Characterization and Selection of Predominant Yeast Strains Involved in Fermentation of the Rwandese Traditional Sorghum Beer "Ikigage"

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Abstract

The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of the Rwandese traditional sorghum beer «ikigage». A total of 127 yeast strains were isolated at different steps of *ikigage* fermentation. These strains belong to species of *Saccharomyces cerevisiae, Issatckenkia orientalis, Candida humilis, Candida incuspicua, Candida magnoliae, Candida krusei* and *Pichia membranifaciens*. But, *S. cerevisiae* and *I. orientalis* were predominant yeast strains. *I. orientalis* strain (named RG1) was able to survive in 15% ethanol and fast convert glucose into ethanol while *S. cerevisiae* strains (named RV6, RB2 and RK1) produced high final ethanol content (5.45 – 6.7% v/v). The yeast strains selected are non-flocculent (RK1 strain), low flocculent (RV6 and RB2 strains) and medium flocculent (RG1strain) strains. The selected *S. cerevisiae* strains were Newflo phenotype while *I. orientalis* RG1 strain was MI (mannose insensitive) phenotype. These yeast strains were found suitable for improving the quality of Rwandese traditional beer «ikigage».

KEYWORDS: Sorghum beer, *Ikigage*, Fermentation, *Umuseburo*,Yeasts.

INTRODUCTION

The African traditional alcoholic beverages such as *ikigage, pito, dolo, tchoukoutou* and *burukutu* are prepared from sorghum malt. These spontaneously fermented beverages, apart from serving as inebriating drinks, are also important in fulfilling social obligations (e.g., marriage, birth, baptism, dowery, etc.) and constitute a source of economic return for the women manufacturer.

In Rwanda, during *ikigage* manufacturing, the malted wort sorghum is inoculated by a traditional leaven *umusemburo* as fermentation starter, which preparation is described by Lyumugabe *et al.* (2010). After 12 to 24 h of fermentation, *ikigage* is ready for consumption. The traditional methods of *umusemburo* production involve the preparation of malted wort sorghum (*igikoma*), the addition of local plants (e.g. *Vernonia amygdalina*) and the spontaneous fermentation of *igikoma*. The microorganisms involved in this fermentation are dominated by yeasts and Lactic acid bacteria (Lyumugabe *et al.*, 2010). However, yeasts are mainly responsible for alcohol content of the alcoholic beverages; *Saccharomyces cerevisiae* was found to predominate in African

sorghum beers (Demuyakor *et al*., 1991; Jespersen, 2003; Naoumova *et al.*, 2003; Lyumugabe *et al*., 2010; Kayode *et al*., 2011).

Selecting the best yeast strain with the desired brewing characteristics has always been brewer's dream. The use of selected local yeasts is believed to be much more effective, since these yeasts are presumed to be more competitive because better acclimated to environmental conditions. Moreover, the selection of suitable local yeasts assures the maintenance of the typical sensory properties of the fermented products produced in any given region (Querol *et al*., 1992, Degre, 1993). The criteria for yeast selection will vary according to the requirements of the brewing equipment and the beer style, but they are likely to include the fermentation speed, yeasts stress tolerance, appropriate flocculation avoiding incomplete attenuation, rate of attenuation at the desired temperature, balanced flavour compounds, efficient conversion of wort sugars to alcohol and genetic stability (Goldammer, 2008).

Currently, the manufacture of *ikigage* beer is declining because of poor hygienic quality, unsatisfactory conservation and poor yield of ethanol and variations of organoleptic quality (Lyumugabe *et al*., 2010). To improve the quality of this beer as it has done for traditional fermented foods such as *Kivude* (Teniola *et al*., 2001) or *Orgi* (Mugula *et al*., 2003), the approach using the predominant yeast strains as single or mixed starter cultures should be considered for a more predictable fermentation outcome. The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of Rwandese traditional sorghum beer *ikigage*. The selection of yeasts was accomplished by acidification power test, tolerance test to alcohol and the efficiency of ethanol production in natural medium. The study of flocculation capacity of selected yeasts and their flocculation phenotypes was also performed.

MATERIAL AND METHODS

Samples

A total of 12 samples of traditional leaven *umusemburo* were obtained from local producers of traditional sorghum beer *ikigage* in the northern (Ruhengeri and Byumba sites) and southern (Tumba and Muhanga sites) province of Rwanda. The local producers of *umusemburo* (one per site) were selected on the basis of frequency of their production. The samples were collected in screw-capped bottles, packed in an insulated icebox, transported to the laboratory and analyzed immediately for microbiological analysis.

Enumeration of microorganisms

Duplicate aliquots of *umesmburo* (10 mL) were homogenized with 90 mL sterile peptone physiological saline solution (1g Peptone, 8.5 g NaCl and 1000 mL distilled water). The homogenate was decimal diluted and the relevant dilutions surface plated. Total accounts aerobic mesophilic flora, lactic acid bacteria, yeasts and moulds were enumerated as described by Lyumugabe *et al*. (2010).

Wort fermentation using umusemburo

Malted sorghum worts collected from the traditional local brewers were dispensed into a 1000 mL Erlenmeyer flask, equipped with a gas trap and autoclaved at 121° C for 15

min. After cooling to about 45°C, 50 mL of *umusemburo* were added with 500 mL of malted sorghum wort and incubated at 30°C for 48h. After each 8h, the growth of total counts aerobic mesophilic flora, lactic acid bacteria, yeasts and moulds were evaluated during the fermentation. The yeasts isolated during fermentation were characterized and identified.

Characterization and identification of yeast isolates

Isolation was done by spread-plating 0.1 mL of suitable dilution of sample on YPD-Chloramphenicol (10 g yeast extract, 10 g peptone, 20 g glucose, 20 g agar, 0.5 g chloramphenicol and 1000 mL distilled water) after 48 to 72 h of incubation at 30°C. For characterization and identification of yeasts, isolates from *ikigage* fermentation were purified by successive sub-culturing on YPD. Preliminary confirmation was based on microscopic observation. The isolates were tested for the fermentation of glucose, sucrose, maltose, lactose and raffinose according the description of Van Der Walt and Yarrow (1984). The assimilation of nitrogen source (nitrate, ethylamine hydrochloride, L-lysine and cadavarine) was also tested. Physiological and biochemical characterization of the isolated yeasts were also determined by investigating the assimilation reactions of sugars using the API 20 C Aux. kit (BioMérieux, Lyon, France) according to the manufacturer's instructions. For the spore morphology, cells were grown on sodium acetate agar and Gorodkowa medium were used to induce the sporulation of yeasts whilst the hyphae growth was also determined by using RAT medium. The identification of yeasts according phenotypic characteristic was done using API taxon 2004 software.

Yeasts identified by phenotypic tests were confirmed by PCR – Sequencing of internal transcribed spacer (ITS) region of rDNA. The ITS1 - 5.8S - ITS2 regions of rDNA were amplified by PCR using the primer ITS1 (5P- TCCGTAGGTGAACCTGCGG-) and ITS4 (5P- TCCTCCGCTTATTGATATGC-) according to White *et al* (1990). The purified PCR products were directly sequenced using ABI 3130 genetic analyzer. Blast searches of sequences were performed at the National Centre for Biotechnology Information (NCBI) Gen Bank data library.

Acidification power test

The method of Kara *et al*. (1998) was used to determine the acidification power of yeasts strains. The pH meter was calibrated using the two-buffer method before each series of assays. Deionised water pH was adjusted to pH 6.5 for AP studies. Sterile deionised water (15 mL) was placed in a 50 ml conical centrifuge tube containing a conical stir bar. The pH of the water was monitored for 5 min with constant stirring. At the end of 5 min, a pH reading was recorded (AP0) and 5 mL of concentrated yeast slurry (10^9 cells/mL) was added to the centrifuge tube. The yeast suspension was allowed to stir for 10 min, after which the pH was recorded (AP10). Immediately after the recording of the AP 10, 5 mL of 20% glucose solution was added to the yeast suspension and allowed to incubate for 10 min. At the end of 10 min the final pH reading was recorded (AP20). The acidification power was calculated by subtracting the AP20 from the AP0 reading.

Wort fermentation using the yeast strains selected

The wort (SB) used is a mixture of sorghum malted wort (70%) from local brew and banana juice (30%). Worts were dispensed into a 100 mL and 500 mL Erlenmeyer flask and sterilized by autoclaving at 121° C for 15 min. After cooling to about 45° C, 0.1 mL of yeast strain selected was cultivated on 25 ml of wort in 100 mL Elernemeyer flask at 30° C for 24 h and then the aliquots of yeast culture were inoculated in 500 mL of wort. Initial cell number of yeast was adjusted to 10^7cfu/mL. Erlenmeyer flask (1000 mL), equipped with a gas trap, was incubated at 30° C for 72h. The yeast growth and ethanol were appreciated every 15 h.

Ethanol tolerance

Yeast strains were inoculated into 100 ml conical flask containing 40 mL of YPD liquid and incubated at 30° C for 48h. 1 mL of 48h old yeast culture were then inoculated into 100 mL containing 40 mL of YPD liquid supplemented with 5, 10 and 15 % of ethanol. Samples were taken after 24 and 48 h of incubation, diluted in 0.1% peptone broth when necessary followed by spread-plating (0.1 mL) on YPD- Agar plates. The colonies were enumerated after incubation at 30 °C for 48 h and colony forming unit (Cfu/mL) obtained.

Flocculation study

The flocculation rate was determined by spectrophotometric method (Hautcourt *et al.*, 1999). The yeast strains were inoculated in 10 mL of wort SB, sterilized by autoclaving at 121° C for 15 min, and incubated at 30° C for 72 h. The cell cultures obtained by the procedure describedabove were centrifuged and the cells were re-suspended in 5mL of Helm's tampon (0.51 g calcium sulfate, 6.8g sodium acetate, 4.05g acetic acid, 4% ethanol, 1000 mL distillated water, pH =4.5) and in 10 mL EDTA solution (46.5g EDTA, 0.35 sodium dihydrogenophasphate, pH=7.3 , 250mL distillated water). The degree of flocculation of the different strains was determined in terms of the ratio between the optical density at 620nm of the culture suspension in Helm's tampon (OD_B) and culture suspension in EDTA solution ((OD_A-OD_B/OD_A) x 100). The following flocculation scale was established: ratio $\leq$ 5%, 0 (no flocculent); ratio between 5% and 20% (low flocculent); ratio between 20% and 50% (medium flocculent); ratio $\geq$ 50% (high flocculent).

For the flocculation inhibition by sugars, the flocculent strains were cultivated at 30° C in sterile YPD medium for 72 h under shaking. The cultures were centrifuged at 5000 g for 5 min at room temperature and washed once with distilled water. Cells were suspended to a final density of 10^6 cfu/mL in 10 mL of Helm's tampon supplemented with glucose or mannose 1M. The cell suspension was maintained at room temperature for 20 min, and flocculation capacity in the presence of these sugars was determined. Flocculation of a strain of yeast is inhibited by sugar if the value of its rate in medium containing sugars solution is lower than the rate of the same strain suspended in calcium.

RESULTS AND DISCUSSION

Microbial population during *ikigage* fermentation

The results of the microbial content of traditional leaven "*umusemburo*" from Rwanda are indicated in **Table 1.** These results showed the predominance of yeasts and lactic acid bacteria in *umusemburo* leaven.

The evolution of microbial population during the fermentation of malted wort sorghum initiated by *umusemburo* leaven in shown in **Figure 1**. Total mesophilic aerobic flora and moulds increase after 8h and disappear respectively after 32 and 40h of fermentation. Yeasts and lactic acid bacteria resist until the end of fermentation, although the progressive reduction of lactic acid bacteria start after 40h. The disappearance of total flora and moulds would be due on the one hand to the acidity of sorghum wort during fermentation and on the other hand with the scarcity of oxygen. The increase of total acidity and decrease of pH during *ikigage* fermentation is accompagned with increase of lactic acid bacteria.

Yeasts and lactic acid bacteria are the predominant microorganisms involved in most African traditional foods and beverage fermentations (Odunfa, 1985; Mugula *et al*., 2003). The quantities relatively higher of yeasts were also reported in traditional "*ikigage*" beers (Lyumugabe *et al*., 2010), "*pito*" beers (Glover *et al.*, 2005) and "*tchoukoutou*" beer (Kayodé *et al.*, 2011) from several African countries. This suggests the existence of a double fermentation (alcoholic and lactic) indicated by several authors (Novellie, 1976; Munyaja *et al.*, 2003).

Characteristics of yeast strains isolated during *ikigage* fermentation

A total of 127 yeast strains were isolated during *ikigage* fermentation and subjected to morphological, fermentation and assimilation tests. The profiles of carbohydrate and fermentation and assimilation of yeasts isolates are shown in **Table 2** and **3**. None of the yeast isolates can ferment lactose, but they were able to ferment glucose (89 %), sucrose (87.7 %), maltose (51.6 %) and raffinose (24.2 %). 41.7 % of yeasts isolates were also able to assimilate ethylamine, L-lysine and cadaverine whereas none of the isolates can assimilate nitrate. Based on their assimilation of carbon compounds, 12 assimilation profiles were distinguished (62.3 %), galactose (56 %) maltose (51.6%) and glycerol (40.7 %). A minor part of the isolates were able to assimilate raffinose (29.7 %), N-acetyl – glucosamine (18.7 %), trehalose (14.3 %), methyl D-glucopyranoside (8.8%) and calcium 2-cetogluconate (6.6 %). None of them assimilated arabinose, xylose, adonitol, xylitol, inositol, sorbitol, cellobiose, lactose and melezitol. According to their phenotypic characters and also molecular identification (**Table 4**), the yeasts isolated during *ikigage* fermentation were found to belong to *S. cerevisiae*, *I. orientalis*, *C. humilis*, *C. incuspicua*, *C. magnoliae*, *C. krusei* and *P. membranifaciens*. The percentages of each species at various stages of *ikigage* fermentation are shown in **Table 5**. When the *ikigage* fermentation were initiated, we found *S. cerevisiae* (41.8 %), *C. incospicua* (21 %), *C. magnolia* (15.8 %), *P. membranifaciens* (15.8 %) and *C. krusei* (10.5 %). *S. cerevisiae* was the most frequent species at the various stages while *I. orientalis* and *C. humilis* were

observed after 8h of fermentation. At the end of fermentation, only *S. cerevisiae* (61 %), *I. orientalis* (29. 3 %), *C. humilis* (7 %) and *C. incospicua* (3.7 %) were found in the fermented product. These results indicate clearly the dominance of *S. cerevisiae* (53.5 %), follow - up by *I. Orientalis* during the *ikigage* fermentation process. The dominance of *S. cerevisiae* is a well indication of occurrence of the alcoholic fermentation during fermentation of *ikigage* beer. Other similar studies realized by reported *S. cerevisiae* as being the predominant yeasts species associated in the alcoholic fermentation of *pito* (Sefa-Deheh *et al*., 1999), *dolo* (Konlani *et al*., 1996; Van der Aa Kuhle *et al*., 2001) and *tchoukoutou* (Koyodé *et al.*, 2011). These authors reported that *S. cerevisiae* was often associated with other yeast species which may contribute to the organoleptic characters of African sorghum beers.

However, while agreeing on the dominance of *S. cerevisiae*, the occurrence of the non – Saccharomyces species seems to vary. *Candida tropicalis, Torulaspora delbrueckii, Kloeckera apiculata, Hansenula anomala, Schizosaccharomyces pombe* and *Kluyveromyces africanus* were found in sorghum beers from Togo, Burkina Faso and Ghana (Demuykor *et al.*, 1991; Konlani *et al*., 1996; Sefa-Deheh *et al.*, 1999). In *Burukutu* from Nigeria, Sanni *et al.* (1993) reported also the presence of *H. anomala, kloeckera apiculata, C. tropicalis, C. krusei, C. castelli, Geotricum candidum, P. membraefaciens* and *klyveromyces africanus*. Except *P. membraefaciens* and *C. krusei*, these yeasts species were not found in this work. We found the presence of *I. Orientalis, C. magnolia, C. humulis* and *C. incospicua* during *ikigage* fermentation.

Contrary to Westen African sorghum beers, where *Candida tropicalis* is predominant yeast strains after *S. crevisiae, I. orientalis* was predominant non-*Saccharomyces cerevisiae* yeast involved in fermentation of Rwandese sorghum beer. *I. orientalis* was also more dominant yeast specie in togwa, Tanzanian fermented food manufactured from sorghum (Mugulu *et al*., 2003).

Table 1. Microbial content (cfu/mL) of traditional leaven from Rwanda "umusemburo"

Sample origin	**Total mesophilic aerobic flora**	**Yeasts**	**Lactic acid bacteria**	**Moulds**
Byumba	78. 2 x 10^6	61. 2 x 10^6	81. 3 x 10^6	2.6 x 10^5
Muhanga	119.7 x 10^6	114.9 x 10^6	59. 2 x 10^6	5. 2 x 10^5
Ruhengeri	210.5 x 10^6	107.3 x 10^6	11. 2 x 10^6	5.5 x 10^5
Tumba	134.8 x 10^6	75.8 x 10^6	58. 2 x 10^6	5.5 x 10^5
Mean	134.8 x 10^6	89.8 x 10^6	52. 6 x 10^6	3.7 x 10^5

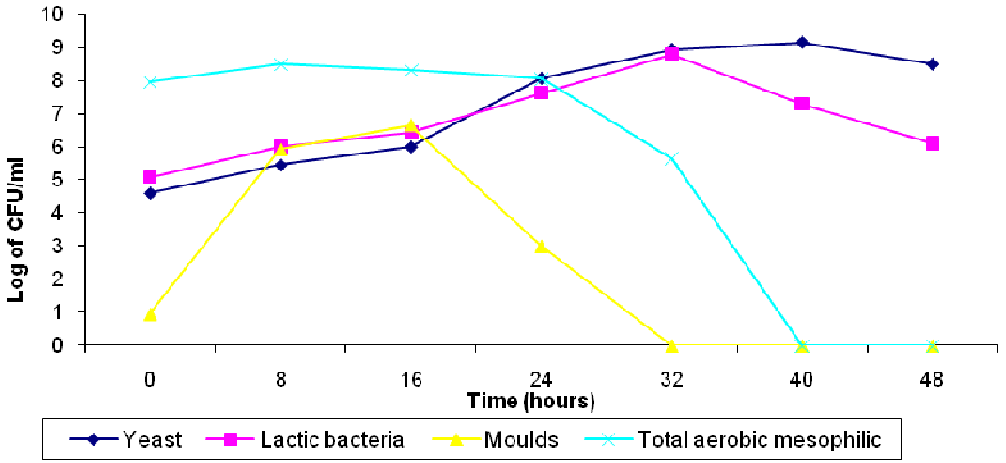

Figure 1. Evolution of microbial popualion of *umusemburo* during ferementation

Table 2. Carbohydrate assimilation profiles of yeasts isolated during *ikigage* fermentation

	*A	B	C	D	E	F	G	H	I	J	K	L	Total (%)
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	100
Glycerol	+	+	-	-	+	+	-	-	-	-	-	-	40.7
Calcium 2-ceto-gluconate	-	-	-	-	-	-	+	-	-	-	-	-	5.5
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	0
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	0
Adonitole	-	-	-	-	-	-	-	-	-	-	-	-	0
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Galactose	-	+	+	-	+	-	-	+	+	-	+	-	56
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	0
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Methyl D – glucopyranoside	-	-	-	-	-	-	-	-	-	-	+	-	8.8
N-acetyl – glucosamine	+	-	-	-	-	+	-	-	-	-	-	-	18.7
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	0
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	0
Maltose	-	-	+	+	+	-	-	+	-	+	+	-	51.6
Sucrose	-	+	+	+	+	-	+	+	+	+	+	-	62.3
Trehalose	-	-	-	-	-	-	-	-	+	-	+	-	14.3
Melezitol	-	-	-	-	-	-	-	-	-	-	-	-	0
Raffinose	-	-	-	-	-	-	-	+	+	+	-	-	29.7
Number of isolates (%)	7	17.3	11.8	10.2	7	5.5	6.3	8	8	8.6	8	2.4	

***A:** isolates L3, N1, K4, R3, A4, M2, N2, R8, R9; **B:** isolates RG1, F2, L6, RG2, R6, G7, V8, RT1, G6, B7, M6 ; A5, N3 ; G9, V11, A6, A7, M8, F5, F9, K12, T15; **C :** isolates RV6, RK1, G10, K8, RN4, RL1, K3, L5, R2, F1, T2, L11, L12, F7, V12 **; D :** isolates RT8 , B8, A8, RN6, RF3, V4, RT4, A1, N10, V3, L13, B5, N9 ; **E :** isolates RV2, F8, F6,RG3,RK5,V10, F4, L10, T7 ; **F:** isolates R4, T12, R7, R5, B3, L9, K7; **G:** isolates T6, T9, G4, T3, A10, R11, M7, M11; **H:** isolates RB2, V1, T14, T13, RA3, RB1, RN7, K6, V9, K10 ; **I:** isolates RR1, RT5, M5, G5, RT4, F10, A9, R10, R12, N8; **J:** isolates RM1, RM3, RM4, V5, RV7, A2, B4, K9, K11, G12, V13 ; **K :** isolates L7, M9, M10, B6, L8, L4, L2, K2, N5, G8. **L**: isolates T10, T11, G11.

Table 3. Fermentation characteristics and nitrogen assimilation profile of yeast isolated during *ikigage* fermentation

Isolates	**Fermentation of carbohydrate**					**Assimilation of nitrogen**			
	Glucose	Lactose	Maltose	Sucrose	Raffinose	Nitrate	Ethylamine	L-lysine	Cadaverine
L3, N1, K4, R3, A4, M2, N2, R8, R9	-	-	-	-	-	-	+	+	+
RB2, V1, RA3, RB1, RN7, K6, V9, RM1, RM3, RM4, V5, RV7, A2, B4, RF3, RK5, RK1, T14, T13, K10, K11, G12, V13, K9	+	-	+	+	+	-	-	-	-
RR1,RT5, M5, G5, RT4, F10,A9,R12, N8	+	-	-	+	+	-	-	-	-
RV6,G10,K8, RN4, RL1, K3, L5, R2, F1, T2, RT8 , RN6, V4, RT4, A1, V3, B5, RV2,RG3,V10, F4, L10, L7, B6, L8, L4, L2, K2, N5, G8, L11, L12, F7, V12, B8, A8, N10, N9, L13, F8, F6, M9, M10	+	-	+	+	-	-	-	-	-
RG1, L6, RG2, R6, G7, V8, RT1, G6, B7, M6 ; A5, N3 ; G9, V11, R4, T12, R7, R5, B3, L9, K7, T6, T7, T9, G4, T3, F2, K12, T15, F9, A7, M8, A6, F5, T12, A10, R11, M7	+	-	-	+	-	-	+	+	+
T10, T11, G11	+	-	-	-	-	-	+	+	+
Frequency (%)	89	0	51.6	87.7	24.2	0	41.7	41.7	41.7

Table 4. **Table 4**. Yeasts identified according to sequences of 5.8S rDNA-ITS region

Yeast isolates	Number of nucleotides compared	Percent homology with GenBank	GenBank accession number of corresponding sequence	Species
RB2	615	100	AM262829	*S. cerevisiae*
RL1	544	98	AB279747	*S. cerevisiae*
RK1	399	99	AY796193	*S. cerevisiae*
RG1	545	99	AF262033	*I.orientalis*
RN4	552	99	AM262830	*S. cerevisiae*
RT1	397	97	AF417255	*I.orientalis*
RG2	447	98	AB365318	*I.orientalis*
RR1	470	98	AY493349	*C. humilis*
RT5	470	98	AY493349	*C. humilis*
RV6	445	99	AM262824	*S. cerevisiae*
RT8	499	97	AB280539	*S. cerevisiae*
RM1	455	98	EU145764	*S. cerevisiae*

Table 5. **Table 5**. Distribution of yeast species (%) isolated during *ikigage* fermentation

	Fermentation time						
	0h	8h	16h	24h	32h	40h	48h
S. cerevisiae	41.8	50	47	50	66.6	63.2	61.5
I. orientalis	-	5.5	17.7	18.7	30.1	26.3	29.3
C. krusei	5.5	9.6	5.7	-	3.3	-	-
C. magnoliae	15.8	16.6	0	12.5	-	-	-
C. humilis	-	7	17.6	12.5	-	7.5	7
C. incospicua	21	7.9	11.7	6.2	-	3	3.7
P. membranifaciens	15.8	3.1	-	-	-	-	-

Selection of predominant yeast strains

Acidification power

Fourteen strains of majority yeast species (*S. cerevisiae* and *I. orientalis*) isolated at the end of *ikigage* fermentation were pre-selected and subjected to acidification power test. The results of acidification power test are represented in **Figure 2**. On the 23 yeast strains tested in this work, 7 strains (named RB2, RK1, RV6, RN4, RT8, RL1 and RG1) are highly active with good fermentation potential (AP value between 1.5 and 3), while 6 yeast strains (named RG2, RR1, RT5, RT8, RM3 and RN7) with AP values between 1 and 1.5 indicated a reduced metabolic activity and 10 yeast strains indicate low metabolic competence (AP value below 1) that could result in sluggish fermentations.

Acidification power indicates glycolytic activity and endogenous reserves of the yeast cell to maintain a fixed ratio between intracellular and extracellular hydrogen ion concentrations (Sigler *et al.*, 1981; Opekarova *et al*., 1982). It is closely related to viability and fermentation performance of yeast strains (Mathieu *et al*., 1991; White *et al*., 2003; Gabriel *et al*., 2008).

Ethanol tolerance

The effect of different concentrations of ethanol on the growth of 7 yeast strains (RB2, RK1, RV6, RN4, RT8, RL1 and RG1) previously selected was studied at 30 °C. The results obtained (**Figure 3**) show that all yeast strains studied resist very well to the concentration of 5% ethanol. There was a progressive decrease in the viable counts of all the isolates when the concentration of the ethanol was increased. *S cerevisiae* strains RV6, RB2 and RK1, and I. *orientalis* strains RG1 exhibited good tolerance at 10% ethanol. Only *I. orientalis* RG1 was able to survive in 15% ethanol.

Similar results have been reported by Day *et al.* (1975) for yeast brewing. They have found that the ability of different yeasts to tolerate high levels of ethanol varies widely. But, *Saccharomyces* strains brewing are fairly uniform in their response to ethanol, tolerating ethanol concentrations of 7 to 13%. *I. orientalis* is very well known as first yeast with high ethanol tolerance (Okuma *et al*., 1995; Isono *et al.*, 2012). The plasma membrane composition was identified as being central to the ethanol tolerance of yeast strains, with yeast responding to increased ethanol concentration, in a dose-dependent manner, by increasing the unsaturation index, and hence fluidity, of their membranes (Beaven *et al*., 1982 ; Odumeru *et al*., 1993. Alexandre *et al*., 1994).

Although ethanol tolerance and ethanol production ability are not directly correlated (Benitez *et al*., 1983), *S. cerevisiae* RK1, RV6 and RB2 strains and *I. orientalis* RK1 strain can be employed for optimization of *ikigage* beer processing with higher ethanol content. However, fermentable sugars in sorghum wort are limited (Palmer 1989; Dufour *et al*., 1992), addition of adjunct or development of

sorghum with improved malting characteristics will be necessary before sorghum beer with increased alcohol percentage can be produced.

Ethanol production

In this work, the banana juice was added as adjuvant before to produce *ikigage* beer. The ethanol produced by each yeast strains is shown in **Figure 4**. The final ethanol content of *I. orientalis* strain RG1 (2.38 % v/v) was lower than those of *S. cerevisiae* RV6, RK1 and RB2 strains (5.45 – 6.7% v/v), but *I. orientalis* produced high ethanol concentration after 15 and 30h. Ethanol production rates reflected the cell growth pattern. The faster cell growth of RG1 after 15 and 30h coincided with faster ethanol production rate because high ability of this strain to assimilate and fermente glucose. Recently, Isono *et al*. (2012) demonstrated that *I. orientalis* strains have a very high ability to ferment glucose to ethanol under high stress conditions (high temperature, such as acid, salt) comparatively to *S. cerevisiae*. Lower final ethonal concentration can be explained by not maltose assimilation and fermentation by *I. orientalis*. This also explains the weak attenuation limit (**Table 6**) compared to *S. cerevisiae* RV6, RB2, RK1 strains.

Flocculation

The results of floculation ability yeast strains and characteristics of flocculent strains are indicated in **Table 6**. The flocculation degrees of RV6, RB2, RK1 and RG1 were 14.7, 18.8, 1.5 and 29.2%, respectively. According to Gilliland (1951) classification, RK1 is non - flocculent strain ($\leq$ 5%) while RB2 and RV6 are low flocculent strains (between 5 and 20 %). RG1 is considered as medium flocculent strain (between 20 and 50%). *S. cerevisiae* RV6, RB2 and RK1 strains were glucose – mannose sensitive while *I. orientalis* RG1 strain was mannose insensitive.

Flocculating is a very important property in itself in the brewing industry as well as in biotechnological applications due to the fact that it is often the primary method of yeast separation. Premature yeast flocculation cause significant financial and logistical problems to the brewer due to incomplete conversion of sugars to alcohol resulting in higher residual extract and lower alcohol than specification demands, flavour abnormalities, disruption of process cycle times and potential issues with the re-use of the yeast in subsequent fermentations (Axell, 2003; Koizumi, 2006; 2009). While non – flocculation of yeast cause many problems for beer clarification (Speers *et al*., 2006). From this point of view, RV6, RB2 and RG1 are considered as good yeast strains for *ikigage* production. However, the flocculation involves lectin – like protein – carbohydrate recognition and interaction in a manner of calcium – dependent and sugar sensitive (Speers and Ritcey, 1995). The lectins are proteins in the walls of flocculent cells able to bind to carbohydrates on neighboring cells. These are specific proteins that require the presence of calcium to maintain their active conformation. Calcium has the property to activate the lectin while decreasing the repulsion between cells and increasing the

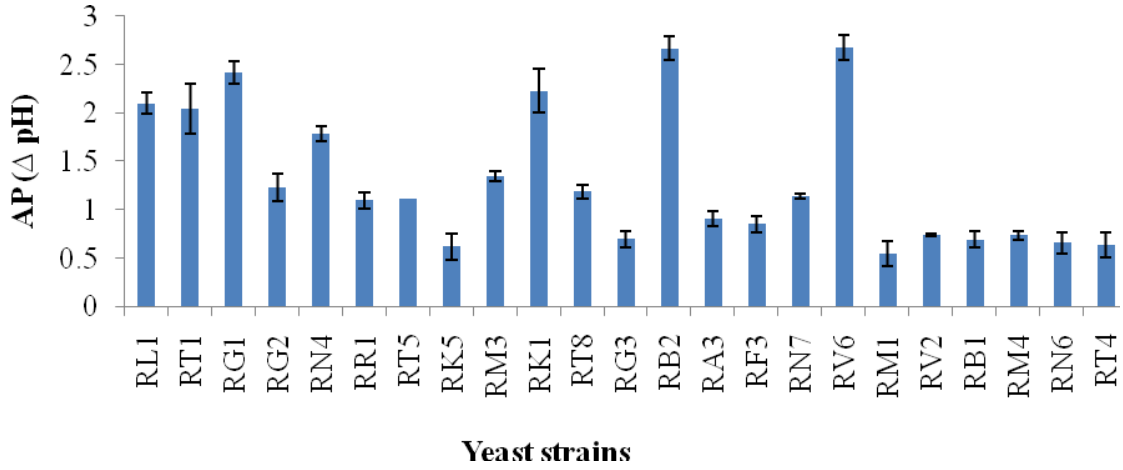

Figure 2. Acidification power of yeast strains pre-selected from *ikigage* fermentation

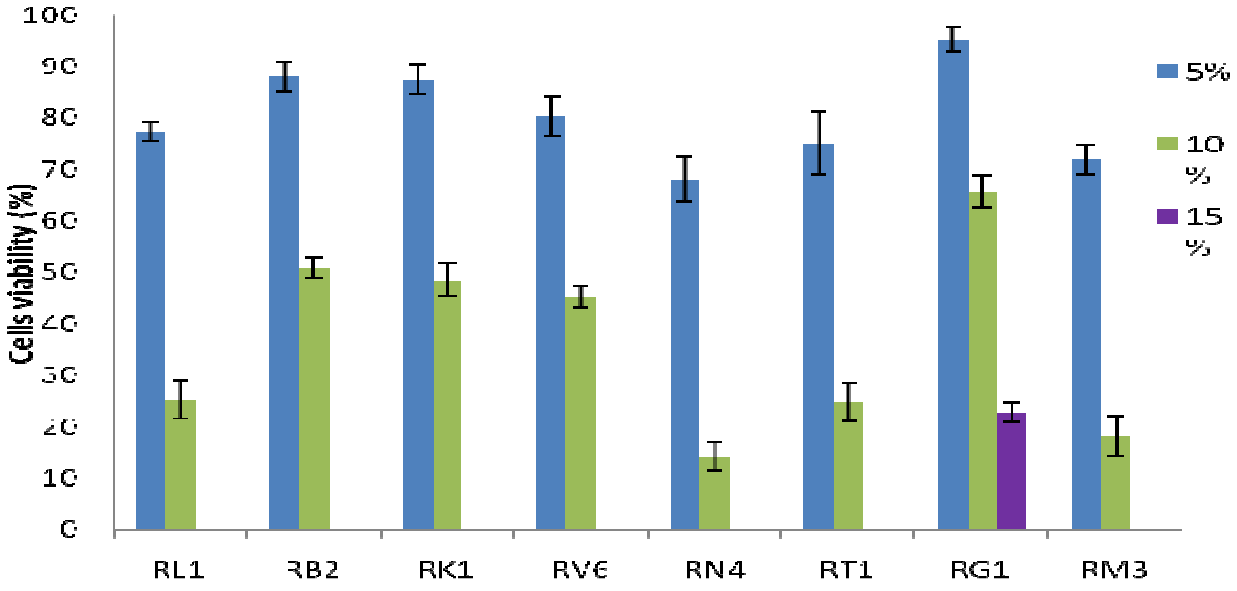

Figure 3. Effect of different concentrations of ethanol on growth of *S. cerevisiae* strains (named RL1, RB2 RK1, RV6, RN4, RT8 and RM3) and *I. orientalis* RG1 strain at 30 °C after 48h.

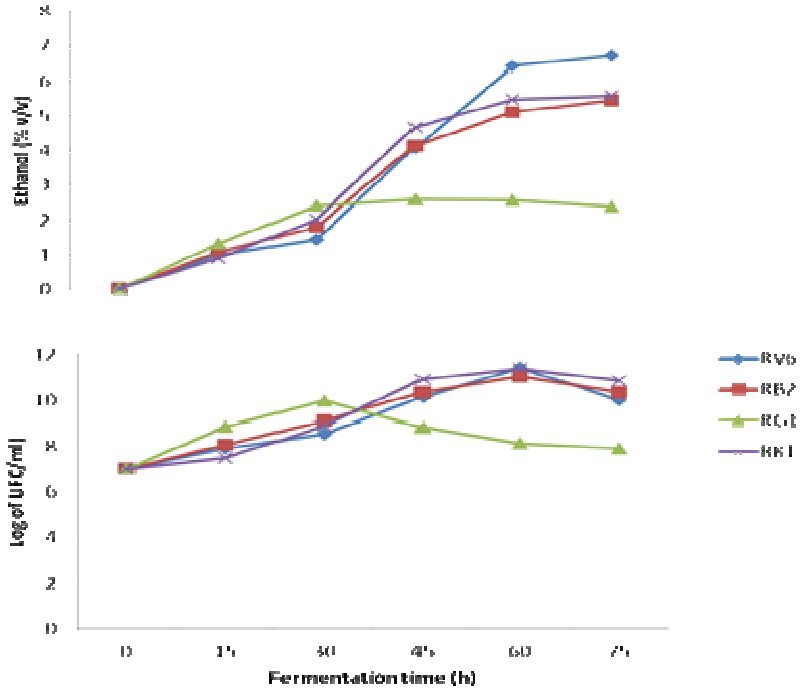

Figure 4. Ethanol production and yeast cell growth during fermentation

Table 6. Attenuation and flocculation characteristics of yeast strains isolated from *umusemburo* leaven

	RV6	RB2	RG1	RK1
Flocculation rate (%)	14.7	18.8	29.2	1.45
Flocculation inhibition by sugar	Newflo or GMS	Newflo or GMS	MI	Newflo or GMS

hydrophobicity of cell surfaces. The fixing of calcium ions on lectin site adjacent to the binding site for sugars passes them to their active form (Stratford, 1992; Yu-Lai *et al*., 1998; Stan *et al*., 2000). Three flocculation phenotypes have been described according to sugar specificity: Flo 1, flocculation of yeast was only inhibited by mannose (MS); the NewFlo phenotype, inhibited by glucose and mannose (GMS); and the mannose insensitive MI phenotype, following Stratford (1989) and Masy *et al*., 1992. The majority of brewery yeast strains belong to the NewFlo phenotype (Soares &Vroman, 2003). In our wort, the selected *S. cerevisiae* strains were glucose – mannose sensitive while *I. orientalis* strains were mannose insensitive. According to Masy *et al*. (1992), flocculation of yeasts classified as mannose insensitive could be produced by hydrophobic

interactions or specific interactions not involving mannans (protein–protein, protein–lipid).

CONCLUSION

The present study proposes the characterization and the selection of powerful yeasts involved in the fermentation of *ikigage.* The yeasts involved in *ikigage* fermentation belong to species of *S. cerevisiae*, *I. orientalis*, *C. humilis*, *C. incuspicua*, *C. magnoliae*, *C. krusei* and *P. membranifaciens*. *S. cerevisiae,* followed by *I. orientalis*, was predominant at the end of fermentation. Seven strains belong to *S. crevisiae* (6) and *I. orientalis* (1) were selected because their high acidification power and ethanol tolerance. *I. orientalis* RG1 strains was able to survive in 15% ethanol and fast convert glucose into ethanol while *S. crevisiae* strains (RV6, RB2 and RK1) produced high final ethanol content. The yeast strains selected are non – flocculent (RK1), low flocculent (RV6 and RB2) and medium flocculent (RG1) strains. *S. cerevisiae* RV6, RB2 and RK1 were Newflo phenotype while *I. orientalis* RG1 strain was MI phenotype. Except RK1, which can cause problems of beer clarification, these yeast strains revealed suitable for improving the quality of *ikigage* beer. However, further researches are needed to establish the flavour of *ikigage* beer produced from these yeast strains.

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