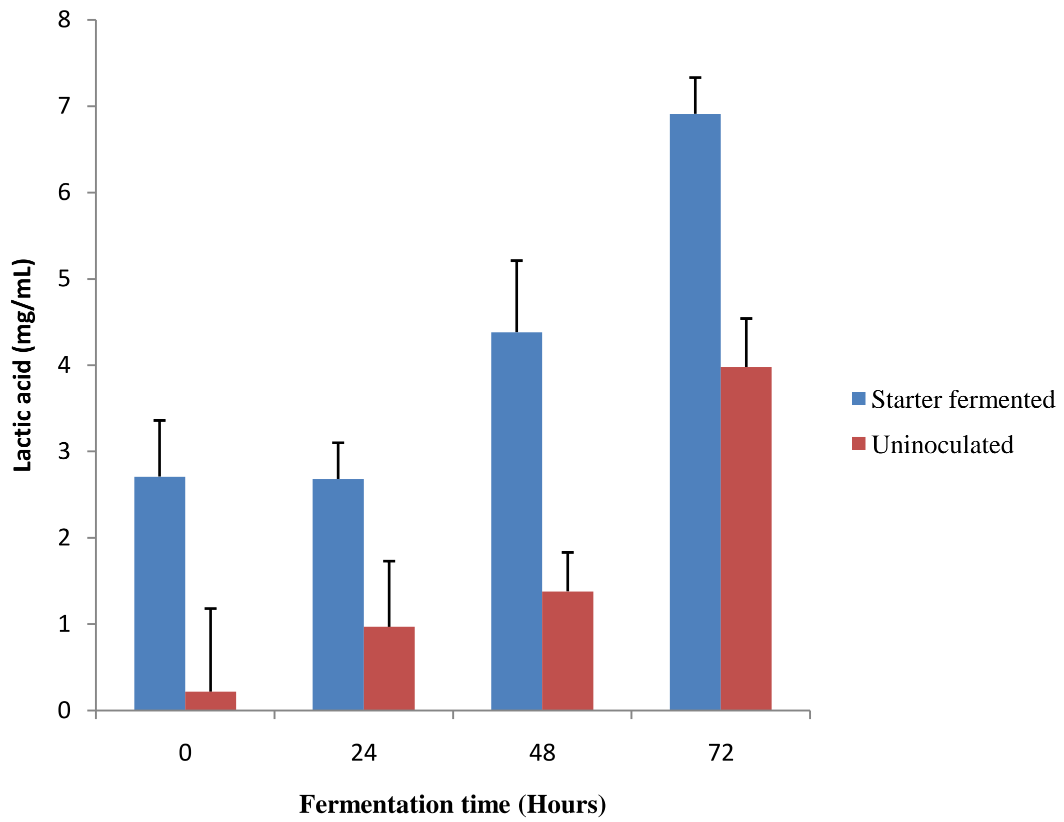

Figure 4.8: Lactic acid quantities at different time intervals during the spontaneous and starter (CGI) fermentation for *usi*.

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Xylose, arabinose, fructose, glucose and sucrose were all detected during the analysis of the cassava mashes using HPLC. Other sugars such as ribose, rhamnose, maltose and lactose, even though present, had quantities below the detectable level in both *fufu* and *usi.*

Figures 4.9 and 4.10 showed the results of the obtained sugar quantities. Xylose quantity increased from 1.95µg/mL at the beginning of fermentation to 3.09 µg/mL at 24 hours after which it further decreased to 1.28 µg/mL and 0.43 µg/mL at 48 and 72 hours respectively during starter (CGI) fermentation for *fufu*. The quantities produced in *usi* were lower except at 72 hours, with values ranging between 0.47 µg/mL and 3.2 µg/mL. However, in both fermentations, the un-inoculated (control) samples recorded increase in xylose quantity with increasing fermentation time. There was gradual increase from 0.38 µg/mL at the beginning of fermentation to 0.42 µg/mL at 24 hours and further to 1.77 µg/mL and 2.14 µg/mL at 48 and 72 hours respectively during spontaneous *fufu* fermentation. The values for *usi* also increased from 0.46 µg/mL to 0.64 µg/mL, 2.34 µg/mL and 2.48 µg/mL with increase in fermentation time. It was however observed that overall highest xylose (3.20 µg/mL) was produced at 72 hours in starter (CGI)-fermented *usi* samples.

Quantities of arabinose produced in the starter (CGI)- fermented samples of both *fufu* and *usi* had slight increase from 0-24 hours with values ranging between 0.39 µg/mL and 0.61 µg/mL, however, a decrease, from 0.35µg/mL to 0.15µg/mL at 48 and 72 hours were observed in *fufu* sample while an increased peak value (1.37 µg/mL) was recorded in *usi* sample. Gradual increase in quantity with an increase in fermentation time characterized the un-inoculated *usi* fermentation with values ranging between 0.41 µg/mL and 1.13 µg/mL.

Much higher quantities of fructose, glucose and sucrose were observed in all experimental batches. Fructose quantity decreased with increasing fermentation time in the starter (CGI)-induced fermentation for both products. *Fufu* had values ranging between 21.9 µg/mL and 25.4 µg/mL, while *usi* had between 24.1 µg/mL and 26.1 µg/mL respectively. There was an increase in quantities produced, with increasing fermentation time in the un-inoculated *usi* sample, with the highest value (26.2 µg/mL) at 72 hours.

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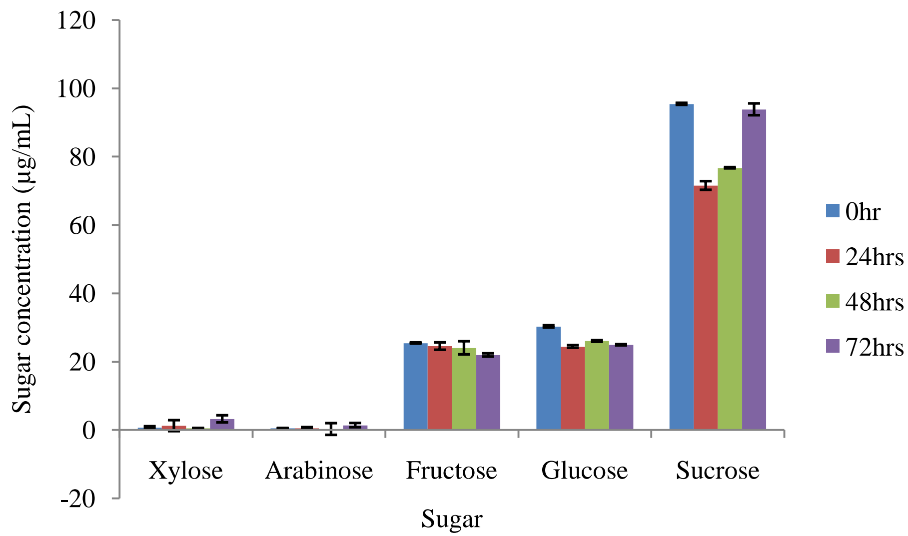

Figure 4.9a: Sugar quantities at different time intervals during starter (CGI) fermentation for *usi*.

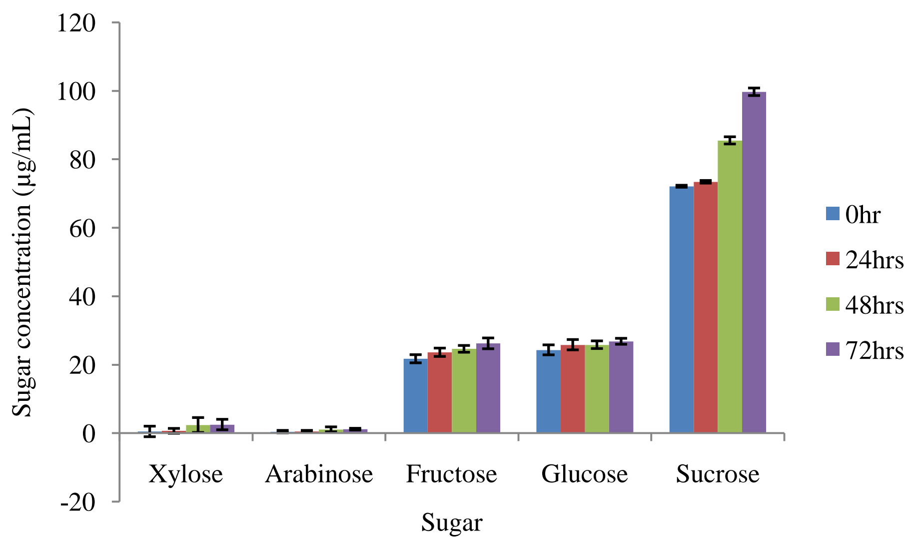

Figure 4.9b: Sugar quantities at different time intervals during spontaneous fermentation for *usi* production.

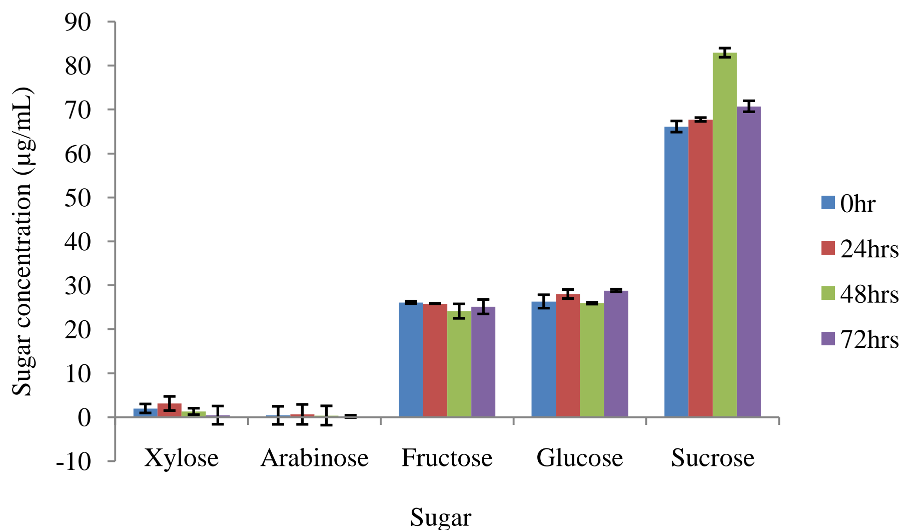

Figure 4.10a: Sugar quantities at different time intervals during starter (CGI) fermentation for *fufu* production.

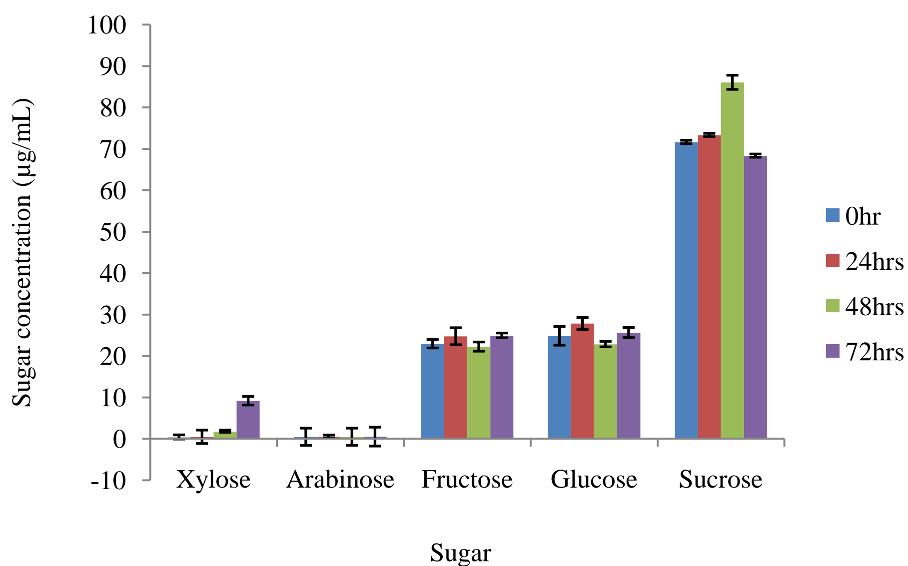

Figure 4.10b: Sugar quantities at different time intervals during spontaneous fermentation for *fufu* production.

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Furthermore, the starter (CGI)-fermented samples had higher fructose quantities than the un-inoculated batches except at 48 and 72 hours during un-inoculated *usi* fermentation where values higher than those of the starter-fermented samples were observed.

During *usi* fermentation, highest glucose quantity (30.3 µg/mL) was recorded at the beginning of the fermentation while the least (24.4 µg/mL) was at 24 hours. The least glucose (25.9 µg/mL) quantity was produced at 48 hours while the highest produced (28.8 µg/mL) was observed at 72 hours. However, a gradual increase in quantity was observed with increase in fermentation time in the un-inoculated *usi* samples with values ranging between 24.3 µg/mL and 26.8 µg/mL. The starter (CGI)-fermented samples had higher glucose quantities than the un-inoculated during *fufu* fermentation.

Sucrose quantities were the highest in both fermentations, with peak values (82.9 µg/mL and 86 µg/mL) at 48 hours during starter (CGI)-fermented and un-inoculated *fufu* samples respectively. The highest value observed during *usi* fermentation was 99.7 µg/mL at 72 hours, in the un-inoculated sample.

4.7 Optimization of growth conditions for selected starter

4.7.1 Effect of different pH values on the growth of selected starter (CGI)

Different pH ranges (3.5 – 7.5) supported the growth of the starter culture except at pH 3.5 when observed at 600 nm. There was a gradual increase in growth with increasing pH values, ranging between 0.22±0.13 and 1.73±0.15. Optimal starter growth (1.73±0.15) was observed at pH 7.5, closely followed by 1.24±0.17, 1.14±0.21 and 0.22±0.13 at pH 6.5, 5.5 and 4.5 respectively (Figure 4.11).

4.7.2 Effect of incubation temperature on the growth of selected starter (CGI)

The starter culture (CGI) grew within incubation temperature range of 25°C and 45°C with growth at 600 nm ranging from 0.07±0.04 to 1.68±0.05. Optimal temperature was at 30°C because it recorded the highest growth (1.75±0.05). This was closely followed by 1.68±0.05 at 37°C. Lower growth values were observed at 40°C (0.24±0.35) and 45°C (0.07±0.04) (Figure 4.12).

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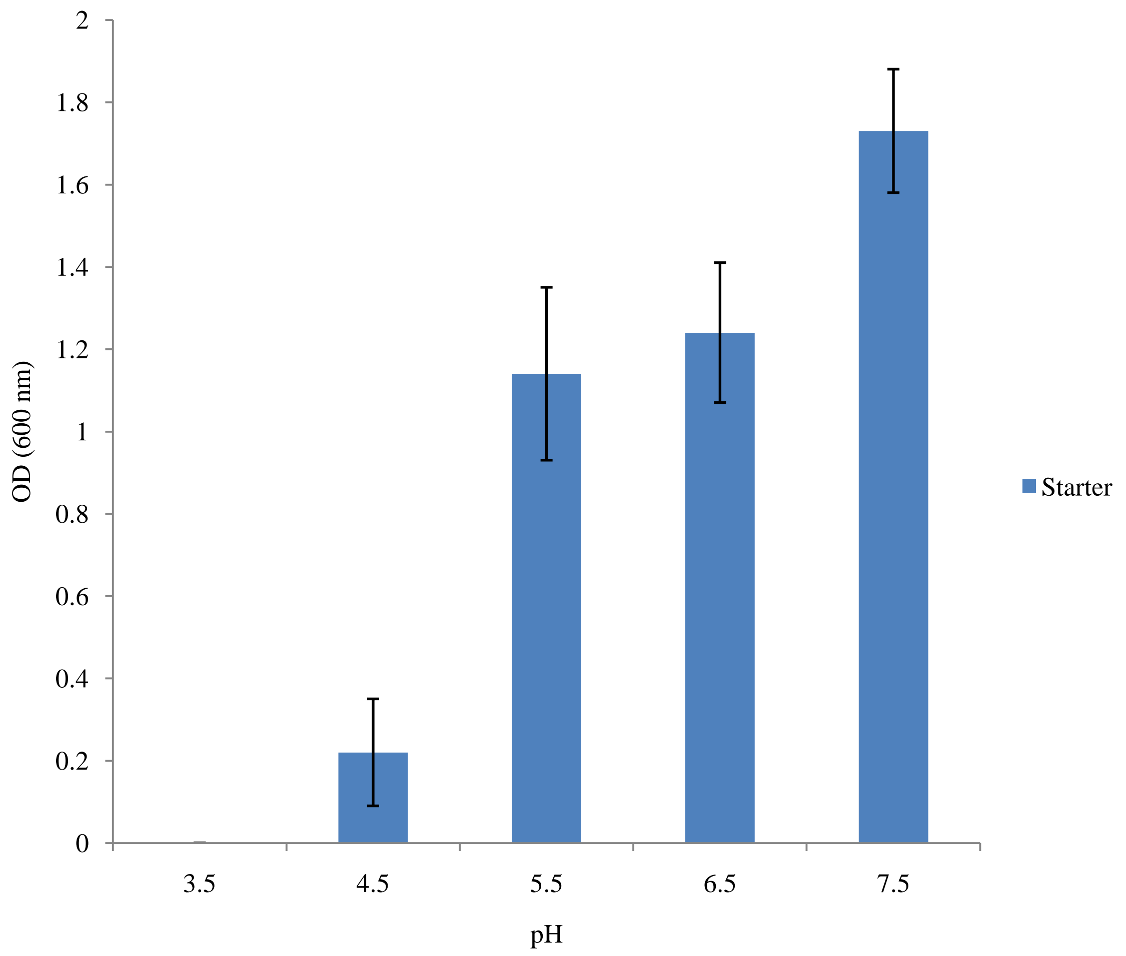

Figure 4.11: Effect of different pH values on the growth of selected starter CGI.

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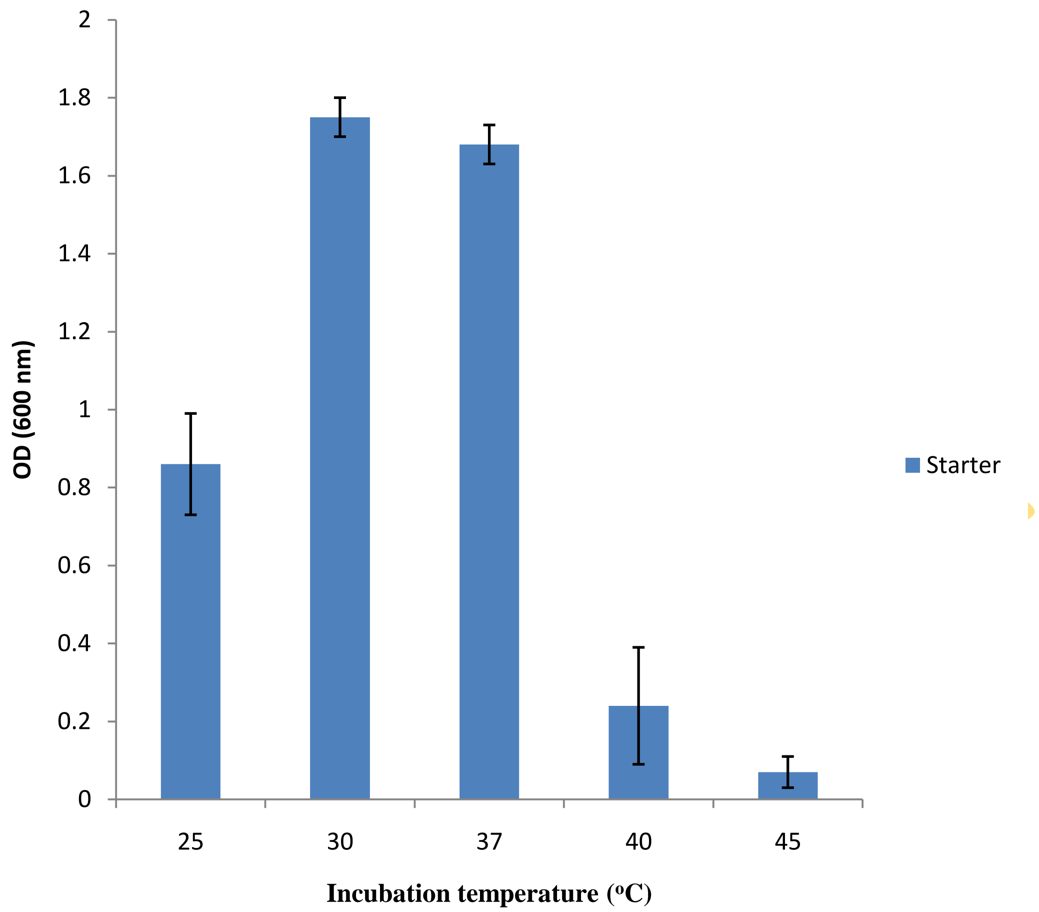

Figure 4.12: Effect of different incubation temperature on the growth of selected starter CGI.

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4.7.3 Effect of salt concentrations on the growth of selected starter (CGI)

Figure 4.13 showed the effect of various NaCl concentrations on starter growth. The optimal salt concentration for the growth of the starter was 2%, with the highest growth value 0.88±0.45 at 600 nm. Next to this was 10% salt concentration with growth measurement of 0.51±0.01 after which much less growth values were obtained at 4% (0.18±0.02), 6% (0.21±0.01) and 8% (0.17±0.01), respectively.

4.7.4 Effect of agitation on the growth of selected starter (CGI)

The effect of agitation on microbial growth was monitored within the range of 50 to 250 revolutions per minute. Growth ranged between 0.89±0.02 and 1.86±0.04 at 600 nm. The optimum agitation speed, resulting in highest growth (1.86±0.04) was 100 rpm. This was closely followed by speed at 50 rpm. Speed at 200, 150 and 250rpm produced starter growth of 1.03±0.13, 0.07±0.04 and 0.89±0.02 respectively (Figure 4.14)

4.7.5 Effect of different carbon sources on the growth of selected starter (CGI)

Figure 4.15 showed the effect of the different carbon sources (glucose, maltose, starch, lactose and galactose) that could support the growth of the starter optimally at 500 nm optical density. Lactose gave the least growth value (0.06±0.1), followed by galactose (0.14±0.02), starch (0.18±0.05), glucose (0.22±0.07) and then maltose, with the highest growth value (0.34±0.22), thus, making maltose the optimal carbon source for the growth of the starter culture (Figure 4.16).

4.7.6 Effect of different nitrogen sources on the growth of selected starter (CGI)

Figure 4.16 showed the effect of different nitrogen sources on the growth of the starter at 500 nm optical density. The highest growth (0.59±0.07) was detected when yeast extract was used as the sole nitrogen source. This was closely followed by casein, having 0.45±0.04. No significant difference was observed in starter growth (0.31) when urea and $(NH_4)_2SO_4$ were used.

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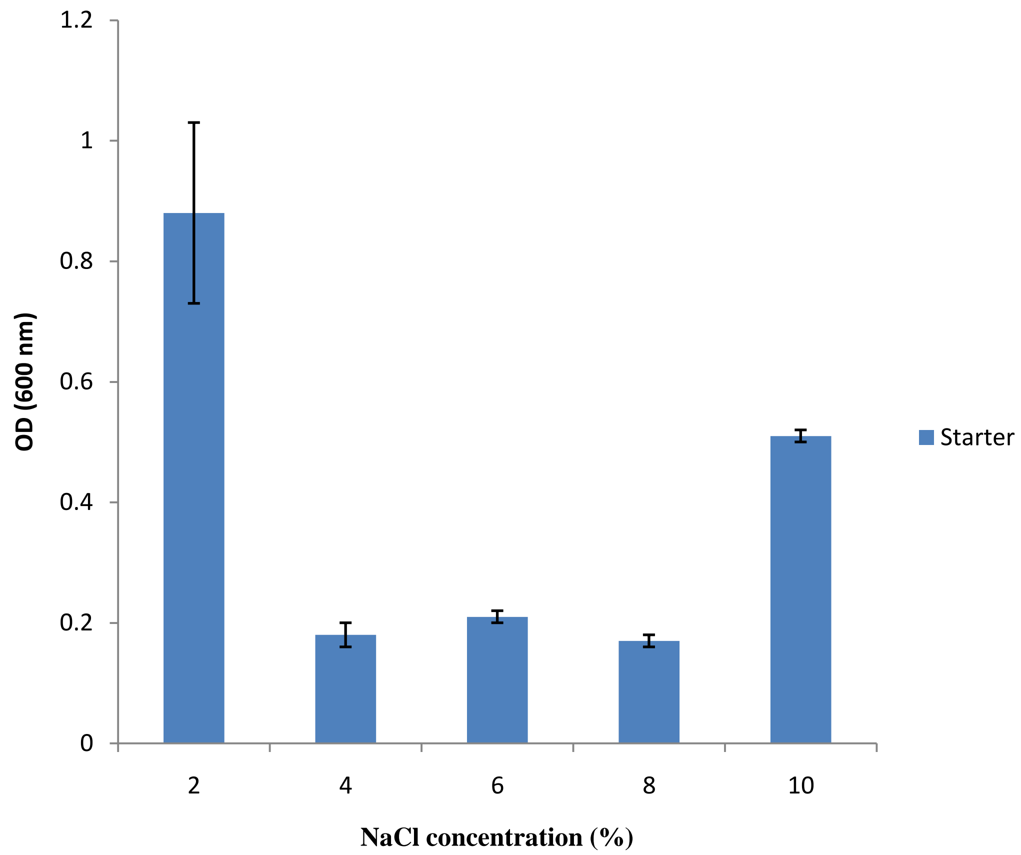

Figure 4.13: Effect of NaCl concentrations on the growth of selected starter CGI

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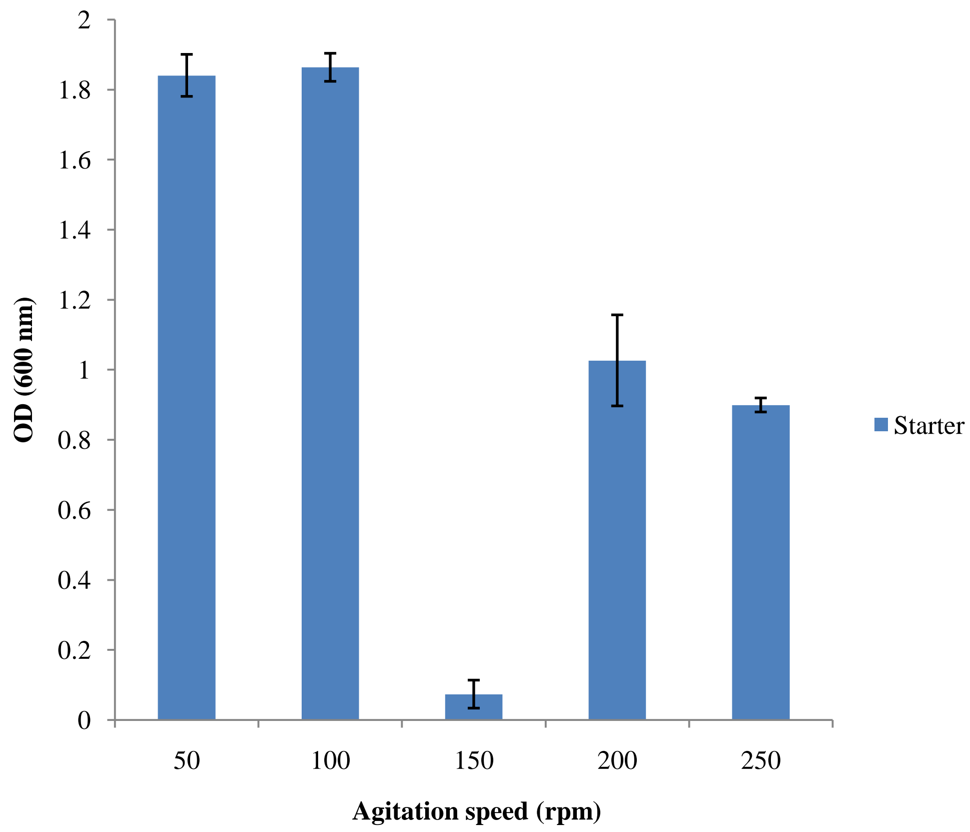

Figure 4.14: Effect of different agitation speed on growth of selected starter CGI.

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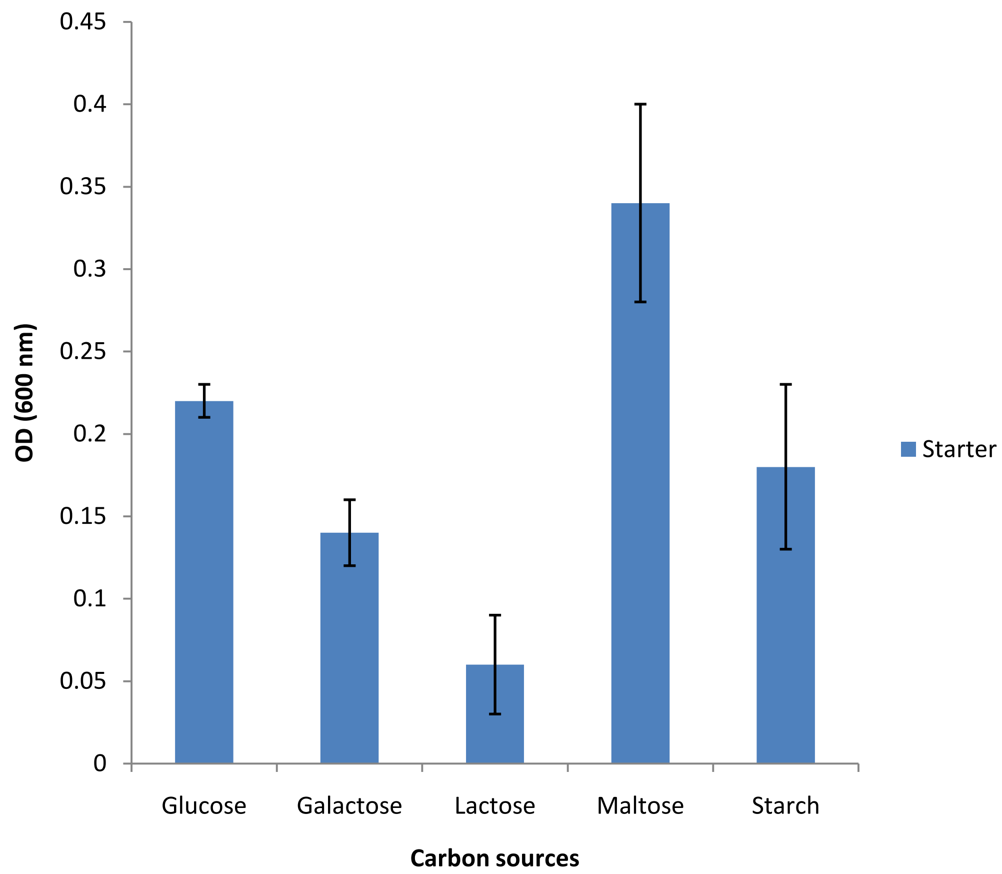

Figure 4.15: Effect of different carbon sources on the growth of selected starter CGI.

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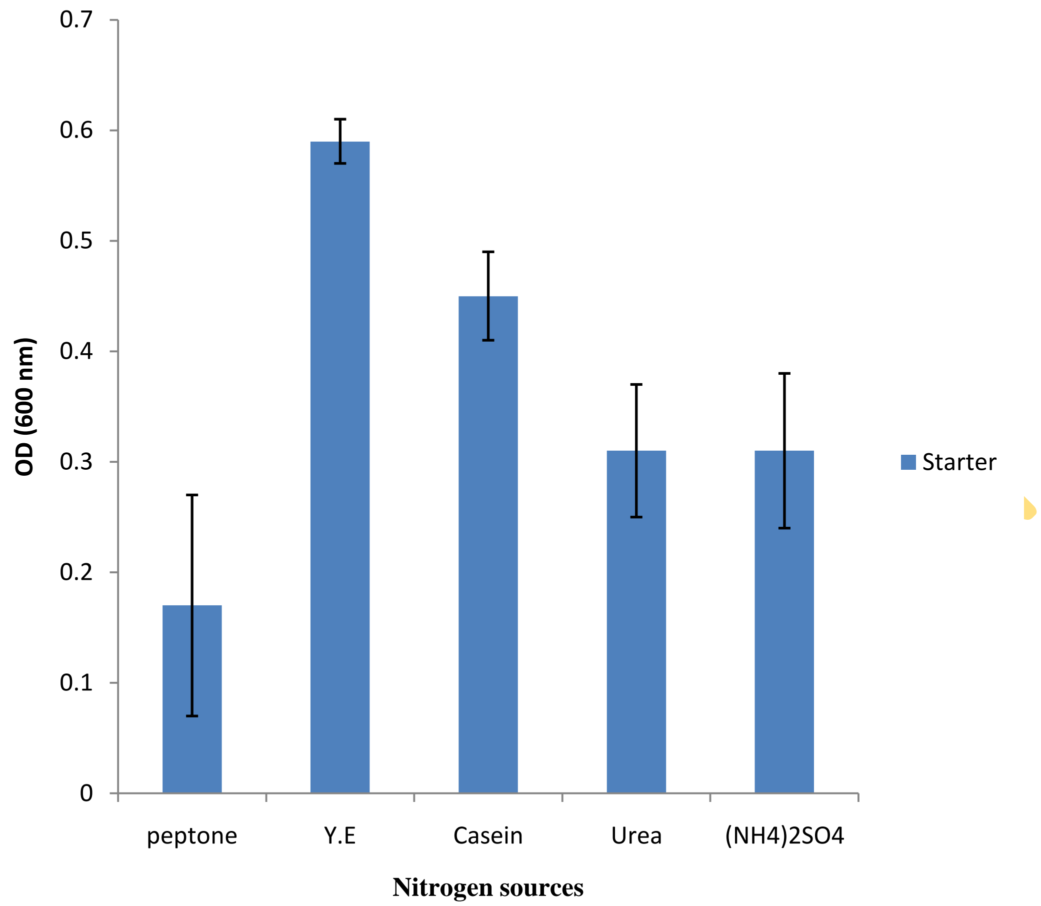

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Figure 4.16: Effect of different nitrogen sources on the growth of selected starter CGI.

4.7.7 Effect of different incubation time on growth of starter

Gradual increase in growth at 620nm with increasing incubation time was observed. Growth increased from 1.46±0.24 to 1.67±0.08 and eventually 1.83±0.08 at 24, 48 and 72 hours, respectively, thus, making 72 hours the optimal incubation time for the cultivation of the starter (Figure 4.17)

4.8 Effect of optimization of starter growth conditions on the proximate and anti-nutritional factors (%) of fermented *fufu* and *usi* mashes

Proximate analysis of the cassava mashes fermented with starter that was cultivated using the optimal growth conditions was evaluated. Significantly improved nutitional content was generally observed when compared with the starter that was not subjected to optimal growth conditions during *fufu* fermentation. When the starter that was subjected to optimal growth conditions was used for *fufu* fermentation, moisture content of the mash was reduced (5.32% to 5.00%), protein content increased (1.28% to 1.52%), crude fat was reduced (0.73% to 0.60%), crude fibre and ash content increased (1.89% to 1.90% and 1.19% to 1.21%) respectively, while reduction in carbohydrate content (92.1% to 91.0%) was also observed. Meanwhile, the results obtained for *usi* also indicated an increase in protein content (1.82% to 1.98%) and slight decrease in crude fibre (2.35% to 2.32%). However, moisture, fat and ash contents increased in samples that were fermented using the starters cultivated under the optimized growth conditions. Results were presented in Table 4.20.

The effect of optimization of on the anti-nutritional factors was also analyzed. As shown in Table 4.21, there was no significant difference at 5% level of probability in the phytate content of both optimized and non-optimized *usi* fermentations, whereas, the *fufu* mash had an increased value of 0.30mg/g in sample fermented with starter grown using optimal growth conditions. Significant reductions were observed in the tannin content of *usi* (33.2 mg/g to 33.0 mg/g) and *fufu* (35.8mg/g to 34.9 mg/g) while there was no significant difference in the cyanide content (0.05mg/g) of both optimized and non-optimized *fufu* fermentations. Cyanide content was not detected in *usi* samples. However, samples fermented with starter grown using the optimal growth conditions as well as those of the non optimised growth conditions had reduced values of anti-nutrients than in the fresh cassava.

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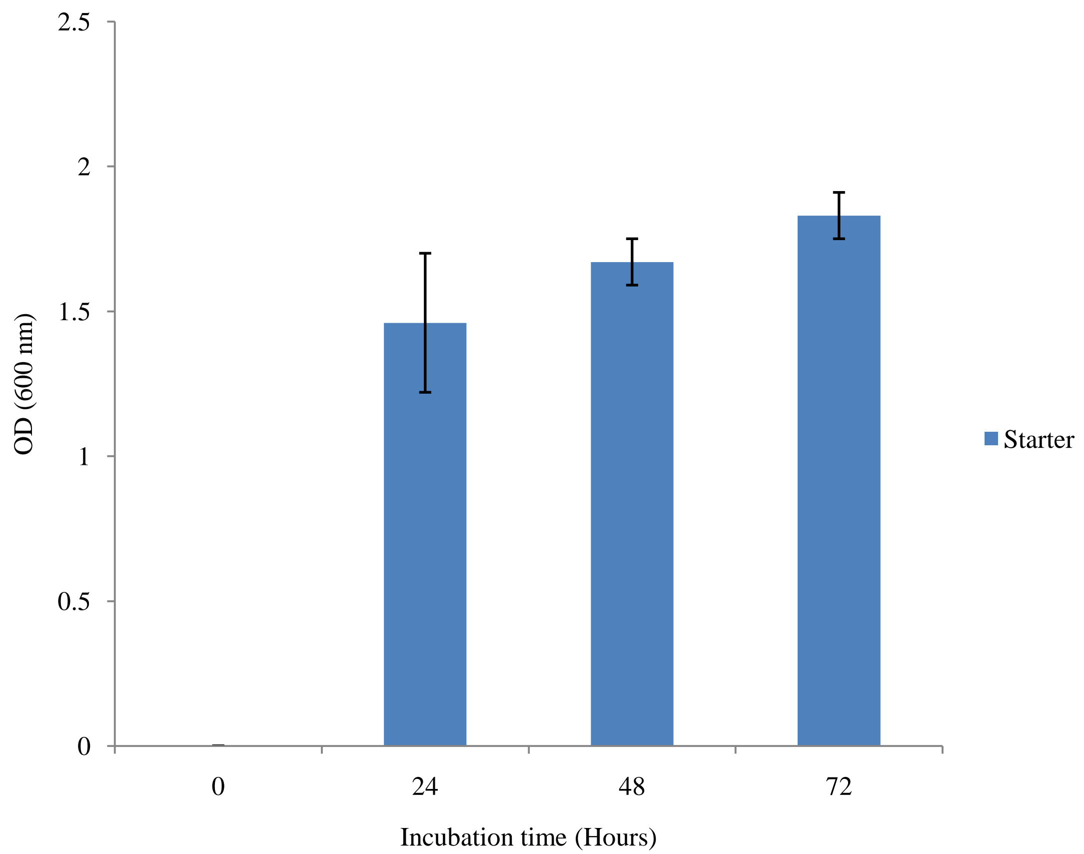

Figure 4.17: Effect of different incubation time on the growth of selected starter CGI

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Table 4.20: Effect of optimization of starter growth conditions on nutritional content (%) of fermented *fufu* and *usi* mashes

Proximate	Fresh cassava	Fermentation with starter (CGI)		Fermentation using optimal growth conditions	
		Fufu	*Usi*	*Fufu*	*Usi*
Moisture	6.82 ± 0.04^{a}*	5.32 ± 0.07^{d}	5.36 ± 0.04^{c}	5.00 ± 0.1^{e}	5.38 ± 0.06^{b}
Protein content	1.12 ± 0.15^{e}	1.28 ± 0.06^{d}	1.82 ± 0.13^{b}	1.52 ± 0.07^{a}	1.98 ± 0.15^{c}
Crude fat	0.50 ± 0.03^{c}	0.73 ± 0.08^{a}	0.36 ± 0.03^{e}	0.60 ± 0.05^{b}	0.41 ± 0.05^{d}
Crude fibre	1.72 ± 0.02^{e}	1.89 ± 0.02^{d}	2.35 ± 0.05^{a}	1.90 ± 0.12^{c}	2.32 ± 0.1^{b}
Ash	1.39 ± 0.07^{a}	1.19 ± 0.05^{c}	0.69 ± 0.02^{e}	1.21 ± 0.02^{b}	0.81 ± 0.04^{d}
Carbohydrate	89.10 ± 0.19^{c}	92.10 ± 0.11^{a}	87.77 ± 0.08^{d}	91.00 ± 0.11^{b}	87.2 ± 0.1^{e}

Data are means±SD, n=3.

*The means reported with the same superscript in each row indicated no significant difference ($p\leq0.05$)

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Table 4.21: Effect of optimization of startergrowth conditions on anti-nutritional factor (mg/g) of fermented *fufu* and *usi* mashes

Parameter	Unfermented cassava	Fermentation with starter (CGI)		Fermentation using optimal growth conditions	
		Fufu	*Usi*	*Fufu*	*Usi*
Phytic acid	$0.34 \pm 0.00^{a*}$	0.20 ± 0.002^{d}	0.29 ± 0.00^{c}	0.30 ± 0.00^{b}	0.29 ± 0.004^{c}
Tannin	36.1 ± 0.05^{a}	35.8 ± 0.19^{b}	33.2 ± 0.03^{d}	34.9 ± 0.22^{c}	33.0 ± 0.01^{e}
Cyanide content	0.10 ± 0.02^{a}	0.05 ± 0.00^{b}	0.00^{c}	0.05 ± 0.003^{b}	0.00^{c}

Data are means±SD, n=2.

*The means reported with the same superscript in each row indicated no significant difference ($p \leq 0.05$)

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4.9 Sensory evaluation of *fufu* and *usi*

Using a 7-point Hedonic scale with a 20-man panel for the analysis of product quality, both products (Plate 2) were generally accepted by the panellists. Results shown in Table 4.22a indicated that the colour, odour and texture of the starter-fermented *usi* were well accepted than that of the spontaneously-fermented product (control). However, there was no significant difference in the taste and elastic quality of both products. Furthermore, the overall acceptability of the starter-fermented *usi* was higher than the control product with 5.29 points as against 5.21.

Starter-fermented *fufu* had more acceptable colour and odour while no significant difference was observed in taste. The control product had a stronger elastic quality and texture even though the overall acceptability was higher in the starter-fermented *fufu* (Table 4.22b).

4.10 Microbial load during *fufu* and *usi* storage at room temperature.

The estimation of microbial load during storage of *fufu* and *usi* at room temperature for a period of seven days was presented in Tables 4.23a and 4.23b, respectively. Total bacterial count was found to increase with increasing storage period in both starter-fermented and control products with values ranging between 5.82×10^9CFU/g on day 1 to 8.51×10^9 CFU/g on day 7 in the starter-fermented *fufu* (SFF) and 5.24×10^9 CFU/g (day 1) to 7.51×10^9 CFU/g (Day 7) in the starter-fermented *usi* (SFU). The total count obtained for the control products was higher, ranging from 6.19×10^9 CFU/g to 9.28×10^9 CFU/g and 6.52×10^9 CFU/g to 8.78×10^9 CFU/g in the *fufu* (CF) and *usi* (CU), respectively.

Lactic acid bacteria count increased in all the products as storage period progressed with values within the range of 0.93×10^9 CFU/g and 2.91×10^9 CFU/g from the first, to the last storage day. Coliform count was higher within the first and second days, but slightly reduced on the 3rd and 4th day, beyond which an increase was observed, mostly with higher values in the control products, till the last storage day. The count ranged between 0.87×10^9 CFU/g to 3.44×10^9 CFU/g (SFF), 2.22×10^9 CFU/g to 3.21×10^9 CFU/g (CF), 1.01×10^9 CFU/g to 3.73×10^9 CFU/g (SFU) and1.93×10^9 CFU/g to 4.09×10^9 CFU/g (CU).

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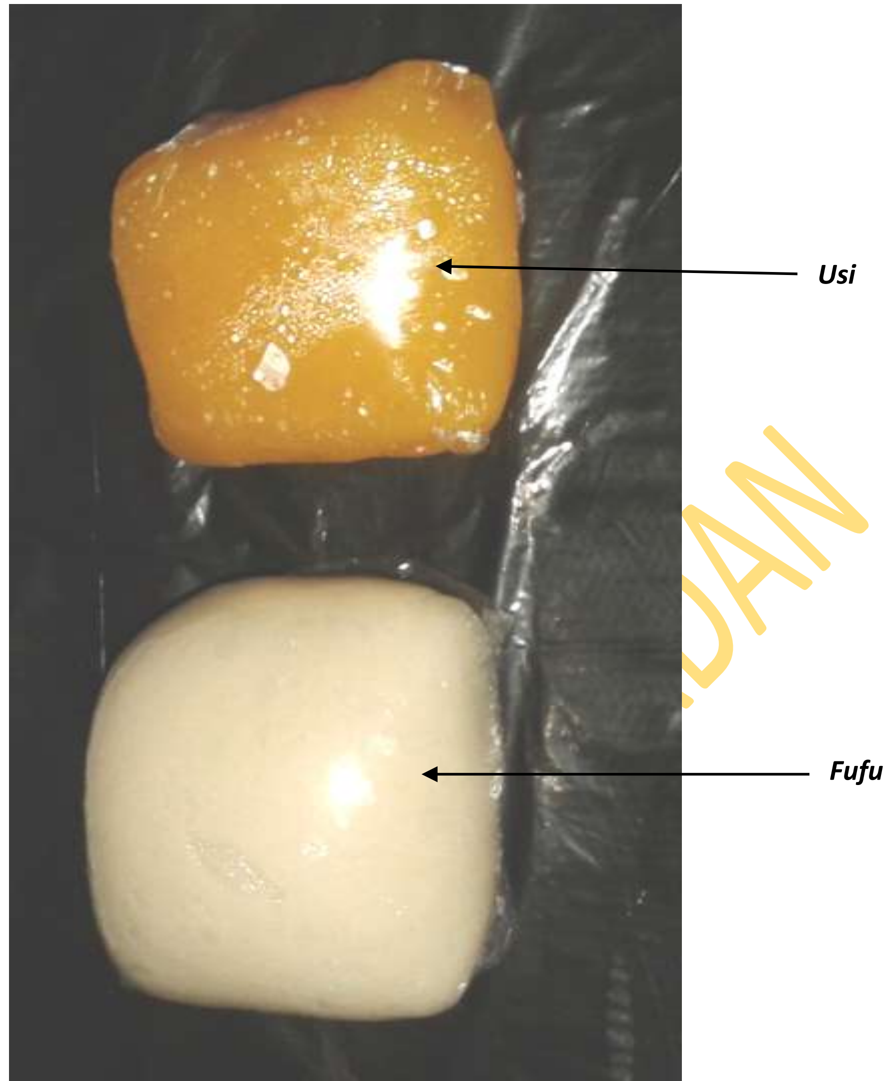

Plate 4.2: Laboratory prepared starter-fermented *usi* and *fufu*.

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Table 4.22a: Sensory evaluation of the starter-fermented and spontaneously-fermented *usi*

Sensory parameters	Starter-fermented *usi*	Spontaneously-fermented *usi*
Taste	4.25±0.5[a]*	4.26±0.5[a]
Colour	6.43±0.5[a]	5.86±0.9[b]
Odour	5.07±0.5[a]	5.00±0.8[b]
Texture	5.21±1.1[a]	5.14±0.6[b]
Elastic quality	5.63±0.8[a]	5.64±0.5[a]
Overall acceptance	5.29±0.5[a]	5.21±0.4[b]

Data are means±SD, n=20.

*The means reported with the same superscript in each row indicated no significant difference ($p \leq 0.05$)

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Table 4.22b: Sensory evaluation of starter-fermented and spontaneously fermented *fufu*

Sensory parameters	Starter-fermented *fufu*	Spontaneously-fermented *fufu*
Taste	4.57±0.5[b]	5.07±0.4[a]
Colour	5.71±0.5[a]	4.79±0.7[b]
Odour	5.14±0.7[a]	4.29±0.8[b]
Texture	4.43±0.5[b]	5.00±0.8[a]
Elastic quality	5.29±0.5[b]	5.71±0.5[a]
Overall acceptance	5.24±0.7[a]	5.21±0.6[b]

Data are means±SD, n=20.

*The means reported with the same superscript in each row indicated no significant difference ($p \leq 0.05$)

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Table 4.23a: Microbial load during *usi* storage at room temperature over a 7-day period

Samples		Storage period (Days)/Microbial count (x10^9 CFU/g)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Starter-fermented *usi*	TBC	5.24	5.97	5.90	6.32	6.91	7.28	7.51
	LC	1.72	1.51	1.82	2.31	2.90	2.78	2.93
	CC	1.01	2.0	1.10	1.02	2.35	2.93	3.73
	YC	0.91	1.28	1.54	2.02	1.97	2.0	2.23
	MC	ND	ND	ND	ND	0.11	0.31	0.64
	SC	0.31	0.72	1.05	1.39	1.52	1.71	1.79
Spontaneously-fermented *usi*	TBC	6.52	8.11	8.36	9.35	9.91	8.41	8.78
	LC	1.31	1.68	2.0	2.91	2.33	2.9	3.1
	CC	1.93	1.99	2.38	3.81	3.89	3.8	4.09
	YC	0.87	1.51	1.73	1.31	2.51	2.72	1.93
	MC	ND	ND	ND	0.72	0.96	1.23	1.82
	SC	0.58	1.31	2.84	1.77	2.93	1.95	2.32

TBC-Total bacteria count; LC-LAB count; CC-Coliform count; YC- Yeast count; MC-Mold count; SC- *Staphylococcus* count, ND-Not detected

Table 4.23b: Microbial load during *fufu* storage at room temperature over a 7-day period

Samples		Storage period (Days)/Microbial count (x10^9 CFU/g)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Starter-fermented *fufu*	TBC	5.82	6.38	6.79	7.13	7.01	7.62	8.51
	LC	1.51	1.98	2.36	2.77	2.86	2.88	2.61
	CC	0.87	2.91	2.11	1.53	1.84	2.65	3.44
	YC	0.34	1.53	1.73	1.91	2.01	1.89	2.02
	MC	ND	ND	ND	0.13	1.51	1.71	1.53
	SC	1.72	1.90	0.8	1.31	1.64	1.82	1.84
Spontaneously-fermented *fufu*	TBC	6.19	7.34	7.91	8.28	8.53	9.01	9.28
	LC	0.93	0.98	1.23	1.38	2.51	2.35	2.91
	CC	2.22	2.73	2.21	1.62	1.71	2.53	3.21
	YC	0.73	1.81	2.0	2.53	2.48	2.45	2.99
	MC	ND	ND	0.75	1.12	2.31	2.7	2.91
	SC	2.15	2.62	1.98	1.89	2.51	2.93	2.81

TBC-Total bacteria count; LC-LAB count; CC-Coliform count; YC- Yeast count; MC-Mold count; SC- *Staphylococcus* count, ND-Not detected

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Staphylococci count was generally not as high as the coliform count. Increasing growth was observed, with slight decrease on the 3rd and 4th day in SFF, CF and CU, after which the count increased until the last storage day

Mold growth was not detected until the 4th and 5th day in SF and SU, but was observed as early as the 3rd day in the spontaneously-fermented products.

Spoilage symptoms were not observed in all products on the first day, till the third storage day. On the fourth day, visible spoilage symptoms were observed in the form of whitish moldy growth, watery film and off colour in the spontaneously-fermented *fufu* while *usi* had off-yellow colour with soft and loosened texture. The symptoms were more prominent as storage period progressed, with slight visible microbial growth on starter-femented *fufu* and *usi* as well towards the end of day 5. By the end of the 7th day, profuse spoilage was evident as off odour, slime and moldy growth in all products, brown coloured patches in *fufu* and off-yellow in *usi* as well as loosened texture (Plate 3).

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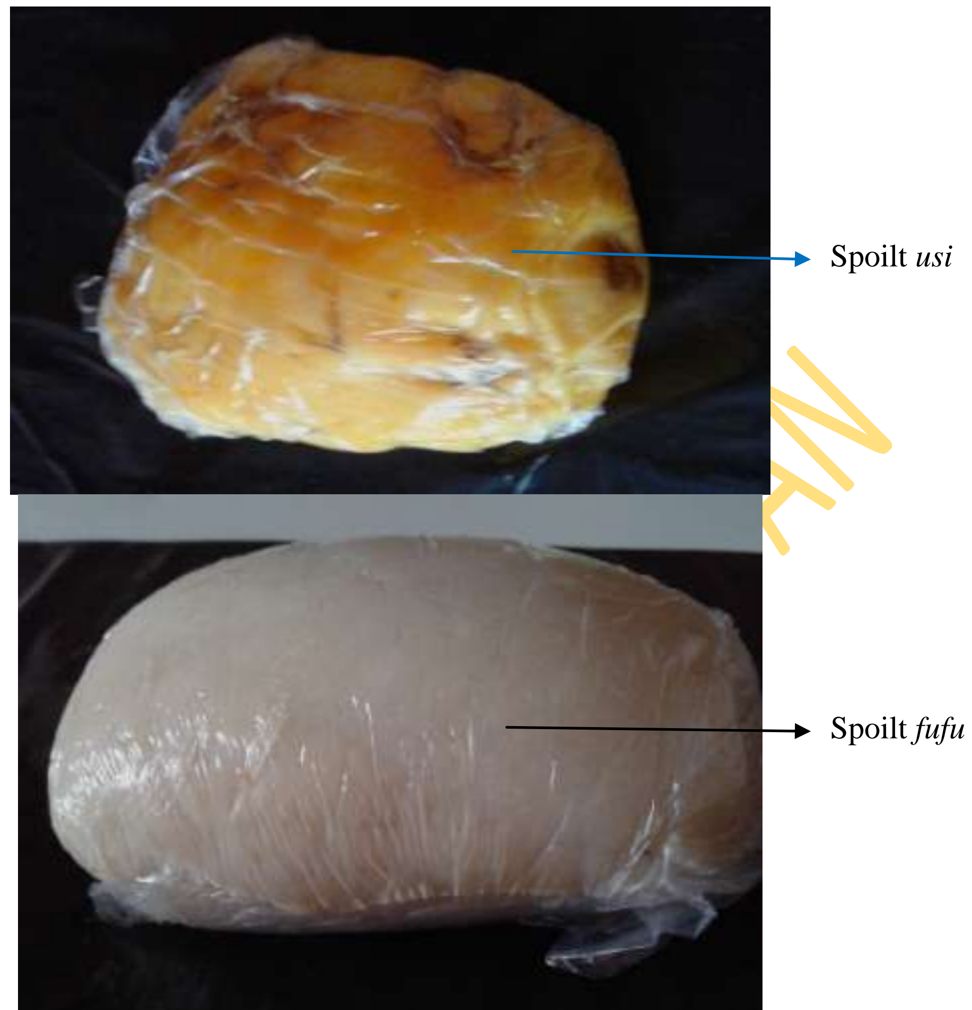

Plate 4.3: *Usi* and *Fufu* showing spoilage symptoms such as whitish moldy growth, watery film, loose texture and off colour on the 7th storage day.

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CHAPTER FIVE

5.0 DISCUSSION

Cassava still remains an important root crop especially in developing countries, where it had been utilized by processing into various food products through different procedures. Fermentation of cassava has been reported to be the most important and widely used means of processing the roots (Oyewole, 1992; Nweke *et al*., 2002) into different fermented cassava products. Numerous authors have fermented cassava into various products like *gari* (Okafor, 1977; Abe and Lindsay, 1978; Ngaba and Lee, 1979; Oyewole and Odunfa, 1988; Osho *et al*., 2010; Edward *et al*., 2012), *fufu* (Oyewole and Odunfa, 1989, 1992; Oyewole, 1990; Oyewole and Sanni, 1995; Brauman *et al*., 1996; Oyewole *et al*., 2001; Obadina *et al*., 2006; Henshaw and Ikpoh, 2010), *lafun* (Oyewole and Odunfa, 1988; Oyewole, 1991; Nwabueze and Odunsi, 2007; Padonou *et al*., 2009) and so on.

The submerged fermentation of cassava roots have been reported to be associated with reduction in pH of the fermenting medium, thus, increasing the acidity and acidification as a result of production of organic acids (Olukoya *et al.*, 2011). Rapid acidification is advantageous for the process because it creates unsuitable environment for spoilage and pathogenic organisms, furthermore, hastening the fermentation process. During the spontaneous fermentation of cassava for *fufu* and *usi* production in this study, the reduction in pH values with increasing fermentation time and increasing acidity observed has previously been reported in numerous literatures. Obadina (2006) observed a decrease from an initial pH of 6.95 to 3.78 after 96 hours of cassava fermentation for *fufu*. During cassava fermentation to *lafun* and *fufu*, initial pH of 6.50 and 6.90 were reduced to 3.80 and 4.10, respectively after the fermentation process (Oyewole, 1995). Kobawila *et al.* (2005) also reported a decrease in initial pH of 7.20 to 3.80. Similar observations were reported with other substrates as well. Edema and Sanni (2008), observed a reduction in initial pH from 5.62 to 3.05, 3.37

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and 3.65 after fermentation of maize sour dough by different microorganisms during starter selection experiment. They earlier reported a final pH of 3.71 during the spontaneous fermentation of maize meal (Edema and Sanni, 2006). The reduced pH at the end of the fermentation could be as a result of the metabolic breakdown of starch into different organic acid metabolites. Mathew *et al.* (1995) had earlier linked the increasing acidity which results in lowering of pH values to microbial activities which converts carbohydrates to organic acids. This was also in accordance with Oyewole (1990) and Malonga *et al.* (1993, 1996) who attributed it to the production of mixed acids by lactic acid bacteria. Lactic acid fermentation resulting in pH levels lower than 4.2 has been reported to constitute a major food safety factor (Holzapfel, 1997). Lower pH values observed in *usi* could be due to the effect of grating prior to fermentation since Oyewole and Odunfa (1992) and Okafor (1984) earlier linked rate of acidification during fermentation to the root cut size.

The indigenous fermentation microflora is a complex and mixed colony of microorganisms (Kobawila *et al*., 2005; Ekundayo and Okoroafor, 2012). The growth and succession pattern of these organisms were reported to be dependent on factors such as water activity, pH, substrate, temperature and so on (Blandino *et al*., 2003), thus, the microbial as well as resulting physicochemical interactions eventually regulates the number and types of microorganisms that survives to the end of the fermentation process (Brauman *et al*., 1996; Padonou *et al*., 2009).
Gradual increase in LAB count with increasing fermentation time in *fufu* up till the end of fermentation as observed was similar to the reports of Wakil and Osamwonyi (2012), Tetchi *et al*. (2012) as well as Ekundayo and Okoroafor (2012). This could either be due to increase in acidity or low oxygen tension which favours growth of facultative anaerobes in submerged fermentation. It was, however, not surprising that subsequent drop in pH could lead to increase in LAB count since it has been reported that LAB predominate at low pH of fermenting medium (Soomro *et al*., 2002).

Increase in total bacteria count reported by Ekundayo and Okoroafor (2012) during *fufu* fermentation contrasted with the result obtained in this study where highest total count was at thebeginning of the fermentation while the least was observed at 72 hours. An increasing total counts as observed by Tetchi *et al*. (2012) also contradicted those observed in this study. Similar to Ekundayo and Okoroafor (2012), a decrease in coliform count was observed during fermentation. Furthermore, while studying the

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survival of enteropathogens during sausage and cassava fermentations by Maftar *et al.* (1993) and Ogunbanwo *et al.* (2004) respectively, decrease in non-lactic acid bacteria with time was also reported. Olsen *et al.* (1995) however, linked this to the interaction among the microbial flora of the indigenous fermented foods that usually caused an apparent reduction in the total count of non lactics when reduction after 24 hours was observed in steeped maize.

It could therefore be said that increase in the number of more acid-tolerant organisms which in turn creates an unfavourable environment for non acid-tolerant ones might be the reason for the decrease in number of non-lactics, whereas, their high counts at the onset of fermentation could be linked to the fermentation water, materials and handling.

Yeast counts decreased throughout the fermentation process. This was contrary to Brauman *et al.* (1996), who reported that yeasts appeared at the end of retting during cassava lactic acid fermentation as well as Kobawila *et al.* (2005) and Tetchi *et al.* (2012), who observed yeasts to have increased and becomes important at the end of retting. The observed decrease after 48 hours of fermentation could be due to a more acidic environment, brought about by increasing number of Lactic Acid Bacteria.

Lactic Acid Bacteria are described as an important group of organisms during fermentation and numerous species have consistently been isolated (Okafor, 1978; Abe and Lindsay, 1978; Ngaba and Lee, 1979; Oyewole and Odunfa, 1988; Oyewole, 1995; Kobawila *et al.*, 2005). Oyewole and Odunfa, (1990) studied their spectrum since it was established that more than one specie was involved. Apart from other bacterial genera associated with cassava fermentation, the different lactic acid bacteria species isolated in this study (*Lactobacillus plantarum, L. fermentum, L. brevis, L. delbrueckii, L. acidilactici, L. lactis* and *Leuconostoc mesenteroides*) had earlier been reported to be involved in fermentation of cassava into numerous products.

It has also been established that it is important to isolate predominant strains from fermentation batches for starter development. The observation of 50% each of randomly selected organisms from each fermentation being identified as *Lactobacillus plantarum* which was also the most prevalent was in line with reports of numerous authors that confirmed the predominance of lactic acid bacteria especially *Lactobacillus plantarum* during cassava fermentation (Ngaba and Lee, 1979; Oyewole

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and Odunfa, 1988, 1990; Amoa-Awua *et al*., 1996; Ben Omar *et al*., 2000; Lacerda *et al*., 2005; Kostinek *et al*., 2007; Adetunde *et al*., 2011; Oyedeji *et al*., 2013). Kobawila *et al*. (2005) reported the predominance of *Lactobacillus sp*. to be 73.3% during the fermentation of cassava roots and leaves to produce bikedi and ntoba mbodi, cassava products from Congo. 99% of the total microflora during traditional lactic acid fermentation for *fufu* was observed to be LAB, as reported by Brauman *et al*. (1996). Also, Olukoya, (1995) detected the prevalence of *L. plantarum* (28.6%) while isolating from nine (9) different African fermented foods with similar report by Ishola and Adebayo-Tayo (2012) during the isolation of LAB from different foods. Apart from fermented foods, Ogunjobi *et al*. (2010) observed 72.5% predominance of *L. plantarum* from the fermenting core of silage. All this could be attributed to the fact that lactic acid bacteria can withstand the acidic environment created due to the gradual decrease in pH through the production of various organic acids during the fermentation process. Furthermore, *L. plantarum* has been shown to have less complex nutritional requirements when compared to other *Lactobacillus* spp. (Hammes *et al*., 1992)

Screening of organisms for starter development during cassava fermentation involves the ability of the microorganisms to produce microbial enzymes (amylase, linamarase, pectinase) which are essential for starch hydrolysis, cyanide detoxification and tissue disintegration, the ability to rapidly acidify the fermentation process as well as production of antimicrobial compounds which antagonize unwanted pathogens (Kostinek *et al*., 2007; Edward *et al*., 2012). Screening of strains in this study for starter development showed all isolates to be negative to starch hydrolysis. This result was similar to earlier reports of Ketiku and Oyenuga, (1972) which confirmed most LAB as non-amylolytic even though, 84% of the cassava carbohydrate is in the form of starch. Amylase activity during fermentation could then be said to induced or constitutive as well as from other micro flora. Sanni *et al*. (2002) also reported the isolation of only a few amylolytic LAB. However, contrary to earlier reports and that of this study, Oyewole, (1995) reported over 80% of the total screened lactobacilli from fermented cassava as being amylolytic and Giraud *et al*. (1993) showed that *L. plantarum* strain A6 isolated from cassava produced an extracellular amylase.

All the screened strains produced linamarase, an enzyme which is responsible for detoxification. Hydrolysis of cyanogenic glucosides was earlier reported (Okafor *et*

al., 1986; Vasconcelos *et al*., 1990; Louembe *et al*., 1997; Kobawila *et al*., 2003) during lactic fermentation of cassava. Several microorganisms including *Bacillus* sp. (Amoa- Awua and Jakobsen, 1995), lactic acid bacteria (Cohen, 1994), Lactobacilli, *Leuconostoc*, Streptococci and yeasts (Obilie *et al*., 2004), *Aspergillus, Fusarium*, *Penicillium* and *Trichoderma* strains (Yeoh *et al*., 1995) are known for their detoxification activities during cassava fermentation. It could however be ascertained that the selected screened isolates can hydrolyse cassava cyanogens through their linamarase production even though, an endogenous linamarase could be inherent in the cassava.

Good pH reduction (a fast lowering of the pH) is important to accelerate fermentation process as well as reduce the levels of contaminating microorganisms which can compete with the starters for nutrients (Holzapfel, 2002) and also a critical factor in developing flavour and aroma of foods (Montet *et al*., 2006; Panda *et al*., 2007). The decrease in pH values during the fermentation of cassava roots resulted from the production of organic acids by lactic acid bacteria, which constitute the dominant micro flora (Malonga *et al*., 1993; Malonga *et al*., 1996). Similar pH reduction as observed in the screened isolates was reported (Kobawila *et al*., 2005; Coulin *et al.,* 2006) with a decrease in values with increasing fermentation time. This trend could result from the fact that LAB dominated till the end of the process, thus the production of acid metabolites most especially lactic acid. A faster acidification rate, than the previous spontaneous fermentation was observed in which the final pH value after 72 hours fermentation was between 4.43 and 4.97, whereas, the starter fermented experiment had between 3.53 and 3.80 even though both initial pH was within the neutral level. Same trend was also observed in the un-inoculated batches.

It has been established that the preservative action of starter culture in food is being attributed to a wide range of metabolites produced during fermentation (Caplice and Fitzgerald, 1999). The potential of LAB to produce antimicrobial compounds has attracted much attention as such compounds can be used to prevent food spoilage and inhibit the growth of food pathogens (Foulquie *et al*., 2006). LAB has been reported to release toxic substances such as hydrogen-peroxide, diacetyl, organic acids and bacteriocins into the fermenting medium during food fermentation (Mataragas *et al*., 2002). However, varied antimicrobial compound (lactic acid, diacetyl and hydrogen peroxide) concentrations as observed in this study might be as a result of being

UNIVERSITY OF IBADAN

produced by different strains since Tannock, (2004) linked production level and proportion to be dependent on strains, medium compounds and physical parameters.

The inhibition zone of the selected potential starters in this study against pathogens ranged between 2 mm and 12 mm. Similar antagonistic effect has been reported from LAB isolated from different sources; dairy products (Saranya and Hemashenpagam, 2011), fermented maize products (Omemu and Faniran, 2011), fermented fish (Liasi *et al*., 2009), poultry meat (Adesokan *et al.,* 2008) and cow milk (Olanrewaju, 2007). As observed in this work, similar antagonistic effect was reported by Adesokan *et al*. (2008) where LAB species were tested against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Escherichia coli* and *Proteus vulgaris.* Obadina *et al.* (2006) also showed the antagonistic effects of *Lactobacillus plantarum* on *Salmonella thyphii*, *S. aureus*, *E.coli* and *Bacillus substilis.* The ability of LAB to inhibit the pathogenic organisms however, could to be due to the fact that they produce certain antagonistic substance which interferes with the metabolic activities of such pathogens, thus, inhibiting their growth.

The molecular identification of the five (5) identified potential starters further confirmed their identification to strain level. The genus *Lactobacillus* consists of a genetically and physiologically diverse group of Gram positive, rod shaped, catalase negative, non-spore forming bacteria (MacFaddin, 2000). Nucleotide base-sequences of 16S ribosomal DNA (rDNA) of *Lactobacillus* spp. has been reported to provide accurate basis for phylogenetic identification and analysis (Tabatabace *et al*., 2005).

The use of starter cultures has been described as an appropriate control and optimization of fermentation problems of variations in organoleptic quality and microbiological stability observed in African indigenous fermented food (Sanni, 1993; Kimaryo *et al*., 2000). Inoculation of starters (singly and in-combination) to initiate both fermentations, had similar trend of decreasing pH values with increase in fermentation period. The least pH values (3.68 and 3.53) for *fufu* and *usi* respectively, after 72 hours fermentation, was in accordance with the report of Henshaw and Ikpoh (2010) who observed decrease in pH values from 6.20 to 3.67 after 72 hours fermentation of cassava with *L plantarum* starter. Kristek *et al*. (2004) however reported a higher range during the inoculation of *L. mesenteroides* and *L. lactis* ssp. *lactis* starters on sauerkraut fermentation. They obtained a final pH within the range 3.86-3.90. This is still based on the fact that fermentation pH reduces (due to increasing hydrogen ion content) as acidity increases, with increase in fermentation

UNIVERSITY OF IBADAN

time. Meanwhile, variation in the pH of *fufu* and *usi* could be as a result of the cassava cut size since Oyewole (1990) associated the rate of acidification to be affected by the size which the tubers were cut. It was however noted that the un-inoculated fermentations had higher pH values than the starter-fermented experiments. This confirms one of the properties of starter cultures which involves rapid rate of acidification.

Furthermore, starter cultures have been shown to have significant effect on fermented foods (Adegbehingbe, 2014). At the end of 72 hours *fufu* fermentation, some of the starters used increased the protein content significantly while most of the starters did same during *usi* fermentation as well. This, according to Erukainure *et al*. (2010), could be attributed to the ability of the organisms to secrete extracellular protease during the fermentation process. Similar increase was reported by Oboh and Elusiyan (2007) and Adeyemi *et al.* (2012) while fermenting cassava tubers with single starters of *Sacharomyces cerevisiae* and *Rhizopus oligosporus* as well as during the fermentation of *Chrysophyllum albidum* seeds with *Aspergillus niger*. Other authors that reported an increased protein content during fermentation using different substrate includes Egounlety (1994), Michodjehoun *et al.* (2005) and Ogunlakin *et al.* (2012). Tortora *et al.* (2002) linked it to be due to the structural proteins that are an integral part of the microbial cell, but Igbabul *et al*. (2014) attributed it to the increase in microbial mass during fermentation, causing extensive hydrolysis of protein molecules to amino acid and other simple peptides. However, *usi* had higher increased values than *fufu* even though, same cassava roots were used. This makes such effect to be attributed to the starters.

Most of the starter fermented mashes had reduced moisture contents which could be an indicator for stable shelf life. Similar reduction, as observed in this study, had earlier been linked to activities of fermenting microorganisms, since water is essential for growth and cell metabolism as well as loss of moisture along with the leaching of nutrients (Tiruha *et al*., 2014). Igbabul *et al.* (2014) reported that the decrease in moisture after fermentation may probably be due to the soft and porous texture after fermentation resulting in maximum moisture loss. However, Wang *et al*. (1999) reported moisture reduction as a function of many factors such as temperature, time, humidity, etc. Contrary results were reported (Omafuvbe *et al*., 2004; Ogueke *et al*., 2010; Adegbehingbe, 2014) during the fermentation of African locust bean and melon seed where moisture content was confirmed to have increased and it was related

UNIVERSITY OF IBADAN

to hydrolytic activity of the fermenting organisms releasing moisture as part of their metabolic product. Oladunmoye (2007) and Fadahunsi *et al*. (2011) also corroborated that the increase could be as a result of decompositon of the substrates by the fermenting organisms which released moisture as one of their end products.

In both fermentations, ash content was reduced when fermented with majority of the starters, but with higher values in *fufu*. Ash content is a factor of mineral availability in the fermented food, so such decrease could be ascribed to leaching of soluble mineral elements into the fermenting medium or as a result of enzymatic hydrolysis of food components into their absorbable forms. Similar decrease in ash content was reported by Atti (2000) during fermentation of millet. It was also consistent with the report of Michodjehoun *et al*. (2005) during fermentation of "Gowe" a traditional food made from sorghum, millet or maize, but contrary report by Sefa-Dedeh and Kluvitse (1995), observed an increase in ash content of fermented maize cowpea blends. Sanusi *et al.* (2013) and Adeyemi *et al*. (2012) also observed an increase in ash content when fermenting *Jatropha curcas* and *Candida albidum* seeds with fungi.

A general increase in crude fibre was observed after both fermentations with very few decreases in *fufu* fermentation. This was similar to the findings of Oyewole and Ogundele (2001) who also reported an increase in crude fibre of *fufu* as fermentation progressed. However, Adeyemi *et al.* (2012) suggested that increasing crude fibre interferes with other nutrients thereby, making such nutrients unavailable for use. On the contrary, Igbabul *et al.* (2014), during the fermentation of cocoyam, observed a decrease in crude fibre which was reported as an indication of softening of the fibrous tissues during fermentation and the decrease was attributed to microbial bio-conversion of carbohydrates and lignocelluloses into protein. Similar findings were reported by Hwei-Ming *et al*. (1994) and Balagopalan (1996).

General increase in total carbohydrate was observed in both fermentations even though, some samples had reduced values. The decrease observed in this study might be as a result of breakdown of carbohydrates into simple sugars and organic acids. Oboh (2006) reported a decrease in carbohydrate content during the fermentation of cassava peels for nutrient enrichment. Meanwhile, Igbabul *et al.* (2014) attributed increase in carbohydrate to the decrease in moisture content as fermentation time increased during the fermentation of cocoyam flour.

UNIVERSITY OF IBADAN

Cassava contains anti-nutritional compounds that affect the digestibility and absorption of nutrients. Since fermentation has been known to denature anti-nutritional factors and increase nutritional values (Montagnac *et al.*, 2009b), decrease in contents would be expected and such was attributed to leaching and microbial activities (Nwosu, 2010). Tannin, phytic acid and cyanide were considered in this study. Tannin contents in *fufu* increased after fermentation with all the starters except one whereas, some decrease in values were observed for *usi.* A general reduction in cyanide content and phytic acid were observed in both fermentations. Since the quantity of anti-nutritional compounds has been linked to the varieties and root maturity (Nambisan and Sundareson, 1994; Montagnac *et al.*, 2009a; Bandna, 2012/2013), inconsistent values obtained when compared with numerous works was justified. Meanwhile, variations among starters indicated varied enzyme activities. Significant reduction observed had been reported by many authors on other substrates as well (Fardiaz and Markakis , 1981; Sutardi and Buckle, 1985; Marfo *et al.*, 1990; Chelule *et al.,* 2010; Azeke *et al*., 2011; Efiong and Umoren, 2011; Adegbehingbe *et al.*, 2014; Igbabul *et al*., 2014).

Reduction in phytic acid below the initial 0.3 mg/g as observed in this study was lower than the value (7.05 mg/g and 97.07 mg/g) obtained by Oboh, (2006) and Igbabul *et al*. (2014) during the fermentation of cassava peels and cocoyam flour respectively. Phytate reduction has been attributed to the activity of the endogenous phytase enzyme from the raw ingredient and inherent microorganisms which are capable of hydrolyzing the phytic acid in the fermented food preparations into inositol and orthophosphate (Sandberg and Andlid, 2002).

The few increase in cyanide content observed in this study was inherent in the fermentation with single starters, however, reduction could be said to be due to combined effect of the combined starters. Majority of the fermented mashes had reduced or no detectable cyanide at all after 72-hour fermentation. This trend had earlier been established, that cassava fermentation using water, is the simplest method to reduce cyanide content (Cumbana *et al.,* 2007; Bradbury and Denton, 2010) as the water will swell the cells and allow linamarase to come into contact with linamarin, thus, initiating hydrolysis (Bradbury, 2006). Furthermore, Guyot *et al.* (1998) reported such effect to have emanated from the use of linamarase producing organisms during fermentation. Meryandini *et al.* (2011) reported a 50% decrease in hydrogen cyanide during the addition of cellulolytic bacteria to improve the quality of cassava flour

UNIVERSITY OF IBADAN

while (70-75%) and 85% decrease was observed by Kobawila *et al*. (2005) and Achinewhu *et al*. (1998) after 72 hours fermentation, respectively. This observation was also in line with that of Agbor-Egbe *et al*. (1995) confirming fermentation to be a very effective process for eradication of endogenous cyanic compounds in cassava roots. Lower values of cyanide 0.01 mg/g was observed by Igbabul *et al*. (2014) when compared to those obtained in this study (0.00, 0.05 and 0.08 mg/g). The reduction from an initial 0.1mg/g to (0-0.05 mg/g) by some starters satisfied the minimum tolerant level 0.05 mg/g (SON, 1985), even though, Tweyongyere and Katongole, (2002) puts the deleterious level at 0.03mg/g and FAO/WHO (1991) recommended 0.01 mg/g dry matter to avoid acute toxicity in humans. However, observed values deviated from the findings of Oboh *et al*. (2002) as well as Oboh and Akindahunsi, (2003) who put the usual cyanide content of cassava products in Nigeria as 0.019 mg/g for *gari,* 0.025 mg/g for *fufu*, and that of the cyanide content of some micro-fungi fermented cassava products was between 9.1 - 17.2 mg/kg. It was noted that cyanide was not detected in most of the *usi* mashes after fermentations unlike for *fufu* and this could be as a result of the effect of grating during *usi* production since Dixon *et al.* (1994) considered grating of cassava roots to be an important factor in linking the hydrolytic enzyme linamarase to intimate contact with linamarin.

Since controlled fermentation enriches nutritive value of foods with respect to shelf-life, hygienic quality, nutrient improvement and anti-nutrient reduction (Adeyemi *et al*., 2012), the results of the anti-nutritional/nutritional composition as well as acidification of the fermentation processes were pooled together, analyzed statistically and a starter, common to both fermentations was selected. The selected starter (CGI) was made up of the combination of *Lactobacillus plantarum* F2C, *Lactobacillus plantarum* U2A and *Lactobacillus paraplantarum* U2C.

The starter CGI showed a considerable reduction in pH values (3.70 and 3.71) for *usi* and *fufu* respectively. This could be termed safe, since Holzapfel (1997) reported that lactic acid fermentation resulting in pH levels lower than 4.2 constitutes a major food safety factor. This pH value after 72-hour fermentation was in agreement with Oyedeji *et al*. (2013) who obtained a pH value of 3.70 during cassava retting, thereby concluding that the low pH would make such food safe for consumption. It was also expressed that the acidic fermentation would be responsible for inactivation of toxin-producing and foodborne infectious pathogens (Lorri, 1993; Byaruhanga *et al*., 1999; Kunene *et al*., 1999; Ana *et al*., 2006).

UNIVERSITY OF IBADAN

The starter combination expressed a significant reduction in the anti-nutritional compounds inherent in the roots after fermentation. Phytic acid has been known to form insoluble salts with metals thus, making them unavailable for absorption in the body (Igbabul *et al*., 2014), so reduced level of phytic acid in foods improves the availability and absorption of required metal ions in the body. The starter reduced initial phytate of 0.30 mg/g to 0.1 mg/g during *fufu* fermentation and to 0.27 mg/g in *usi*. Adegbehingbe (2014) reported higher values (3.17, 3.18 and 3.23 mg/g) than those observed in this study when fermenting Lima beans with different starter cultures. The minimum values (32.3 mg/g and 34 mg/g) in tannin content for both fermentations were observed in the samples fermented with the selected potential starter. Reduced cyanide content of 0.05 mg/g was observed in *fufu* while no cyanide in *usi* characterized the selected starter-fermentation as well.

Despite being a cheap source of food calories, cassava is nutritionally deficient in protein and its high consumption without adequate supplements exposes the vulnerable group to incidence of protein-energy malnutrition (FAO, 1984). Cassava contains about 1-2% protein which makes it a predominantly starchy food (Charles *et a*l., 2005). Certain processing techniques have been employed to improve the protein content of cassava, among which was the utilization of starter for fermentation as it was expected that the organisms will secrete protease enzymes useful in the hydrolysis of protein molecules into simple absorbable amino acids (Erukainure *et al*., 2010; Igbabul *et al*., 2014). The selected starter combination had the most improved protein content after both fermentations from an initial 1.02% to 1.82% and 1.34% for *usi* and *fufu*, respectively. Furthermore, it also showed the least moisture content in both products and reduced moisture in foods was linked to a more stable and prolonged shelf life as this hinders the proliferation of spoilage microorganisms (Padonou *et al*., 2010; Harris and Koomson, 2011). Least moisture levels of 5.10% and 5.36% were observed. This was lower than the recommended standard 13% for edible cassava flour (Sanni *et al*., 2005) and it could be due to the fact that the samples in this study are in the wet form. Reduced carbohydrate content was observed when the starter combination was used. Even though cassava is a high caloric food crop, efforts has been made to improve it nutritionally, so carbohydrate reduction observed could be as a result of the starters hydrolyzing available carbohydrate in the form of starch into simpler absorbable nutrients. The mashes had reduced fibre content and this could be

UNIVERSITY OF IBADAN

due to the fact that the organisms utilized soluble fibre. Similar observation was reported by Srinorakutara *et al.* (2006).

In view of this, the selected potential starter could be said to have satisfied the aspect of reduction of anti-nutritional compounds in both fermentations more than any other observed combination, thus, its selection.

Monitoring the enzyme activities during fermentation with the selected starter gives a more insight into the process. The optimal activity with respect to fermentation time of amylase, linamarase and pectinase enzymes was analyzed. Interestingly, starch hydrolysis was negative during the screening for starters in growth medium, but amylase activity was observed during fermentation with starters. This could be ascribed to the fact that the amylase enzyme was not produced by the organisms as most LAB have been proven to be non amylolytic even as 84% of cassava carbohydrate was stored as starch (Ketiku and Oyenuga, 1972). Numerous authors listed *Bacillus* spp. (Olafimihan and Akinyanju, 1999; Pandey *et al*., 2000; Gupta *et al*., 2003), *Escherichia* spp, *Pseudomonas, Proteus, Serratia* and *Rhizobium* (Oliviera *et al*., 2007), *Aspergillus, Rhizopus, Mucor, Neurospora, Penicillium* and *Candida* (Pandey *et al*., 2000; Gupta *et al*., 2003) as prominent organisms used in the commercial production of amylase, and as expected, LAB was not that prominent. The amylase activity might be as a result of the enzyme production being induced in the presence of starch.

During *fufu* fermentation, the highest amylase activity 10.1 U/mL observed was at 24 hours after which there was subsequent reduction till the end of fermentation. More carbohydrate in the form of starch would still be available at the beginning of fermentation which provides a more substrate for induction since the starters are non amylolytic, thus leading to maximal activity. As fermentation progresses, available starch reduces and this could account for the reduced activity at 48 and 72 hours respectively. Activity in the un-inoculated control increases with increasing fermentation time with more reduced values, except at 72 hours. Reduced activity at the beginning of fermentation could be linked to the fact that development and colonization of a spontaneous fermentation process will be slower than an inoculated experiment, thus, a slower metabolic activity. Meanwhile, a higher activity in the control at 72 hours might be as a result of the development of more amylolytic microflora.

UNIVERSITY OF IBADAN

Pectinases, during cassava fermentation are involved with root softening and it has been reported that cassava softening seems to be mediated by bacteria (Okafor *et al.,* 1984; Oyewole, 1990; Brauman *et al.,* 1995) since sterile roots soaked into sterile water did not soften. Pectinase activity reduced with increase in fermentation time in both starter fermented and un-inoculated *fufu* fermentation. Earlier reports of Ampe and Brauman (1995) showed similar decrease in pectinase activity with increase in fermentation time. High activity in both fermentations at 24 hours indicated that tissue disintegration was maximal at such time since pectinase enzyme was attributed to tissue disintegration, that is, breakdown of cassava texture (Amoa- Awua and Jakobsen, 1995). During *usi* fermentation, the detection of pectinase activity at 24 hours, unlike in the control experiment could be as a result of faster metabolic activities in the starter inoculated batch.

Linamarase or β-D-glucosidase converts the cyanide containing compounds in cassava into acetone cyanohydrins, which spontaneously decomposes to hydrogen cyanide HCN (Rolle, 1998), then further dissolves readily in water or is released into the air. This detoxification process is by the endogenous linamarase released during fermentation (Ikediobi & Onyike, 1982) or the bacteria involved in retting (Okafor & Ejiofor, 1985; Giraud & Raimbault, 1992). A higher linamarase activity observed in both fermentations when compared with the uninoculated controls indicated that starter cultures produced more linamarase than the spontaneous microflora. Decrease in activity after 48 hours during starter inoculated *fufu* fermentation could be as a result of rapid detoxification of cyanogenic glucosides within the early hours of fermentation. Linamarase activity in *usi* was higher than in *fufu* fermentation at 24 hours and this might be linked to the fact that cassava for *usi* was grated, thus, enabling rapid contact of hydrolytic linamarase with linamarin since grating has been described as an important factor during cassava fermentation (Mkpong *et al*., 1990; Dixon *et al.,* 1994). Brauman *et al.* (1996) also ascertain the slower rate of linamarin removal in *fufu* when compared to *gari* as a result of grating before processing. The observed increased activity in *fufu* at 48 hours was in agreement with the findings of Cooke *et al*. (1978) who reported elimination of linamarin after 48 hours and it was suggested that linamarase reached optimal pH at this stage. It might also be as a result of increased tissue disintegration in *fufu*. It was however observed that enzyme activities in the starter fermented batches were higher than in the un-inoculated cassava mashes.

UNIVERSITY OF IBADAN

Fermentation metabolites have a role to play in flavour enhancement, increased nutritive and organoleptic quality, suppression of undesirable organism which in turns leads to a stable shelf life and the general acceptability of fermented foods. Organic acid, which is among the numerous fermentation metabolites are naturally present in foods or they are synthesized during biochemical or bacteria metabolism (Akalin *et al*., 2002; Soyer *et al*., 2003; Karadeniz, 2004). They have important roles because they affect the organoleptic properties, stability, nutrition, acceptability and in maintaining quality (Santalad *et al*., 2007). They also improve the safety and preservation of foods (Soomro *et al*., 2002). Quantitative determination of lactic acid is important in fermented products for technical, nutritional, sensorial, and microbial reasons (Vodnar *et al*., 2010)

During fermentation with the selected starter, lactic acid was the main organic acid produced throughout the fermentation, even though, mild traces of acetic and butyric acids were discovered with few other starters. This was in contrast to Aka *et al.* (2008) who observed numerous organic acids (lactate, fumarate, oxalate, propionate) during the production of a traditional sorghum beer. Miguel *et al*. (2014) reported the production of citric, succinic, acetic, lactic, malic, tartaric, propionic and oxalic acids, with lactic and acetic acid dominating, thus, linked to the presence of LAB throughout the fermentation. The range (0.04 mg/mL to 1.23 mg/mL) of the lactic acid produced in the starter fermented fufu was higher than the un-inoculated batch and this confirms the fact that a starter must have a rapid acidification rate through the production of acids. Bustos *et al.* (2004) predicted a maximum lactic acid concentration (58.9 g/L) after 96 hour fermentation while Narita *et al.* (2004) obtained 14.7 g/L lactic acid (73.5% recovery) from raw starch. Javanainen and Linko (1995) utilized mixed culture of *L. amylovarus* and *L. casei* to produce lactic acid (36 g/L) using barley flour as substrate. Decrease in quantity of lactic acid with increase in fermentation time in the un-inoculated control might be as a result of the proliferation of non-lactic microflora since spontaneous fermentation is invaded by numerous unwanted organisms. The non detection of the acid at zero hour in both starter fermented and control experiment indicated that metabolic activity at such stage is minimal.

Increase in quantity produced with increasing fermentation time both in the starter fermented and un-inoculated experiments in usi could be due to the fact that LAB predominates and increases till fermentation lapse. This agrees with Aka *et al.* (2008) who reported an increase in lactate concentration (0.17 - 7.75 g/L) till the end

UNIVERSITY OF IBADAN

of beer fermentation as well as Farook *et al*. (2012) during the production of lactic acid using sugarcane molasses.

Lactic acid fermentation has been studied since 1935 using different types of microorganism and fermentation operation conditions such as pH, carbon source, temperature, inoculum size, initial substrate conditions and nitrogen source (Hofvendal and Hagerdal, 1997) as these parameters and operating condition affect the optimal process. Research has therefore concerned the influence of carbon and nitrogen sources, other growth factors (amino acids and vitamins), and culture conditions such as temperature, pH, level of aeration etc., on microbial growth (Polak-Berecka *et al*., 2010).

Even though the starter grew at different pH range (3.5-7.5), the observed optimal being 7.5 in this study was slightly higher than an optimal pH of 7.0, reported by Barbu (2008) for the growth of LAB. Hofvendhal *et al*. (1999) obtained a maximum growth rate for *Lactococcus lactis* at pH 5.75 while Kandler and Weiss (1986) however emphasized that except for some species of *Lactobacillus* and *Leuconostoc*, lactic acid bacteria are neutrophiles i.e., their optimal pH for growth lies between 5 and 9, attributing a much lower pH to be harmful to the group of bacteria as this causes acidification of cytoplasmic pH below a threshold value and subsequent inhibition of cellular functions (Kashket, 1987). Yusuf and Abdul-Hamid (2012) also reported an optimal pH of between 9 and 10 for the growth of *Enterococcus faecium* B3L3. Similar report was by Ishikawa *et al*. (2005) who observed an optimal pH of 9.5 for halophilic and alkaliphilic marine LAB, but a contrary report (Guyot *et al*., 2000) indicated that *L. plantarum* WSO and *L. plantarum* A6 grew at pH values as low as 4.5. This feature allows *L. plantarum* strains to participate in the last stage of natural lactic acid fermentations of plant material (Daeschel and Nes, 1995) because, even though highest growth was recorded at pH 7.5, the starter expresses a gradual increase in growth from pH value 4.5.

Optimum temperature is the basic characteristic of all Lactic Acid Bacteria which helps to differentiate them from each other because temperature controls the growth of bacteria. It affects bacterial generation time according to the phase of growth as each species has a unique optimum growth temperature (Ahmed *et al.,* 2006). The optimum growth temperature for *Lactobacilli* was reported to be between 30°C and 40°C, but they can grow at temperatures ranging from as low as 5°C to an upper limit of 53°C depending on species (http//www.positiveaction.co.uk). Unlike the optimum

growth temperature (30°C) for *L. plantarum* strains observed in this study, Ahmed *et al*. (2006) discovered that different strains of *L. lactis, S. cremoris* and *L. acidophilus* isolated from camel milk showed maximum growth at 37°C and 40°C. The variations in optimum temperature even among strains have been reported to be due to their genetic makeup, as it was observed by Yu *et al.* (1983) that if the plasmid profile of two organisms is similar or even identical, there may be differences in their nucleotide composition, sequence or even both. Barbu (2008) also reported that even though *Lactobacillus* spp isolated from wheat epiphyte microbiota grew within temperature range of 28- 45°C, optimal growth was observed at 37°C. However, these optimum temperatures are still within the 30-40°C range and this could be attributed to their mesophilic nature. Meanwhile, Leroi *et al*. (2012) reported that *Lactococcus piscium* CNCM I-4031, a new species described by Williams *et al.* (1990), grew well at low temperature (0°C) and reached maximum growth below 25°C, which is very uncommon among lactic acid bacteria. Garnier *et al*. (2010) had earlier explained such psychrotrophic behaviour to be as a result of over-expression of cold-adaptation proteins during growth at low temperature.

Salt concentration is another important growth factor for Lactic Acid Bacteria. NaCl have been confirmed to affect the metabolism and growth of LAB which are involved in fermentation processes (Chikthimma *et al*., 2001; Johanningsmeier *et al.*, 2012). The optimal salt concentration of the selected starter was 2%. This was in agreement with the report of Barbu (2008) who observed an optimal salt concentration for *Lactobacillus* strains to be between 2-4% after which a decline in growth occurred even though they grew up till 10% concentration. Lee (2010) however, discovered that LAB in doenjang, a fermented soybean paste was survivable between 0- 40% with optimal value at 30%. This value was way above those obtained in this study even though a range of 2- 10% was used. It has been reported that bacterial cells cultivated in a high salt concentration would experience a loss of turgor pressure, which would then affect the physiology, enzyme activity, water activity and metabolism of the cells (Liu *et al*., 1998) even though, some cells overcome this effect by regulating the osmotic pressure between the inside and outside of the cell (Kashket, 1987).

Rao *et al.*(2004) concluded that a 4% salt concentration was optimal for *Lactobacillus plantarum* strains 541 and A6 to be used as starters. Similar to this, was the observation made by Abdulla *et al*. (2013) in which optimal salt concentration of 4% was discovered for *Lactobacilli* strains. Ishikawa *et al*. (2005) who worked with

halophilic and alkaliphilic marine LAB, reported an optimal concentration of between 2.5-3.0%. However, it should be noted that the selected starter was tolerable to concentration 2-10% and this was in accordance with the findings of Elizete and Carlos (2005) that *Lactobacilli* isolated from gastrointestinal tract of swine were tolerable to 4-8 % Nacl even though they are from different sources. It thus gave an indication of the osmo-tolerance level of the LAB strains.

The effect of oxygen on cell growth is important because during the cell metabolism, oxygen has a significant effect on the physiological characteristics of the cells (Pearce *et al*., 2003) and agitation tends to transfer such oxygen within the growth medium rather than it being limited to the medium surface, it also helps in avoiding cell culture to sediment at the bottom of flasks and become rapidly oxygen depleted, thus, maintaining homogeneity and fluid-to-particle mass transfer (Khadijah *et al*., 2009; Gupta *et al*., 2010). However, stationary conditions for growth and agitation mode for fermentation have also been reported in some earlier studies (Roy *et al*., 1986) and differences in growth rates has been attributed to differences in metabolic pathways under aerobic and anaerobic conditions (Murphy and Condon, 1984). Similar to the results obtained in this study, was the findings of Yu *et al.* (2013) during the development of Fed-batch process for high cell density cultivation of *Lactobacillus fermentum* L1. They observed that cells grew stronger with agitation speeds of 50 and 100rpm, the optimal being 100rpm, but when agitation speed was increased to150 or 200 rpm, cell density declined and this was attributed to the fact that suitable agitation helped the mixing of cells and nutrients, meanwhile, strong agitation was proven to cause high DO (dissolved oxygen) or shear force which inhibited cell growth (Yu *et al*., 2013). Also in accordance were the reports of Ibrahim *et al*. (2010), who obtained same speed of 100rpm during their study on *Lactococcus lactis* and that of Gupta *et al.* 2010, who observed maximum growth of *L plantarum* at 100rpm during the fermentation of edible Irish brown seaweeds. However, contrary to these was the report of Chauhan *et al*. (2013) who varied agitation speed for growth of *Acetobacter tropicalis* between 150-600 rpm and discovered the optimal growth at 450rpm, during dextransucrase production.

The evaluation of cell viability and dominance during fermentation is another important characteristic of a starter culture and dominance is usually exerted by fast and dominant growth that is, generation time (Soro-Yao *et al*., 2014). In contrast to the result of the effect of incubation time on growth of the starter in which increasing

incubation period led to increase in LAB growth, Ketut *et al*. (2012) observed that increasing incubation periods from 24 to 48 and 72 hours, respectively reduced the number of lactic acid bacteria. They ascribed the reductions to have appeared due to the drop in pH of kefir samples and longer incubation period affects the intracellular pH of the lactic acid bacteria, which inhibit the enzyme activity, ion transport and nutrient uptake, thus, retarding the growth and counts of the lactic acid bacteria. However, a similar report was that of Enitan *et al*. (2011) where LAB isolates showed an increase in growth as incubation period increases even though, a decline was observed after 72 hours.

Another critical step in the development of economical fermentation process is selection and optimization of carbon and nitrogen sources (Naveena *et al*., 2005) since high growth activity of *Lactobacilli* has been reported to be affected by medium formulation (Polak-Berecka *et al*., 2010). LAB, according to Amrane, (2000) has complex nutritional requirements, due to their limited ability to synthesize their own growth factors such as B vitamins and amino acids. Therefore, they require some elements for growth, such as carbon and nitrogen sources, in the form of carbohydrates, amino acids, vitamins, and minerals. The cost, availability and stability of fermentation substrates are of interest, as they can represent up to 68% of the production costs of fermentation products (Kwon *et al.,* 2000; Serna-Cock and Rodríguez-De Stouvenel, 2007). Yeast extract was found to be the most suitable nitrogen source in this study and this was corroborated by Tejayadi and Cheryan (1995), in an economic study for *Lactobacilli* growth, who discovered the largest contributor to be yeast extract accounting for about 38% of total production medium cost whereas, Monteagudo *et al*. (1993) had earlier related such to be as a result of having profound influence on the cell concentration. Furthermore, it was reported that buffering capacity of yeast extract could prevent excessive drops in pH, which would inhibit cell growth (Gaudreau *et al*., 1997). Enitan *et al.* (2011) also discovered highest *L. casei* growth when the medium was supplemented with yeast extract. In contrast, Fung *et al*. (2008) showed that meat extract, vegetable extract and peptone significantly influenced the growth of *L. acidophilus.*

Maltose, closely followed by glucose was the best carbon source observed in this study. Other reports have shown the effect of glucose on cell numbers (Liew *et al*., 2005; Ibrahim *et al*., 2010). Bajpaj-Dikshit *et al*. (2003) and Polak-Berecka *et al.* (2010) however confirmed the combination of glucose and pyruvate to be more

suitable because it leads to higher biomass yield. Galactose was reported to be most suitable carbon source for *L. bulgaricus* growth during production of hydrogen peroxide (Enitan *et al*., 2011).

Utilizing the optimal conditions to cultivate the starter and further utilization in cassava fermentation for *fufu* and *usi*, recorded an improved proximate composition as well as anti-nutritional factors, however, some insignificant effects were noticed. This might be as a result of the optimization effect (improved metabolism) on the starter.

Evaluation of the starter-fermented *fufu* and *usi* in this study, alongside the spontaneously-fermented products by trained panellists, indicating overall acceptance for the starter-fermented products justified the effective utilization of *Lactobacillus plantarum* F2C, *Lactobacillus plantarum* U2A and *Lactobacillus paraplantarum* U2C for both food products. This resulted in drastic reduction in foul odour and improved colour. Although, texture and elastic quality of the products had been linked to high dry matter (Etudaye *et al*., 2012), they could still be dependent on preparation method. These observations thus, placed utilization of starter cultures in food fermentation above the traditional processes. Similar findings were reported (Ojokoh, 2009; Henshaw and Ikpoh, 2010; Adetunde *et al*., 2011; Singh *et al*., 2012; Opere *et al*., 2012; Ogunbanwo *et al*., 2013) in which utilization of starters have been linked to control of acid content of the fermenting medium, inhibit and discourage undesirable bacteria, control fermentation time, improve odour, flavour and nutritional value of fermented products.

Ready-to-eat foods are increasingly becoming the main source of inexpensive, convenient and nutritious foods with immediate consumption or consumption at a later time without further processing or preparation (Mensah *et al*., 2002). The highly perishable nature of ready-to-eat *fufu* has emerged as a recurrent and cross cutting issue over time. The negative effect of this includes spoilage of food products and contamination by pathogenic microorganisms (Adeyemi, 2012). By increasing their numbers, utilizing nutrients, causing enzymatic changes, and contributing off-flavours through breakdown of a product or synthesis of new compounds, they can spoil a food, thus, leading to reduction in demand as well as inability to expand operations size. The microbial load of starter-fermented and spontaneously fermented *fufu* and *usi*, studied over a storage period of seven (7) days at room temperature, which indicated increase in total bacteria counts with increasing storage period was similar to the report of Odom *et al*. (2012). This was attributed to have been as a result of storage temperature,

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pH and acidity which favour growth of microbes. Furthermore, in accordance with the works of Onilude *et al.* (2002, 2004) and Odom *et al.* (2012), LAB counts increased as storage period progressed. However, coliform count and other spoilage organism count were evident in the food products on the first and second storage days, after which they slightly decrease in number on the 3rd and 4th day. Beyond this, they grew profusely till the last day, leading to spoilage of the food samples. The acidity of stored foods was reported to increase with increasing number of days (Aderiye *et al.*, 2006), but Odom *et al*. (2012) reported increase in the pH of stored foods with increasing storage days. This had earlier been attributed to the production of ammonia (Sarkar *et al.*, 2006; Olawepo *et al.*, 2001) which favours the proliferation of more spoilage organisms as well as fungal/yeast growth. The initial decrease could be due to inhibitory action of antimicrobial compounds by LAB.

Spoilage symptoms were visible in starter-fermented *fufu* and *usi* towards the end of the 5th day, unlike in the spontaneously-fermented products that had observable spoilage properties on the 4th day of storage. Obadina *et al.* (2009) reported no visible changes on day 1 and 2, change in colour and odour on day 3 and more profuse spoilage till the 7th day during their study on the spoilage of *fufu*, *lafun* and *gari.* The fact that the starter produced products stayed more than the control product could be ascribed to the effectiveness of the starter acting as preservative agent during storage.

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Summary and Conclusion

This study investigated the possibility of developing a common starter culture for *fufu* and *usi* fermentation, monitoring product characteristics.

Ninety eight (98) Lactic Acid Bacteria were isolated from spontaneous cassava fermentation, with *Lactobacillus plantarum* having the highest frequency of occurrence. They were characterized and screened (starch hydrolysis, linamarase, pectinase and antimicrobial compounds production, rate of acidification and antagonistic effect on pathogens for possible use as starter cultures. The obtained screened isolates were genotypically identified as *Lactobacillus pentosus* F2A, *L. plantarum* F2B, *L. plantarum* F2C, *L. plantarum* U2A *and L. paraplantarum* U2C. They were further utilized as potential starters singly and in-combination to initiate the fermentation of *fufu* and *usi.*

The best starter combination, comprising of *L. plantarum* F2C, *L. plantarum* U2A *and L. paraplantarum* U2C (CGI) gave rapid acidification rate from 7.10 to 3.7, improved moisture and protein contents and reduced anti-nutritional contents in both *fufu* and *usi* fermentations. Lactic acid was the major organic acid produced while sugars such as xylose, arabinose, glucose, sucrose and fructose were detected at different fermentation stages, with sucrose having the highest quantity. Amylase activity decreased with inceasing time in both fermentations and pectinase activity were higher during *fufu* fermentation.

The optimal growth conditions for the selected starter was at pH 7.5, 30°C incubation temperature, 2% NaCl concentration, 100rpm agitation speed, maltose as well as yeast extract as carbon and nitrogen sources. Optimization of starter growth conditions had a more improved effect on the nutritional and also reduced the anti-nutritional contents of both mashes.

When compared with spontaneously-fermented products, the starter-fermented *fufu* and *usi* had increased overall acceptance and a more stable shelf-life.

In conclusion, a starter combination, comprising *L. plantarum* F2C, *L. plantarum* U2A *and L. paraplantarum* U2C could be used to initiate the fermentation of both *fufu* and *usi* to yield products with improved nutritional contents, shelf life and reduced anti-nutrients.

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Contribution of the study to knowledge

Utilisation of starter culture for fermented foods over the years has been focused on the development of a starter for a particular food product. This study established the use of a single starter for two related food products which tends to be more economical to producers.

Furthermore, this will be the first work to report the utilisation of starter culture for *usi* fermentation.

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APPENDIX

I MRS Agar

	g/L
Peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Glucose	20
Di-pottasium phosphate	2.0
Sodium acetate	5.0
Ammonium citrate	2.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Tween 80	1.08
Agar	15

II Violet Red Bile Glucose Agar

	g/L
Yeast extract	3.0
Peptone	7.0
NaCl	5.0
Bile salt	1.5
Glucose	10
Crystal violet	0.002
Neutral red	0.03
Agar	12

III Malt Extract Agar

	g/L
Malt extract	30.0
Peptone	5.0
Agar	15.0

IV Plate Count Agar

	g/L
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar	12

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V **Modified MRS with arginine**

	g/L
Peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Glucose	20
Di-pottasium phosphate	2.0
Sodium acetate	5.0
Sodium citrate	2.0
Arginine	3.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Tween 80	1.08

VI **Amplified nucleotide sequence**

Lactobacillus plantarum subsp. *argentolarensis* F2B

AATTGTGCTTAACACATGCATGTCGAACGAACTTTGGTATTGATTGGTGCA
TCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACCGTGGGAAAC
CTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATA
ACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTGCTATCACTTTT
GGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCTCACCATG
GCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGA
GACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT
GGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC
TCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGG
TATGACGGTATTTAACCAGAAGTACGTTATGCCCG

Lactobacillus plantarum U2A

CGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAAT
ACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAG
AGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGA
CTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGG
CCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACGTAT
CATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGAC
CATCCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCGGTCC
AAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTCTG
GGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAA
TCATGATGCAAGCACCAATCAATACCAGAGTTCGTTCGACTTGCATGTGTT
AGGCA

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Lactobacillus pentosus F2A

GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATA
CCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGA
GTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGAC
TTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGC
CGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACGTATC
ATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGACC
ATCCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCGGTCCA
AGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTCTGG
GCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAAT
CATGATGCAAGCACCAATCAATACCAGAGTTCGTTCGACTTGCATGTGTTA
GGCAA

Lactobacillus paraplantarum U2C

TCTCGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTA
AATACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAA
CAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATC
AGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTT
GGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACG
TATCATTGCCATGGTGAGCCGTTACCTCACCATCTAGCTAATACGCCGCGG
GACCATCCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCG
GTCCAAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCT
TCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATG
TAAATCATGATGCAAGCACCAATCAATACCAGAGTTCGTTCGACTTGCATG
TGTTAGGCA

Lactobacillus plantarum F2C

GTATTACCCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAAT
ACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAG
AGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGA
CTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGG
CCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACGTAT
CATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGAC
CATCCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCGGTCC
AAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTCTG
GGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAA
TCATGATGCAAGCACCAATCAATACCAGAGTTCGTTCGACTTGCATGTGTT
AGGCAAGAT

VII Chemically-defined medium for pectinase production

$(NH_4)_2SO_4$	2g
KH_2PO_4	4g
Na_2HPO_4	6g
$FeSO_4.7H_2O$	1mg
$MgSO_4$	0.2g
$CaCl_2$	1mg
H_3BO_3	10µg
$MnSO_4$	10µg
$ZnSO_4$	70µg
$CuSO_4$	50µg
MoO_3	10µg
Pectin	5g
Agar	15g
Distilled water	1000mL

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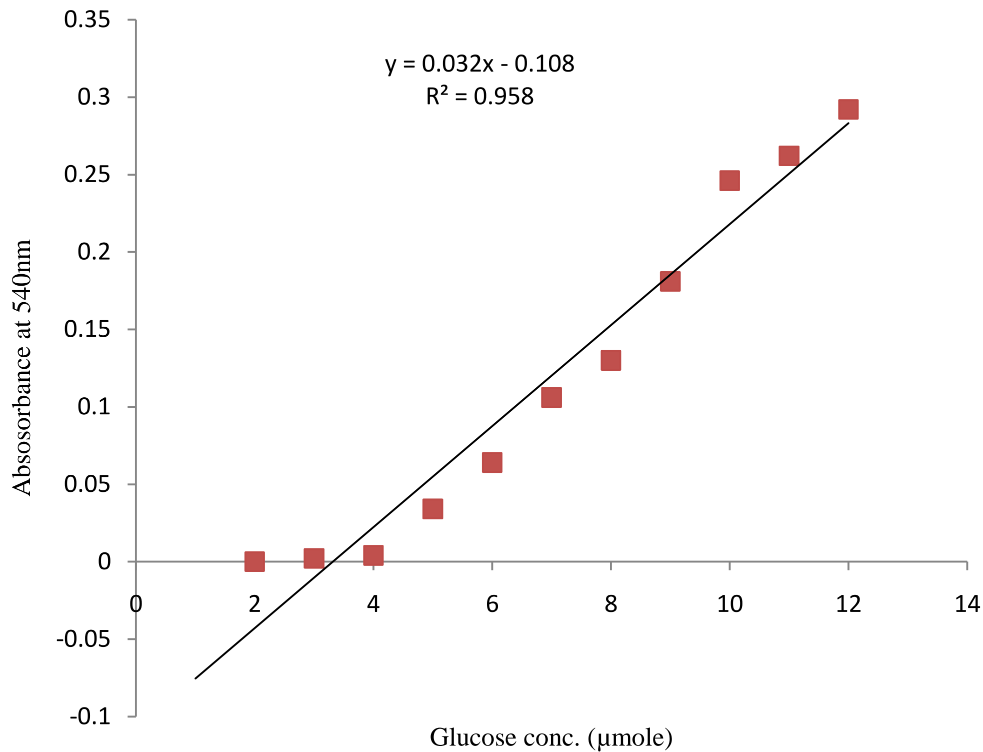
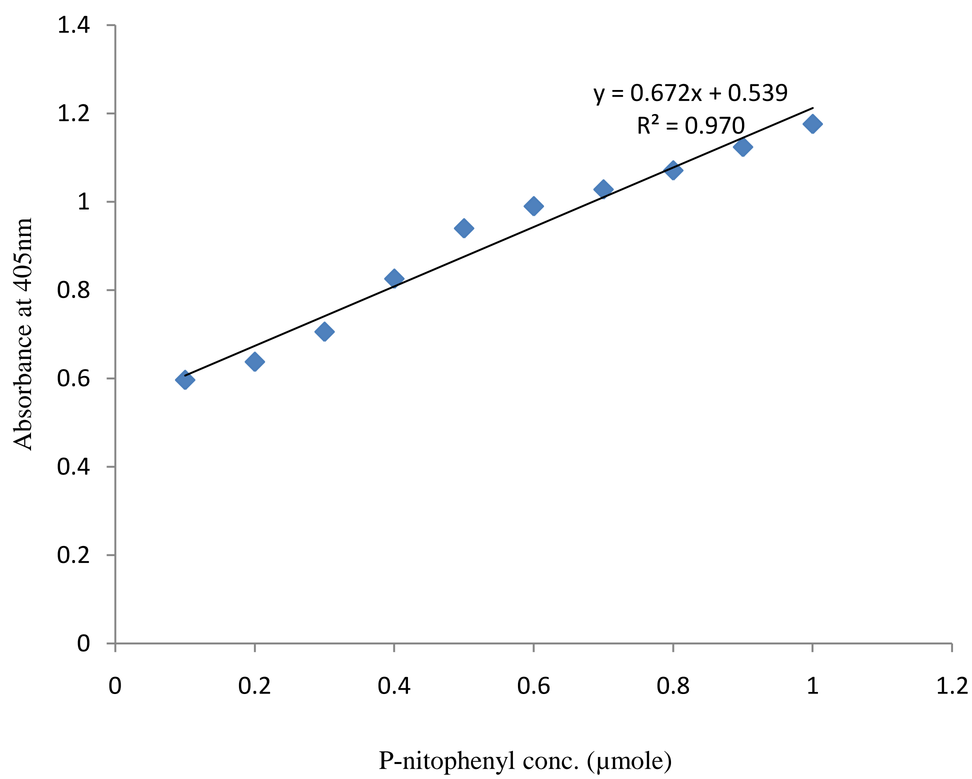
VIII Glucose standard curve



IX PNPG standard curve



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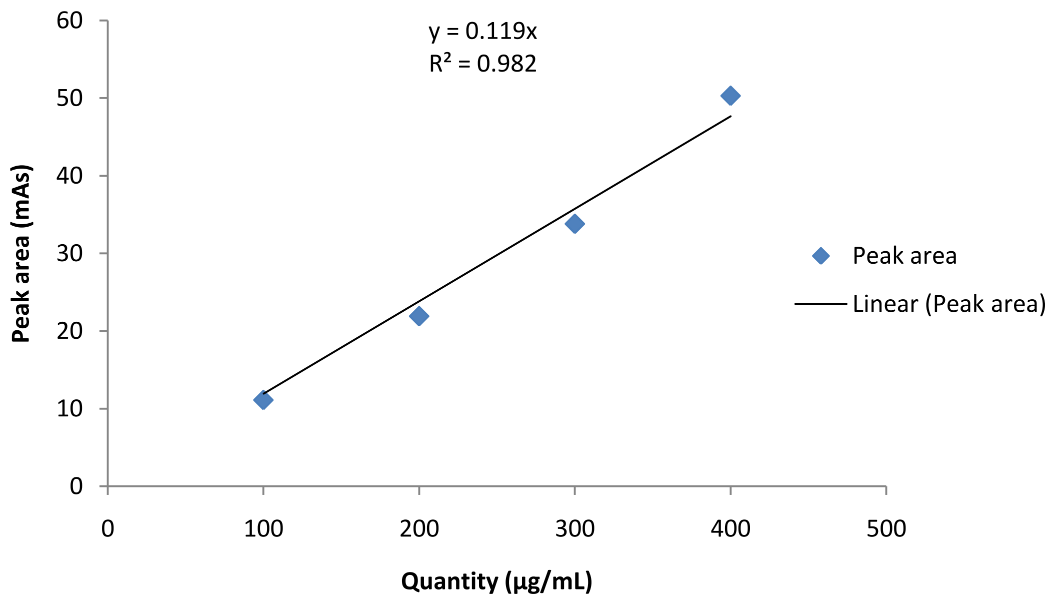
X Lactic acid calibration curve (Retention time 02mins:06secs)



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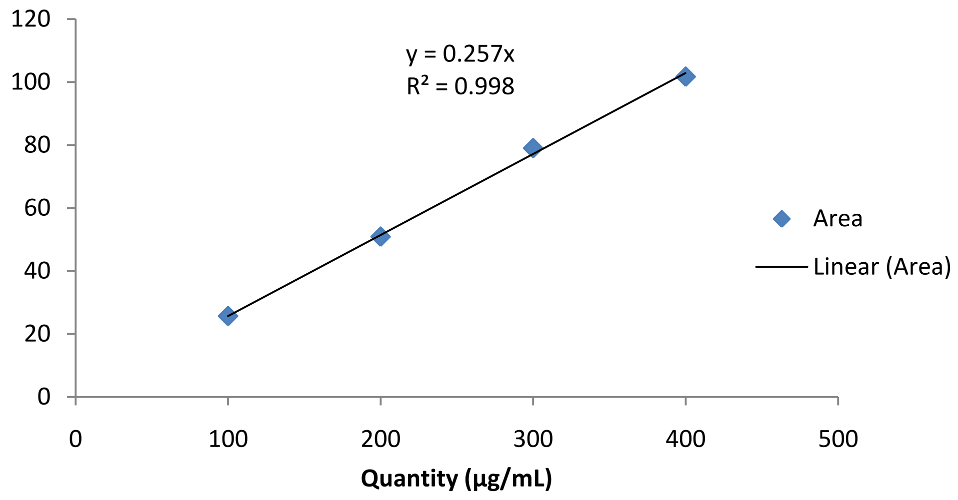
XI Acetic acid calibration curve (Retention time 02mins:11secs)



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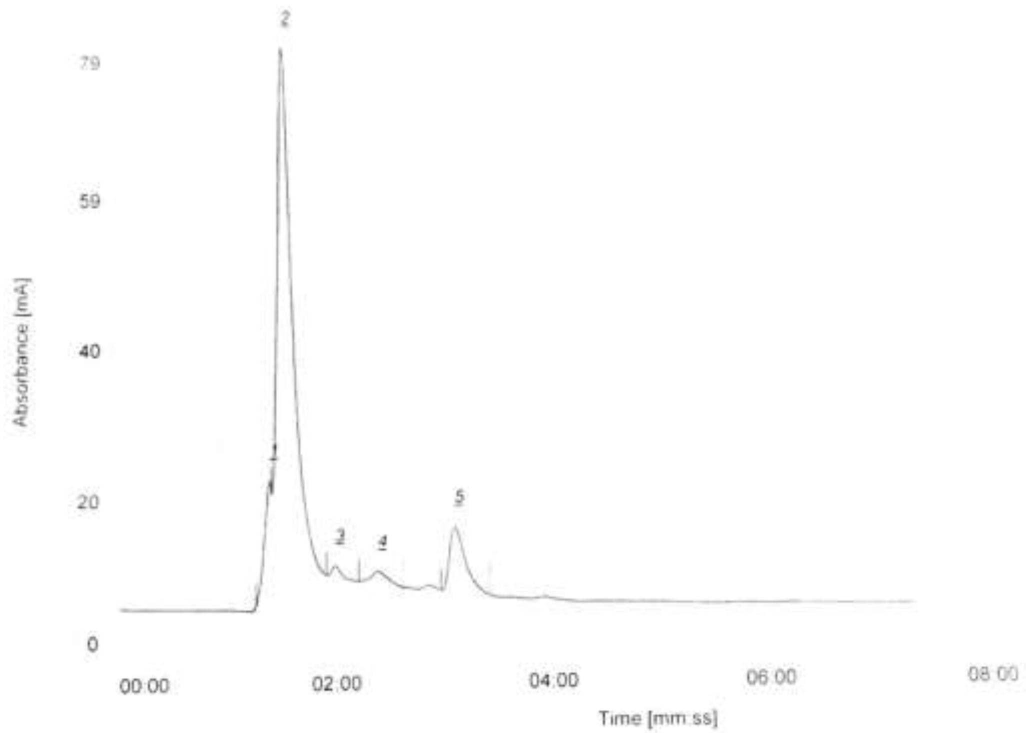
XII: Representative chromatograms showing different lactic and organic acid peak areas



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:22.9	01:15.0	01:23.9	70.1	15.9
002	* * *	01:31.8	01:23.9	01:54.3	787.2	72.5
003	* * *	01:59.0	01:54.3	02:12.2	45.2	3.9
004	ACETIC ACID	02:22.8	02:12.2	02:37.1	35.4	2.5
005	* * *	03:05.8	02:57.7	03:25.4	84.9	8.4

HPLC chromatogram of starter-fermented *fufu* at 0 hour

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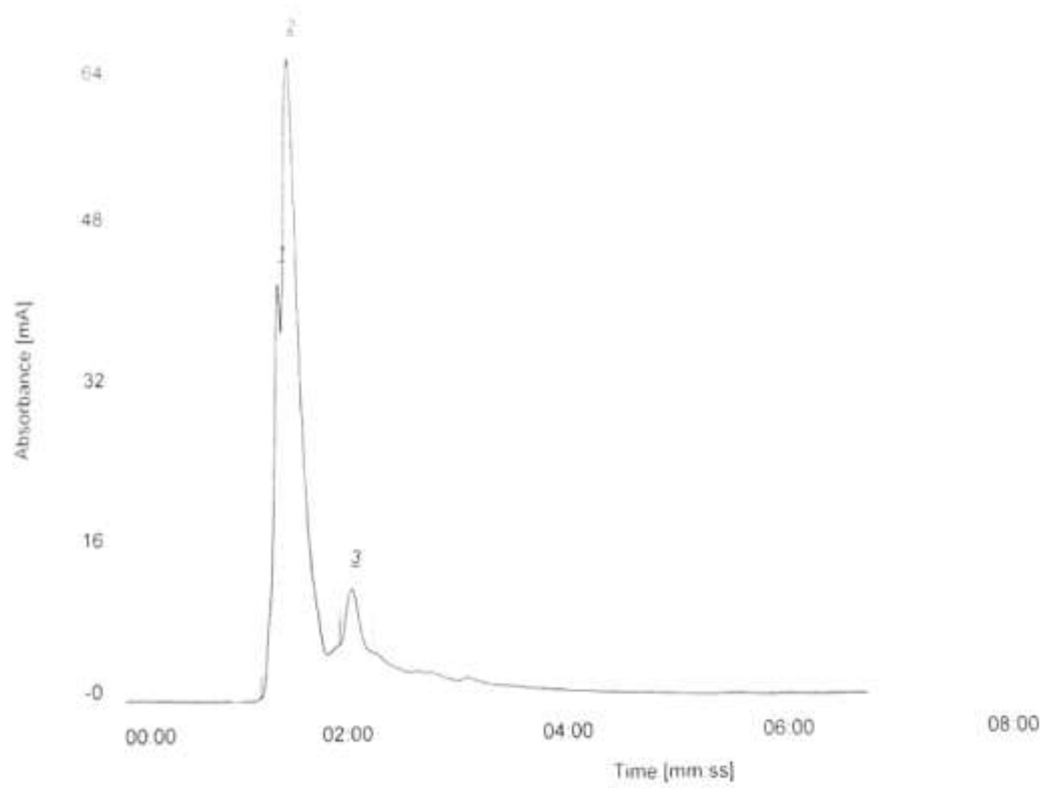

No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	...	01:24.2	01:13.2	01:25.7	209.0	40.2
002	...	01:31.1	01:25.7	01:56.1	657.4	62.5
003	LACTIC ACID	02:02.5	01:56.1	02:13.9	61.7	7.1

HPLC chromatogram of starter-fermented *fufu* at 24 hours

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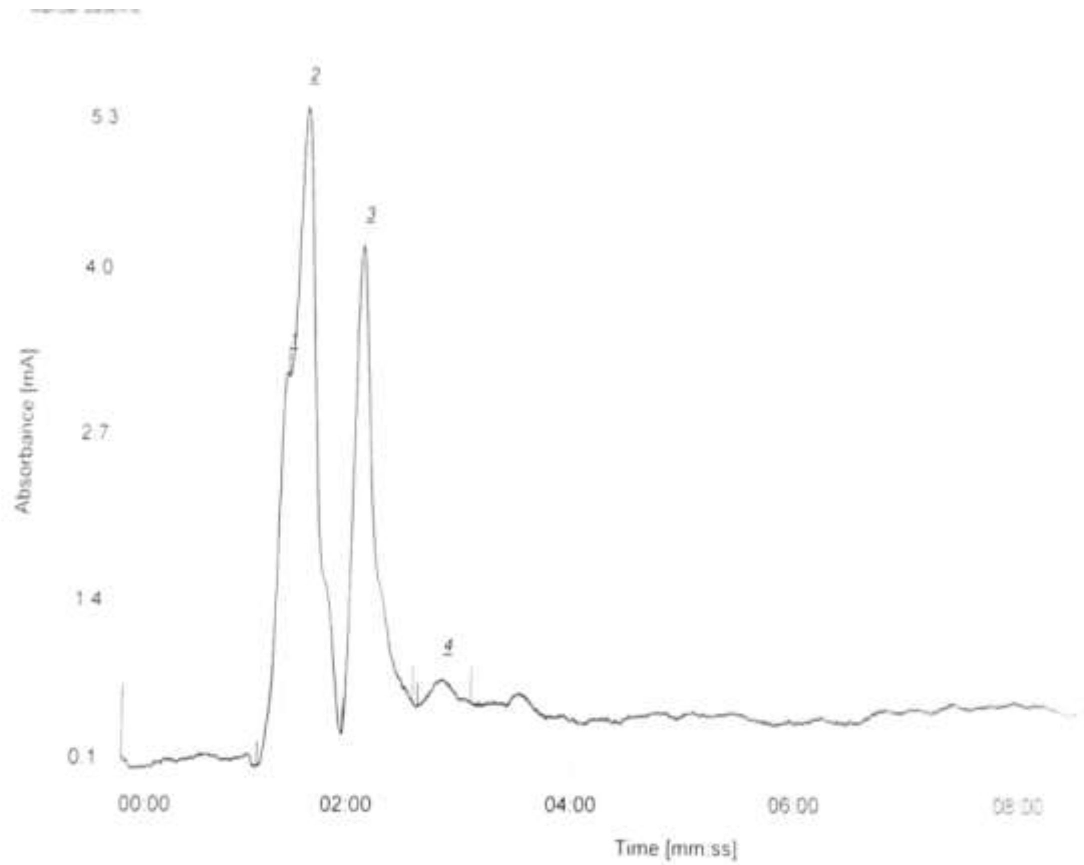
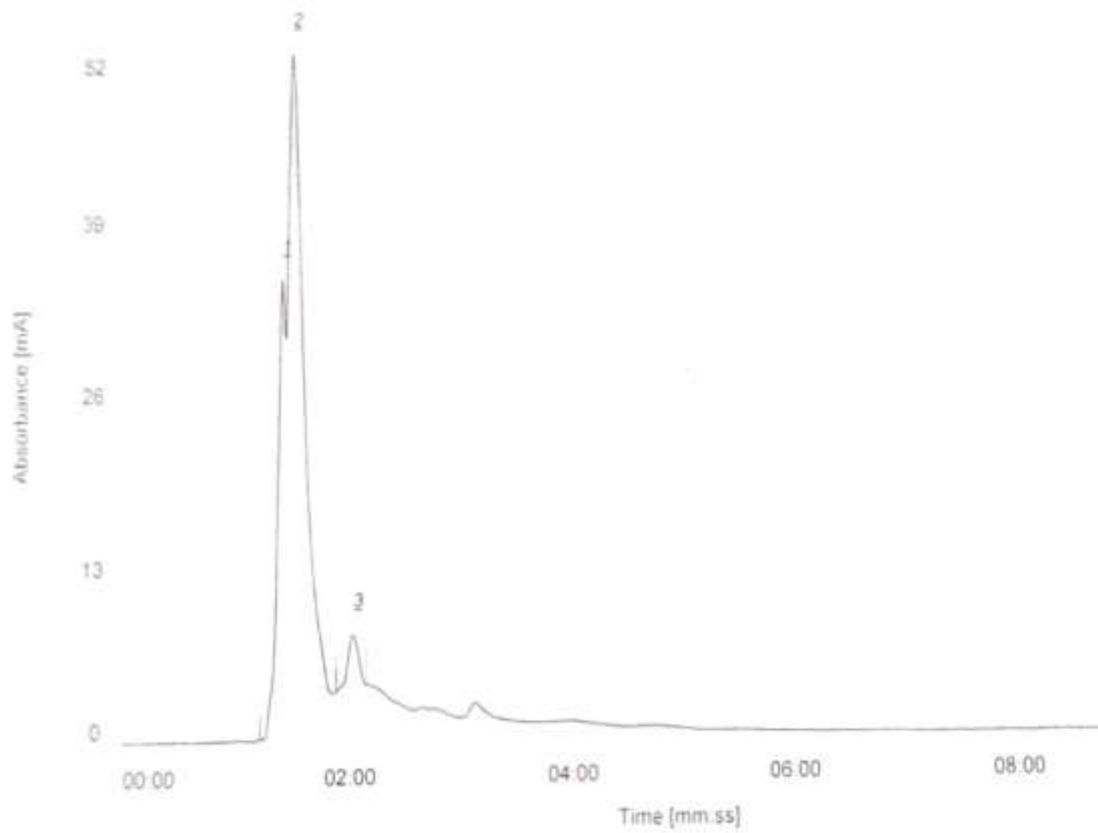


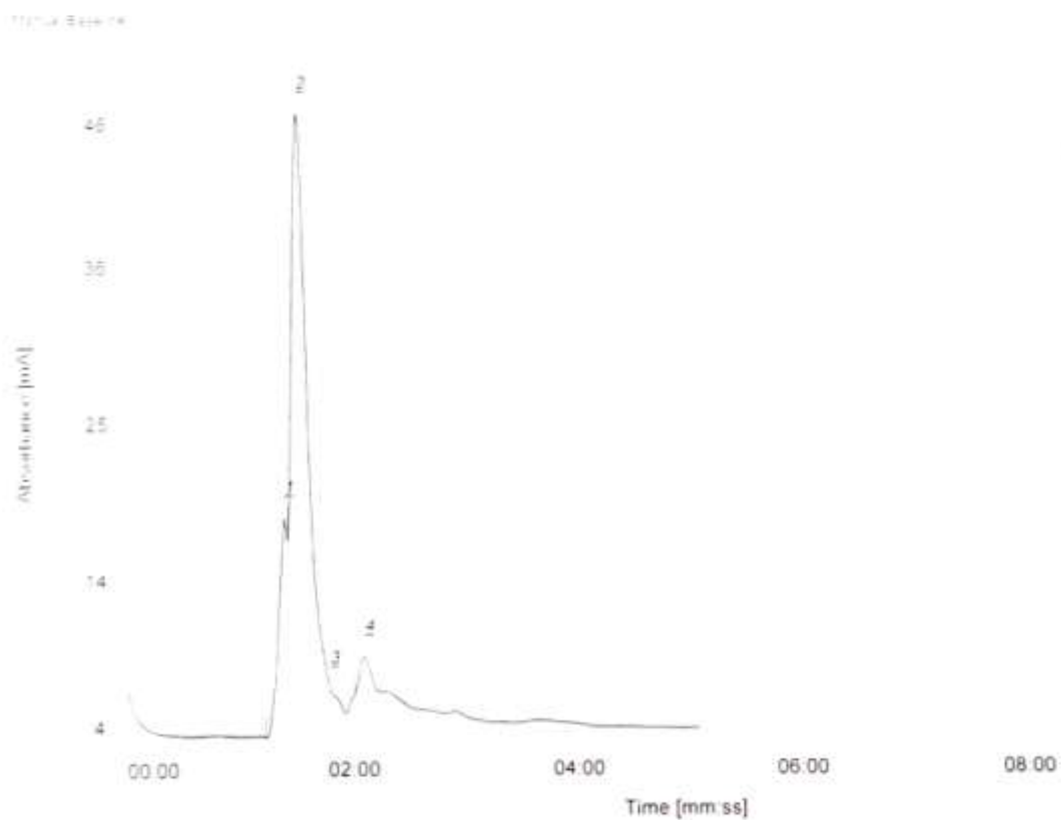
No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	***	01:24.6	01:12.3	01:25.7	15.3	3.0
002	***	01:33.5	01:25.7	01:57.0	73.3	5.0
003	LACTIC ACID	02:05.1	01:57.0	02:34.5	45.7	3.7
004	***	02:50.2	02:37.1	03:05.7	2.5	0.2

HPLC chromatogram of starter-fermented *fufu* at 48 hours



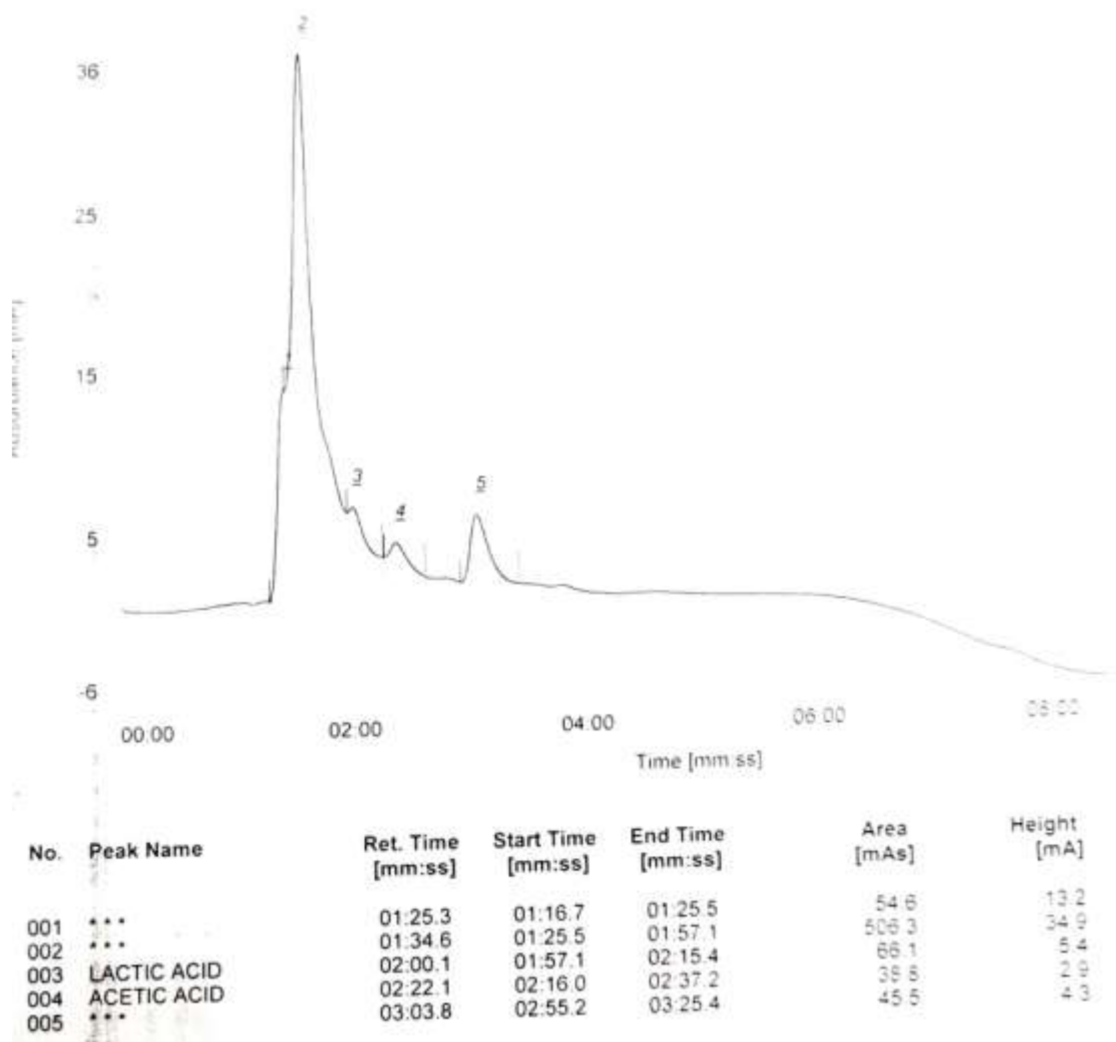
No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	. . .	01:24.4	01:12.3	01:26.6	175.8	33.3
002	. . .	01:31.0	01:26.6	01:52.5	484.1	49.9
003	LACTIC ACID	02:01.8	01:52.5	02:08.6	30.3	4.2

HPLC chromatogram of spontaneously-fermented *fufu* at 24 hours



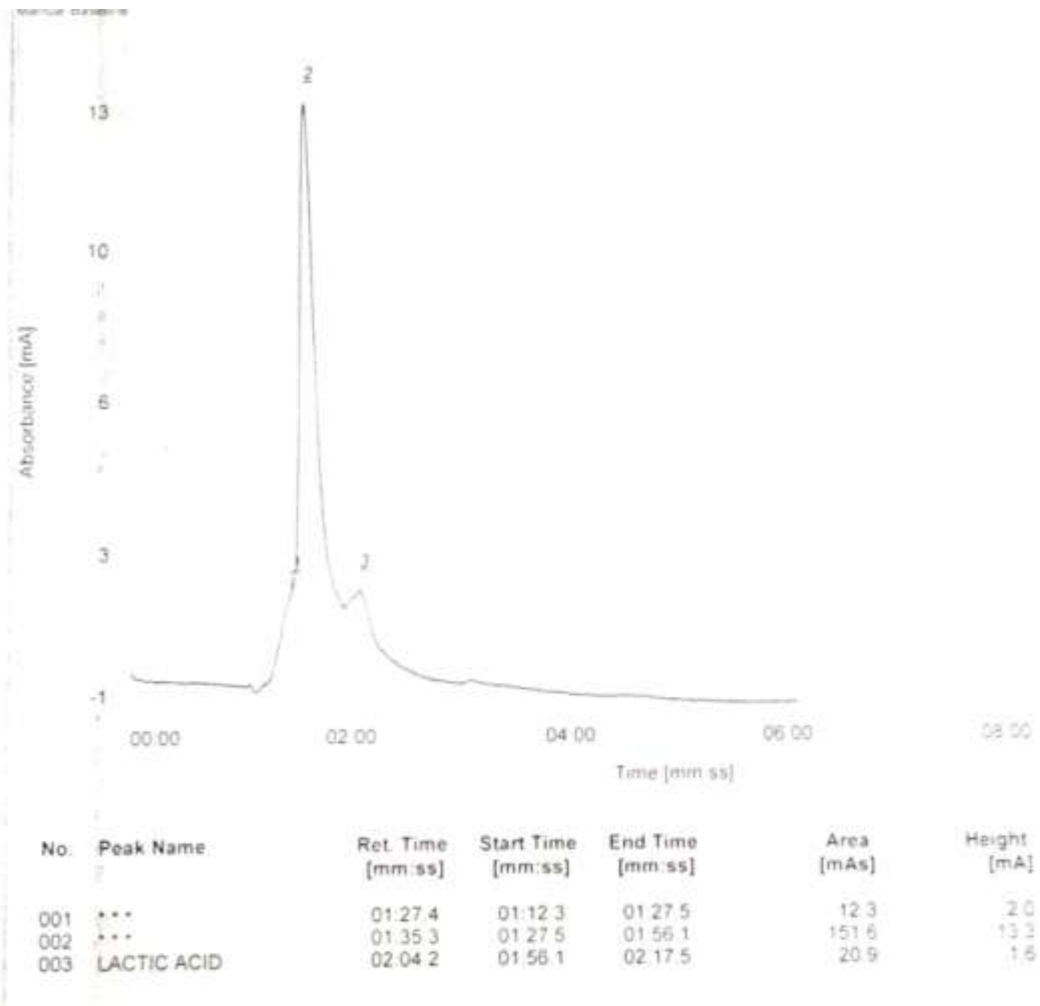
No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:23.1	01:13.2	01:24.8	72.1	14.2
002	* * *	01:31.1	01:24.8	01:47.1	416.7	41.4
003	* * *	01:47.1	01:47.1	02:00.6	-1.1	1.2
004	LACTIC ACID	02:05.6	02:00.6	02:16.6	15.4	2.4

HPLC chromatogram of spontaneously-fermented *fufu* at 72 hours



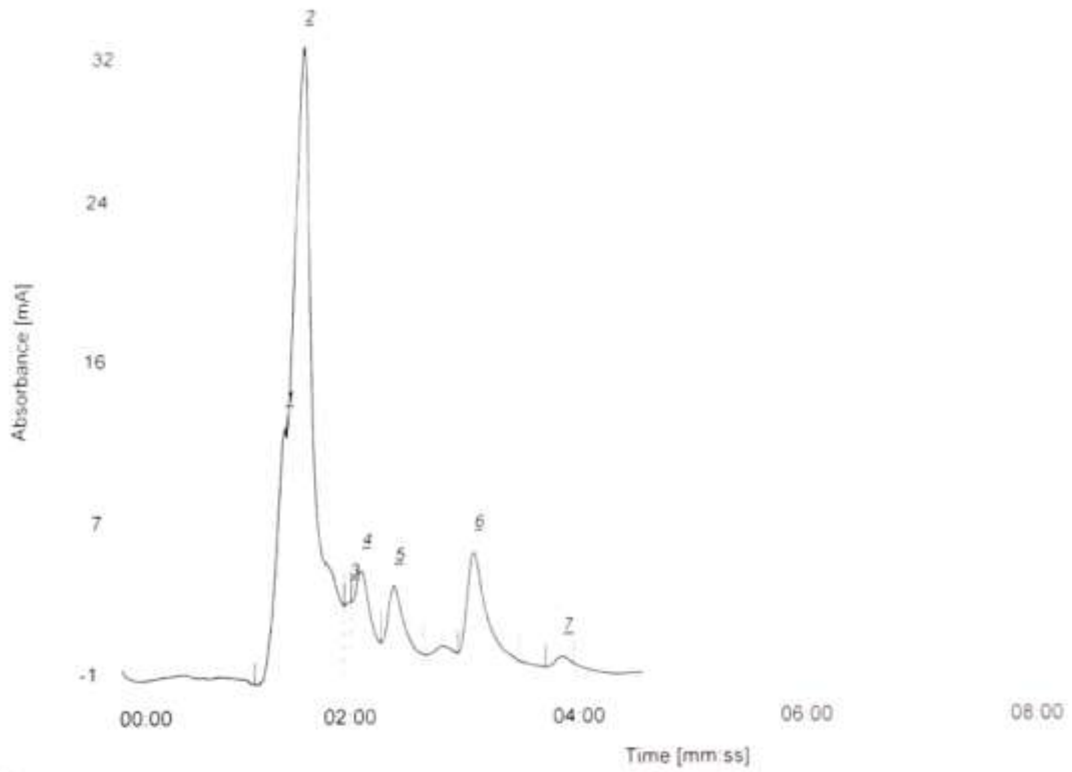
No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:25.3	01:16.7	01:25.5	54.6	13.2
002	* * *	01:34.6	01:25.5	01:57.1	506.3	34.9
003	LACTIC ACID	02:00.1	01:57.1	02:15.4	66.1	5.4
004	ACETIC ACID	02:22.1	02:16.0	02:37.2	39.8	2.9
005	* * *	03:03.8	02:55.2	03:25.4	45.5	4.3

HPLC chromatogram of starter-fermented *usi* at 0 hour



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:27.4	01:12.3	01:27.5	12.3	2.0
002	* * *	01:35.3	01:27.5	01:56.1	151.6	13.3
003	LACTIC ACID	02:04.2	01:56.1	02:17.5	20.9	1.6

HPLC chromatogram of starter-fermented *usi* at 72 hours



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:23.5	01:09.9	01:24.6	64.1	13.0
002	* * *	01:31.7	01:24.6	01:55.9	428.7	32.9
003	* * *	01:58.6	01:56.2	01:59.5	12.6	3.8
004	LACTIC ACID	02:04.7	01:59.8	02:15.4	56.0	5.4
005	ACETIC ACID	02:22.0	02:15.4	02:37.8	52.3	4.4
006	* * *	03:03.6	02:55.9	03:28.1	69.5	5.8
007	* * *	03:51.2	03:42.2	03:57.0	1.7	0.4

HPLC chromatogram of spontaneously-fermented *usi* at 24 hours

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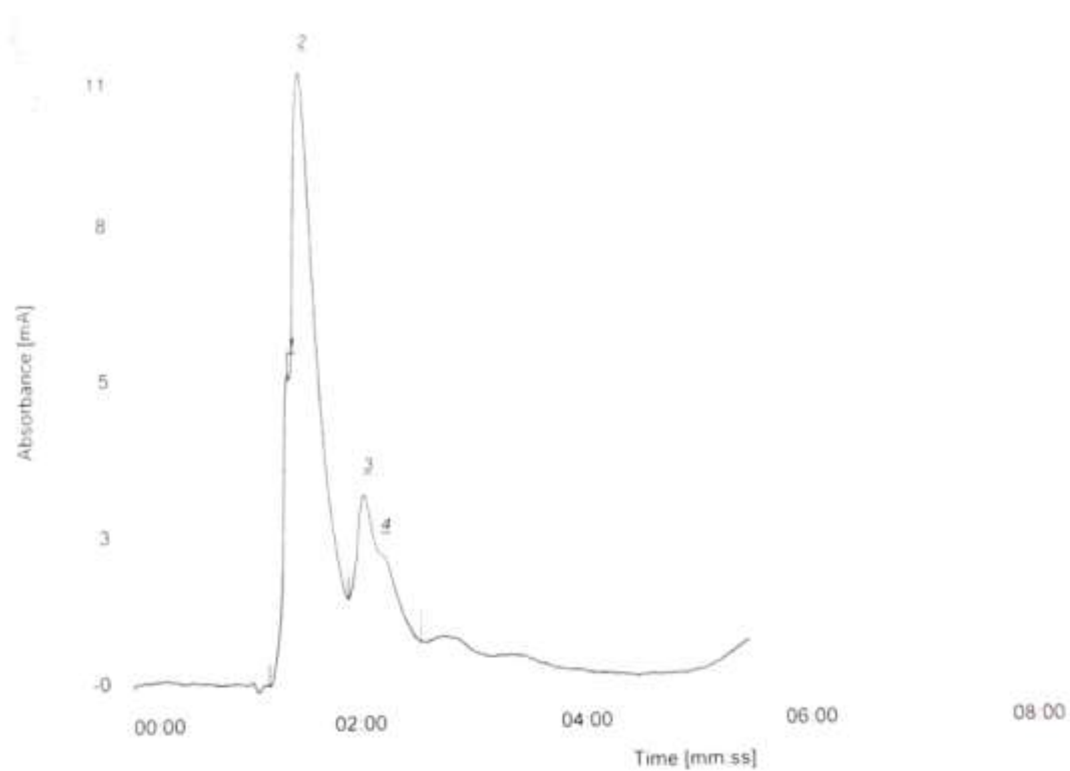
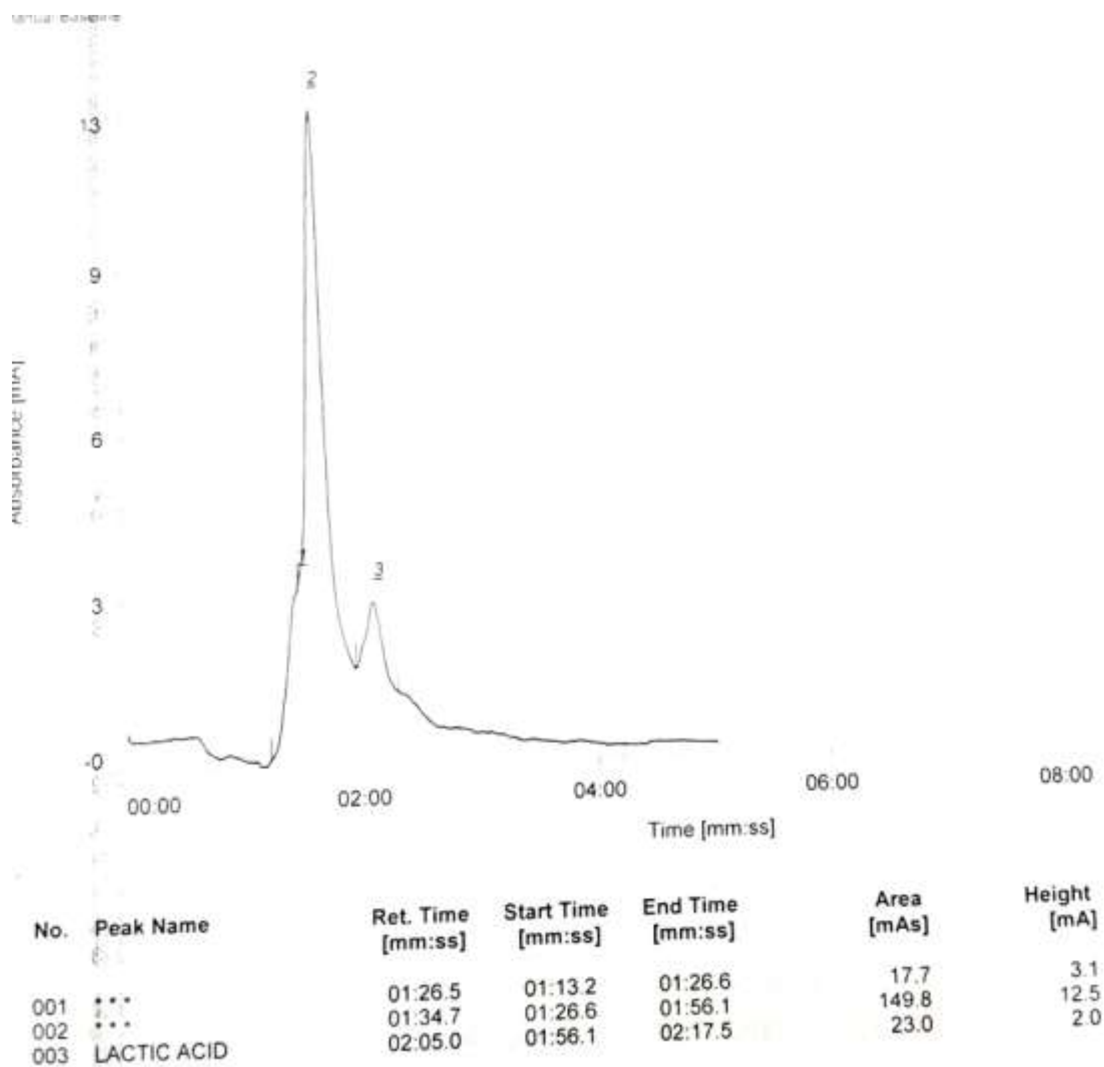


No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	* * *	01:23.4	01:12.3	01:23.9	20.5	5.2
002	* * *	01:32.4	01:23.9	01:54.3	163.9	10.3
003	LACTIC ACID	02:03.1	01:54.3	02:12.2	35.5	2.8
004	* * *	02:12.2	02:12.2	02:32.6	15.3	1.7

HPLC chromatogram of spontaneously-fermented *usi* at 48 hours



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]
001	...	01:26.5	01:13.2	01:26.6	17.7	3.1
002	...	01:34.7	01:26.6	01:56.1	149.8	12.5
003	LACTIC ACID	02:05.0	01:56.1	02:17.5	23.0	2.0

HPLC chromatogram of spontaneously-fermented *usi* at 72 hours

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