Fermentation profile of *Saccharomyces cerevisiae* and *Candida tropicalis* as starter cultures on barley malt medium

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Abstract *Saccharomyces cerevisiae* C8-5 and *Candida tropicalis* F0-5 isolated from traditional sorghum beer were tested for kinetic parameters on barley malt extract, YPD (863 medium) and for alcohol production. The results showed that *C. tropicalis* has the highest maximum growth rate and the lowest doubling time. Values were 0.22 and 0.32 h⁻¹ for maximum growth rate, 3 h 09 min and 2 h 09 min for doubling time respectively on barley malt extract and YPD. On contrary, glucose consumption was the fastest with *S. cerevisiae* (−0.36 and −0.722 g/l/h respectively on barley malt extract and YPD). When these two yeasts were used as starters in pure culture and co-culture at proportion of 1:1 and 2:1 (cell/cell) for barley malt extract fermentation, we noticed that maltose content increased first from 12.12 g/l to 13.62–16.46 g/l and then decreased. The highest increase was obtained with starter *C. tropicalis* + *S. cerevisiae* 2:1. On contrary, glucose content decreased throughout all the fermentation process. For all the starters used, the major part of the ethanol was produced at 16 h of fermentation. Values obtained in the final beers were 11.4, 11.6, 10.4 and 10.9 g/l for fermentation conducted with *S. cerevisiae*, *C. tropicalis*, *C. tropicalis* + *S. cerevisiae* 1:1 and *C. tropicalis* + *S. cerevisiae* 2:1. Cell viability measurement during the fermentation by using flow cytometry

revealed that the lowest mean channel fluorescence for FL3 (yeast rate of death) was obtained with *C. tropicalis* + *S. cerevisiae* 2:1 after 48 h of fermentation.

Keywords Barley malt extract · *Candida tropicalis* · *Saccharomyces cerevisiae* · Kinetic culture · Flow cytometry

Abbreviations

YPD Yeast Peptone Dextrose
FSC Forward Scatter
SSC Side Scatter

Introduction

In sub-Saharan African countries, traditional fermented beverages such as *ikigage* (Lyumugabe et al. 2010), *tchoukoutou* (Kayodé et al. 2007), *dolo* (Dicko et al. 2006) *pito*, *bili bili* (Maoura et al. 2005), *tchapalo* (N'guessan et al. 2010) are prepared from sorghum and/or millet malts. The fermentation of these beverages is uncontrolled and the microorganisms that intervene come from the raw materials, equipment and local environments or from residues of previous fermentation batch. These microorganisms, by virtue of their metabolic activities, play an active role in physical, nutritional and organoleptic modification of starting material. However, the wide variety of microorganisms present in a spontaneously fermented food gives a product with widely varying quality.

A very varied yeast and lactic acid bacteria has been found in African sorghum beers, although *S. cerevisiae* and heterofermentative “*Lactobacillus* sp.” usually predominate (Sefa-dedeh et al. 1999; Chamunorwa et al. 2002; Kayodé et al. 2007; Lyumugabe et al. 2010). Traditional African sorghum beers are very rich in calories, nutrition value B-

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group vitamins including thiamine, folic acid, riboflavin, and nicotinic acid, and essential amino acids such as lysine (Chevassus-Agnes et al. 1979). The beers are consumed at various festivals and African ceremonies (e.g., marriage, birth, baptism, the handing over of a dowry, etc.) and constitute a source of economic return for the female beer producers. However, in the majority of African countries, traditional sorghum beers are less attractive than Western beers brewed with barley malt because of their poor hygienic quality, low ethanol content, organoleptic variation and unsatisfactory conservation (Sanni et al. 1999; Lyumugabe et al. 2010). The use of starter culture was so suggested as the appropriate approach to alleviate the problems of variations in organoleptic quality and microbiological stability (Sanni 1993; Holzapfel 2002). The use of pure strains or starters requires knowledge of the viability of these strains. Flow cytometry is used to measure and analyze multiple physical characteristics of individual cells and other particles within heterogeneous suspensions. It is used for a wide range of applications including immunophenotyping (Hwang et al. 2012; Liu et al. 2012), cancer diagnosis (Lacombe and Belloc 1996; McCoy and Carey 1990), cell sorting (Fromm and Wood 2012), and cell counting (Lazzarin et al. 2005). Cell viability is probably the most widely used parameter in this respect. A comprehensive study of viability assessment by flow cytometry and cell sorting has been described by Nebe-von Caron et al. (1998).

The purpose of this study was to investigate the kinetics of pure yeasts *S. cerevisiae* and *C. tropicalis* on barley malt extract and on 868 medium in order to know cell behaviour to improve cell mass and ethanol productivity without agitation for 48 h. This study reports the effect of co-culture on the ethanol production and yeast viability rate as a part of the ultimate goal of optimizing growth rate and ethanol yield for two yeasts.

Materials and methods

Materials

Glucose monohydrate yeast extract, Peptone casein (Organotechnie, France), Agar (Merck, Germany) 1.5 %, barley malt extract, propidium iodide and HPLC column (C-610H Supelco Gel) were purchased from sigma Aldrich, Belgium. All others chemicals reagents were of analytical grade or higher.

Yeast strains and culture conditions

Yeast species of *C. tropicalis* and *S. cerevisiae* used as starters in this study were belonged to the culture collection of the Food Technology Department (University of Nangui Abrogoua). They were isolated from traditional sorghum beer

from the district of Abidjan (Southern Côte d'Ivoire). They were identified by PCR-RFLP of the ITS region and sequencing of D1/D2 domains of the 26S rRNA gene. The amplification was performed with the thermal cycler Omnigene TR3 CM 220 (UK) and the restriction enzymes used for RFLP analysis were *Hae* III, *Hinf*I and *Cfo* I (N'guessan et al. 2011). Before the alcoholic fermentation, yeasts were cultivated on 868 medium with chloramphenicol at 30 °C for 48 h. This medium contained (w/v): glucose monohydrate 2 %, yeast extract (Organotechnie, France) 1 %, peptone casein (Organotechnie, France) 1 % and agar (Merck, Germany) 1.5 %.

Yeast culture from medium 868-Chloramphenicol plate was harvested with a loop to prepare a dense suspension in 5 ml of sterile peptone water (peptone casein and water). The suspension was added to 50 ml of sterile 863 medium (the medium 868 without agar). The mixture was incubated at 30 °C for 24 h.

Experimental procedure

Before alcoholic fermentation, kinetic study of the two yeasts was realized on the same medium. About 250 ml Erlenmeyer containing 50 ml of medium 863 or malt extract medium were inoculated with cultures on solid medium and incubated for 48 h at 30 °C under agitation at 120 rpm. At regular times during the culture (0, 2, 4, 8, 10, 12, 14, 24, 28, 40 h), samples were withdrawn for dextrose rate, cell count and optic density measurements. According to Eqs. (1 and 2) kinetic parameters such as growth rate and doubling time were calculate.

$$\mu_{\max} = \frac{2,303(\log N_0 - \log N_1)}{t_1 - t_0} \quad (1)$$

$$g = \frac{\ln 2}{\mu_{\max}} \quad (2)$$

$\mu_{\max}$	maximal growth rate (h^{-1})
N_0	cell concentration at the beginning of the exponential growth phase (CFU/ml)
T_0	time of beginning of the exponential growth phase (h)
N_1	cell concentration at the end of the exponential growth phase (CFU/ml)
T_1	time at the end of the exponential growth phase (h)
g	generation time or doubling time (h).

The fermentation medium was made from final malt extract. Erlenmeyer flasks (250 ml) containing 50 ml of sterile malt extract medium (30 g/l malt extract and 5 g of yeast extract) were saturated 30 min before inoculation with starter

culture to obtain 10^6 CFU/ml and were incubated for 48 h at 30 °C. Four fermentation systems were constituted as follows: (1) individual pure culture fermentations with *C. tropicalis* and *S. cerevisiae*; (2) mixed culture fermentations of both yeast strains, respectively, in the ratios of 2:1 and 1:1 (cell/cell). At 0, 4, 8, 16 and 48 h of fermentation, samples were withdrawn for microbial counts, total soluble solids, organic acids and volatile compounds measurements. The experiments were replicated two times.

Analytical method

The microbial growth of *C. tropicalis* and *S. cerevisiae* was evaluated by optical density measurements at a wavelength (λ) of 600 nm on a GENESYS 2 spectrometer (Thermo Scientific, Waltham, MA). The dextrose concentration was determined by an enzymatic method (model 2700 Select; YSI, Yellow Springs, OH). Cell concentration was determined by microscopic observations on a Bürker counting chamber (mean of 10 counts) after dilution.

Absence of aerobic contaminants during fermentation was verified by spreading 0.1 mL of culture on 868 medium plates and incubating for 48 h at 30 °C. Cell concentration was determined by microscopic observations on a Bürker counting chamber (mean of 10 counts) after dilution.

Collected liquid samples were centrifuged at 13,000g for 10 min and the supernatants obtained were filtered through a 0.2 µm cellulose acetate membrane (Minisart Sartorius) and analysed by HPLC for glucose, ethanol, lactate, acetate, succinate and propionate. The HPLC analysis was carried out using an Agilent 1110 series (HP Chemstation software) with a Supelcogel C-610H column preceded by a Supelguard H pre-column (oven temperature 40 °C), 0.1 % H_3PO_4 (in milliQ water) as the isocratic mobile phase at a flow rate of 0.5 mL·min⁻¹ and a differential refractive index detector (RID, heated at 35 °C). The process lasted for 35 min at a maximum pressure of 60 bars as described formerly (Hamilton et al. 2010).

Analysis of the cell viability was performed by Flow cytometry employed a Becton Dickinson FAC scan instrument flow cytometry on a FACScan (Becton Dickinson) instrument. Samples were taken directly from the flask and were diluted in 900 µL of PBS and 100 µL of a cycloheximide solution (1 mg/mL) in order to stop protein synthesis. For each measurement, 30,000 cells were analyzed. The FSC (Forward Scatter) and FL3 channels were logarithmically amplified with the following settings: FSC E00 and FL3 600. Propidium iodide (PI) was used to determine membrane permeability. After staining with PI, cells with compromised membranes were detected by flow cytometry using the following settings: FSC E00, FL3 600 previously described by Brognaux et al. (2013).

Statistical analysis

The data obtained were subjected to analysis of variance (Statistica, 99 Edition) and mean differences determined by Duncan's multiple range test ($P < 0.05$).

Results

Kinetic studies before alcoholic fermentation

The aim of the kinetic culture test was to know the different phases and parameters of yeasts in malt extract medium. The parameters studied were maximal growth rate, glucose degradation, and generation time comparatively to reference medium (863).

As shown in Fig. 1a, our experimentation find three periods: 0–4 h was latency phase for both of the yeast; 4–8 h and 4–10 h were exponential phase respectively for *C. tropicalis* and *S. cerevisiae*; 8–10 h or 10–40 h was attributed to stationary phase. *C. tropicalis* growth started faster than *S. cerevisiae* one. This led to a higher cell concentration in the culture medium, a higher maximum growth rate and a lower doubling time for this yeast on the both media tested. Values were 0.22 and 0.32 h⁻¹ for maximum growth rate, 3 h 09 min and 2 h 09 min for doubling time respectively on barley malt extract and YPD (Table 1). On contrary, glucose consumption was faster with *S. cerevisiae* than with *C. tropicalis*. In addition, all the kinetic parameters studied were higher on YPD than on barley malt medium. As example, *S. cerevisiae* doubling time was 5 h 09 min on barley malt extract while it was 2 h 18 min on YPD.

Metabolites synthesis during alcoholic fermentation on malt extract with starters

Figure 2 illustrates the time course of sugars (maltose, glucose) and organics acids (succinate, lactate, acetate, propionate) in fermenting malt extract inoculated with four starter cultures. At the beginning of the fermentation (0–4 h), maltose content increased in all the fermentation trials, passing from 12.12 to 13.62–16.46 g/L. The highest increase was obtained with starter *C. tropicalis* + *S. cerevisiae* 2:1. From 4 h of fermentation, maltose content decreased in all the fermentation systems. After 48 h of fermentation, all the maltose in the medium was transformed to other metabolites such as glycerol, organic acids and alcohol.

On contrary to maltose, glucose content decreased throughout all the fermentation process with rate varying according to the starter cultures. The fastest decrease during the first 16 h of fermentation was brought about *S. cerevisiae* in pure culture. After 48 h of fermentation, contents in glucose were

Fig. 1 Yeasts growth on barley malt extract medium (a) and 863 medium (b). SC: *S. cerevisiae*; CT: *C. Tropicalis*

respectively 0.22, 0.09, 0.06 and 0.1 g/l for *S. cerevisiae*, *C. tropicalis*, *C. tropicalis* + *S. cerevisiae* 1:1 and *C. tropicalis* + *S. cerevisiae* 2:1.

Fluctuations in organic acids were not regular for each compound regardless of starter cultures. Indeed, succinate contents were respectively 0.08, 0.08, 0.05 and 0.06 g/l in barley beer produced with *S. cerevisiae*, *C. tropicalis*, *C. tropicalis* + *S. cerevisiae* 1:1 and *C. tropicalis* +

S. cerevisiae 2:1. In the same way, lactate values were 0.14, 0.15, 0.08 and 0.2 g/l.

For all the starters used, the major part of the ethanol was produced at 16 h of fermentation (Fig. 3). Until 8 h, *C. tropicalis* in pure culture was the lowest ethanol producer and *S. cerevisiae* in pure culture, the highest producer. But after 8 h, *C. tropicalis* produced higher ethanol than *S. cerevisiae*. Values obtained in the final beers were 11.4,

Table 1 Kinetic parameters of *S. cerevisiae* and *C. tropicalis* on barley malt extract and 863 medium (YPD)

Medium/strains	Glucose consumption rate (g/l/h)	Maximum growth rate (h ⁻¹)	Doubling time (h)
<i>S. cerevisiae</i> on barley malt extract	-0.36	0.134	5 h 09
<i>C. tropicalis</i> on barley malt extract	-0.125	0.22	3 h 09
<i>S. cerevisiae</i> on 863 medium	-0.722	0.30	2 h 09
<i>C. tropicalis</i> on 863 medium	-0.58	0.32	2 h 09

Fig. 2 Sugars (maltose, glucose) and organic acids content (succinate, lactate, acetate and propionate) during barley malt extract fermentation with the four yeast starters. For each fermentation time, histograms with the same letter are not significantly different, $P \geq 0.05$. SC: *S. cerevisiae*, CT: *C. tropicalis*, CT/SC 1:1: *C. tropicalis* + *S. cerevisiae* (1:1), CT/SC 2:1: *C. tropicalis* + *S. cerevisiae* (2:1)

Fig. 3 Ethanol rate in the fermenting barley malt extract carried out with four yeast starter cultures. For each fermentation time, histograms with the same letter are not significantly different, $P \geq 0.05$. SC: *S. cerevisiae*, CT: *C. tropicalis*, CT/SC 1:1: *C. tropicalis* + *S. cerevisiae* (1:1), CT/SC 2:1: *C. tropicalis* + *S. cerevisiae* (2:1)

11.6, 10.4 and 10.9 g/l for fermentation conducted with *S. cerevisiae*, *C. tropicalis*, *C. tropicalis* + *S. cerevisiae* 1:1 and *C. tropicalis* + *S. cerevisiae* 2:1.

Cell viability measurement by flow cytometry during alcoholic fermentation

Flow cytometry is a powerful technique for the study of single cells, and thus it is of particular utility in the study of heterogeneity in microbial populations. Cell viability measurement is done after production in order to know and to evaluate yeasts capacities to resist in presence of ethanol for measurement on a new production medium. Table 2 shows that yeast rate of death (FL3) values was low 10^3 , precisely starters with *C. tropicalis* + *S. cerevisiae* 2:1 presented lowly FL3 mean

Table 2 FSC and FL3 values of yeasts starters during alcoholic fermentation on barley malt

Starter cultures	Mean FSC-A	CV FSC-A%	Median FSC-A	Mean FL3-A	CV FL3-A%	Median FL3-A
SC at 8 h	1 654 964.75	96.77	1 232 732.5	9 868.02	798.58	3 498.0
CT at 8 h	1 728 642.96	100.99	1 202 864.0	8 963.84	246.16	5 205.0
CT/SC 1:1 at 8 h	1 794 412.90	94.69	1 316 115.0	16 653.44	539.93	4 077.0
CT/SC 2:1 at 8 h	1 783 817.98	100.64	1 303 066.0	8 032.05	356.85	4 059.0
SC at 16 h	1 727 549.62	75.07	1 411 737.5	3 921.28	478.73	2 040.0
CT at 16 h	1 567 484.59	71.20	1 319 768.0	3 755.47	329.89	2 259.0
CT/SC 1:1 at 16 h	1 736 815.19	68.52	1 446 290.0	4 549.92	505.38	1 775.0
CT/SC 2:1 at 16 h	1 626 159.41	69.07	1 403 440.0	5 129.73	523.43	2 464.0
SC at 48 h	1 617 626.66	62.90	1 419 663.0	2 628.43	1 073.59	1 403.0
CT at 48 h	1 554 611.86	69.19	1 349 347.0	3 187.77	727.67	1 963.0
CT/SC 1:1 at 48 h	1 470 952.80	41.61	1 402 319.5	2 269.45	498.51	1 436.049
CT/SC 2:1 at 48 h	447 426.06	389.83	184 616.0	966.16	473.13	535.0

SC: *S. cerevisiae*, CT: *C. tropicalis*, CT/SC 1:1: *C. tropicalis* + *S. cerevisiae* (1:1), CT/SC 2:1: *C. tropicalis* + *S. cerevisiae* (2:1)

values than the others. The values of FSC mean and median show a significant difference (CV %) between the starters.

Discussion

In this study, we used *C. tropicalis* and *S. cerevisiae* in pure and co-culture (1:1) and (2:1) in order to know the profile fermentation and to evaluate their viability on the malt extract beer in non-agitated batch fermentation saturated in oxygen.

Before beer production, kinetic study was achieved on agitated fermentation and show clearly that the two yeast species presented different characteristics during fermentations on malt extract medium and on YPD. Kinetic parameters such as maximum growth rate and doubling time show that *C. tropicalis* grew faster than *S. cerevisiae* in the two media. The unavailability of a high amount of glucose in the malt extract medium may explain the highest doubling time (3 to 5 h) for yeasts which is favourable for non-Saccharomyces yeasts grow (Jeffries 1981). According to Boekhout and Robert (2003), *C. tropicalis* grew on medium that contains sugars (C5 and C6). Several studies noted that kinetic parameters are depending to the medium culture, the microbial and the experimental conditions. Doubling time of *S. cerevisiae* on YPD was 2 h. This result is in according to Boekhout and Robert (2003) who confirm that *S. cerevisiae* as a single celled organism is small with a short generation doubling time 1.25–2 h at 30 °C and can be easily cultured.

S. cerevisiae grew slowly conversely *C. tropicalis* grew first until the end of the fermentation. This result was briefly observed by N'guessan et al. (2010) during the fermentation of sorghum wort for tchapalo production. The different on

yeast behaviour observed at the early hours of fermentation could be attributed to the composition of medium. Malt extract contained majoritary maltohexaose, maltose glucose, and xylitol at the start of fermentation. Maltose and glucose values were significantly different $P < 0.05$ with yeasts during fermentation. Indeed, it has been reported that oxygen availability during the first stages of alcoholic fermentation favoured the growth of non-Saccharomyces yeasts (Hansen et al. 2001). Bourgeois and Mafart (1996) also reported a death of *S. cerevisiae* at the start of fermentation for the modern type beer production. Results (Fig. 2) showed organic acids production such as succinic acid, lactic acid, acetic acid and propionic acid (significantly different $P < 0.05$) by all starters. But higher production of lactic acid by pure *C. tropicalis* at 16 h of culture was observed. This is approved with previous results which reported that non-Saccharomyces yeasts are characterized as secondary metabolite producers (Lema et al. 1996; Lambrechts and Pretorius 2000). Ethanol production with four starters showed that there was a stimulatory effect for ethanol at the end of fermentation with *C. tropicalis* in single culture, which led to higher ethanol concentration (11.57 g/l). Without agitation (0 rpm) ethanol production was privileged during fermentation (Arisra et al. 2008). In according to these authors, ethanol production decreased when agitation increased, but cell concentration rate increased. On contrary, N'guessan et al. (2010) noted high production of lactic acid (20.7 g/l) by *C. tropicalis* on sorghum medium. Flow cytometry measurement confirmed that during alcoholic fermentation high concentration of ethanol didn't destroy yeast cells, but their viability has been preserved after 48 h on barley malt extract medium. Starter (2:1) showed the best viability rate (FL3 < FSC). In according to Reisa et al. (2005), the flow cytometry approach is continuing to be useful for the study of bacterial population dynamics, providing important

physiological information at the individual cell level that is difficult to obtain in any other way.

Conclusion

The results of the present study showed that *C. tropicalis* grows better on barley malt extract than *S. cerevisiae* although glucose consumption rate is higher with *S. cerevisiae* than with *C. tropicalis*. These two yeasts can be used as starters for barley beer production. But co-inoculation at proportion of 2:1 (cell/cell) is the best starter culture for beer production as it showed similar ethanol content to *S. cerevisiae* in pure culture and the best organic acid profile and viability rate. As *C. tropicalis* is now quoted as an opportunistic pathogen, further investigations are needed before it uses for beer production.

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