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Biochemical characterization and growth patterns of new yeast isolates

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Abstract African sorghum opaque beers play a vital role in the diet of millions of consumers. In the current study we investigated the growth profiles of yeast strains isolated from kpete-kpete, a traditional starter used to produce tchoukoutou, an opaque sorghum beer in Benin. 10 yeast strains were isolated from sorghum beer starters and cultivated under both liquid and solid media for phenotypic growth characterization. All yeast isolates were able to grow both on solid and liquid media. Based on their growth profiles, the isolates were clustered into three groups: (i) the aggressive growth pattern (30 %), (ii) the moderate growth pattern (50 %), and (iii) the slow growth pattern (20 %). Based on gene expression pattern, absorbance (A_{600nm}) and diameter of growth in both liquid and solid media respectively, yeast strains YK34, YK15 and YK48 were clustered in the first group, and referred to as the most aggressive growth strains, followed by group 2 (YK24, YK5, YK12, YK20, YK2) and group 3 (YK37, YK41). This growth pattern was confirmed by *Invertase* gene expression profiling of the yeasts showing group 1 with high level of *Invertase* gene expression followed by group 2 and group 3 respectively. Our results suggest that YK34, YK15 and YK48 and YK2 yeast strains constitute the best candidates in fermentation of sorghum beer production based on growth rate and assimilation of carbon and nitrogen sources.

Keywords Yeast · *Saccharomyces cerevisiae* · Growth · Sorghum beer · Tchoukoutou · Invertase gene · Benin

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Introduction

All over the world people have learnt to culture and use essential microorganisms for production of alcoholic beverages and fermented foods. Fermentation is a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidizing agent [1]. In general, that process proceeds under the influence of activities exerted by enzymes and microorganisms [2]. Even though the scientific explanation and the identity of these beneficial microorganisms mostly lactic acid bacteria, filamentous moulds and yeasts were unknown to people in the past, they cultured them traditionally for production of foods for consumption [3]. In Europe, America and Africa fermented foods are prepared exclusively using bacteria or bacteria-yeasts mixed culture [4].

Yeasts serve a critical role in the production of many traditionally fermented staple foods and beverages across the world [5]. Numerous studies have examined the major role of yeast genera in different sorts of fermented foods process: fermented meat products [6], fermented milk products [7] fermented cereals products [8, 9] and alcoholic beverages [10, 11]. Alcoholic beverages represent a vast diversity of products ranging from table wines, sake, cider, fruit wines, beer and distilled alcoholic products [12, 13]. Cereals are the main substrates for fermentation [14]. In

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Africa some beers are made from cereals, especially from sorghum which is the major crop used to produce traditional opaque beers [15]. These beers are known as Kaffir in South Africa [16], Otika or burukutu in Nigeria [17], mtama in Tanzania [18] doro or chibuku in Zimbabwe [19], bili bili in Tchad [20], dolo in Burkina Faso [21], ikigage in Rwanda [22] and tchoukoutou in Togo and Benin [23]. African sorghum opaque beer are mainly consumed by the poorest people in rural communities [24], and they play a significant role as a source of dietary nutrients for millions of consumers [25, 26]. However, African sorghum opaque beers are still produced using traditional manufacturing processes where mixtures of wild yeasts and bacteria conduct the fermentation [3]. In brewing of tchoukoutou, an opaque sorghum beer largely produced and consumed in Benin, the yeasts involved in the fermentation process are uncharacterized.

The fermentation process performance depends on a controlled management of fermentation microorganisms [14]. Up to now little is known about the diversity and growth pattern of yeasts involved in fermentation of sorghum wort during tchoukoutou production. To obtain a desired beverage in terms of quality and flavor, a careful combination of fermentative yeast strains is necessary. Therefore, in this work we studied the phenotypic characterization of yeasts isolated from different sorghum beer starters to improve the production of traditional beer (tchoukoutou). Yeast strains responsible for the fermentation process in tchoukoutou production were here characterized on basis of their growth pattern in both liquid and solid media.

Materials and methods

Microorganisms

A total of ten yeast strains previously characterized in our lab as *Saccharomyces cerevisiae* and *Candida heliconiae* were used in this study. These were isolated from sorghum beer starters (kpete-kpete). Kpete-kpete is the traditional starter used in the fermentation of tchoukoutou, a local beverage made from sorghum in Benin, West Africa. Samples of starters were collected from ten commercial processing sites in the central regions of Benin (West Africa) and screened for microbial strains according to Hounhouigan et al. [27].

The yeast strains were purified by successive sub-culturing on a selective growth medium made of Oxytetracycline Glucose Yeast Agar (OGYA, CM0545, Basingstoke Hampshire, England). Preliminary identification was made according to Barnett et al. [28] and Kurtzman et al. [29]. Glycerol stocks of the strains were made and stored at −80 °C.

Media and culture growth conditions

For liquid culture, yeast cells were grown on 5 mL yeast peptone dextrose (YPD: 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) and incubated at 30 °C with shaking at 270 rpm for 24 h [30]. For solid culture, YPD agar (1 % yeast extract, 2 % peptone, 2 % dextrose, 1.2 % agar) was prepared, inoculated with yeast strain and incubated at 30 °C.

Phenotypic characterization of yeasts on liquid growth media

In liquid culture experiments, 1 mL of pre-culture with equal optical density (OD = 0.6 at 600 nm) was used to inoculate 10 mL fresh liquid media and allowed to grow for phenotypic characterization of the strains. Yeasts were grown in liquid culture (as described above) and monitored by measuring the optical density 24 h after inoculation. For data analysis, 200 µL of a 24 h-old liquid culture were diluted with 800 µL ddH_2O and the optical density was taken at 600 nm, using a spectrophotometer (Beckman Coulter DU 530 UV/Vis.).

Phenotypic characterization of yeasts on solid medium

For solid media, pre-cultures of yeast strains (20 µL) of identical growth stage (OD = 0.6 at 600 nm) were used to inoculate the center of YPD-agar petri dishes and allowed to grow for 3 days, while being observed daily for phenotypic characterization. Data was recorded using a digital camera. After 72 h of incubation, the yeast growth was measured by taking the diameter of the colonies on the plates.

Microscopic characterization of yeast strains

For microscopy, overnight liquid growth culture of yeasts was harvested by spinning (8,000 rpm) the culture at room temperature for 10 min and the pellet washed once with sterile double distilled water (ddH_2O). The yeast cells were then re-suspended into 1 mL $sddH_2O$ and stained with 1 % W/V Congo-Red to obtain contrast during microscopy. The cells were mounted on slides and covered with cover-slips and observed under transmission microscope mounted with digital camera. The cell images were recorded and processed with Photoshop CS4 image software.

Biochemical characterization of yeast strains

The strains were tested for the fermentation of sucrose, lactose, glucose and raffinose, as well as the assimilation of selected nitrogen sources i.e. nitrate, ethylamine, L-lysine,

Table 1 Sequences of oligonucleotide primers used in this study

Name	Primer sequence	Description	Accession number	Chromosome
InVertase-F	5′CAAGAGTGGCTTCTGGGATAA3′	For RT-PCR	ABSV0100117	Chr # 9
Invertase-R	5′TCCGGGCTCTACTAAGGATAC3′	For RT-PCR	ABSV0100117	Chr # 9
Actin-F	5′GGAACGGAGGAATCCTGATAAC3′	For RT-PCR	ABSV01000750	Chr # 6
Actin-R	5′CCAACACTTCGTGGTCAACTA3′	For RT-PCR	ABSV01000750	Chr # 6

Table 2 Growth pattern characterization of yeast strains

Groups	Sample codes	Yeast name	Growth on liquid media (absorbance A_{600})	Growth on solid media (diameter in cm)	Aspects (color)
1	YK34	*S. cerevisiae*	0.514 ± 0.012^{a}	3.8 ± 0.0^{a}	Reddish
	YK48	*S. cerevisiae*	0.493 ± 0.004^{a}	4.0 ± 0.0^{a}	Reddish
	YK15	*S. cerevisiae*	0.471 ± 0.002^{a}	3.4 ± 0.1^{a}	Reddish
2	YK24	*S. cerevisiae*	0.062 ± 0.000^{b}	2.4 ± 0.1^{b}	Whitish
	YK5	*S. cerevisiae*	0.061 ± 0.003^{b}	2.4 ± 0.1^{b}	Whitish
	YK12	*S. cerevisiae*	0.058 ± 0.002^{b}	2.0 ± 0.0^{b}	Whitish
	YK20	*S. cerevisiae*	0.058 ± 0.002^{b}	2.8 ± 0.1^{b}	Whitish
	YK2	*C. heliconiae*	0.057 ± 0.000^{b}	2.2 ± 0.0^{b}	Whitish
3	YK37	*S. cerevisiae*	0.033 ± 0.000^{c}	1.0 ± 0.1^{c}	Whitish
	YK41	*S. cerevisiae*	0.032 ± 0.009^{c}	1.0 ± 0.1^{c}	Whitish

Values with the same letter are not significantly different from each other ($p < 0.05$)

cadaverine, and creatine. The assimilation of carbon sources was performed using API 20 C AUXstrips (BioMérieux, Lyon, France) according to the manufacturer's instructions.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Invertase gene expression level, known to be important during fermentation process of yeasts was assessed via qRT-PCR analysis to further characterize the strains at genetic level. Total RNA from yeast strains was extracted using Trizol and reverse transcribed into cDNA using qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD, USA). Real-time qPCR was then performed on Eco real-time PCR system (Illumina, San Diego, CA, USA) using PerfeCTa SYBR green FastMix (Quanta BioScience, Gaithersburg, MD, USA) to analyze the relative Invertase gene expression levels. Actin gene was used as the internal reference control. The specific Invertase and Actin gene primer pairs (Table 1) were used to perform the qRT-PCR analysis, which was performed in triplicate for each gene [31].

Statistical data analysis

Unless otherwise stated, the experiments were performed at least in three replicates throughout this work. The statistical significance of the data was analyzed using a univariate analysis of variance ($p < 0.05$) (one-way ANOVA; SPSS for Windows, version 16.0, Chicago, IL, USA).

Results and discussion

Growth profiles of the yeast strains

Yeasts have some advantages including rapid growth, ease of cultivation and the ability to be grown in an inexpensive medium [32]. These growth properties can have a real relevance in the fermentation process during the production of alcoholic beverages. Fermentation is the cumulative effect of growth of yeast on wort, ultimately resulting in production of alcohol in the growth medium, [30]. We therefore focused on characterizing growth parameters of yeast strains isolated from kpete-kpete in this study.

Ten strains of yeasts studied in this work (Table 2) were cultivated on both solid and liquid media. The data revealed three growth clusters as shown in Table 2. All yeast strains were able to grow on solid media. However their growth patterns showed different growth rates, which we used to classify them into three groups. Three (YK15, 34, 48) yeast strains (30 %) representing the first group grew after 20 h, five (YK2, 5, 12, 20, 24) (50 %) clustering in the second group grew after 48 h and (20 %) (YK37, YK41) representing the third group grew after 72 h (Table 2). In liquid culture, all yeast strains grew after

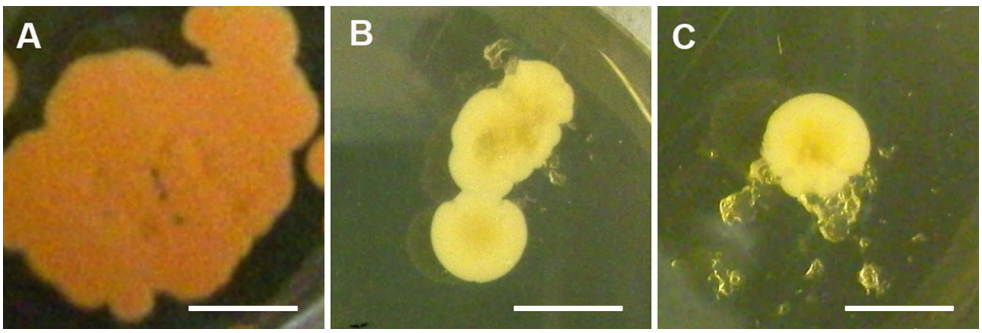

Fig. 1 Growth pattern of yeast strains on solid media. Representative of each group was depicted in **a** (group 1), **b** (group 2) and **c** (group 3). *Bars* 1 cm

24 h. They were also clustered under the same groups (groups 1, 2, 3). The first group three strains (YK15, 34, 48) (30 %) grew at 18 h after incubation, while the seven other strains (YK2, 5, 12, 20, 24, 37, 41) (70 %) were able to grow after 24 h and therefore grouped under a big cluster 2. However, based on the optical density, cluster 2 could be subdivided into two sub-groups where sub-group 1 was represented by YK2, 5, 12, 20, and YK24 with higher optical density values and sub-group 2 was represented by YK37 and 41 with lower optical density. Overall, the growth patterns of the yeasts were clustered into three groups under both solid and liquid conditions (Table 2; Fig. 1a–c).

We next carried out structural characterization of the strains under the microscope. Yeast cells, easily recognizable through their reproductive budding system were easily identified as depicted in Fig. 2(a–c, arrow heads), demonstrating that the organisms characterized in this study were indeed yeast strains. The growth pattern on solid media showed colonies of varying sizes and colors (Fig. 1; Table 2).

Under the same growth conditions, all the yeast strains were able to grow both on solid and liquid media. However, they displayed significant phenotypic growth differences ($p < 0.05$) in terms of growth diameters, absorbance, as well as growth period. These differential growth patterns indicate that the isolates have different sensitivity to growth conditions [33]. It has been reported that microbial growth depends on several factors which include physical factors, such as pH and temperature, chemical factors which include nutrient, redox potential, antimicrobial agents, etc., as well as biotic factors such as antagonism [34]. More importantly, in biotechnological applications, the growth of yeasts can be affected by carbohydrates utilization, nitrogen uptake, mineral requirements, presence of oxygen, pH and temperature [30, 35]. Hanscho et al., [36] found that BY strain of *Saccharomyces cerevisiae* grew to different cell densities when cultivated in a defined medium composition. In addition, growth of yeast strains can be arrested after depletion of lysine and other specific amino acids [36], suggesting that standard recipes for yeast growth medium are required for optimum growth adaptation and ultimate fermentation by yeast strains.

Group 1 of yeast strains: the aggressive growth pattern

Yeast Strains YK34, YK15 and YK48 are ranked into aggressive growth pattern (Table 2) because of some characteristics that distinguish them from others strains. They had the significantly ($p < 0.05$) largest diameters (4.0–3.4 cm), the highest absorbance at 600 nm (0.514–0.471), the shortest growth period on plates (20 h) and in liquid culture (18 h). They have a reddish color on solid media (Fig. 1a). Yeast strains of aggressive growth pattern were essentially characterized by their rapid growth. That is due not only to their ability to adapt faster to physical factors but also their capability to utilize carbohydrates, nitrogenous, mineral compounds contained in the YPD medium. These results are in agreement with those reported by Jang et al. [37], who cultivated *Saccharomyces cerevisiae* in YPD medium and obtained maximum growth in liquid culture after about 20 h.

Another characteristic of strains with an aggressive growth pattern was their reddish color. Chatterjee et al. [35] reported that yeast strain BUSCY1 grown on YPS (yeast extract, peptone and sucrose) exhibited reddish color and the medium has been suspected to support the synthesis of the reddish pink color pigment. In the present study, strains were cultured in YPD and cells were able to synthesize the pigment. According to findings by Lieckefeld et al. [38], stress or environmental stimuli can cause metabolic changes in an organism, acting as expression activator for genes involved in the synthesis of specific compounds that protect the organism. However, a molecular characterization may be necessary to confirm this hypothesis. The studied yeast strains (YK15, 34, and 48) produced the reddish pigment without being under stress conditions, suggesting that the production of the reddish pigment by these yeasts is part of their normal metabolism.

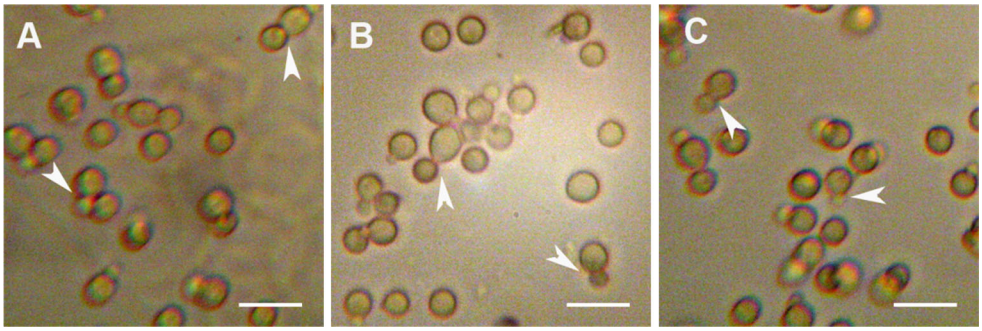

Fig. 2 Microscopic characterization of yeast strains. Representative of each cluster was depicted in **a** (cluster 1), **b** (cluster 2) and **c** (cluster 3). *Arrow heads* show budding dividing yeast cells. *Bars* 40 μm

Table 3 Fermentation and nitrogen source assimilation of yeasts strains

Groups	Samples codes	Yeast name	Fermentation				Assimilation of nitrogen source		
			Glucose	Sucrose	Rafinose	Nitrate	Ethylamine	Lysine	Cadaverine
1	YK 34	*S. cerevisiae*	+	+	+	+	+	+	+
	YK 15	*S. cerevisiae*	+	−	−	+	+	+	+
	YK 48	*S. cerevisiae*	+	+	−	−	−	−	−
2	YK 24	*S. cerevisiae*	+	+	−	−	−	−	−
	YK 5	*S. cerevisiae*	+	−	−	−	−	−	−
	YK 12	*S. cerevisiae*	+	+	−	+	−	−	+
	YK 20	*S. cerevisiae*	+	+	−	+	−	+	−
	YK 2	*C. heliconiae*	+	−	−	+	+	+	+
3	YK 37	*S. cerevisiae*	+	+	+	+	+	+	+
	YK 41	*S. cerevisiae*	+	+	−	−	−	−	−

(+) = assimilation of nitrogen source, (−) = unable to assimilate the nitrogen source

Group 2 of yeast strains: the moderate growth pattern

Out of the ten yeast strains, five strains (YK24, YK5, YK12, YK20, YK2) (50 %) had a moderate growth pattern based on their optical density and growth diameter values compared to the yeast strains in group 1 (Table 2; Fig. 1a). The colony diameters of strains in group 2 were significantly ($p < 0.05$) smaller (2.8–2.0 cm) and their absorbance (600 nm) was lower (0.062–0.057) compared to group 1 (Table 2). Their growth on solid and liquid media was slower than that of group 1. In addition, yeast colonies in group 2 were white in color, which is a common characteristic of yeasts. This observation suggests that group 1 strains are the first to vigorously ferment/use the carbon sources in the media, producing less complex carbohydrates that will allow yeasts in group 2 to feed on during the production of sorghum beer. The contribution of group 2 yeast strains to the fermentation process is minimal compared to group 1. However, they could be important in flavor and other properties of the final beverage.

Group 3 of yeast strains: the slow growth pattern

The third group of yeast strains (YK37, YK41) (20 %) are characterized by a slow growth pattern (Table 2; Fig. 1c), based on their smaller colony diameter (1.0 cm), lower absorbance (0.033–0.032), and slower growth on solid medium (72 h) compared to groups 1 and 2. Like group 2, group 1 yeasts displayed a whitish color (Fig. 1b, c). This data suggests that group 3 yeasts could only feed and grow easily on the less complex carbohydrate sources produced from group 1 and 2 during production of sorghum beer. However, our data suggests also that group 3 is able to growth in the same carbon sources without the cooperation of group 1 and 2, but they will require longer time for growth since their growth rate is slower (Table 2).

Assimilation of carbon and nitrogen sources

More importantly, we examined the fermentation profile of these strains under different carbon and nitrogen sources to

Table 4 Assimilation profiles of yeasts strains

Sample code	YK 34	YK 15	YK 48	YK 24	YK 5	YK 12	YK 20	YK 2	YK 37	YK 41
Tentative identification	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*C. heliconiae*	*S. cerevisiae*	*S. cerevisiae*
D-glucose	+	+	+	+	+	+	+	+	+	+
Glycerol	−	−	−	+	−	−	−	+	−	−
Potassium 2-cétogluconate	−	−	−	−	−	−	−	+	−	−
L-Arabinose	−	−	−	+	−	−	−	+	−	−
D-Xylose	−	−	+	+	–	+	−	−	−	−
D-Galactose	+	+	+	–	+	+	+	+	+	+
D-Sacharrose	−	−	+	−	−	−	−	+	−	−
N-Acétyl-glucosamine	−	−	−	+	+	+	−	+	−	−
Lactic acid	−	−	−	+	−	+	−	+	−	−
D-Raffinose	−	−	−	−	−	−	−	+	−	−
D-Maltose	+	+	+	−	−	−	+	+	+	+
D-Trehalose	−	−	−	−	−	−	−	+	−	−
Methyl-αD-glucopyranoside	−	−	−	+	−	+	−	+	−	−
D-Mannitol	−	−	−	+	−	+	−	+	−	−
D-Lactose	−	−	+	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	+	−	−	−	−
D-Ribose	−	−	−	+	−	−	−	−	−	−
Palatinose	−	−	+	+	+	−	−	+	−	−
Erythritol	−	−	−	+	−	−	−	−	−	−
D-Melibiose	−	−	−	+	−	−	−	−	−	−
Sodium glucuronate	−	−	−	+	−	−	−	−	−	−
D-Melezitose	−	−	−	+	−	−	−	−	−	−
Potassium gluconate	−	−	−	+	−	−	+	−	−	+
Levulinic acid	−	−	−	+	+	−	−	−	−	−

(+) = assimilation of carbon/nitrogen source, (−) = unable to assimilate carbon/nitrogen source

establish their biochemical characterization. As shown in Table 3, group 1 yeast strains were able to use almost all carbon and nitrogen sources examined with the exception of strain YK48. The carbon and nitrogen assimilation profile of some members in group 3, not those of group 2, was more similar to the pattern seen in group 1 (Table 2). However, group 3 was composed of slow growing yeast strains (Table 2). In order to further understand this discrepancy in carbon and nitrogen assimilation pattern, we assessed their ability to use different sugars and nitrogen metabolites (Table 4). These sugar and nitrogen sources ranged from simple to relatively complex molecular structures to gain understanding on the ability of the yeasts to ferment complex carbon/nitrogen sources. Our data suggest that group 1 and group 2 were able to use the simple as well as the complex carbon/sugar sources more than group 3 (Table 4). Interestingly, yeast strain YK2 from group 2 seems to be able to use almost all the sugars and nitrogen sources tested followed by YK24 of group 2 (Table 4). Our data suggest that the yeasts with moderate growth pattern (group 2) are better carbon and nitrogen assimilators than group 1 and group 3. A combination of group 1 and group 2 yeast strains were found to be the best combination to assimilate all carbon and nitrogen sources tested in this study (Table 4).

Invertase gene expression profile

Invertase is the enzyme that catalyzes the hydrolysis of sucrose into an equimolar mixture of glucose and fructose during yeast fermentation process. It represents a key enzyme in a wide range of fermentation beverages and bakery industries. We assessed the expression level of Invertase gene in the Kpete-Kpete isolated yeasts to further characterize them at genetic level and to determine their ability to utilize sucrose for their growth. As expected, members of group 1 showed a significantly higher level of invertase transcript accumulation compared to group 2 and group 3 when growing in media containing sucrose as carbon source (Fig. 3). Likewise, invertase gene expression from members of group 2 was higher than that of members of group 3 (Fig. 3), suggesting that group 1 can better assimilate sucrose better than group 2, and group 2 better than group 3, respectively. Invertase enzyme in yeast is an extra-cellular, glycoprotein localized at the periplasmic space (inter-space between the plasma membrane and its outer cell wall). This plays the important biological function of cleaving sucrose from the outside of the cell (in the cultural media) into monosaccharides that can be transported (and subsequently metabolized) in the cytoplasm. This might justify why group 1 has the fastest growth pattern, followed by group 2 and then by group 3 (Table 2).

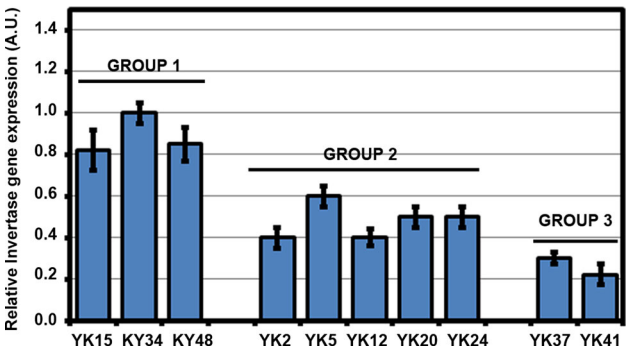

Fig. 3 Expression profile of Invertase gene from the yeast isolates. The expression pattern within the yeast groups is depicted *A.U.* arbitrary unit

Conclusions

In this study, yeast strains YK15, YK34 and YK48 of group 1 are considered the faster growing yeasts for traditional sorghum starter based on their high adaptation capacity and their ability to grow rapidly on liquid and solid media and gene (Invertase) expression data profiling. In addition, our data suggest that a combination of groups 1 and 2 might be the best for sorghum beer fermentation process. In particular, YK34 and YK2 were suggested to be the best strains among all the ten yeast strains studied in this work based on faster growth and different carbon and nitrogen assimilation sources. These strains have the highest potential to be promising yeast candidates for starter cultures and fermentation process during sorghum beer preparation.

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Competing interests The authors declare that they have no competing interests.

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