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IDENTIFICATION OF YEASTS ISOLATED FROM *MUKUMBI*, A ZIMBABWEAN TRADITIONAL WINE

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Abstract

The use and identity of microbes in traditional fermented beverages has attracted the interest of researchers and industrialists world-wide, probably due to their potential to reduce transmission of bacterial enteric pathogens, as well as improving their nutritional value. In the work done, yeasts were isolated from samples of Zimbabwean traditional wine, mukumbi, collected from rural households in Mberengwa, Zimbabwe. Isolates were enumerated, characterised, and identified using morphological, physiological, and biochemical properties of strains. Yeast counts ranged from 7.60 to 7.74 log colony forming units/ml of mukumbi. Saccharomyces cerevisiae (two isolates) were the predominant species identified in mukumbi. Other yeast species identified were Pichia anomala, Pichia guilliermondii, Candida tropicalis, and Candida intermedia. The study showed that the yeast flora in mukumbi is diverse. The occurrence of C. tropicalis, a known human pathogen, emphasised the need to develop a starter culture to produce safer and nutritious mukumbi.

Introduction

Various substrates, such as cereals and fruits, are used in the production of traditional alcoholic fermented beverages in Zimbabwe (Sanni & Lonner, 1993; Mugochi *et al*., 1999; Benhura & Chingombe, 1989). The beverages, apart from serving as inebriating drinks, are important at social and cultural gatherings, such as weddings, religious ceremonies, and for income generation (Mutasa & Ayebo, 1993; Okagbue, 1995; Gadaga *et al*., 1999). *Mukumbi* is a popular beverage in many Zimbabwean villages in semi arid regions. The beverage, a wine, is traditionally prepared by spontaneously

fermenting a mash prepared from ripe fruits of the *marula* plant (*Sclerocarya birrea* subspecies *caffra*). Reports show that the preparation of *mukumbi* varies from producer to producer (Madovi, 1981; Okagbue, 1995; Mugochi *et al*. 1999).

The preparation of *mukumbi* is basically a traditional family art passed on from generation to generation. The micro-organisms responsible for the fermentation are poorly studied and are thought to include wild yeasts and bacteria from fermentation pots passed from previous fermentations (Gadaga *et al.*, 1999), and from the microflora of the *marula* fruit. Due to chance inoculation, there is likely to be a wide variety of micro-organisms involved in the production of *mukumbi,* leading to variability in quality. Micro-organisms associated with the fermentation contribute to variations in brews from different local brewers (Owuama, 1997).

Although the production of *mukumbi* is a result of mixed, spontaneously generated micro-organisms, the yeast flora are responsible for the alcohol content of the wine. There is need, therefore, to isolate, identify, and characterize the yeasts involved in the traditional fermentations for potential use as starter cultures for the production of a consistent quality product and for understanding the role of the various yeasts in the fermentation process and final product quality.

Materials and Methods

Samples of *mukumbi* were randomly collected from three rural households in Mberengwa district, Southern Zimbabwe. Fermenting marula juice samples were collected from traditional 20 liter (L) earthenware pots during preparation and placed in sterile plastic screw-capped bottles before being plated out within 3 hours after sampling.

Traditional preparation of mukumbi and sample collection: Traditional preparation was conducted by villagers in Mataga village, Mberengwa, a semi-arid communal area in

Southern Zimbabwe. Ripe *marula* fruits (10 kg) from the wild were pierced with a wooden spoon and the juice was squeezed out of the skins. *Marula* seeds were pounded in a wooden mortar and pestle to completely extract the juice and the flesh. The slurry mixture composed of the flesh and the juice was poured into a 20 L earthenware pot. An equal volume of water was added and the pot was covered with a wooden plate. The mixture was left to ferment naturally at room temperature (approximately 25 ^{o}C) for 72 hours. The fermented slurry was filtered through a 435 micrometer (μm) sieve and the filtrate, now called *mukumbi*, was ready for consumption. A thick residue formed mainly from the flesh was collected on top of the sieve and discarded. Samples were collected for yeast isolation.

Enumeration and isolation of yeasts: Samples (1 ml) of *mukumbi* were serially diluted using sterile peptone water (Oxoid) and 0.1 ml quantities of the appropriate dilutions were spread onto wort agar (Oxoid) plates. Plates were incubated at 30^{o}C for 48 hours. Microbial load was expressed as log colony forming units per millilitre (log cfu/ml) of *mukumbi*.

Yeasts were enumerated on spread plates of wort agar using a colony counter (Stuart Scientific). Colonies with distinct morphological differences, such as colour, shape, and size were picked and purified by streaking three times on wort agar. Colonies were examined for cellular morphology, using a Zeiss phase contrast microscope. Purified isolates were stored on wort agar slants at 4^{o}C. For long-term storage, the purified isolates were cryopreserved in 10% glycerol in a -70^{o}C freezer and also at –196^{o}C under liquid nitrogen. Cultures were deposited with the University of the Free State yeast culture collection.

Identification of yeasts: Yeasts were identified using the conventional methods described by Van der Walt and Yarrow (1984), Kurtzman and Fell (1998), and Barnett *et*

al. (2000). Morphological, physiological, and chemotaxonomic tests were used for yeast identification.

Morphological characteristics of yeasts: Actively growing yeast cells were streaked onto yeast-malt extract (YM) agar plates and 5% malt extract (ME) agar plates and incubated at 25°C and at 30°C, respectively, for 5 days. Wet mount slides of the yeasts were prepared from single colonies picked from the YM agar plates and examined microscopically to determine cellular morphology and modes of reproduction.

Ascospore formation was examined on 5% ME agar and YM agar according to the method described by Van der Walt & Yarrow (1984). Actively growing yeast cells were streaked onto plates, which were incubated at 25°C for 3 weeks. Ascospores were examined after 3 days and weekly thereafter, to determine variation in shape, ornamentation, colour, dimensions, mode of production, and the number formed per ascus. Formation of pseudohyphae and true hyphae was examined on corn meal agar using the Dalmau plate technique (Kurtzman & Fell, 1998; Barnett *et al.,* 2000).

Fermentation of sugars: The fermentation of D-glucose, sucrose, D-galactose, lactose, maltose, and raffinose was tested, according to the method of Van der Walt and Yarrow (1984). A visible suspension of yeast suspended in sterile distilled water was used to inoculate the fermentation medium in sterile test tubes containing Durham tubes. The tubes were incubated at 25°C on a Rolldrum for 2 weeks and examined after 3 days, 1 week, and 2 weeks. A positive result was indicated by accumulation of gas in the Durham tubes, as described by Van der Walt & Yarrow (1984).

Liquid assimilation of carbon compounds: An aliquot (0.5 ml) of filter-sterilized yeast nitrogen base (Difco) containing 5% of the compound under test was aseptically added to sterile distilled water (4.5 ml) in test tubes. The tubes were inoculated by aseptically adding 2 drops of a visible suspension of an actively growing culture in sterile

distilled water. The tubes were incubated at 25°C on a Rolldrum and examined after 3 days, 1 week, 2 weeks, and 3 weeks. A positive reaction was detected by visual inspection for an increase in the turbidity of the solution on examination at the different intervals, as described by Van der Walt & Yarrow (1984). The carbon compounds tested were D-galactose, D-glucose, sorbose, D-glucosamine, D-ribose, D-xylose, L-rhamnose, sucrose, lactose, L-arabinose, D-arabinose, arabinitol, maltose, D-mannitol, melibiose, melezitose, raffinose, soluble starch, inulin, ά,ά trehalose, ά-methyl-D-glucoside, D-gluconate, 2-keto-D-gluconate, cellibiose, erythritol, xylitol, salicin, L-arabinitol, dulcitol, adonitol, glycerol, methanol, ethanol, 1,2 propanediol, 2,3 butanediol, meso (i) erythritol, succinate, citrate, and DL-lactate.

Assimilation of nitrogen compounds: The procedure was as described by Barnett *et al.* (2000). Aliquots (20 ml) of sterile, synthetic basal medium (0.5% ammonium sulphate, 0.1% potassium dihydrogen phosphate, 0.05% magnesium sulphate heptahydrate, 2% purified high grade agar), devoid of a nitrogen source, were melted and cooled to about 40°C then poured into petri dishes containing about 2 ml suspension of the yeasts under test in sterile peptone water. After solidification, the plates were kept, lid side up, at 25°C for a few hours to obtain a dry surface agar. Small amounts of the various nitrogen compounds were then deposited at different, evenly spaced sites on the agar. Sites were marked on the outside of the smaller dishes. Plates were then incubated at 25°C, lid side down, and results were observed after 3 days. The nitrogen compounds tested were nitrate, nitrite, ethylamine, L-lysine, creatine, and creatinine. Ammonium sulphate was used as a positive control.

Determination of yeast lipid profiles: Yeast strains were inoculated onto YM plates and incubated at 30°C for 7 days. Single colonies from the plates were used to inoculate 1 L Erlenmeyer flasks containing 400 ml synthetic medium made up of yeast

nitrogen base (2.6 g) and glucose (40 g) dissolved in 1 L of distilled water. The cultures were incubated at 30°C on a rotary shaker for 48 hours. Biomass was harvested by centrifugation at 5116g for 10 min. Lipids in the biomass were extracted using chloroform/methanol (2:1). The organic phase was evaporated and the lipids were dried in a vacuum oven (50°C) over P_2O_5 for 12 hours. Chloroform (2 ml) was added to the vials and the mixture was shaken. The mixture (200 µl) was placed in a Gas Chromatograph (GC) vial. Trimethyl sulphonium hydroxide (TMSOH) (200 µl) was added to the GC vial to transesterify the fatty acids to give methyl esters of the fatty acids, which are volatile and can be analyzed by gas chromatography.

Vials were closed with crimp caps, marked, and placed on the GC autosampler. Fatty acids in the lipids were determined by an HP5890 Series II Gas Chromatograph equipped with a Supelcowax 10 column (30 m x 0.55 mm). Samples of 1 µl were injected onto the GC. The column temperature was held at 140°C for 5 minutes, then raised at 3°C/min to 245°C. Nitrogen was used as the carrier gas at a flow rate of 3 ml/min. Fatty acid profiles detected for the yeast strains were compared with known fatty acid profiles of yeast strains for their identification.

Other tests for yeast identification: The diazonium blue B (DBB) test was carried out to distinguish between ascomycetous and basidiomycetous yeasts (Kurtzman & Fell, 1998). Yeasts were grown as spot cultures on YM agar plates and incubated at 25°C for 10 days. Freshly prepared chilled DBB reagent (1-2 drops) was then applied directly to the surface of the colonies. A positive reaction was recorded when the colonies developed a dark red or violet red colour within 2 minutes at room temperature.

The use of the ability or inability to grow in a mineral medium devoid of vitamins was used as a diagnostic property. Actively growing yeast cells from a slant were inoculated into tubes containing mineral medium devoid of vitamins and incubated at

25°C for 7 days. Growth was observed after 7 days. A second vitamin-free tube was inoculated with a standard loop from the first to override growth, which may be due to vitamins carried over from the inoculum.

The ability of yeasts to grow on high concentrations of sugar was tested by growth on agar media containing 50% (w/w) glucose (50 g glucose, 49.5 ml distilled water, 1.5 g yeast extract, 3 g purified agar) and 60% (w/w) glucose. Actively growing cultures were lightly inoculated as a streak on the glucose agar in tubes. Tubes were incubated at 25°C and growth was examined after 5 days. Agar slants were prepared by dispensing 5-6 ml aliquots in 16 mm plugged tubes, which were autoclaved for 10 minutes at 121°C, then slanted and cooled.

To determine yeast growth at various temperatures, actively growing yeasts were streaked onto YM agar plates and incubated at various temperatures; 25°C, 30°C, 35°C, 37°C, and 42°C. Growth was observed after 5 days. In case of weak growth, subcultures were made which were incubated at the same temperature for 2-4 days. Results of the latter test were taken as decisive.

For the determination of cycloheximide resistance, actively growing yeasts were inoculated in the same manner as for the carbon assimilation tests, into tubes containing filter sterilized 0.01% and 0.1% solutions of cycloheximide. Tubes were incubated at 25°C with agitation for 3 weeks and examined after 3 days, 2 weeks, and 3 weeks.

The urease test was carried out, as described by Van der Walt and Yarrow (1984), using commercial Christensen's urease agar medium. The test was taken to be positive when there was development of a deep red colour in the agar. The starch test was carried out by adding 2 drops of Lugol's iodine reagent to the carbon assimilation tubes after 21 days of incubation and noting the colour change.

Results and discussion

Table 1 shows mean yeast counts of six yeast isolates from *mukumbi* samples. Yeast counts ranged from 7.60 to 7.74 log cfu/ml in *mukumbi*. Sanni and Lönner (1993) reported a range of yeast counts of 4.90 to 6.79 log cfu/g in Nigerian traditional fermented beverages. Variable counts are expected for traditional fermentations, as they depend on spontaneous, uncontrolled fermentations and the use of variable raw materials.

Table 1: **Yeast Colony Counts of Six Yeast Isolates from *Mukumbi* Samples**

Yeast Isolate Reference Number	*Yeast Count (log cfu/ml)*
1	7.60
2	7.60
3	7.74
4	7.73
5	7.64
6	7.60

All of the six yeast isolates from *mukumbi* were identified to species level. Yeast isolates 2 and 5 from *mukumbi* were identified as *Saccharomyces cerevisiae*. All isolates had cream to pale brown colonies on malt extract (ME) agar. Cells were round to ovoid in shape after 3 days at 25^{o}C. Multipolar budding was observed on wet smears. Two to four spheroidal ascospores were visible per ascus on YM agar after 21 days incubation at 25^{o}C on YM agar. Most of the isolates were able to ferment D-glucose, D-galactose, sucrose, and raffinose, while none could ferment lactose (Table 2).

Table 2: **Fermentation of Different Substrates by Yeasts Isolated from *Mukumbi***

	Yeast Reference Number					
Fermentation Substrate	**1**	**2**	**3**	**4**	**5**	**6**
Glucose	+	+	+	+	+	+
Galactose	+	+	-	+	+	+
Maltose	+	+	-	+	+	+
Sucrose	+	+	+	+	+	+
Lactose	-	-	-	-	-	-
Raffinose	+	+	+	-	+	+

The *Saccharomyces cerevisiae* species plays an important role in fermented alcoholic beverages because of its ability to ferment several sugars to produce ethanol. *Saccharomyces cerevisiae* has been previously isolated from wines, beers, fruits, fruit-juice, fruit flies, and soil (Kurtzman & Fell, 1998). Gadaga *et al,* (2000) isolated *Saccharomyces cerevisiae* from Zimbabwe traditional fermented milk. The species is used commercially in the production of beer, wine, and bread.

Table 3 shows the assimilation pattern of different nitrogen compounds by the *Saccharomyces cerevisiae* yeast isolates. All of the isolates were able to assimilate ammonium sulphate, which was included as a positive control for the nitrogen assimilation tests.

Table 3: Assimilation of Different Nitrogen Compounds by Yeasts Isolated from *Mukumbi*

	Yeast Reference Number					
Nitrogen Compound	**1**	**2**	**3**	**4**	**5**	**6**
Nitrate	+	-	-	-	-	+
Nitrite	+	-	-	-	-	+
Ethylamine	+	-	+	+	-	+
L-lysine	+	-	+	+	-	+
Creatine	-	+	-	-	-	-
Creatinine	-	-	-	-	-	-

Saccharomyces cerevisiae species were not able to use any of the other nitrogen sources studied as sole sources of nitrogen. Isolate 2, unlike the description of *Saccharomyces cerevisiae* by Barnett *et al*. (2000) and Kurtzman and Fell (1998), managed to assimilate creatine.

There was variation in the carbon assimilation patterns of the *Saccharomyces cerevisiae* species, with both being capable of assimilating D-glucose, D-galactose, sucrose, maltose, ά, ά-trehalose, and raffinose (Table 4).

Table 4: **Assimilation of Different Carbon Compounds by Yeasts Isolated from *Mukumbi***

	Yeast Reference Number					
Carbon Compound	**1**	**2**	**3**	**4**	**5**	**6**
D-Glucose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
L-Sorbose	-	-	+	+	-	+
D-Glucosamine	-	-	+	+	-	+
D-Ribose	+	-	+	-	-	+
D-Xylose	+	-	+	+	-	+
L-Arabinose	-	-	+	-	-	+
D-Arabinose	-	-	+	-	-	+
L-Rhamnose	-	-	+	-	-	-
Sucrose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
ά, ά, Trehalose	+	+	+	+	+	+
Me ά Glucoside	+	-	+	+	-	+
Cellobiose	-	-	+	+	-	+
Salicin	+	-	+	+	-	+
Salicin	-	-	+	-	-	-
Salicin	-	-	-	-	-	+
Lactose	+	+	+	-	+	+
Raffinose	+	+	+	+	+	+
Melezitose	+	-	+	+	-	+
Soluble starch	+	-	-	+	-	-
Glycerol	+	-	+	-	-	-
Meso erythritol	+	-	-	-	-	-
Adonitol	+	-	+	+	-	+
Xylitol	+	-	+	-	-	+
L-Arabinitol	-	-	+	-	-	-
D-Mannitol	+	-	+	+	-	+
Dulcitol	-	-	+	-	-	+
Inositol	-	-	-	-	-	-
2 keto D-Gluconate	-	-	+	-	-	-
D-Gluconate	+	-	+	+	-	+

Cont.

DL-Lactate	+	+	-	-	-	-
Succinate	+	+	+	+	-	+
Citrate	+	-	+	+	-	-
Methanol	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+
Propan 1,2 diol	+	-	-	-	-	-
Butan 2,3 diol	+	-	-	-	-	-

Both the *Saccharomyces cerevisiae* strains were capable of growing at temperatures from 25°C to 37°C (Table 5). Isolates 2 and 5 were thermo-tolerant and were able to grow at 40°C. Isolate 5 was the only *Saccharomyces cerevisiae* yeast isolate capable of growth at 42°C, and such a thermo-tolerant would be very useful in industrial production of ethanol, where high temperatures are employed.

Table 5: **Growth at Different Temperatures by Yeasts Isolated and *Mukumbi***

	Yeast Reference Number					
Temperature	**1**	**2**	**3**	**4**	**5**	**6**
25°C	+	+	+	+	+	+
30°C	+	+	+	+	+	+
37°C	-	+	+	+	+	+
40°C	-	+	+	+	+	-
42°C	-	-	+	+	+	-
45°C	-	-	-	-	-	-

Saccharomyces cerevisiae isolates showed characteristic high fatty acid ratios of 16:1 and 18:1 fatty acids in their fatty acid profiles (Table 6A).

Table 6A: **Fatty Acid Ratios (%) of *Saccharomyces cerevisiae* Yeasts Isolated from *Mukumbi***

	Yeast Reference Number	
Fatty Acid	**2**	**5**
16:0	14.20	11.70
18:0	40.60	44.70
16:1	10.20	7.50
18:1	31.10	34.30
18:2	3.90	1.80
18:33	0.00	0.00
18:36	0.00	0.00

Table 6b: Fatty Acid Ratios (%) of Yeasts Isolated from *Mukumbi*

	Yeast Reference Number			
Fatty Acid	**1**	**3**	**4**	**6**
16:0	22.50	26.50	17.90	23.90
18:0	10.10	7.90	8.50	12.80
16:1	12.30	14.10	8.60	3.20
18:1	40.20	29.90	48.50	40.60
18:2	12.20	18.00	14.30	17.30
18:33	2.70	3.50	2.20	2.00
18:36	0.00	0.00	0.00	0.12

Further studies are necessary to determine the use of yeast fatty acid profiles as markers in yeast identification studies. Both isolates 2 and 5 were capable of growth in vitamin-free media. None of the isolates were able to grow at the two cycloheximide concentrations used (Table 7), which is characteristic of the *Saccharomyces cerevisiae* species (Kurtzman & Fell, 1998). None of the *Saccharomyces cerevisiae* isolates displayed urease activity or were able to split arbutin (Table 7).

Table 7: **Results of Additional Tests Carried out on Yeasts Isolated from *Mukumbi***

	Yeast Reference Number					
Growth In/On:	**1**	**2**	**3**	**4**	**5**	**6**
0.01% cycloheximide	-	-	+	+	-	+
0.1% cycloheximide	-	-	+	+	-	-
1% acetic acid	-	-	-	-	-	-
50% glucose	-	-	-	-	-	-
60% glucose	-	-	-	-	-	-
Arbutin	+	-	+	+	-	+
Vitamin free medium	+	+	+	-	+	-
Test:						
Diazonium Blue B test	-	-	-	-	-	-
Starch test	-	-	-	-	-	-
Urease test	-	-	-	+	-	+
Ascospore/basidiospore	+	+	+	-	+	-
Pseudo/ true hyphae	-	-	-	-	-	-

Isolates 2 and 5 from *mukumbi*, were identified as belonging to the genus *Candida*. Isolate 4 was identified as *Candida tropicalis* and isolate 6 was identified as *Candida intermedia* (Barnett *et al*., 2000). The morphological and cultural characteristics of the two *Candida* species showed that the species had smooth, flat, cream colonies on ME agar. On a wet smear, individual cells were round to ovoid in shape after three days of incubation at 25^{o}C on YM agar. No ascospores were observed in this species. *Candida intermedia* (isolate 6) failed to grow at temperatures above 37^{o}C. *Candida tropicalis* (isolate 4) was able to grow at temperatures up to 42^{o}C (Table 5). *Candida tropicalis* (isolate 4) failed to ferment lactose and raffinose, whilst *Candida intermedia* (isolate 6) failed to ferment lactose only (as shown in Table 2). Both *Candida intermedia* and *Candia tropicalis* were able to utilise ethylamine and lysine but failed to utilise creatine and creatinine (Table 3). *Candida intermedia* (isolate 6) and *Candida tropicalis* (isolate 4) gave positive results to the urease test while. *Candida tropicalis* has been previously isolated from humans in Austria and Egypt, fodder yeast in Germany and rotten pineapple in Iran (Kurtzman & Fell, 1998). *Candida intermedia* have been previously isolated from washed beer bottles in Sweden, grapes in Brazil, soil and beer in South Africa (Kurtzman & Fell, 1998).

Isolates 1 and 3, from *mukumbi*, were identified as belonging to the genus *Pichia*. Isolate 1 was identified as *Pichia anomala* and isolate 3 was identified as *Pichia guilliermondii*. The yeast cells were round to ovoid in shape and characterised by the observation of hat-shaped ascospores. Table 2 shows that both species managed to ferment glucose, sucrose, and raffinose and failed to ferment lactose. *Pichia anomala* (isolate 1) was able to ferment galactose and maltose, whilst *Pichia guilliermondii* (isolate 3) was not. *Pichia guilliermondii* (isolate 1) was more versatile than *Pichia*

anomala (isolate 3) in its ability to assimilate carbon sources (Table 4), but was less versatile in its ability to assimilate nitrogen sources (Table 3). *Pichia anomala* (isolate 1) failed to grow at temperatures from 37^{o}C and above, while *Pichia guilliermondii* (isolate 3) was able to grow at 42^{o}C but not at 45^{o}C (Table 5). Both species managed to grow in media without vitamins, indicating that they were able to synthesize their own vitamins (Table 7). Such yeasts can be exploited commercially in the manufacture of vitamins. Both species managed to split arbutin. *Pichia anomala* has been previously isolated from raw Cuban sugar in the United Kingdom, tuberculosis patients in Italy, on cucumbers and sauerkraut in USA, brewer's yeast and clay in United Kingdom, and banana skin in Indonesia (Kurtzman & Fell, 1998).

Conclusion

The results of this study indicate that the Zimbabwean traditional alcoholic beverage, *mukumbi* has a complex community of yeasts with variable characteristics. Further studies are necessary to determine the role of the various yeast species in determining the overall product quality of the alcoholic beverages. The study showed that the yeast flora in *mukumbi* is diverse. The occurrence of *C. tropicalis*, a known human pathogen, emphasised the need to develop a starter culture to produce safer *mukumbi*.

If the yeasts are to be used as starter cultures for the controlled production of the beverages, it would be necessary to do additional characterisation using molecular techniques and to study the ethanol production capacity of the isolated yeasts and their contribution to the flavour characteristics of the fermented beverages.

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