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Fermentation characteristics of yeasts isolated from traditionally fermented *masau* (*Ziziphus mauritiana*) fruits

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ABSTRACT

Yeast strains were characterized to select potential starter cultures for the production of *masau* fermented beverages. 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*, *Pichia fabianii* and *Saccharomycopsis fibuligera* were tested for production of flavor 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 four tested 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. *S. cerevisiae* strains produced higher amounts of ethanol and flavor compounds as compared to the other species, especially fatty acid ethyl esters that provide the major aroma impact of freshly fermented wines. The developed library of characteristics can help in the design of mixtures of strains to obtain a specific melange of product functionalities.

1. 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* (sand apple, *hacha*), *Uapaca kirkiana* (*mazhanje*) and *Ziziphus mauritiana* (*masau*). *Masau* fruits are not only eaten raw, but also processed into products such as porridge, traditional cakes, fermented beverages and jam. The *masau* fruit ripen from mid June and are available until the end of September in Zimbabwe. The fruit is first green, turning yellow to brown as it ripens. The *masau* fruit contains glucose and fructose in equal amounts. Organic acids such as citric acid, tartaric, malic, succinic and oxalic acids are found in the fruits (Nyanga et al., 2008). Nyanga et al. (2013) reported that *masau* fruits contain nutritionally significant levels of important nutrients including minerals, fiber, crude protein and vitamin C. In Zimbabwe, *masau* fruit pulp is naturally fermented and distilled into a spirit called *kachasu* (Gadaga et al., 1999; Nyanga et al., 2007). *Kachasu* is a universal name given to spirits by the rural communities in Zimbabwe for which substrates such as wild fruits and cereals can be used. Nyanga et al. (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. Therefore, coming up with a controlled process with known ingredients might lead to the production of *masau* fruit fermented beverages such as wine and spirit. In other countries, e.g., in Malawi, beverages 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 (Maghembe and Seyani, 1992).

The use of yeast as starter cultures for wine fermentation and distillates has led to the production of wines 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 micro-organisms for use as starter cultures is the first crucial step of technology development and scale-up. Therefore, the aim of the study was to select yeast strains for potential use as starter cultures to produce *masau* fermented beverages.

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2. Materials and methods

2.1. 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 −80 °C in 300 ml l^{-1} glycerol prepared in peptone physiological saline (PPS) [NaCl 8.5 g l^{-1} (Merck, Darmstadt, Germany), and neutral peptone 1 g l^{-1} (Oxoid, Basingstoke, UK), pH = 7.2].

2.2. Preparation of inocula

The preparation of inocula was performed according to the method describe by Dung et al. (2004). Yeast cultures were incubated for two days at 30 °C in 10 ml malt extract broth. Next, suspensions of 10^8 cells ml^{-1} were made in sterile PPS as checked by microscopic counts.

2.3. 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 sterilized at 121 °C for 15 min. 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 30 °C for five days under non-aerated conditions achieved by replacing the cotton plug with a water lock. The other series was incubated at 30 °C under aerated conditions for five days in a shaking incubator (Innova™ 4335, Refrigerated incubator shaker, New Brunswick scientific, Nijmegen, Netherlands) at 200 rpm. At the end of the fermentation, alcohol content, residual sugar, biomass and pH were measured.

2.3.1. Determination of sugar and ethanol

Fermented standard broth was centrifuged at 10,164 ×*g* for 10 min. Protein precipitation was done by means of Carrez reagents (Barnett, 1997) as follows. An aliquot of 0.5 mL of the supernatant was deproteinated by adding 0.25 ml of Carrez A reagent (42.20 g $K_4Fe(CN)_6$ $3H_2O$ l^{-1} demineralized water) in an Eppendorf tube and vortexed well. Then 0.25 of Carrez B reagent (57.50 g $ZnSO_4$ $7H_2O$ l^{-1} of demineralized water) was added and vortexed well. The mixture was centrifuged for 5 min at 13,900 ×*g*. The supernatant was analyzed 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 × 7.8 mm^2) at an oven temperature of 40 °C 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.

2.3.2. Determination of biomass

The traditional standard method for direct determination of biomass was used as described by Li and Mira de Orduña (2010) with some modifications. The fermented broth was transferred to a pre-weighed sterile 50 ml centrifuge tube and centrifuged at 10,164 ×*g* for 10 min. The microbial pellet was washed twice with demineralized 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.

2.3.3. 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 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.

2.4. Production of volatile metabolites in masau fruit must

2.4.1. Preparation and fermentation of masau fruit must

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 2 h at 25 °C. The fruits (500 g ± 1) were weighed and crushed in a Waring blender after which 1 l of demi-water was added to thin the fruit juice and adjusted to 5.2°Brix. Equal amounts of glucose (25 g) and fructose (25 g) were added to bring the must to 12.2°Brix, pH 3.8. The must was distributed (100 g portions) into 250 ml volumetric flasks, and steam heated at 10 °C for 30 min. After cooling, an inoculum of 10^8 cells ml^{-1} was used to inoculate the must. The juice was fermented for 72 h. A non-inoculated *masau* must (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 −20 °C until further analyses. The experiment was done twice.

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

Capturing of HS volatiles by SPME was performed according to the method described by Mallouchos et al. (2002) with some modifications. Two SPME fibers (Supelco, Bellefonte, PA) [carboxen/polydimethylsiloxane, 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. We also determined the optimum fiber exposure time to the sample headspace and desorption time. 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 + 0.4 g NaCl 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 25 °C on a magnetic stirrer for 15 min. Then the fiber was exposed to the headspace for another 15 min, under the same conditions. Prior to the first analysis, the fiber was conditioned for 1 h at 270 °C in the injector port of a Thermo Scientific Trace GC Ultra gas chromatograph. For adsorption of volatiles the fiber was exposed to the headspace of sample vials for 15 min at 25 °C. For thermal desorption the needle was inserted into the injection port at 270 °C of the GC-MS system for 3 min. Prior to the next analysis, the fiber was reconditioned for 10 min at 270 °C in the injector of another Thermo Scientific Trace GC Ultra gas chromatograph to avoid carry-over of compounds between samples.

2.4.3. 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) were 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 min at 80 °C then rising to 280 °C at a rate of 10 °C min^{-1}, at which it was held for 5 min.

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, 2003b). Analyses were done in duplicate.

2.5. Statistical analysis

The data was analyzed 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.

3. Results and discussion

3.1. 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 (Fig. 1). The standard broth medium was supplemented with fructose and glucose to mimic the composition of the free sugars found in *masau* fruits. In order to assess the efficiency of the microorganisms to convert the sugars to desirable products there was a need to calculate the carbon balance, hence, standard broth as opposed to *masau* juice was used to enable determination of biomass.

Most of the *Saccharomyces cerevisiae* strains were superior to the other species in terms of the production of ethanol under both experimental conditions. Most *Pichia kudriavzevii* strains yielded intermediate levels of ethanol. *Saccharomyces* species are known as the main agent in alcoholic fermentations (Alfenore et al., 2002; Battcock and Ali, 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 and Langkjaer, 2004). Almost all the *S. cerevisiae* strains studied were able to utilize all glucose and fructose provided (Tables 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 · 100 ml^{-1} of alcohol from glucose and fructose fermentation, respectively. These yeast strains belonged mostly to *S. cerevisiae* and *P. kudriavzevii*. Most of the yeasts also produced ethanol under aerated conditions (Fig. 1), a phenomenon known as the "Crabtree Effect".

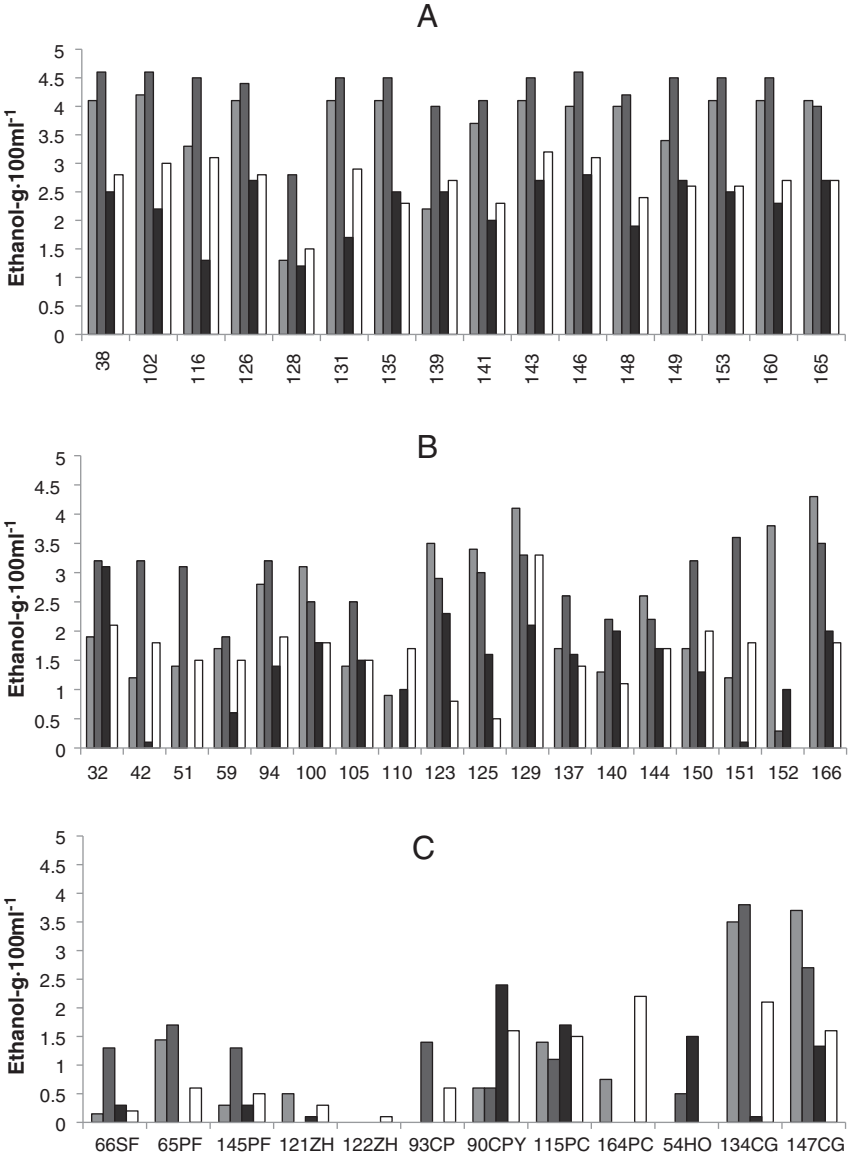

Fig. 1. Ethanol produced after five days at 30 °C by yeasts under aerated and non-aerated conditions in standard broth containing 10% sugar (glucose or fructose). A = *Saccharomyces cerevisiae* strains; B = *Pichia kudriavzevii* strains and C = other yeast strains. ▨ = glucose fermentation (non-aerated),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

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 also "Crabtree Negative" for fructose. A variation in the utilization of sugars was noted and, hence, the difference in the production of ethanol and biomass (Fig. 1 and S1, respectively) among 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 *Zygoascus hellenicus* strains. *Z. hellenicus* strains produced 1.3 and 1.9 $g \cdot 100\ ml^{-1}$ (dry weight) of biomass from fructose, whereas the highest amounts produced by *S. cerevisiae* and *P. kudriavzevii* were 0.58 and 0.77 $g \cdot 100\ ml^{-1}$ (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 micro-organisms (Nowakowska-Waszczuk and Sokolowski, 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 — Tables 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 and Baumberger, 1947). The discrepancy of C-mol unaccounted for ranged from 6.7 to 18% and 13.9 to 25% for fructose and glucose, respectively. This discrepancy can be attributed to the other carbon metabolites that are produced during fermentation; these may include enzymes, organic acids and volatile compounds.

3.2. Pre-selection of yeasts as starter cultures for fermented masau beverages

The production of high concentrations of ethanol from both glucose and fructose, and the ability to assimilate all of the sugars were used as criteria to select candidate yeasts to be used as starter cultures for *masau* fermented beverages. Six strains of *S. cerevisiae* (strains no. 38, 102, 131, 135, 143 and 153) were selected to analyze the formation of aromatic volatile compounds during fermentation of *masau* must. In addition, three strains of *P. kudriavzevii* (strains no. 125, 129 and 166) were selected given that it was the most predominant yeast isolated from the traditionally fermented *masau* fruit (Nyanga et al., 2007) One *Saccharomycopsis fibuligera* and one *Pichia fabianii* were also included in this comparative test. Although these species had a low ethanol production, 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 flavor diversity and complexity to wines (Fleet, 2008). *Candida glabrata*, which showed the best alcohol production among the non-Saccharomyces yeasts was not selected because it has been reported as a human pathogen in literature (Groot et al., 2008; Krcmercy and Barnes, 2002; Li et al., 2007).

3.3. Fermentation of masau must by selected yeasts isolates

Saccharomyces cerevisiae strains exhausted the glucose present in the must and left some traces of fructose (Table 1) during the fermentation period of 72 h. The other species and strains could not consume all of the glucose and fructose present in the juice during the 72 h. Fructose was found to be the most abundant residual sugar in the fermented must. 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 et al. (2004) explained this discrepancy on the basis of differences in kinetic properties of sugar transporters and hexokinases.

3.4. Production of volatile metabolites during fermentation of masau must

The volatile compounds produced by the selected yeast species during fermentation of *masau* must are shown in Table 2. The differences in the final amounts of some volatiles were statistically significant among the strains. These differences among strains and species could be attributed to their differences in genetic material as shown in the DNA polymorphisms seen in Amplified Fragment Length Polymorphism fingerprinting of the genomes and variability in physiological properties of the yeasts as reported by Nyanga et al. (2007).

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* must. 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.

3.4.1. 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. At concentrations below 300 $mg\ l^{-1}$, higher alcohols contribute to the complexity of wine, but when their concentrations exceed 400 $mg\ l^{-1}$ higher alcohols have a negative quality factor (Valinova and Martinez, 2006).

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 et al. (2008) reported that fusel alcohols at high concentrations impart off-flavors, but low concentrations of these compounds and their esters make an essential contribution to the flavors and aromas of fermented foods and beverages.

3.4.2. 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 $mg\ l^{-1}$). *P. kudriavzevii* has a potential role and contribution to the product flavor and quality since it was the most predominant yeast isolated from the traditionally fermented *masau* fruit pulp (Nyanga et al., 2007). Acetate esters are reportedly produced at relatively high concentrations by non-*Saccharomyces* species (Rojas et al., 2001, 2003; Trinh et al., 2011). The amounts of ethyl acetate

Table 1
Sugars, ethanol and organic acids determined by HPLC in laboratory fermented *masau* juice (72 h, 30 °C).

Sample	Residual sugars (g 100 ml^{-1})		Ethanol (g 100 ml^{-1})	Organic acids (g l^{-1})				
Fermentation by:	Glucose	Fructose		Citric	Malic	Acetic	Oxalic	Succinic
Saccharomyces cerevisiae								
143	Nd	0.38^{a}	6.4^{a}	0.78^{a1}	1.15^{a}	0.12^{a}	0.43^{a}	1.35^{a}
102	Nd	0.18^{b}	5.6^{b}	0.57^{b}	0.76^{b}	0.14^{b}	0.36^{b}	0.89^{b}
131	Nd	0.16^{c}	6.4^{a}	0.66^{c}	0.87^{c}	0.21^{c}	0.38^{b}	1.22^{c}
153	Nd	0.29^{d}	8.4^{c}	0.71^{d}	0.89^{c}	0.18^{d}	0.52^{c}	1.23^{c}
38	Nd	0.25^{e}	7.4^{d}	0.72^{d}	0.9^{c}	0.23^{c}	0.46^{dg}	1.28^{d}
135	Nd	0.5^{f}	5.8^{b}	0.84^{e}	1.21^{d}	0.17^{e}	0.45^{d}	1.27^{d}
Pichia kudriavzevii								
125	2.3^{a}	3.6^{g}	2.0^{e}	0.74^{f}	1.59^{e}	0.19^{f}	0.38^{b}	1.42^{e}
129	1.9^{b}	3.4^{g}	3.0^{f}	0.70^{g}	1.69^{f}	0.17^{e}	0.22^{e}	1.29^{f}
166	2.4^{a}	4.2^{h}	2.6^{f}	0.96^{h}	2.11^{g}	0.29^{g}	0.59^{f}	1.65^{g}
Saccharomycopsis fibuligera								
66	4.0^{c}	4.8^{i}	0.6^{g}	0.9^{i}	1.95^{h}	0.15^{b}	0.48^{g}	1.14^{h}
Pichia fabianii								
65	3.7^{c}	4.4^{h}	0.4^{g}	0.85^{e}	1.73^{i}	0.21^{c}	0.51^{h}	1.57^{i}
Masau juice (control)	6.7^{d}	6.7^{j}	0.2^{g}	0.47^{j}	1.09^{j}	0.09^{h}	0.34^{i}	0.83^{j}

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

produced by non-*Saccharomyces* species may contribute to the fruity character of wine when their concentration exceeds the odor 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., 2003a). As for isoamyl acetate, the amounts produced by all strains are above the odor threshold. Isoamyl acetate was reported to have an odor threshold of 0.03 mg l^{-1} and it contributes a fruity, banana, sweet flavor (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 odor thresholds. Ethyl hexanoate has a fruity, green apple flavor (Chaves-Lopez et al., 2009) and a threshold of 0.005 mg l^{-1} (Guth, 1997). The odor threshold of ethyl octanoate is 0.002 mg l^{-1} (Guth, 1997; Palomo et al., 2007) and contributes a fruity, banana, pineapple flavor (Palomo et al., 2007). Ethyl decanoate has been reported to have a sweet fruity flavor, 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 odors and often occur at concentrations much higher than their respective odor thresholds (Mallouchos et al., 2003a).

Table 2
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* 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^{a*}	29.2^{b}	32.6^{c}	45.6^{d}	38.2^{e}	46.5^{f}	55.5^{g}	7.9^{h}	9.4^{i}	10.0^{j}	1.8^{k}	2.0^{l}
Propanol[1]	0.23^{a}	1.6^{b}	1.3^{c}	2.2^{d}	1.3^{c}	0.6^{e}	1.1^{c}	0.9^{c}	1.0^{c}	0.7^{e}	0.5^{e}	0.3^{a}
Isoamyl alcohol[1]	0.01^{a}	7.6^{b}	7.2^{b}	8.9^{c}	7.5^{b}	7.1^{b}	7.3^{b}	2.7^{d}	4.4^{e}	4.6^{e}	0.7^{f}	0.4^{g}
Acetate esters												
Isoamyl acetate[1]	0.06^{a}	0.9^{b}	0.9^{b}	0.9^{b}	0.7^{be}	1.2^{cd}	1.4^{d}	0.5^{e}	0.8^{b}	1.1^{c}	2.8^{f}	3.3^{g}
Ethyl acetate[1]	0.38^{a}	2.4^{b}	1.5^{c}	2.4^{b}	1.6^{c}	4.0^{d}	2.7^{b}	9.4^{e}	18.4^{f}	17.7^{g}	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^{b}	0.8^{c}	0.9^{d}	0.9^{d}	0.6^{e}	1.2^{b}	0.4^{f}	0.6^{e}	0.5^{e}	1.4^{g}	1.4^{g}
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^{b}	28.7^{c}	92.0^{d}	28.6^{c}	21.3^{e}	23.2^{e}	1.4^{f}	2.5^{g}	2.5^{g}	1.3^{f}	1.8^{f}
Ethyl octanoate[2]	0.35^{a}	31.9^{b}	30.3^{c}	58.0^{d}	20.3^{e}	18.8^{f}	21.0^{e}	14.5^{g}	11.6^{h}	2.7^{i}	0.3^{a}	0.9^{j}
Ethyl decanoate	0.02^{a}	1.8^{b}	1.0^{c}	1.3^{d}	0.6^{e}	0.7^{ef}	0.8^{f}	nd	nd	0.1^{a}	nd	nd
Fatty acids												
Octanoic acid[2]	0.2^{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]	2.08^{a}	0.8^{b}	0.5^{c}	0.7^{b}	0.5^{c}	0.6^{bc}	0.6^{bc}	2.8^{d}	0.5^{c}	0.3^{e}	3.1^{f}	3.0^{f}
Isovaleric acid[2]	0.2^{a}	0.6^{b}	0.5^{b}	0.5^{b}	0.4^{c}	0.4^{c}	0.4^{c}	0.3^{a}	0.3^{a}	0.4^{c}	0.4^{c}	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^{c}	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]	0.6^{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

Means in the same row with the same letter are not significantly different according to the LSD at the 0.05 level. 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.

Therefore, ethyl esters produced by the yeasts may have a profound impact on the aromatic flavor 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.

3.4.3. 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 flavor (Palomo et al., 2007). The levels of octanoic acid detected for all the yeast species were below the odor threshold of 0.5 mg l^{-1} (Ferreira et al., 2000). Isovaleric acid has a rancid flavor and all the fermented juices showed values above the odor threshold (0.7 mg l^{-1}, Lambrechts and Pretorius, 2000). The presence of fatty acids in amounts above the odor thresholds might cause undesirable effects to the aroma of the wine.

Of the organic acids (Table 1), citric, malic, acetic, oxalic and succinic acids were detected in higher amounts in most of the fermented *masau* must than in the non-fermented must. 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. The levels of succinic acid detected in the fermented musts were above the threshold of 0.034–0.035 g l^{-1}. This might have a negative impact on the wine flavor as succinic acid has been reported to have an unusual salty, bitter taste in wine (Swiegers et al., 2005). Acetic acid is of particular importance as it imparts a vinegar-like character to wine at concentrations above the threshold (0.7–1.1 g l^{-1}) (Swiegers et al., 2005; Lambrechts and Pretorius, 2000). However, organic acids can contribute positively to the organoleptic character of wine when in balance with the other wine compounds, and they also contribute to the physical, biochemical and microbial stability of the wine (Volschenk et al., 2006).

4. 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* must, 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* fermented beverages as they are the best 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.08.003.

References

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. Appl. Microbiol. Biotechnol. 60, 67–72.

Barnett, J.A., 1997. Sugar utilisation by Saccharomyces cerevisiae. In: Zimmermann, F.K., Entian, K.D. (Eds.), Yeast Sugar Metabolism. Technomic Publishing Co., United States, pp. 35–43.

Battcock, M., Ali, S.A., 1993. Fermented fruits and vegetables. Agric. Serv. Bull. 134, 13–28.

Berthels, N.J., Otero, R.R.C., Bauer, F.F., Thevelein, J.M., Pretorius, I.S., 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. 4, 683–689.

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

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 Microbiol. 21, 149–155.

Crabtree, H.G., 1929. Observations on the carbohydrate metabolism of tumors. Biochem. J. 23, 536.

De Deken, R.H., 1966. The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44, 149–156.

Dung, N.T.P., Rombouts, F.M., Nout, M.J.R., 2004. Defined Fungal Starter Granules for Purple Glutinous Rice Wine. (PhD Thesis) Wageningen University, Wageningen, The Netherlands.

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 Microbiol. 23, 331–340.

Fales, F.W., Baumberger, J.P., 1947. The anaerobic assimilation of glucose by yeast cells. J. Biol. Chem. 173, 1–8.

Ferreira, V., Lopez, R., Cacho, J.F., 2000. Quantitative determination of the odorants of young red wines from different grape varieties. J. Sci. Food Agric. 80, 1659–1667.

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 Food Sci. Technol. 21, 77–84.

Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Res. 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. J. Agric. Food Chem. 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. Int. J. Food Microbiol. 53, 1–11.

Groot, P.W.J., Kraneveld, E.A., Yuan Yin, Q., Dekker, H.L., Groß, U., Crielaard, W., De Koster, C.G., Bader, O., Klis, F.M., Weig, M., 2008. The cell wall of the human pathogen *Candida glabrata*: differential incorporation of novel adhesin-like wall proteins. Eukaryot. Cell 7, 1951–1964.

Guth, H., 1997. Quantitation and sensory studies of character impact odorants of different white wine varieties. J. Agric. Food. Chem. 45, 3027–3032.

Hazelwood, L.A., Daran, J., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Erhlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol. 74, 2259–2266.

Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75, 197–212.

Krcmercy, V., Barnes, A.J., 2002. Non-albicans *Candida* spp. causing fungaemia: pathogenicity and antifugal resistance. J. Hosp. Infect. 50, 246–260.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeasts and its importance to wine aroma — a review. S. Afr. J. Enol. Vitic. 21, 97–129.

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. J. Biol. Chem. 286, 20267–20274.

Li, E., Mira de Orduña, R., 2010. A rapid method for determination of microbial biomass by dry weight using a moisture analyser with an infrared heating source and an analytical balance. Lett. Appl. Microbiol. 50, 283–288.

Li, L., Redding, S., Dongari, A., 2007. Candida glabrata, an emerging oral opportunistic pathogen. J. Dent. Res. 86, 204–215.

Maghembe, J., Seyani, J., 1992. Multipurpose trees used by small-scale farmers in Malawi. Results of an Ethnobotanical Survey. AFRENA report No. 42ICRAF, Nairobi.

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. J. Agric. Food Chem. 50, 3840–3848.

Mallouchos, A., Komaitis, M., Koutinas, A., Kanellaki, M., 2003a. Evolution of volatile byproducts during wine fermentations using immobilized cells on grape skins. J. Agric. Food Chem. 51, 2402–2408.

Mallouchos, A., Skandamis, P., Loukatos, P., Komaitis, M., Koutinas, A., Kanellaki, M., 2003b. Volatile compounds of wines produced by cells immobilized on grape skins. J. Agric. Food Chem. 51, 3060–3066.

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

Nowakowska-Waszczuk, A., Sokolowski, A., 1987. Application of carbon balance to submerged citric acid production by *Aspergillus niger*. Appl. Microbiol. Biotechnol. 26, 363–364.
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. Int. J. Food Microbiol. 120, 159–166.
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. Ecol. Food Nutr. 47, 95–107.
Nyanga, L.K., Nout, M.J.R., Smid, E.J., Boekhout, T., Zwietering, M.H., 2013. Nutritive value of *masau* (*Ziziphus mauritiana*) fruits from Zambezi valley in Zimbabwe. Food Chem. 138, 168–172.
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.
Piskur, J., Langkjaer, R.B., 2004. Yeast genome sequencing: the power of comparative genomics. Mol. Microbiol. 53, 381–389.
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. Int. J. Food Microbiol. 70, 283–289.
Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol. 86, 181–188.
Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139–173.
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.
Valinova, M., Martinez, C., 2006. First study of determination of aromatic compounds of red wine from Vitis vinifera cv. Castanal grown in Galicia (NW Spain). Eur. Food Res. Technol. 224, 431–436.
Volschenk, H., van Vuuren, H.J.J., Viljoen-Bloom, M., 2006. Malic acid in wine: origin, function and metabolism during vinification. S. Afr. J. Enol. Vitic. 27, 123–136.
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. Biotechnol. Bioeng. 108, 1776–1787.