Ziziphus mauritiana (*masau*) fruits fermentation in Zimbabwe: from black-box to starter culture development

Loveness Kuziwa Nyanga

Thesis committee

Promotor
Prof. dr. ir. M. H. Zwietering
Professor of Food Microbiology
Wageningen University

Co-promotors
Dr. ir. M. J. R. Nout
Associate professor, Laboratory of Food Microbiology
Wageningen University

Dr. T. Boekhout
Senior scientist, Fungal Biodiversity Centre
CBS-KNAW, Utrecht

Other Members
Prof. dr. J. T. Pronk, Delft University of Technology, the Netherlands
Prof. dr. L. Jespersen, University of Copenhagen, Denmark
Dr. ir. I. D. Brouwer, Wageningen University
Prof. dr. ir. M. A. J. S. van Boekel, Wageningen University

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Ziziphus mauritiana (*masau*) fruits fermentation in Zimbabwe: from black-box to starter culture development

Loveness Kuziwa Nyanga

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 4 December 2012
at 1:30 p.m. in the Aula.

Loveness Kuziwa Nyanga
Ziziphus mauritiana (*masau*) fruits fermentation in Zimbabwe: from black-box to starter culture development,
124 pages.

Thesis, Wageningen University, Wageningen, The Netherlands (2012)
With references, with summaries in English and Dutch

ISBN 978-94-6173-375-7

To Ernest, Unite, El, Tadie
and baby Adiel Kuku

"Fight the good fight, finish the race and keep the faith"

Contents

ABSTRACT

This thesis reports on studies of microbiological and biochemical properties of *masau* (*Ziziphus mauritiana*) fruit fermentation and the development of starter cultures for the production of *masau* beverages.

A survey to document the traditional processing techniques was conducted using a questionnaire and focus group discussions in each of the three districts, i.e., Mudzi, Mt Darwin and Muzarabani in Zimbabwe. The survey results showed that the *masau* fruit is usually gathered by women and children, and eaten raw or processed into products such as porridge, traditional cakes, *mahewu* (non-alcoholic fermented beverage), and jam, which are sold at local markets. It is also naturally fermented under uncontrolled conditions and distilled into *kachasu*. The nutritional composition of the *masau* fruit was analysed. The fruits are good sources of nutrients such as carbohydrates, protein, and essential micronutrients such as calcium, potassium, phosphorus, copper, iron, zinc and vitamin C.

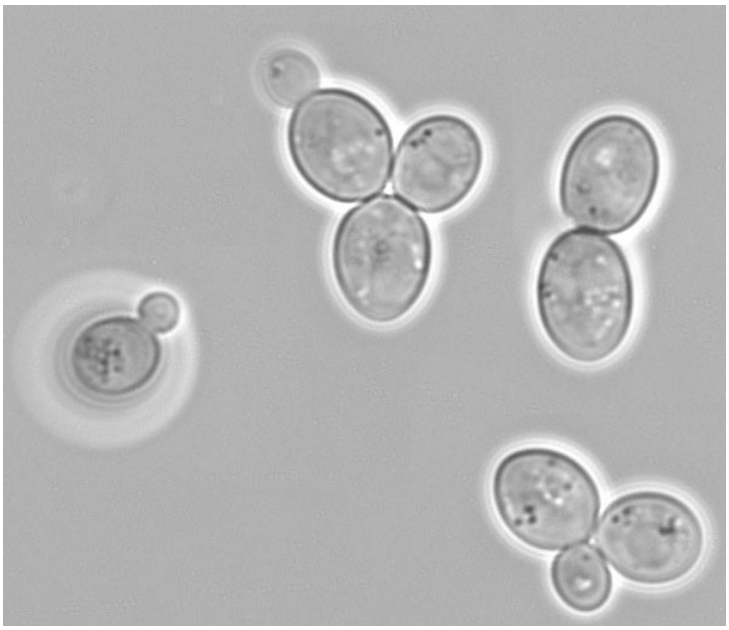
In order to enable the selection of starter cultures for the production of *masau* wine and distillate, yeasts, yeast-like fungi, and lactic acid bacteria present on the unripe, ripe and dried fruits, and in the fermented *masau* fruits were isolated and identified using physiological and molecular methods. The predominant species were identified as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *P. fabianii*, *Aureobasidium pullulans*, *Lactobacillus agilis* and *L. plantarum*. The yeast species were then characterised with respect to ethanol and flavour compounds production. Significant differences in the production of ethanol and other volatile compounds were observed during fermentation of *masau* juice among and within the tested *Saccharomyces*, *Pichia* and *Saccharomycopsis* species. Alcohols and esters were the major volatiles detected in the fermented juice. Ethyl hexanoate and ethyl octanoate were produced in highest amounts as compared to the other flavour compounds.

Two traditional low-tech methods for preserving starter cultures, i.e., stabilisation of yeast cultures in dried plant fibre strands, and in rice cakes, were compared with standard lyophilisation. Viable cell counts made during six months storage at 4 °C and 25 °C of lyophilised yeasts, and yeast cultures preserved in dry rice cakes and dry plant fibre strands showed that the rice cake method performed significantly better than lyophilisation.

The developed library of fermentation characteristics of yeasts can help in the design of mixtures of strains to obtain a specific melange of *masau* product functionalities. The defined starter cultures could be preserved using the traditional approaches, which are suitable for small-scale, low-tech applications.

CHAPTER 1

INTRODUCTION



I. BACKGROUND

1. Fermented foods

Fermentation to produce food and beverages is one of the oldest ways of food processing. It is defined as bioprocessing using microorganisms and their enzymes to achieve desirable quality characteristics (Nout, 2005). The functional microorganisms in food fermentations include bacteria, yeasts and moulds. Popular fermented food products include beer, bread, wine, yoghurt, cheese, sauerkraut and tempe.

Fermented foods obtained by traditional fermentation techniques constitute an important part of the diet in many communities in the world (Gadaga et al., 1999; Steinkraus, 1996). However, many of the fermentation processes are complicated, produce variable product quality, and are time consuming which has led to the replacement of some of these indigenous foods by industrially processed and convenience foods often based on technology developed in industrialised countries. The unfortunate outcome of this replacement is the inevitable loss of the traditional technologies before they are fully understood and harnessed for the future generations (Gadaga et al., 1999). Another challenge is that the industrial scale production has to be done without losing the unique flavour and other traits associated with the traditional products from which they are derived (Caplice et al., 1999). It is therefore important to study, document and upgrade these traditional fermented foods.

The traditional fermented foods include the fermented beverages from indigenous fruits. In Africa, there are more than 50 wild indigenous tree species which bear highly valued edible fruits (Karachi et al., 1991; Maghembe et al., 1992). Indigenous fruits are essential for food security, health and nutrition (Akinnifesi et al., 2004; Saka et al., 2007; Tembo et al., 2008b). Many of the miombo (dry wooded area with deciduous growth) indigenous fruits ripen and are often available during the dry season when food availability is low (Akinnifesi et al., 2006; Campell, 1987). These indigenous fruits are an important source of income for poor people since entry barriers for collection and use are relatively low (Tembo et al., 2008b). The wild fruits such as *Adansonia digitata* (baobab), *Ziziphus mauritiana* (Indian plum) and *Sclerocarya caffra* (yellow plum) provide micronutrients such as vitamin C and minerals (Achinewhu et al., 1995). These seasonal wild fruits are collected and consumed either fresh or made into various products such as jams, chutneys and spontaneously fermented juices.

The production of African traditional fermented foods and beverages mostly relies on natural fermentation, which is an uncontrolled process. Microorganisms present from the raw materials, the environment, equipment and utensils are responsible for the fermentation. However, because the fermentations are spontaneous and uncontrolled the product microbiota is inconsistent and the product quality is variable (Gadaga et al., 1999; Halm et al., 1993; Sanni et al., 1994). It is expected that the fermentation processes can be improved to better controlled processes in which appropriate starter cultures are used. Optimisation of the production processes should result in consistent and high quality fermented products. In Zimbabwe there are many traditional fermented beverages and/or wines made from indigenous fruits, which are

produced through uncontrolled processes. The fermented beverages include *mukumbi*, *mudetemwa*, *mazhanje* and *masau* wines. The microbiological and biochemical processes that occur during the production of the beverages have not been studied, except for *mukumbi*. This study focused on the fermentation of *Ziziphus mauritiana* fruits, locally called *masau* in Shona language.

2. The *Ziziphus mauritiana* tree

Description, distribution and uses

The genus *Ziziphus* belongs to the Rhamnaceae (buffalo or buck thorn) family (Johnston, 1963; Moll, 1997) and contains about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (Johnston, 1963). Some species, like *Z. mauritiana* and *Z. jujuba* occur nearly on all continents, whereas other species, like *Z. nummularia*, *Z. spina-christi* and *Z. mucronata* are restricted in their distribution to distinct areas (Johnston, 1963).

The *Z. mauritiana* tree (Figure 1) is a medium sized thorny tree that has a rapidly developing taproot, a necessary adaptation to drought conditions (Fact, 1998). It occurs in arid and semi-arid lands. The young leaves and stem are covered with a whitish dawn. The tree is thought to have originated from Middle or East India where it is widely cultivated (Cherry, 1985). In both India and China *Ziziphus* trees have a long tradition of agronomic selection and cultivation with the result that the species occurring in these countries (i.e. *Z. mauritiana*, *Z. jujuba*) are better known and more widely researched than those occurring in other regions (Cherry, 1985). The use of *Z. mauritiana* in India can be traced back to as early as 1,000 BC (Morton, 1987). Extensive research has been done on changes in the physical and chemical characteristics of jujube fruit during growth and development (Abbas et al., 2002). Extensive research on the chemical and physiological properties was first undertaken in India and has expanded to Israel, Malawi, Senegal and Zimbabwe (Kaaria, 1998). The species is widely cultivated in Mozambique, spreading up the Zambezi valley and has become naturalized in many areas of Southern Africa (Moll, 1997), including Zimbabwe where it is regarded as indigenous (Palgrave, 1990).

Ziziphus mauritiana is a valuable tree. An apt description of its value is that *Z. mauritiana* produces the three vital 'F's' that desert dwellers require - Fruit, Fodder and Fuel (Kaaria, 1998). A great majority of the rural population in arid regions meet their daily requirements of biomass or biomass-based products, such as food, fuel (firewood), fodder, fertilizer (organic manure, forest litter), building materials (poles) and medical herbs (Morton, 1987) from this tree. In many regions, *Ziziphus* is grown as a hedge with its spines creating effective live-fencing, and with its highly nutritious fruits providing a valuable source of energy, vitamins and income when sold at the local market. In addition, extracts from the fruits, seeds, leaves, roots and bark of the trees are used in traditional medicine to alleviate the effects of insomnia, skin diseases, inflammatory conditions and fever (Morton, 1987). For these reasons, *Ziziphus* trees play an important role in the integrated economy of the arid lands.

Figure 1. The *masau* (*Ziziphus mauritiana*) tree, Muzarabani rural community in the Zambezi valley, Zimbabwe (Photo taken by L. K. Nyanga).

Yield and nutritional composition of the fruit

Ziziphus mauritiana fruit is also known as 'ber', 'Indian jujube', 'Indian plum', or 'desert apple' (Kaaria, 1998). *Z. mauritiana* fruit (Figure 2) varies in shape and size. The fruit can be round, oval or oblong, large, medium or small and has a comparatively large stone. The size of the fruit can be as small as 1.8 to 2.5 cm in diameter, for fruit from wild trees or as large as 5 cm (plum sized) from improved cultivars. The fruit is first green, turning yellow to brown as it ripens. *Ziziphus mauritiana* fruits ripen from mid June and are available until the end of September in the Zambezi valley in Mashonaland central province (17º 0' S, 31º 0' E), Zimbabwe. The ripe fruit is sweet to sour in taste, and is thirst quenching. Both flesh texture and taste are reminiscent of apples (Kaaria, 1998). Yields of 80 to 130 kg/tree/year have been reported in the Sahel region, Africa (Von Maydell, 1986). From the naturally growing, scattered wild trees in Zimbabwe, a yield of 4-5 tons has been obtained from a 3-4 hectare area per year (Maposa et al., 1998). In the semi-arid subtropical climate of northern India under irrigated conditions, the fruit yield per tree ranges from 80 to 200 kg depending on the varieties and management practices during the prime bearing age of 10 to 20 years (Bakhshi et al., 1974).

The fruits are nutritious and are usually eaten fresh (Khoshoo et al., 1985; Von Maydell, 1986). The nutritional composition of the fruit depends on the source and/or cultivar of the fruit. Tables 1 and 2 show some cited data on the nutritional composition of *Z. mauritiana* fruits.

Figure 2. The *Ziziphus mauritiana* (*masau*) fruits. Scale: 1:2 (Photo taken by L. K. Nyanga).

Non-fermented fruit products

Ziziphus mauritiana fruit can also be consumed as dried, candied or pickled fruits, juice or as *masau* butter (Fact, 1998). In India the ripe fruits are mostly consumed raw, but sometimes stewed. Slightly under-ripe fruits are candied by a process of pickling, immersing in a salt solution of which the salinity is gradually raised from 2 to 8%. The solution is then drained and another solution of 8% salt and 0.2% potassium metabisulphite is added. The mixture is stored for one to three months, after which the fruits are rinsed and cooked in sugar syrup with citric acid (Morton, 1987). In the Zambezi valley in Zimbabwe the powder from dried fruits is used for baking and to prepare jam (Kalikiti, 1998; Maposa et al., 1998) and a traditional loaf (Kadzere, 1998). The pulp of ripe fruits of *masau* is commercially made into jam and sun dried fruit slices (*masau* snacks) by Tulimara – Speciality Foods for Africa (SFA), Private Limited Zimbabwe. Cakes, which resemble gingerbread, are made from dried and fermented pulp in Western Sudan (Egceling, 1951) and in Zambia (Kalikiti, 1998).

3. Traditionally fermented beverages from indigenous fruits in Zimbabwe

Makumbi is the generic name in Zimbabwe for beverages made from wild fruits (Gadaga et al., 1999; Gomez, 1989). In general, over 10% of recorded major food plants of Zimbabwe, which include cereals, are fermented into some type of beverage or wine (Okagbue, 1995).

Table 1. Proximate composition of *Ziziphus mauritiana* fruits

Nutrient	Content (g 100 g^{-1})[1]	Source of fruit	Reference
Dry matter	17.0-18.4	India	(Morton, 1987)
(F.w., basis)[2]	4.6	Nigeria	(Lockett et al., 2000)
	14.8	Malawi	(Saka et al., 1994)
Ash	3.5	Nigeria	(Lockett et al., 2000)
	10.1	Malawi	(Saka et al., 1994)
	0.3-0.6 (F.w.)	India	(Morton, 1987)
Total Sugars	5.4 – 23.0	Semi-arid lowlands, West Africa	(Leakey, 1999)
	12.8	Malawi	(Kaaria, 1998)
	21.7	India	(Morton, 1987)
	17.9	Iraq	(Abbas, 1997)
	20.0 -23.0	Senegal	(Danthu et al., 2002)
Carbohydrates	73.0	Malawi	(Saka et al., 1994)
	66.0	Nigeria	(Lockett et al., 2000)
Crude Protein	≤ 2.5	Malawi	(Kaaria, 1998)
	4.1	Malawi	(Saka et al., 1994)
	1.4	India	(Morton, 1987)
	2.4	Nigeria	(Lockett et al., 2000)
Crude fat	9.6	Malawi	(Saka et al., 1994)
	1.6	Nigeria	(Lockett et al., 2000)
	0.3 (F.w.)	India	(Rathore, 2009)
	0.1 (F.w.)	India	(Morton, 1987)
Crude fibre	3.4	Malawi	(Saka et al., 1994)
	0.6 (F.w.)	India	(Morton, 1987)

[1]All parameters are based on dry weight. Exceptions are indicated. [2]F.w., fresh weight basis.

Mukumbi beverage

Mukumbi is a traditional Zimbabwean wine prepared in many rural homes in the arid regions of Zimbabwe. The wine is prepared from a fruit called *mapfura* (*Sclerocarya caffra*, Anacardiaceae) in Shona and *amaganu* in Ndebele. The fruit tree is called *marula* in Sub-Sahara Africa. *Mukumbi* is traditionally produced by spontaneous fermentation of the mashed *mapfura* fruits. The ripe fruits are washed and the skins removed. The seeds, which are coated by a mucilaginous flesh, are pounded in a wooden mortar and pestle to completely extract the juice (Gadaga et al., 1999; Madovi, 1981; Mpofu et al., 2002). The slurry mixture composed of flesh and the juice are then poured into an earthenware pot. An equal volume of water is added and the mixture is allowed to ferment naturally at room temperature for three days.

Table 2. Micronutrient composition of *Ziziphus mauritiana* fruits

Micronutrient	Content (mg 100 g^{-1})[1]	Source of fruit	Reference
Vitamin C	96.0 – 500.0	Semi-arid lowlands, West Africa	(Leakey, 1999)
(F.w., basis)[2]	65.8 – 76.0	India	(Morton, 1987)
	300.0 – 500.0	Senegal	(Danthu et al., 2002)
	13.6	Malawi	(Saka et al., 1994)
Carotene	21.0 -28.0	Semi-arid lowlands, West Africa	(Leakey, 1999)
	0.02 (F.w.)	India	(Morton, 1987)
Calcium	13.5	Malawi	(Saka et al., 1994)
	499.0	Nigeria	(Lockett et al., 2000)
	712.5	Nigeria	(Eromosele et al., 1991)
	25.6	India	(Morton, 1987)
	4.0	Rajasthan, India	(Rathore, 2009)
Copper	0.2	Nigeria	(Lockett et al., 2000)
	0.6	Nigeria	(Eromosele et al., 1991)
Iron	6.3	Nigeria	(Eromosele et al., 1991)
	17.9	Nigeria	(Lockett et al., 2000)
	0.76-1.8 (F.w.)	India	(Morton, 1987)
	1.8 (F.w.)	Rajasthan, India	(Rathore, 2009)
Magnesium	49.9	Nigeria	(Lockett et al., 2000)
	227.0	Nigeria	(Eromosele et al., 1991)
	0.5	Malawi	(Saka et al., 1994)
Manganese	3.5	Nigeria	(Eromosele et al., 1991)
	1.14	Nigeria	(Lockett et al., 2000)
Phosphorus	13.0	Nigeria	(Eromosele et al., 1991)
	2.2	Malawi	(Saka et al., 1994)
	144.0	Nigeria	(Lockett et al., 2000)
	9.0 (F.w.)	Rajasthan, India	(Rathore, 2009)
Potassium	17.3	Malawi	(Saka et al., 1994)
Sodium	426.0	Malawi	(Saka et al., 1994)
Zinc	0.6	Nigeria	(Lockett et al., 2000)
	1.55	Nigeria	(Eromosele et al., 1991)

[1]All the parameters are based on dry weight. Exceptions are indicated. [2]F.w., fresh weight basis.

Mukumbi is yellow in colour with a tart-sour taste and a slight turpentine-like aroma (Mpofu et al., 2002). Changes occurring during the fermentation of *mapfura* juice to produce *mukumbi* have been studied by Mpofu and co-workers (2002). Results from this study indicated that *mukumbi* can be classified under the sweet dessert wines according to the definition of Amerine and Ough (1980).

The production of a *mapfura* alcoholic beverage on an industrial scale has been developed in South Africa. The following yeast species and related flora have been isolated and identified from the ripe mapfura fruits in Zimbabwe: *Aureobasidium pullulans*, *Geotrichum capitatum*, *Trichosporon brassicae*, *Rhodotorula*

mucilaginosa, *Hansenula anomala*, *Hansenula jadinii* and other *Hansenula* species. The physiological characteristics of these organisms suggest that, generally, they are not important in traditional *marula* wine fermentation as they are not fermentative (Okagbue et al., 2002). However, the *Hansenula* species are fermentative and may play a role in *marula* wine making.

***Mudetemwa* beverage**

Parinari curatellifolia (sand apple, Chrysobalanaceae) fruit is called *hacha* in Shona, a local language in Zimbabwe. The *hacha* fruits are seasonal and available during the months of August to December. Sand apple juice, made by manually squeezing pounded fruits, is boiled, then left to ferment for four days, after which it is distilled to make a potent spirit called *mudetemwa* (Gadaga et al., 1999; Tredgold, 1986). Alternatively, the sand apple juice is fermented overnight, boiled and drunk as a beer after cooling (Gadaga et al., 1999; Tredgold, 1986).

***Mazhanje* wine**

Mazhanje (*Uapaca kirkiana*, Phyllanthaceae*)* are wild fruits that are abundant seasonally and can be fermented into a sweet wine called *mutandavira* (Gadaga et al., 1999; Tredgold, 1986). *Mazhanje* fruits are pounded to rupture the skins and extract the seeds. The pulp is left to ferment for three days (Tredgold, 1986). Alternatively, the pulp is mixed with cold water and then left to ferment until the water turns grey or opaque. The liquid is then used to make a thin porridge by mixing with ground maize-meal. In Zambia, *masuku* wine from *U. kirkiana* is being produced on a commercial scale (Leakey, 1999; Mwamba, 1989).

***Masau* fermented products**

Masau wine is made by soaking *Z. mauritiana* fruits for several hours and allow them to ferment at ambient temperature (Chivero, 2001). A distillate called *kachasu* is made from *masau* fruits that have been fermented for 4-7 days. *Kachasu* is a common name given to traditionally fermented and distilled alcoholic spirits which are potent. Although *kachasu* can be produced from wild fruits, it is usually made from maize meal, bulrush or finger millet (Gadaga et al., 1999). In Zimbabwe, *kachasu* consumption is illegal because it is alleged to be toxic hence it can cause ill health and is also associated with cases of sudden death (Brett et al., 1992; Gadaga et al., 1999). In Malawi, dried *Z. mauritiana* fruits are fermented and then distilled to make a potent alcoholic beverage (Kaaria, 1998) and a wine called *mlunguzi* is produced from a combination of *Uapaca kirkiana* and *Z. mauritiana* (Maghembe et al., 1992). There are no published data on the microbial and biochemical changes taking place during the traditional fermentation of *masau* fruits to produce wine. Other wild fruits which are fermented into alcoholic beverages in Zimbabwe are presented in Table 3.

Table 3. Other wild fruits which are fermented into alcoholic beverages in Zimbabwe (Gadaga et al., 1999).

Botanical name	Family name	English name	Shona/Ndebele Name
Balanites aegyptica	Balanitaceae	Torchwood	*nyahoko*
Bequeritiodendron magalismontanum	Sapotaceae	milk plum	*muhorongwa/umhlautshwa*
Berchemia discolour	Rhamnaceae	bird plum	*munyii/umcaga*
Cassia petersiana	Fabaceae	monkey pod	*muremberembe*
Diospyros mespiliformis	Ebenaceae	jackal berry	*mushenje/umdlawu*
Garcinia huillensis	Clusiaceae	granite garcinia	*mutunduru*
Grewia monticola	Malvaceae	donkey berries	*mutongoro/umtewa*
Pappea capensis	Sapindaceae	indaba tree	*chitinunu/uzagogwane*
Popowia obovata	Annonaceae	monkey fingers	*munyani/umkozombo*
Rhus tenuinervis	Anacardiaceae	nana berry	*mudzambuya/umkungu*
Syzygium cordatum	Myrtaceae	water berry	*mukute/umdori*

4. Yeasts and lactic acid bacteria

Yeasts are unicellular fungi that reproduce vegetatively by budding or fission, and form sexual states that are not enclosed in a fruiting body (Boekhout et al., 2003). The taxonomy and specification of yeast is usually based on morphology of the vegetative and sexual stages, and on certain physiological, biochemical and DNA characteristics. Yeast can utilize a wide range of food substrates under a variety of environmental conditions (Deak et al., 1996). Plant materials containing fermentable sugars provide substrates for yeast species belonging to genera such as *Saccharomyces*, *Candida*, *Torula* and *Hansenula* (Holzapfel, 1997).

Yeasts have found application in several industries such as the pharmaceutical, brewing and dairy industries, and also in the production of biomass and ethanol. They have been used to produce wines and other alcoholic beverages for thousands of years and were found predominating in traditional fermented foods such as Korean *nuruk*, Nigerian *fufu* and Indian *idli* (Nout, 2003).

Lactic acid bacteria (LAB) are defined as a group of microaerophilic, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. LAB play a prominent role in the world food supply, performing the main bioconversions in fermented dairy products, meats, and vegetables (Miller et al., 2000). LAB are also important in the production of wine, coffee, sourdough, and numerous indigenous food fermentations such as *ogi*, *uji* and *mahewu* (Holzapfel, 1997; Oyewole, 1997; Sanni et al., 1994). Some LAB produce exopolysaccharides that contribute to the consistency and rheology of fermented milk products (Ruas-Madiedo et al., 2002).

Associations of yeasts and lactic acid bacteria (LAB) are often encountered or used in the production of beverages and fermented foods such as cheese, kefir, wine and sausages (Gobbetti, 1998). Fermented foods are generally appreciated for attributes such as pleasant flavour, aroma, texture, and improved cooking and processing properties. Microorganisms, by virtue of their metabolic activities,

contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life and safety of the fermented foods (Holzapfel, 2002).

5. Starter Cultures

Traditional fermentation processes relied on the transfer of knowledge and methodologies associated with the manufacturing from generation to generation. Techniques such as 'back-slopping', which involves the use of a residue from a previous batch of acceptable quality, and impregnation of a cloth or other inert material with the fermented product, followed by drying and storage in a dry place, were used for inoculation (Holzapfel, 1997). These traditional approaches are still practiced in developing countries, and even in industrialized countries the technique of 'back slopping' may still be applied in the processing of sauerkraut and sourdough.

The industrialization of food production together with the blossoming of microbiology in the middle of 19th century led to the optimization and upscaling of many food fermentation processes (Leroy et al., 2004) and hence, development of starter cultures. A starter culture may be defined as a preparation or material containing large numbers of variable microorganisms, which may be added to accelerate a fermentation process (Holzapfel, 2002). There are three categories of starter cultures which include single-strain cultures, multiple strain cultures and undefined mixed starter cultures which contain two or more strains. The direct addition of selected starter cultures to raw materials has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end product (Leroy et al., 2004). Originally, industrial starter cultures were maintained by daily propagation. Later they became available as frozen concentrates and dried or lyophilized preparations (Sandine, 1996).

Lactic acid bacteria starter cultures are well developed for the dairy industry. The genera of great importance in the dairy industry include *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* (Collingwood, 1995). Reasons for unsuccessful application of starter cultures in non-dairy fermentations are the following: (i) lack of knowledge of suitable strains, (ii) fermentation technology not properly adapted to the requirement of starters, (iii) pasteurisation is inapplicable, (iv) some products require growth of microbial populations in succession which cannot be reproduced with starters (Hammes, 1991). To scale up the production of traditional fermented food, the development of starter cultures is pre-requisite. The use of starter cultures will reduce the fermentation time, and improve and stabilise the nutritional, sensory properties and quality of fermented food. Therefore, the development of starter cultures has become an important science with a driving force to search for microorganisms especially suited for particular food fermentations.

II. RATIONALE OF STUDY

The optimisation and control of the processing of traditionally fermented foods, such as indigenous fruit products, has the potential of multiple benefits, including the improvement of the socio-economic status of rural communities through

employment creation, augmenting family income, providing a non-seasonal supply of safe and quality beverages, environmental rehabilitation, domestication of the fruit trees as well as retaining an important food source. Studies have shown that indigenous fruits increasingly contribute a sizeable portion of the income of rural communities in Zimbabwe (Cavendish, 2000). In the harvesting season, the fruits (e.g. *Z. mauritiana* and *U. kirkiana*) are sold at urban and rural markets in Zimbabwe. It was also reported that *masau* constitute a significant part of the diet in some areas of Zambia and were commonly exchanged for grain during the 1995/96 drought (FEWS Bulletin, 1996). Though the fruit does not find much favour with the upper classes (it is considered a "poor man's fruit"), it has a high nutritional value and great commercial potential. Consequently, in several regions of the world, *Z. mauritiana* fruits are sold on local markets, generating income for people of the rural areas and improving family nutrition (Saka et al., 1994). The latter authors also reported that indigenous fruits play a role in people's diet and contribute to the economy of the rural communities in Malawi. Therefore, the fruit has contributed to the reduction of poverty as the local communities where they grow or can be cultivated, have alternative sources of income. In Zimbabwe, rural people in Rushinga district collect the fruit and perform some preliminary processing for Speciality Foods for Africa (SFA) that commercially processes the fruit pulp into jam. The cooperation between SFA and Southern Alliance for Indigenous Resources (SAFIRE) ensures that a fair price is paid to the communities and that the highest quality fruit is used for the jam.

To date, the majority of research on African fermented foods occurs in East and West Africa. Some of the products, e.g., *gari*, *dawa dawa*, and *ogi,* have been semi-industrialized because of the extensive studies done. The traditional fermented foods of Zimbabwe have not been systematically studied, except for products such as fermented milk, *mahewu*, *masvusvu*, *mangisi* and *mukumbi* (Mpofu et al., 2002). *Masau* fermented products are one of the popular traditional fermented beverages consumed by many rural households in the Zambezi valley of Zimbabwe. However, with the increasing trend of rural-urban migration, and the adoption of western culture by the younger generation, most of these fermentation techniques will die out at a time when other countries in Asia, Latin America and West Africa are developing and upgrading the processing of their respective indigenous fermented foods and beverages (Mwesigye et al., 1995).

No attempts have been made so far to develop fermentation technology suitable for the production of *masau* wine and/or beverage. Little efforts have been made to investigate and document the traditional processes. The microorganisms involved and physicochemical changes associated with the fermentation process have not been studied at all. Accordingly, this study aims to measure, understand and document the traditional processing of *masau* beverage/wine and study the microbiological and biochemical changes that take place during the traditional fermentation of *masau* fruits. This will include enumeration, isolation and identification of the microorganisms involved in the uncontrolled fermentation of the *masau* fruits, followed by the selection and development of starter cultures.

To achieve this goal, the specific objectives of the thesis were:

- To document and understand the traditional processing of *masau* fruits.
- To determine the proximate composition and mineral content of the fruits.
- To isolate and identify yeasts and lactic acid microbiota from *masau* fruits and their fermented fruit pulp.
- To screen and characterize the yeast isolates with respect to their production of ethanol and flavour compounds.
- To study the feasibility of two traditional low-cost, low-technology methods for drying starter cultures, and compare them with standard lyophilisation methods.

III. OUTLINE OF THE THESIS

This research project aimed to study the microbiological and biochemical properties of *masau* fruit fermentation with prospects for starter culture development. Information on the traditional processing of the fruits was gathered and documented in **Chapter 2**. In **Chapter 3**, the nutrient composition of the fruit is described. The fruits were reported to be naturally fermented to produce an unattractive product which is distilled into a potent product called *kachasu*. In pursuit of the production of consistent quality fermented products from *masau* fruits, yeasts and lactic acid bacteria microbiota from *masau* fruits and their fermented fruit pulp were isolated and identified as described in **Chapter 4**. Consequently, the yeast isolates were screened and characterized to enable selection of starter cultures for the production of *masau* wine and distillate (**Chapter 5**). Production and storage of starter cultures for use in traditionally fermented foods is a great challenge since standard methods such as lyophilisation cannot be applied due to economic constraints and low-tech infrastructure in the developing world. Hence, in **Chapter 6**, a comparative study on the effect of two low-tech traditional methods for drying starter cultures and standard lyophilisation was done. Finally, in **Chapter 7**, a general discussion is presented.

CHAPTER 2

Traditional processing of *masau* (*Ziziphus mauritiana*) fruits in Zimbabwe

L. K. Nyanga, M. J. R. Nout, T. H. Gadaga, T. Boekhout and M. H. Zwietering:
Ecology of Food and Nutrition (2008) 47, 95-107.

ABSTRACT

A survey of the traditional processing techniques of *masau* was conducted using a questionnaire and focus group discussions in each of the three districts, i.e., Mudzi, Mt Darwin and Muzarabani in Zimbabwe. *Masau* fruits form part of the family diet and generate additional income by selling at local markets. Surplus fruits are sun dried and can be transformed into various products such as porridge, traditional cakes, *mahewu* and also fermented to produce a spirit called *kachasu*. The ethanol content of the fermented fruit pulp ranged from 2.1 – 3.7 ml·100ml^{-1}, whereas the traditionally made distillate contained 23.8 – 45.6 ml·100ml^{-1}.

INTRODUCTION

The *masau* (*Ziziphus mauritiana*) tree occurs in arid and semi-arid regions in the world. Its fruit is one of the most commonly utilized wild fruits in Zimbabwe. A significant rural population in arid regions derive various products such as wood fuel (firewood), fodder, fertilizer (organic manure, forest litter), building material and herbs from *masau* trees (Morton, 1987). In some places, the *masau* tree is grown as a hedge with its spines creating effective live fencing. Its highly nutritious fruits provide a valuable source of energy, vitamins and also income (Bakhshi et al., 1974; Saka et al., 1994). Extracts from the fruits, seeds, leaves, roots and bark of the *masau* tree are used as traditional medicines to treat the effects of insomnia, skin diseases, inflammatory conditions and fever. Therefore, for these reasons, the *masau* tree plays an important role in the integrated economy of arid regions (Morton, 1987).

In Zimbabwe, the fruit undergoes spontaneous fermentation and is then distilled into a potent spirit called *kachasu* (Gadaga et al., 1999; Tredgold, 1986). The optimisation and control of the processing of indigenous fruit products such as traditionally fermented foods has the potential for multiple benefits, including the improvement of socio-economic status for rural communities through employment creation, augmenting family income and providing a non-seasonal supply of safe and quality beverages. In addition, environmental rehabilitation, domestication of the fruit trees as well as retaining an important food source (Cavendish, 2000) are reasons to develop the utilisation processes of wild fruits.

The objective of this study was to document the traditional processing techniques of *masau* fruits in Zimbabwe, and to study the physical and chemical properties of the fruit, the fermented pulp and its distillate.

MATERIALS AND METHODS

Survey of the traditional processing of *masau* fruits

A survey to document the handling and processing of *masau* fruits and products thereof was conducted in Muzarabani (16º 20' S, 31º 21' E), Mudzi (17º 17' S, 32º 35' E) and Mt Darwin (16º 27' N, 31º 53' E) districts of Mashonaland Central Province, Zimbabwe. A semi-structured questionnaire was administered to 30 randomly selected households in Muzarabani, 10 in Mudzi and 10 in Mt Darwin. Focus group discussions were also performed with women who sold *masau* fruits in Harare (two groups of between 10 and 8 women, in each of the three districts).

Sample collection and preparation

During the survey, 2 kg samples of fresh ripe and dried *masau* fruits were harvested from villages in Muzarabani district. Fermented *masau* pulp and the distilled product (200 ml samples) were collected from seven households that had been identified as brewers of *kachasu* in the same area. Police clearance was sought since *kachasu* is an illicit beverage.

Fermented pulp and distilled product were collected in 250 ml sterile bottles (Schott Duran, Elmsford, NY, USA) from brewers in Muzarabani communal area.

Samples were transported in a cooler box and kept at 5ºC on arrival at the laboratory, and analysed the following day.

Samples of fermented fruit pulp were centrifuged at 1207 x *g* (MSE Super Minor Centrifuge, Sussex, England). The supernatant was collected and then frozen at –18ºC until further analysis. The distillate was also kept at –18ºC until further analysis.

Composite samples of each set of fresh and dried fruit *masau* samples were prepared. The seed was extracted from the fruit pulp and skin by cutting open the fruit using a knife. The fruit pulp and skin (100 g) were homogenised using a blender (Waring® Commercial blender, Torrington, Connecticut, USA). A juice was made by mixing 100 g of the homogenate with 100 ml of distilled water. The juice was then centrifuged at 1207 x *g* and the supernatant was stored at -18ºC until further analysis.

Determination of pH

pH of the fermented fruit pulp and fruit juice was carried out using a Metrohm model 744 (Metrohm Ltd, Herisau, Switzerland) pH meter, with a combination glass electrode and calibrated using commercial buffers (Merck, Darmstadt, Germany) at pH 4 and 7.

Titratable acidity

A portion of the fruit juice (10 ml) was titrated against 0.1M NaOH (Soyer et al., 2003). The results were expressed as g of citric acid $100g^{-1}$ of fruit juice dry matter.

Determination of sugar, ethanol and organic acids

Fermented pulp, fruit juice and the distillate were analysed by High Performance Liquid Chromatography (HPLC), fitted with Refractive Index and UV/VIS detectors (Spectra System Thermo separation products, Reviera, Florida, USA). The separation was done on an Aminex HPX-87H ion exclusion column (300 x7.8mm^2) at an oven temperature of 40ºC and a flow rate of 0.6 ml min^{-1}. Juice and fermented fruit pulp were filtered through a 0.45 µm Millipore filter (Schleider & Schuell GmbH, Dassel, Germany). Standards for the organic acids (tartaric, malic, citric, succinic and oxalic) were obtained from ALDRICH Co. (Sigma-Aldrich Chemie, Steinheim, Germany) and sugars (sucrose, fructose and glucose) were obtained from Merck (Damstardt, Germany). The standard solutions were prepared individually by dissolving in double distilled water. The mobile phase was 5mM degassed H_2SO_4.

Statistical analysis

The analytical data were analysed using the statistical program SPSS 13.0 for Windows (Apache Software Foundation, USA) and the one-way ANOVA model was used applying the LSD test to evaluate significant difference among means.

RESULTS AND DISCUSSION

Survey of the traditional processing of *masau* fruits

Masau fruits ripen from mid-May and are available until the end of September in Muzarabani, Mudzi and Mt Darwin districts in Zimbabwe. The fruit is first green (Figure 1), turning yellow to brown as it ripens, and it is generally considered to have a sweet-sour taste. Two categories, sweet and sour, of the *masau* fruit are distinguished by the communities. Fruit sizes range from 1 - 2.5 cm in diameter and are influenced by location of the tree. The trees that grow in river banks have relatively larger fruits with a small stone, compared to those that grow in drier areas. Most respondents (96%, n=50) consume fresh *masau* fruits every day during the season. Women and children usually gather the fruits in the morning, and most families (92%) spend at most two hours per day in gathering *masau*. About 2 - 4 buckets of 20 litre capacity (approximately 30 - 60 kg in total) of *masau* fruits are collected per day per family depending on the number of persons gathering the fruit. Occasionally the *masau* fruit trees are located at homesteads and in fields belonging to particular families. The harvested fruits are consumed fresh by the local people, and also sold at rural and urban markets. Most respondents (80%) gather *masau* fruits for sale to retailers in urban markets. The prices of *masau* fruits range from approximately € (Euro) 1 - 3 per 15 kg depending on the market. The fruits can also be exchanged for soap, salt, sugar, clothes, kitchenware and tea leaves. Saka and Msonthi (1994) reported that indigenous fruits play a role in people's diet and contribute to the economy of the rural communities in Malawi, and we observed a similar situation in Zimbabwe.

In the study districts, surplus fruits are sun dried and processed later into various products such as porridge, traditional cake, *mahewu* and instant powder drink. The porridge for children and adults is made by mixing water and *masau* powder followed by boiling while stirring. The traditional cake (Figure 2) is made from *masau* powder by mixing it with a bit of water to enable it to be moulded into desired shapes, ready to be consumed as a snack. For *mahewu*, the dried *masau* fruits together with the seeds are pounded using pestle and mortar, and mixed with water to make slurry. This is left in the sun for a few hours and then consumed as a beverage. In Western Sudan (Egceling, 1951), cakes resembling gingerbread are made from a mixture of dried and fermented pulp. In India, the ripe fruits are mostly consumed raw, but sometimes candied fruits are made after pickling in a salt solution for 1 to 3 months (Morton, 1987). The latter products were not encountered during our survey. The practices of producing a fermented *masau* beverage in Muzarabani, Mudzi and Mt Darwin are the same. The dried *masau* pulp is mixed with water (approximately 1:10 ratio of pulp:water) and left to ferment spontaneously for 6 - 7 days. The fermented pulp (Figure 3) is not consumed as such because of its unattractive exterior and smell.

Figure 1. The *masau* (*Ziziphus mauritiana*) fruit tree found in Zimbabwe, showing ripe (yellow/ orange) and unripe fruits (green) (Photo taken by L.K. Nyanga).

Figure 2. Traditional *masau* (*Ziziphus mauritiana*) cake made by rural communities in Muzarabani, Zimbabwe. Scale: 1:1.5 (Photo taken by L. K. Nyanga).

The fermented pulp is transferred to a drum and distilled to obtain the spirit called *kachasu* using a set up shown in Figure 4. An outline of the fermentation and the distillation processes is presented in Figure 5. The sour type of *masau* is preferred for making *kachasu* because it is considered to give the spirit a better taste. Although *kachasu* can be made from other wild fruits such as *Adansonia digitata*, *Tamarindus indica* and *Ziziphus mucronata,* the respondent brewers preferred *kachasu* from *masau* because it had a better taste and flavour.

Figure 3. Mass of fermented *masau* (*Ziziphus mauritiana*) fruits from rural community in Muzarabani, Zimbabwe (Photo taken by L. K. Nyanga).

Figure 4. Women distilling fermented *masau* (*Ziziphus mauritiana*) fruit pulp to produce *kachasu* (Photo taken by L. K. Nyanga).

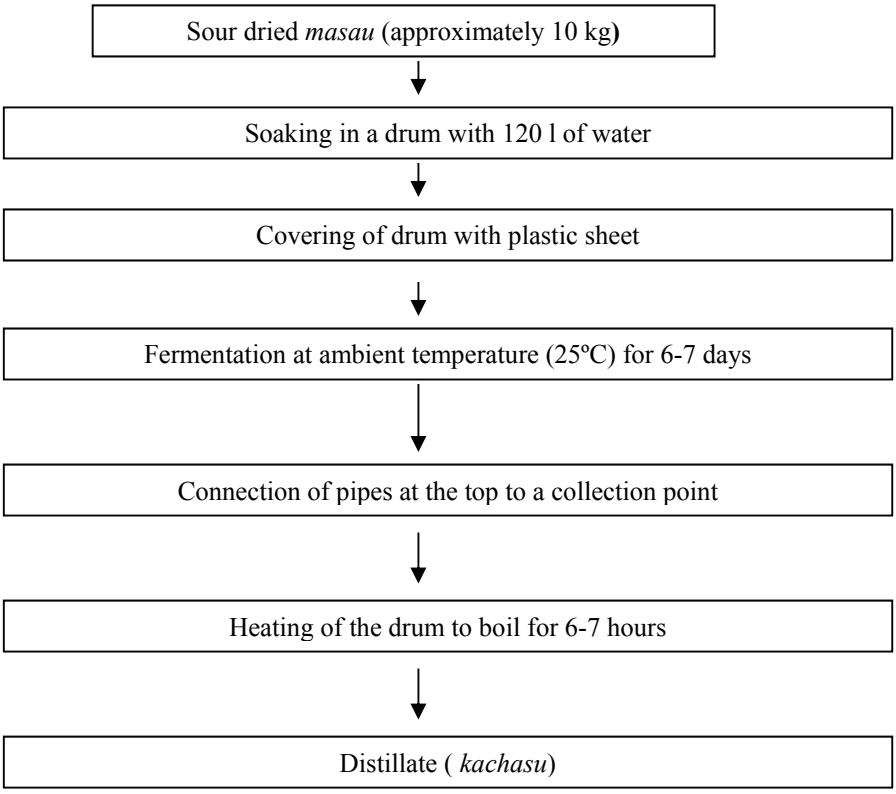


Figure 5. Flow diagram for the fermentation and distillation process of *masau* (*Ziziphus mauritiana*) fruit pulp to produce *kachasu* liquor.

Kachasu can be made from other substrates including maize meal, bulrush or finger millet meal, various fruits and banana peels. Usually other non-food ingredients such as the bark of the *masau* tree are added to make the distillate more intoxicating and this is one of the reasons this product was banned in 1971 (Brett et al., 1992). *Kachasu* is similar to *waragi* (Mwesigye et al., 1995) and *chang'aa* (Nout, 1979) which are produced in Uganda and Kenya, respectively. It was observed that the production of *kachasu* in the districts surveyed is done in private by certain families for income generation and livelihood. However, in other countries e.g. Malawi, spirits made from indigenous fruits have been promoted. For instance, distilled alcoholic liquors called *mlunguzi* are produced at an industrial scale from a combination of *Uapaca kirkiana* and *Ziziphus mauritiana* fruits (Maghembe et al., 1992). In Venezuela, a jujube liquor is made and sold as *Crema de ponsigue* (Morton, 1987). And in Uganda, spirits are produced by licensed communities and then sent to the main distillery where it is then triple-distilled to produce a bottled spirit (Mwesigye et al., 1995). Several variations in the use and pre-treatment of the ingredients used for the fermentation of *masau* were observed. Whole *masau* fruits are occasionally added to the pounded fruits before fermentation. In addition, other ingredients such as salt, the bark of the *masau* tree, crushed *usika* (*Tamarindus indica*) fruits, and malted millet and sorghum meal are added to the mixture. It was observed that in some cases, the fermented mixture is first sieved to remove the solid particles prior to the distillation process.

From 10 kg of dried *masau* fruits, the distillation process of its fermented pulp yields about 15 l of alcoholic spirit (*kachasu*). The spirit is collected in three batches according to their alcoholic strength. During consumption of the spirit, the last batch collected is often used to dilute the first batch. Soft drinks or water are also used to dilute the spirit. Male adults aged from about 20 years and older are the main consumers although some women aged about 40 and older also consume the spirit. Most respondents (98%) produce the spirit as a source of family income. A 750 ml bottle of spirit sells at approximately €1 – 2 depending on the demand.

The survey also reveals that besides providing food to the rural community, the *masau* tree and its fruits are important as traditional medicine and livestock feed. Of the 50 respondents, 65% reported that the bark of the tree is used to cure scorpion bites and the roots are traditionally used to cure colic problems in babies. The roots can also be boiled and the extract is ingested to reduce high blood pressure. The *kachasu* was reported to be a good remedy for persistent cough. The fruits are traditionally used to treat cold, flu and indigestion and also to stimulate appetite.

Composition of *masau*, fermented pulp and distillate (*kachasu*)

The levels of titratable acidity, pH, sugars, organic acids and ethanol in *masau* fruits, fermented pulp and distillate are shown in Tables 1 and 2. The values are reported on a dry matter basis.

The predominant acids in both fresh and dried fruits are citric, malic, oxalic and succinic acids (Table 1). Tartaric and acetic acids occur at considerably lower levels. Morton (1987) also detected the presence of citric, malic and oxalic acids in *Z. mauritiana* but did not quantify them. Muchuweti et al., (2005) detected citric, malic and malonic acids, but did not quantify their concentration. The citric acid content we found in fresh *masau* fruits is comparable to that of lemons and lime (4.2 – 8.3 $g \cdot 100g^{-1}$) (Nielsen, 1998). The sour taste of *masau* fruits is thus explained by presence of these organic acids.

In the fermented pulp, the major organic acids were citric acid and lactic acid ranging from 1.2 – 3.6 $g \cdot 100ml^{-1}$ and 1.5 – 3.3 $g \cdot 100ml^{-1}$, respectively (Table 2). Acetic and malic acids were found in lesser amounts, along with traces of oxalic acid. Lactic acid was found only in fermented pulp indicating that it is produced during fermentation. Malic acid levels were lower in fermented pulp than in *masau* fruits. Probably malic acid is assimilated or converted during the fermentation.

Glucose and fructose but not sucrose were identified in *masau* fruits. The equal amounts of glucose and fructose found in the fruits suggest the presence of invert sugar, which is found naturally in fruits and honey. The ethanol content of the fermented pulp ranged from 2.1 – 3.7 $ml \cdot 100ml^{-1}$. The distillate (*kachasu*) had alcohol levels ranging from 23.8 – 45.6 $ml \cdot 100ml^{-1}$.

Table 1. Sugars, organic acids, pH and titratable acidity of *masau* fruits in Zimbabwe.

Parameter (dry weight basis)	**Sample**	
	A[1]	**B**
Sugars ($g \cdot 100g^{-1}$)[3]		
Glucose	6.7 ± 0.14^{a}	7.5 ± 0.4^{a}
Fructose	6.8 ± 0.6^{a}	7.9 ± 0.7^{a}
Organic acids ($g \cdot 100g^{-1}$)		
Citric	3.8 ± 0.5^{a}	4.9 ± 0.5^{a}
Tartaric	0.5 ± 0.02	nd[2]
Malic	3.4 ± 0.13^{a}	4.8 ± 0.6^{b}
Succinic	2.6 ± 0.11^{a}	2.4 ± 0.02^{a}
Acetic	0.2 ± 0.11^{a}	0.3 ± 0.06^{a}
Oxalic	4.6 ± 0.8^{a}	4.6 ± 0.5^{a}
pH	3.6 ± 0.3^{a}	3.8 ± 0.5^{a}
Titratable Acidity (as $g \cdot 100g^{-1}$ citric acid)	5.6 ± 0.3^{a}	6.6 ± 0.02^{b}

[1]A, Fresh *masau* composition, B, dried *masau* fruit composition used for the fermentation process. [2]nd – not detected. [3]Means ± standard deviation, means in the same row having the same letter are not significantly different according to the LSD at the 0.05 level.

CONCLUSION

Masau fruit plays an important role in the livelihood of the rural communities in Zimbabwe. The fruit is consumed fresh and also sold at local markets. The sweet-sour taste of *masau* fruits is attributed to the presence of glucose and fructose, and citric, malic, oxalic and succinic acids. Surplus fruits are preserved by sun drying. Dried fruits can be transformed into various products such as porridge, traditional cake and *mahewu*. The dried fruit pulp can be spontaneously fermented and distilled to produce *kachasu*. The fermented *masau* pulp is not consumed because it smells and looks unattractive. However, it may be of interest to improve the fermentation process in order to produce a consistent and attractive quality product. Further studies of the microbiological properties of the fruit and the fermented pulp are needed to select and possibly develop starter cultures for improved fermentation processes.

Table 2. Sugars, organic acids, ethanol, pH and total acidity of fermented *masau* fruit pulp.

Sample	Sugars (g·100ml^{-1})			Ethanol (ml·100 ml^{-1})		Organic acids (g·100ml^{-1}) in fermented pulp					pH	Titratable acidity as (g·100g^{-1} citric acid)
	sucrose	glucose	fructose	pulp	distillate	citric	malic	acetic	lactic	oxalic		
F1[1]	0.27^{c2}	nd^{3a}	nd^{a}	3.7^{d}	29.6^{d}	2.4^{b}	nd^{a}	0.17^{b}	1.5^{a}	0.11^{b}	3.66^{b}	5.25^{c}
F2	0.37^{d}	0.12^{b}	0.64^{d}	4.0^{e}	31.1^{e}	3.6^{c}	1.31^{f}	0.12^{a}	3.3^{c}	nd^{a}	3.56^{a}	5.47^{c}
F3	nd^{a}	nd^{a}	0.19^{b}	2.9^{b}	25.7^{b}	1.5^{a}	0.45^{c}	0.20^{b}	2.6^{b}	nd^{a}	3.92^{c}	4.45^{a}
F4	0.01^{a}	nd^{a}	0.05^{a}	2.1^{a}	23.8^{a}	1.4^{a}	nd^{a}	0.14^{a}	2.4^{b}	nd^{a}	3.67^{b}	4.60^{a}
F5	nd^{a}	nd^{a}	0.05^{a}	3.2^{c}	27.7^{c}	3.6^{c}	0.53^{d}	0.12^{a}	2.1^{b}	nd^{a}	3.66^{b}	5.33^{c}
F6	0.23^{b}	nd^{a}	0.53^{c}	3.6^{d}	31.4^{e}	1.2^{a}	0.13^{b}	0.20^{b}	2.2^{b}	nd^{a}	3.55^{a}	5.29^{c}
F7	nd^{a}	nd^{a}	0.02^{a}	4.0^{e}	45.6^{f}	3.6^{c}	0.61^{e}	0.18^{b}	2.1^{b}	nd^{a}	3.51^{a}	5.17^{b}
Mean	0.13	0.02	0.21	3.4	30.7	2.5	0.4	0.16	2.3	0.06	3.65	5.08
STDEV	0.15	0.04	0.26	0.7	7.0	1.1	0.5	0.04	0.5	0.04	0.14	0.39

[1]F1 – F7 fermented *masau* samples from seven different households. [2]Means in the same column and with the same letter are not significantly different according to the LSD at the 0.05 level. [3]nd – not detected.

ACKNOWLEDGEMENTS

The authors thank the Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation for Science (IFS Grant No. C/3737) for financial support. We thank Julius Mutseka, Ernest Nyanga and Mr Mutemeri for their concerted efforts during the survey. We are grateful to Birgit Hasenack for assistance with the HPLC analyses.

CHAPTER 3

Nutritional potential of *masau* (*Ziziphus mauritiana*) fruits from Zambezi Valley in Zimbabwe

L. K. Nyanga, M. J. R. Nout, T. H. Gadaga, T. Boekhout, E. J. Smid and M. H. Zwietering. Food Chemistry. Accepted for publication.

ABSTRACT

Ziziphus mauritiana (masau) fruits are consumed by many people in Zimbabwe. The fruits contribute significantly to the people's diet when they are in season. The objective of this study was to determine the nutritional content of the fruits and, hence, quantify their contribution to the diet. Dry matter content ranged from 21.1±0.23 to 24.1±0.34 g·100g^{-1} of edible portion of the sweet and sour fruits, and 84.8±0.21 to 87.2± 0.24 g·100g^{-1} for the dried fruit. Crude protein per 100 g edible portion of dry weight ranged between 7.9±0.02 and 8.7±0.02 g, crude fat from 0.8±0.02 to 1.5±0.02 g, crude fibre from 4.9±0.02 to 7.3±0.02 g, ash between 3.0±0.01 and 4.3±0.02 g and carbohydrate between 79.5±0.03 and 83.2±0.03 g. The fruits were rich in vitamin C (15.0±0.02 – 43.8±0.02 mg·100g^{-1}) and the energy values ranged between 1516.0±1.73 and 1575.0±2.33 kJ·100g^{-1}. Furthermore, the fruits contained (mg·100g^{-1}of dry weight) potassium from 1865.0±1.31 to 2441.0±1.14, calcium from 160.0±0.31 to 254.0±0.14, sodium between 185.0±0.12 and 223.0±0.23, magnesium between 83.0±0.04 and 150.0±0.13 and phosphorous from 87.0±0.14 to 148.0±0.52. Manganese and copper contents ranged between 0.7±0.03 and 1.6±0.03, while iron and zinc ranged between 2.1±0.43 and 4.3±0.12, and 0.6±0.01 – 0.9±0.01 mg·100g^{-1}of dry weight, respectively. The *masau* fruit is, therefore, a good source of carbohydrates, proteins and micronutrients, such a calcium, potassium, sodium, phosphorous, copper, iron, vitamin C and zinc.

INTRODUCTION

Indigenous fruits are essential for food security, health and nutrition, and economic welfare of rural communities in the developing world (Saka, Rapp, Akinnifesi, Ndolo, & Mhango, 2007). *Ziziphus mauritiana* (Rhamnaceae), *Sclerocarya birrea* (Anacardiaceae), *Uapaca kirkiana* (Phyllanthaceae), *Strychnos cocculoides* (Loganiaceae) and *Adansonia digitata* (Bombaceae) are amongst the commonly utilized indigenous fruits in Africa.

In Zimbabwe, the collecting, processing, storing and marketing of indigenous fruits are notable coping strategies adopted by rural communities to reduce hunger, improve nutrition and generate income (Mithofer, Waibel, & Akinnifesi, 2006). *Ziziphus mauritiana* fruits, locally called *masau* in the Shona language, are extensively gathered, processed and marketed (Nyanga, Nout, Gadaga, Boekhout, & Zwietering, 2008). *Masau* fruits ripen from mid-June and are available until the end of September. Although the trees can grow in many different parts of the country, they normally grow and fruit in warmer climates as is found in the low lying areas of the Zambezi valley. Rural communities in Zimbabwe distinguish the fruits into sour and sweet categories (Nyanga et al., 2008). The fruit is first green, turning yellow to brown as it ripens, and it is generally considered to have a sweet-sour taste. Fruit sizes range from 1 – 3cm in diameter.

Some research has been done in Zimbabwe to improve the postharvest quality of the *masau* fruit (Tembo, Chiteka, Kadzere, Akinnifesi, & Tagwira, 2008), and on the traditional processing of the fruit (Nyanga et al., 2008). Microorganisms, such as lactic acid bacteria and yeasts, have been isolated from the fruits and were identified (Nyanga et al., 2007). Other studies have shown that these fruits are potentially a rich source of vitamin C and minerals (Morton, 1987). Rathore (2009) also reported that *Z. mauritiana* was richer than apple in protein, phosphorus, calcium and vitamin C, and contained more phosphorus, iron, vitamin C, calorific value and carbohydrates than oranges. However, a great variation has been recorded in the fruit's nutritional content depending on the source and/or cultivar of the fruit. Although the fruit is widely sold at rural and urban markets, information on the nutritional content of the *masau* fruits found in Zimbabwe is scarce. Availability of nutritional data can improve the perception of the fruit, which is usually considered as a poor man's fruit. The aim of the current study, therefore, was to determine the nutritional value of *masau* fruits obtained in Zimbabwe over a period of two seasons. The information will be a useful guide in nutrition assessments because the fruits are an important part of the diet in some rural and urban communities in Zimbabwe, and will aid in marketing efforts, so as to improve the livelihood of rural families.

MATERIALS AND METHODS

Sampling and Processing

Mature, fresh, sour, and sweet *masau* fruits were procured from villages in Muzarabani (16º 20' S, 31º 21' E) rural community in 2006 and 2007, in the month of August when the *masau* fruits are in season. The fruits were taken from fruit trees in fields belonging to certain households identified by the village extension officer. The same households were visited for each sampling trip. Samples of the dried *masau* fruit were obtained from the same households. Fruits collected from different villages were put into three categories; i.e. sweet fresh, sour fresh and sour dried. Ten samples were collected for each category of fruits per season. The fruits were packed in polyethylene bags and transported in a cooler box to the laboratory at the University of Zimbabwe. The fruits were kept overnight at 4^{o}C and processed the following day. The fruits were washed with distilled water before analysis. The fruits from each category from the same year were pooled to make a composite sample of 1 kg. Composite samples of each fruit category were made to get a representative sample of what the rural and urban people consume. The fruits are usually gathered from different areas and trees to fill up 10-20 kg buckets and are transported home, to the rural markets, or as truck loads to the urban markets. The fruits were sorted based on quality and size. Mature, unblemished fruits were used for analysis. For the fresh fruits, 10 to 15 fruits (100 g) from each of the ten collected samples were taken for processing and 40 to 50 fruits (100 g) for the dried samples. The seeds were carefully removed by knife from pulp and skin, which were then homogenised using a Waring blender. The samples were stored at 4^{o}C until required for analysis. All analyses were done in triplicate.

Chemical analysis: (i) Determination of proximate composition

The homogenised fruit samples were analysed for dry matter, ash, crude fibre, fat, and crude protein according to the A.O.A.C. (1990) official methods. Briefly, dry matter was determined by drying a portion (2 g) from the composite sample for each year to constant weight in an oven at 105^{o}C. Ash was determined by incinerating the dried sample (2 g) in a muffle furnace at 600^{o}C for 6 hours. Crude protein was estimated by the macro-Kjeldahl method, and calculated by multiplying the measured nitrogen by 6.25 (method 978.04, A.O.A.C, 1990). An aliquot of 3 g was used to determine crude fat by extracting with petroleum ether (40 – 60°C) in a Soxhlet apparatus. The crude fibre was determined by alternately digesting the dried, defatted sample (2 g) in 1.25% HCl and 1.25% NaOH (method 930.10, A.O.A.C, 1990). The digested sample was then ashed in a muffle furnace at 600^{o}C. The crude fiber was then expressed as percent weight loss on ignition at the ashing temperature. Carbohydrate levels were calculated by subtracting the total sum of crude protein, crude fat, and crude fibre from 100% dry weight sample. The fruit calorific value (expressed in kJ) was estimated by multiplying the percentages of

crude protein, crude fat and carbohydrate by the factors 16.7, 37.7 and 16.7 respectively (A.O.A.C., 1990).

(ii) Determination of vitamin C

Vitamin C was estimated by titration with 2,6-dichlorophenolindophenol (DCPIP, 0.05%, w/v, Sigma-Aldrich Chemie, Steinheim, Germany) (Paul & Pearson, 1967). Briefly, the fruit juice made from a portion of the homogenised composite sample was diluted with an equal volume of 1% oxalic acid (Merck, Damstardt, Germany). To 10 ml of the diluted juice acetone (2.5 ml) was added and the mixture was allowed to stand for 10 minutes in the dark. The mixture was then titrated with dichlorophenylindophenol solution until a weak pink colour was observed.

(iii) Determination of mineral content

Potassium and sodium were determined by a method described by Bonire, Jahil, & Lori, (1990). The ash of the pulp (0.5 g) was digested by adding a mixture of perchloric acid (60%, 1ml), nitric acid (70%, 5 ml) and sulphuric acid (98%, 0.5 ml) in a Kjeldahl flask. The digestion was begun at low heat with swirling until evolution of brown fumes subsided, and continued at steadily increasing temperatures controlling the rest of digestion until the solid was dissolved. After cooling, the digest was made up to 100 ml with distilled water. Sodium and potassium were then determined using flame emission (FAAES, SHIMADZU Corporation, AA 6406 series). Calcium, magnesium, iron, zinc, copper and manganese were determined using atomic absorption spectroscopy according to the A.O.A.C. method 968.08 (1990). The sample was prepared in a similar way to that of sodium and potassium above. In order to avoid phosphate and ionisation interferences, an additional 1/10 (v/v) dilution was performed in lanthanum chloride (1.8%, w/v) for determination of calcium and magnesium. Sample concentrations of each element were determined by comparing absorbance to a standard linear regression curve from standard solutions. Phosphorus was determined using a Scanning UV-VIS-NIR Spectrophotometer (UV- 3101PC, SHIMADZU Corporation, Japan) according to the method described by Lazano-Calero, Martin-Palomeque, & Madueno-Loriguillo (1996). Standards for the minerals (Ca, Mg, K, Na, P, Fe, Cu, Mn and Zn) and other reagents (perchloric acid, nitric acid, sulphuric acid and lanthanum chloride) were obtained from ALDRICH Co. (Sigma-Aldrich Chemie, Steinheim, Germany).

Statistical Analysis

One way analysis of variance (ANOVA) was used to compare means of samples of the *masau* fruit categories (i.e. sweet fresh, sour fresh and sour dried) and between the two years. Least Significant Difference (LSD) was used to identify significant differences at $\alpha = 0.05$ levels using Statistical Package for Social Sciences (SPSS) version 16.

RESULTS AND DISCUSSION

Proximate and vitamin C composition

Dry matter, moisture, carbohydrate, crude protein, crude fat and fibre, vitamin C and energy content of *masau* fruits gathered during two consecutive years, 2006 and 2007, were analysed. The proximate and vitamin C composition of the fruits are presented in Table 1. While the dry matter content and vitamin C were determined on a fresh weight basis, all the other parameters were based on dry weight. Overall, there was no significant variation ($p< 0.05$) in the proximate composition among samples in the same category collected in the 2006 and 2007 seasons. The major components of the fruit were moisture and carbohydrates. The moisture content of the fresh fruits ranged from 75.9±0.32 – 78.9±0.24 g·100g^{-1} (value not shown in the table). The range of the moisture content of the fruit was similar to that of the conventional fruits which ranges between 75 – 95% (Ruiz-Rodríguez et al., 2011).

Dry matter content of fresh sweet and fresh sour fruits ranged between 21.1±0.23 – 24.1±0.34 g·100g^{-1} and for the dried sour fruit it was 87.2±0.24 and 84.8±2.1 g·100g^{-1} for 2006 and 2007 seasons, respectively. The dry matter contents which are 21.1±0.23 and 24.1±0.34 g·100g^{-1} for the sour fruit, and 22.5±0.32 and 23.2±0.21 g·100g^{-1} for sweet fruit (for the two seasons, respectively) were higher than the 14.8 g·100g^{-1} reported by Saka & Msonthi (1994) from *Z. mauritiana* fruits from Malawi. This is probably due to different environmental conditions, such as water availability, sunlight and wind exposure which contribute to fruit desiccation (Ruiz-Rodríguez et al., 2011). However, the dry matter values are similar to those of other edible wild fruits such as *Strychnos innocua* and *Strychnos spinosa*, with values of 21.8 and 22.1 g·100g^{-1}, respectively (Saka & Msonthi, 1994).

The ash content ranged between 3.0±0.01 and 3.6±0.11 g·100g^{-1} for both the sour and sweet fruits. These values are similar to those reported by Lockett, Calvet, & Grivetti (2000). In that particular study, *Z. mauritiana* from Nigeria was found to have about 3.53 g·100g^{-1} ash content. However, the ash content values in the current study were lower than the 10.1 g·100g^{-1} reported by Saka et al. (1994).

The *masau* fruit had a crude protein ranging between 8.0±0.13 and 8.7±0.02 g·100 g^{-1} for year 2006, and 7.9±0.02 and 8.6±0.02 g·100g^{-1} for year 2007 for sour and sweet fruits, respectively. The crude protein contents for the sour fruits were similar for the years 2006 and 2007 (7.9±0.0 g·100g^{-1}). These values are relatively high considering that fruits are generally not good sources of protein (Agrahar-Murugkar & Subbulakshmi, 2005). Osman (2004) reported a value of 3.2 g·100g^{-1} of crude protein for baobab fruit pulp. Other wild edible fruits, namely *Adansonia digitata*, *Sclerocarya birrea*, *Strychnos spinosa* and *Vanguenia infausta* from Botswana were reported to have crude protein contents ranging from 1.3 to 3.7 g·100g^{-1} (Amerteifio & Mosase, 2006). Rathore (1999) reported that fruits such as orange, mango, grapes, banana and papaya have crude protein contents of 0.7, 0.6, 0.5, 1.2 and 0.6, respectively. Fresh sweet *masau* fruit contained

significantly ($p< 0.05$) more crude protein (8.7±0.02 and 8.6±0.01 g·100 g^{-1}) than the fresh sour fruit (8.0±0.13 and 7.9±0.02 g·100g^{-1}) in both seasons. There were no significant differences ($p>0.05$) in the crude protein contents between seasons for each category of *masau* fruit. The crude protein values recorded for sweet and sour *masau* fruits were much higher than 2.42 g·100g^{-1} as reported by Lockett et al. (2000), 4.1 g·100g^{-1} reported by Saka et al. (1994) and 0.46 g·100g^{-1} reported by Mahapatra, Mishra, Basak & Panda (2012) for *Z. mauritiana* from other regions.

The crude fat content ranged from 0.8±0.02 to 1.5±0.01 g·100g^{-1} for the fruits during the two seasons. The crude fat content of dried fruits (0.8±0.02 g·100g^{-1}) was significantly ($p<0.05$) lower than the contents (1.5±0.01 and 1.5±0.02 g·100g^{-1}) found in both fresh sweet and fresh sour fruits. The lower value in the dried fruit could be attributed to some degradation of the lipids during drying (Lewicki, 1998).

The sweet fruits had significantly higher amounts of crude fibre (7.3±0.02 g·100 g^{-1}) as compared to the sour fruits (5.2±0.02 and 5.3±0.02 g·100g^{-1}) and dried (4.9±0.02 and 5.0±0.01 g·100g^{-1}) fruits. The levels of crude fibre in the *masau* fruits in this study was higher compared to 3.4 g·100g^{-1} reported by Saka et al. (1994).

The *masau* fruits were rich in carbohydrates and the values ranged from 79.5±0.03 to 83.2±0.03 g·100g^{-1} for the two seasons. The carbohydrate values were similar among the samples studied. Lockett et al. (2000) reported a lower carbohydrate value of 66.02 g·100g^{-1} for *Z. mauritiana* fruits found in Nigeria. The energy values, which ranged from 1516±1.73 to 1575±2.42 kJ·100g^{-1}, are indicative of the high carbohydrate content. In another study, Saka et al. (1994) reported a similar energy value of 1588 kJ 100g^{-1}.

The vitamin C content was found to be highest in sweet fruit samples (40.7±0.02 and 43.8±0.02 mg·100g^{-1}), followed by sour fruits (27.8±0.24 and 28.6±0.82 mg·100g^{-1}) and the lowest amount was found in dried fruits (15.0±0.20 and 18.2±0.10 mg·100g^{-1}). There were significant differences ($p< 0.05$) in vitamin C content among the sweet, sour and dried samples. The difference in vitamin C content among the sweet and sour fruits samples can probably be due to the fact that the sweet *masau* trees are found mainly along river banks which are cooler than the drier areas where the sour *masau* fruit trees are located. Naggy (1980) reported that vitamin C content in fruits is affected by production factors, environmental conditions, maturity state and position on the tree, type of fruits (species and variety), handling and storage. As expected, the dried sour fruits had low vitamin C content. Tembo et al. (2008) also showed that the vitamin C content in *masau* fruits was negatively affected by drying. Moreover, temperature and duration of storage have also been reported to affect vitamin C content in *masau* fruits (Tembo et al., 2008).

Table 1. Nutrient composition of *masau* fruits from Muzarabani rural community in Zimbabwe

***Masau* fruit**	**Dry matter* (g)**	**Ash (g)**	**Crude protein (g)**	**Crude Fat (g)**	**Crude Fibre (g)**	**Carbohydrate **(g)**	**Energy Value (kJ)**	**Vitamin C* mg**
Sour								
2006	24.1±0.34^{a}	3.6±0.11^{a}	8.0±0.13^{a}	1.5±0.01^{a}	5.3±0.02^{a}	79.5±0.03^{a}	1517.0±1.24^{a}	28.6±0.82^{a}
2007	21.1±0.23^{a}	3.1±0.02^{b}	7.9±0.02^{a}	1.5±0.02^{a}	5.2±0.02^{a}	79.6±0.03^{a}	1516.0±1.73^{a}	27.8±0.24^{a}
Sweet								
2006	23.2±0.21^{a}	3.0±0.01^{b}	8.7±0.02^{b}	1.5±0.01^{a}	7.3±0.02^{b}	81.7±0.03^{b}	1566.0±2.33^{a}	43.8±0.02^{b}
2007	22.5±0.32^{a}	3.1±0.03^{b}	8.6±0.01^{b}	1.5±0.02^{a}	7.3±0.03^{b}	82.3±0.03^{b}	1575.0±2.42^{a}	40.7±0.02^{b}
Sour-Dried								
2006	87.2±0.24^{c}	4.3±0.02^{a}	7.9±0.03^{a}	0.8±0.02^{b}	4.9±0.02^{a}	82.1±0.03^{b}	1535.0±1.43^{a}	18.2±0.10^{c}
2007	84.8±0.21^{c}	3.1±0.01^{b}	7.9±0.02^{a}	0.8±0.02^{b}	5.0±0.01^{a}	83.2±0.03^{b}	1550.0±1.32^{a}	15.0±0.20^{c}

*Dry matter and Vitamin C based on 100g fresh weight, all other parameters based on 100g dry weight. **Calculated by difference. Values are means of three analyses. Means with standard deviations in the same column having the same letter are not significantly different (p<0.05).

The vitamin C contents obtained in this study, however, were lower than the values (65.8 – 76 mg·100g^{-1}) reported in fruits from India, and those from semi-arid lowlands in West Africa (96 – 500 mg·100g^{-1}) (Morton, 1987; Leakey, 1999). The amount of vitamin C in the sweet fruits was comparable to that found in grapes (38 mg·100g^{-1}) but less than in oranges (50 mg·100g^{-1}) and strawberries (59 mg·100g^{-1}) (Eromosele, Eromosele, & Kuzhkuzha, 1991). The sweet fruit vitamin C content (40.7±0.02 and 43.8±0.02 mg·100g^{-1}) is also comparable to that of *Vitex mombassae* (40.4 mg·100g^{-1}) and higher than the values reported for *Sclerocarya birrea, Uakapa kirkiana* and *A. digitata* (20.1, 20.4 and 20.3 mg·100g^{-1}, respectively) wild fruits, as reported by Ndabikunze et al. (2010). Vitamin C is the primary water soluble antioxidant in the human body, eliminating free radicals and preventing damage in the aqueous environment both inside and outside cells (Pattison et al., 2004).

Mineral content of the fruits

The mineral composition of *masau* fruit classes based on dry weight are shown in Table 2. Macroelements (Ca, Mg, K, Na and P) and microelements (Fe, Cu, Mn and Zn) were analysed. Amongst the macroelements, potassium was the most abundant mineral present in the fruits ranging from 1865±1.31 to 2441±1.14 mg·100g^{-1}, followed by calcium (160.0±0.31 – 254.0±0.14 mg·100g^{-1}), then sodium (194.0±0.22 – 223.0±0.23 mg·100g^{-1}). Magnesium and phosphorus were the least abundant mineral elements with values ranging between 83.0±0.04 to 150.0±0.13 mg·100g^{-1}. There was significant variation ($p<0.05$) in the amount of calcium and potassium amongst the years (2006 and 2007) for both sour and sweet fruits. The calcium content of both sweet and sour fruits (160.0±0.31 and 238.0±0.14 g·100g^{-1}, respectively) in 2006 were lower than the values (171.0±0.34 and 248.0±0.2 g·100g^{-1}, respectively) obtained in 2007. Nutrient content in the fruits can be influenced from year to year by weather conditions and the local environment (Feyssa, Njoka, Asfaw, & Nyangito, 2011). However, no variation was observed for the other elements for the two seasons.

The content of potassium (1865±1.31 to 2441±1.14 mg·100g^{-1}) found in this study is many folds higher that found in fruits, such as guava (417 mg·100g^{-1}), orange (200 mg·100g^{-1}), apple (90 mg·100g^{-1}) and banana (358 mg·100g^{-1}) (Mahapatra, Mishra, Basak & Panda, 2012). Potassium has diverse roles in the human metabolism and body functions and is essential for proper functioning of cells, tissues and organs. The calcium content of sweet fruits (160±0.31 and 171±0.34 mg·100g^{-1}) was significantly ($p< 0.05$) lower than that of sour and dried fruits (238±0.14 and 248.0±0.23, and 251.0±0.34 and 254±0.14 mg·100g^{-1}, respectively), for the two seasons. Calcium content (160.0±0.31–254.0±0.14 mg·100g^{-1}) of the *masau* fruits was also found to be much higher than known values of fruits such as guava (18 mg·100g^{-1}), orange (11 mg·100g^{-1}), pears (4 mg·100g^{-1}) and strawberry (22 mg·100g^{-1}) as reported by Mahapatra et al. (2012). The sour and dried fruits had higher amounts of magnesium (90±0.14 and 92.0±0.12 mg·100g^{-1}, and 149.0±0.83 and 150±0.13 mg·100g^{-1}, respectively) compared to the sweet fruits (83±0.04 and 85±0.23 mg·100g^{-1}), for the two seasons. The range of values (83.0±0.04 –

2441.0±1.14 mg·100g^{-1}) of macroelements found in *masau* fruits in this study for calcium, magnesium, potassium, sodium and phosphorus are much higher than the range of values (1.2±0.4 – 175.7±73.5 mg·100g^{-1}) reported by Ndabikunze et al. (2010) for other wild fruits such as *V. mombasse*, *U. kirkiana*, *S. birrea* and *A. digitat*a. The calcium, magnesium and potassium have essential roles in a variety of body functions including bone health and heart, muscle, nerve and immune systems maintenance.

Regarding the microelements, no variation among samples of the two seasons was observed. Iron was the main element showing highest values (2.1±0.54 – 4.3±0.12 mg·100g^{-1}). The sweet fruits had significantly higher amounts of iron (3.8±0.14 and 4.3±0.12 mg·100g^{-1}) compared to the sour and dried fruits (2.1±0.54 – 2.9± 0.03mg·100g^{-1}). Copper and manganese contents were of similar ranges (0.7±0.12 – 1.5±0.32 and 0.7±0.03 – 1.6±0.03 mg·100g^{-1}, respectively). Zinc content was the lowest with values ranging between 0.6±0.03 and 0.9±0.01 mg·100g^{-1}. Mahapatra et al. (2012) reported lower values for iron (1.04 mg·100g^{-1}), copper (0.17 mg·100g^{-1}), manganese (0.52 mg·100g^{-1}) and zinc (0.49 mg·100g^{-1}) contents of *Z. mauritiana* from India as compared to iron (2.1±0.54 – 4.3±0.12 mg·100g^{-1}), copper (0.7±0.12 – 1.5±0.32 mg·100g^{-1}), manganese (0.7±0.03 – 1.6±0.03 mg·100g^{-1}) and zinc (0.6±0.03 – 0.9±0.01 mg·100g^{-1}) contents obtained from the current study. The *masau* fruit contents of iron (2.1±0.54 – 4.3±0.12 mg·100g^{-1}) and zinc (0.6±0.03 – 0.9±0.01 mg·100g^{-1}) are much higher than the values of iron (0.09 – 0.11 mg·100g^{-1}) and zinc (0.02 – 0.22 mg·100g^{-1}) reported by Amerteifio et al. (2006) for other wild fruits like *A. digitata*, *S. birrea*, *S. spinosa* and *Vangueria infausta*. The iron content of *masau* fruits is also higher than the content found in fruits such as mango, orange, grapes, banana and papaya, as reported by Mahapatra et al. (2012). There is, therefore, value in consuming *masau* fruits when they are available.

Table 2. Mineral content in *masau* fruits from Muzarabani rural community in Zimbabwe

***Masau* fruits**	**Minerals (mg·100g^{-1})***								
	Ca	**Mg**	**K**	**Na**	**P**	**Fe**	**Cu**	**Mn**	**Zn**
Sour									
2006	238.0±0.14^{a}	92.0±0.12^{a}	2133.0±1.42^{a}	198.0±0.53^{a}	145.0±0.24^{a}	2.1±0.54^{a}	0.9±0.44^{a}	1.6±0.03^{a}	0.6±0.03^{a}
2007	248.0±0.23^{b}	90.0±0.14^{a}	2317.0±1.23^{b}	185.0±0.12^{b}	148.0±0.52^{a}	2.1±0.43^{a}	1.0±0.23^{a}	1.5±0.01^{a}	0.7±0.01^{a}
Sweet									
2006	160.0±0.31^{c}	85.0±0.23^{b}	1865.0±1.31^{c}	216.0±0.24^{c}	90.0±0.33^{b}	4.3±0.12^{b}	0.7±0.12^{b}	0.8±0.02^{b}	0.6±0.01^{a}
2007	171.0±0.34^{d}	83.0±0.04^{b}	1934.0±2.42^{d}	223.0±0.23^{c}	87.0±0.14^{b}	3.8±0.14^{b}	0.8±0.33^{b}	0.7±0.03^{b}	0.6±0.02^{a}
Sour-Dried									
2006	254.0±0.14^{e}	150.0±0.13^{c}	2416.0±1.34^{e}	197.0±0.24^{d}	119.0±0.42^{c}	2.7±0.04^{c}	1.5±0.32^{c}	1.1±0.02^{c}	0.8±0.02^{b}
2006	251.0±0.34^{e}	149.0±0.83^{c}	2441.0±1.14^{e}	194.0±0.22^{d}	120.0±0.23^{c}	2.9±0.03^{c}	1.5±0.21^{c}	1.1±0.02^{c}	0.9±0.01^{b}

*Based on dry weight. Values are means of three analyses. Means with standard deviations in the same column having the same letter are not significantly different ($p< 0.05$).

CONCLUSION

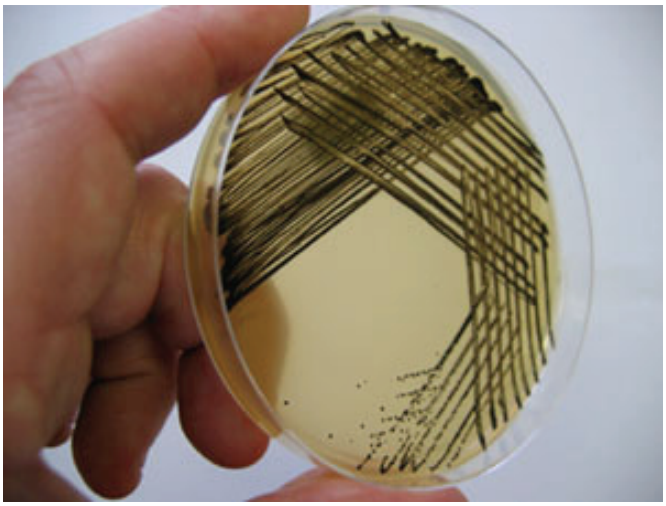
The *Z. mauritiana* (*masau*) fruits from the Zambezi valley in Zimbabwe contain nutritionally significant levels of important nutrients including minerals, fibre, carbohydrates and crude protein. The sweet *masau* fruit were found to be richer in vitamin C than the sour fruit, whereas the sour fruit were richer in minerals. The fresh sweet fruits were particularly richer in vitamin C than the sour fruits. However, there was very little variation in nutrient content with season. The results show that the nutrient value of *masau* wild fruits can even be higher than some domesticated popular fruits, such as mango, guava, orange and strawberry in terms of protein, carbohydrates and the micronutrient contents. This makes the fruit a potential contributor towards a balanced diet for children and adolescents in Zimbabwe and surrounding countries, considering their low cost and high abundance. Therefore, consumption of *masau* fruits needs to be promoted as they can contribute significantly to the diet and income of the rural people of Zimbabwe in the areas where the fruits are found.

ACKNOWLEDGEMENTS

The financial support by the MacGillavry Fund of the Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation for Science (IFS Grant No. C/3737), is gratefully acknowledged. The authors thank the technical assistance given by Evan Nyatanga and Power Gombiro.

CHAPTER 4

Yeasts and lactic acid bacteria microbiota from *masau* (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe



L. K. Nyanga, M. J. R. Nout, T. H. Gadaga, B. Theelen, T. Boekhout and M. H. Zwietering: International Journal of Food Microbiology (2007) 120, 159-166.

ABSTRACT

Masau are Zimbabwean indigenous fruits, which are usually eaten raw and/ or processed into products such as porridge, traditional cakes, *mahewu* and jam. Yeasts, yeast-like fungi, and lactic acid bacteria (LAB) present on the unripe, ripe and dried fruits, and in the fermented *masau* fruits collected from Muzarabani district in Zimbabwe were isolated and identified using physiological and molecular methods. The predominant species were identified as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *P. fabianii* and *Aureobasidium pullulans*. *Aureobasidium pullulans* was the dominant species on the unripe fruits but was not isolated from the fermented fruit pulp. *S. cerevisiae* and *P. kudriavzevii* were predominant in the fermented fruit pulp but were not detected in the unripe fruits. *Saccharomyces cerevisiae*, *P. kudriavzevii*, *P. fabianii* and *S. fibuligera* are fermentative yeasts and these could be used in the development of starter cultures to produce better quality fermented products from *masau* fruit. The predominant lactic acid bacteria identified were *Lactobacillus agilis* and *L. plantarum*. Other species identified included *L. bifermentans*, *L. minor*, *L. divergens*, *L. confusus*, *L. hilgardii*, *L. fructosus*, *L. fermentum* and *Streptococcus* sp. Some of the species of LAB can potentially be used in a mixed-starter culture with yeasts and might contribute positively in the production of fermented *masau* fruit products.

INTRODUCTION

Ziziphus mauritiana fruit, which is locally called *masau* fruit in Zimbabwe, is one of the wild fruits that are traditionally fermented into beverages through spontaneous and uncontrolled processes. Other wild fruits that are spontaneously fermented include *mapfura* (*Sclerocarya birrea* subspecies *caffra*), *hacha* (*Parinari curatellifolia*) and *mazhanje* (*Uapaca kirkiana*). However, because the fermentations are spontaneous and uncontrolled, the product microbiota are inconsistent and products' quality is variable (Gadaga et al., 1999; Halm et al., 1993; Sanni et al., 1994). The fermentations mainly rely on the microbiota from the fruit surfaces and to some extent from the utensils used during the fermentation process. The diverse microbial flora on fruit surfaces may play an important role during the spontaneous fermentation process (Fleet, 2003). The type of microorganisms includes coliforms, lactic acid bacteria, yeasts and moulds (Mbugua, 1985). The spontaneous fermentation of grape juice into wine, as observed by Louis Pasteur about 150 years ago, established the broadly accepted view that yeasts have a natural association with fruits and fruit products (Fleet, 2003). *Ziziphus mauritiana* was also reported previously to be among the Zimbabwean indigenous fruits that are associated with high numbers of microbes which contribute to the natural fermentation following ripening (Chivero et al., 2001).

In Zimbabwe, *masau* fruits are spontaneously fermented and distilled into a potent spirit called *kachasu* (Gadaga et al., 1999; Tredgold, 1986), that is highly intoxicating and is regarded as illegal beverage (Brett et al., 1992). The fermented fruit pulp is not consumed as such, because of its unattractive exterior and smell.

The microbial changes taking place during the spontaneous fermentation of *masau* have not been recorded. The objective of this study was therefore, to isolate and identify yeasts and LAB microbiota that are involved in the spontaneous fermentation of *masau* fruit pulp.

MATERIALS AND METHODS

Sample collection

The collection and analysis of samples was done in two consecutive years 2004 and 2005 during the month of August when *masau* fruits are in season. Both ripe and unripe *masau* fruits were harvested from Muzarabani (16º 20' S, 31º 21' E) district in Northern Zimbabwe. Dried fruit samples were obtained from the local people who ferment the fruits. The fruits were collected into sterile stomacher bags (Stomacher® Lab System, London, UK) and ferried to the laboratory in a cooler box. Fermented samples (200 ml) (n=7 for each year) were collected into sterile 250 ml screw capped bottles (Schott Duran, Elmsford, NY, USA) from the local people in Muzarabani. Samples of distilled product made from *masau* fruits were also collected into 250 ml sterile bottles (Schott Duran). These samples were transported to the laboratory in a cooler box and were kept at 5ºC on arrival and processed the following day.

Microbiological analyses

Isolation of yeasts and LAB from *masau* fruits and the fermented fruit pulp

Masau fruits (100 g) were aseptically weighed and transferred into sterile 250 ml bottles containing Peptone Physiological Saline (100 ml) [0.8% NaCl (Merck, Darmstadt, Germany) 0.1% neutral peptone (Oxoid, Basingstoke, UK); PPS]. The bottles were vigorously shaken by hand for 10 minutes. Appropriate serial dilutions were plated in duplicate on Malt Extract Agar (MEA) (Oxoid) containing 0.1% oxytetracycline Sigma-Aldrich Co., St Louis, MO, USA), and on de Man, Rogosa, Sharpe (MRS) agar (Oxoid) containing 0.1% natamycin (Aldrich Chemical Co., Gillingham, Dorset, England). Similarly, appropriate serial dilutions were also made from the fermented fruit pulp samples and plated in duplicate on MEA containing 0.1% oxytetracycline and MRS agar containing 0.1% natamycin. The MEA plates were incubated at 25ºC for 48 h. MRS agar plates were incubated at 37ºC for 48 h under anaerobic conditions using the BBL® Gas Pak® Anaerobic systems (Becton Dickinson Company, Maryland, USA) according to manufacturer's instructions. After the incubation a differential count based on colony colour was made for yeasts of different colony morphology, whereas presumptive LAB colonies were counted on the MRS agar plates.

Purification of yeasts and LAB

Morphologically distinct yeast and LAB colonies were selected and purified by making streak plates on MEA and MRS respectively. The MEA and MRS plates were incubated as mentioned above.

Morphological characterization of yeasts

Yeasts strains were streaked on Glucose Peptone Yeast Extract Agar [2% D-glucose (Merck), 0.5% bacto peptone (Oxoid), 0.5% yeast extract (Oxoid), and 2% agar (Oxoid); GPYA] plates and incubated at 25ºC for 7 days and examined for characteristics such as colony appearance, cell shape and presence of filaments (Kurtzman et al., 2003).

Phenotypical identification of yeasts

The phenotypic characteristics of the yeast isolates were examined using conventional tests as described by Kurtzman et al. (2003). The tests included ability of yeasts to ferment sugars, assimilation of nitrogen compounds, diazonium blue B test, production of acetic acid and growth at different temperatures. The ability of yeasts to ferment sugars was detected by examining the cultures for production of CO_2 gas. A filter sterilised solution of 6% of any of the sugars [2 ml; glucose, galactose, sucrose, maltose, lactose and raffinose (Sigma Chemical Co., St Louis, MO, USA)] was gently mixed with sterile 2% (w/v) yeast extract broth (4 ml) in a test tube containing an inverted Durham tube. A yeast cell suspension (0.2 ml) was then added and the tube was incubated at 25^{o}C. Observations were made after every two days for 28 days. Assimilation of nitrate (Merck), nitrite (Merck), ethylamine (Acros Organics, New Jersey, USA), L-lysine (Acros), cadavarine (Acros), creatine (Merck),

imidazole (Merck) and ammonium sulphate (Merck) by the yeast isolates was tested using the auxanogram method. To test production of acetic acid, a small amount of yeast inoculum was streaked onto plates of GPYA with 0.5% w/v $CaCO_3$ (Merck). The production of acetic acid was observed as a clear zone around the culture. The growth of yeasts at different temperatures was tested by streaking the culture on a slant of GPYA and incubating for 5 days at the following temperatures: 25°C, 30°C, 37°C, 42°C and 45°C. In addition, the ability of the yeast isolates to assimilate carbon compounds were tested using the API 20 C kit (Bio-Meriex, Marcy I'Etoile, France) according to the manufacturer's instructions.

Genotypical identification of yeasts

DNA extraction

DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method. Yeast cells (500 µl) were transferred to a 1.5 ml Eppendorf tube, before adding CTAB buffer (800 µl) (O'Donnel et al., 1997). The suspension was mixed using a vortex mixer and incubated for 1 h at 65°C, with vigorous shaking at 15 min intervals. The suspension was centrifuged at 14,000 rpm in an Eppendorf bench top centrifuge for 30 min at 4°C. The supernatant (700 µl) was transferred to a fresh 1.5 ml Eppendorf tube. Subsequently, chloroform-isoamylalcohol (700 µl, 24:1 by volume) was added, and the solution was shaken vigorously. The solution was then centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant (500 µl) was transferred to a fresh Eppendorf tube. To this solution, chloroform-isoamylalcohol (500 µl) was added, and the suspension was centrifuged again at 14,000 rpm for 10 min at 4°C. From this suspension, a portion of the aqueous layer (350 µl) was pipetted and mixed with CTAB buffer (150 µl). To this, ice-cold isopropanol (300 µl, kept at –20°C) was added, and the DNA was precipitated by centrifugation at 14,000 rpm for 10 min at 4°C. The pellet obtained was washed with 70% alcohol. It was dried and then suspended in sterile water (100 µl) containing RNase (4µl; 10 mg ml^{-1}) (USB Corp., Cleveland, Ohio). The samples were stored at –20°C.

PCR amplification and sequencing of Internal Transcribed Spacer (ITS) and Large Subunit (LSU) regions

The sequencing of the ITS 1 + 2 regions of the rDNA and the D1/D2 domains of the 26s rRNA was done using the procedures described by Gupta et al. (2004). The nucleotide sequences obtained were identified using BLAST.

Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP analysis (Vos et al., 1995) was performed according to the manufacturer's instructions in the AFLP microbial fingerprinting protocol (Applied Biosystems), with some modifications according to the method described by Gupta et al. (2004) for the construction of the dendrogram.

Physiological identification of LAB

The 35 isolates of LAB were confirmed using the regular Gram stain method, the catalase and oxidase tests, growth at different temperatures and assimilation of carbon compounds. The presence of catalase activity was observed by formation of gas bubbles after suspension of bacterial cells in a droplet of 3% hydrogen peroxide. The oxidase reaction of LAB was determined using BBL DrySlide Oxidase (Difco) according to the manufacturer's instructions. The growth of LAB was tested by inoculating the culture into MRS broth and incubating for 5 days at 15^{o}C and 45^{o}C. The carbohydrate fermentation profiles of the LAB isolates were investigated using API 50 CHL strips and API CHL medium according to the manufacturer's instructions (Bio-Merieux). Using the data obtained from the above mentioned tests the LAB were then identified using Intelligent Bacteria Identification System (IBIS) software (Wijtzes et al., 1997).

RESULTS AND DISCUSSION

Enumeration of yeasts

Yeast strains (107 isolates) were isolated from the unripe, ripe and dried fruits, and the fermented fruit pulp. The yeasts had different morphological characteristics and could be grouped according to the colour of their colonies, namely red/pink and cream/white as shown in Table 1. Generally, there was a decrease in the red/pink yeast population and an increase in the cream/white yeast population from the unripe fruit to the fermented fruit pulp. The yeast populations obtained in the ripe fruits are comparable to the literature values (ranging from 2 – 6 log CFU g^{-1} of fruit) obtained for yeast populations on different types of ripe fruit surfaces such as apples, grapes and strawberries (Beech, 1993; Dennis et al., 1977; Rosini et al., 1982). Fleet (2003) also reported that the yeasts responsible for the fermentation originate from the surface of the fruit, contact with processing equipment and other environmental sources, and developed into communities as dense as 7 – 8 log CFU g^{-1}, which is similar to yeast population counts (approximately 9.3 log CFU g^{-1}) obtained in the fermented fruit pulp in this study.

Table 1. Yeast counts from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp from Muzarabani district in Zimbabwe.

Fruit sample	Yeasts (log CFU g^{-1})	
	Red/pink	Cream/white
Unripe (n=10)	3.7 ± 0.09[1]	3.3 ± 0.1
Ripe (n=10)	3.0 ± 0.03	3.9 ± 0.04
Dried (n=10)	1.2 ± 0.04	4.3 ± 0.02
Fermented (n=14)	<4	9.3 ± 0.4

[1]Values are means ± standard deviation of n determinants (two harvests); each determinant was calculated as the average of a duplicate.

Identification of yeasts

Morphologically distinct colonies of yeasts were picked and identified (Tables 2 and 3) using the methods as described by Deak (1993; 2003), Kurtzman (2003) and

Gupta et al., (2004). From the yeast strains isolated, 14 species were identified namely: *Saccharomyces cerevisiae* (21 strains), *Pichia kudriavzevii* (25 strains), *Pichia fabianii* (12 strains), *Aureobasidium pullulans* (26 strains), *Candida glabrata* (3 strains), *Pichia ciferrii* (3 strains), *Saccharomycopsis fibuligera* (2 strains), *Hanseniaspora opuntiae* (1 strain), *Zygoascus hellenicus var. hellen* (2 strains), *Cryptococcus flavus* (1 strain), *Cryptococcus magnus* (1 strain), *Candida parapsilosis* (1 strain), *Candida pyralidae* (1 strain) and *Rhodotorula mucilaginosa* (1 strain) (Table 4). Seven of the isolates could not be identified. However, according to the data obtained by sequencing the ITS and LSU regions, the closest identification for the isolates 002, 008, 98, 97, 112, 92 and 95 were *Cryptococcus heveanensis*, *Bullera dendrophila*, *Fusarium lichenicola* (mould), *Filobasidium floriforme*, *Cryptococcus chernovii*, *Cryptococcus flavus* and *Myrothecium roridum* respectively. The red/pink yeasts enumerated in Table 1 represent *A. pullulans*, *C. flavus* and *R. mucilaginosa* which were found dominantly on the unripe fruits; these are all non-fermentative yeasts. The cream/white group that was predominant includes all the other identified yeast species and comprised both fermentative and non-fermentative yeasts. These results represent yeast isolates selected from the two harvests and it was observed that *H. opuntiae*, *Cr. magnus*, and yeasts 002 and 008 were only encountered in the samples collected in 2004. *Candida glabrata, P. ciferrii, Cr. flavus, C. paraplosis, C. pyralidae, R. mucilaginosa, Z. hellenicus*, and the yeasts 92, 95, 97, 98 and 112 were found only in the samples collected in 2005. The reason why other yeast species were not found in year one and also in year two could be that they were missed during the selection since they occurred less frequently when compared to the other yeast species found in both harvests. All the other species were common in both years. However, the comparison could not be done in terms of CFU's because in 2004 less of the cream colonies were picked for identification since they appeared the same on solid media, and in 2005 more cream colonies were picked for identification.

The fruit surface presents an environment of limited nutrient availability, depending on the concentration of sugars, organic acids and amino acids, which leach from the underlying tissue. Many factors affect the populations and community structure of yeasts that are present on any one type of fruit, including fruit cultivars, geographic location, and fruit developmental stage (Fleet, 2003). In this study the unripe fruit surface was colonised mainly by *A. pullulans*. Chand-Goyal and Spotts (1996), and also Fleet (2003) reported *A. pullulans's* relative abundance as highest among the yeast/yeast like fungi colonising the surface of pears and apples. The fungus causes russeting of apples (Gildemacher et al., in press; Okagbue et al., 2002). *A. pullulans* has also been isolated from tropical plant leaves, marula fruits, flowers, pulp and juices in Zimbabwe (Okagbue et al., 2001). The fungus is an important microorganism in applied microbiology and biotechnology because of the extracellular enzymes it produces, that have industrial applications (Deshpande et al., 1992; Okagbue et al., 2001).

Table 2. Identification of yeasts isolated from *masau* fruits and fermented *masau* fruit pulp from Muzarabani district in Zimbabwe.

Substrate	Identity and strain reference number						
	Saccharomyces cerevisiae (38, 46, 102, 116, 124, 126, 128, 130, 131, 135, 139, 141, 142, 143, 146, 148, 149, 153, 160, 165)	*Pichia kudriavzevii* (3, 27, 30, 32, 42, 51, 55, 56, 91, 94, 100, 105, 110, 123, 125, 129, 132, 137, 138, 140, 144, 150, 151, 152, 166)	*Pichia fabianii* (1a, 2a, 4a, 6a1, 6a2, 8a1, 8a2, 70, 145, 167)	*Saccharomycopsis fibuligera* (3a, 66)	*Hanseniaspora opuntiae* (54)	*Aureobasidium pullulans*(3,5, 6, 7, 9, 10, 13, 16, 19, 20, 37, 56, 57, 58, 61, 63,64, 72, 73, 74, 75, 99, 104, 109, 119)	*Candida glabrata (*133, 134, 147)
Fermentation of:							
Glucose	+	V	+	+	+	−	V
Sucrose	+	−	+	+	+	−	−
Maltose	+	Nd	V	+	+	−	−
Galactose	+	−	−	Nd	+	−	−
Raffinose	+	−	−	Nd	−	−	−
Assimilation of:							
Glucose	+	+	+	+	+	+	+
Galactose	V	V	V	−	+	V	−
MDG	V	−	V	−	−	V	−
Cellobiose	−	V	+	+	−	+	−
Lactose	−	−	−	−	+	V	−
Maltose	+	−	V	+	+	+	−
Saccharose	+	−	+	+	+	+	−
Trehalose	V	−	+	−	+	+	V
Melezitose	V	−	+	−	+	+	−
Raffinose	V	−	+	−	+	+	−
L-lysine	−	+	+	+	Nd	+	V
Cadavarive	−	+	+	+	Nd	+	−
Creatine	V	V	+	+	Nd	+	V
Growth at 37 °C	+	+	+	+	+	+	+
	Pichia ciferii (115, 164, 1690)	*Cryptococcus flavus* (111)	*Cryptoccus magnus* (71)	*Zygoascus hellenicus* (121, 122)	*Candida parapsilosis* (93)	*Candida pyralidae* (90)	*Rhodotorula mucilaginosa* (117)
Fermentation of:							
Glucose	+	−	−	+	+	−	−
Sucrose	+	−	−	+	−	−	−
Maltose	Nd	Nd	−	Nd	−	Nd	−
Galactose	−	Nd	−	+	−	−	−
Raffinose	−	Nd	−	Nd	Nd	Nd	−
Assimilation of:							
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
MDG	−	+	+	−	+	−	−
Cellobiose	−	+	+	+	+	−	−
Lactose	−	+	+	−	−	−	−
Maltose	+	+	+	+	+	−	+
Saccharose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Melezitose	+	+	+	−	+	−	+
Raffinose	+	+	+	−	+	−	+
L-lysine	+	Nd	Nd	+	−	+	Nd
Cadavarive	−	Nd	+	+	−	+	Nd
Creatine	+	Nd	+	+	−	+	Nd
Growth at 37 °C	+	Nd	−	W	W	W	+

Notes: Nd = no data, w = weak, + = positive, − = negative, V = variable, MDG = methyl-D-glucose. Results for macro- and micro-morphology, acetic acid production, diazonium, blue B test, glycerol, xylose, adonitol, xylitol, inositol, sorbitol, *N*-acetyl-glucosamine, imidazole, ethylamine, growth at 42°C and 45°C are not included in the table.

Table 3. The diversity of yeasts isolated from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp from Muzarabani rural community in Zimbabwe.

Yeast species	**No of isolates detected in fruits**			
	Unripe	**Ripe**	**Dried**	**Fermented**
Fermentative:				
S. cerevisiae	-	1[a]	-	20
P. kudriavzevii	-	3	-	22
P. fabianii	-	-	8	4
Sm. fibuligera	-	-	1	1
H. opuntiae	-	-	-	1
Non-Fermentative:				
A. pullulans	19	1	6	-
C. glabrata	-	-	-	3
P. ciferrii	-	-	3	-
Cr. magnus	-	1	-	-
Cr. flavus	1	-	-	-
Z. hellenicus	-	2	-	-
C. parapsilosis	-	1	-	-
C. pyralidae	-	1	-	-
R. mucilaginosa	1	-	-	-

- : not found. [a]Values are combined data from the two harvests.

Other yeast species which were identified on the unripe fruits included *Cr. flavus* and *R. mucilaginosa*. The ripe fruits were colonized by a wide range of yeast species (Table 3) comprising fermentative as well as non-fermentative yeasts. On the ripe *masau* fruits, *S. cerevisiae* was found less frequently than in the fermented fruit pulp. The gradual transition from non-fermentative species in unripe fruits, through mixed populations in ripe fruit, towards predominantly fermentative species in fermented pulp is evident, and we assume that this is strongly related with the evolving degree of maturity and ensuing chemical composition and softening of consistency. This phenomenon is similar as in grape fermentation for wine production (Fleet, 2003; Heard et al., 1985; Rosini et al., 1982). Difficulties to detect or isolate *S. cerevisiae* from either immature or mature grapes, have been reported elsewhere (Martini et al., 1996; Van der Westhuizen et al., 2000). *Pichia fabianii* was recorded highest on the dried fruits probably because it is able to thrive under reduced water activity.

The fermented fruit pulp harboured a predominance of *S. cerevisiae*, *P. kudriavzevii* and *P. fabianii* and other minor species like *Sm. fibuligera*, *C. glabrata* and *H. opuntiae*. These are all fermentative yeasts, which have also been reported as predominating in traditional fermented foods such as Korean *nuruk*, Nigerian *fufu* and Indian idli (Nout, 2003).

The yeast species were grouped based on their physiological properties and molecular data obtained from sequencing of D1/D2 domains and ITS 1 + 2 regions as well as AFLP fingerprinting. The sequencing results in Table 4 show the accession

number and percentage similarity for both the LSU and ITS of the different yeast species.

Table 4. Identification of yeasts isolated from *masau* (*Z. mauritiana*) fruits and fermented *masau* fruit pulp by D1/D2 domains of 26S rRNA (LSU) and ITS sequence analysis.

Species and group no.	No of strains	ITS accession no.	LSU accession no.	% Similarity of ITS sequences	% Similarity of LSU sequences
S. cerevisiae					
group 1[1]	5	AB212260.1	AJ746340.1	99	100
group 2	7	AB212260.1	AJ746340.1	99	99
group 3	3	AB212260.1	AJ746340.1	99	100
group 4	3	AB212260.1	AY601161.1	99	99
group 5	2	DQ167471.1	AJ746340.1	99	99
group 6	1	AB212260.1	Z7332.1	99	99
P. kudriavzevii					
group 1	9	AY939808.1	AY707865.1	100	100
group 2	5	AY939808.1	AY601160.1	99	99
group 3	3	AY939808.1	AY707865.1	100	99
group 4	5	AY939808.1	AY707865.1	99	100
group 5	3	AY939808.1	AY707865.1	99	99
P. fabianii					
group 1	1	AF335967.1	AF335967.1	100	100
group 2	1	AF335967.1	AF335967.1	99	99
group 3	2	AF335967.1	AF335967.1	100	99
group 4	7	AF335967.1	AF335967.1	99	100
group 5	1	AF335967.1	AF335967.1	99	99
Sm. fibuligera					
group 1	1	AF335940.1	U40088.1	99	100
group 2	1	U410409.1	U40088.1	99	99
A. pullulans					
group 1	13	AY225166.1	AF050239.1	99	100
group 2	4	AY225166.1	AF050239.1	99	100
group 3	3	AY225166.1	AF050239.1	99	100
group 4	4	AY139394.1	AF050239.1	99	100
group 5	1	Ay225166.1	AF050239.1	99	100
group 6	1	AY625057.1	AY18811.1	99	96
C. glabrata					
group 1	2	AY939749.1	AY198398.1	99	99
group 2	1	Nd	AJ617300.1	Nd	100
P. ciferrii	3	Nd	U74587.1	Nd	100
Cr. flavus	1	AF444338.1	AF075497	Nd	99
Cr. magnus	1	AF444450.1	AY362182.1	99	100
Z. hellenicus	2	AY447022.1	AY447006.1	99	100
C. parapsilosis	1	AJ49821.1	AY391843.1	99	99
C. pyralidae	1	AY013715	AY498864.1	98	99
H. opuntiae	1	Nd	AY267820.1	Nd	100
R. mucilaginosa	1	DQ386306.1	AB02610.2	98	99

Nd = no data. [1]The groups are based on the sequencing consensus similarity.

The molecular identification techniques were used in combination with conventional methods of identification in order to confirm the identities and to assess

relatedness among the yeast species. A dendrogram based on the AFLP analysis representing all yeast species isolated is depicted in Figure 1. The AFLP patterns clearly show that each species forms a distinct cluster. Common bands as well as different bands were observed thus show DNA polymorphisms among the isolates of the same yeast species. This is particularly clear for isolates belonging to *A. pullulans*. The biodiversity seen in our isolates of *S. cerevisiae* has also been observed in other studies on African indigenous fermented foods and beverages (Jespersen, 2003). Not all the strains had assimilation profiles typical for *S. cerevisiae* and this could probably be explained by the polymorphisms seen in Figure 1. Some of the strains were shown to be weak in fermenting both sucrose and galactose. Variability was also shown in acetic acid production, assimilation of carbon compounds (galactose, inositol, methyl α-D-glucoside, N-acetyl-glucosamine, saccharose, melezitose and raffinose) and growth at 42ºC and 45ºC. In total, six assimilation profiles were distinguished.

Lactic acid bacteria

Yeasts are not the only organisms of importance in the microbiology of fruit and fruit products. Bacteria, especially lactic acid bacteria (LAB) and acetic acid bacteria, are prominent in the spoilage of some fruits and fruit products, and certain species of LAB can have a positive contribution in the production of wines (Fleet, 2003). In most indigenous fermentation processes, yeasts occur in association with LAB (Caplice et al., 1999; Fleet, 1999; Gobbetti et al., 1994; Jespersen, 2003; Nout, 2003; Sanni, 1993). This was also observed in this study whereby LAB and yeasts were found co-existing in *masau* fruits and the fermented fruit pulp.

LAB populations found on unripe, ripe and dried fruits were approximately 3.0 and 2.9 log CFU g^{-1} (Table 5). Just like the yeasts, the LAB multiply during fermentation and therefore larger numbers of LAB were found in fermented fruit pulp compared with the unprocessed fruits (Table 5).

Table 5. Lactic acid bacteria from *masau* (*Ziziphus mauritiana*) fruits and fermented *masau* fruit pulp.

Fruit	Lactic acid bacteria[1] (log CFU g^{-1})
Unripe (n=10)	< 2
Ripe fruits (n=10)	3.0 ± 0.07[2]
Dried fruits (n=10)	2.9 ± 0.11
Fermented samples (n=14)	9.2 ± 0.58

[1]Grown on MRS agar with 0.1 % natamycin, incubated anaerobically, Gram-positive and catalase-negative, [2]Values are means ± standard deviation of n determinants (two harvests) and each determinant was calculated as the average of duplicate counts.

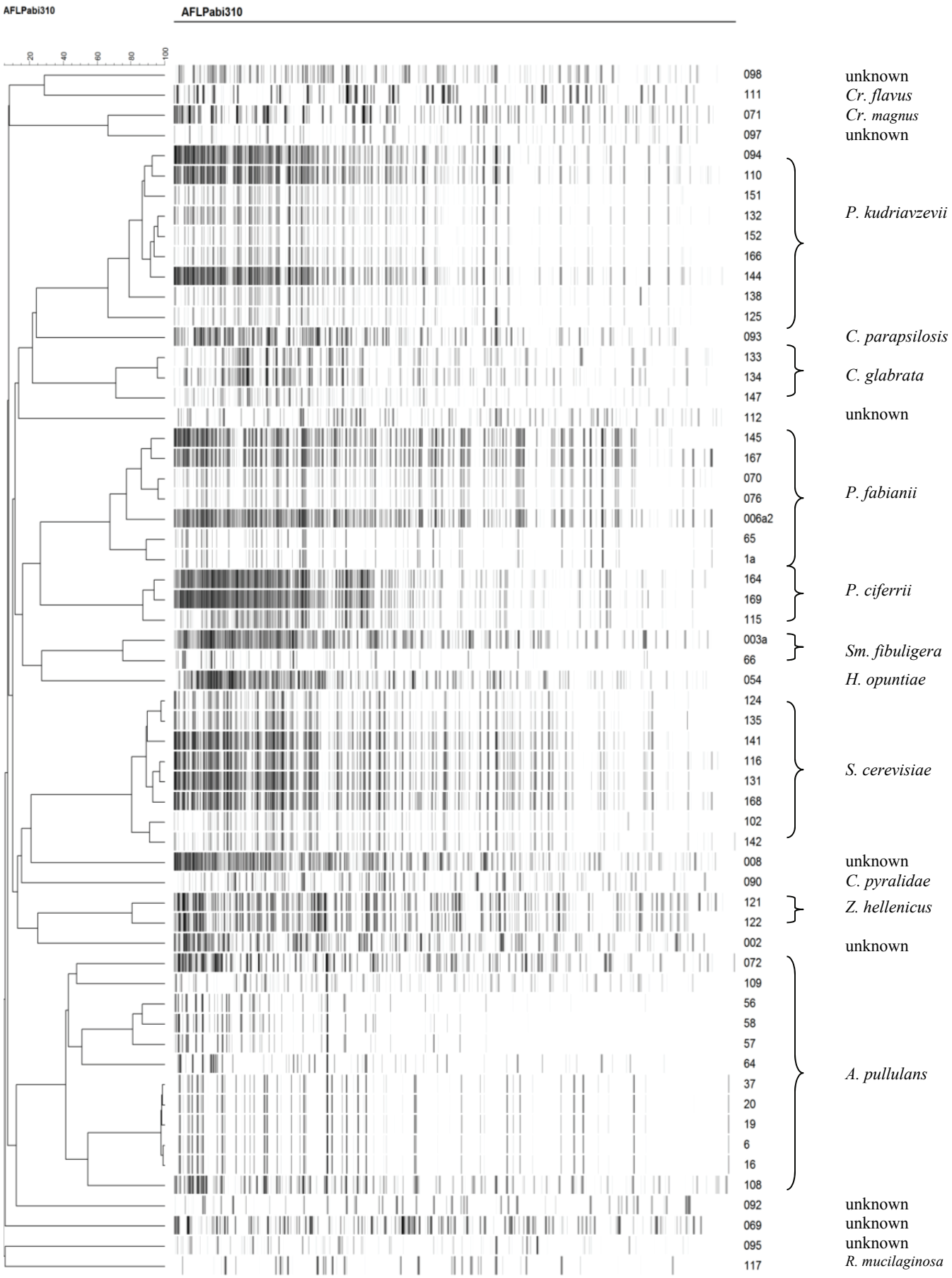


Figure 1. AFLP dendrogram representing the identified and unidentified yeast species isolated from *masau* (*Z. mauritiana*) fruits and the fermented fruit pulp.

A representative number of predominant LAB isolated from fruits harvested in two consecutive seasons were characterised. For each fruit type 10 samples were analysed in duplicate and LAB isolates from both years showed similar phenotypic properties. The LAB found in ripe fruits were preliminary identified as *Lactobacillus agilis* (2 strains), *L. minor* (2 strains), *L. confusus* (1 strain) and *L. fructosus* (1 strain). In dried fruits *L. minor* (1 strain) and *L. divergens* (2 strains) were found. The fermented fruit pulp harboured mostly *L. agilis* and *L. plantarum*. The other species found included *L. bifermentus* (2 strains), *L. divergens* (2 strains), *L. fermentum* (1 strain), *L. hilgardii* (1 strain), *L. minor* (1 strain), and *Streptococcus* spp. (2 strains) and 1 unidentified strain. The LAB results are preliminary being based on phenotypic properties, and need to be confirmed by molecular identification methods.

L. plantarum has been reported to be involved in many cereal-based African fermented foods such as *ogi*, *uji* and *mahewu* (Halm et al., 1993; Nyanga et al., 2007; Sanni, 1993) as well as other fermented foods (Caplice et al., 1999; Hammes et al., 1994; Holzapfel, 1997; Leisner et al., 1999). Certain strains of *L. plantarum* and *L. agilis* have been reported to have probiotic effects (Lee et al., 1995).

CONCLUSION

This study showed that there is a transition from the predominance of non-fermentative yeasts to fermentative yeast species as the *masau* fruit matures until it is fermented. *A. pullulans,* a non-fermentative yeast-like fungus was dominant on the unripe fruit surface. The ripe and dried fruits had a mixture of non-fermentative and fermentative yeasts. The fermentative yeasts *S. cerevisiae*, *P. kudriavzevii* and *P. fabianii* were dominant in the fermented *masau* fruit pulp. These yeasts could be useful for the development of starter cultures to produce better quality fermented *masau* fruit products. The yeasts were found co-existing with LAB in the ripe fruits and the fermented fruit pulp suggesting that some of the LAB could contribute positively in the development of starter cultures to produce a fermented fruit product of stable and consistent quality.

ACKNOWLEDGEMENTS

The authors are grateful to the MacGillavry fund of The Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation for Science (IFS Grant No C/3737) for financial support. We are also grateful to Mr Mutseka, Mr. E. Nyanga and Mr. J. Mutemeri for their assistance during the collection of samples.

CHAPTER 5

Fermentation characteristics of yeasts isolated from *masau* (*Ziziphus mauritiana*) fruits and their traditionally fermented fruit pulp in Zimbabwe

L. K. Nyanga, M. J. R. Nout, T. Boekhout, E. J. Smid and M. H. Zwietering. Submitted.

ABSTRACT

Yeast strains were characterised to select potential starter cultures for the production of *masau* wine and distillate. The yeast species originally isolated from *Ziziphus mauritiana* (*masau*) fruits and their traditionally fermented fruit pulp in Zimbabwe, were examined for their ability to ferment glucose and fructose using standard broth under aerated and non-aerated conditions. Most *Saccharomyces cerevisiae* strains were superior to other species in ethanol production. The best ethanol producing *S. cerevisiae* strains, and strains of the species *Pichia kudriavzevii*, *Saccharomycopsis fibuligera* and *Pichia fabianii* were tested for production of flavour compounds during fermentation of *masau* fruit juice. Significant differences in the production of ethanol and other volatile compounds during fermentation of *masau* juice were observed among and within the tested four species. Alcohols and esters were the major volatiles found in the fermented juice. Trace amounts of organic acids and carbonyl compounds were detected. Ethyl hexanoate and ethyl octanoate were produced in highest amounts as compared to the other volatile compounds. The *S. cerevisiae* strains produced the highest concentrations of flavour compounds. The developed library of characteristics can help in the design of mixtures of strains to obtain a specific melange of product functionalities.

INTRODUCTION

Many traditional fermented food products rely on uncontrolled, natural fermentation with unpredictable outcome. In Africa, traditional fermented foods include fermented beverages from indigenous fruits such as *Parinari curatellifolia* (*hacha)*, *Uapaca kirkiana* (*mazhanje*) and *Ziziphus mauritiana* (*masau*). In Zimbabwe, *Z. mauritiana* fruit pulp is naturally fermented and distilled into a spirit called *kachasu*. Nyanga and co-workers (2008) reported that the fermented fruit pulp is not consumed as such because of its unattractive exterior and smell. They also reported that the production of *kachasu* is done in private by families for income generation and livelihood. It is done in privacy because making and drinking *kachasu* is illegal in Zimbabwe. In other countries, e.g., in Malawi, spirits made from indigenous fruits have been commercially promoted. For instance, a distilled alcoholic liquor and a wine called *mluguzi* are produced at an industrial scale from a combination of *U. kirkiana* and *Z. mauritiana* fruits (Gadaga et al., 1999; Maghembe et al., 1992).

The use of yeast as starter culture for wine fermentation and distillates has led to the production of beverages of more consistent quality with a commercial value (Fundira et al., 2002). Yeasts from traditionally fermented *masau* fruit pulp and the fruits have been isolated and identified (Nyanga et al., 2007). These yeasts may be of interest as ecologically adapted, potential starter cultures to produce good quality fermented *masau* fruit products. Microbes isolated from mixed microbial populations obtained from traditional fermented foods exhibit a diversity of metabolic activities, that vary among strains (Holzapfel, 2002). Fleet (2008) also reported that profiles for volatile production vary significantly, thus screening of candidate microorganisms for use as starter cultures, is the first crucial step of technology development and scale-up.

The aim of the present study was to characterise the yeast isolates belonging to *Saccharomyces cerevisiae*, *Pichia kudriavzeii*, *Saccharomycopsis fibuligera*, *P. fabianii, P. ciferrii, Zygoascus hellenicus*, *Candida glabrata, C. parapsilosis*, *C. pyralidae* and *Hanseniaspora opuntiae* species that were isolated from traditionally fermented *masau* fruit pulp and the fruits, with respect to their formation of ethanol and flavour compounds, and tolerance to ethanol. The results of this characterization can subsequently be used for the rational selection of suitable starter cultures for the future production of *masau* wine and distillate.

MATERIALS AND METHODS

Cultures

In this study, 46 previously identified yeast strains originating from *masau* fruits and traditionally fermented fruit pulp (Nyanga et al., 2007) were investigated. The strains were cultured on Malt Extract Agar (MEA) and were maintained routinely at -80ºC in 300 ml l^{-1} glycerol prepared in peptone physiological saline (PPS) [NaCl 8.5 g l^{-1} (Merck, Darmstadt, Germany), neutral peptone 1 g l^{-1} (Oxoid, Basingstoke, UK), pH = 7.2].

Preparation of inocula

Yeast cultures were incubated for two days at 30ºC in 10 ml malt extract broth. Suspensions of 10^8 cells ml^{-1} were then made in sterile PPS as confirmed by microscopic counts.

Fermentation tests

Two standard broths of 100 ml containing 100 g l^{-1} sugar (glucose or fructose) and 10 g l^{-1} yeast extract were distributed in 250 ml volumetric flasks. The flasks were plugged with cotton and sterilised at 121ºC for 15 minutes. The sterile broths were inoculated with 10^6 yeast cells ml^{-1} in duplicate for each sugar. One series of inoculated broth was incubated at 30ºC for five days under non-aerated conditions achieved by replacing the cotton plug with a water lock. The other series was incubated at 30ºC under aerated conditions for five days in a shaking incubator (Innova™ 4335, Refrigerated incubator shaker, New Brunswick scientific, Nijmegen, The Netherlands) at 200 rpm. At the end of the fermentation, alcohol content, residual sugar, biomass and pH were measured.

Determination of sugar and ethanol

Fermented standard broth was centrifuged at 10,164 x g for 10 minutes. Protein precipitation was achieved by means of Carrez reagents (Barnett, 1997) as follows. An aliquot of 0.5 ml of the supernatant was then deproteinated by adding 0.25 ml of Carrez A reagent (42.20 g $K_4Fe(CN)_6$ $3H_2O$ l^{-1} demineralised water) in an Eppendorf tube and vortexed well. Then 0.25 ml of Carrez B reagent (57.50 g $ZnSO_4$ $7H_2O$ l^{-1} of demineralised water) was added and vortexed well. The mixture was centrifuged for 5 minutes at 13,900 x g. The supernatant was analysed by high performance liquid chromatography (HPLC), fitted with Refractive Index and UV/VIS detectors (Spectra System Thermo separation products, Reviera, Florida, USA). The separation was done on an Aminex HPX-87H ion exclusion column (300 x 7.8 mm^2) at an oven temperature of 40ºC and a flow rate of 0.6 ml min^{-1}. The mobile phase was 5 mM H_2SO_4 (degassed). Standards for the sugars (fructose and glucose) were obtained from Merck (Darmstadt, Germany). The standard solutions were prepared individually in double distilled water.

Determination of biomass

The fermented broth was transferred to a pre-weighed sterile 50 ml centrifuge tube and centrifuged at 10,164 x g for 10 minutes. The microbial pellet was washed twice with demineralised water and centrifuged again. The tubes were then oven dried at 80°C until a constant weight was obtained. The tubes were equilibrated to room temperature before weighing.

Calculation of the carbon balance

The carbon balance calculation was based on the assumption that during fermentation, the C-source sugars are converted mainly into ethanol, biomass and carbon dioxide. The carbon balance was expressed as carbon (C) moles. The following chemical formula was used for biomass: $CH_2O_{0.5}N_{0.2}$ (Roels, 1983). Carbon dioxide (CO_2) was assumed to be equal to half of the produced ethanol in C-moles.

Ethanol tolerance

The ethanol tolerance test was done according to the method described by Swinnen et al. (2012).

(i) Yeast culturing

Yeast isolates were grown on yeast extract peptone glucose agar (YPG) and yeast extract fructose agar (YPF) for two days. A sterile 96-well micro-titre plate was filled with 150 µl of YPG broth and colonies from different yeast strains were transferred from the YPGA and YPFA plates into different wells in the 96-well plate. The micro-titre plates were incubated on a plate shaker at 25ºC for two days. After incubation, the OD_{650} of each culture plate was measured and corrected to ± 1.0 by diluting the culture with sterile PPS under aseptic conditions ready to be used in the spot test.

(ii) Preparation of ethanol plates

YPG agar at ± 50ºC was poured into four sterile 250 ml bottles containing absolute ethanol to make YP-ethanol agar of 100, 140, 160 and 180 ml l^{-1} ethanol under sterile conditions. The YP-ethanol agar was immediately poured into square Petri dishes which were immediately closed to minimize evaporation of ethanol. The gar was dried at 25°C.

(iii) The spot test

A replicator (Boekel Microplate replicator 384-Pin, Boekel Scientific, Pennsylvania, USA) was used to transfer the yeast isolates from the 96-well plate in duplicate to YPG, YPF and the YP-ethanol plates under aseptic conditions. The strains were spotted away from the sides of the plate because here the alcohol evaporates faster. The plated cultures were incubated at 25ºC. Growth was scored after 1 day for plates without ethanol and after 2 to 11 days for plates containing ethanol. All the spot tests were repeated at least twice.

Production of volatile metabolites in *masau* juice: (i) Preparation and fermentation of *masau* fruit juice

Frozen samples of fresh *masau* fruits were thawed at room temperature**.** The fruits were washed under running tap water and left to dry on a paper towel for two hours at 25°C. The fruits (500 g±1) were weighed and crushed in a Waring blender after which 1 l of demineralised water was added to thin the fruit juice (5.2°Brix). Equal amounts of glucose (25 g) and fructose (25 g) were added to bring the juice to 12.2°Brix, pH 3.8. The juice was distributed (100 g portions) into 250 ml volumetric flasks, and steam heated at 103°C for 30 minutes. After cooling, an inoculum of 10^8 cells ml^{-1} was used to inoculate the juice. The juice was fermented for 72 hours. A non-inoculated *masau* juice (12.2°Brix) was used as a control. After fermentation the juice was portioned into 10 ml samples and put into 50 ml tubes. The tubes were tightly closed and kept at -20ºC until further analyses. The experiment was done twice.

(ii) Capturing of Head Space (HS) volatiles by Solid Phase Micro Extraction (SPME)

Two SPME fibres (Supelco, Bellefonte, PA, USA) [carboxen/polydimethyl-siloxane, 85 µm, (CAR/PDMS-85), and divinylbenzene/carboxen/polydimethylsiloxane, 50/30 µm (DVB/CAR/PDMS-50/30)] were evaluated for their selectivity in absorbing volatile alcohols and esters from the headspace of the samples. The optimum fibre exposure time to the sample headspace and desorption time was also determined. DVB/CAR/PDMS-50/30 gave better response for simultaneous analysis of alcohol and esters, and this was used in all subsequent analyses. The conditions of the head space SPME sampling used were as follows: each sample

was prepared by measuring 2 g of fermented *masau* juice and 0.4 g NaCI in a 10 ml GC vial containing a magnetic stirrer and 250 µl of 4-methyl-1-pentanol (5 mg l^{-1} in final solution) as an internal standard. The vial was sealed with a septum (Butyl/PTFE gray, AChroma, Müllheim, Germany) and an aluminium cap. Equilibrium was achieved by stirring the vial at 25ºC on a magnetic stirrer for 15 minutes. The fibre was then exposed to the headspace for another 15 minutes, under the same conditions. Prior to the first analysis, the fibre was conditioned for 1 hour at 270ºC in the injector port of a Thermo Scientific Trace GC Ultra gas chromatograph. For adsorption of volatiles the fibre was exposed to the headspace of sample vials for 15 minutes at 25ºC. For thermal desorption the needle was inserted into the injection port at 270ºC of the GC-MS system for 3 minutes. Prior to the next analysis, the fibre was reconditioned for 10 minutes at 270ºC in the injector of another Thermo Scientific Trace GC Ultra gas chromatograph to avoid carry-over of compounds between samples.

(iii) Analysis of volatiles

A Thermo Scientific Trace GC Ultra gas chromatograph, equipped with a split-splitless injector and a Thermo Scientific DSQII detector, with a fused silica column (Supelco, MDN-5S, 60 m, 0.25 mm internal diameter, 0.25 µm film thickness) was used for analysis of volatiles. Helium was used as a carrier gas, at a flow rate of 2.05 ml min^{-1}. The oven temperature was programmed as follows: 2 minutes at 80ºC then rising to 280ºC at a rate of 10ºC min^{-1}, at which it was held for 5 minutes.

Identification of compounds was obtained by comparing the retention times with those of authentic compounds and the spectral data from Wiley and NIST libraries. The volatile compounds were semi-quantified after correction for the internal standard (Mallouchos et al., 2002; Mallouchos et al., 2003). Analyses were done in duplicate.

(iv) Distillation of the fermented *masau* juice

Fermented *masau* juices (250 ml) were distilled in flame heated round-bottomed 2 l flasks fitted with a Vigreux column and water condenser. The fermented juices were distilled until 40 ml of distillate was collected. The distillates were kept at -20ºC until analysis.

Statistical Analysis

The data was analysed using the statistical program SPSS 13.0 for windows (Apache Software Foundation, USA) and the one-way ANOVA model was used applying the LSD test to evaluate significant difference among means.

RESULTS AND DISCUSSION

Screening for alcoholic fermentation in standard broth

The yeast strains isolated from *masau* fruits and their fermented products (Nyanga et al., 2007) were screened for the production of ethanol under controlled conditions using standard broth containing 100 g l^{-1} sugar, either fructose or glucose, and 10 g l^{-1} yeast extract (Figure 1). Most of the *S. cerevisiae* strains were superior to the other species in terms of the production of ethanol under both experimental conditions. Most *P. kudriavzevii* strains yielded intermediate levels of ethanol. *Saccharomyces* species are known as the main agent in alcoholic fermentations (Alfenore et al., 2002; Battcock et al., 1993; Clemente-Jimenez et al., 2004; Dung

et al., 2006; Yang et al., 2011), because of their fast growth, good ability to produce ethanol and tolerance for several environmental stresses, such as high ethanol concentration and low oxygen levels (Ferreira et al., 2010; Piskur et al., 2004). Almost all the *S. cerevisiae* strains studied were able to utilize all glucose and fructose in the fermentation broth (Table S1 and S2), which explains their higher alcohol production as compared to the other species. Most (52% and 67%, n = 46) of the yeasts were able to produce more than 2.0 $g \cdot 100ml^{-1}$ of alcohol from glucose and fructose fermentation, respectively. These yeast strains belonged mostly to *S. cerevisiae* and *P. kudriavzevii* and fermented fructose better than glucose.

There was significant correlation between the amounts of ethanol produced during glucose and fructose as shown in the scatter plot in Figure 2. The yeast strains located at the top right hand corner of the scatter plot were the highest producers of ethanol from both glucose and fructose. The *P. kudriavzevii* strain 152 clearly behaved differently from all the other strains as it produced clearly ethanol from glucose (3.8 $g \cdot 100ml^{-1}$) and very little from fructose (0.3 $g \cdot 100ml^{-1}$).

Most of the yeasts produced ethanol under aerated conditions (Figure 1), a phenomenon known as the "Crabtree Effect". Under these conditions respiration is repressed in the presence of high concentrations of sugar (Crabtree, 1929; De Deken, 1966; Lee et al., 2011). Nevertheless, the ethanol contents were lower than those found under non-aerated conditions. Interestingly, *P. kudriavzevii* strain 152, which produced very little ethanol from fructose, was "Crabtree Negative" for fructose. A variation in the utilization of sugars was noted and, hence, the difference in the production of ethanol and biomass (Figures 1 and 3, respectively) amongst the species investigated. The quantity of biomass produced from glucose and fructose was similar for most strains except for *S. cerevisiae* strains 38, 135, 143 and 165; *P. kudriavzevii* strains 123, 129, 152 and 166; and *Hanseniaspora opuntiae* which all produced more biomass from glucose than from fructose.

Another remarkable exception was the high production of biomass from fructose by *Z. hellenicus* strains. *Z. hellenicus* strains produced 1.3 and 1.9 $g \cdot 100ml^{-1}$ (dry weight) of biomass from fructose, whereas the highest amounts produced by *S. cerevisiae* and *P. kudriavzevii* were 0.58 and 0.77 $g \cdot 100ml^{-1}$ (dry weight), respectively. The former species could not utilize glucose or fructose under non-aerated conditions and, therefore, *Z. hellenicus* is most likely an obligate oxidative yeast.

The mass-energy balance has been used to check the consistency of experimental measurements and to evaluate the efficiency of the conversion of organic substrates to desired products by microorganisms (Nowakowska-Waszczuk et al., 1987). In this study, the carbon balance was used to evaluate the efficiency of the conversion of the sugars to ethanol under non-aerated conditions (supplementary data in Table S1 and S2 for glucose and fructose, respectively). Most *S. cerevisiae* strains yielded at least 50% of the used C-mol as ethanol from both glucose and fructose. Thus, *S. cerevisiae* proved to be more efficient in the conversion of the sugars to ethanol than any other tested species. The percentage yields of ethanol and CO_2 (77 – 80%) by most *S. cerevisiae* strains are within the same range as reported in the literature (Fales et al., 1947). The discrepancy of C-mol that was unaccounted for ranged from 6.7 – 18% and 13.9 – 25% for fructose and glucose, respectively. The discrepancy can be attributed to the other carbon metabolites that are produced during fermentation; these may include enzymes, organic acids and volatile compounds.

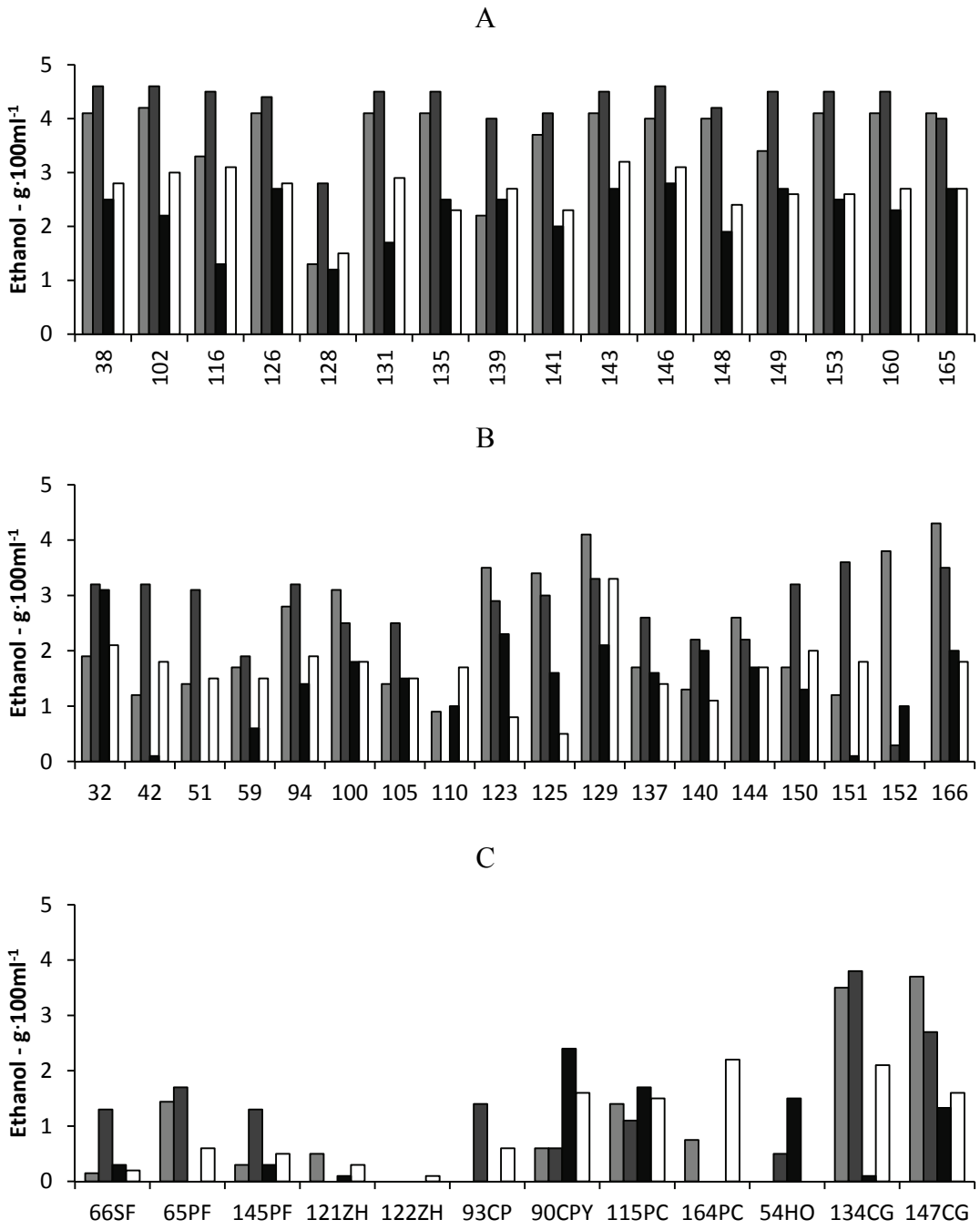


Figure 1. Ethanol produced after five days at 30°C by yeasts under aerated and non-aerated conditions in standard broth containing 10% sugar (glucose or fructose). A = *Saccharomyces cerevisiae* strains; B = *Pichia kudriavzevii* strains and C = Other yeast strains. ■ = Glucose fermentation (non-aerated), ■ = Fructose fermentation, ■ = Glucose assimilation (aerated) and □ = Fructose assimilation. (SF), *Saccharomycopsis fibuligera*; (PF), *Pichia fabianii*; (ZH), *Zygoascus hellenicus*; (CP), *Candida parapsilosis*; (CPY), *Candida pyralide*; (PC), *Pichia ciferii*; (HO), *Hanseniaspora optuntiae*; and (CG), *Candida glabrata*.

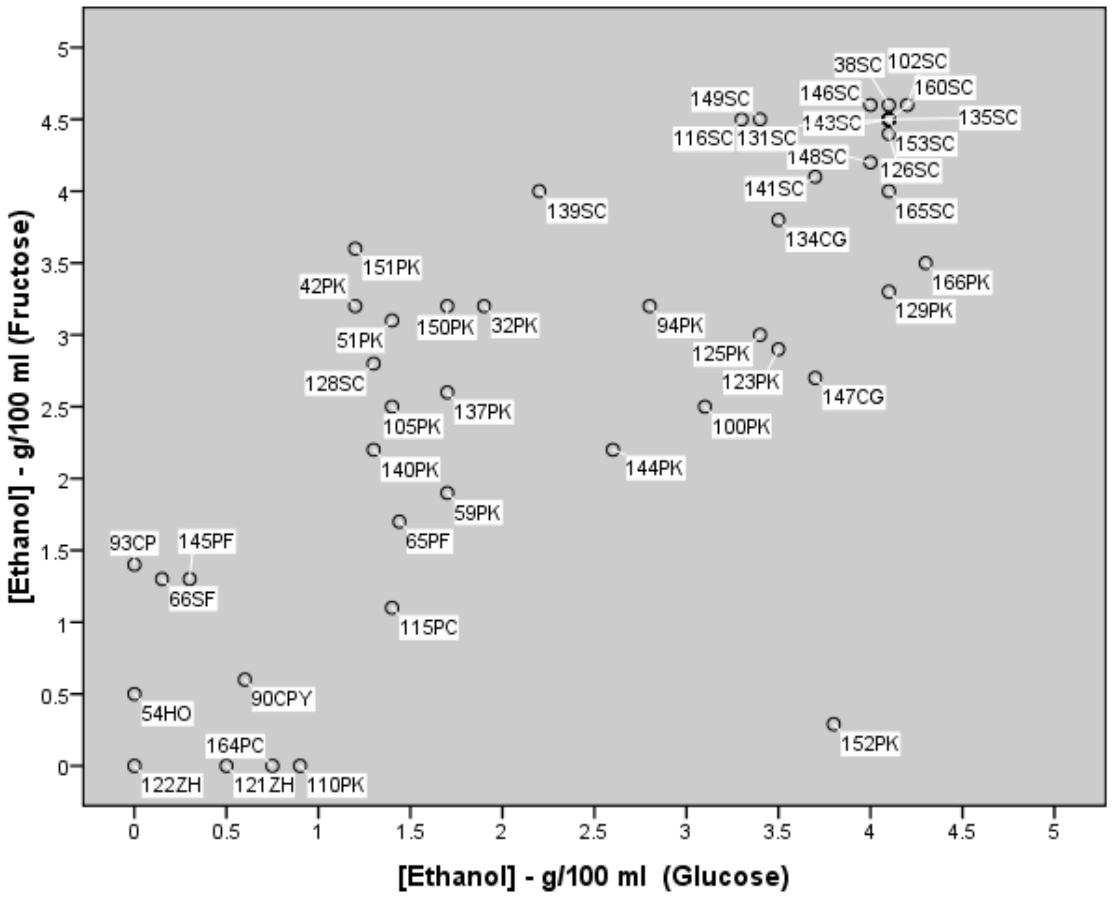


Figure 2. Scatter plot showing correlation between ethanol content produced after five days at 30°C by yeasts from fructose and glucose fermentation**. (**SC), *Saccharomyces cerevisiae*, (PK), *Pichia kudriavzevii*, (SF), *Saccharomycopsis fibuligera*; (PF), *Pichia fabianii*; (ZH), *Zygoascus hellenicus*; (CP), *Candida parapsilosis*; (CPY), *Candida pyralide*; (PC), *Pichia ciferii*; (HO), *Hanseniaspora optuntiae*; and (CG) *Candida glabrata.*

Screening for ethanol tolerance

The accumulation of ethanol to toxic concentrations during fermentation is a major stress factor that causes reduced ethanol production and eventual stuck fermentations (Gibson et al., 2007; Yang et al., 2011). Hence, in this study the yeast species were also compared for their ethanol tolerance as shown in Table 1.

The *S. cerevisiae* yeast strains were able to tolerate up to 160 ml l^{-1} alcohol and few strains were able to show weak growth even at 180 ml l^{-1} ethanol. There are several factors involved in the ethanol tolerance of *S. cerevisiae* as reported by Ding et al. (2009). These include changes in the plasma membrane composition of unsaturated fatty acids which increase stability and antagonize the fluidity caused by ethanol. Most *P. kudriavzevii* strains were able to tolerate up to 140 ml l^{-1} ethanol, and showed variation of ethanol tolerance at 160 ml l^{-1} and 180 ml l^{-1} ethanol. As an exception, growth of *P. kudriavzevii* strain 152 was suppressed at 100 ml l^{-1} ethanol, and also differed from the other *P. kudriavzevii* strains in its utilization of fructose under aerated and non-aerated conditions.

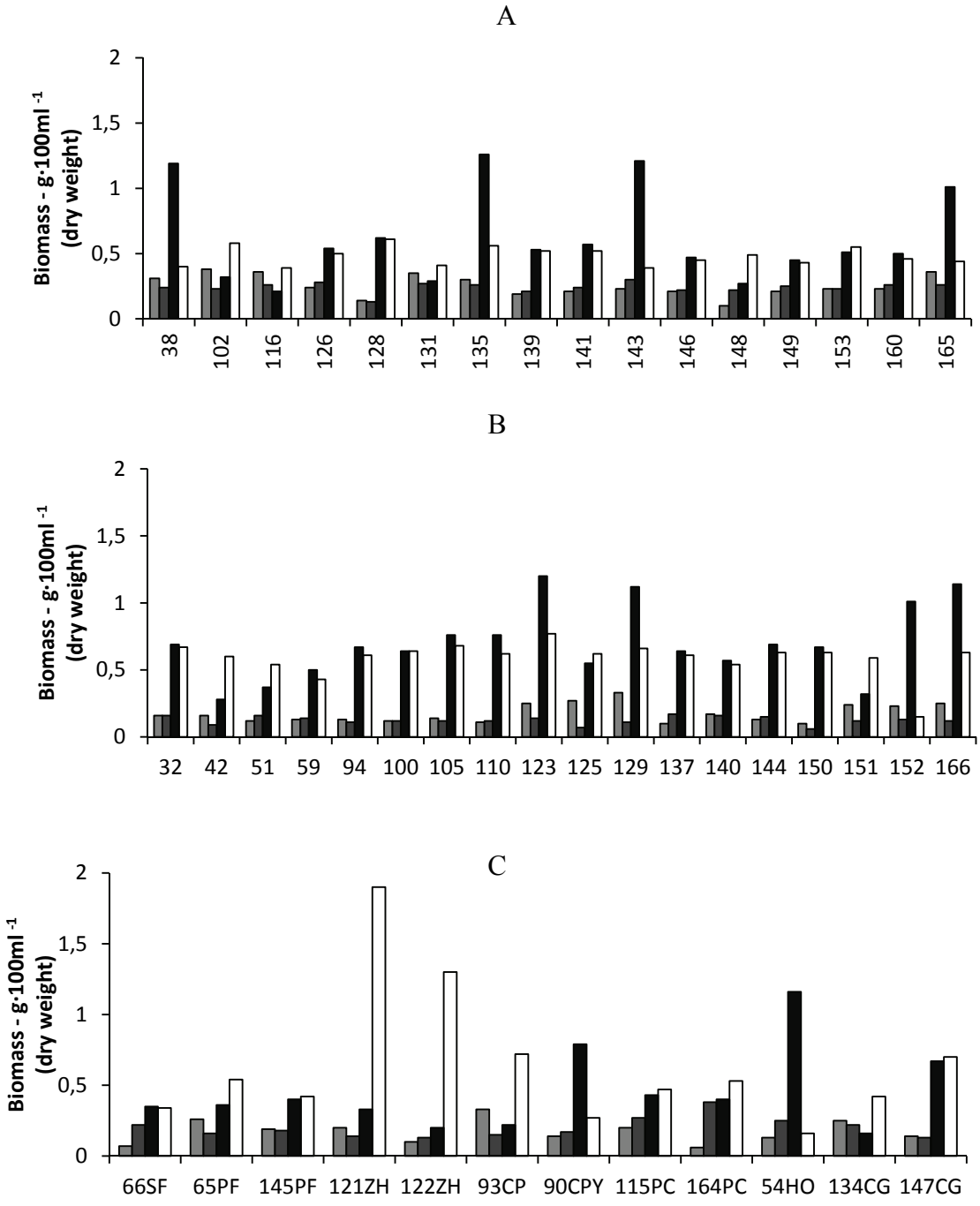


Figure 3. Biomass produced after five days at 30°C by yeasts under aerated and non-aerated conditions in standard broth containing 100 ml l^{-1} sugar (glucose or fructose). A = *Saccharomyces cerevisiae* strains; B = *Pichia kudriavzevii* starins and C = Other yeast strains. ▒ = Glucose fermentation (non-aerated), ■ = Fructose fermentation, ■ = Glucose assimilation (aerated) and □ = Fructose assimilation. (SF), *Saccharomycopsis fibuligera*; (PF), *P. fabianii*; (ZH), *Zygoascus hellenicus*; (CP), *Candida parapsilosis*; (CPY), *Candida pyralide*; (PC), *Pichia ciferii*; (HO), *Hanseniaspora optuntiae*; and (CG), *Candida glabrata.*

Table 1. A summary of the growth of yeast strains showing their alcohol tolerance at various concentrations of ethanol.

Yeast strain	Growth of yeasts in media containing various concentrations of ethanol: 100 ml l^{-1}		140 ml l^{-1}		160 ml l^{-1}		180 ml l^{-1}	
	YPF[1]	YPG[1]	YPF	YPG	YPF	YPG	YPF	YPG
Saccharomyces cerevisiae (38, 102, 116, 126, 128, 131, 135, 139, 141, 143, 146, 148, 149, 153, 160, 165)	+	+	+	+	v	+	-	V
Pichia kudriavzevii (32, 42, 51, 59, 94, 100, 105, 110, 123, 125, 129, 137, 140, 144, 150, 151, 166)	+	+	+	v	v	v	v	v
Pichia kudriavzevii (152)	-	-	-	-	-	-	-	-
Saccharomycopsis fibuligera (66)	+	+	w	w	-	-	-	-
Pichia fabianii (65, 145)	+	+	+	+	w	v	-	-
Zygoascus hellenicus (121,122)	-	w	-	v	-	-	-	-
Candida parapsilosis (93)	-	+	-	+	-	w	-	w
Candida pyralide (90)	+	+	-	-	-	-	-	-
Pichia ciferrii (115,164)	+	+	-	v	-	-	-	-
Hanseniaspora optuntiae (54)	+	+	+	+	+	+	w	-
Candida glabrata (134, 147)	+	+	+	+	w	w	-	-

Notes: w = weak, + = positive, - = negative, v = variable. A control of media containing no ethanol showed growth for all the yeast strains, the results are not included in the table. [1]YPF = yeast extract peptone fructose agar; YPG = yeast extract peptone glucose agar.

Comparison of candidate yeasts for wine starter cultures

The production of high concentrations of ethanol from both glucose and fructose, the ability to assimilate all of the sugars supplied, and a high ethanol tolerance were used as criteria to select candidate yeasts for *masau* wine and distillate starter cultures. Six strains of *S. cerevisiae* (strains no. 38, 102, 131, 135, 143 and 153) were selected to analyse the formation of aromatic volatile compounds during fermentation of *masau* juice. In addition, three strains of *P. kudriavzevii* (strains no. 125, 129 and 166) were selected. The selected *S. cerevisiae* and *P. kudriavzevii* strains are all located at the top right hand corner of the scatter plot (Figure 2). *Sm. fibuligera* and *P. fabianii* strains were also included in this comparative test. Although these species had a low ethanol tolerance they are frequently encountered in traditional fermented

foods (Nout, 2003), and they may produce interesting volatiles. For example, *Pichia* spp. were reported to contribute additional flavour diversity and complexity to wines (Fleet, 2008).

Fermentation of *masau* juice by selected yeast isolates

Saccharomyces cerevisiae strains exhausted the glucose present in the juice and left some traces of fructose (Table 2) during the fermentation period of 72 hours. The other species and strains could not exhaust all of the glucose and fructose present in the juice during the 72 hours. Fructose was found to be the most abundant residual sugar in the fermented juice. Apparently, these yeasts showed a discrepancy in glucose and fructose utilization as described by Berthels et al. (2004). Although fructose is used concomitantly with glucose, the latter is depleted first from the medium. Berthels and co-workers (2004) explained this discrepancy on basis of differences in kinetic properties of sugar transporters and hexokinases.

Production of volatile compounds

The volatile compounds produced by the selected yeast species are shown in Table 3 (fermented juices) and Table 4 (distillates). The differences in the final amounts of some volatiles were statistically significant among the strains.

The naturally occurring volatile compounds in *masau* juice generally showed increased concentrations after fermentation. These included propanol, isoamyl alcohol, isoamyl acetate, ethyl propanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, octanoic acid, hexanoic acid, isovaleric acid and 4-nonanone. The other compounds, 3-hydroxyl-2-butanone, furaldehyde and N-acetyl guanidine, were not detected in the fermented juices and heptyl propyl ketone was detected in lesser amounts compared to the initial quantity present in the natural *masau* juice. They could have been modified or were still present but in amounts below the detection threshold. The latter was the case with 3-hydroxyl-2-butanone and furaldehyde as they could be detected after concentration in the distilled products.

Production of higher alcohols

Propanol and isoamyl alcohol were the most abundant higher alcohols produced by the yeast strains. The amounts ranged from 0.3 to 2.2 mg l^{-1} and from 0.4 to 8.9 mg l^{-1} for propanol and isoamyl alcohol, respectively. *S. cerevisiae* strains produced higher amounts compared to the other species. Isoamyl alcohol appeared in higher amounts in the fermented juice than propanol but was not detected in the distillates. Due to the concentration effect caused by the distillation process the propanol content increased from 1.7 to 13.4 fold in the distillates depending on the strain, if compared to the amounts detected in the fermented juice. *S. cerevisiae* strain 153 had formed double the amount of propanol (2.2 mg l^{-1}) compared with *S. cerevisiae* strain 38 and *P. kudriavzevii* strain 129 (1.1 mg l^{-1} and 1.0 mg l^{-1}, respectively) in the fermented juices, but the distillate of *S. cerevisiae* strain 153 had a lower propanol concentration (28.5 mg l^{-1}) compared with these two strains with 34.5 mg l^{-1} and 30.2 mg l^{-1}, respectively. Propanol and isoamyl alcohol have boiling points < 200 °C, are soluble in alcohol, and are completely or partially soluble in water. They therefore distil mainly in the heart fraction of the distillate. The process of distillation and yeast lees present in fermented juice at the time of distillation, contribute both to the composition of the distillate. This is attributed to the cell wall polysaccharides with the ability to bind to particular compounds (Fundira et al., 2002; Steger et

al., 2000), which may explain the absence of isoamyl alcohol and the difference in increases of propanol concentration in the distillates.

Table 2. Sugars, ethanol and organic acids determined by HPLC in laboratory fermented *masau* fruit juice (72 hours, 30°C) produced by yeasts isolated from traditionally fermented fruit pulp.

Sample	**Residual Sugars ($g \cdot 100ml^{-1}$)**		**Ethanol ($g \cdot 100ml^{-1}$)**	**Organic acids ($g \cdot 100ml^{-1}$)**				
Fermentation by:	**Glucose**	**Fructose**		**Citric**	**Malic**	**Acetic**	**Oxalic**	**Succinic**
Saccharomyces cerevisiae								
143	nd	0.38^{e1}	3.2^{e}	0.78^{g}	1.15^{d}	0.12^{b}	0.43^{d}	1.35^{f}
102	nd	0.18^{b}	2.8^{d}	0.57^{b}	0.76^{a}	0.14^{c}	0.36^{c}	0.89^{b}
131	nd	0.16^{a}	3.2^{e}	0.66^{c}	0.87^{b}	0.21^{d}	0.38^{c}	1.22^{d}
153	nd	0.29^{d}	4.2^{g}	0.71^{e}	0.89^{b}	0.18^{f}	0.52^{g}	1.23^{d}
38	nd	0.25^{c}	3.7^{f}	0.72^{e}	0.90^{b}	0.23^{d}	0.46^{ef}	1.28^{e}
135	nd	0.50^{f}	2.9^{d}	0.84^{h}	1.21^{e}	0.17^{e}	0.45^{e}	1.27^{e}
Pichia kudriavzevii								
125	2.3^{b}	3.6^{g}	1.0^{b}	0.74^{f}	1.59^{f}	0.19^{f}	0.38^{c}	1.42^{g}
129	1.9^{a}	3.4^{g}	1.5^{c}	0.70^{d}	1.69^{g}	0.17^{e}	0.22^{b}	1.29^{e}
166	2.4^{b}	4.2^{h}	1.3^{c}	0.96^{j}	2.11^{j}	0.29^{g}	0.59^{g}	1.65^{i}
Saccharomycopsis fibuligera								
66	4.0^{c}	4.8^{i}	0.3^{a}	0.90^{i}	1.95^{i}	0.15^{c}	0.48^{e}	1.14^{c}
Pichia fabianii								
65	3.7^{c}	4.4^{h}	0.2^{a}	0.85^{h}	1.73^{h}	0.21^{d}	0.51^{f}	1.57^{h}
Masau juice (control)	6.7^{d}	6.7^{j}	0.1^{a}	0.47^{a}	1.09^{c}	0.09^{a}	0.34^{a}	0.83^{a}

[1]Means in the same column with same letter are not significantly different according to the LSD at the 0.05 level. nd = not detected.

Propanol and isoamyl alcohol are fusel alcohols derived from amino acid catabolism via a pathway named after Ehrlich (Hazelwood et al., 2008). The ratio of ethanol to fusel alcohol is much higher for *Saccharomyces* strains (3.1 – 6.0) compared to non-*Saccharomyces* species (1.5 – 2.8), which means that the latter are utilizing the Ehrlich pathway more than the former. However, the levels of fusel alcohol produced by *Saccharomyces* strains are higher than those produced by the other species. Hazelwood and co-workers (2008) reported that fusel alcohols at high concentrations impart off-flavours, but low concentration of these compounds and their esters make an essential contribution to the flavours and aromas of fermented foods and beverages.

Production of esters

Isoamyl acetate, ethyl acetate and butyl acetate were the major acetate esters produced. Butyl acetate was only produced by *P. fabianii* and *Sm. fibuligera* strains. These two species also produced the highest amounts of ethyl acetate (20.7 mg l^{-1} and 20.9 mg l^{-1}, respectively) and isoamyl acetate (2.8 and 3.3 mg l^{-1}, respectively) compared with the other species. *P. kudriavzevii* strains produced higher amounts of ethyl acetate (9.4 mg l^{-1}, 17.1 mg l^{-1} and 18.4 mg l^{-1}) than the *S. cerevisiae* strains (1.5 – 4.0 mg l^{-1}). Acetate esters are reportedly produced at relatively high concentrations by non-*Saccharomyces* species (Rojas et al., 2001; Rojas et al., 2003; Trinh et al., 2011). The amounts of ethyl acetate produced by non-*Saccharomyces* species may contribute to the fruity character of wine when their concentration exceeds the odour threshold (7.5 mg l^{-1}) (Guth, 1997) and is lower than the level considered to have a negative impact on wine aroma (>150 – 200 mg l^{-1}) (Mallouchos et al., 2003). As for isoamyl acetate, the amounts produced by all strains are above the odour threshold. Isoamyl acetate was reported to have an odour threshold of 0.03 mg l^{-1} and it contributes a fruity, banana, sweet flavour (Chaves-Lopez et al., 2009). Thus, the levels of isoamyl acetate detected may contribute to the sensory quality of wine produced.

Ethyl propanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate were the fatty acid ethyl esters produced by the yeasts. *S. cerevisiae* strains produced ethyl hexanoate and ethyl octanoate in highest amounts (21.3 – 110 mg l^{-1} and 18.8 - 58 mg l^{-1} respectively) relative to the other ethyl esters and amounts produced by other yeast species (1.3 – 2.5 mg l^{-1} and 0.3 – 14.5 mg l^{-1}). However, all the yeast species produced ethyl hexanoate, ethyl octanoate and ethyl decanoate amounts which are above their odour thresholds. Ethyl hexanoate has a fruity, green apple flavour (Chaves-Lopez et al., 2009) and a threshold of 0.005 mg l^{-1} (Guth, 1997). The odour threshold of ethyl octanoate is 0.002 mg l^{-1} (Guth, 1997; Palomo et al., 2007) and contributes a fruity, banana, pineapple flavour (Palomo et al., 2007). Ethyl decanoate has been reported to have a sweet fruity flavour, resembling dry fruits (Chaves-Lopez et al., 2009), and a threshold of 0.2 mg l^{-1} (Ferreira et al., 2000). Mallouchos et al. (2003a) reported that fatty acid ethyl esters are the volatiles providing the major aroma impact of freshly fermented wines. They have fruity odours and often occur at concentrations much higher than their respective odour thresholds (Mallouchos et al., 2003). Therefore, ethyl esters produced by the yeasts may have a profound impact on the aromatic flavour of the *masau* wine bouquet.

Isoamyl acetate, ethyl acetate, ethyl hexanoate and ethyl octanoate were present in the distillates at elevated levels, depending on the strain. All other esters found in the fermented juice were not detected in the distillates.

Production of acids

Some fatty acids including hexanoic, octanoic and isovaleric acids occur naturally in the fruit. The juices that were fermented with strains *P. fabianii* 65 and *Sm. fibuligera* 66 contained hexanoic acid at a concentration close to the threshold (3 mg l^{-1}). Hexanoic acid has a cheese flavour (Palomo et al., 2007). The levels of octanoic acid detected for all the yeast species were below the odour threshold of 0.5 mg l^{-1} (Ferreira et al., 2000). Isovaleric acid has a rancid flavour and all the fermented juices showed values above the odour threshold (0.033 mg l^{-1}).

Table 3. Volatile compounds (mg l^{-1}) determined by HS-SPME GC-MS in laboratory fermented *masau* fruit juices produced by yeasts isolated from traditionally fermented *masau* fruit pulp and *masau* fruits.

Volatile compounds		Yeast Strains										
	MJ	**102SC**	**131SC**	**153SC**	**143SC**	**135SC**	**38SC**	**125PK**	**129PK**	**166PK**	**65PF**	**66SF**
Alcohols												
Ethanol[1]	0.33[a3]	29.2[f]	32.6[g]	45.6[i]	38.2[h]	46.5[j]	55.5[k]	7.9[c]	9.4[d]	10.0[e]	1.8[b]	2.0[b]
Propanol[1]	0.23[a]	1.6[d]	1.3[c]	2.2[e]	1.3[c]	0.6[b]	1.1[c]	0.9[c]	1.0[c]	0.7[b]	0.5[b]	0.3[a]
Isoamyl alcohol[1]	0.01[a]	7.6[f]	7.2[f]	8.9[g]	7.5[f]	7.1[f]	7.3[f]	2.7[d]	4.4[e]	4.6[e]	0.7[c]	0.4[b]
Acetate esters												
Isoamyl acetate[1]	0.06[a]	0.9[c]	0.9[c]	0.9[c]	0.7[bc]	1.2[de]	1.4[e]	0.5[b]	0.8[c]	1.1[d]	2.8[f]	3.3[g]
Ethyl acetate[1]	0.38[a]	2.4[c]	1.5[b]	2.4[c]	1.6[b]	4.0[d]	2.7[c]	9.4[e]	18.4[g]	17.7[f]	20.7[h]	20.9[h]
Butyl acetate[1]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2[a]	0.2[a]
Ethyl esters												
Ethyl propanoate[2]	0.05[a]	1.2[e]	0.8[d]	0.9[d]	0.9[d]	0.6[c]	1.2[e]	0.4[b]	0.6[c]	0.5[c]	1.4[f]	1.4[f]
Ethyl butanoate[2]	0.37[a]	0.6[b]	0.6[b]	0.9[c]	0.5[b]	0.6[b]	0.6[b]	0.3[a]	0.5[b]	0.5[b]	0.5[b]	0.5[b]
Ethyl hexanoate[2]	0.12[a]	110[g]	28.7[e]	92.0[f]	28.6[e]	21.3[d]	23.2[d]	1.4[b]	2.5[c]	2.5[c]	1.3[b]	1.8[b]
Ethyl octanoate[2]	0.35[a]	31.9[i]	30.3[h]	58.0[j]	20.3[g]	18.8[f]	21.0[g]	14.5[e]	11.6[d]	2.7[c]	0.3[a]	0.9[b]
Ethyl decanoate	0.02[a]	1.8[e]	1.0[c]	1.3[d]	0.6[b]	0.7[b]	0.8[b]	nd	nd	0.1[a]	nd	nd
Fatty acids												
Octanoic acid[2]	0.20[a]	0.3[a]	0.2[a]	0.4[b]	0.2[a]	0.3[a]	0.2[a]	0.3[a]	0.3[a]	0.3[a]	0.2[a]	0.2[a]
Hexanoic acid[1]	2.1[d]	0.8[c]	0.5[b]	0.7[c]	0.5[b]	0.6[bc]	0.6[bc]	2.8[e]	0.5[b]	0.3[a]	3.1[f]	3.0[f]
Isovaleric acid[2]	0.20[a]	0.6[c]	0.5[c]	0.5[c]	0.4[b]	0.4[b]	0.4[b]	0.3[a]	0.3[a]	0.4[b]	0.4[b]	0.3[a]
Carbonyl compound												
3-hydroxyl-2-butanone[2]	0.31	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4- Nonanone[2]	0.02[a]	0.6[b]	0.6[b]	0.7[b]	0.5[b]	0.6[b]	0.5[b]	0.3[d]	0.5[b]	0.6[b]	0.5[b]	0.6[b]
Heptyl propyl ketone[2]	0.60[a]	0.2[b]	0.2[b]	0.4[c]	0.1[b]	0.1[b]	0.3[c]	0.1[b]	0.1[b]	0.2[b]	0.1[b]	0.2[b]
Furaldehyde[2]	2.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Other												
N-acetyl guanidine[2]	0.33	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

MJ = *Masau* juice, nd = not detected, SC = *Saccharomyces cerevisiae,* PK = *Pichia kudriavzevii*, PF = *Pichia fabianii* and SF = *Saccharomycopsis fibuligera*. [1]Identification by comparison of retention times and mass spectral data with those of authentic compounds; [2]Tentative identification. [3]Means in the same row with the same letter are not significantly different according to the LSD at the 0.05 level.

Table 4. Volatile compounds (mg l^{-1}) determined by HS-SPME GC-MS in distilled product of laboratory fermented *masau* fruit juices produced by yeasts isolated from traditionally fermented *masau* fruit pulp and *masau* fruits.

Volatile compounds		**Yeast Strains**			
	MJ	**153SC**	**38SC**	**129PK**	**66SF**
Alcohols					
Ethanol[1]	0.33[a3]	942.5[d]	1052.3[e]	142.1[c]	14.4[b]
Propanol[1]	0.23[a]	28.5[c]	34.5[e]	30.2[d]	0.5[b]
Isoamyl alcohol[1]	0.01[a]	nd	nd	nd	nd
Acetate esters					
Isoamyl acetate[1]	0.06[a]	3.7[b]	3.7[b]	19.1[d]	5.4[c]
Ethyl acetate[1]	0.38[a]	17.2[b]	22.0[c]	237[e]	35.7[d]
Butyl acetate[1]	nd	nd	nd	nd	nd
Ethyl esters					
Ethyl propanoate[2]	0.05[a]	nd	nd	nd	nd
Ethyl butanoate[2]	0.37[a]	nd	nd	nd	nd
Ethyl hexanoate[2]	0.12[a]	256.2[d]	228.7[c]	4.7[b]	nd
Ethyl octanoate[2]	0.35[a]	342.5[d]	402.9[e]	87.9[c]	4[b]
Ethyl decanoate[2]	0.02[a]	nd	nd	nd	nd
Fatty acids					
Isovaleric acid[2]	0.2[a]	1.9[b]	3.2[d]	24.6[e]	2.8[c]
Carbonyls					
3-hydroxyl-2-butanone[2]	0.31[a]	2.8[b]	3.0[b]	2.9[b]	nd
4- Nonanone[2]	0.02[a]	15.3[c]	9.4[b]	26.2[e]	23.3[d]
Heptyl propyl ketone[2]	0.6[a]	nd	nd	nd	nd
Furaldehyde[2]	2.53[b]	8.6[d]	4.7[c]	37.2[e]	1.9[a]

MJ = *Masau* juice, SC = *Saccharomyces cerevisiae,* PK = *Pichia kudriavzevii*, SF = *Saccharomycopsis fibuligera* , and nd = not detected. [1]Identification by comparison of retention times and mass spectral data with those of authentic compounds; [2]Tentative identification; [3]Means in the same row with same letter are not significantly different according to the LSD at the 0.05 level.

Of the organic acids (Table 1), citric, malic, acetic, oxalic and succinic acids were detected in higher amounts in most of the fermented *masau* juices than in the non-fermented juice. Apparently, the yeast strains produced these organic acids, though in small amounts. Succinic acid was the major acid produced by *S. cerevisiae* strains whereas the non-*Saccharomyces* strains produced malic and succinic acids in similar amounts. The other acids were found in trace amounts.

CONCLUSION

Yeast strains belonging to the same species and isolated from the same habitat exhibited significant differences in the production of ethanol and volatile compounds during fermentation of the standard broth and *masau* fruit juice, emphasizing the importance of screening yeasts isolates. The selected *S. cerevisiae* strains (102, 131, 153, 143,135 and 38) may be further used as starter cultures for the production of *masau* wine and distillate as they are the highest producers of ethanol and aromatic compounds, especially fatty acid ethyl esters that provide the major aroma impact of freshly fermented wines. There is also a possibility of using the *S. cerevisiae* strains in mixed starters with selected non-*Saccharomyces* yeasts such as *P. kudriavzevii* (125, 129), *P. fabianii* (65) and *Sm. fibuligera* (66) which produced even higher amounts of ethyl acetate. Ethyl acetate has a fruity, sweet aroma that can contribute to the wine's olfactory complexity, thus enhancing the *masau* wine bouquet. The developed library of characteristics can help in the design of mixtures of strains to obtain a specific melange of product functionalities.

ACKNOWLEDGEMENTS

The authors are grateful to the MacGillavry fund of The Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation of Science (IFS Grant No. C/3737) for financial support. We are also grateful to Judith Wolkers-Rooijackers and Peter de Gijsel for assistance with GC-MS analyses.

Supplementary data:
Table S1. The carbon (C) balance during fermentation (5 days, 30°C) of 100 g l^{-1} glucose in standard broth by yeasts under non-aerated conditions.

Yeast strains	**Carbon moles (moles 100ml^{-1})**						
	Biomass	**Ethanol**	**CO_2**	**Total C moles produced**	**Sugar used (C moles)**	**% Yield of Ethanol (C moles)**	**% Yield of C moles**
Saccharomyces cerevisiae							
38	0.011	0.178	0.089	0.278	0.333	53.5	83.4
102	0.013	0.183	0.091	0.287	0.333	54.8	86.1
116	0.012	0.143	0.072	0.228	0.303	47.5	75.0
126	0.008	0.178	0.089	0.276	0.333	53.5	82.7
128	0.005	0.057	0.028	0.090	0.157	36.1	57.2
131	0.012	0.178	0.089	0.279	0.333	53.5	83.8
135	0.010	0.178	0.089	0.278	0.333	53.5	83.3
139	0.007	0.096	0.048	0.150	0.243	39.3	61.7
141	0.007	0.161	0.080	0.249	0.300	53.6	82.8
143	0.008	0.178	0.089	0.275	0.333	53.5	82.6
146	0.007	0.174	0.087	0.268	0.333	52.2	80.4
148	0.003	0.174	0.087	0.264	0.333	52.2	79.3
149	0.007	0.148	0.074	0.229	0.293	50.4	78.1
153	0.008	0.178	0.089	0.275	0.333	53.5	82.6
160	0.008	0.178	0.089	0.275	0.333	53.5	82.6
165	0.012	0.174	0.087	0.273	0.333	52.2	82.0
Pichia kudriavzevii							
32	0.006	0.083	0.041	0.129	0.190	43.5	68.1
42	0.006	0.052	0.026	0.084	0.170	30.7	49.3
51	0.004	0.061	0.30	0.095	0.133	45.7	71.6
59	0.004	0.074	0.037	0.115	0.237	31.2	48.7
94	0.004	0.122	0.061	0.187	0.287	42.5	65.3
100	0.004	0.135	0.067	0.206	0.230	58.6	89.7
105	0.005	0.061	0.030	0.096	0.193	31.5	49.7
110	0.004	0.039	0.020	0.062	0.107	36.7	58.6
123	0.009	0.152	0.076	0.237	0.333	45.7	71.1
125	0.009	0.148	0.074	0.231	0.287	51.6	80.6
129	0.011	0.178	0.089	0.279	0.330	54.0	84.5
137	0.003	0.074	0.037	0.114	0.230	32.1	49.7
140	0.006	0.057	0.028	0.091	0.233	24.2	38.8
144	0.004	0.113	0.057	0.174	0.243	46.5	71.5
150	0.003	0.074	0.037	0.114	0.163	45.1	70.0
151	0.008	0.052	0.026	0.087	0.167	31.3	51.9
152	0.008	0.165	0.083	0.256	0.333	49.6	76.7
166	0.009	0.187	0.093	0.289	0.333	56.1	86.7
Other strains							
66SF	0.002	0.007	0.003	0.012	0.047	14.0	26.1
65PF	0.009	0.063	0.031	0.103	0.130	48.2	79.1
145PF	0.007	0.013	0.007	0.026	0.100	13.0	26.1
121ZH	0.007	0.022	0.011	0.040	0.100	21.7	39.5
122ZH	0.003	0.0	0.0	0.003	0.043	0.0	8.0
93CP	0.011	0.0	0.0	0.011	0.100	0.0	11.4
90CPY	0.005	0.026	0.013	0.044	0.133	19.6	33.0
115PC	0.007	0.061	0.030	0.098	0.127	48.1	77.5
164PC	0.002	0.033	0.016	0.051	0.093	34.9	54.6
54HO	0.004	0.0	0.0	0.004	0.084	0.0	8.4
134CG	0.009	0.152	0.076	0.237	0.330	46.1	71.8
147CG	0.005	0.161	0.080	0.246	0.333	48.3	73.8

Notes: CO_2 was calculated based on ethanol. (SF), *Saccharomycopsis fibuligera*; (PF), *Pichia fabianii*; (ZH), *Zygoascus hellenicus*; (CP), *Candida parapsilosis*; (CPY), *Candida pyralide*; (PC), *Pichia ciferii*; (HO), *Hanseniaspora optuntiae*; (CG), *Candida glabrata.*

Table S2. The balance of carbon during fermentation (5 days, 30°C) of 100 g l^{-1} fructose in standard broth by yeasts under non-aerated conditions.

Yeast strains	Carbon content (C moles 100ml^{-1})						
	Biomass	Ethanol	CO_2	Total C moles produced	Sugar used (C moles)	% Yield of ethanol (C moles)	% Yield of C moles
Saccharomyces cerevisiae							
38	0.008	0.200	0.100	0.308	0.333	60.0	92.5
102	0.008	0.200	0.100	0.308	0.330	60.6	93.3
116	0.009	0.196	0.098	0.302	0.333	58.7	90.7
126	0.010	0.191	0.096	0.297	0.333	57.4	89.0
128	0.004	0.122	0.061	0.187	0.333	36.5	56.1
131	0.009	0.196	0.098	0.303	0.333	58.7	90.8
135	0.009	0.196	0.098	0.302	0.330	59.3	91.6
139	0.007	0.174	0.087	0.268	0.307	56.7	87.4
141	0.008	0.178	0.089	0.276	0.333	53.5	82.7
143	0.010	0.196	0.098	0.304	0.333	58.7	91.1
146	0.008	0.200	0.100	0.308	0.333	60.0	92.3
148	0.008	0.183	0.091	0.281	0.333	54.8	84.4
149	0.009	0.196	0.098	0.302	0.333	58.7	90.6
153	0.008	0.196	0.098	0.301	0.333	58.7	90.4
160	0.009	0.196	0.098	0.302	0.330	59.3	91.6
165	0.009	0.174	0.087	0.270	0.327	53.2	82.6
Pichia kudriavzevii							
32	0.006	0.139	0.070	0.214	0.243	57.2	88.0
42	0.003	0.139	0.070	0.212	0.243	57.2	87.0
51	0.006	0.135	0.067	0.208	0.300	44.9	69.2
59	0.005	0.083	0.041	0.129	0.157	52.7	82.2
94	0.004	0.139	0.070	0.212	0.253	54.9	83.9
100	0.004	0.109	0.054	0.167	0.200	54.4	83.6
105	0.004	0.109	0.054	0.167	0.197	55.3	85.0
110	0.004	0.0	0.0	0.004	0.037	0.0	11.3
123	0.005	0.126	0.063	0.194	0.250	50.4	77.6
125	0.002	0.130	0.065	0.198	0.260	50.2	76.2
129	0.004	0.143	0.072	0.219	0.257	55.9	85.3
137	0.006	0.113	0.057	0.175	0.207	54.7	84.9
140	0.006	0.096	0.048	0.149	0.187	51.2	79.8
144	0.005	0.096	0.048	0.149	0.177	54.1	84.1
150	0.002	0.139	0.070	0.211	0.237	58.8	89.1
151	0.004	0.157	0.078	0.239	0.270	58.0	88.5
152	0.004	0.013	0.006	0.023	0.043	29.1	54.0
166	0.004	0.152	0.076	0.232	0.243	62.5	95.5
Other strains							
66SF	0.008	0.057	0.028	0.092	0.110	51.4	84.0
65PF	0.006	0.074	0.037	0.116	0.170	43.5	68.5
145PF	0.006	0.057	0.028	0.091	0.113	49.9	80.3
121ZH	0.005	0.0	0.0	0.005	0.067	0.0	7.2
122ZH	0.004	0.0	0.0	0.004	0.013	0.0	33.6
93CP	0.005	0.061	0.030	0.096	0.133	45.7	72.4
90CPY	0.006	0.026	0.013	0.045	0.073	35.6	61.4
115PC	0.009	0.048	0.024	0.081	0.167	28.7	48.6
164PC	0.013	0.0	0.0	0.013	0.127	0.0	10.3
54HO	0.009	0.022	0.011	0.041	0.243	8.9	16.9
134CG	0.008	0.165	0.083	0.255	0.287	57.6	89.1
147CG	0.004	0.117	0.059	0.181	0.333	35.2	54.2

Notes: CO_2 was calculated based on ethanol. (SF), *Saccharomycopsis fibuligera*; (PF), *Pichia fabianii*; (ZH), *Zygoascus hellenicus*; (CP), *Candida parapsilosis*; (CPY), *Candida pyralide*; (PC), *Pichia ciferii*; (HO), *Hanseniaspora optuntiae*; (CG), *Candida glabrata.*

CHAPTER 6

Yeasts preservation: alternatives for lyophilisation

L. K. Nyanga, M. J. R. Nout, E. J. Smid, T. Boekhout and M. H. Zwietering: World Journal of Microbiology and Biotechnology. Accepted for publication.

ABSTRACT

The aim of the study was to compare the effect of two low-cost, low-technology traditional methods for drying starter cultures with standard lyophilisation. Lyophilised and dry rice cakes preserved yeast cultures were examined for viable cell counts during six months storage at 4°C and 25°C. Yeast cultures preserved in dry plant fibre strands were stored at 25°C. None of the yeast cultures showed a significant loss in viable cell count during six months of storage at 4°C upon lyophilisation and preservation in dry rice cakes. During storage at 25°C in the dark, yeast cultures preserved in dry rice cakes, and lyophilised cultures of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* showed no significant loss of viable cells up to four months of storage. Yeast cultures preserved in dry plant fibre strands had the greatest loss of viable count during the six months of storage at 25°C. Preservation of yeasts cultures in dry rice cakes provided better survival during storage at 4°C than lyophilisation. The main challenge of having controlled fermentation is the availability of reliable economic starter storage especially for small and medium enterprises in the developing countries that cannot afford standard culture preservation methods such as lyophilisation. The current study demonstrated that traditional methods can be useful as defined starter culture preservation methods in small-scale, low-tech applications.

INTRODUCTION

The basic concept of microbial preservation of starter cultures is to avoid any change in the genetic, physiological and morphological characteristics of the microorganisms during storage and to promote a complete depression of all metabolic activity (Cheong et al., 2008). Lyophilisation is one of the most successful methods for preserving bacteria, yeasts and sporulating fungi (Berny et al., 1991; Spadaro et al., 2010; Tan et al., 2007). This method offers convenience of storage and postage, and it keeps the microorganisms viable for long periods of time (Miyamoto-Shinohara et al., 2006). However, lyophilisation is relatively expensive as it requires sophisticated equipment and adequate power supply.

Most small and medium-sized enterprises (SME's) in developing countries cannot afford lyophilisation of starter cultures. Traditional methods for starter culture preservation could be an economical and dependable alternative to lyophilisation in low-tech infrastructure conditions. Traditional fermentation of *pito,* a traditional alcoholic beverage brewed and drunk by people from the West African sub-region (Demuyakor et al., 1993), uses yeast cells from a previous brew trapped in the interstices of a traditional woven belt (Sefa-Dedeh et al., 1999). In East Asian countries rice starter cakes which contain complex mixtures of fungi are used for rice wine production (Nout et al., 2002). However, the use of traditional methodologies can result in unpredictable fermentation products as the inocula contain uncontrolled mixed microbiota. As a result, even when the fermentation process is successful, its outcome could show considerable variation in product quality. These traditional starter preservation methods could be harnessed for dependable and low-cost preservation of defined fermentation starter cultures. Since the traditional methods are usually economically feasible and can be applied under rural conditions, the basic work flow of these processes is preferably kept intact.

In Zimbabwe, *Ziziphus mauritiana* (*masau*) fruit pulp is naturally fermented and distilled into a spirit called *kachasu* (Nyanga et al., 2008). In order to improve the product quality and its consistency, certain aspects of the process can be potentially changed, like changing from an uncontrolled natural fermentation to a fermentation with an undefined starter culture. Even more consistent quality can be obtained in certain cases with a defined starter culture. In this respect yeasts from the traditionally fermented fruit pulp have been isolated (Nyanga et al., 2007) and characterised (Chapter 5) to enable development of starter cultures for *masau* wine and distillate production. *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and *Saccharomycopsis fibuligera* species were selected as candidate yeasts for starter cultures. A further challenge in the move from uncontrolled natural fermentation to the use of stable defined starter cultures is the availability of reliable economic starter storage. The current study was, therefore, designed to assess two traditional methods for drying starter cultures, i.e., stabilization of yeast cultures in plant fibre strands and in rice cakes, for subsequent use in *masau* wine production and compare these methods with lyophilisation. We analysed the impact of the different drying processes on survival of yeast cells during six months of storage.

MATERIALS AND METHODS

Preparation of the inoculum

Cultures used in the study were *Saccharomyces cerevisiae* (strains 38 and 153), *Saccharomycopsis fibuligera* (66) and *Pichia kudriavzevii* (129). These strains were previously isolated from traditionally fermented *masau* fruit pulp (Nyanga et al., 2007) and were

maintained routinely at -80°C in 300 ml l^{-1} glycerol prepared in peptone physiological saline (PPS) [NaCl 8.5 g l^{-1} (Merck, Darmstadt, Germany), neutral peptone 1 g l^{-1} (Oxoid, Basingstoke, UK)].

Yeast cells were grown on Malt Extract Agar (MEA) (Oxoid, Basingstoke, UK) slants at 30°C for 48 hours. A suspension of yeast cells was made by adding 2 ml of sterile PPS onto each pure culture slant. The biomass was gently scraped off the agar by means of an inoculating loop. The yeast cell suspension was then transferred to a sterile tube and preserved by lyophilisation, on rice cakes and plant fibre strands. A fresh yeast culture was made for each method.

Preservation methods

For each preservation method two independent experiments were performed as described below.

Lyophilisation: Yeast suspensions of 1 ml volume were transferred to sterile Eppendorf tubes and centrifuged for 10 minutes at 2600 x *g* (Cheong et al., 2008). The cells were then washed and centrifuged thrice in 1 ml sterile PPS. The pellet was then suspended in 1 ml sterile solution of 120 g l^{-1} fat free instant milk powder (Clover SA Pty Ltd, Roodepoort, South Africa) prepared in sterile distilled water supplemented with 70 g l^{-1} trehalose (Sigma-Aldrich Co., St Louis, MO, USA) (Cheong et al., 2008; Tan et al., 2007; Wiemken, 1990). The yeast-milk suspension (0.2 ml) was transferred to sterile cryotubes. The cryotubes were kept at -58°C for three hours and were then lyophilised for 3 hours in a lyophilisation apparatus (Edwards Freeze dryer-Modulyo model 4k, Crawley, West Sussex, England). After freeze-drying the cryotubes were sealed.

Rice cakes: Rice flour was made from white polished rice using a pulveriser mill (Siebtechnik GmbH, Mülheim Ruhr, Germany). Portions of rice flour (50 g) were made into a 40% moisture content dough using sterile water according to Dung et al. (2005). The dough from each flask was inoculated with 8 ml of yeast suspension and incubated at 30°C for 24 hours. The inoculated rice dough was then aseptically made into cakes of about 3 - 4 cm diameter and 5 - 6 mm thickness. The cakes were aseptically dried at 40°C in a ventilated oven for 5 hours to reach a moisture content of about 4 - 5% w/w and then placed in tightly closed containers.

Plant fibre strands: The plant fibre belt named *Tafanta* in *Biali* language, and made of twined baobab (*Adansonia digitata*) fibres was obtained from Benin. The plant fibre belt was cut into strands (7 – 8 cm length and 6 mm thickness) that were wrapped in aluminium foil and sterilized at 121°C for 15 minutes. Two hundred ml of Glucose-Yeast extract broth (GYEB) [glucose 100 g l^{-1}, yeast extract 10 g l^{-1}] was brought into 500 ml volumetric flasks and sterilized at 12°C for 15 minutes. Sterile strands were added to GYEB aseptically, inoculated with 1 ml of yeast suspension, whereas a control flask was not inoculated. Broths were fermented for five days at 30°C under non-aerated conditions, plugged with a water-lock. After fermentation the strands were covered and impregnated with sedimented yeast biomass. They were taken out of the flask under aseptic conditions and dried at 40°C in a ventilated oven for 3 hours. The dried and yeast impregnated fibre strands were placed in tightly closed containers.

Storage conditions

The lyophilised yeast cells and dry rice cakes were stored at 4°C and room temperature (≈ 25°C) in a dark cabinet. The dry plant fibre strands were stored at room temperature (≈ 25°C) in a dark cabinet.

Rehydration and yeast viable cell counts

The survival of the yeast cells was determined by sampling once a week for one month and then once every month for six months. Viable cells were also determined immediately after each drying method. The number of viable cells was determined as colony forming units per ml (CFU ml^{-1}) for the lyophilised yeast cells and as colony forming units per gram (CFU g^{-1}) for rice cakes and plant fibre strands.

Lyophilisation: The total content of one cryotube was resuspended in 1 ml of PPS and vortexed for 1 minute to disintegrate any cell clumps.

Rice cakes: A sample of 1 g of rice cake was aseptically transferred into sterile 250 ml bottles containing 99 ml of PPS. The bottles were vigorously shaken at intervals by hand for 10 minutes to dissolve the cakes.

Plant fibre strands: One strand (0.5 g ± 0.02) was transferred into a sterile 250 ml bottle containing 50 ml of PPS. The bottle was vigorously shaken at intervals by hand for 10 minutes.

Plating: Pour plates were made using glucose peptone yeast extract agar [2% (w/v) D-glucose (Merck), 0.5% (w/v) bacto peptone (Oxoid), 0.5% (w/v) yeast extract and 2% (w/v) agar (Oxoid); (GPYA)] with appropriate dilutions in duplicate. The inoculated plates were incubated for 48 hours at 30°C and colonies were counted.

Survival rate and calculation of D-values

Loss of viability was calculated as log N_o/N_t, where N_o represents the counts of viable microorganisms immediately after drying, and N_t the counts of viable microorganisms after a given storage period. Linear regression analysis was carried out on numbers of surviving cells at each storage temperature against time. The negative reciprocal of the slope of regression lines was calculated to obtain D-values (months). D is the time of the first decimal reduction (i.e. time required to reduce the population by 1 log unit from initial level at $t = 0$).

Statistical Analysis

The analytical data were analysed using the statistical program SPSS16.0 for Windows (Apache Software Foundation, USA) and the one-way ANOVA model was used applying the LSD test to evaluate significant differences among means.

RESULTS

Lyophilised, dry rice cake and dry plant fibre strand preserved yeast cultures were examined for viable cell counts during six months storage at 4ºC and 25ºC in a dark cabinet. During storage at 4ºC, cultures of the different yeast species all showed a similar survival behaviour with a viable cell count reduction of less than 1 log unit after six months storage upon either lyophilisation or drying in rice cakes (Figure 1).

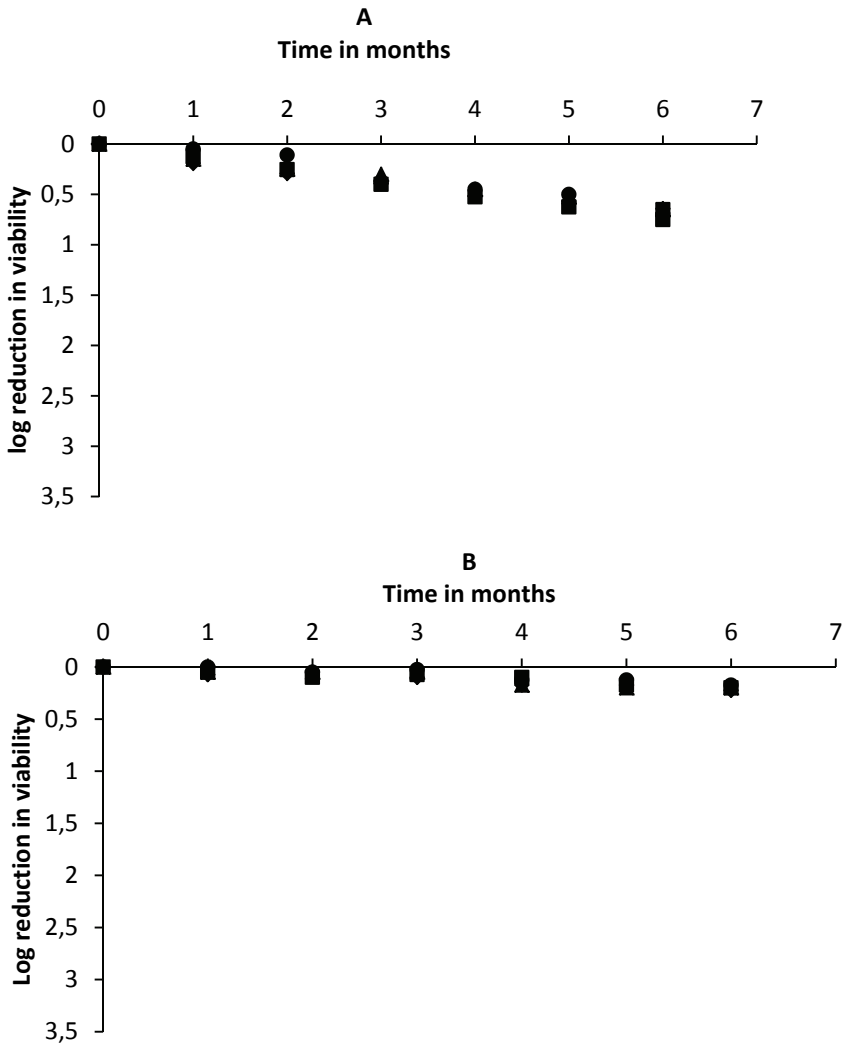


Figure 1. Log reduction in viable counts of each yeast species in lyophilised (A) and dry rice cake (B) cultures during six months storage at 4ºC. ◆ , *Saccharomyces cerevisiae* (38); ■ , *Saccharomyces cerevisiae* (153); ▲ , *Pichia krudriavzevii* (129) and ●, *Saccharomycopsis fibuligera* (66).

Interestingly, dry rice starter cakes showed minimal losses of viable counts for all strains (*viz.* between 0.2 and 0.3 logs) as compared to lyophilised cultures, which had higher losses of viable counts (viz. between 0.6 and 0.8 logs). The observation was supported by the calculated D-values ranging from 28 to 40 months (Table 1), which clearly showed that yeast cells preserved in dry rice cakes survived significantly ($p<0.05$) better than lyophilised yeast cells which had D-values ranging from 8 to 10 months. Cultures of the yeast species showed different D-values. For the yeast cultures preserved in dry rice cakes, *Sm. fibuligera* had the highest D-value and *S. cerevisiae* strain 153 had the lowest value. Lyophilised yeast cultures of *S. cerevisiae* strain 38 and *P. kudriavzevii* had the highest D-value followed by *Sm. fibuligera* and lastly *S. cerevisiae* strain 153.

Table 1. Estimated D-values (months) of yeast strains preserved by lyophilisation, in dry rice cakes and dry fibre strands stored at 4°C and 25°C .

Preservation Method and Yeast strains		**Storage Temperature Linear regression D-value[1]**	
Lyophilised	Strain No.	4ºC	25ºC
Saccharomyces cerevisiae	38	10.0±0.7[c2]	4.0±0.6[c]
Saccharomyces cerevisiae	153	8.0±1.0[a]	4.0±0.1[c]
Pichia kudriavzevii	129	10.0±0.5[c]	3.0±0.3[b]
Saccharomycopsis fibuligera	66	9.0±1.0[b]	3.0±0.0[b]
Dry Rice Cakes			
Saccharomyces cerevisiae	38	33±1.9[f]	5.0±1.4[d]
Saccharomyces cerevisiae	153	28±1.4[d]	5.0±1.7[d]
Pichia kudriavzevii	129	31±1.7[e]	4.0±1.0[c]
Saccharomycopsis fibuligera	66	40±2.0[g]	5.0±0.9[d]
Dry Plant Fibre Strands			
Saccharomyces cerevisiae	38	ND	2.0±0.0[a]
Saccharomyces cerevisiae	153	ND	2.0±0.1[a]
Pichia kudriavzevii	129	ND	2.0±0.2[a]
Saccharomycopsis fibuligera	66	ND	2.0±0.0[a]

[1]D-values ± standard deviation shown are means of two replicate experiments, the enumeration was done in duplicate. [2]Means in the same column with same letter are not significantly different according to the LSD at 0.05 level. ND, not determined.

There was a significant ($p < 0.05$) decrease in viable counts of yeast cultures during the six months of storage for all preservation methods for yeasts stored at 25ºC (Figure 2). It was noted that yeast cultures preserved in dry rice cakes had the best retention of viable cell counts with no significant loss up to four months of storage for all the strains. Lyophilisation was second best with *S. cerevisiae* (strains 38 and 153) and *P. kudriavzevii* cultures showing no significant decrease in viable cell counts up to four months. On the other hand, lyophilised *Sm. fibuligera* cultures performed differently showing a slight loss in viable cell counts during three months of storage. Yeast cultures preserved in dry fibre strands had the greatest loss of viable counts as there was significant decrease in viable cell count (between 1.2 and 1.3 log CFU/g) after three months of storage.

DISCUSSION

Starter culture preservation, maintenance and distribution are important as the quality of the final fermented product strongly depends on the preservation technologies employed that are required to guarantee long-term maintenance of stable cultures in terms of viability and activity.

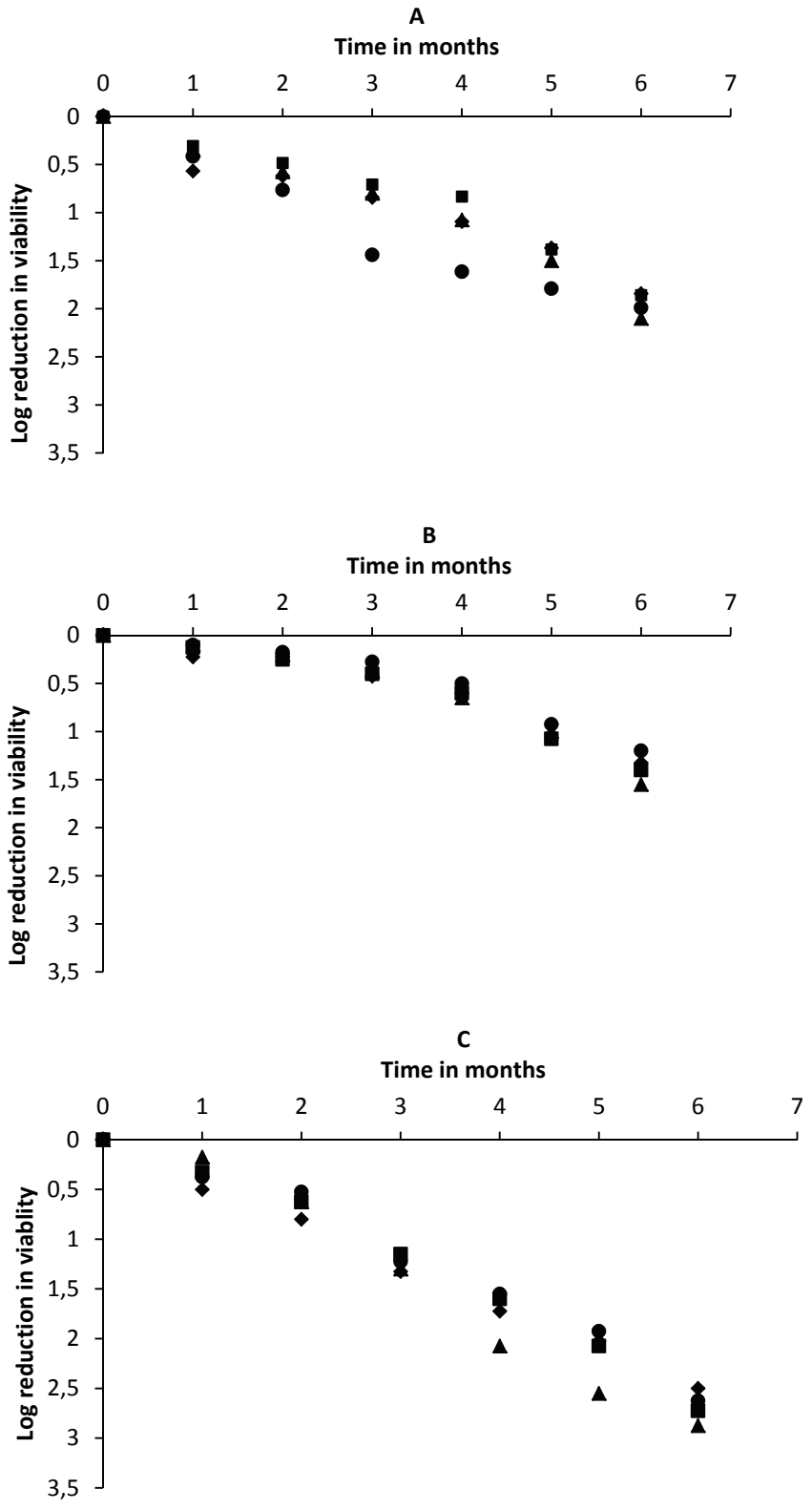


Figure 2. Log reduction in viable count of each yeast species in lyophilised (A), dry rice cake (B) and dry plant fibre strand (C) cultures during six months storage at 25ºC. ◆ , *Saccharomyces cerevisiae* (38); ■ , *Saccharomyces cerevisiae* (153); ▲ , *Pichia krudiavzevii* (129) and ●, *Saccharomycopsis fibuligera* (66).

This demands logistic infrastructure and economic affordability particularly for small-scale, low-tech applications. In this study we compared the survival of yeast starter cultures preserved by two low-tech traditional methods (i.e., stabilization of yeast cultures in plant fibre strands and in rice cakes), with lyophilisation.

The results indicated that survival of the yeasts was better at 4ºC than at 25ºC for both the lyophilised cultures and yeast cultures preserved in dry rice cakes. According to Spadaro et al. (2010) this could be due to the fact that low temperature helps to keep the metabolic activity at a low level which is expected to contribute to an increase in storage and shelf-life. Better survival of lyophilised microorganisms stored at 4ºC than at 25ºC was reported in several studies (Abadias et al., 2001; De Valdez et al., 1993; Li et al., 2007). Storage in the presence of air, high moisture content, and high temperature has been reported to be detrimental to freeze-dried microorganisms (Coulibaly et al., 2009). Loss of viability during storage of dried cultures of microbial cells has been attributed to lipid oxidation of cell membrane fatty acids (Coulibaly et al. 2009). This would be initiated by loss of water that increases the ionic concentration, which can lead to the formation of reactive oxygen species. Subsequently, these oxygen species can damage proteins, modify nucleobases and sugars in deoxyribonucleic acid and eventually cause lipid oxidation. The enhanced survival of air dried lactic acid bacteria in the presence of mannitol was also explained by the anti-oxidative properties of this polyol (Efiuvwevwere et al., 1999).

Skimmed milk and trehalose were used in this study as cryoprotectants for the yeast cultures during lyophilisation. The combination of skimmed milk with disaccharides has been reported to improve the viability and stability of microorganisms during lyophilisation and storage (Hamoudi et al., 2007; Li et al., 2007; Tan et al., 2007). Direct interactions between sugar molecules and membrane phospholipids, or sugar molecules and proteins, and vitrification of sugars in the dry state are thought to be the main protective mechanisms (Crowe et al., 1998).

The criteria for the choice of solid support of microorganisms are that the carrier substance should not be toxic to the microorganisms and should not affect their metabolism (Isu et al., 2000). The mechanisms by which some polyhydroxyl compounds provide protection have been attributed to the formation of hydrogen bonds with sensitive components when water is removed, maintaining the structural integrity of membranes and proteins (Crowe et al., 1987; Mazzobre et al., 1999). A second hypothesis is related to the ability of saccharides to form a glassy structure (Mazzobre et al., 1999), in which the sensitive components of the membrane are embedded.

In this study, dry rice cakes provided the best retention of viable cell counts for the yeast cultures. Rice contains starch, which comprises amylose and amylopectin that could have provided hydroxyl groups as reactive groups for attachment to the yeast cells. It could also form a glass structure, protecting the yeast cells from damage.

The preservation of starter cultures by a simple and adoptable technology using locally available substrates such as grains and pulses that are familiar to the consumers, would be compatible with the existing low level of technology (Isu et al., 2000) in most developing countries.

CONCLUSION

Dry rice cake cultures retained viable cell counts significantly better as compared to lyophilised cultures. Traditional culture preservation methods, such as the dry rice cakes and dry plant fibres can be used to preserve defined starter cultures for traditional fermented foods. In order to safeguard the quality of the preserved yeast, the preservation should be carried out under hygienic and controlled conditions. The use of dry rice cakes and plant fibre strands as preservation methods are of interest in small-scale, low-tech applications.

ACKNOWLEDGEMENTS

The financial support by the McGillavry Fund of the Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation for Science (IFS Grant No. C/3737), is gratefully acknowledged. The authors thank Dr. Polycarpe Kayodé, University of Abomey-Calavi, Benin, for kindly providing the plant fibre belt. We are also grateful to the technical assistance given by Mr. P. Gombiro.

CHAPTER 7

DISCUSSION

FINDINGS OF THE THESIS AND FUTURE PROSPECTS

Indigenous fruit trees are an important part of diet and livelihood in Africa. The indigenous fruits are a source of energy and nutrients such as vitamins and minerals. Many of the wild fruits are traditionally fermented into beverages. Studies in this thesis focused on one of the most utilised wild fruits in Zimbabwe, *Ziziphus mauritiana* (*masau*). The study documented the traditional processing and analysed the nutritional composition of the fruit. Yeasts, yeast-like fungi and lactic acid bacteria microbiota were isolated from the fruits and their fermented pulp. The yeasts were characterised for their fermentation properties with regard to their production of ethanol and volatile compounds, to select potential starter cultures for the production of *masau* wine and distillate. Starter culture maintenance using two low-cost 'low-tech' traditional starter drying methods was compared with standard lyophilisation. The major conclusions of the thesis were:

- The survey conducted in the Zambezi valley in Zimbabwe, revealed that the *masau* fruits contribute significantly to the people's livelihoods through subsistence use and as a safety net in drought periods.
- The *masau* fruits are nutritionally valuable to the people as they are a potential source of energy and nutrients such as carbohydrates, proteins and micronutrients like vitamin C, calcium, potassium, phosphorus, iron and zinc.
- The microbiological analyses of yeasts occurring on the fruit and in the fermented fruit pulp showed a gradual transition from non-fermentative species in unripe fruits, through mixed population in ripe fruits and predominantly fermentative species in fermented fruit pulp.
- Yeasts were found to co-exist with lactic acid bacteria (LAB) in ripe fruits and the fermented fruit pulp. *Saccharomyces cerevisiae* and *Pichia kudriavzevii* were predominant in the fermented fruit pulp.
- Characterisation of the yeasts to select potential starter cultures for the production of *masau* wine and distillate showed that *S. cerevisiae* strains were superior to other species in ethanol and flavour compounds production especially fatty acid ethyl esters that provide the major aroma impact of freshly fermented wines.
- *Saccharomyces cerevisiae* strains can potentially be used in mixed starters with selected non-*Saccharomyces* yeasts such as *P. kudriavzevii*, *Saccharomycopsis fibuligera* and *P. fabianii.*
- A comparative study of two low-cost low-tech traditional methods for drying starter cultures and standard lyophilisation showed that yeast cultures preserved in dried rice cakes survived significantly better than lyophilised cultures at both 4°C and 25°C. The traditional methods can be used to preserve defined starter cultures for traditional fermented foods.

In the following sections the results and future prospects are discussed.

Documentation of the traditional processing techniques and analyses of nutritional composition of *masau* fruits

Knowledge of traditional foods is important for sustaining their development and usage (Ohiokpehai, 2003). It is important for people to know the prevailing traditional foods in their

areas and how they can be improved for better sustainable food security and nutrition. Documentation of the indigenous knowledge (Chapter 2) was done through a survey in August, a period in which the fruit is available. The survey showed that the fruit has a short shelf-life and therefore it is preserved by sun drying. The people from the community make most *masau* products from the dried fruits. The products are made manually and hygienic standards are low. The development of appropriate processing devices will be helpful for the processing of *masau* fruits in a hygienic manner and this could attract customers and receive better prices on the markets for the *masau* fruit products.

The nutritional composition of the fruit is important since the fruit is consumed fresh when in season and in dried form throughout the year, which means it plays an important role in the diet. The study showed that the fruit is a good source of energy, carbohydrate, protein and micronutrients such as vitamin C, calcium, phosphorus, iron and zinc. The bioavailability of the nutrients needs to be determined to ensure that *masau* fruits and their food products could be useful in complementing other alleviation programmes of malnutrition. The food and nutrition council of Zimbabwe (2010) reported that 34% of children under five years of age in the country suffer from chronic malnutrition. The use of local resources for local solutions is a better way of dealing with malnutrition since it is economical and sustainable because the wild fruits are easily accessible.

In addition to the sweeter taste, the sweet *masau* fruit variety was significantly richer in vitamin C than sour fruit while the latter was richer in minerals. This is useful information in selection of cultivars during domestication of the fruit tree. *Z. mauritiana* was reported to be amongst the indigenous fruit trees that are included in the tree domestication programme of the Southern Africa Development Community (SADC) (Akinnifesi et al., 2006).

Isolation, identification and characterisation of microorganisms from *masau* fruits and their fermented fruit pulp

The survey revealed that the *masau* fruits are naturally fermented and distilled to produce *kachasu*. The fermented fruit pulp is not consumed as it is characterised by bad smell and unattractive surface due to decomposition by fungus and other undesirable microorganisms, having been naturally fermented under unhygienic conditions. Yeasts were isolated, identified (Chapter 4) and characterised with regard to their fermentation characteristics in order to select starter cultures (Chapter 5), to upgrade the traditional fermentation of *masau* fruits to a controlled fermentation process. The use of starter culture has clear advantage over the traditional spontaneous fermentation (Holzapfel, 2002; Siebenhandl et al., 2001) because of more consistent quality product and food safety. The local yeasts are presumed to be more competitive than commercial yeasts because they are better adapted to the ecological and technological features of their own growing area (Lopes et al., 2007).

The choice of alcoholic fermentation starters is one of the most important factors influencing the yield and quality of the final products. The criteria for selection and development of yeasts for wine fermentation have evolved over many years (Fleet, 2008). It is important that the yeast does not give slow, sluggish or stuck fermentations (Bisson, 1999; Fleet, 2008). In this study yeasts were pre-selected according to consumption of sugar, ethanol yield and alcohol tolerance. This is important because desirable wine yeasts should assimilate all fermentable sugars and produce high ethanol yields. In addition, the yeast should be tolerant

to alcohol so that it does not cause stuck fermentations. The pre-selected yeasts were tested for the production of volatile compounds because these are the ones that determine wine flavour and aroma, and hence, its quality and character. Flavour is the wine's most important characteristic. A wine's flavour could, in its widest sense, be said to be the overall sensory impression of both aroma (as sensed by nose and from mouth) and taste compounds (Lambrechts et al., 2000).

The literature on the influence of yeasts on volatile composition of wines showed that yeast strains vary greatly in volatile compounds production (Fundira et al., 2002; Lurton et al., 1995; Romano et al., 2003; Steger et al., 2000). It was found in this study that the yeast strains belonging to the same species and isolated from the same habitat exhibited significant differences in the production of ethanol and volatile compounds. In addition, the results of this study clearly show how misleading it can be to choose a yeast strain for the production of a distillate on the basis of the performance of the yeast in the fermented juice as reported by Fundira and co-workers (2002). For instance, *S. cerevisiae* strain 153 had formed double the amount of propanol compared to *S. cerevisiae* strain 38, but the distillate of *S. cerevisiae* strain 153 had a lower propanol concentration compared to the other strain.

The characterised species which are potential starter cultures for *masau* wine and distillate production, *S. cerevisiae*, *P. kudriavzevii*, *Sm. fibuligera* and *P. fabianii* need to be explored further in their functionality during the fermentation of *masau* juice by carrying out sensory evaluation of the fermented juice and distillate. Single and mixed cultures can be tried in order to find the best combination and/or culture. Combinations of *S. cerevisiae* with non-*Saccharomyces* yeasts in starters may be important. Fleet (2008) reported that non-*Saccharomyces* yeasts contribute significantly to the overall character of wine. Other studies also confirmed the important contribution that non-*Saccharomyces* species make to the overall kinetics of yeast growth during both spontaneous and *S. cerevisiae* inoculated wine fermentations (Combina et al., 2005; Comitini et al., 2011; Jolly et al., 2003; Zott et al., 2008). In this study the *S. cerevisiae* strains were the best producers of volatile compounds, but the non-*Saccharomyces* yeast species *Sm. fibuligera* and *P. fabianii* produced the highest levels of acetate esters that contribute to wine flavour complexity.

LAB isolated and identified were not characterised in this study but they could be of interest for the production of non-alcoholic beverages from *masau* fruits. Most of the physiological properties which hitherto are recognised to be essential for different food fermentations are present in the various species of lactobacilli (Buckenhüskes, 1993). LAB are associated with several potential health and nutritional benefits such as control of intestinal infections, some types of cancer and serum cholesterol levels (Gillilan, 1990).

The *masau* fruits are perishable and sun drying is not the best way of preserving them as it results in loss of some nutrients (Tembo et al., 2008a). Preservation by fermentation could be an alternative as it enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein and fibre digestibility, the micronutrient bioavailability and by degrading antinutrients (Giraffa, 2004). The *masau* fruit could be fermented in the following ways:

- ✓ Yeast-based fermentation to produce an alcoholic beverage,
- ✓ LAB-based fermentation to produce a non-alcoholic beverage,
- ✓ A combination of yeasts and LAB to produce a non-alcoholic beverage.

Fermentation is an advantageous technology in the sense that extension of shelf-life, enhancement of sensory properties, safety and improved nutritional value are achieved by a technique which is affordable at the household level (Oyewole, 1997). Application of post harvest handling techniques to reduce microbial spoilage and extend shelf-life could be difficult and costly to some parts of the rural community. Postharvest studies of *masau* fruits indicated that low temperature storage can improve the shelf life of the fruits (Tembo et al., 2008b), but low temperature storage is not applicable under rural conditions, whereas fermentation is an economical method of producing and preserving food (Mensah, 1997).

Up-scaling of the production of traditional fermented foods from indigenous fruits can support environmental and social sustainability by providing food as well as promoting economic growth. The current studies offer an opportunity for product development from *masau* fruits and this is an important argument in favour of the domestication of the fruit tree. The domestication of trees for agroforestry as an approach towards poverty alleviation and environmental rehabilitation in the tropics depends on the expansion of the market demand for non-timber forest products (Leakey, 1999). It has been recognised that an important factor in improving the viability of rural livelihood in developing countries is the promotion of sustainable agriculture. As opposed to relying solely on cash crops, sustainable agriculture can be more easily achieved through the domestication of various indigenous fruit trees that can be cultivated and owned by smallholder farmers (Mithofer, 2003).

A comparative study of two low-cost low-tech traditional starter culture drying methods and lyophilisation

Traditional food processes produce a good percentage of the staple foods and condiments consumed in the third world countries (Isu et al., 2000). Hence, it is important to upgrade the traditional technologies to meet the challenges of modern food processing. The introduction of appropriate starter culture techniques may constitute one major step toward improved safety, quality, and security of traditional small-scale fermentation in Africa, Asia and Latin America (Coulibaly et al., 2009). Lack of experience in the application of starter cultures in small-scale operations and under rural conditions presents a major obstacle but also an exciting challenge to food microbiologists and technologists (Holzapfel, 2002). Proper maintenance of the starter cultures by employing dependable preservation technologies is required to warrant long-term delivery of stable cultures in terms of viability and functional activity. Two methods which are commonly used for the preservation and storage of most microbial starters are freezing and lyophilisation. However, dried cultures have an advantage over frozen cultures due to easy handling and storage (Hamoudi et al., 2007). This study compared the effect of two traditional drying methods for starter cultures (i.e., stabilisation of yeast cultures in plant fibre strands and rice cakes) with lyophilisation (Chapter 6). Although all three drying methods used electricity, lyophilisation requires sophisticated equipment whereas the traditional methods use simple equipment. The current study demonstrated that traditional drying methods for starter cultures are economic and dependable technologies for 'low-tech' food fermentations because the carrier substrates are inexpensive, readily available and familiar to the consumers. Dried starters offer great opportunities for improvement of traditional fermentation processes which are mostly practised under rural conditions. However, in this study viable cell counts were used to determine the shelf-life and stability of dried cultures.

Future studies should include testing the preserved cultures by allowing them to ferment *masau* juice and carry out sensory evaluation of the fermented juice and distillate to determine whether they have maintained their fermentation characteristics hence their functionality.

CONCLUDING REMARKS

Masau fruits are of social, economic and nutritional significance in Zimbabwe. *S. cerevisiae* strains and non-*Saccharomyces* species such as *Sm. fibuligera*, *P. fabianii* and *P. kudriavzevii* isolated from the *masau* fruits and their traditionally fermented fruit pulp are potential starter cultures for the upgrading of the traditional fermentation of *masau* fruit into beverages. LAB strains were identified but not characterised in this study but they could be of interest in the production of *masau* fruit products. Traditional culture preservation methods are of interest in small-scale, low-tech applications.

REFERENCES

REFERENCES

Amarteifio, J. O., Mosase, M. O., 2006. The chemical composition of selected indigenous fruits of Botswana. Journal of Applied Science and Environmental Management, 10, 43-47.

A.O.A.C., 1990. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, Virginia, USA.

Abadias, M., Teixido, N., Usall, J., Benabarre, A., Vinas, I., 2001. Viability, efficacy, and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. Journal of Food Protection 64, 856-861.

Abbas, M.F., 1997. Jujube. In: Mitra, K.S. (Ed.), Postharvest Physiology and Storage of Tropical and Subtropical Fruits. CAB International, Wallingford, pp. 405-415.

Abbas, M.F., Fandi, B.S., 2002. Respiration rate, ethylene production and biochemical changes during fruit development and maturation of jujube (*Ziziphus mauritiana* Lamk.). Journal of the Science of Food and Agriculture 82, 1472-1476.

Achinewhu, S.C., Ogbonna, C.C., Hart, A.D., 1995. Chemical composition of indigenous wild herbs, spices, fruits, nuts and leafy vegetables used as food. Plant Foods For Human Nutrition 48, 341-348.

Agrahar-Murugkar, D., Subbulakshmi, G., 2005. Nutritive values of wild edible fruits, berries, nuts, roots and spices consumed by the Khasi tribes of India. Ecology of Food and Nutrition, 44, 207-233.

Akinnifesi, F.K., Kwesiga, F., Mhango, J., Chilanga, T., Mkonda, A., Kadu, C.A.C., Mithofer, D., Saka, J.D.K., Sileshi, G., Ramadhani, T., Dhliwayo, P., 2006. Towards the development of miombo fruit trees as commercial tree crops in Southern Africa. Forests, Trees and Livelihoods 16, 103-121.

Akinnifesi, F.K., Kwesiga, F., Mhango, J., Mkonda, A., Chilanga, T., Swai, R., 2004. Domesticating priority miombo indigenous fruit trees as a promising livelihood option for smallholder farmers in Southern Africa. Acta Horticulturae 632, 15-30.

Alfenore, S., Molina-Jouve, C., Guillouet, S.E., Uribelarrea, J.L., Goma, G., Benbadis, L., 2002. Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process. Applied Microbiology and Biotechnology 60, 67-72.

Bakhshi, J.C., Singh, P., 1974. The ber - a good choice for semi-arid and marginal soils. India Horticulture 19, 27-30.

Barnett, J.A., 1997. Sugar utilization by *Saccharomyces cerevisiae*. In: Zimmermann, F.K., Entian, K.D. (Ed.), Yeast sugar metabolism. Technomic Publishing Co., United States, pp. 35-43.

Battcock, M., Ali, S.A. (1993). Fermented fruits and vegetables., Agricultural Services Bulletin, 134, 13-28.

Beech, F.W., 1993. Yeasts in cider-making. In: Rose, A.H., Harrison, J.S. (Ed.), The Yeasts. Academic Press, London, UK, pp. 169-214.

Berny, J.F., Hennebert, G.L., 1991. Viability and stability of yeast cells and filamentous fungus spores during freeze-drying: effects of protectants and cooling rates. Mycologia 83, 805-815.

Bisson, L.F., 1999. Stuck and sluggish fermentations. American Journal of Enology and Viticulture 50, 107-119.

Boekhout, T., Phaff, H., 2003. Yeast biodiversity. In: Boekhout, T., Robert, V. (Ed.), Yeasts in Foods: Beneficial and Detrimental Aspects. B. Behr's Verlag GmbH and Co., Hamburg, pp. 1-38.

Bonire, J.J., Jahil, N.S., Lori, J.A., 1990. Sodium and potassium content of two cultivars of white yam (*Dioscorea rotundata*) and their source soils. Journal of the Science of Food and Agriculture 53, 271-274.

Brett, T.R.L., Nyamupingidza, E.N., Gurira, R.C., 1992. The analysis, identification and determination of toxic substances in *Kachasu*. Transactions of the Zimbabwe Science Association 66, 25-29.

Buckenhüskes, H.J., 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiology Reviews 12, 253-273.

Campell, B.M., 1987. The use of wild fruits in Zimbabwe. Economic Botany 41, 375-385.

Caplice, E., Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology 50, 131-149.

Cavendish, W., 2000. Empirical regularities in the poverty-environment relationship of rural households: evidence from Zimbabwe. World Development 28, 1979-2003.

Chand-Goyal, T., Spotts, R.A., 1996. Enumeration of bacterial and yeast colonists of apple fruits and identification of epiphytic yeasts on pear fruits in the Pacific Northwest United States. Microbial Research 151, 427-432.

Chaves-Lopez, C., Serio, A., Osorio-Cadavid, E., Paparella, A., Suzzi, G., 2009. Volatile compounds produced in wine by Colombian wild *Saccharomyces cerevisiae* strains. Annals of Microbiology 59, 733-740.

Cheong, C., Wackerbauer, K., Beckmann, M., Jang, K.H., Kang, S.A., 2008. Effect of cultivation conditions on trehalose content and viability of brewing yeast following preservation via filter paper or lyophilization methods. Biotechnology and Bioprocess Engineering 13, 690-696.

Cherry, M., 1985. The needs of the people. In: Wickens, G.E., Goodin, J.R., Field, D.V. (Ed.), Plants for Arid Lands. Unwin Hyman, London, pp. 1-8.

Chivero, E.T., Mutukumira, A.N., Zvauya, R., 2001. Partial purification and characterisation of a xylanase enzyme produced by a micro-organism isolated from selected indigenous fruits of Zimbabwe. Food Chemistry 72, 179-185.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Heras-Vázquez, F.J.L., Rodríguez-Vico, F., 2004. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiology 21, 149-155.

Collingwood, K., 1995. International dairy of lactic acid bacteria conference. Trends in Food Science and Technology 6, 212-213.

Combina, M., Elia, A., Mercado, L., Catania, C., Ganga, A., Martinez, C., 2005. Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. International Journal of Food Microbiology 99, 237-243.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiology 28, 873-882.

Coulibaly, I., Amenan, A.Y., Lognay, G., Fauconnier, M., Thonart, P., 2009. Survival of freeze-dried *Leuconostoc mesenteroides* and *Lactobacillus plantarum* related to their cellular fatty acids composition during storage. Applied Biochemistry and Biotechnology 157, 70-84.

Crabtree, H.G., 1929. Observations on the carbohydrate metabolism of tumors. Biochemistry Journal 23, 536.

Crowe, J.H., Carpenter, J.F., Crowe, L.M., 1998. The role of vitrification in anhydrobiosis. Annual Review of Physiology 60, 73 - 103.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Wistrom, A., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochemistry Journal 242, 1 -10.

Danthu, P., Soloviev, P., Totté, A., Tinee, E., Ayessouf, N., Gayeb, A., Niang, T.D., Seck, M., Falle, M., 2002. Jujube trees in Senegal: a comparison between the organoleptic and physico-chemical characteristics of the wild fruits and the introduced *Gola* variety. Fruits 57, 173-182.

De Deken, R.H., 1966. The Crabtree effect: a regulatory system in yeast. Journal of General Microbiology 44, 149-156.

De Valdez, G.F., Diekmann, H., 1993. Freeze-drying conditions of starter cultures for sourdoughs. Cryobiology 30, 185-190.

Deak, T., 1993. Simplified techniques for identifying foodborne yeasts. International Journal of Food Microbiology 19, 15-26.

Deak, T., 2003. Detection, enumeration and isolation of yeasts. In: Boekhout, T., Robert, V. (Ed.), Yeasts in Food: Beneficial and Detrimental Effects. Behr's Verlag GmbH and Co., Hamburg, pp. 39-59.

Deak, T., Beuchat, L.R., 1996. Yeasts in specific types of food. In: Deak, T., Beuchat, L.R. (Ed.), Handbook of Food Spoilage. CRC Press, London, pp. 61-95.

Demuyakor, B., Ohta, Y., 1993. Characteristics of single and mixed culture fermentation of *pito* beer. Journal of the Science of Food and Agriculture 62, 401-408.

Dennis, C., Davis, R.P., 1977. Susceptibility of strawberry varieties to postharvest fungal spoilage. Journal of Applied Bacteriology 42, 197-206.

Deshpande, M.S., Rule, V.B., Lynch, J.M., 1992. *Aureobasidium pullulans* in applied microbiology. A status report. Enzyme and Microbial Technology 14, 514 - 527.

Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D., Zhang, K., 2009. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology 85, 253-263.

Dung, N.T.P., Rombouts, F.M., Nout, M.J.R., 2005. Development of defined mixed-culture fungal fermentation starter granulate for controlled production of rice wine. Innovative Food Science and Emerging Technologies 6, 429-441.

Dung, N.T.P., Rombouts, F.M., Nout, M.J.R., 2006. Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. Food Microbiology 23, 331-340.

Efiuvwevwere, B.J.O., Gorris, L.G.M., Smid, E.J., Kets, E.P.W., 1999. Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. Applied Microbiology and Biotechnology 51, 100-104.

Egceling, W.J., 1951. The indigenous trees of the Uganda protectorate. The Government printer, Uganda.

Eromosele, I.C., Eromosele, C.O., Kuzhkuzha, D.M., 1991. Evaluation of mineral elements and ascorbic contents in fruits of some wild plants. Plants Foods for Human Nutrition 41, 151-154.

Fales, F.W., Baumberger, J.P., 1947. The anaerobic assimilation of glucose by yeast cells. The Journal of Biological Chemistry 173, 1-8.

Ferreira, I.M.P.L.V.O., Pinho, O., Vieira, E., Tavarela, J.G., 2010. Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. Trends in Food Science and Technology 21, 77-84.

Ferreira, V., Lopez, R., Cacho, J.F., 2000. Quantitative determination of the odorants of young red wines from different grape varieties. Journal of the Science of Food and Agriculture 80, 1659-1667.

Feyssa, D.H., Njoka, J.T., Asfaw, Z., Nyangito, M.M., 2011. Wild edible fruits of importance for human nutrition in semi-arid parts of East Shewa Zone, Ethiopia: Associated indigenous knowledge and implications to food security. Pakistan Journal of Nutrition 10, 40-50.

Fleet, G.H., 1999. Microorganisms in food ecosystems. International Journal of Food Microbiology 50, 101-117.

Fleet, G.H., 2003. Yeasts in fruit and fruit products. In: Boekhout, T., Robert, V. (Ed.), Yeasts in Food: Beneficial and detrimental Aspects. B. Behr's Verlag GmbH and Co., Hamburg, pp. 267-287.

Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Research 8, 979 -995.

Fundira, M., Blom, M., Pretorius, I.S., van Rensburg, P., 2002. Selection of yeast starter culture strains for the production of marula fruit wines and distillates. Journal of Agricultural and Food Chemistry 50, 1535-1542.

Gadaga, T.H., Mutukumira, A.N., Narvhus, J.A., Feresu, S.B., 1999. A review of traditional fermented foods and beverages of Zimbabwe. International Journal of Food Microbiology 53, 1-11.

Gibson, B.R., Lawrence, S.J., Leclaire, J.P.R., Powell, C.D., Smart, K.A., 2007. Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiology Reviews 31, 535-569.

Gildemacher, P., Heijne, B., Silvestri, M., Houbraken, J., Hoekstra, E., in press. Interactions between yeasts, fungicides and apple fruit russeting. FEMS Yeast Research.

Gillilan, S.E., 1990. Health and nutritional benefits populations during food fermentation. FEMS Microbiology Reviews 87, 175-188.

Giraffa, G., 2004. Studying the dynamics of microbial populations during food fermentation. FEMS Microbiology Reviews 28, 251-260.

Gobbetti, M., 1998. The sourdough microflora: Interactions of lactic acid bacteria and yeasts. Trends in Food Science and Technology 9, 267-274.

Gobbetti, M., Corsetti, A., Rossi, J., 1994. The sourdough microflora interaction between lactic acid and yeasts: metabolism of amino acids. World Journal of Microbiology and Biotechnology 10, 275-279.

Gomez, I., 1989. A resource inventory of indigenous and traditional foods in Zimbabwe. University of Zimbabwe, Harare.

Gupta, A.K., Boekhout, T., Theelen, B., Summerbell, R., Batra, R., 2004. Identification and typing of *Malassezia* species by amplified fragment length polymorphism and sequence analyses of the internal transcribed pacer and large-subunit regions of ribosomal DNA. Journal of Clinical Microbiology 42, 4253-4260.

Guth, H., 1997. Quantitation and sensory studies of character impact odorants of different white wine varieties. Journal of Agricultural and Food Chemistry 45, 3027-3032.

Halm, M., Lillie, A., Sorensen, A.K., Jakobsen, M., 1993. Microbiological and aromatic characteristics of fermented maize doughs for *kenkey* production in Ghana. International Journal of Food Microbiology 19, 135-143.

Hammes, W.P., 1991. Fermentation of non-dairy foods. Food Biotechnology 5, 293-303.

Hammes, W.P., Tichaczek, P.S., 1994. The potential of lactic acid bacteria for the production of safe and wholesome food. Lebensmittel Untersuchung und Forschung 198, 193-201.

Hamoudi, L., Goulet, J., Ratti, C., 2007. Effect of protective agents on the viability of *Geotrichum candidum* during freeze-drying and storage. Journal of Food Science 72, M45-M49.

Hazelwood, L.A., Daran, J.M., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Applied and Environmental Microbiology 74, 2259-2266.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Applied and Environmental Microbiology 50, 727-728.

Holzapfel, W.H., 1997. Use of starter cultures in fermentation on household scale. Food Control 8, 241-258.

Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. International Journal of Food Microbiology 75, 197-212.

Isu, N.R., Abu, G.O., 2000. An evaluation of the effect of *Bacillus* cells and *Bacillus* spores in association with cowpea granules as starter cultures for the fermentation of African oil bean (*Pentaclethra macrophylla* Bentham) to 'ugba'. Plant Foods For Human Nutrition 55, 127-138.

Jespersen, L., 2003. Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. FEMS Yeast Research 3, 191-200.

Johnston, M.C., 1963. The species of *Ziziphus* indigenous to United States and Mexico. American Journal of Botany 50, 1020-1027.

Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2003. The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape must from four

production regions of the Western Cape, South Africa. South African Journal of Enology and Viticulture 24, 35-42.

Kaaria, S. (1998). *Ziziphus mauritiana* - a valuable tree for arid and semi-arid lands. Fact sheet. A quick guide to multipurpose trees from around the world (p. 4). Morrilton, Arkansas: A publication of the Forest, Farm and Community Tree Network (FACT Net), Winrock International.

Kadzere, I. (1998). Role of *Ziziphus mauritiana* in the livelihood of some communities in Zimbabwe. In: International Workshop on *Ziziphus mauritiana*. Harare, Zimbabwe.

Kadzere, I., Hove, L., Gatsi, T., Masarirambi, M.T., Tapfumaneyi, L., Maforimbo, E., Magumise, I., 2004. Current status of post-harvest handling and traditional processing of indigenous fruits in Zimbabwe. In: Kwesiga, R.M.R. (Ed.), Proc Regional Agroforestry Conference on Agroforestry impacts on Livelihoods in Southern Africa: Putting Research into Practice. World Agroforestry Centre (ICRAF), Nairobi, Kenya, pp. 353 - 363.

Kalikiti, F. (1998). *Ziziphus mauritiana* in Siavonga district, Zambia. In: International Workshop on *Ziziphus mauritiana*. Harare, Zimbabwe.

Karachi, M., Ruffo, C., Lema, N., Minae, S. (1991). Use of trees in western Tanzania: results of ethanobotanical survey of multipurpose trees in the Tabora region. AFRENA Report No. 40. Nairobi: ICRAF.

Khoshoo, T.N., Subrahmanyam, G.V., 1985. Ecodevelopment of arid lands in India with non-plants for arid lands. In: Wickens, G.E., Goodin, J.R., Field, D.V. (Ed.), Plants for Arid Lands. Unwin Hyman, London, pp. 243-265.

Kurtzman, C.P., Boekhout, T., Robert, V., Fell, W.J., Deak, T., 2003. Methods to identify yeasts. In: Boekhout, T., Robert, V. (Ed.), Yeasts in Food: Beneficial and Detrimental Aspects. Behr's Verlag, Hamburg, pp. 69-117.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeasts and its importance to wine aroma - a review. South African Journal of Enology and Viticulture 21, 97-129.

Lazano-Calero, D., Martin-Palomeque, P.I., Madueno-Loriguillo, S., 1996. Phosphorus in cola drinks. Journal of Chemical Education 73, 1173-1174.

Leakey, R.R.B., 1999. Potential for novel food products from agroforestry trees: a review. Food Chemistry 66, 1-14.

Lee, Y.-K., Salminen, S., 1995. The coming age of probiotics. Trends in Food Science and Technology 6, 241-244.

Lee, Y.J., Burlet, E., Galiano, F., Circu, M.L., Aw, T.Y., Williams, B.J., 2011. Phosphate and succinate use different mechanisms to inhibit sugar-induced cell death in yeast. The Journal of Biological Chemistry 286, 20267-20274.

Leisner, J.J., Pot, B., Christensen, H., Rusul, G., Olsen, J.E., Wee, B.W., Muhammad, K., Ghazali, H.M., 1999. Identification of lactic acid bacteria from Chili Bo, a Malaysian food ingredient. Applied and Environmental Microbiology 65, 599-605.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science and Technology 15, 67-78.

Lewicki, P.P., 1998. Effect of pre-drying treatment, drying and rehydration on plant tissue properties: A review. International Journal of Food Properties 1, 1-22.

Li, B.Q., Tian, S.P., 2007. Effect of intracellular trehalose in *Cryptococcus laurentii* and exogenous lyoprotectants on its viability and biocontrol efficacy on *Penicillium expansum* in apple fruit. Letters in Applied Microbiology 44, 437-442.

Lockett, C., Calvet, C.C., Grivetti, L.E., 2000. Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, Northeastern Nigeria. International Journal of Food Sciences and Nutrition 51, 195 - 208.

Lopes, C., Rodríguez, M.I., Sangorrín, M., Querol, A., Caballero, A.C., 2007. Patagonian wines: the selection of an indigenous yeast starter. Journal of Industrial Microbiology and Biotechnology 34, 539-546.

Lurton, L., Snakkers, G., Roulland, C., Galy, B., 1995. Influence of the fermentation yeast strain on the composition of wine spirits. Journal of the Science of Food and Agriculture 67, 485-491.

Madovi, P.B., 1981. Food handling in Shona villages of Zimbabwe. Ecology of Food and Nutrition 1, 133- 144.

Maghembe, J., Seyani, J. (1992). Multipurpose trees used by small-scale farmers in Malawi. Results of an ethnobotanical survey. AFRENA report No. 40. Nairobi: ICRAF.

Mahapatra, A. K., Mishra, S., Basak, U. C., Panda, P. C., 2012. Nutrient analysis of some selected wild edible fruits of deciduous forests of India: an explorative study towards non conventional bio-nutrition. Advanced Journal of Food Science and Technology 4, 15-21.

Mallouchos, A., Komaitis, M., Koutinas, A., Kanellaki, M., 2002. Investigation of volatiles evolution during the alcoholic fermentation of grape must using free and immobilized cells with the help of solid phase microextraction (SPME) headspace sampling. Journal of Agricultural and Food Chemistry 50, 3840-3848.

Mallouchos, A., Komaitis, M., Koutinas, A., Kanellaki, M., 2003. Evolution of volatile byproducts during wine fermentations using immobilized cells on grape skins. Journal of Agricultural and Food Chemistry 51, 2402-2408.

Maposa, M., Chisuro, D. (1998). Importance of *Ziziphus mauritiana* (*masau*) in the Mukumbura area of Zimbabwe- From a farmer's and extensionist's point of view. In: International Workshop on Ziziphus mauritiana. Harare, Zimbabwe.

Martini, A., Ciani, M., Scorzetti, G., 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. American Journal of Enology and Viticulture 47, 435-440.

Mazzobre, M.F., Lodato, P., De Huergo, M.S., Buera, M.P., Chirife, J., 1999. The performance of maltodextrins as protective excipients in freeze-dried biomaterials subjected to thermal treatment, in comparison with trehalose systems. In: Colonna, P., Guilbert, S. (Ed.), Biopolymer Science: Food and Non Food Applications. Colloques de I'INRA, pp. 321-325.

Mensah, P., 1997. Fermentation - the key to food safety assurance in Africa? Food Control 8, 271-278.

Miller, N., Wetterstrom, W., 2000. The beginnings of agriculture: the ancient near East and North Africa. In: Kiple, K.F., Ornelas, K.C. (Ed.), Cambridge World History of Foods. Cambridge University Press, Cambridge, pp. 1123-1139.

Mithofer, D., 2003. Income and labour productivity of collection and use of indigenous fruit tree products in Zimbabwe. Agroforestry Systems 59, 295-305.

Mithofer, D., Waibel, H., Akinnifesi, F.K. (2006). The role of food from natural resources in reducing vulnerability to poverty: a case study from Zimbabwe. In: Proceedings of the German development economic conference, vol. 28. Berlin: Research Committee Development Economics.

Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., Nakahara, T., 2006. Survival curves for microbial species stored by freeze-drying. Cryobiology 52, 27-32.

Moll, E.J., 1997. Trees of Southern Africa. C. Struik Publishers, Cape Town, Johannesburg.

Morton, J.F., 1987. Indian jujube. In: Morton, J.F. (Ed.), Fruits of Warm Climates. Miami, Florida, pp. 272-275.

Mpofu, A., Zvauya, R., 2002. Microbial and biochemical changes occurring during fermentation of *marula* (*Sclerocarya birrea* subspecies *caffra*) juice to produce mukumbi, a traditional Zimbabwean wine. Advances in Food Sciences 24, 116-120.

Muchuweti, M., Zenda, G., Ndlala, A.R., Kasiamhuru, A., 2005. Sugars, organic acids and phenolic compounds of *Ziziphus mauritiana* fruit. European Food Research and Technology 221, 570 - 574.

Mwamba, C.K., 1989. Natural variation in fruits of *Uapaca kirkiana* in Zambia. Forest Ecology and Management 26, 299-303.

Mwesigye, P.K., Okurut, T.O., 1995. A survey of the production and consumption of traditional Alcoholic beverages in Uganda. Process Biochemistry 30, 497-501.

Naggy, S. (1980). Vitamin C contents of citrus fruits and their product: a review. Journal of Agricultural and Food Chemistry, 28, 8-18.

Ndabikunze, B. K., Masambu, B. N., & Tiisekwa, B. M. (2010). Vitamin C and mineral contents, acceptability and shelf life of juice preparation from four indigenous fruits of the Miombo woodlands of Tanzania. Journal of Food, Agriculture and Environment 8, 91-96.

Nielsen, S.S., 1998. Food Analysis Second Edition. A Chapman and Hall Food Science Title, Aspen Publishers, Inc., Gaihersburg, Maryland.

Nout, M.J.R., 1979. The manufacture and composition of *Changa'a* (Nubian gin). Lebensmittel Wissenschaft und Technologie 12, 212-216.

Nout, M.J.R., 2003. Traditional fermented products from Africa, Latin America and Asia. In: Boekhout, T., Robert, V. (Ed.), Yeasts in Foods: Beneficial and Detrimental Effects. B. Behr's Verlag GmbH and Co., Hamburg, pp. 451-473.

Nout, M.J.R., 2005. Food Fermentation: an introduction. In: Nout, M.J.R., De Vos, W.M., Zwietering, M.H. (Ed.), Food Fermentation. Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 13-18.

Nout, M.J.R., Aidoo, K.E., 2002. Asian fungal fermented food. In: Osiewacz, H.D. (Ed.), The Mycota. Vol. X "Industrial applications". Springer-Verlag, Berlin, pp. 23-47.

Nowakowska-Waszczuk, A., Sokolowski, A., 1987. Application of carbon balance to submerged citric acid production by *Aspergillus niger*. Applied Microbiology and Biotechnology 26, 363-364.

Nyanga, L.K., Nout, M.J.R., Gadaga, T.H., Boekhout, T., Zwietering, M.H., 2008. Traditional processing of *masau* fruits (*Ziziphus mauritiana*) in Zimbabwe. Ecology of Food and Nutrition 47, 95-107.

Nyanga, L.K., Nout, M.J.R., Gadaga, T.H., Theelen, B., Boekhout, T., Zwietering, M.H., 2007. Yeasts and lactic acid bacteria microbiota from *masau* (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. International Journal of Food Microbiology 120, 159-166.

O'Donnel, K.E., Cigelnik, N.S., Weber, N.S., 1997. Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequences analysis. Mycologia 89, 48-65.

Ohiokpehai, O., 2003. Promoting the nutritional goodness of traditional food products. Pakistan Journal of Nutrition 2, 267-270.

Okagbue, R.N., 1995. Microbial biotechnology in Zimbabwe: current status and proposals for research and development. Journal of Applied Science in Southern Africa 1, 148-158.

Okagbue, R.N., Mwenje, E., Kudanga, T., Siwela, M., Sibanda, T., 2001. Isolation of *Aureobasidium pullulans* from Zimbabwean sources and glucosidase activities of selected isolates. South African Journal of Botany 67, 157-160.

Okagbue, R.N., Siwela, M., 2002. Yeasts and related microorganisms isolated from ripe marula fruits (*Sclerocarya caffra*). South African Journal of Science 98, 551-552.

Osman, M. A. (2004). Chemical and nutrient analysis of baobab (*Adansonia digitata*) fruit and seed protein solubility. Plants for Human Nutrition 59, 29-33.

Oyewole, O.B., 1997. Lactic fermented foods in Africa and their benefits. Food Control 8, 289-297.

Palomo, E.S., Diaz-Maroto, M.C., Vinas, M.A.G., Soriano-Perez, A., Perez-Coello, M.S., 2007. Aroma profile of wines from Albillo and Muscat grape varieties at different stages of ripening. Food Control 18, 398-403.

Pattison, D.J., Silman, A.J., Goodson, N.J., Lunt, D., Bunn, R., Luben, A., Welch, A., Bingham, S., Khaw, K.T., Day, N., Symmons, D.P., 2004. Vitamin C and the risk of developing inflammatory polyarthritis, prospective nested case control study. Annals of the Rheumatic Diseases 63, 843-847.

Paul, G., Pearson, W.N., 1967. The Vitamins: Chemistry, Physiology, Pathology, Methods. Academic Press, New York, USA.

Piskur, J., Langkjaer, R.B., 2004. Yeast genome sequencing: the power of comparative genomics. Molecular Microbiology 53, 381-389.

Rathore, M., 2009. Nutrient content of important fruit trees from arid zone of Rajasthan. Journal of Horticulture and Forestry 1, 103 - 108.

Roels, J.A., 1983. Energetics and Kinetics in Biotechnology. Elsevier Biomedical, New York.

Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. International Journal of Food Microbiology 70, 283-289.

Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. International Journal of Food Microbiology 86, 181-188.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A., 2003. Function of yeast species and strains in wine flavour. International Journal of Food Microbiology 86, 169-180.

Rosini, G., Federici, F., Martini, A., 1982. Yeast flora of grape berries during ripening. Microbial Ecology 8, 83-89.

Ruas-Madiedo, P., Hugenholtz, J., Zoon, P., 2002. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. International Journal of Food Microbiology 12, 163-171.

Ruiz-Rodríguez, B., Morales, P. Fernández-Ruiz, V., Sánchez-Mata, M., Cámara, M., Díez-Marqués, C., Pardo-de-Santayana, M., Molina, M., & Tardío, J. (2011). Valorization of wild strawberry-tree fruits (*Arbutus unedo* L.) through nutritional assessment and natural production data. Food Research International 44, 1244-1253.

Saka, K.J.D., Msonthi, J.D., 1994. Nutritional value of edible fruits of indigenous wild trees in Malawi. Forest Ecology and Management 64, 245-248.

Saka, K.J.D., Rapp, I., Akinnifesi, F.K., Ndolo, V., Mhango, J., 2007. A comparative study of the physicochemical and organoleptic characteristics of *Uapaca kirkiana*, *Strychnos cocculoides*, *Adansonia digita* and *Mangifera indica* products. International Journal of Food Science and Technology 42, 836 - 841.

Sandine, W.E., 1996. Commercial production of dairy starter cultures. In: Cogan, T.M., Accolas, J.P. (Ed.), Dairy starter cultures. Wiley-VCH, New York.

Sanni, A.I., 1993. The need for process optimisation of African fermented foods and beverages. International Journal of Food Microbiology 18, 85-95.

Sanni, A.I., Lonner, C., Makinder, I., Johasson, M.L., Molin, G., 1994. Starter cultures for the production of *ogi*, a fermented infant food from maize and sorghum. Chemie Mikrobiologie Technologie der Lebensmittel 16, 29-33.

Sefa-Dedeh, S., Sanni, A.I., Tetteh, G., Sakyi-Dawson, E., 1999. Yeasts in the traditional brewing of pito in Ghana. World Journal of Microbiology & Biotechnology 15, 593-597.

Siebenhandl, S., Lestario, L.N., Trimmel, D., Berghofer, E., 2001. Studies on Tape Ketan - an Indonesian fermented rice food. International Journal of Food Science and Nutrition 52, 347-357.

Soyer, Y., Koca, N., Karadeniz, F., 2003. Organic acid profile of Turkish white grapes and grape juices. Journal of Food Composition and Analysis 16, 629-636.

Spadaro, D., Ciavorella, A.A., Lopez-Reyes, J.G., Garibaldi, A., Gullino, M.L., 2010. Effect of culture age, protectants, and initial cell concentration on viability of freeze-dried cells of *Metschnikowia pulcherrim*a. Canadian Journal of Microbiology 56, 809-815.

Steger, C.L.C., Lambrechts, M.G., 2000. The selection of yeast strains for the production of premium quality South African brandy base products. Journal of Industrial Microbiology & Biotechnology 24, 431-440.

Steinkraus, K.H., 1996. Handbook of Indigenous Fermented Foods. Marcel Dekker, Inc, New York.

Swinnen S., K. Schaerlaekens, T. Pais, J. Claesen, G. Hubmann, Y. Yang, M. Demeke, M.R. Foulquié-Moreno, A. Goovaerts, K. Souvereyns, L. Clement, F. Dumortier and J.M. Thevelein 2012. Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. Genome Research 22, 975-984

Tan, C.S., van Ingen, C.W., Stalpers, J.A., 2007. Freeze-drying fungi using a shelf freeze-drier. cryopreservation and freeze-drying protocols. Methods in Molecular Biology 368, 119 -125.

Tembo, L., Chiteka, Z.A., Kadzere, I., Akinnifesi, F.K., Tagwira, F., 2008a. Blanching and drying period affect moisture loss and vitamin C content in *Ziziphus mauritiana* (Lamk.). African Journal of Biotechnology 7, 3100 - 3106.

Tembo, L., Chiteka, Z.A., Kadzere, I., Akinnifesi, F.K., Tagwira, F., 2008b. Storage temperature affects fruit quality attributes of Ber (*Ziziphus mauritiana* Lamk.) in Zimbabwe. African Journal of Biotechnology 7, 3092 - 3099.

Tredgold, M.H., 1986. Food plants of Zimbabwe. Mambo Press, Gweru.

Trinh, T.T.T., Yu, B., Curran, P., Liu, S.Q., 2011. Dynamics of volatile compounds during Longan juice fermentation by three yeasts from the genus *Williopsis*. Acta Alimentaria 40, 385-395.

Umaru, H.A., Adamu, R., Dahiru, D., Nadro, M.S., 2007. Levels of antinutritional factors in some wild edible fruits of Nothern Nigeria. African Journal of Biotechnology 6, 1935-1938.

Van der Westhuizen, T.J., Augustyn, O.P.H., Khan, W., Pretorius, I.S., 2000. Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa. South African Journal of Enology and Viticulture 21, 10-16.

Von Maydell, H.-J., 1986. Trees and shrubs for the Sahel, their characteristics and uses. Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ), Federal Republic of Germany.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407-4414.

Wiemken, A., 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie van Leeuwenhoek 58, 209-217.

Wijtzes, T., Bruggeman, M.R., Nout, M.J.R., Zwietering, M.H., 1997. A computerised system for the identification of lactic acid bacteria. International Journal of Food Microbiology 38, 65-70.

Yang, J., Bae, J.Y., Lee, Y.M., Kwon, H., Moon, H.Y., Kang, H.A., Yee, S.B., Kim, W., Choi, W., 2011. Construction of *Saccharomyces cerevisiae* strains with enhanced ethanol tolerance by mutagenesis of the TATA-binding protein gene and identification of novel genes associated with ethanol tolerance. Biotechnology and Bioengineering 108, 1776-1787.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., Masneuf-Pomarede, I., 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stage in winemaking. International Journal of Food Microbiology 125, 197-203.

SUMMARY

Food fermentation is one of the oldest 'biotechnology' processes which is used in food production for preservation, improvement of flavour and texture, digestibility and nutritional enrichment. Examples of fermented foods are yoghurt, cheese, tempe, beer, sausages, bread and sauerkraut. During fermentation microorganisms modify biological material, resulting in a desirable product. Several indigenous fruits are traditionally fermented into beverages at household level in Zimbabwe. These include *Ziziphus mauritiana*, *Uacapa kirkiana, Parinari curatellifolia* and *Sclerocarya caffra* fruits. The indigenous fruits are naturally fermented under uncontrolled conditions and the quality of the beverages produced is variable. In this thesis, the microbiological and biochemical properties of *masau* fruit and its fermented fruit pulp were described.

Information on the traditional processing techniques of *masau* fruits was gathered and documented in Chapter 2. A survey was conducted using a semi-structured questionnaire and focus group discussions in each of the three districts, i.e., Mudzi, Mt Darwin and Muzarabani in Zimbabwe. The survey revealed that *masau* fruits form part of the family diet and generate additional income by selling at local markets. The people distinguish the fruit into sour and sweet varieties. The sweet-sour taste of *masau* fruits was attributed to the presence of glucose, fructose, citric acid, malic, oxalic and succinic acids. Surplus fruits are sun dried and can be transformed into various products such as porridge, traditional cakes, *mahewu* and are also fermented to produce a spirit called *kachasu*. The ethanol content of the fermented fruit pulp ranged from 2.1 – 3.7 ml·100 ml^{-1}, whereas the traditionally made distillate contained 23.8 – 45.6 ml·100 ml^{-1}.

Masau fruits are valuable sources of nutrients. Macronutrient and some micronutrient contents of the *masau* fruit were analysed in Chapter 3. Dry matter content ranged from 21.1±0.23 to 24.1±0.34 g·100g^{-1} of edible portion of the sweet and sour fruits, and 84.8±0.21 to 87.2± 0.24 g·100g^{-1} for the dried fruit. Crude protein per 100 g edible portion of dry weight ranged between 7.9±0.02 and 8.7±0.02 g, crude fat from 0.8±0.02 to 1.5±0.02 g, crude fibre from 4.9±0.02 to 7.3±0.02 g, ash between 3.0±0.01 and 4.3±0.02 g and finally carbohydrate between 79.5±0.03 and 83.2±0.03 g. The fruits were rich in vitamin C (15.0±0.02 – 43.8±0.02 mg·100 g^{-1}) and the energy values ranged between 1516.0±1.73 and 1575.0±2.33 kJ 100g^{-1}. Furthermore, the fruits contained (mg·100$g^{-1}$of dry weight) potassium from 1865.0±1.31 to 2441.0±1.14, calcium from 160.0±0.31 to 254.0±0.14, sodium between 185.0±0.12 and 223.0±0.23, magnesium between 83.0±0.04 and 150.0±0.13 and phosphorous from 87.0±0.14 to 148.0±0.52. Manganese and copper contents ranged between 0.7±0.03 and 1.6±0.03, while iron and zinc ranged between 2.1±0.43 and 4.3±0.12, and 0.6±0.01 – 0.9±0.01 mg·100$g^{-1}$of dry weight, respectively.

In Chapter 4, yeasts, yeast-like fungi, and lactic acid bacteria present on the unripe, ripe and dried fruits, and in the fermented *masau* fruits collected from Muzarabani district were isolated and identified using physiological and molecular methods. The predominant species were identified as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *P. fabianii* and *Aureobasidium pullulans*. *Aureobasidium pullulans* was the dominant species on the unripe fruits but was not isolated from the fermented fruit pulp. *S. cerevisiae* and *P. kudriavzevii* were predominant in the fermented fruit pulp but were not detected on the unripe fruits. The less predominant species included *Candida glabrata*, *C. magnus*, *C. parapsilosis*, *C. pyralidae, P.*

ciferrii, *Saccharomycopsis fibuligera*, *Hanseniaspora opuntiae*, *Zygoascus hellenicus*, *Cryptococcus flavus*, and *Rhodotorula mucilaginosa*. Lactic acid bacteria from fruits and pulp were preliminary identified and the predominant species found were *Lactobacillus agilis* and *L. plantarum*. Other species identified included *L. bifermentans*, *L. minor*, *L. divergens*, *L. confusus*, *L. hilgardii, L. fructosus*, *L. fermentum* and *Streptococcus* spp.

In Chapter 5, yeast isolates belonging to *S. cerevisiae*, *P. kudriavzevii*, *Sm. fibuligera*, *P. fabianii, Z. hellenicus*, *C. parapsilosis*, *C. glabrata, C. pyralidae*, *P. ciferrii*, *H. opuntiae* and *C. glabrata* isolated from traditionally fermented *masau* fruit pulp and the fruits (fresh and dried) were characterised with respect to the formation of ethanol and flavour compounds, and their tolerance to ethanol. The yeasts were examined for their ability to ferment glucose and fructose using standard broth under aerated and non-aerated conditions. Most *S. cerevisiae* strains were superior to other species in ethanol production. The best ethanol producing *S. cerevisiae* strains, and strains of the species *P. kudriavzevii*, *Sm. fibuligera* and *P. fabianii* were tested for production of flavour compounds during fermentation of *masau* fruit juice. Significant differences in the production of ethanol and other volatile compounds during fermentation of *masau* juice were observed among and within the tested four species. Alcohols and esters were the major volatiles detected in the fermented juice. Trace amounts of organic acids and carbonyl compounds were detected. Ethyl hexanoate and ethyl octanoate were produced in highest amounts as compared to the other volatile compounds.

Production and storage of starter cultures for use in traditionally fermented foods is a great challenge since standard methods such as lyophilisation cannot be applied due to economic constraints and low-tech infrastructure in the developing world. Hence, in Chapter 6, a comparative study on the effect of two low-tech traditional methods for drying starter cultures and standard lyophilisation was done. Strains of *S. cerevisiae, P. kudriavzevii* and *Sm. fibuligera* that are potential starter cultures for the production of *masau* wine and distillate were used in this experiment. Lyophilised and dry rice cakes preserved yeast cultures were examined for viable cell counts during six months storage at 4°C and 25°C. Dry plant fibre preserved yeast cultures were stored at 25°C. None of the yeast cultures showed a significant loss in viable cell count during six months of storage at 4°C upon lyophilisation and preservation in dry rice cakes. During storage at 25°C in the dark, yeast cultures preserved in dry rice cakes, and lyophilised cultures of *S. cerevisiae* and *P. kudriavzevii* showed no significant loss of viable cells up to four months of storage. The rice cakes performed significantly better than lyophilisation. Yeast cultures preserved in dry plant fibre strands suffered the greatest loss of viable count during the six months of storage at 25°C.

In conclusion, *masau* fruits are of social, economic and nutritional significance in Zimbabwe. The traditional fermentation of the fruit can potentially be improved by application of starter cultures obtained from the fruit and its fermented pulp to produce beverages. These cultures include strains of *S. cerevisiae* and non-*Saccharomyces* yeast species such as *P. kudriavzevii, P. fabianii* and *Sm. fibuligera*. LAB strains were not characterised in this study but they could be of interest in the production of *masau* fruit products. This study demonstrates that traditional approaches can be useful as starter culture preservation methods in small-scale, low-tech applications.

SAMENVATTING

Fermentatie wordt van oudsher gebruikt voor de conservering en verbetering van aroma, consistentie, verteerbaarheid en voedingswaarde van voedsel. Voorbeelden van gefermenteerde voedingsmiddelen zijn yoghurt, kaas, tempé, bier, rauwe worsten, brood en zuurkool. Tijdens de fermentatie worden door micro-organismen voedselbestanddelen omgezet waardoor een aantrekkelijk product ontstaat. In Zimbabwe worden diverse inheemse vruchten zoals *Ziziphus mauritiana* (*masau*), *Uacapa kirkiana, Parinari curatellifolia* en *Sclerocarya caffra* in de huishouding gefermenteerd tot drankjes. Deze natuurlijke fermentaties leiden tot wisselende kwaliteit van de eindproducten. In dit proefschrift worden de microbiologische en biochemische eigenschappen van *masau*-vruchten en hun gefermenteerde moes beschreven.

In hoofdstuk 2 wordt een overzicht gegeven van de traditionele verwerking van *masau*-vruchten in drie districten in Zimbabwe, nl. Mudzi, Mt Darwin en Muzarabani, resultaat van veldwerk door middel van vragenlijsten en groepsdiscussies. Naar bleek, is *masau* onderdeel van de gezinsvoeding en op de plaatselijke markten wordt geld verdiend met de verkoop van *masau*. Men onderscheidt zure en zoete *masau*-vruchtvarianten. De zoet-zure smaak kan worden toegeschreven aan de aanwezigheid van de suikers glucose en fructose, en de organische zuren citroenzuur, appelzuur, oxaalzuur en barnsteenzuur. Overschotten van *masau* vruchten worden in de zon gedroogd en er worden pap, cakejes, *mahewu* en gefermenteerde moes van gemaakt. Van de laatste wordt de sterke drank *kachasu* gedestilleerd. Het alcoholgehalte van gefermenteerde moes is 2,1 – 3,7 volume %, en van kachasu 23,8 – 45,6 volume %.

Masau-vruchten zijn voedzaam. In hoofdstuk 3 wordt de nutriëntenanalyse beschreven. Het eetbare gedeelte van verse zoete en zure vruchten bevatte per 100 g respectievelijk 21,1±0,23 – 24,1±0,34 g·droge stof, en gedroogde vruchten 84,8±0,21 – 87,2± 0,24 g. Verder bevatten de vruchten per 100 g eetbare droge stof: ruw eiwit 7,9±0,02 – 8,7±0,02 g, ruw vet 0,8±0,02 – 1,5±0,02 g, ruw vezel 4,9±0,02 – 7,3±0,02 g, as 3,0±0,01 – 4,3±0,02 g, koolhydraten 79,5±0,03 – 83,2±0,03 g, veel vitamine C 15,0±0,02 – 43,8±0,02 mg, energie 1516,0±1,73 – 1575,0±2,33 kJ. De volgende mineralen werden aangetoond: kalium 1865,0±1,31 – 2441,0±1,14 mg, calcium 160,0±0,31 – 254,0±0,14 mg, natrium 185,0±0,12 – 223,0±0,23 mg, magnesium 83,0±0,04 – 150,0±0,13 mg, fosfor 87,0±0,14 – 148,0±0,52 mg, mangaan en koper beiden 0,7±0,03 – 1,6±0,03 mg, ijzer 2,1±0,43 – 4,3±0,12 mg, en zink 0,6±0,01 – 0,9±0,01 mg.

Hoofdstuk 4 beschrijft de isolatie en identificatie m.b.v. fysiologische en moleculaire methoden van gisten, schimmels en melkzuurbacteriën die werden aangetroffen op onrijpe, rijpe en gedroogde vruchten, en in de gefermenteerde *masau*-moes uit Muzarabani district. *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Pichia fabianii* en *Aureobasidium pullulans* waren veel voorkomende gisten. De laatste soort overheerste op onrijpe vruchten maar was afwezig in gefermenteerde moes. *S. cerevisiae* en *P. kudriavzevii* zaten echter vooral in gefermenteerde moes, maar niet op onrijpe vruchten. In mindere mate aanwezig waren de gistsoorten *Candida glabrata*, *C. parapsilosis*, *C. pyralidae, P. ciferrii*, *Saccharomycopsis fibuligera*, *Hanseniaspora opuntiae*, *Zygoascus hellenicus*, *Cryptococcus flavus*, *Cr. magnus*, en *Rhodotorula mucilaginosa*. Melkzuurbacteriën van vruchten en moes betroffen voornamelijk *Lactobacillus agilis* en *L. plantarum,* en in mindere mate *L. bifermentans*, *L. minor*, *L. divergens*, *L. confusus*, *L. hilgardii, L. fructosus*, *L. fermentum* en *Streptococcus* spp.

Hoofdstuk 5 beschrijft de productie van alcohol en aroma, alcoholresistentie, en het vermogen glucose en fructose te assimileren en fermenteren van een aantal gisten uit gefermenteerde pulp en verse en gedroogde vruchten. Het betrof stammen van de soorten *S. cerevisiae*, *P. kudriavzevii*, *Sm. fibuligera*, *P. fabianii, Z. hellenicus*, *C. parapsilosis*, *C. glabrata, C. pyralidae*, *P. ciferrii* en *H. opuntiae.* De meeste *S. cerevisiae* stammen produceerden meer alcohol dan de overige soorten. De beste alcoholvormende *S. cerevisiae* stammen en ook stammen van *P. kudriavzevii*, *Sm. fibuligera* en *P. fabianii* werden onderzocht naar de vorming van aromabestanddelen in *masau*-vruchtensap. Significante verschillen in alcohol en aromavorming werden waargenomen tussen, maar ook binnen de onderzochte soorten. De belangrijkste vluchtige aromastoffen waren alcoholen en esters, maar er werden ook sporen van organische zuren en carbonylverbindingen gedetecteerd. De hoogste concentraties vluchtige aromastoffen betroffen de esters ethylhexanoaat en ethyloctanaat.

Fabricage en bewaring van startercultures voor gebruik in traditionele voedingsmiddelenfermentaties is in ontwikkelingslanden een uitdaging, gezien de minder ontwikkelde technische infrastructuur en de hoge kosten van vriesdroogapparatuur die in meer ontwikkelde landen vaak wordt gebruikt. In hoofdstuk 6 wordt een vergelijking gemaakt van de gangbare vriesdroogmethode voor de conservering van startercultures, met twee eenvoudige – uit andere ontwikkelingslanden geïnspireerde – droogmethoden. Potentieel bruikbare startercultures van de stammen *S. cerevisiae, P. kudriavzevii* en *Sm. fibuligera* werden gekweekt en gedroogd op drie manieren (vriesdrogen, gedroogd in rijsttabletjes, en gedroogd op plantenvezel), waarna hun overleving werd onderzocht tijdens 6 maanden bewaring. Gevriesdroogde en in rijsttabletjes gedroogde starters werden in het donker bewaard bij 4°C en 25°C, en op plantenvezel gedroogde starters alleen bij 25°C. Bij 4°C trad bij geen van de starters vermindering van overleving op binnen 6 maanden. Bij 25°C konden zowel gevriesdroogde als in rijsttabletjes gedroogde *S. cerevisiae* en *P. kudriavzevii* 4 maanden zonder een significant verlies van vitaliteit worden bewaard, waarbij de rijsttabletjes zelfs betere overleving gaven dan vriesdrogen. Op plantenvezel gedroogde gisten gingen tijdens de 6 maanden bij 25°C het sterkst achteruit.

Concluderend kan gesteld worden dat *masau*-vruchten in maatschappelijk, economisch en voedingskundig opzicht van belang zijn in Zimbabwe. Hun traditionele fermentatie kan worden verbeterd door toepassing van startercultures, zoals *S. cerevisiae* en andere soorten zoals *P. kudriavzevii, P. fabianii* en *Sm. fibuligera*. Melkzuurbacteriën werden in dit onderzoek niet verder gekarakteriseerd, maar hebben wellicht een toekomst in de productie van gefermenteerde *masau*-producten. Dit onderzoek laat tevens zien dat in ontwikkelingslanden gangbare kleinschalige en eenvoudige methoden voor de conservering van startercultures niet per se onder hoeven te doen voor meer geavanceerde technieken.

ACKNOWLEDGEMENTS

My PhD has been a remarkable journey and an experience of lifetime. It wasn't an easy road but today, I say EBENEZER!! The challenges I faced developed me academically and as a person. To mention a few, I learnt to persevere, to be patient, innovative and to live each day as it comes.

I would like to express my sincere gratitude to everyone who walked with me in this remarkable journey.

Dr Gadaga and Dr Mutukumira you inspired me to embark on this PhD journey, thank you. Dr Gadaga, I am grateful for the daily supervision, encouragement and the support you gave me.

Assoc. Prof. R. Nout, I am greatly indebted to you. You made the completion of this thesis possible and you also secured funding. You encouraged me, you sharpened my intellectual skills and you were very patient with me. It took me longer than usual to complete this journey but you continued with your excellent and untiring enthusiastic guidance. My sincere gratitude goes to you and Frances, your wife, for the hospitality the times I visited you at your home. Frances you were my first port of call when I broke my leg.

Prof. M. Zwietering, your constructive criticism and comments were valuable to the completion of this thesis. Thank you.

Dr. T. Boekhout thank you for your untiring and excellent supervision throughout this project.
Assoc. Prof. E. Smid you came in during my last lap of this thesis but you indeed added great value to my work. I am thankful for the strong suggestions and discussions.

My colleagues, Augustine, Xiaowei, Jianfen, Lidia, Polycarpe and Diah, I greatly appreciate your friendship during my PhD journey.

To Bart, Birgit, Ingrid, Judith, Power, Evidence and Evan, thank you for your technical support. You were there whenever I needed your help.

To Farie, Memo and Cathe you stood in for me when I could not take my lectures. Thank you. Farie I am also grateful for your support, you read through my draft thesis and the propositions.

Staff members and colleagues at IFNFS and from other Departments, Faculty of Science, University of Zimbabwe, and Laboratory of Food Microbiology, Wageningen University, thank you for the wonderful support during my PhD journey.

Gerda thank you for the administrative work.

To Farai, Busi and kids, I depended so much on you. Thank you for your kindness, prayers and being there for me each time I came to the Netherlands and when I broke my leg. I indeed counted on you.

To Elton, Ednah and kids, you are kind-hearted, you accepted me in your home the time when I could not do much for myself.

Letty, thank you for everything you did for me. You were more than a friend to me.
Munashe, Nathan, Gamu, Orlin and Taka, I greatly appreciate the times you visited me in Holland. I cherish those moments.

To my fellow Zimbabweans whom I met during my PhD journey you made a difference in my life in Dutch land. The times we spent together were great.

To my parents, I count on your love, prayers, support and encouragement in my life as a whole. You gave me a wonderful foundation. Thank you.

To the wonderful maids, Patricia and the late Alice, thank you for your tender love, taking care of my children during my absence.

My sisters, brothers, my dear in-loves, friends, relatives, Amazing Grace Parish and Waterfalls Corps, thank you for your wonderful support and encouragement. I can finally answer your question, I have finished *chikoro chacho.*

Ernie my love, it was not easy for you and me. We went through difficult times. I thank you because I finally made it. Your took care of our kids at such a tender age, never told me when they were sick, spoiled them with pizza and beautiful gifts. I have depended on you and I still depend on you. Your excellent communications with Rob and the printing of drafts. That was great! Your confidence in me and your great love made it possible for me to successfully complete this chapter of life.

To Una, El, Tadie and baby Kuku Adiel, you are great and excellent. Continue with the excellence!! I will always cherish you!

Stay Blessed!
Loveness Kuziwakwashe

List of publications

Peer-reviewed papers

Nyanga, L. K., Nout, M. J. R., Smid, E. J., Boekhout, T. and Zwietering, M. H. 2012. Yeasts preservation: alternative for lyophilisation. World Journal of Microbiology and Biotechnology, **In Press**, DOI 10.1007/s11274-012-1118-y.

Nyanga, L. K., Nout, M. J. R., Gadaga, T. H., Boekhout, T. Zwietering, M. H. 2008. Traditional processing of *masau* fruits (*Ziziphus mauritiana*) in Zimbabwe. Ecology of Food and Nutrition, 47, 95-107.

Nyanga, L. K., Nout, M. J. R., Gadaga, T. H., Theelen, B., Boekhout, T., Zwietering, M. H.. 2007. Yeasts and lactic acid bacteria microbiota from *masau* (*Ziziphus mauritiana*) fruits and their fermented fruit pulp in Zimbabwe. International Journal of Food Microbiology, 120, 159-166.

Ndlovu, P. D., Mduluza, T., Kjetland, E. F., Midzi, N., **Nyanga, L**. **K**., Gundersen, S. G., Friis, H. and Gomo, E. 2006. Prevalence of urinary schistosomiasis and HIV in females living in rural community of Zimbabwe: does age matter? Transaction of the Royal Society of Tropical Medicine and Hygiene, 101, 433-438.

Vogel, R. F. and **Nyanga, L. K.** 2005. Sourdough Fermentation. In Food Fermentation ed. Nout, M. J. R., de Vos, W. M. and Zwietering, M. H., pp 53-60. Wageningen Academic Publishers, the Netherlands. ISBN 9076998833.

Gadaga, T. H., **Nyanga, L.K**. and Mutukumira A. N. 2004. The occurrence, growth and control of pathogens in African fermented foods. African Journal of Food, Agriculture, Nutrition and Development, 4:1-13.

Midzi, N., Ndlovu, P. D., **Nyanga, L. K.,** Kjetland, E. F., Reimert, C. M., Vennervald, D., Mudenge, G., Friis, H., Gundersen, S. G., and Mduluza, T. 2003. Assessment of eosinophil cationic protein as a possible diagnostic marker for female genital schistosomiasis in women living in a *Schistosoma haematobium* endemic area. Parasitology Immunology, 25:581-588.

Papers submitted

Nyanga, L. K., Nout, M. J. R., Smid, E. J., Boekhout, T. and Zwietering, M. H. Nutritive values of *masau* (*Ziziphus mauritiana*) fruits from Zambezi valley in Zimbabwe.

Nyanga, L. K., Nout, M. J. R., Gadaga, T. H., Smid, E.J., Boekhout, T. and Zwietering, M. H. Fermentative characteristics of yeasts isolated from *masau* (*Ziziphus mauritiana*) fruits and their traditionally fermented fruit pulp in Zimbabwe.

Non peer-reviewed scientific papers

Nyanga, L. K., Nout, M. J. R., Smid, E. J., Boekhout, T. and Zwietering, M. H. 2012. Genotypic and phenotypic characteristics of yeasts isolated from *masau* (*Ziziphus mauritiana*) fruits and their fermented products in Zimbabwe. T*he 23rdInternational ICFMH Symposium on Global Issues In Food Microbiology,* Abstract book , Istanbul, Turkey, pp 128.

Nyanga, L. K., Nout, M. J. R., Gadaga, T. H., Boekhout, T. and Zwietering, M. H. 2006. Isolation and identification of yeasts from *masau* (*Ziziphus mauritiana*) fruits and their fermented products in Zimbabwe. T*he 20th International ICFMH Symposium on Food safety and food biotechnology: diversity and global impact,* Abstract book, Bologna, Italy, pp 250.

Curriculum Vitae

Loveness Kuziwa Nyanga (nee Manyumwa) was born on 15 December, 1973 in Nyanga, Zimbabwe. She finished her secondary school in 1993 at St David's Girls High Bonda boarding school in Mutare, Zimbabwe. In 1994 she was enrolled for undergraduate studies for Bachelor of Science general degree in Geology, Biology and Chemistry subjects at the University of Zimbabwe. During her second year she majored in Biochemistry and Biology and she graduated with BSc Honours in Biochemistry, in 1996. In 1997 to 1998 she did a Master's degree in Biotechnology at the same university. Upon completion of her Master's degree she worked as biology teacher at Queen Elisabeth school in Harare for half a year in 1999 and was then employed within the same year by the Ministry of Health and Child Welfare at the National Institute of Health Research (previously known as Blair Research Institute) as a researcher in Immunology – HIV and AIDS. She participated in the following research projects: Female genital schistosomiasis and its association with HIV, and Micronutrient supplementation for pregnant and lactating mothers: prevention of mother to child transmission. In 2001 she joined the University of Zimbabwe as a teaching assistant and was appointed as a lecturer in 2003. In March 2004, Loveness started her sandwich PhD project entitled: "*Ziziphus mauritiana* (*masau*) fruits fermentation in Zimbabwe: from black-box to starter culture development" at the laboratory of Food Microbiology, Wageningen University. The results of this thesis project are described in this thesis. Her PhD research was mainly sponsored by the Royal Netherlands Academy of Arts and Sciences (KNAW) MacGillavry Fund (Grant No. ISK/9495/MacGillavry) and partly by the International Foundation of Science (IFS Grant No. C/3737).

Overview of completed training activities

Discipline specific activities

Courses

Food Fermentation, VLAG, Wageningen, 2004
Food Perception and Food Preference, VLAG, Wageningen, 2005
Molecular Biology techniques for Yeast identification, KNAW CBS, Utrecht, 2005
Management of Microbiological Hazards in Foods, VLAG, Wageningen, 2006
Food Safety in Africa – ICFMH, Stellenbosch University, South Africa, 2007

Conferences/Meetings

Biotechnology Society of Zimbabwe, Harare, 2004-2006
Biochemistry Society of Zimbabwe, UZ, Harare, 2004-2011
Food Inspection Workshop, IFNFS, UZ, Harare, 2005
Food Safety Workshop for Catering Industry, IFNFS, UZ, Harare, 2005
The 20th International ICFMH Symposium Food Microbiology, Bologna, 2006
Exhibition Research and Intellectual Expo, UZ, Harare, 2010
Food Safety Workshop for Food Handlers, IFNFS, UZ, Harare, 2011
Sports Nutrition, IFNFS, UZ, Harare, 2011
Food Fermentation Symposium, Wageningen, 2011

General courses

Teaching and learning in Higher Education, UTLC, UZ, Harare, 2005
Wageningen UR Digital Library Introduction, 2010
Social Moulding Symposium, UZ, Harare, 2011

Other activities

Preparation of PhD research proposal, 2004
Laboratory of Food Microbiology PhD trip South Africa, 2005
Seminars Laboratory of Food Microbiology, 2004 - 2011

The research work was carried out at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands, and the Institute of Food, Nutrition and Family Sciences, Faculty of Science, University of Zimbabwe, Harare.

The research described in this thesis was funded by The Royal Netherlands Academy of Arts and Sciences (KNAW, Grant No. ISK/9495/MacGillavry) and the International Foundation for Science, Stockholm, Sweden (IFS Grant No. C/3737).

Financial support for the printing of thesis was obtained from the Dr. Judith Zwartz Foundation and Wageningen University, Wageningen, The Netherlands.

Cover picture and design by Patron printers and Loveness K. Nyanga.
Printed by GVO printers & designers B.V. | Ponsen & Looijen, Ede, The Netherlands.