EFFECT OF VARYING GRILLED SORGHUM CONCENTRATION ON PHYSICO-CHEMICAL QUALITY PARAMETERS OF *URWAGWA* AND IDENTIFICATION OF INDIGENOUS YEASTS INVOLVED IN ITS FERMENTATION

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any college, institution or university.

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DEDICATION

This work is wholeheartedly dedicated to my beloved parents, my fiancé Divine Mukeshimana, aunt and relatives, who have been a source of inspiration and gave me strength when I thought of giving up; they continually provided their moral, emotional and financial support.

To my friends and classmates who shared their words of advice and encouragement to finish this study.

And lastly I dedicate this work to the Almighty God, thank you for guidance, strength, power of mind, protection and for giving me a healthy life.

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ABBREVIATIONS AND ACRONYMS

AAA-EAHB:	East Africa highland Banana cultivar
AAB:	Acetic Acid Bacteria
AOAC:	Association of Official Analytical Chemists
BC:	Before Christ
BLAST:	Basic Local Alignment Search Tool
CFRDTA:	Centre de Formations et des Recherches pour le Développement des Technologies Appropriées
DDP:	District Development Plan
DNA:	Deoxyribonucleic Acid
EAC:	East African Community
ERIC:	Enterobacterial Repetitive Intergenic Consensus
FAO:	Food and Agriculture Organization of The United Nations
ITS:	Internal Transcribed Spacer
LAB:	Lactic Acid Bacteria
min:	Minute
ml:	Mililitre
MT:	Metric Tonne
NISR:	National Institute of statistics of Rwanda
PCR:	Polymerase Chain reaction
pH:	Potential of Hydrogen
REP:	Repetitive Extragenic Palindromic
RFLP:	Restriction Fragment Length Polymorphism

rRNA:	Ribosomal Ribonucleic Acid
SNPs:	Single Nucleotide Polymorphism
TSS:	Total Soluble Solids
UNCTAD:	United Nations Conference on Trade and Development
WHO:	World Health Organization
YPD:	Yeast Extract Peptone Dextrose
RAB:	Rwanda Agriculture Board
MRS:	De Man, Rogosa and Sharpe agar.
TAE:	Tris- Acetate-EDTA
g/L:	Gram per litre

ABSTRACT

The alcoholic beverage called *Urwagwa* in Rwanda is one of the most popular drinks traditionally processed from banana. Besides being a popular beverage during various social gatherings, it is also a primary source of income for farmers in some of the banana producing agricultural zones in Rwanda. Due to limited information on the aspect of microorganisms involved in the fermentation process and the effect of grilled *ground sorghum vulgaris sp* on physico-chemical changes that take place during fermentation. This study was carried out to determine how varying grilled sorghum concentration affected both proximate composition of banana juice-sorghum composite mixture before fermentation and the physico-chemical quality parameter of *Urwagwa*. The study also sought to identify the indigenous yeasts involved in the spontaneous fermentation of this alcoholic beverage. Both proximate composition of banana juice-sorghum composite mixtures and physico-chemical characteristics of *urwagwa* were determined include the protein, pH, sugar content, ethanol and titratable acidity in natural fermentation. Identification of indigenous yeast involved in fermentation was carried out using both the conventional (morphological identification) and the molecular (PCR) techniques. The results obtained at the end of fermentation indicated that the ethanol content in all fermenters ranged from 10.3-12.2% v/v, total soluble solids 8.1-9.7°Brix, pH 4.0-4.3 and titratable acidity 0.64-0.93%. The highest fermentation rate was observed in fermenters containing ground grilled sorghum compared to the control (0% sorghum added). The highest total soluble solid at the completion of fermentation was found in banana-based alcoholic beverage with zero percent of sorghum while highest pH

was generally found in fermenters with sorghum combinations. High titratable acidity was observed in fermenter with zero percent of sorghum. The concentration of 5% of ground grilled sorghum was found to result to the most desirable banana-based alcoholic beverage as it gave the best quality parameters of banana-based alcoholic beverage. The morphological characteristics of the isolates recovered from *urwagwa* matched those of yeast (circular, umbonate, smooth and white to cream color). Molecular identification through BLAST analysis of partial sequences of isolates showed that they were from genus *Saccharomyces cerevisiae* with 97 to 100% similarities of previously known sequences in GenBank database. These findings seem to suggest that a sorghum concentration of 5% would give the best quality *urwagwa* under the study conditions reported here and can therefore be recommended for adoption by traditional *urwagwa* producers. The study has also confirmed that yeast species *Saccharomyces cerevisiae* is involved in the spontaneous fermentation of this alcoholic beverage. *Saccharomyces cerevisiae* strain 1000 (LC269108.1) identified in fermenter with 0%, 2%, 5% and 15% of sorghum was found to be the best strain which can be used in banana-based alcoholic beverage production.

CHAPTER ONE
INTRODUCTION

1.1 General background information

Alcoholic beverages are among the leading drinks in many African communities in terms of consumption (Shale *et al*., 2014). The high consumption levels of these beverages have vitalized both domestic and industrial production of those beverages. Almost all African societies have well established domestic production industries for the fermented alcoholic beverages, which to a great extent depends on traditional techniques (Mohapatra *et al*., 2010). Domestic production is often adopted to meet subsistence demands, with these beverages common in social functions such as weddings and rites of passage celebrations, among others. Commercial production is of a larger scale, adopting an industrial outlook that is often oriented towards mass production for consequent economic value. Despite the lower production threshold and traditional outlook of domestic production of fermented alcoholic beverages, it is strange that standard of specificity in terms of the quality, diversity, and appeals for these products are higher than that adopted in commercial production (Shale *et al*., 2014).

African countries, particularly the East African countries had historically embraced fermented beverages made from various raw materials including bananas. For instance, Rwanda, Uganda, and Tanzania have elaborated fermented banana beverage industries functioning at domestic capacities, but with emerging attempts towards commercial-level production (Wilson, 2012). In Rwanda particularly, *Urwagwa*, a popular traditional banana-based alcoholic beverage, has had a long historical significance for the society. This alcoholic beverage, which is made from

banana juice blended with ground grilled sorghum through spontaneous fermentation, is mainly confined to domestic production with annual production of 700 million litres and an average consumption per capita of about 1.2 litres per day (Immaculate, 2013). During its production, ground grilled sorghum which is thought to accelerate the fermentation process and enhance flavour development in the end product is included as part of the composite mixture (Munyanganizi, 1975). This processing is however yet to be evaluate and documented through scientific studies (Wilson, 2012).

The vibrancy within the fermented banana beverage market may be attributed to socio-cultural primers as drivers of consumption of banana-based beverage products (Ouma & Jagwe, 2010). Further, successes in banana farming have played a role in the consequent availability of good-quality resources to support the production of fermented banana-based alcoholic beverages. However, production standards regarding quality and capacity for alcoholic banana-based beverages continue to be low based on a general unavailability of effective evidence-based production procedures (Satav & Pethe, 2017). In particular, there is limited knowledge on production-based characteristics of banana and yeast species as regards to the production of fermented banana beverages remains scanty. Traditional production of banana-based alcoholic beverages has often relied on nature as a conduit for the relevant microorganism to spur the fermentation process (Wilson, 2012). Various studies have highlighted the dangers of such uninformed approaches that include the production of unhealthy products (Shale *et al*., 2014). Modern alcoholic production techniques are centered on better understanding of the relevance of characteristic-based specificities of the microorganism (Hierro *et al*. 2004). Various bacterial and

fungal species have featured in several studies for their unique efficacies within the fermentation process. Some yeast species that include *Saccharomyces pastorianus*, *Saccharomyces bayanus*; and *Saccharomyces cerevisiae* have had a long historical utility in fermented alcoholic beverage production, with *S. cerevisiae* sustaining a prevailing dominance within the suggested industry. These species are largely valued for their high ethanol tolerance, proliferation in high sugar concentration and resistance to the production of sulphur-based compounds (Hutkins, 2006).

However, emphasis on the need to achieve value from an effective production process necessitates an effective understanding of intricate changes and process embodied in banana-based alcoholic beverage production cycle. An understanding of biochemical and microbial activities provides an effective way of ensuring a proper the fermentation process and identifying plausible inconsistencies (Satav *et al*, 2017). As observed by Wilson, (2012), knowledge on the product (substrate) and microorganism-based differences as regards fermented banana-based alcoholic beverage manufacturing process may aid in determining the most effective approach that can be adopted for the production of quality and consistent banana-based alcoholic beverage. Therefore, an understanding of the particular indigenous yeast species involved in the fermentation of the traditional banana-based alcoholic beverage, *Urwagwa*, and the physico-chemical changes taking place during the fermentation process could aid in providing an effective framework for better banana-based alcoholic beverage production.

1.2 Statement of the problem

Despite traditional alcoholic banana beverage production being an expansive industry of long historical existence in Rwandan society, there is little documented research conducted on it. The few studies carried out on the fermentation of banana to produce alcoholic beverages have not reports on the aspect of microorganisms involved in the fermentation process and the effect of grilled ground sorghum on physico-chemical changes that take place during fermentation (Wilson, 2012). Such information about banana-based alcoholic beverage production is deemed essential in promoting best practices with regard to raw materials inclusion, processing controls, and quality of end-product. It is therefore necessary to isolate, identify and characterize the indigenous yeast involved in the production of banana-based alcoholic beverage in an attempt to improve the traditional banana-based alcoholic beverage production so as to enhance consistent quality product for future industrial purpose (Wilson, 2012). Further, there is need to determine intrinsic physico- chemical changes occurring during the fermentation process. Despite the trends cited in other established beer production processes, the economic and aesthetic values of banana-based alcoholic beverage will only be realized through documentation of the relevant key components involved in the banana-based alcoholic beverage production process.

1.3 Justification and significance of the study

Rwanda is ranked among top ten banana producing countries in the world according to the United Nation Conference on Trade and Development report (UNCTAD, 2016). The country is one of the major producers of bananas in the East African Great Lakes region after Uganda. In Rwanda, bananas come second after roots and

tubers in terms of the production levels and they are grown by 80% of smallholders (RAB, 2018). The commodity occupies 23% of the country's arable land and contribute more than 27.6% of annual crop production in terms of produce (NISR, 2016). *Urwagwa* is one of the oldest and major alcoholic beverages traditionally produced from banana and is popular in Rwanda as well as in other East Africa countries. In Rwandan culture, traditional banana-based alcoholic beverage is important in various social and ceremonial roles, where no ceremony is complete without these beverage being served (Shale *et al.*, 2014).

Production of *Urwagwa* also serves as an important source of income and employment among many farmers. Rwanda is facing postharvest losses of bananas which is estimated to be up to 30 to 40% (RAB, 2018). This problem can be solved through value-addition by processing banana into banana-based alcoholic beverage. But the processing methods of *Urwagwa* are mainly done using traditional methods. Improving the production standards and quality of banana-based alcoholic beverage can result in a product of higher quality, with higher economic and potential for the farmers and entrepreneurs. It is therefore important to understand the production process, identify the microorganisms that are naturally involved in the fermentation and understand the effect of ingredients such as grilled ground sorghum on physico-chemical changes that take place and its influence on the overall product quality.

1.4 Objectives

1.4.1 General objective

The general objective of this study was to determine the effect of varying grilled *sorghum vulgaris sp* levels on the physico-chemical properties of *urwagwa* and to identify indigenous yeast involved in the fermentation process.

1.4.2 Specific objectives

1. To determine the proximate composition of the banana juice-sorghum composite mixture used in production of *Urwagwa*.
2. To determine the effect of grilled sorghum concentration on the physico-chemical changes (pH, sugar concentration, ethanol accumulation and titratable acidity) during the fermentation process.
3. To isolate and identify the indigenous yeast species involved in the fermentation process.

1.5 Research hypothesis

1. Proximate compositions of ground grilled sorghum do not affect the composition of banana juice-sorghum composite mixture used in production of *Urwagwa*.
2. Varying grilled sorghum concentration has no effect on the physico-chemical changes occurring during the fermentation process.
3. There is no variation in indigenous yeast strains involved in fermentation of *urwagwa*.

1.6 The scope of the study

The study was limited to Rwandese banana-based alcoholic beverage *Urwagwa* context as the domain of interest. The research included an analysis of the proximate composition of banana juice-sorghum composite mixtures before fermentation and their effect on fermentation process, with particular emphasis on physico-chemical changes occurring during the beverage production process. Further, the study was conducted on the molecular identification of indigenous yeast linked to the banana-based alcoholic beverage production.

CHAPTER TWO

LITERATURE REVIEW

2.1 Banana origin description

The banana plant originated from South-East Asia and has been cultivated for nearly 10,000 years. The first traces, which date back to 7 000 B.C., were found in Papua New Guinea. This giant herb belongs to the Monocotyledon class and the Musaceae family (UNCTAD, 2016).

Banana *(Musa spp.)* is an edible fruit produced by large herbaceous flowering plants which belong to the family *Musaceae*, genus *Musa* and species *acuminate* (UNCTAD, 2016). Bananas are perennial tropical and subtropical crops which grow in a wide range across the globe and are an important staple and nutritional food which play a key role in food security in the producing countries. Bananas contain a high water succulent stem which helps resist drought. Beer and cooking banana varieties vary in both physical and chemical characteristics. Normally both varieties mature about three months after flowering.

Banana height averages from 2-9 m while the fruits are variable in size, shape and color (Nelson, 2006). Generally fruits are elongated-cylindrical, straight to strongly curved 3-40 cm long and 2-8 cm diameter. About 12-20 flowers are estimated per cluster and the collection of flowering parts and fruits are referred as bunch. According to Byarugaba-bazirake, (2008) *Mbidde* (AAA-EA) cultivar has hard bunches with starchy banana fingers which require an expert to differentiate them from cooking banana cultivars. *Kisubi* cultivars are usually shorter compared to cooking varieties whereas sweet *Ndiizi* cultivars have both smaller banana fingers

and stems in the mother crop. The cooking banana cultivars are normally harvested while still green whereas banana beer cultivars are harvested at full maturity when the first fingers nearest the stalk turn to yellow.

Banana fruits are excellently starchy and are very rich in carbohydrate. However their chemical compositions vary a great deal depending on different factors such as location ecology, nutrition and location of bunch from which bananas fingers are sampled for analysis as well as maturity (IITA, 1993). Different cultivars also differ considerably in terms of chemical content such as sugar, fats, minerals, proteins, moisture content and acidity.

Bananas and plantains feature among the top ten crops produced worldwide, ranking behind maize, rice, wheat, cassava, and potatoes, but ahead of sorghum, millet and sweet potatoes (German et al., 2015). World production of banana is estimated to be about 133,691,965 tonnes (Table 2.1). Main production zones are located in Asia, representing 44 percent of world volumes, followed by Africa at 25 per cent and central and South America at 22 per cent. Rwanda, as one of East African countries is ranked among top ten producers of bananas worldwide with an estimated production of 3,263,462 tonnes (UNCTAD, 2016). According to the National Institute of statistics of Rwanda (NISR, 2016) banana occupies 314705Ha of cultivated land which represent 23.2 per cent of arable land. The large number of clones grown in Rwanda are the local East African highland cooking and beer cultivars (AAA-EAHB) and introduced beer (AB, ABB) and desert (AAA, AB) types (Gaidashova, 2005). For the last 15 years, beer cultivars have dominated banana production increasing from 67% to 71% at the expense of dessert types (Gaidashova,

2005). But previous study done by (Nsabimana *et. al.*, 2008) reported two major banana subgroups determined at all sites, Lujugira-Mutika with 77.8% and Pisang Awak with 11.9% of abundance respectively. A total of 104 cultivar names were recorded, with 53 synonyms identified for 51 cultivars. Forty cultivars belonged to Lujugira-Mutika subgroup, with 'Intuntu', 'Intokatoke', 'Injagi, 'Mbwaziruma' being the most abundant cultivars, while eleven cultivars are exotic. Gisubi (ABB), Gros Michel (AAA), and 'Kamaramasenge' (AAB) are the most abundant. Farms with a higher proportion of Gisubi contained fewer of other cultivars (Nsabimana *et al.* 2008).

Table 2.1: Estimated banana production (dessert and cooking banana), Metric Tonnes (MT) per annum.

N°	Country	Total Production	Dessert banana	Cooking banana
1	India	27575 000	17 075 000	10 500 000
2	China	12075238	11 506 238	569 000
3	Uganda	8 926 308	500 000	8 426 308
4	Philippines	8 645 749	5 790 091	2 855 658
5	Brazil	6 892 622	6 402 622	490 000
6	Ecuador	6 739 739	6 145 527	594 212
7	Colombia	5 405 365	2 587 625	2 817 740
8	Indonesia	5 359 115	3 289 115	2 070 000
9	Rwanda	3 263 462	250 000	3 013 462
10	Nigeria	3 222 000	315 000	2 907 000

Source: UNCTAD, 2016

2.2 Production zone of banana in Rwanda

Banana is the second main staple food after beans in Rwanda with widespread cultivation, consumption and cultural acceptance. The main production zone and quantities produced are shown in Figure 2.1.

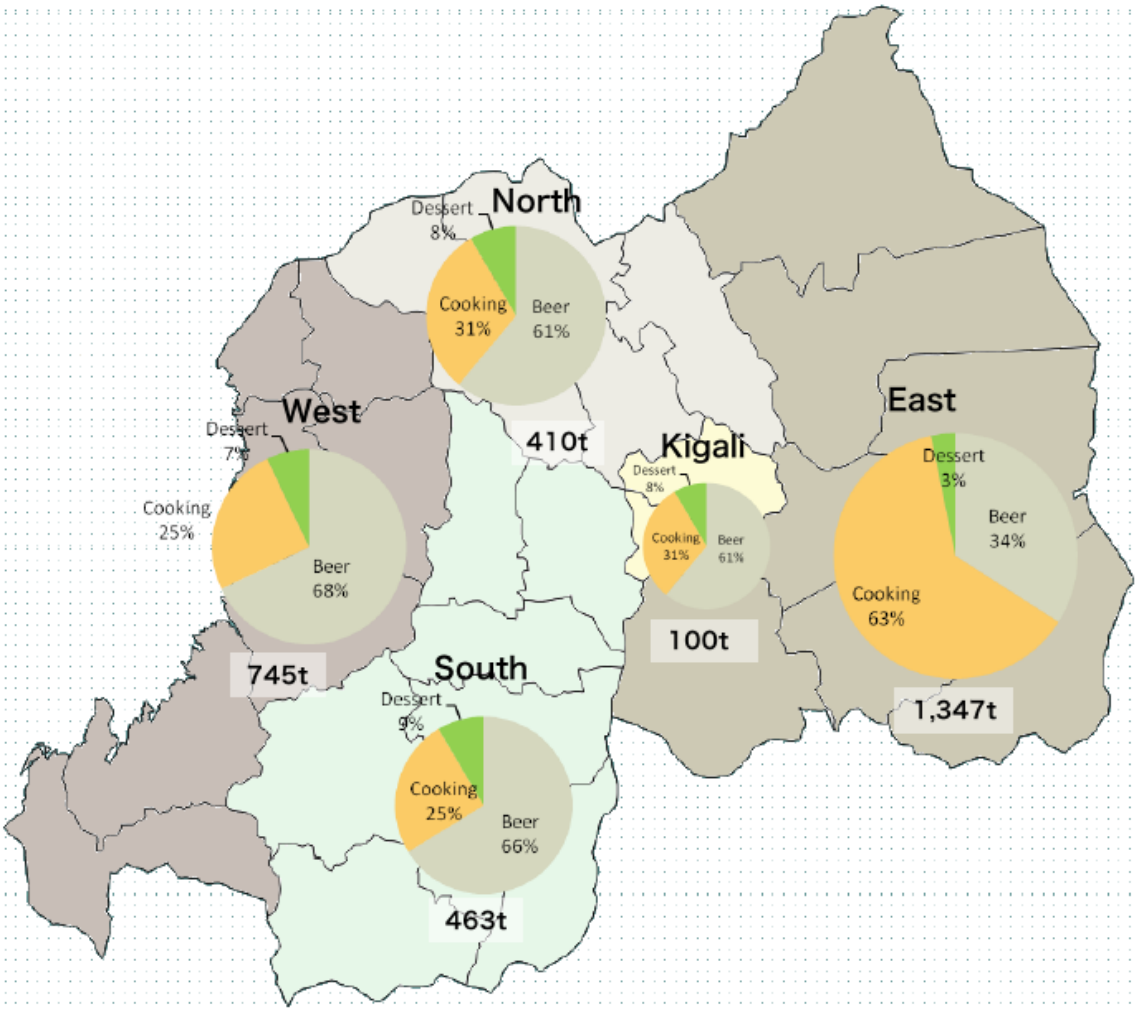


Figure 2.1: Rwanda's banana producing zones and their quantity in tonnes.
Source: Ministry of Agriculture, 2012.

Based on Figure 2.1, the Western province is the leading producer of beer banana in Rwanda with 68% of total banana production which show the dominance of beer banana over cooking banana, followed by Southern and Northern Province with 66%

and 61% respectively. The least prevalence of beer banana production is seen Eastern province with 34% (Ministry of Agriculture, 2012) .

2.3 Banana cultivars used for beer production in Rwanda

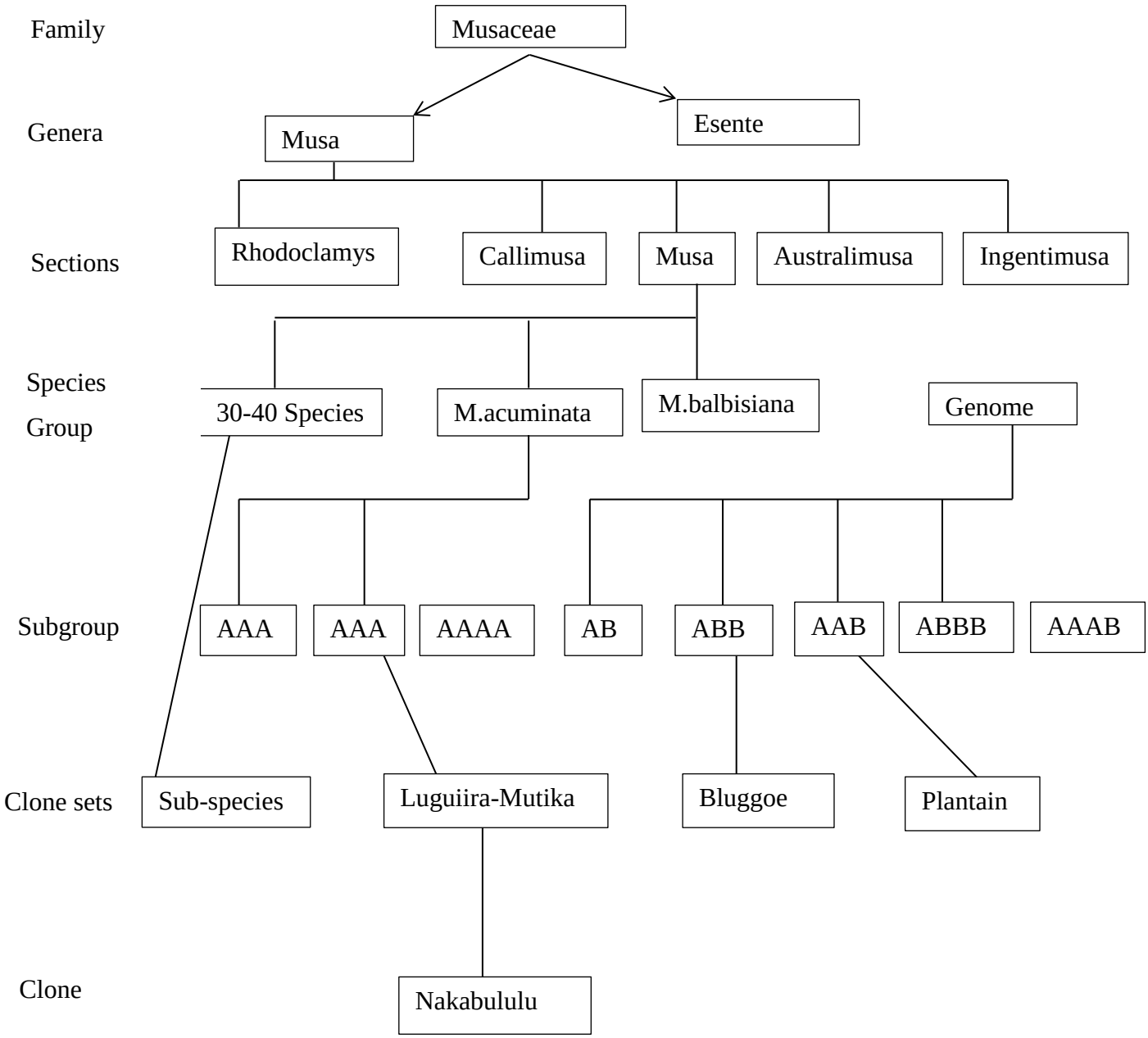


Figure 2.2: The classification of banana. (Source: Karamura, 1998)

2.4 Nutritional composition of banana

As one of the main staple crops in most East African countries bananas, are nutritious and often complement cereals and roots as well as tubers. Bananas are consumed as starchy food, sweet fruit and are used in brewing for banana-based alcoholic beverages. Bananas are good source of vitamins and minerals as well as rich sources of fibre and carbohydrates (Table 2.2). Bananas contain minerals such as potassium, copper, magnesium, iron, sodium, manganese and calcium (Table 2.2). Bananas contain dopamine; a powerful antioxidant and all B group' vitamins especially vitamin B6.

Table 2.2: Nutritional composition of banana

Nutrient	Unit	Value per 100 g
Water	g	74.91
Energy	kcal	89
Energy	kJ	371
Protein	g	1.09
Total lipid	g	0.33
Ash	g	0.82
Carbohydrate	g	22.84
Total dietary fibre	g	2.6
Total sugars	g	12.23
Sucrose	g	2.39
Glucose	g	4.98
Fructose	g	4.85
Lactose	g	0
Maltose	g	0.01
Galactose	g	0
Starch	g	5.38
Minerals		
Calcium	mg	5
Iron	mg	0.26
Magnesium	mg	27
Phosphorus	mg	22
Potassium	mg	358
Sodium	mg	1
Zinc	mg	0.15
Copper	mg	0.078
Manganese	mg	0.27
Selenium	µg	1
Fluoride	µg	2.2
Vitamins		
Vitamin C	mg	8.7
Thiamin	mg	0.031
Riboflavin	mg	0.073
Niacin	mg	0.665
Pantothenic acid	mg	0.334
Vitamin B-6	mg	0.367

Source : USDA National Nutrient Database, (2016**)**

2.5 Fermentation

According to modern industrial microbiology fermentation is a process of biotransformation carried out by microorganisms or their enzymes, irrespective of

whether it is based on fermentation in the classic sense (anaerobic and catabolism of organic substrates without the involvement of exogenous electron acceptors) or oxidative metabolism respiration (Forschungsgemeinschaft, 2010).

Alcoholic fermentation refers to the biochemical conversion of sugars within a particular medium to ethanol and carbon dioxide as pre-dominant fermentation products following action by micro-organisms species. Alcoholic fermentation is an anaerobic process mainly performed by yeasts such as that of the *S. cerevisiae* species (Stewart, 2014).

Fermentation is a practice enshrined in human tradition, with studies such as that of (Chambers & Pretorius, 2010) indicating the practice as being evident in the 3000B.C era. The uses of fermentation vary according to individual contexts and relevance, but a general depiction for the practice is that of increasing the consumption longevity, improving standards of quality (such as taste, aroma and other sensory properties), and reducing susceptibility to contamination by microbial species within the environment. Chambers & Pretorius, (2010) add that fermentation may also be a value addition mechanism for food products, especially when considered in terms of improving standards of quality as well as increasing the various ways or designs to present and consume a product. The relevance of fermentation is further underscored by actions aimed at eliminating wastage or losses of food as indicated in the study by (Zilberman & Kim, 2011). The fermentation functions as one way of utilizing excess food products or a solution towards preserving the suggested commodities for future utility (Zilberman & Kim, 2011). In the case of *Urgwagwa*, the role of alcoholic fermentation is seen as that of providing

an alternative utility domain for bananas, especially with regard to handling surplus stocks of the commodity.

2.6 Traditional alcoholic beverage production in Africa

Some of the most common and popular African alcoholic beverages are shown in Table 3. As it can be seen in the table, the most dominating fermentation substrate is often cereal-based. In general, banana beer production (even at traditional contexts) remains largely unexplored, with the exceptions of countries such as Rwanda where the industry shows greatest promise based on the availability of the raw material and consumption market.

Table 2.3: Some traditional African beer

Product	Raw material	Region
Agadagidi	Plantain	Nigeria
Borde	Maize or wheat	Ethiopia
Chikokiyana	Maize and millet	South Africa/Zimbabwe
Dolo	Sorghum	Togo/Bukinafaso
Doro	Sorghum	Zimbabwe
Ikigage	Sorghum	Rwanda/Burindi
Kaffir beer	Sorghum	South Africa
Merissa	Sorghum and millet	Sudan
Palm wine	Palm, trees	West Africa
Pito	Sorghum	Ghana/Togo
Tchapelo	Sorghum	Ivory Coast

Source: Shale *et al*., 2014

2.7 Sorghum as banana-based alcoholic beverage adjunct

Sorghum is an ancient grain which contributes to the number of elite grains to supply about 85% of world's food energy (Lindsay, 2010). Sorghum is valued to both its grains and stalk. Sorghum is widely used in food production system and it's known to be a rich source of macro and micronutrient which count 74.6 g/100g of carbohydrate, 11.3 g/100 g of protein, 350 mg/100g of potassium, iron, zinc and copper content as 2.24 mg/100g, 0.75mg/100g and 0.61mg/100g respectively (Lindsay, 2010). Sorghum is used in production of different fermented food products particularly in traditional brewing where it is processed into sorghum beer throughout Africa (Lyumugabe, Uyisenga, Songa, & Thonart, 2014; Konfo *et al*., 2015). In banana-based alcoholic beverage context sorghum is added as source of flavor and colour (Munyanganizi, 1975; Wilson, 2012).

2.8 Banana-based alcoholic beverages production

Use of bananas as a primary ingredient in banana-based alcoholic beverage called *Urwagwa* has largely been confined in rural and traditional contexts, hence the perception that it is largely a domestic production industry (Satav & Pethe, 2017). *Urwagwa* production uses traditional technology processing procedures which involve the use of spear grass (*Imperata cylindrica*) for juice extraction. Grilled ground sorghum flour is subsequently added to the extracted juice as adjunct to spur fermentation and also to impart colour and flavour to the product. Traditionally the mixture is approximately blended at a rate of 1kg of grilled ground sorghum flour and 10L of banana juice (Byarugaba-bazirake, 2008). Figure 2.5 shows a flow diagram of traditional *urwagwa* production process. Banana-based alcoholic

beverage quality depends to a great extent on the degree of dilution of the juice, amounts and quality of sorghum added as well as the conditions for fermentation.

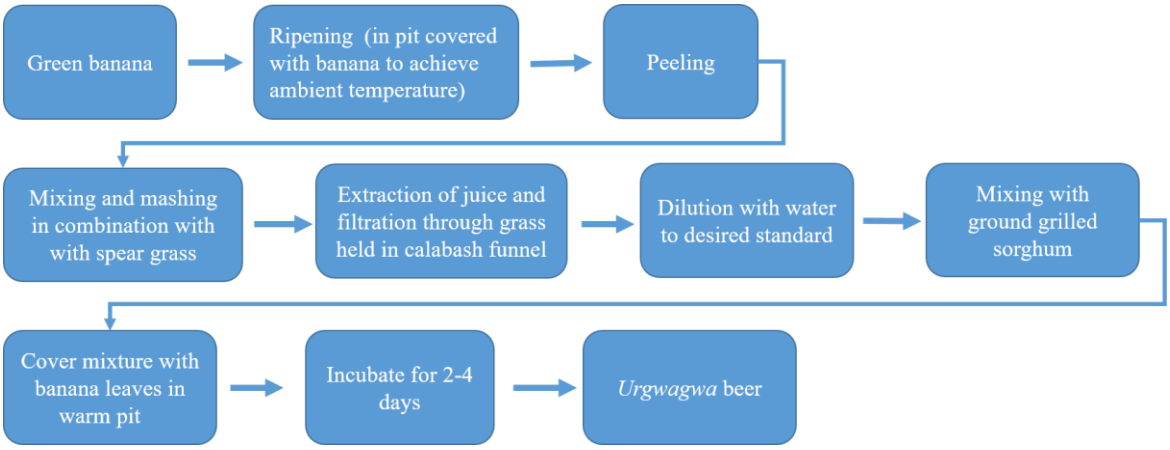


Figure 2.3: The General flow chart of traditional banana beer production
source: Shale *et al*., 2014

Normally, a banana-based beverage of 2 to 5% of ethanol is produced from diluted juice with the best flavor, bitter taste and golden color (Byarugaba-bazirake, 2008). According to Davies, (1993) a banana-based alcoholic beverage of 11-15% of alcohol is produced from undiluted juice. The main sugar in banana is glucose (19-22% of total sugar), followed by fructose (12-17% of total sugar) and sucrose (62-68% of total sugar) (Hammond *et al*.,1996).

The various banana beers brands in existence around the East African region are often brewed as a mixture of composites that include sorghum and other cereals as well as other fruit juices and water as a diluting or clarifying agent (Mohapatra *et al*., 2010). Rwanda's *Urwagwa* combines *Igikashi* (bitter) and *Kamaramasenge (Musa paradisiaca*) sweeter banana varieties, indicating a plausible need to meet taste specificities within the suggested product (Anderson & Moo-Young, 2008). Similarly, some brewing regions within the country supplement the banana-based

alcoholic beverage with juices for improving taste and quality (Dieu et al., 2017). Satav & Pethe, (2017) indicate that the traditional brewing methodologies that emphasized the use of natural materials persist in modern brewing circles. Additives such as processed sugar and flavouring chemicals are often discouraged, alternatives such as natural honey, herbal mints, and spices being encouraged as flavouring. However, commercial production of banana-based alcoholic beverage may require that the aforementioned additives be incorporated, as they aid in improving the efficacy of the production process (Karamura et al., 1998). Despite the envisioned changes in production style, current brewers and consumers of good banana generally agree that the product is derived from nearly-ripe (to ripe) bananas as the main substrate, the inclusion of cereal substrate(s) as a fermenting agent.

Of all the banana spp cultivated in Rwanda, M. *acuminate* is the predominant, hence a widely used banana species for banana-based alcoholic production (Brickell *et al*., 2009). The suggested species has the Asiatic regions as its endemic domain, but has had success as a consumable plant within the East African region. Brickell e*t al*., (2009) noted that there are various cultivars developed from the *M. acuminate* species that grow and are consumed in different regions around East Africa. The *Mdidde* cultivars (for instance *Entundu*) common in Rwanda, Burundi, and Uganda are generally unpalatable with bitter secretions making them only applicable in banana-based alcoholic beverage production (Karamura *et al*., 1998).

2.9 Factors influencing the fermentation in alcoholic beverage production

2.9.1 Juice clarification

Juice clarification before fermentation by settling is an important step which helps the removal of high amounts solid materials and maintain desired fruitiness flavour

of substrate (Byarugaba-bazirake, 2008). High level of suspended solids was found to impact the fermentation through increasing hydrogen sulphide production (Amerine *et al*., 1980). Juice clarification involves also the removals of suspended colloidal sulphur materials, thus avoiding the problems of hydrogen sulphide production during fermentation (Ribéreau-Gayon & Glories, 2006). Due to high nutrient removal during clarification the optimum growth of yeast is undermined and may lead to stuck of fermentation. The suspension of solids materials does not affect only the fermentation process but lead to haze formation which spoils the presentation of alcoholic beverages (Byarugaba-bazirake, 2008). Elimination of suspended solids materials in juice is the most crucial step prior fermentation which reduces the microbial load adhering on them.

2.9.2 Free available nitrogen

The presence of free available nitrogen is one of main factors that affects the fermentation and it has a strong relationship with ethanol produced (Picki-reix *et al*., 1986). High concentration of free amino acids corresponds to high ethanol production (Picki-reix & Ryburgh, 1986). Nitrogen available is needed by yeast cell for both growth and glycolytic pathway enzyme synthesis among which permease is the first enzyme responsible for transportation of amino acids and sugars into the cell (Jiranek , Langridge, 1995). Low nitrogen is linked to low cellular activity as well as stuck of fermentation kinetics. According to Jiranek , Langridge, (1995) yeast assimilable nitrogen is normally in form of ammonium and amino acids. However yeast cell growth preferably in the substrate supplemented by ammonium compared to amino acids. In particular ammonium and amino acids are consumed within 24-48

hrs, which means that the fermentation of sugar in the late phase occur in the presence of nitrogen starvation (König *et a*l., 2017). Amino acids such as glutamate and glutamine are excellently preferred by yeast for (Jiranek et al.,1993)

The supplementation of nitrogen materials during fermentation is the best way yeasts obtain free assimilable nitrogen. Different authors have highlighted different concentrations of nitrogen in various substrates. According to Sablayrolles *et al*.,(1996) the nitrogen concentration in grape must ranges from 60 to 400 mg/L. As the fermentation taking place the nitrogen concentration start to decrease and yeast cell would require the nitrogen supplementation for optimum growth. (Ribéreau-Gayon & Glories, 2006) reported an increase in fermentation rate with an increase of nitrogen supplementation (with diammonium phosphate and /or amino acids) of 800-2000 mg/L.

2.9.3 Juice composition

Normally fruit juice prepared for alcoholic beverage contains most of all nutrients required to fuel fermentation by fermenting microorganisms. However different procedures of juice preparation which include pressing may affect the composition of various fruit juices which may lead to higher phenol and tannin concentration, low acidity, higher pH as well as higher concentration of polysaccharides (Byarugaba-bazirake, 2008). Pressed juices prove to have higher levels of oxidative enzymes due to higher to the solids content (Byarugaba-bazirake, 2008). Nitrogen deficiency in juice composition is one of limiting factor that may lead stuck fermentation. Sugar concentration in fruit juice has also reported as a factor that influences the growth of yeast especially in lag-phase. The most optimum growth seemed to happen between 15 to 20 ^{o}Brix (Byarugaba-bazirake, 2008). Fruits juice with low sugar concentration

have been shown to result to fast and complete fermentation while those high in sugar concentration inhibit fermentation due to high osmotic pressure which pull out the water from yeast cells (König *et al*., 2017).

2.9.4 Acidity and pH

The pH levels are an essential component in the fermentation process as they have an impact on the quality of the fermenting process as well as outcome of products following the process. Studies testing osmo-tolerance thresholds yeast species indicate point to requisite pH values (between4.5-5.5) as being necessary for effective fermenting activity by yeast (Rosa, 2005). Other studies have also measured the effects of varying pH levels with key results indicating an increase in pH lowers sucrose metabolism, hence a reduction in the fluidity of the fermenting process (Kudo *et al*., 1998). The aforementioned studies underscore the relevance of investigations to ascertain pH during the fermenting process. Testing may be conducted through the isolation of a sample in which an indicator is introduced to verify pH levels. Changes in pH levels during the fermentation process also emerge as an important factor within the context of banana beer quality and yield. As such, the pH decreases with consequent increase in titratable acidity and ethanol. However, studies indicate that different substrate matters exhibit unique variations in pH changes, which could be suitable in determining different fermenting qualities of substrates added to banana juice for alcoholic beverage brewing purposes (Tropea *et al*., 2016). Hydrogen ion concentration has a significant influence on industrial fermentation due to its importance in controlling bacterial contamination which can have an effect on yeast growth, fermentation rates and by-product formation (Rangel & Tóth, 1998). The best ethanol yields are generally obtained at pH 4.5-4.7 and at

higher pH, more glycerol and organic acids are formed at the expense of ethanol (Lewis & Young, 2001). Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0 (Lewis & Young, 2001). As, the gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast (Pascal Ribereau-Gayon, Denis Dubourdieu, 2006).

This increased conversion is independent of the presence of nutrient supplements in the medium (Campbell, 1999). If the pH is adjusted to 7 or above, acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Lewis & Young, 2001)

2.9.5 Temperature

Temperature changes during the fermentation process have a drastic effect on the activity of microorganisms as well as the quality of product following the culmination of the brewing process. The fermentation temperature affect yeast cells growth, fermentation and metabolism process. According to Zhu, (2013) the fermentation temperature can regulate the gene expression of aroma compounds metabolism. Fermentation temperature has been used also to differentiate yeast for fermentation purpose. For lager fermentation, yeast the optimum temperature averages from 8 to 15 ^{o}C while the optimum temperature for ale fermentation yeast ranges from 15 to 22 ^{o}C (Stewart, 2014, Nachel, 2008). However most brewery

yeast is grown at optimum fermentation temperature between 22 ^{o}C (71.6^{o}F) to 27^{o}C (80.6 ^{o}F) (Schanderl, 1959). Margalit, (1997) reported that fermentation can take place at 5^{o}C to 38^{o}C because below and above that temperature range may led to enzymes dysfunction. It is advised to not allow fermentation temperature to exceed 33^{o}C as it may stop fermentation. The fermentation temperature may affect the fermentation rate by which a warm fermentation (25 to 30^{o}C) may lead to the completion of fermentation within four to six days while cool to dry fermentation (about 8 to 10 ^{o}C) may take up to three to four weeks (Margalit, 1997).

2.9.6 Sulphur dioxide

Substrate for alcoholic beverage production is a favourable media for both desirable and undesirable source of microorganism, hence the addition of Sulphur dioxide (SO_2) at the beginning of fermentation to limit the propagation of undesirable microorganisms (yeast, lactic acid bacteria, to lesser extent acetic acid bacteria). Although sulphur SO_2 is highly toxic to bacteria and moulds and indigenous yeast, commercial yeast strain are selected to have a higher tolerance to SO_2 (Ribereau-Gayon, Pascal, Denis Dubourdieu, Bernard Doneche, 2006). The supplementation of (20 to 30 mg/L) of sulphur before fermentation is known to slow fermentation but; eventually it complete fermentation rapidly (Ribereau-Gayon, Pascal, Denis Dubourdieu, Bernard Doneche, 2006).

2.9.7 Aeration

Even though the fermentation process is carried out in anaerobic condition the introduction of oxygen in fermentation substrate prior to fermentation impact on vigor of yeast performance (Margalit, 1997). Under fermentation conditions yeast

does not require oxygen for energy production but for efficient growth a significant amount of oxygen is required (Ribéreau-Gayon, 1999). According to *(*Sablayrolles *et al.*, 1996) an average of 5 to 10 mg/L of oxygen is required for optimum yeast growth. The decrease in oxygen availability prior fermentation may results to decrease in biomass production and glycolysis rate because of inhibition of fatty acids and sterol synthesis in yeast cell.

2.9.8 Ethanol and carbon dioxide

Ethanol and carbon dioxide are main fermentation end products from simple sugar. Ethanol itself is one of an inhibiting effect that increasing the stress condition to yeast cell (Pascal Ribereau-Gayon, Denis Dubourdieu, 2006). According to Margalit,(1997), a slight inhibition of yeast growth by ethanol is started at the concentration of 2% (v/v) and full inhibition happens at 16-17% (v/v) while on the other hand, yeast growth starts slowing at the concentration of 3-4 % (v/v) of ethanol and ceases at 8 to 15% (v/v) based on yeast strains (Alexandre & Charpentier, 1998). Compared to ethanol, carbon dioxide effect on alcoholic fermentation is less considered and it is not preventing yeast growth. Although the carbon dioxide content of 15 g/L was found to result to yeast growth stoppage (Amerine *et al*.,1980).

2.9.9 Minerals

Minerals are important factors for yeast growth and alcoholic fermentation. For normal alcoholic fermentation minerals such as potassium, magnesium, manganese, zinc and cobalt are required for optimum fermentation (Ferreira, 2004). Normally bananas are rich source of minerals that ensure a satisfactory of fermentation. Minerals concentration in bananas can however vary depending on growing

environment, location on the bunch, as well as soil nutrient content (IITA, 1993). For example a deficiency in zinc and magnesium in fermentation substrate may directly affect sugar metabolism (Ferreira, 2004). The presence of magnesium in substrate was found to involve in stabilization of nucleic acid, proteins, polysaccharides and plays a key role in cell growth and proliferation (Walker, 1994). Kudo *et al*.,(1998) have reported the important role played by potassium in early fermentation of fermenting substrate through acceleration of glucose consumption by yeast. In addition, potassium which is found in high concentration in banana plays a key role in pH tolerance by *Saccharomyces* (Asli, 2010). Potassium deficiency may lead to the reduction of fermentation capacity of individual yeast cells or fermentation capacity of culture through the loss of yeast cell viability (Ferreira, 2004).

2.9.10 Sugar levels

The fermentation process is characterized by the conversion of simple sugars (glucose, fructose, and sucrose) into ethanol. As such, assessing the availability of sugars in comparison to expected yields and properties of the product are essential (Stewart, 2014). The initial sugar concentration is the main parameter which drives the fermentation process. This parameter is highly dependent upon the fermenting substrate. During the fermentation process, the uptake of fermentable sugars by yeast is an ordered process by which glucose and fructose are consumed first, with any sucrose present being hydrolysed extracellularly by β-fructosidase (invertase) secreted by yeast (Stewart, 2014). As wort is an aqueous mixture of high fermentable sugar, wort density is usually measured as a fermentation progress indicator (Stewart, 2014). The decline in density observed in fermentation process is directly related to sugar consumption and subsequent ethanol production. On another

hand the concentration of sugar in wort is expressed in degree Brix which equivalent to 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by weight (Wilson, 2012).

Changes in sugar levels are also essential in determining the microbial properties of organism involved in fermentation. For instance, a slow decline in sugar levels may be an indicator of poor fermentation qualities by microorganisms based on low population numbers or impeded metabolic functionality (Pascal Ribereau-Gayon, Dubourdieu, 2006).

2.10 Microorganisms associated with banana-based alcoholic beverage production

Microorganisms are involved in chemical and physical changes that occur during banana-based alcoholic beverage production and are equally important for the sensory characteristics of the end-product. Shale *et al*. (2014) reported that the chemical composition (including sugars) of banana as a substrate for beer production makes it a suitable material for different microbial species. Munyanganizi, (1983) reported 82% of yeast strains which belongs to 4 genera: *Hansenula*, *Kluyveromyces*, *Pichia* and *saccharomyces* in banana juice and banana wine. *Saccharomyces cerevisiae* and *saccharomyces italicus* species constituted 54% and 21% respectively of *saccharomyces* genera and they are the main species fuelling fermentation of *Kivu* banana wine. Although yeasts are main primers of alcoholic fermentation, there are other microbial groups such as acetic acid bacteria, lactic acid bacteria and moulds implicated in the fermentation process.

2.10.1 Yeasts

Generally yeasts are ubiquitous unicellular microorganisms whose existence in environment is highly dependent on physiological adaptability (Rosa, 2005). For optimum growth yeast require a simple media containing a fermentable carbohydrate, to supply adequate nitrogen for protein synthesis, minerals salts and some growth factors (Campbell, 1999). Yeasts are normally recognized as non-pathogenic and they as have been exploited by humankind since the beginning of Neolithic period around 8000 years BC (König *et al*., 2017). Yeasts are recognized because of their importance in fermentation process where they convert sugar to alcohol, carbon dioxide and other compounds which influence alcoholic beverage flavor (Stewart, 2014). According to Stewart, (2014), all yeast strains used in fermentation belong to the genus *saccharomyces* and *cerevisiae* species. *Saccharomyces* species have a numbers of unique traits that make them different from other yeast species. In fact majority of yeast species which belong to *saccharomyces* genera can survive without oxygen (using fermentation process), but other yeast species do not have this ability (Labagnara, 2009). However based on industrial brewing purpose yeast are classified into ale yeast (*saccharomyces cerevisiae*) and lager yeast (S. *carlsbergensis*). Ale yeast operate at room temperature (18-22 ^{o}C), ferment quickly and produce fruitiness characteristics while lager yeast works at cold temperature (8-15 ^{o}C) (Stewart, 2014). Up to 15 species have been reported to be involved in alcoholic fermentation (Ribereau-Gayon *et al*., 2006). *Saccharomyces cerevisiae* are primary microorganisms that are responsible for conversion of sugar to alcohol in fermentation process but other yeast have been reported to contribute to aroma and flavour profile of beer (Hutkins, 2006). Some of these native yeast include

Hanseniaspora (anamorph: *Kloekera apiculate*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*) and *candida stellata*. *Candida, Hansenula, Pichia, Hanseniaspora, Rhodotorula, T. delbrueckii, K. gamospora* are recognized as higher producers of esters compound (König *et al*., 2017). Although the aforementioned species have been reported to contribute to wine profile flavour, their evaluation in fermentation have been found to reduce ethanol content of wine. Some yeast such *brettanomyces, zygosaccharomysces bailii, Hanseniaspora uvarum, Kloeckera apiculata* and *Candida krusei* have been reported to contribute to high acetic acid production (König *et al*., 2017).

2.10.2 Acetic acid bacteria

Acetic acid bacteria (AAB) are a group of microorganisms featured in sugar-rich and alcohol-rich environments. Acetic acid bacteria are subdivided into *Acetobacter* and *Gluconobacter* genera and they comprise an important industrial group of Gram-negative bacteria capable of converting ethanol to acetic acid. According to (Campbell, 1999) the family of *acetarobacteraceae* has a unique characteristics of oxidizing alcohol and glycerol to acetic acid and glycose to gluconic acid.

The metabolic activity and optimum growth of AAB are enhanced by the presence of oxygen at pH of 5.5-6.3 and the temperature of 25-30 ^{o}C but majority of strains are able to multiply at low acidic environment of pH 3.5 (Ribereau-Gayon *et al*., 2006). This feature has made acetic acid bacteria to be useful in biotechnological application such as ascorbic acid and cellulose production. In food industry, AAB are used in production of several beverages and food such as cocoa, kombucha, vinegar and other fermented beverages (Sigmon, 2003). Although the acetic acid bacteria are

completely inhibited by ethanol concentration higher than 6% v/v, there are strains such as *Acetobacter* spp.BSO5 and *Gluconobacter oxydans* subsp. *oxydans* strain NCIB9013 that are able to survive 12-13% v/v of ethanol produced from high gravity brews (Campbell, 1999). However presence of acetic acid bacteria activity leads to the spoilage of foods including soft drinks, fruits, wine, and beer.

2.10.3 Lactic acid bacteria

Lactic acid bacteria (*Bacterium lactis*) (LAB) are characterized by production of lactic acid as major catabolic end product from glucose. Lactic acid bacteria have been used by human as starter culture in food fermentation for more than 5000 years (König *et al*., 2017). Lactic acid bacteria are divided into obligate homofermentative strains like *Pediococcus* and l*actobacillus* which produce lactic acid as sole end product while wine-related obligate heterofermentative LAB strains like *Leuconostoc*, *Oeconostoc*, and *Weissella* produce lactic acid, CO_2, ethanol and acetate from glucose. Most lactic acid bacteria featured in alcoholic fermentation belong to the genera of *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Ribereau-Gayon *et al*., 2006).

Some of LAB play an important role in food preservation by preventing food spoilage and growth of pathogenic microorganisms because of acidification (König *et a*l., 2017). Wilson, (2012) reported the presence of a high number of lactic acid bacteria (4.12×10^{13}cfu/ml) in *urwagwa* which shows that the spontaneous fermentation procedures mixture of alcoholic and lactic acid products. Lactic acid bacteria are most desirable for their role in malolactic fermentation during fermentation but the can produce several off-flavours in wine. While other

microorganisms are inhibited by the concentration of above 4% v/v of ethanol, some LAB species can grow at higher ethanol concentration (König *et al*., 2017).

2.11 Yeast identification and characterization

Identification of yeast is based on physiological and molecular approaches for species identification. According to Dilip, Berka, & Singh, (2002), a major challenge affecting such research studies is the need to undertake identification procedures amidst confined parameters on rapidity and efficiency. As such, a holistic approach that includes both physiological and molecular approaches may be convenient, but the latter emerges as the most convenient in such study contexts.

2.11.1 Morphological identification of yeast

Morphology is an essential tool in classification and identification of yeast which includes the description of shape, size and internal structures of individual yeast cells (George, 1955). Colony characterizations of yeast are based on appearance of yeast growth visible to naked eye such as texture, elevation, margin, and colony color. According George, (1955), yeast cells and its structural parts are described as tri-dimensional objects such as sphere, eggshaped, oliveshaped, globe, cylindrical or as two-dimensional like bottle shape, circular, triangular and elliptical as they appear when viewed through a microscope. *Saccharomyces cerevisiae* which are most domesticated yeast, appear as eggshaped, elliptical and occasionally spherical under the microscope. Other yeast species of genus *Torulopsis* (Torula) form a perfect spheres; *saccharomyces ellipsoideus* (a variant of *S.cerevisiae*), *saccharomyces pastorianus* and *Schizosaccharomyces* species are usually more cylindrical shaped cells (George, 1955).

Based on macroscopic characterization yeasts that belong to *saccharomyces* genera are generally flat, smooth, moist, glistening or dull, and cream to tannish cream color, *Endomyces* spp are large white colonies while , *candida* spp are flat, smooth and large colonies (Omar-Zahid, 2013). On other hand, the morphology of *S.cerevisiae* is evidenced by circular colony and a variable color from cream to green. Generally most of yeast colony has an umbonate elevation (Labagnara, 2009).

2.11.2 Molecular identification of yeast

The identification of specific bacterial or fungal species, including yeasts has developed greatly as the techniques based on DNA analyses have become available. Molecular identification of indigenous yeast especially *saccharomyces cerevisiae* strains is an important step towards a better conservation and employment of microbial diversity. Utilization of selected indigenous yeast involved in fermentation would be a powerful tool to enhance organoleptic and sensory property of alcoholic beverage. Different molecular techniques have been used for more reliable taxonomic studies. A variety of techniques have been successfully applied to identify yeast species involved in fermentation, including the qPCR, restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, multiplex reverse transcription PCR (RT-PCR), and Single Nucleotide Polymorphisms (SNPs) (Barbosa *et al*., 2015).

The genome of *Saccharomyces*

Generally *Saccharomyces cerevisiae* has a relatively small genome, a large numbers of chromosomes, little repetitive DNA and few introns. Some haploid strains contain 12-13 megabases of nuclear DNA distributed along 16 linear chromosomes whose sizes vary from 250 to 2000 kb. Mitotic crossing over and gene conversion promotes

a faster adaptation to environmental changes than spontaneous mutations, which occur at comparatively very low rates. Each strain displays specific phenotypic traits. It is largely demonstrated that wine yeasts have a high level of chromosomal length polymorphism. The comparison of ribosomal RNA (rRNA) and its template, generally, allow to differentiate yeast taxonomy based on ribosomal DNA (rDNA) and this technique has been used extensively in recent years. It allows finding the relationships among many yeast species identification. Some of these molecular technologies are based on sequence analysis; one of the first was the amplification of the 26S rDNA D1/D2 domain. However, these methods now are impractical for the routine screening concerning the identification of yeast isolates (Stielow *et al*., 2015). According to Esteve-Zarzoso *et al*.,(1999) the amplification and restriction analysis of non-coding internal transcribed spacers region (ITS) and 5.8 SrRNA gene was found to be the most rapid identification method allowing the identification of more than 300 yeast species including *Saccharomyces* sensu stricto group (*S.cerevisiae, S.bayanus, S.pastorianus, S.paradoxus, S.mikatae, S.kudriavzevii* and *S.carianus*).

Internal transcribed spacer (ITS) as fungal barcode

As mentioned before yeast are taxonomically classified and identified based on parts of DNA region such as sections of nuclear ribosomal RNA large subunit (D1-D2 domains of 26/28S), Based on the strength, impact and its higher taxonomic resolution, the rDNA ITS was suctioned as universal fungal DANA barcode (Stielow *et al*., 2015). Fungal internal transcribed spacers (ITS) region varies roughly, with some exception approximately between 450 and 750 base pairs in length and consists of three subregions including variable spacers ITS1 and ITS2 and intercalary 5.8 gene (Blaalid *et al*., 2013). While the 5.8S gene is highly conserved, the ITS1 and

ITS2 spacers normally provide resolution at a within- genus and often within-species level. Although the ITS region has been selected as the fungal DNA barcode, it lacks the necessary resolution in some groups of fungi (Blaalid *et al*., 2013). Different universal primers are used to identify yeast species in wine fermentation by amplification rDNA repeat unit that include 5.8SrRNA gene with two non-coding region, but the analysis of ITS region of yeast isolates was found to be successfully amplified by ITS1-ITS4 primers. In fact PCR products showed different size which means the presence of diverse yeast in wine fermentation and the amplified ITS fragments length varied from 400bp in *Candida* and *Metschnikowia* to 850 bp in *Saccharomyces* (Labagnara, 2009).

On the other hand, the ITS1 and ITS2 in *Saccharomyces cerevisiae* is spanning 361and 232bp long respectively, while in *Saccharomyces pombe* corresponds to 412-420 bp and 300 bp (Korabecna, 2007). Specific primers to amplify ITS region in genomic DNA of fungal community including yeast involved fermentation have been validated among which ITS1F-ITS2 and ITS3-ITS4 resulted into the best resolution with 484 and 850 bp of amplicons respectively (Op De Beeck *et al*., 2014). A study done by (Op De Beeck *et a*l., 2014) using the PCR-ITS technique has identified different yeast species by using the primers that included ITS 3:5'-TCC GTA GGT GAA CCT GCC G-3' and ITS 4:5'-TCC TCC GCT TAT TGA TAT GC-3' as being commonly used.

CHAPTER THREE
MATERALS AND METHODS

3.1 Research Design

The study was used a completely randomized design to determine the proximate composition of the banana juice-sorghum composite mixture used in production of *Urwagwa*, determination of physical and chemical changes during the suggested fermentation process and specific yeast involved in the fermentation of banana-based alcoholic beverage called *Urwagwa*. The research approach was included collecting unripened banana from farmers and making ripen, juice squeezing, determination of the proximate composition of the banana-sorghum composite mixture and effect of ground grilled *sorghum vulgaris* sp concentration on physico-chemical change during a fermentation process and finally, there was an isolation and identification of indigenous yeast involved in the fermentation process.

3.2 Collection and ripening of banana for beer production

Four physiologically mature banana cultivars namely: Kayinja (*Musa* genus), Indaya, Intuntu or Igikashi *musa* groups (AA, AB, AAA, AAB, ABB) and Poyo *musa robusta (AAA group),* in which Intuntu represented 67 percent were collected from a local market around Rugende in Gasabo District of Rwanda and transported in plastic crates under normal conditions to Centre de Formations et des Recherches pour le Développement des Technologies Appropriées (CFRDTA) for experimentation.

The bananas were placed in plastic container and covered by spear grass (*Imperata cylindirica*) and green banana leaves in order to ensure the warm conditions and promote ethylene production and accumulation necessary for ripening. The bananas took 6 days to ripen indicated by their change in peel colour to yellow and softening

3.3 Juice extraction

Ripe bananas (*musa acuminata*) were peeled manually by hand. Juice extraction was mechanically done by mixing 69 kg of peeled banana with 6.9 kg of special grass (*Imperata cylindirica)* which had been previously washed and dried in order to remove dust and other miscellaneous materials that could contaminate the beverage. The banana juice extraction, which was aided by *Imperata cylindirica,* was achieved by use of a fabricated juice extracting machine. The juice was filtered using a plastic sieve and the remaining juice in banana-grass pulp was exhausted by pressing using a fabricated juice extracting machine pressor.

3.4 Determination of composition of banana juice-sorghum composites mixture

3.4.1 Banana juice-sorghum combinations used in *Urwagwa* production

The obtained banana juice was pasteurized at 90^{o}C for 30 minutes to prevent premature fermentation caused by indigenous yeast and lactic acid bacteria and other contaminating microorganisms. Lightly roasted *sorghum vulgaris sp*, often red sorghum in ground form is commonly used in the production of banana beer in Rwanda as an adjunct where it is thought to contribute to the flavour and colour of the finished product (Munyanganizi,1975;Wilson, 2012). Different proportions of sorghum were mixed with a constant amount of extracted banana juice as shown in (Table 3.1). The *sorghum vulgaris sp* used in this study which was previously washed, dried, lightly roasted and ground for *urwagwa* production was bought from Kimironko market in Rwanda.

Table 0.1: Banana juice-sorghum combinations used in the production of *Urwagwa* through spontaneous fermentation.

Banana juice (l)	Sorghum (w/v)
3	0
3	2
3	5
3	7
3	10
3	15

3.4.2 Determination of initial pH of banana juice and banana juice-sorghum mixture

The pH of banana juice and banana juice-sorghum composite mixture before fermentation was evaluated with pH meter (PB-11Sortorius, Germany) at 25^{o}C. A volume of 20 ml was taken and the measurement of each sample was done after calibration of pH-meter with a buffer (4-7).

3.4.3 Determination of protein content in banana juice-sorghum mixture before fermentation

The protein content of banana juice-sorghum mixture was determined on the basis of total nitrogen content by using auto titration Kjeltec models 2300 (Denmark). A representative sample of 0.4g of each of the composite mixture was weighted into 100 ml labelled digestion tubes by use of an electronic balance (Sortorius CPA3245). One Kjeltabs Cu 3.5 tablet of catalyst and 6 ml of concentrated H_2SO_4 were added to each digestion tube. The samples were heated at 420°C for 2 hours and after digestion, they were cooled to room temperature (25°C). After cooling the tubes containing the samples were transferred into distillation unit (Foss 2300 Kjeltec) and

protein percentage determined according to the Kjeltec machine manufacturer's instructions where mass of each sample was entered in the Kjeltec system and the protein was reported as percentage and calculated according to the formula by (Muriro,2017). On the basis of early determinations, the average nitrogen (N) content of proteins was found to be about 16 percent, which led to use of the calculation N x 6.25 (1/0.16 = 6.25) to convert nitrogen content into protein content. The following equation was used in the calculation of protein content in the samples.

$$\%\ Nitrogen = \frac{(T - B) * N * 14.007 * 100}{W(mg)}$$

$$\%\ Protein\ = \%\ Nitrogen * F$$

Where;

T = Titration volume for sample (ml)

B = Titration volume for blank (ml)

F = Conversion factor for nitrogen to protein

W = Weight of the sample (mg)

3.4.4 Determination of total soluble solids (TSS) in banana juice-sorghum mixture before fermentation

Total soluble solids of the banana juice and banana juice-sorghum were determined as °Brix according to Serpen, (2012) with some modification by which hand held optical refractometer was replaced by A.Kruss Optronic refractometer GmbDR6100 (Germany) which gives a direct reading of ^{o}Brix, or relative sugar concentration. The refractometer was cleaned using distilled water before sample analysis. Distilled water was used as control in this experiment (0°Brix). The amount of sugar in the sample was directly read from the refractometer.

3.5 Fermentation of banana juice and banana juice-sorghum mixtures

The fermentation was carried out in six fermenters of 3 litres each. One fermenter, with no added sorghum (banana juice only) served as the control. The experiment was carried out in triplicates. The content in fermenters was left to ferment spontaneously at room temperature (25^{o}C) for 6 days. The whole production process, from ripening of the bananas to final fermented alcoholic product took 13 days.

3.5.1 Monitoring of the fermentation process

During fermentation 100 ml of samples was collected, in sterile bottles from each fermenter after every 24 hours for 6 days to determine various physico-chemical parameters (pH, titratable acidity, ethanol accumulation and sugar). After collection the samples were pasteurized at 60^{o}C for 15 minutes to end fermentation after which they were stored at 4^{o}C in the refrigerator before analysis.

3.5.2 Determination of ethanol content

Test for ethanol content was conducted using WineScan™ SO2 (Denmark) from the beginning to the end of the fermentation process. A sample of 100 ml of sample to be analysed was filtered by using Whatman filters of 11µm size and 20 ml of filtrate transferred into vials from where they were sucked by the column of WineScan™ SO2 for analysis. Before analysing each sample, there was auto-cleaning of WineScan™ SO2 through zero setting the machine according to the manufacturer's instructions.

3.5.3 Determination of total soluble solids (TSS)

Total soluble solids of *urwagwa* were determined as described for banana juice-sorghum composite mixture above.

3.5.4 Determination of pH

The pH of the whole fermentation process was evaluated as aforementioned for banana juice and banana juice-sorghum combination before fermentation.

3.5.5 Determination of titratable acidity

The titratable acidity, expressed in percent of lactic acid, was determined by the titrimetric method according to (Association of Official Analytical Chemist[AOAC], 2005). A 5ml subsample from the 100 ml sample was used for titration after decarbonating by shaking. The sample was diluted twice with distilled water (10 ml) which was previously brought to boil at 90^{o}C and cooled at room temperature (25°C). Three drops of 1% phenolphthalein indicator was added to the sample in a conical flask and titrated with 0.0909M NaOH to a persistent faint pink colour compared against a white background. The titre volume was noted and used for calculations of the amount of total titratable acidity as percentage of lactic acid using the equation below.

$$\%\ lactic\ acid = \frac{ml\ of\ 0.0909M\ NaOH * Normality\ of\ NaOH * MW\ of\ acid}{Volume\ of\ sample * 10}$$

Where MW = molecular weight

3.6 Isolation and identification of indigenous yeast species involved in fermentation of *urwagwa*

3.6.1 Isolation of indigenous yeast involved in fermentation of *Urwagwa*

3.6.1.1 Sampling for yeast isolation

One hundred millilitres of *urwagwa* were collected aseptically in 300 ml in sterile bottles from each fermentation plastic containers. A maximum of nine samples, from six fermenters and three from traditional brewers were collected. All samples were stored at 4^{o}C in the refrigerator until use.

3.6.1.2 Isolation of indigenous yeast from *urwagwa* samples

The indigenous yeasts from collected samples were isolated at the end of fermentation process according to Koulougliotis & Eriotou, (2016) methodology with some modification whereby yeast extract peptone dextrose agar was replaced by yeast extract agar . Five tenfold dilution (10^{-1} to 10^{-5}) of the samples was carried out using peptone buffer as the diluent. An aliquot of 100 µl from each dilution was spread plated onto Yeast Extract Agar plates (3% yeast extract, 5% peptone and 15% agar) supplemented by chloramphenicol (50g/ml) (Oxoid England) previously prepared according to manufactures instructions. The plates were incubated at 25° C for 5 days. At the end of the incubation period, the plates were kept at 4° C followed by purification through three repetitive streaking on potato dextrose agar medium plates previously prepared according to manufacturer's instruction. The plates were incubated at 25^{o}C for additional periods of 5 days and one pure colony from each plate was sub-cultured into 9 ml of MRS broth (10.00 g/L proteose peptone, 10.00g/L beef extract, 5.00 g/L yeast extract, 20.00g/L dextrose, 1.00 g/L polysorbate80, 2.00 g/L ammonium citrate, 5.00 g/L sodium acetate, 0.10 g/L magnesium sulphate, 0.05 g/L manganese sulphate, 2.00 g/L dipotassium phosphate) at final pH of 6.5±0.2 HiMedia laboratories Pvt. Ltd. India.

3.6.1.3 Morphological identification of yeast

Isolated yeast colonies and were identified and characterized based on colony features such as colony color, elevation, form, texture and under microscopic examination. The shape of the yeast cell and its structural parts were alternatively described according to George, (1955).

3.6.2 Molecular identification of indigenous yeast involved in fermentation of *Urwagw*a

3.6.2.1 DNA extraction

Yeasts isolates grown in MRS broth were transferred to an Eppendorf tubes and centrifuged for 10 min at 13000 rpm using Thermofisher Scientific centrifuge (model 41930819, Germany) and obtained pellets were washed by re-suspension the cells into equal volume of TE buffer and centrifuged for 5 minutes at 13000 rpm and the supernatant were discarded.

Approximately 50-100mg of the pellets was used for DNA extraction from each sample. The DNA was extracted in duplicate using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit according to manufacturer's instructions (Zymo Research Corp, USA).This resulted in duplicate for each of nine samples and the DNA was stored -20 ^{o}C.

3.6.2.2 DNA amplification

Total genomic DNA sample was used to amplify ITS1-5.8S RNA-ITS2 gene by Applied Biosystems by life technology (Proflex™ base PCR system, Singapore). An internal transcribed spacer was amplified by two set of universal primers synthesised by Inquaba Biotec East Africa ITS1-F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS2 (5-GCTGCGTTCTTCATCGATGC-3) and ITS3 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Op De Beeck et al., 2014) under the following conditions: Initial denaturation for the first pair of primer was set at 95°C for 5 minutes while the second one the initial denaturation time was 2 minutes and remaining conditions were the same. The above conditions were followed by 40 cycles of denaturation at 95°C for 30 seconds,

annealing at 55°C for 1minute and final extension phase was performed at 72°C for 10 minutes. The reactions were carried out in 50 µl volumes and each reaction was contained 25 µl Thermo Scientific PCR Master mix 2x for 200rxns of 0.05u/µl *Taq* DNA polymerase, reaction buffer, 4mM $MgCl_2$, 0.4mM of each dNTP (dATP, dCTP, dGTP and dTTP) 0.5 µl forward and reverse primers of 10 µm/µl, 1µl of template and 23 µl of nuclease free water and the amplification was confirmed by running the gel electrophoresis.

3.6.2.3 Agarose gel electrophoresis

Electrophoresis of PCR products was done on agarose gel. It was made by melting the agarose 2% (w/v) in the presence of 2x TAE buffer supplemented by 5 µl fluorescence dyes (truegel) and the mixture was brought to boil in a microwave for 2 min in order to dissolve completely agarose powder. The agarose was then allowed to cool to about 55^{o}C, and poured into a gel chamber. Combs were inserted and the gel was allowed to polymerise. The gel was then submerged into electrophoresis buffer tank, after removing the combs, wells flashed with buffer and samples loaded. The samples were mixed with dye prior to load into wells. A 1kb ladder was loaded along the samples as DNA molecular size marker. The power of 75 voltage of an electric current was then applied for 45 minutes and samples allowed to migrate until the dye front was near the end of the gel and the gel was subjected to UV transillumination for imaging using UVITEC (Made in France). The bands were visualized by fluorescence dye staining (Sambrook and Russel, 2001).

3.6.2.4 Purification of PCR products

The PCR products were purified using QIAquick PCR purification kit (Quiagen, Germany) according to manufacturer's instructions.

3.6.2.5 Sequencing and phylogenetic analysis

The PCR products from the amplification of *ITS* region of yeast isolates after purification was sent to a commercial sequencing provider (Macrogen Netherland). In this case dye-terminator (Sanger sequencing) technique was used. The obtained sequences in FASTA format were compared to the sequence deposited in GenBank DNA database by using Basic Alignment Search tool (BLAST) on the National Center for Biotechnology (NCBI) (https://www.ncbi.nlm.nih.gov) and those with high similarity were retrieved. Alignment of sequences obtained for isolates and reference sequences retrieved from database was done by using CLUSTAL W software. To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbour-joining Parsimony (Tamura et al.,2013). The ITS1-5.8rDNA-ITS2 gene sequences of isolates and their closest *Saccharomyces cerevisiae* strains were aligned and phylogenetic trees were generated through neighbour-joining statistical method using Bootstrap method for the test of phylogeny 1000 replicates through MEGA7.018 software.

3.7 Data analysis

Proximate composition of banana juice and banana juice-sorghum composite mixture and the physico-chemical data analysis were performed by using descriptive statistic tool (Graphpad prism7). The data obtained from analysis were presented as mean±standard deviation with triplicate in trials for each treatment. Morphological

identification of indigenous yeasts was done by observation through dissecting microscope (x40). The ITS1-5.8rDNA-ITS2 sequences from yeast isolates were viewed and edited using Chromas Pro version 2.1.8 (2018) software package. The sequences were aligned using CLUSTAL W to provide full sequences after which they were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) to find the closely related based on query cover, E-values and percentage of similarity. The sequences of isolates and their closely related were aligned and processed to generate phylogenetic trees using MEGA7 .018 software package.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Proximate composition of banana juice and banana juice-sorghum mixture before fermentation

The addition of grilled sorghum to banana juice marginally increased the pH, soluble sugar concentration and protein content of the composite mixtures, and this change tended to increase with the percentage increase in grilled sorghum added (Table 4.1). Out of the three parameters determined, more change was noticeable with protein concentration than with the changes in pH and soluble sugar concentration on addition of grilled sorghum. The pH ranged from 4.68±0.05 to 4.75±0.03 (0% sorghum added and 15% sorghum added, respectively), soluble sugar concentration ranged from 21.00±0.07 to 21.37±0.17 (0% sorghum added and 15% sorghum added, respectively) and protein content 1.0±0.08 to 2.7±0.20 (0% sorghum added and 15% sorghum added respectively). Total soluble solids and protein are the main nutrients required for growth of yeast in wort fermentation. The slight increase in total soluble solids as well as protein concentration on addition of varying quantities of grilled sorghum can be attributed to the presence of free sugars and protein found in sorghum.

Table 4.1: pH, Sugar and Protein content of banana juice and banana juice-sorghum mixture.

Sorghum concentration	pH	Sugar (°Brix)	Protein (g)
0%	4.68±0.05[a]	21.00±0.07[a]	1.0±0.08 [a]
2%	4.69±0.02[ab]	21.07±0.06 [a]	1.3±0.01 [a]
5%	4.70±0.01[ab]	21.09±0.02 [a]	1.6±0.01 [a]
7%	4.71±0.06[b]	21.10±0.10 [a]	1.8±0.09 [b]
10%	4.73±0.04[c]	21.23±0.12 [a]	2.1±0.14 [c]
15%	4.75±0.03[d]	21.37±0.17 [a]	2.7±0.20 [d]
Significance at P= 0.05	P<0.0001	P=0.2388	P<0.0001

The data represent the means with standard deviations of three replicates (n=3).
Letters on each number indicate mean separation by Turkey'HSD ANOVA post-hoc test. Means not sharing a superscript in a group (Protein, TSS and pH) within a column are significantly different ($p<0.05$).

4.2 Effect of sorghum content on ethanol concentration

Generally, the onset of ethanol production started at the same time in all the six fermenters (including the control), with even quantifiable levels at the start of the experiment (Figure 4.1). It was observed that the production of ethanol for all the composites tended to increase with time as well as the increase in percentage of sorghum added to the banana juice. The trend for the production of ethanol for all the composites except the one containing no sorghum (control) followed a similar pattern. There was a sharp increase in ethanol production for all the composites after the 48th hour except for the one containing no sorghum where the increase in this parameter seemed to occur rather steadily up to the 72nd hour, after which there was a sharp increase. Except for the control and the composite containing 10% sorghum, in all the other combinations, ethanol production tended to start decreasing before or at the start of the 144th hour of fermentation with the composite containing 15% sorghum showing the earliest onset of ethanol production decline. The final ethanol content in all fermenters ranged from (10.3-12.2%) with the control resulting in the

least concentration and the composite containing 10% sorghum resulting into the highest concentration (Figure 1).

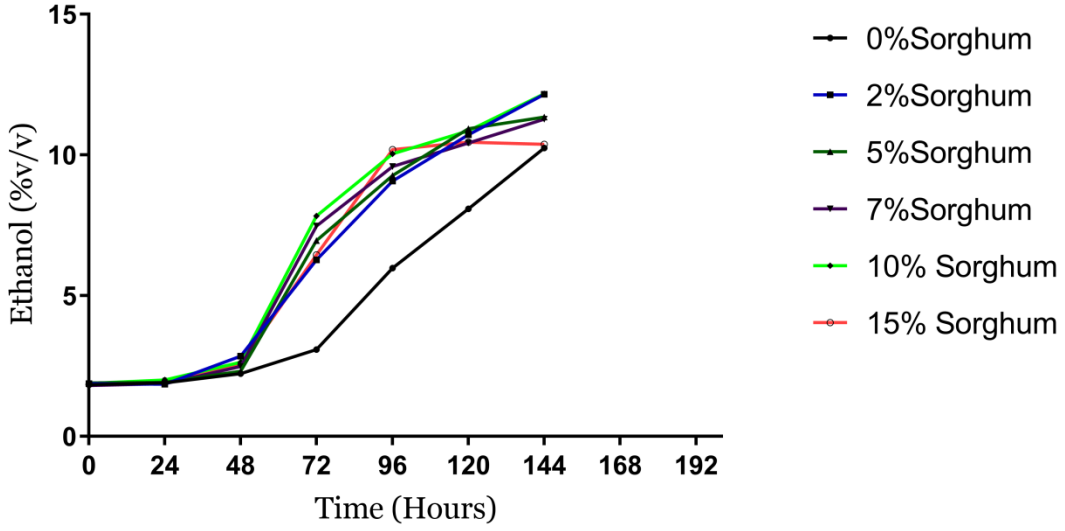


Figure 4.1: Changes in ethanol concentration during the spontaneous fermentation of *urwagwa* with varying sorghum combination.

These results showed that the alcohol content does not only result from banana juice but also from added roasted sorghum. The initial concentration of soluble sugars in banana juice with no added sorghum was the least at 21°Brix (Table 4.1). It is therefore not a surprise that the control (banana juice with no added sorghum) yielded the least ethanol than the fermenters containing the composite mixtures. Sugars are normally converted to ethanol by yeasts during fermentation, and the higher the concentration, the higher the expected ethanol yield (Raikar, 2012). From limited data available, previous studies by Davies, (1993) and Shale *et al*.,(2013) showed that banana beer with an ethanol content of 11-15% (v/v) and 8.7-18.1% (v/v) was produced from undiluted banana juice and from indigenous banana beer respectively. Similar studies done by Byarugaba-Bazirake,(2013) and Dieu *et al*., (2017) reported an alcohol content of 6.4-14.6% (v/v) from banana beverage made

from three banana cultivars and 12% (v/v) ethanol in Rwanda banana beverage respectively.

There was early decline of ethanol in fermenter with 15% of sorghum. According to Ferreira,(2004) the acetic acid bacteria may be responsible for oxidation of ethanol to acetic acid, leading to its reduction. The trend of higher ethanol production in all fermenters in comparison to the control could be attributed to the presence of nitrogen and minerals from sorghum that could have favoured the conditions for yeast growth compared to the fermentation of banana juice with no sorghum added (Deesuth et al., 2012). Similar study by Picki-reix *et al*. (1986) has established the clear relationship between the ethanol production with free amino acids concentration in wort by which high free amino acid corresponded with high ethanol production. According to Carrau et al. (2008), Picki-reix *et al*. (1986), Barrajón-Simancas *et al*. (2011), Jiranek *et al*. (1995) and Sablayrolles *et al*. (1996) nitrogen deficiency in wort may lead to lower fermentation rate the problem of which could be solved by supplementation with a nitrogen source. The need for a source of nitrogen in fermentation is also highlighted by Deesuth *et al*., (2012). Sorghum is known to be a good source of nitrogen as well as other essential nutrients including minerals such as potassium that are reported to be necessary for yeast growth (Lindsay, 2010). Kudo *et al*., (1998) reported the significance of potassium in early fermentation of wort through acceleration of glucose consumption by yeast. In addition, potassium which is found in high concentration in sorghum plays a key role in pH tolerance of *Saccharomyces spp* and high yield of ethanol is produced at pH of 4.5 (Asli, 2010). Potassium inadequacy may lead to the reduction of fermentation

capacity of individual yeast cells or fermentation capacity of culture through the loss of yeast cell viability (Ferreira, 2004).

4.3 Effect of sorghum concentration on soluble sugar during fermentation

Sugar is one of the main substrate in alcoholic fermentation of the wort. The initial concentration of total soluble sugars in all the combinations, at time zero, including in the control was on average, 21°Brix. This remained largely unchanged for all the combinations up to the 48th hour of the fermentation except for the sample containing 15% sorghum which showed a slight reduction (20°Brix). There was a sharp decline in soluble sugar concentration after the 48th hour of fermentation for all combinations except with the control where the decrease was observed to be steady (Figure 4.2)

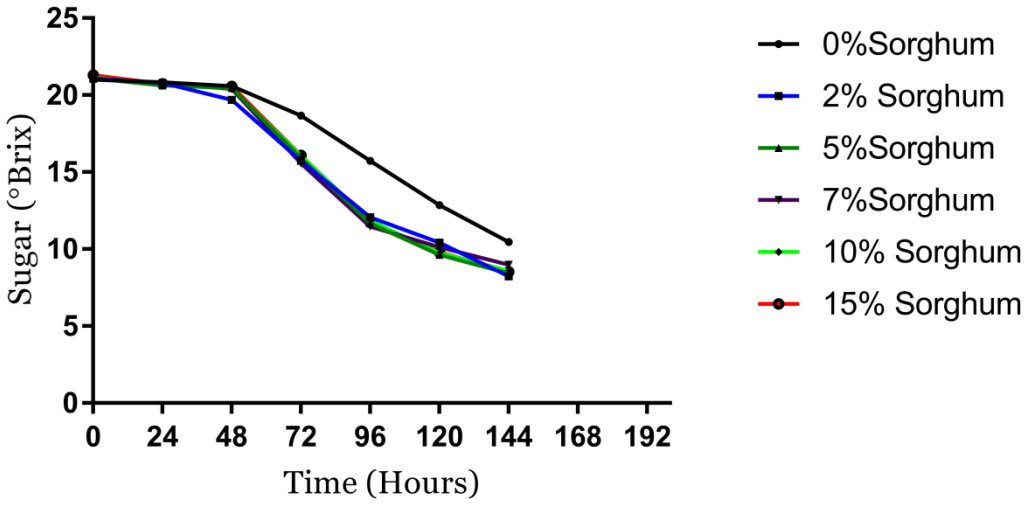


Figure 4.2: Changes in soluble sugar concentration (°Brix) during fermentation of *urwagwa* with varying sorghum concentration.

At the end of the fermentation (144th hour), all the samples seemed to have similar levels of soluble sugars remaining. Previous studies indicate that the main fermentable sugars in ripe bananas are glucose (19-22% of total sugar), fructose (12-17% of total sugar) and sucrose (62-68% of total sugar) (Hammond *et al*., 1996).

According to Hammond *et al*., (1996) the ideal sugar content of unfermented wort and which lead to the production of beverage of 8-12% (v/v) ethanol should be around 15-25% (w/w). This observation agrees with the findings of this study in terms of soluble sugar concentration and ethanol produced during fermentation. Similar findings have also been reported (Byarugaba-Bazirake, 2013). The sugar level at the end of fermentation was slightly higher in fermenter with zero percent of sorghum (9.7°Brix) which is attributable to high residual sugar.

4.4 Effect of sorghum concentration on pH variation during fermentation

The pH level is an essential component of the fermentation process as it can have an impact on the quality of the fermenting process as well as outcome of the final product following the process. The initial pH of all the samples, including the control was similar, ranging from 4.68-4.75 (Table 4.1). From a previous study of Munyanganizi (1974) and (Newilah *et al*., 2009), the pH of fully ripened bananas ranges from 4.2 to 4.9, a finding which is in agreement with the results of this study. There was a common trend for all the samples during the fermentation process; the pH decreased as the fermentation progressed (Figure 4.3).

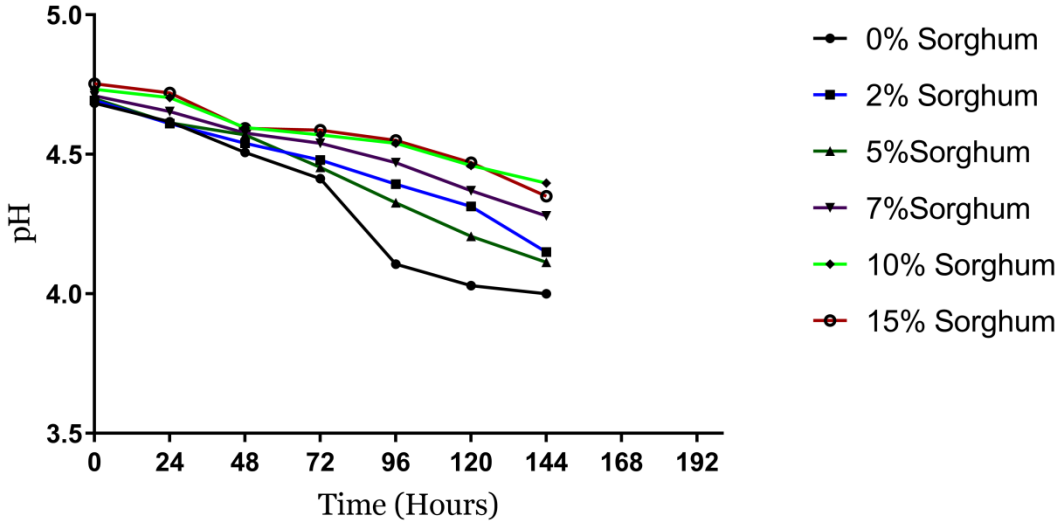


Figure 4.3: Changes in pH during fermentation of *urwagwa* with varying sorghum concentration**.**

With the exception of the control (no sorghum added), the pH of the rest of the samples were found to decrease steadily up to the end of the fermentation period. There was a sharp decrease of pH in the control sample between the 72nd and 96th hour of fermentation. The less sharp drop in pH in samples containing sorghum compared to the control could be explained by higher content of minerals in sorghum that may have led to high buffering of the wort (Munyanganizi,1976). At the end of fermentation period the pH of samples was ranged from 4.0-4.4. According to Rwanda Standard Board, (2017) the pH of banana-based alcoholic beverages should range from 4.0 to 5.0 which means that the final products of this study complies with the country's requirement in terms of this attribute.

4.5 Effect of sorghum concentration on titratable acidity

Titratable acidity reflects the number of protons recovered during titration of acid by strong base at specific end point and it is an approximation of total acidity in a given product (Boulton, 1980). All the samples prior to the fermentation process (time

zero) had quantifiable acidity which averaged to 0.17 % of TA. There was a noticeably exponential increase in titratable acidity for all the samples between time zero and 24 hours during fermentation, after which the increase, for all the samples became gradual (Figure 3).

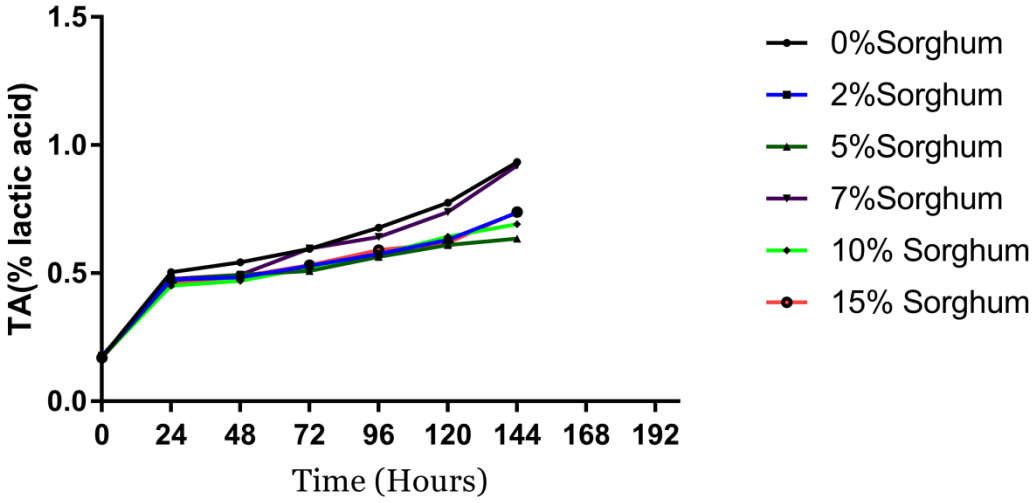


Figure 4.4: Changes in titratable acidity during fermentation of *urwagwa* with varying sorghum concentration

Generally, a similar trend for all the samples in the development of the acidity (increase) was observed throughout the fermentation period. The acidity in the final products ranged from 0.64 and 0.93% of TA respectively. The highest titratable acidity was found in banana juice fermented without sorghum compared to those with added sorghum. High titratable acidity in sample containing zero percent sorghum may be attributed to the possibility of presence of lactic bacteria involved in early fermentation compared to samples with sorghum added as at this point the fermenters with sorghum added had high ethanol content that could reduce the population of lactic acid bacteria (Sigmon, 2003). According to Wilson *et al*., (2012), titratable acidity in traditional alcoholic banana beverage ranges from 0.18 to 0.9%

ile Akubor, Obio, Nwadomere, & Obiomah, (2003) found 0.85% in final banana alcoholic beverage. Byarugaba-Bazirake, (2013) also reported titratable acidity expressed as g/L in banana alcoholic beverage produced by conventional and modern techniques from different banana cultivars which ranged from 5.24 to 8.4, which is in agreement with obtained results of this study.

4.6 Isolation and identification of indigenous yeast involved in fermentation of *urwagwa*

Plate1 shows morphological features of yeast colonies after incubation at 25^{o}C. Plate 1a shows growth on yeast extract agar medium while Plate 1b shows growth on potato dextrose agar.

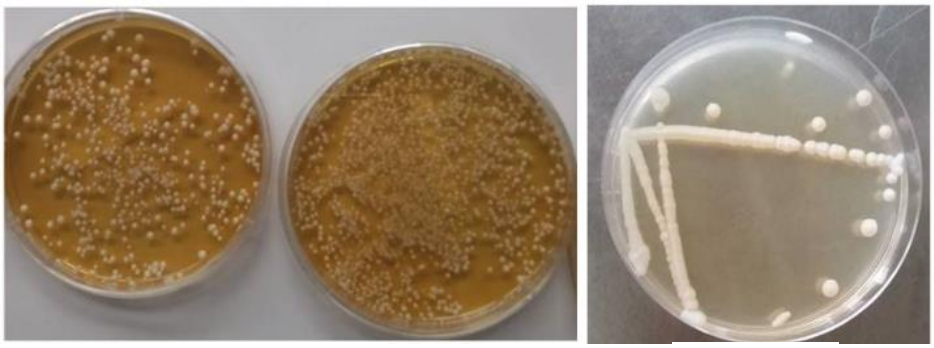


Plate1 a Plate 1b

Plate 4.1: Plate 1a: Yeast extract agar medium for isolation
P1b: Potato dextrose agar medium for colonies purification.

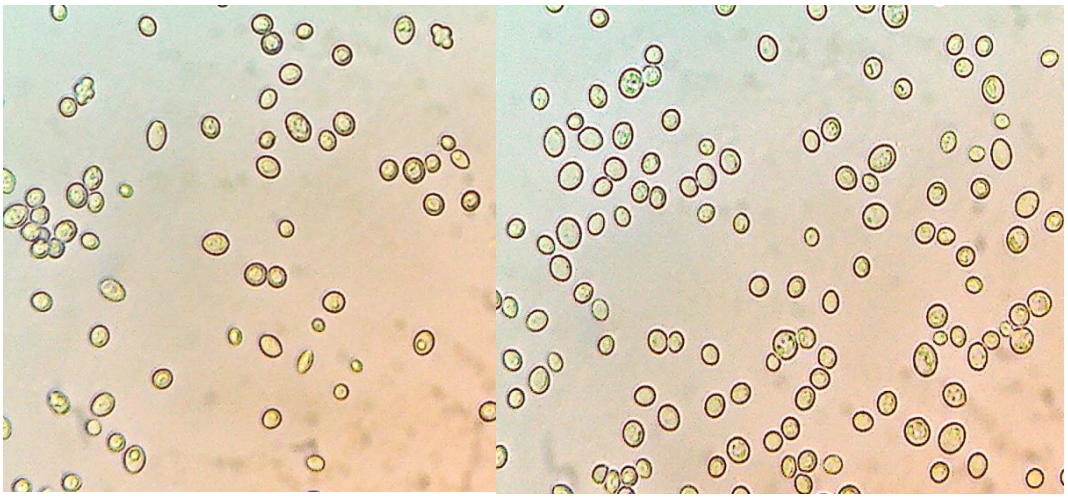


Plate 4.2: Morphological characteristics of yeast cell as viewed under dissecting microscope (x40)

4.6.1 Morphological identification of indigenous yeast

4.6.1.1 Colony and cell morphology

Morphological characterization of indigenous yeast was based on classical macroscopic attributes of colour, shape, elevation and texture of pure colonies (George, 1955). Most colonies were able to grow within 3-4 days of incubation at 25^{o}C. The colony shapes of indigenous yeast isolated from banana-based alcoholic beverages ranged from circular and umbonate (Plate1). The colony texture was smooth and the color ranged from white to cream. The cells ranged from egg-shaped to circular (Table 4.3).

Table 4.2: Morphological characteristic of indigenous yeast involved in fermentation *Urwagwa.*

	Colony characterization				Cell characterization
Isolate	Colony color	Colony form	Colony elevation	Colony margin	Cell arrangement
YBBO	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
YBB2	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
YBB5	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
YBB7	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
YBB10	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
YBB15	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
MB1	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
MB2	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical
BN2	White cream	Circular	Umbonate	smooth	Egg-shaped and cylindrical

YBBO: Indigenous yeast isolated from banana-based alcoholic beverage fermented with zero percent of sorghum.
YBB2: Indigenous yeast isolated from banana-based alcoholic beverage fermented with 2% of sorghum.
YBB5: Indigenous yeast isolated from banana-based alcoholic beverage fermented with 5% of sorghum.
YBB7: Indigenous yeast isolated from banana-based alcoholic beverage fermented with 7% of sorghum.
YBB10: Indigenous yeast isolated from banana-based alcoholic beverage fermented with 10% of sorghum.
YBB15: Indigenous yeast isolated from banana-based alcoholic beverage fermented with 15% of sorghum.
MB1and MB2: Indigenous yeast isolated from Munyanganizi brewery
BN2: Indigenous yeast isolated from Bernadette brewery

4.6.2 Molecular identification of indigenous yeast

4.6.2.1 PCR amplification of ITS1-5.8rDNA-ITS2 by ITS1F-ITS2 and ITS3-ITS4 primers from isolates

Genomic DNA was extracted successfully from all isolates using Zymo research kit procedures. Amplification of ITS1-5.8rDNA-ITS2 gene with fungal universal primers ITS1F-ITS2 and ITS3-ITS4 (Op De Beeck *et al*., 2014) in order get to reliable results and they have yielded an amplification product of approximately 484 bp and 850bp respectively (Plate 4.3 and 4.4).

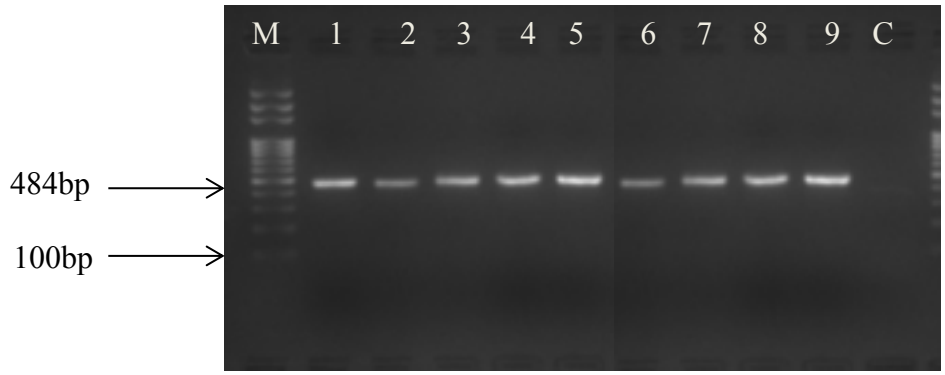


Plate 4.3: PCR products after amplification of ITS1-5.8S rDNA-ITS2 of the isolates from *urwagwa* using universal primers ITS1F and ITS2
Lanes 1(**YBBO**), 2(**YBB2**), 3(**YBB5**), 4(**YBB7**), 5(**YBB10**), 6(**YBB15**),7(**MB1**), 8(**MB2**), 9(**NB2**), (**C**) negative control and (**M**) M- 484bp Molecular marker size.

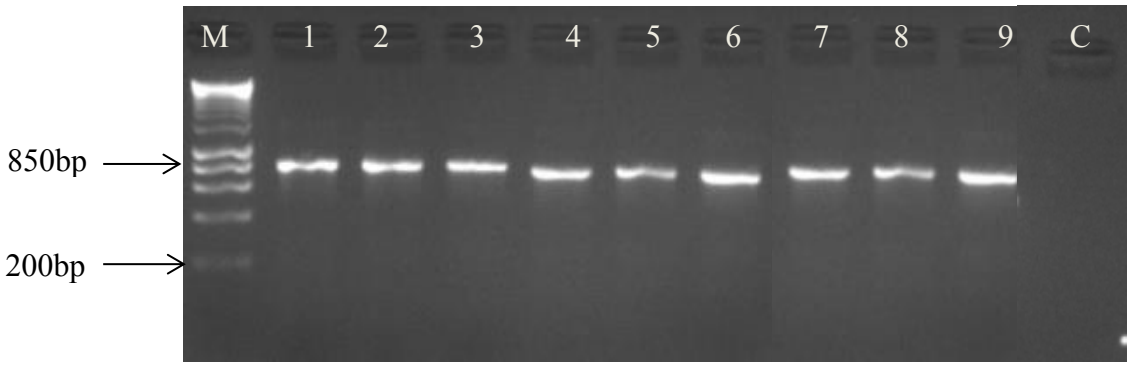


Plate 4.4: PCR products after amplification of ITS1-5.8SrDNA-ITS4 of the isolates from *urwagwa* using universal primers ITS3and ITS4.
Lanes 1(**YBBO**), 2(**YBB2**), 3(**YBB5**), 4(**YBB7**), 5(**YBB10**), 6(**YBB15**),7(**MB1**), 8(**MB2**), 9(**NB2**), (**C**) negative control and (**M**) M- 850bp Molecular marker size.

4.6.2.2 Phylogenetic analysis of sequences

Using BLAST programme the ITS sequences amplified by ITS1F-ITS2 primers of all isolates were compared with ITS sequences available in database showed that all isolates were from genus S*accharomyces* and *cerevisiae* species with similarities between 98% and 100% of previous sequences in data base (NCBI) . Among these were; *Saccharomyces cerevisiae strain 1000, Saccharomyces cerevisiae strain IFM 61207***,** *Saccharomyces cerevisiae strain Udatsuyama-1* and others (Table 4.4).

Five isolates which represent 55% of all isolates belonged to *Saccharomyces cerevisae* strain 1000 with 99 to 100 % similarities. Four isolates had 45% and belonged to genus *Saccharomyces* species *cerevisiae* with different strains with similarities between 98 and 100 % (Table 4.4). All isolates sequences were placed into a phylogenetic tree and phylogenetic analysis (Figure 4.5) showed that all isolates were clustered into genus *saccharomyces sp*, isolates YBBO, YBB2, YBB5, YBB10, YBB15 and BN2 were closely related to *saccharomyces cerevisiae* strain 1000 with 100% sequence similarity while Isolates YBB7 was closely related to *Saccharomyces cerevisiae* strain IFM 58282 with 99% similarity. However, MB1 and MB2 were grouped into different group which were distant to other group but they were clustered into genus *Saccharomyces* sp. *cerevisiae.*

Table 4.3: BLAST analysis results of the isolates from nine fermenters and their percentage of relatedness using ITS1F and ITS2 primers.

Isolate	Next Neighbour	Accession Number	% similarity
YBB0 (ITS1F)	*Saccharomyces cerevisiae strain 1000*	LC269108.1	100
YBB2 (ITS1F)	*Saccharomyces cerevisiae strain 1000*	LC269108.1	100
YBB5 (ITS1F)	*Saccharomyces cerevisiae strain 1000*	LC269108.1	100
YBB7 (ITS1F)	*Saccharomyces cerevisiae strain IFM 61207*	LC413776.1	99
	Saccharomyces cerevisiae strain IFM 58282	LC413774.1	99
YBB10 (ITS1F)	*Saccharomyces cerevisiae strain Udatsuyama-1*	LC215452.1	100
	Saccharomyces cerevisiae strain PUMY065	LC042148.1	100
YBB15 (ITS1F)	*Saccharomyces cerevisiae strain 1000*	LC269108.1	99
	Saccharomyces cerevisiae strain IFM 40026	LC413760.1	99
MB1 (ITS1F)	*Saccharomyces cerevisiae CBS:12552*	KY105006.1	99
	Saccharomyces cf. cerevisiae/paradoxus strain CBS:4456	KY105156.1	99
MB2 (ITS1F)	*Saccharomyces cerevisiae culture CBS:12555*	KY105010.1	98
	Saccharomyces cerevisiae strain ABM51215	HG532088.1	98
BN2 (ITS1F)	Saccharomyces cerevisiae strain 1000	LC269108.1	99
	Saccharomyces cerevisiae strain MUCL51215	MG183699.1	99

The same isolates were amplified by ITS3-ITS4 primers and the sequence analysis (BLAST) of results showed that all isolates belonged to genus *Saccharomyce*s, *cerevisiae* species with 97 and 100% similarities (Table 4.5). Among them were; *Saccharomyces cerevisiae isolate B-WHX-12-48, Saccharomyces cerevisiae isolate 27 18S*, *Saccharomyces cerevisiae isolate B-NC-12-OZ03, Saccharomyces cerevisiae isolate L2M* and others (Table 4.6). Compared to isolates similarities amplified by ITS1F-ITS2 primers, only 3 isolates amplified by ITS3-ITS4 primers which represent 33 % of isolates belonged to genus *Saccharomyces cerevisiae* isolate B-WHX-12-48 and *Saccharomyces cerevisiae* strain D120B, respectively, with 100% similarities. Six isolates had 67% and belonged to genus *Saccharomyces* species *cerevisiae* with different strains with similarities between 97 and 100 % (Table 4.6).

The results after placing sequences on tree (Figure 4.6) showed that all isolates clustered into genus *Saccharomyces* where isolates YBB0, YBB2, YBB10 and YBB15 were closely related to *Saccharomyces cerevisiae* strain (MH459411.1) with 99% similarities. Isolates YBB5 and YBB7 were clustered into the same group and they were related to first big group while MB2, BN2 were in the same group, but a bit distant to the first two group which belonged to genus S*accharomyces* sp. Finally, MB1 isolate was found to be distant from all groups but also belonged to genus *Saccharomyces* sp.

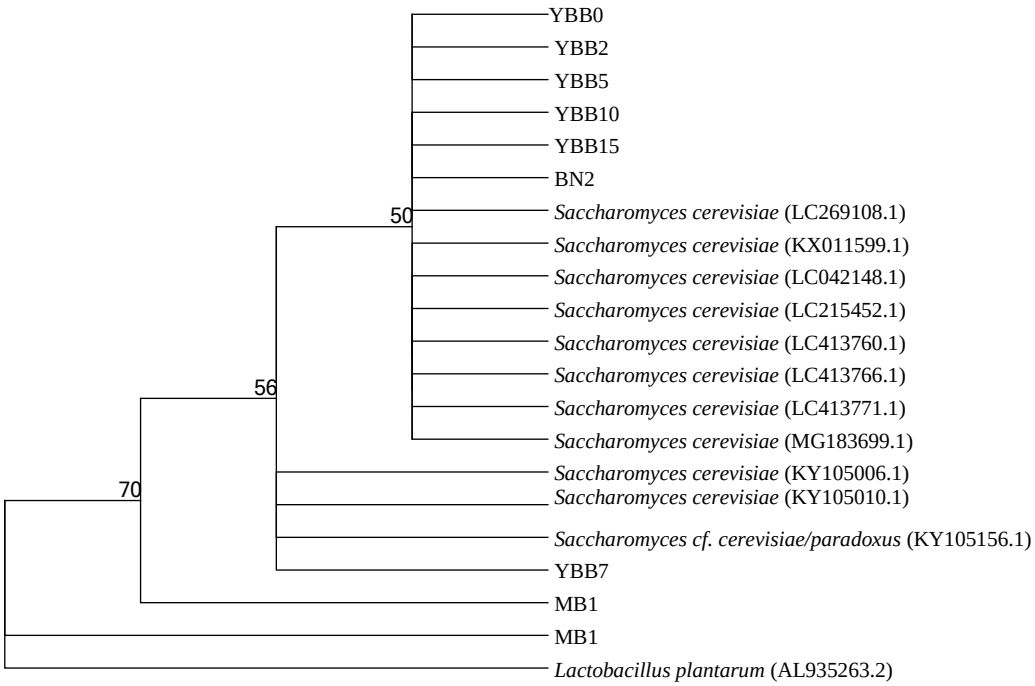


Figure 4.5: Phylogenetic relationship of indigenous yeast sequences of isolates of *Saccharomyces cerevisiae* from *urwagwa* using Neighbor-Joining method.
Lactobacillus plantarum was used as the out group. The evolutionary history was inferred by using the Maximum Likelihood method based on the (Tamura K. *et al.*, 2013) .The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates approximately 5% sequence difference. The analysis involved 21 nucleotide sequences. Evolutionary analyses were conducted in MEGA7**.**

Table 4.4: BLAST analysis results of the isolates from nine fermenters and their percentage of relatedness using ITS3 and IT4 primers.

Isolate	Next Neighbour	Accession Number	% similarity
YBB0 (ITS3)	*Saccharomyces cerevisiae isolate B-WHX-12-48*	KC544501.1	100
YBB2 (ITS3)	*Saccharomyces cerevisiae isolate 27 18S*	KT175188.1	99
	Saccharomyces cerevisiae isolate B-NC-12-OZ03	KF728786.1	99
YBB5 (ITS3)	*Saccharomyces cerevisiae isolate B-WHX-12-48*	KC544501.1	99
	Saccharomyces cerevisiae isolate B-WHX-12-43	KC544486.1	99
YBB7 (ITS3)	*Saccharomyces cerevisiae isolate N8*	KX824758.1	99
	Saccharomyces cerevisiae isolate L2M	KP723678.1	99
YBB10 (ITS3)	*Saccharomyces cerevisiae isolate B-WHX-12-48*	KC544501.1	100
YBB15 (ITS3)	*Saccharomyces cerevisiae strain D120B*	KP674649.1	100
MB1 (ITS3)	*Saccharomyces cerevisiae isolate 2276*	KY596697.1	97
	Saccharomyces cerevisiae strain Husl_FF7	MH459411.1	97
	Saccharomyces cerevisiae strain Husl_FF19	MH459412.1	97
MB2 (ITS3)	*Saccharomyces cerevisiae strain Husl_FF19*	MH459412.1	99
BN2 (ITS3)	*Saccharomyces cerevisiae strain Y1 18S*	KP216389.1	99
	Saccharomyces cerevisiae isolate JRC5	KX668409.1	99

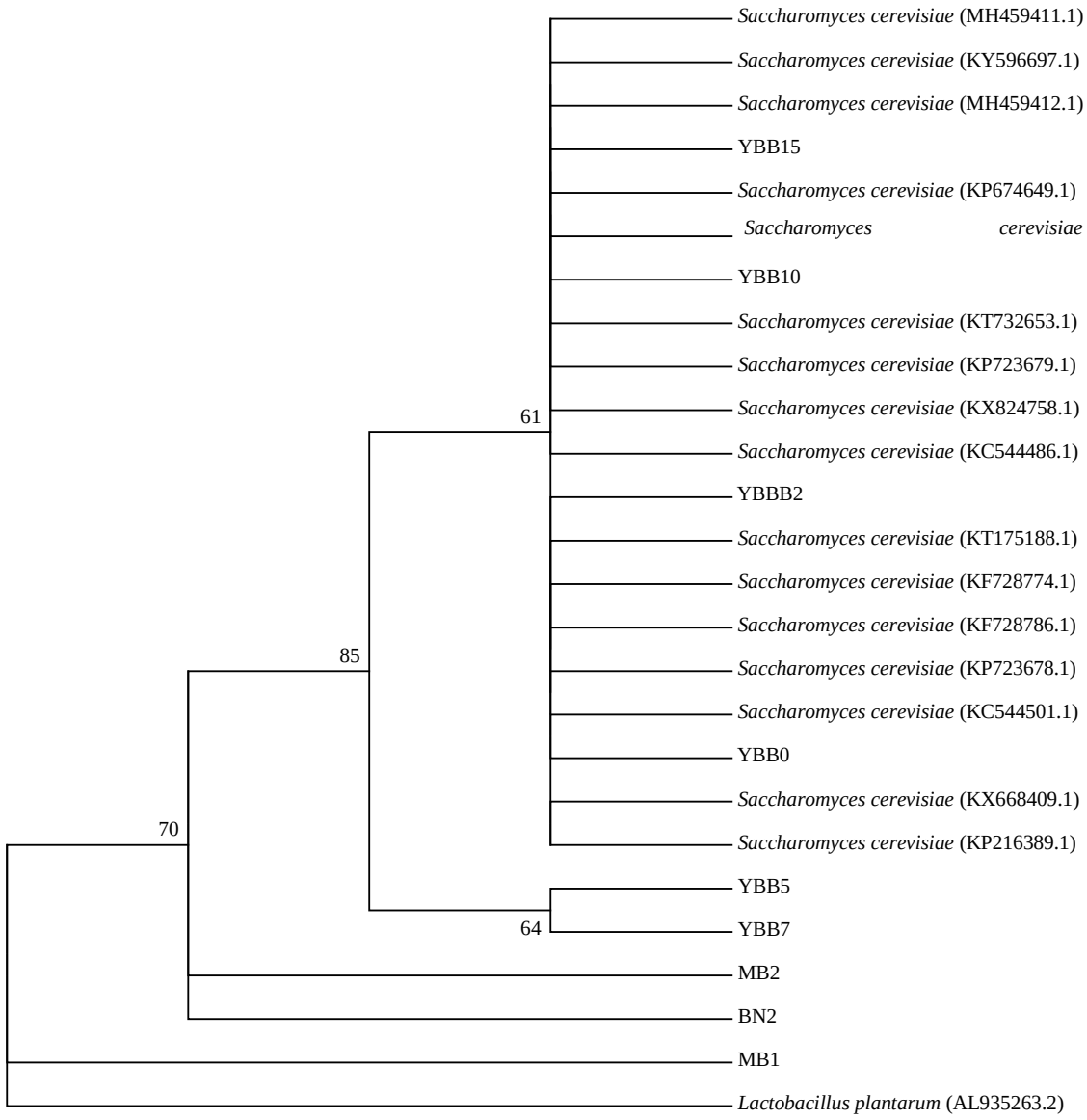


Figure 4.6: Phylogenetic relationship of indigenous yeast sequences of isolates of saccharomyces sp amplified by ITS3-ITS4 primers from urwagwa using Neighbor-Joining method. *Lactobacillus plantarum* was used as the out group. The evolutionary history was inferred by using the Maximum Likelihood method based on the (Tamura K. *et al*., 2013) .The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar indicates approximately 5% sequence difference. The analysis involved 21 nucleotide sequences. Evolutionary analyses were conducted in MEGA7.

Morphological identification of indigenous yeasts based on colony and cell features showed that the indigenous yeast that involved fermentation were clustered into genus *Saccharomyces cerevisiae* which is in accordance with the description given by George, (1955), Labagnara, (2009) and Omar-Zahid, (2013). The presence of indigenous yeast involved in fermentation of *urwagwa* was confirmed by identification based on amplification of ITS region as most variable and conserved region in fungal community by two set of primers ITS1F-ITS2 and ITS3-ITS4 which resulted in the identification of different *Saccharomyces cerevisiae* strains that were present in fermentation of *urwagwa*. The ITS region of all isolates studied showed the same molecular size of about 484 bp in isolates amplified by ITS1F-ITS2 and 850bp in isolates amplified by ITS3-ITS4 respectively.

Molecular identification of indigenous yeast strains based on amplification of ITS1-5.8rDNA-ITS2 by ITS1F-IT2 primers through BLAST analysis of sequences showed that isolates YBB0, YBB2, YBB5 and YBB15 were clustered into genus *saccharomyces cerevisiae* strain 1000 with 99 and 100 % similarities (Table 4.5). The phylogenetic analysis (Figure 4.5) showed that the aforementioned isolates, YBB10 and BN2 isolates are clustered into the same taxa. According to a previous study Nwuche *et al*.,(2018) *Saccharomyces cerevisiae* strain 1000 is described as thermotolerant and acid-tolerant strain. Based on the above characteristics strain 1000 LC269108.1 has been selected for bioethanol production because of its ability to withstand to multiple stress conditions pH ranged 4-5 and 10% w/v ethanol which are similar to the conditions where the above strains were isolated. Isolates YBB7, MB1 and MB2 which were clustered into *Saccharomyces sensu* stricto group

Saccharomyces cf. cerevisiae/paradoxus strain CBS: 4456 were reported among most important strains of genus *Saccharomyces spp* capable to withstand stressful conditions of high ethanol concentration. They have high fermentation efficiency, ability to produce and consume ethanol (Reis *et al*, 2013). On other hand phylogenetic analysis (Figure 4.6) of sequence of isolates amplified by ITS3-ITS4 primers showed that isolates YBB0, YBB2, YBB10 and YBB15 were clustered into the same group and were closely related to *Saccharomyces cerevisiae isolate B-WHX-12-48*. According to Wang *et al*., (2015) *Saccharomyces cerevisiae isolate B-WHX-12-48* is described as osmotolerant strain capable of resisting high osmotic pressure. They also grow fast in high concentration of glucose ranging from 20 to 40% w/v.

Saccharomyces cerevisiae Strain NCIM3186 in the same group was reported to be a suitable strain for bioethanol production from sweet sorghum stalk (Goud & Ulaganathan, 2015). Isolates YBB5 and YBB7 were clustered into the same genus *Saccharomyces cerevisiae* and were closely related to the first group of phylogenetic tree (Figure 4.6) and it is described among robust yeasts capable to withstand stressful condition with high fermentation efficiency, rapid growth and effective sugar use (Reis *et al*., 2013). Isolates MB2, BN2 as well as MB1 (Figure 4.6) were clustered into genus *Saccharomyces cerevisiae*. However they were closely distant to isolates YBB0, YBB2, YBB5, YBB7, YBB10 and YBB15 which could be attributed to the conditions where they were grown. Finally, the phylogenetic analysis using both ITS1F-ITS2 and ITS3-ITS4 primers showed that the isolates from *Urwagwa* alcoholic beverage fermented with 0%, 2%, 5%, 7%, 10% and 15% of sorghum were

closely related compared to isolates MB1, MB2 and BN2 which could be attributed to conditions where they were isolated. Primers ITS1F-ITS2 resulted in large numbers of isolates which represent 55% and were clustered into genus *Saccharomyces cerevisiae* with 100% similarities to those in NCBI repository compared to 33% of ITS3-ITS4. Also ITS1F-ITS2 primer pair was chosen to be among the most efficient primer pair with 82% of amplification of ITS region in fungal community (Op De Beeck *et a*l., 2014).

CHAPTER FIVE
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The proximate composition of banana juice-sorghum composite mixture analyses has shown that concentration of total soluble solids as well as protein content has increased with an increase of the sorghum concentration. *Sorghum vulgaris sp* used in production of *urwagwa* was found to affect the physico-chemical quality parameters of *urwagwa*. It was observed that the trend of ethanol production for all the composites mixtures followed the similar pattern and tended to increase as well as the increase in percentage of sorghum added to the banana juice while pH and sugar seemed to decrease steadily up to the end of the fermentation period.

Based biochemical parameters such as ethanol content, sugar concentration, pH, titratable acidity which are important quality parameter requirement in banana-based alcoholic beverage the concentration of 5% of sorghum was found to be the optimum percentage which result most desirable banana-based alcoholic beverage. Morphological features of indigenous yeast involved in the fermentation of *Urwagwa* showed that all isolates belonged to *Saccharomyces* cerevisiae.

Molecular identification of ITS1-5.8SrDNA-ITS2 by two sets of universal primers (ITS1F-ITS2 & ITS3-ITS4) confirmed that the isolates belong to *Saccharomyces* spp with similarity of 97 to 100%. However, ITS1F-ITS2 showed better results with similarity of 98 to 100% where 5 out of 9 isolates had 100% similarity compared to ITS3-ITS4 with 97 to 100%. The strains isolated from banana-based alcoholic beverages fermented with 0%, 2%, 5%, 7%, 10% and 15% of sorghum were found to

be distant from MB1, MB2 and BN2 of traditional brewery. S*accharomyces cerevisiae* strain 1000 (LC269108.1) identified in fermenter with 0%, 2%, 5% and 15% was found to be the best strain which can be used in production *urwagwa.*

5.2 Recommendation

The concentration 5% of sorghum was found to result the best banana-based alcoholic quality parameters. This portion can be used in production of standardised banana-based alcoholic beverage called *urwagwa*. Economically, there is a big interest to use the minimum possible quantity of sorghum which meets highest quality standards. However, further study can be carried out on sensory characteristics of *urwagwa* fermented with aforementioned sorghum concentration. *Saccharomyces cerevisiae* strain 1000 (LC269108.1) identified in fermenter with 0%, 2%, 5% and 15% of sorghum was found to be the best strain which can be used in banana-based alcoholic beverage production. Therefore there is a need to study its biochemical potentiality in different alcoholic beverage.

REFERENCES

Akubor, P. I., Obio, S. O., Nwadomere, K. A., & Obiomah, E. (2003). Production and Quality Evaluation of Banana Wine, (2), 4–5.

Alexandre, H. & Charpentier, C. (1998). *Biochemical aspects of stuck and sluggish fermentation in grape must*. J. Ind. Microbiol. Biotech. 20(1) 20-27

Amerine, M.A., Kunkee, R., Ough, K.C.S., Singleton, V.L. and Webb, A. . (1980). *The technology of wine making* (4th Ed). AVI, Westport, Connecticut.

Asli, M.S. (2010). A study on some efficient parameters in batch fermentation of ethanol using Saccharomyces cerevesiae SC1 extracted from fermented siahe sardasht pomace. *African Journal of Biotechnology*, *9*(20), 2906–2912. https://doi.org/10.4314/ajb.v9i20.

Association of Official Analytical Chemist. (2005). *Official Methods of Analysis of AOAC International* (18th ed., Vol. 18th Editi). Gaithersburg, Md. : AOAC International, 2005.

Bai, F.W., Anderson, W.A., & Moo-Young, M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances*, *26*(1), 89–105. https://doi.org/10.1016/j.biotechadv.2007.09.002

Barbosa, C., Mendes-Faia, A., Lage, P., Mira, N. P., & Mendes-Ferreira, A. (2015). Genomic expression program of Saccharomyces cerevisiae along a mixed-culture wine fermentation with Hanseniaspora guilliermondii. *Microbial Cell Factories*, *14*, 124. https://doi.org/10.1186/s12934-015-0318-1

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., & Briones, A. (2011). Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. *Food Chemistry*, *127*(2), 441–446.

https://doi.org/10.1016/j.foodchem.2010.12.151

Blaalid, R., Kumar, S., Nilsson, R.H., Abarenkov, K., Kirk, P.M., & Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*, *13*(2), 218–224. https://doi.org/10.1111/1755-0998.12065

Boulton, R. (1980). The Relationships between Total Acidity, Titratable Acidity and pH in Wine. *Vitis*, *19*(1), 113–120.

Brickell, C.D., Alexander, C., David, J.C., Hetterscheid, W.L. a., Leslie, A.C., Malecot, V., & Jin, X. (2009). International Code of Nomenclature for Cultivated Plants. *Scripta Horticulturae*, (10), 184. https://doi.org/10.1099/ijsem.0.000778

Byarugaba-bazirake, G.W. (2008). *The Effect of Enzymatic Processing on Banana juice and Wine (Doctoral dissertation, stellenbosch: University,2008)*.

Byarugaba-Bazirake, G.W.P. van R. and W.K. (2013). The influence of commercial enzymes on wine clarification and on the sensory characteristics of wines made from three bana. *American Journal of Biotechnology and Molecular Sciences*. https://doi.org/10.5251/ajbms.2013.3.2.41.62

Campbell, F.G.P. and I. (1999). *Brewing Microbiology*. *Brewing Microbiology* (Second Edi). Aspen Publishers, Inc. Gaithersburg, Maryland. https://doi.org/10.1016/B978-1-78242-331-7.00019-8

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., & Dellacassa, E. (2008). Production of fermentation aroma compounds by Saccharomyces cerevisiae wine yeasts : e ¡ ects of yeast assimilable nitrogen on two model strains, *8*, 1196–1207. https://doi.org/10.1111/j.1567-1364.2008.00412.x

Chambers, P.J., & Pretorius, I.S. (2010). winemaking , science and yeast research,

11(12).

Davies, G. (1993). Domestic Banana-Beer Production in Mpigi District, Uganda. *The International Magazine on Banana and Plantain*, *2*(1), 12–15.

Deesuth, O., Laopaiboon, P., Jaisil, P., & Laopaiboon, L. (2012). Optimization of nitrogen and metal ions supplementation for very high gravity bioethanol fermentation from sweet sorghum juice using an orthogonal array design. *Energies*, *5*(9), 3178–3197. https://doi.org/10.3390/en5093178

Dieu, H.J. De, Kubana, I., Claudine, M., Claire, I., Eric, K., Labs, T., … Africa, S. (2017). Comparative Study of Physicochemical and Bacteriological Characteristics of Banana Wines Produced by Conventional and Modern Techniques in Southern province of Rwanda, *5*(5), 192–198. https://doi.org/10.12691/ajfst-5-5-4

Dilip, A., Berka, R., & Singh, G. (2002). *Applied Mycology and Biotechnology* (Vol. 3). ELSEVIER Amsterdam - Boston - Heidelberg - London - New York - Oxford. https://doi.org/10.1021/bi035895h

Esteve-Zarzoso B, Belloch C, Uruburu F, and A.Q. (1999). *Esteve-Zarzoso B, Belloch C, Uruburu F, and A Querol. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers*. Int J Syst Bacteriol.

Ferreira, J. (2004). *Factors influencing the fermentation performance of commercial wine yeasts (Masters dissertation, stellenbosch: University,2004)*.

Forschungsgemeinschaft, D. (2010). Microbial food cultures. *DFG Senate Commission on Food Safety*.

Gaidashova, S.V. (2005). Why beer bananas : The case for Rwanda Mycosphaerella

culture, (August 2014).

George, B. (1955). Microbilogical Process Report Yeasts : I . Morphology.

German, G., Staver, C., & Siles, P. (2015). An assessment of global banana production and suitability under climate change scenarios. *Climate Change and Food Systems: Global Assessments and Implications for Food Security and Trade*, 266–291.

Goud, B. S., & Ulaganathan, K. (2015). Draft genome sequence of Saccharomyces cerevisiae strain NCIM3186 used in the production of bioethanol from sweet sorghum. *Genome Announcements*, *3*(4), 6–7. https://doi.org/10.1128/genomeA.00813-15

Hammond, J. B., Egg, R., Diggins, D., & Coble, C. G. (1996). Alcohol from bananas. *Bioresource Technology*, *56*(1), 125–130. https://doi.org/10.1016/0960-8524(95)00177-8

Hierro, N., González, Á., Mas, A., & Guillamón, J. M. (2004). New PCR-based methods for yeast identification. *Journal of Applied Microbiology*, *97*(4), 792–801. https://doi.org/10.1111/j.1365-2672.2004.02369.x

Hutkins, R. (2006). *Microbiology and Technology of Fermented Foods* (First). Blackwell Publishing Ltd. https://doi.org/10.15713/ins.mmj.3

IITA. (1993). *International Institute for Tropical Agriculture, IITA Report,Rome ,Italy*.

Immaculate, E. O. and P. V. A. (2013). Quality assesment of banana juice and beer in Rwanda. Medwell Journals.

Jiranek , Langridge, and P. A. H. (1995). Amino Acid and Ammonium Utilization by Saccharomyces cerevisiae Wine Yeasts From a Chemically Defined

Medium. *Copyright © 1995 by the American Society for Enology and Viticulture. All Rights Reserved.*, *46*(1), 79–85. https://doi.org/10.1016/S1567-1356(03)00157-0

Joseph Sambrook and Russel. (2001). *Molecular Cloning* (3rd ed., Vol. 1). Cold spring harbor laboratory Press,New York.

K. Shale, J. Mukamugema, R. J. Lues, P. Venter, K. K. M. (2013). Characterisation of selected volatile organic compounds in Rwandan indigenous beer ‘ Urwagwa ’ by dynamic headspace gas chromatography-mass spectrometry. *African Journal of Biotechnology*, *12*(20), 2990–2996. https://doi.org/10.5897/AJB12.1173

Karamura, E., Frison, E., Karamura, D. A., & Sharrock, S. (1998). Banana production systems in eastern and southern Africa. *Bananas and Food Security. Proceedings of an International Symposium Held in Douala, Cameroon*, (January 2015), 401–412.

Konfo, C. T. R., Chabi, N. W., DahouenonAhoussi, E., CakpoChichi, M., Soumanou, M. M., & Sohounhloue, D. C. K. (2015). Improvement of African traditional sorghum beers quality and potential applications of plants extracts for their stabilization: a review. *Journal of Microbiology, Biotechnology and Food Sciences*, *5*(2), 196. many ref. https://doi.org/10.15414/jmbfs.2015.5.2.190-196

König, H., Unden, G., & Fröhlich, J. (2017). *Biology of Microorganisms on Grapes, in Must and in Wine*. Springer International Publishing AG 2017. https://doi.org/10.1007/978-3-319-60021-5

Korabecna, M. (2007). The variability in the fungal ribosomal DNA (ITS1, ITS2, and 5.8 S rRNA gene): its biological meaning and application in medical mycology.

Communicating Current Research and Educational Topics and Trends in Applied Microbiology, *108*(43), 783–787. https://doi.org/10.1128/JCM.39.10.3617

Koulougliotis, D., & Eriotou, E. (2016). Isolation and Identification of Endogenous Yeast Strains in Grapes and Must Solids of Mavrodafni kefalonias and Antioxidant Activity of the Produced Red Wine. *Fermentation Technology*, *05*(01), 1–9. https://doi.org/10.4172/2167-7972.1000125

Kudo, M., Vagnoli, P., & Bisson, L. F. (1998). Imbalance of pH and potassium concentration as a cause of stuck fermentations. *American Journal of Enology and Viticulture*, *49*(3), 295–301.

Labagnara, T. (2009). *“ Wine yeast biodiversity during spontaneous fermentation in response to environmental stress ” “ Biodiversità di lieviti vinari nella fermentazione spontanea in risposta a stress ambientali ” Candidate : Tilde Labagnara*.

Lewis, Young, T. (2001). *Brewing* (Second). Springer Science+ Business Media New York. https://doi.org/10.1007/978-1-4615-0729-1

Lindsay, J. (2010). *Sorghum: An Ancient, Healthy and Nutritious Old World Cereal*. Nebraska - Lincoln.

Lyumugabe, F., Uyisenga, J. P., Songa, E. B., & Thonart, P. (2014). Production of Traditional Sorghum Beer “<i>Ikigage</i>” Using <i>Saccharomyces cerevisae</i>,<i> Lactobacillus fermentum</i> and<i> Issatckenkia orientalis&a. *Food and Nutrition Sciences*, *05*(06), 507–515.

https://doi.org/10.4236/fns.2014.56060

Margalit.Y. (1997). *Concepts in wine chemistry*. The Wine Appreciation Guild Ltd,360 Swift Avenue, South San Francisco, CA 94080.

Ministry of Agriculture, F. and F. (2012). Agriculture , forestry and fisheries of Rwanda, 81.

Mohapatra, D., Mishra, S., & Sutar, N. (2010). Banana and its by-product utilisation: An overview. *Journal of Scientific and Industrial Research*, *69*(5), 323–329.

Munyanganizi. (1975). *La technologie de l'extraction du jus de bananes et sa vinification. Thèse de doctorat.* Faculté des Sciences Agronomiques de l'Etat à GEMBLOUX, Belgique.

Munyanganizi. (1983). Les levures des des vins de bananes du Kivu. *Inst.Fac.Sci. Agron. (Yangambi)*, *IV*(1).

Munyanganizi, C. . . (1976). Etude Comparative de deux procédés d'extraction du jus de banane appliqués ā deux Variétés différentes. *Industries Alimentaires et Agricoles*.

Muriro, P.A. (2017). of Crude Catalyst and Steam Distillation Into Document Control :, 1–19.

Nachel, M. (2008). *Homebrewing by DUmmies* (2nd ed.). Daniel Bradford, Publisher.

Nelson, S. C. (2006). Species Profiles for Pacific Island Agroforestry. *Permanent Agriculture Resources (PAR)*, (April), 1–18.

Newilah, G. N., Tomekpe, B. K., Fokou, B. E., & Etoa, B. F.-X. (2009). Physicochemical Changes during Ripening of Bananas Grown in Cameroon. *Fresh Produce 3.1*, *3*(1), 64–70.

NISR. (2016). *Seasonal Agricultural Survey 2016*.

Nsabimana, a, Gaidashova, S. V, Nantale, G., Karamura, D., & Staden, J. V. a N. (2008). Banana cultivar distribution in Rwanda, *16*(1), 1–8.

Nwuche, C. O., Murata, Y., Nweze, J. E., Ndubuisi, I. A., Ohmae, H., Saito, M., & Ogbonna, J. C. (2018). Bioethanol production under multiple stress condition by a new acid and temperature tolerant Saccharomyces cerevisiae strain LC 269108 isolated from rotten fruits. *Process Biochemistry*, *67*(December 2017), 105–112. https://doi.org/10.1016/j.procbio.2018.01.016

Omar-Zahid, L. A. (2013). *Atlas of Food Microbiology LAB*.

Op De Beeck, M., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J., & Colpaert, J. V. (2014). Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS ONE*, *9*(6). https://doi.org/10.1371/journal.pone.0097629

Ouma, E., & Jagwe, J. (2010). Banana Value Chains in Central Africa : Constraints and Opportunities By Banana Value Chains in Central Africa : Constraints and Opportunities.

Pascal Ribereau-Gayon, Denis Dubourdieu, B. D. and A. L. (2006). *Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition* (Vol. 1). John Wiley & Sons,Ltd.

Picki-reix, B. A. T. W., & Ryburgh, G. (1986). e¡ects of yeast assimilable nitrogen ontwo model strains, *92*, 568–571.

RAB. (2018). Postharvest Loss Assessment of green bananas in Rwanda, 7–8.

Raikar, R. V. (2012). Enhanced production of Ethanol from grape waste.

International Journal of Environmental Sciences, *3*(2), 776–783. https://doi.org/10.6088/ijes.2012030132004

Rangel, A. O. S. S., & Tóth, I. V. (1998). Sequential determination of titratable acidity and tartaric acid in wines by flow injection spectrophotometry. *Analyst*, *123*(4), 661–664. https://doi.org/10.1039/a707477h

Reis, V. R., Bassi, A. P. G., da Silva, J. C. G., & Ceccato-Antonini, S. R. (2013). Characteristics of Saccharomyces cerevisiae yeasts exhibiting rough colonies and pseudohyphal morphology with respect to alcoholic fermentation. *Brazilian Journal of Microbiology*, *44*(4), 1121–1131. https://doi.org/10.1590/S1517-83822014005000020

Ribéreau-Gayon, Glories, D. D. (2006). *Hand book of Enology*. John Wiley & Sons,Ltd.

Ribereau-Gayon, Pascal, Denis Dubourdieu, Bernard Doneche, A. L. (2006). *Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications 2 nd Edition Pascal Rib ´. Microbiology* (Vol. 1). https://doi.org/10.1016/j.fm.2007.03.003

Ribéreau-Gayon, P. (1999). *Observations related to the causes and consequences of stuck fermentation in vinification*. J. Int. Sci. Vigne Vin.

Rosa, G. P. (2005). *Biodiversity and Ecophysiology of Yeasts*. Springer-Verlag Berlin Heidelberg.

Rwanda Standard Board. (2017). Uburyo buboneye bwo gukora inzoga z'ibitoki na Divayi zifatiye kubimera, (Ukwakira), 1–99.

Sablayrolles, J.M., Dubois, C., Manginot, C., Roustan, J.L. & Barre, P. (1996).

s*luggish and stuck fermentations. Effectiveness of ammonium nitrogen and oxygen additions*. Vitic. Enol. Sci.

Sablayrolles, J. M., Dubois, C., Manginot, C., Roustan, J. L., & Barre, P. (1996). Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck wine fermentations. *Journal of Fermentation and Bioengineering*, *82*(4), 377–381. https://doi.org/10.1016/0922-338X(96)89154-9

Satav, P.D., & Pethe, A.S. (2017). Production and optimization of wine from banana fruits, *8*(2), 790–794.

Schanderl, H. (1959). *Die Microbiologie des Mostes und Weines,2nd Ed*. Stuttgart, Germany, Eugen Ulmer.

Serpen, J.Y. (2012). Comparison of Sugar Content in Bottled 100 % Fruit Juice versus Extracted Juice of Fresh Fruit. *Food and Nutrition Science*, *3* (November), 1509–1513. https://doi.org/10.4236/fns.2012.311196

Shale, K., Mukamugema, J., Lues, R. J., & Venter, P. (2014). Possible microbial and biochemical contaminants of an indigenous banana beer ' Urwagwa ': A mini review, *8*(July), 376–389. https://doi.org/10.5897/AJFS12.134

Sigmon, K. (2003). *Brewing Microbiology Third Edition*. (G.lain Campbell; Priest; Fergus, Ed.) (Third edit). Kluwer Academic/Plenum Publishers New York in 2003. https://doi.org/10.1007/978-1-4419-9250-

Stewart, G. G. (2014). *Brewing Microbiology Managing Microbes , Ensuring Quality*. (A. E.Hill, Ed.). Woodhead Publishing Series.11-12

Stielow, J. B., Lévesque, C.A., Seifert, K.A., Meyer, W., Irinyi, L., Smits, D., … Robert, V. (2015). One fungus, which genes? Development and assessment of

universal primers for potential secondary fungal DNA barcodes. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, *35*(1), 242–263. https://doi.org/10.3767/003158515X689135

Tamura K., Stecher G., Peterson D., Filipski A., and K. S. (2013a). *Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution*. Molecular Biology and Evolution.

Tamura K., Stecher G., Peterson D., Filipski A., and K. S. (2013b). *Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution*. Molecular Biology and Evolution.

Th.Munyanganizi, R. C. (1974). Extraction du Jus de banane. *Industries Alimentaires et Agricoles*.

Tropea, A., Wilson, D., Cicero, N., Potortì, A.G., La Torre, G.L., Dugo, G., … Waldron, K.W. (2016). Development of minimal fermentation media supplementation for ethanol production using two Saccharomyces cerevisiae strains. *Natural Product Research, 30*(9), 1009–1016. https://doi.org/10.1080/14786419.2015.1095748

UNCTAD. (2016). United Nations Conference on Trade and Development, 1.

USDA National Nutrient Database. (2016). National Nutrient Database for Standard Reference Release 28, Full Report (All Nutrients): 20060, watermelon, raw, (5), 1–6.

Walker, G. . (1994). *The roles of magnesium in biotechnology*. Crit. Rev. Biotech.

Wang, H., Hu, Z., Long, F., Niu, C., Yuan, Y., & Yue, T. (2015). Characterization of Osmotolerant Yeasts and Yeast-Like Molds from Apple Orchards and Apple Juice Processing Plants in China and Investigation of Their Spoilage Potential.

Journal of Food Science, *80*(8), M1850–M1860. https://doi.org/10.1111/1750-3841.12946

Wilson, P. (2012). Microbial and Biochemical Changes Occurring During Production of Traditional Rwandese Banana Beer "Urwagwa." *Fermentation Technology*, *01*(03), 1–4. https://doi.org/10.4172/2167-7972.1000104

Zhu, Y. (2013). *Solid State Fermentation for Foods and Beverages*. (J. C. and Y. Zhu, Ed.). CRC PRESS(Taylor & Francis Group.

Zilberman, D., & Kim, E. (2011). The lessons of fermentation for the new bio-economy. *AgBioForum*, *14*(3), 97–103.

Zvauya R1, Mygochi T, P. W. (1997). Microbial and biochemical changes occurring during production of masvusvu and mangisi, traditional Zimbabwean beverages. *Fermentation Technology*, *01*(03), 308. https://doi.org/10.4172/2167-7972.1000104

APPENDIXES

Appendix 1: Tools used for proximate composition of banana juice and banana juice-sorghum composite mixture and physico-chemical changes during fermentation of Urwagwa.

Banana peeling

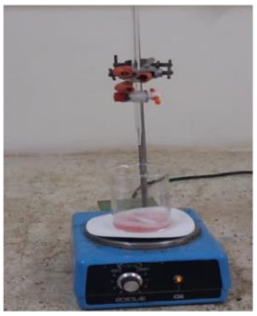
Juice extracting machine

Kjeltec Machine

Refractometer

pH-meter

WineScan™ SO2



Titration

Appendix 2: Sequence listings

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