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Ethanol effect on yeast strains isolated from tchapalo, a traditional sorghum beer from Côte d'Ivoire

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Abstract

The general trend in sorghum beer production is now the use of starter culture to alleviate the problems of variations in organoleptic quality and microbiological stability. In this study, we aimed to select strains of *Saccharomyces cerevisiae* and *Candida tropicalis* to use as starter culture based on their ability to face ethanol toxicity. All the strains exhibited a relatively high resistance up to 5% ethanol. At 7.5% ethanol, *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 were the most resistant strains with viability rate of 80-97% after 24 h of incubation. These two strains showed also the highest unsaturated/saturated fatty acids ratios. Values increased from 68.86% to 80.30% and from 72.97 to 85.96% respectively for *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 cultivated under 0% and 7.5% ethanol. The cellular neutral lipid composition differed markedly according to the yeast strains. With proportion of 36.73% for *S. cerevisiae* F12-7 and 78.75% for *C. tropicalis* C0-7, ergosterol was the most abundant neutral lipid founded in the strains membranes. In addition, phosphatidylethanolamine contents decreased with the increase of ethanol concentration in the culture medium on contrary to phosphatidylcholine contents. These two strains were so suggested as starter cultures for sorghum beer production.

Introduction

In sub-Saharan Africa, sorghum is used abundantly to produce traditional alcoholic beverages named sorghum beers. The brewing is managed by women at small-scale and involves two steps of fermentation: lactic acid fermentation which confers souring taste and storage longevity and alcoholic fermentation (Haggblade and Holzapfel, 1989; Glover *et al*., 2005; Maoura *et al.*, 2005). These fermentations are uncontrolled and the microorganisms involved come from the raw materials, equipment and local environments or from residues of previous fermentation batches. The wide variety of microorganisms present during the fermentations lead to a product with widely varying qualities and unsatisfactory conservation. The use of starter cultures was so suggested as the appropriate approach to alleviate the problems of variations in organoleptic quality and microbiological stability of traditional sorghum beer (Sanni, 1993; Holzapfel, 2002).

The alcoholic fermentation is one of the steps in the traditional process which mainly determines the quality of the final product. It is usually initiated by pitching final wort with a portion of previous brew or dried yeast harvested from previous beer (Yao *et al*., 1995; Sefa-Dedeh *et al*., 1999). Identification studies of yeasts involved in the alcoholic fermentation of the beer have highlighted species of *Saccharomyces cerevisiae, Candida tropicalis, Kloeckera apiculata, Hansenula anomala, Torulaspora delbrueckii, Kluyveromyces marxianus* and other non-*Saccharomyces* yeasts according to countries (Sefa-Dedeh *et al*., 1999; Jespersen, 2003). *Saccharomyces cerevisiae* was reported as the predominant species, making up 33–99% of the yeast cell population (van der Aa Kühle *et al.*, 2001; Naumova *et al.*,

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2003; Glover *et al.*, 2005; Maoura *et al*., 2005; N'guessan *et al*., 2011). In addition, the results from the characterization of the *S. cerevisiae* isolates demonstrated the presence of several strains during the fermentation. Among the non-*Saccharomyces* yeasts, the predominant species varied according to geographic origin of the beer. Thus, *Candida tropicalis* was reported as the predominant non-*Saccharomyces* yeast in pito and tchapalo (Sefa-Dedeh *et al*., 1999; N'guessan *et al*., 2011) while *Clavispora lusitaniae* and *Candida inconspicua* were reported for tchoukoutou and ikigage respectively (Lyumugabe *et al*., 2010; Greppi *et al.*, 2013).

Good fermentation power is obviously related to the capacity of the strain to overcome the stresses associated with the fermentation process, such as the hyperosmotic environment present during the initial phases of the process or the elevated concentrations of ethanol and scarcity of assimilable nitrogen and growth or survival factors during subsequent phases. These stress conditions can dramatically affect the population dynamics and ethanol production (Estruch, 2000; Hohmann, 2002). The effects of ethanol toxicity on yeast physiology are diverse, though cellular membranes appear to be the main sites of ethanol damage. Specific effects include growth inhibition, reduced cell size (Canetta *et al*., 2006), reduced viability, reduced respiration and glucose uptake (Pascual *et al*., 1988), reduced fermentation (Fernandes *et al.*, 1997), enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane (Petrov and Okorokov, 1990; Mizoguchi and Hara, 1997), membrane permeability increase (Marza *et al.*, 2002), lowering of cytoplasmic pH and the induction of respiratory-deficient mutants (Jimenez *et al.*, 1988; Ibeas and Jimenez, 1997; Chi and Arneborg, 1999). So, selection of a strain for the use as starter culture must take into account the ability of this strain to overcome the stresses associated with the fermentation process, especially ethanol toxicity. In this study, we aimed to select strains of *S. cerevisiae* and *C. tropicalis* based on their ability to face ethanol toxicity.

Materials and Methods

Yeast strains

Six strains of *S. cerevisiae* (strains D12-3, D12-10, E4-4, A12-1, C8-5, and F12-7) and three strains of *C. tropicalis* (strains F0-5, C0-7, and C8-10) were tested in this study. They belonged to the culture collection of the Food Technology Department (University of Nangui Abrogoua). They were previously 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 the sequencing of D1/D2 domains of the 26S rRNA gene (N'guessan *et al.,* 2011). The yeast strains were maintained routinely at -20°C in 20% of glycerol.

Analysis of yeast strains tolerance to ethanol

All yeast strains were evaluated for their ability to grow under ethanol stress according to the method described by Carrasco *et al.* (2001) with some modifications. The strains were transferred from Sabouraud-chloramphenicol plates to pre-culture 10 ml YPG broth (10 g/L yeast extract, 10 g/L peptone, 10 g/L glucose) and incubated at 30°C for 24 h. The pre-cultures were used to inoculate at initial concentration of 0.2 OD_{600nm} 50 mL of YPD broth supplemented with 0%, 2.5%, 5% and 7.5% ethanol (v/v). Cultures were incubated at 30°C for 24 h, 150 rpm. At the end of incubation time, cultures were used for viability, fatty acid and lipid analyses. The experiments were replicated three times.

Determination of yeast viability

The cellular 'viability' was determined by the methylene blue method (Postgate, 1967). A 200 µL sterile solution of methylene blue (0.3 mM in 68 mM Na_3 citrate) was mixed with 200 µL of a yeast suspension diluted to reach an $OD_{600\ nm}$ of 0.4–0.7. The mixture was shaken and, after 5 min incubation, placed in a Thoma's counting chamber. The number of stained (non-active cells) or un-stained (active cells) and the number of buds were counted in five different fields with a total of at least 200–300 cells. The percentage of 'viable' cells ('viability') was determined as the number of un-stained cells (live cells) divided by the total number of cells (stained and unstained).

Analysis of fatty acids

The analysis was carried out following the modified procedure of Rozès *et al.* (1992). Twenty milligrams of sedimented cells (12000 rpm, 20 min, 4°C) were placed in sealed tubes with a Teflon-lined screw cap and saponified using 2 mL of the mixture methanol/sulfuric acid 2.5% (v/v). The tubes were placed in a water bath at 80ºC for 2-3 h. Then the saponified material was cooled at room temperature and 1.5 mL of hexane and NaCl 5% were added respectively. The organic phase was collected after centrifugation at 2000 rpm for 2 min. Analytical gas chromatography was performed using a Hewlett-Packard 6850 (Agilent Technologies). One µL of cellular extract was injected (splitless, 1 min) into an

FFAP-HP column (30 m x 0.25 mm x 0.25 µm, from Agilent Technologies) with an HP 6850 automatic injector. The oven temperature was held at 120°C for 1 min, increased by 10°C/min to 180°C, by 0.33°C/min to 183°C and by 15ºC/min up to 250ºC. Injector and detector temperatures were 250ºC and 270ºC, respectively. The carrier gas was helium at a flow rate of 1.2 mL/min. Heptadecanoic acid (100 µg/mL) was added as internal standard before cell saponification. Relative amounts of fatty acids were calculated from their respective chromatographic peak areas using the Chromoleon Dionex software, 6.70 SP2a version. These values were related to the dry weight of cells and expressed as a percentage of the total fatty acid extracted. The ratios of unsaturated to saturated fatty acids (UFA/SFA) were calculated. Analyses were made in duplicate.

Yeast neutral lipid and phospholipid composition by thin-layer chromatography (TLC)

The procedure described by Redón *et al.* (2009) was followed with little modification. Prior to lipid extraction, a solution of 100 µL of cold methanol and 10 µL of EDTA 0.1 mM was added to the yeast cells (15-20 mg dry mass) with 1 g of glass beads (0.5 mm, Biospec Products) in an Eppendorf tube, and then mixed for 5 min in a mini-beadbeater- 8 (Biospec Products, Qiagen). Lipid extraction was performed in five steps: four steps with 300 µL chloroform/methanol (2:1, v/v, for 2 h) and the fifth with 300 µL of chloroform/methanol 1:2 (v/v) overnight. Both organic phases were transferred to a 15 mL glass screw-cap tube and cleaned twice by adding NaCl 0.9% (one fourth of the total volume of the extract). After vortexing and cooling at 4ºC for 10 min, the samples were centrifuged at 2000 rpm for 2 min. The organic phase was collected and finally concentrated to dryness under a nitrogen stream. The residue was dissolved in 500 µL of chloroform and stored at -20ºC until analysis.

The neutral lipid composition of the yeast strains was separated by one-dimensional TLC on silica gel 60F254 plates (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in two steps with (i) hexane, methyl tert-butyl ether (MTBE), glacial acetic acid (70:30:3, v/v/v) applied to the half of the plate and (ii) hexane applied to 9.5 cm (Redón *et al.*, 2009). For phospholipids, separation was conducted in one step with chloroform:acetone:methanol:glacial acetic acid:water (50:15:10:10:5, v/v/v/v/v). The standard lipids ergosterol, ethyl oleate, oleic acid, diolein, triolein, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purchased from Sigma and applied to every plate in the range of 1-4 µg/µL. After TLC, lipids were charred with 10% $CuSO_4$ in 8% H_3PO_4 and heated at 180ºC for 4 min on a TLC Plate Heater (CAMAG). An image of the plate was acquired with an Image Scanner (Amersham Biosciences). Each spot of the image was quantified as an integrated optical density (IOD) with Quantity One software (Bio-Rad) and the calibration curves were constructed by plotting the IOD of the lipid standard versus the amount of lipid loaded.

Statistical analysis

Differences amongst calculated proportions were examined using Fisher's exact test ($P < 0.05$). Statistical analyses were performed using R Version 3.1.2 (R Software, Copenhagen, Denmark).

Results

Ethanol effect on yeast viabilities

All *S. cerevisiae* and *C. tropicalis* strains used in this study exhibit a relatively high resistance to 2.5 and 5% (v/v) ethanol. Indeed, cell viability analysis revealed that cultivation of the yeast strains under 2.5% and 5% ethanol triggered a reduction of viability below 10% in reference to control condition (in the absence of ethanol) (Figure 1). But at 7.5% ethanol, except for *S. cerevisiae* F12-7 and *C. tropicalis* C0-7, reduced levels of viability higher than 20% were observed. *S. cerevisiae* E4-4, *S. cerevisiae* C8-5, *C. tropicalis* F0-5 and *C. tropicalis* C8-10 seemed to be the most sensitive strains to 7.5% ethanol concentration after 24 h of incubation. On contrary, *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 tolerated higher ethanol concentration. The results showed viability rate of 80-97% for these two strains after 24 h of incubation.

Ethanol effect on fatty acids composition of strains

Six cellular fatty acids, namely myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic (18:2) acids, were identified. As shown in Figure 2, the level of each fatty acid varied diversely with ethanol concentration. Oleic acid followed by linoleic acid on one hand and palmitic acid on the other hand were quantitatively the most important unsaturated and saturated fatty acids, respectively. Oleic and linoleic acids level increased with ethanol concentration for all of the yeast strains except for *S. cerevisiae* A12-1 and D12-10. Moreover, among *S. cerevisiae,* the strain F12-7 showed a higher oleic acid content than the other strains at a same ethanol concentration. Values was 42.96% at 5% ethanol against 40.80% for the strain C8-5, 38.26% for the strain A12-1, 34.76% for

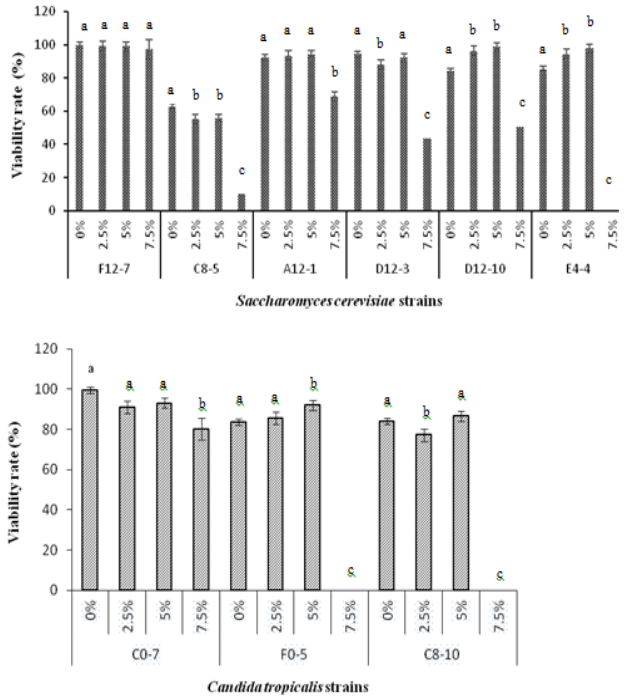

Figure 1. Viability of *Saccharomyces cerevisiae* and *Candida tropicalis* strains after growing for 24 h under different ethanol concentrations (0%, 2.5%, 5% and 7.5%). The errors bars represent the standard deviations from the means of three independent cultures. For each strains, histograms with the same letters are not significantly different ($P < 0.05$).

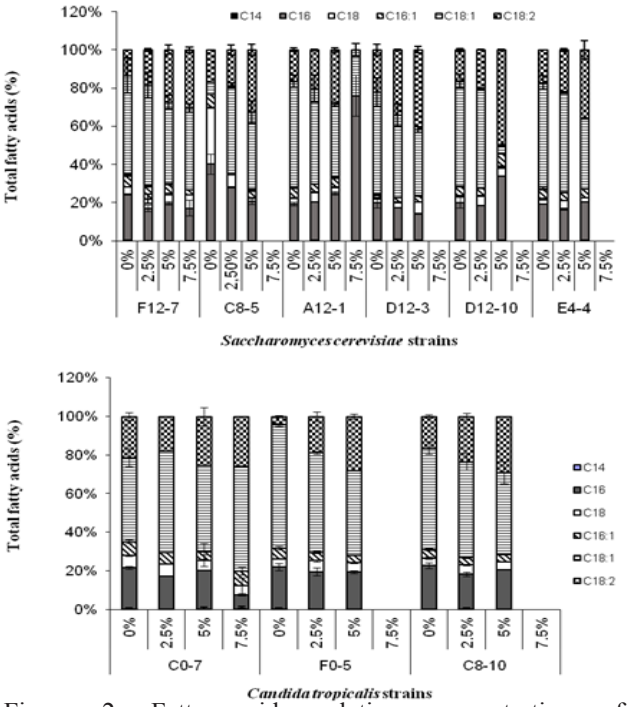

Figure 2. Fatty acids relative concentration of *Saccharomyces cerevisiae* and *Candida tropicalis* strains grown under different ethanol concentrations (0%, 2.5%, 5% and 7.5%).

the strain D12-3, 4.26% for the strain D12-10 and 36.88% for the strain E4-4. For *C. tropicalis* strains, oleic acid levels at 5% ethanol were 44.59%, 44.08% and 42.58% respectively for the strains C0-7, C8-10 and F0-5. Among all the tested strains of *C. tropicalis,* only the strain C0-7 grew under 7.5% ethanol. So, the main fatty acids at this ethanol concentration for the strain C0-7 were oleic and linoleic acids with relative concentrations of 54.13% and 25.86% respectively.

The UFA/SFA ratios increased with ethanol concentrations in the culture medium for all of the strains examined in this study except for *S. cerevisiae* A12-1 and D12-10 (results not shown). In addition, the highest UFA/SFA ratios were obtained with *S. cerevisiae* F12-7 and *C. tropicalis* C0-7. Values increased from 68.86% to 80.30% and from 72.97 to 85.96% respectively for *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 cultivated under 0% and 7.5% ethanol.

Ethanol effect on neutral lipids and phospholipids composition

The cellular neutral lipid composition differed markedly according to the yeast strains (Figure 3). Thus, for *S. cerevisiae* F12-7 and A12-1, ergosterol and free fatty acids were the neutral lipids detected with a higher proportion of free fatty acids (69-84%). For the other strains of *S. cerevisiae*, ergosterol, triacylglycerols, sterol esters and free fatty acids with ergosterol as the most abundant compound (39-69%) were found in their membranes. In the membranes of *C. tropicalis* strains, regularly detected neutral lipids were ergosterol, triacylglycerols and free fatty acids with ergosterol as the most abundant compound too (34-52%).

When the yeast strains were cultured under different ethanol concentrations, the neutral lipid composition varied differently from one strain to another. Thus, *S. cerevisiae* F12-7 and A12-1 proportions of free fatty acids decreased with the increase of the ethanol concentration while ergosterol content increased. Values decreased from 84.75% to 26.75% then to 17.21% for free fatty acids and increased from 15.25% to 16.01% then to 22.71% for ergosterol respectively at 0%, 2.5% and 5% of ethanol for the strain A12-1. On contrary, with *S. cerevisiae* E4-4, the proportion of free fatty acids increased with ethanol concentration while that of ergosterol decreased. For the strains that grew well at 7.5% ethanol, the most abundant neutral lipid was ergosterol with proportion of 36.73% for *S. cerevisiae* F12-7 and 78.75% for *C. tropicalis* C0-7.

Two phospholipids were detected during this study: phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PE contents decreased with the increase of ethanol concentration

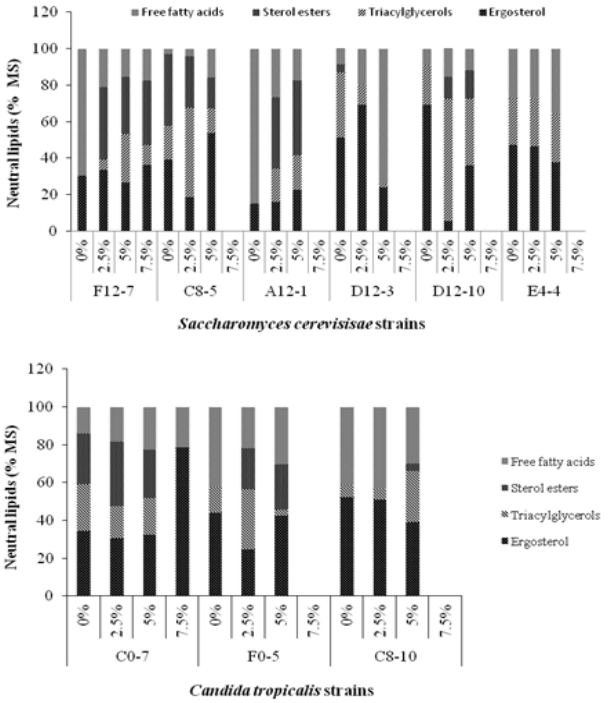

Figure 3. Neutral lipids concentration of *Saccharomyces cerevisiae* and *Candida tropicalis* strains grown under different ethanol concentrations (0%, 2.5%, 5% and 7.5%).

in the culture medium while the contrary was observed with PC for all the tested strains (results not shown). Thus, PE content decreased from 81% (in the absence of ethanol) to 50% (in the presence of 7.5% ethanol) and from 80% to 72% for the strains *S. cerevisiae* F12-7 and *C. tropicalis* C0-7, respectively.

Discussion

Brewery fermentation imposes a variety of stresses on the yeast cell among with ethanol concentration remains the most stress (Stanley *et al.*, 2010). Ethanol primarily targets membranes, increasing their fluidity and permeability, thus affecting the transport system of essential compounds such as amino acids and glucose. Its accumulation compromises a wide range of cellular functions leading to the reduction of cell metabolic rate, growth and viability, ultimately promoting a sluggish fermentation (Alexandre *et al*., 2001; Gibson *et al*., 2007). Of the nine yeast strains studied in this work, *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 were the most resistant yeasts to high ethanol concentration. The higher ethanol tolerance exhibited by these two strains could be due to a greater capability to consume ethanol in the presence of oxygen. In fact, it was reported that the ethanol resistance of yeasts greatly depended on mitochondria, and that the ethanol tolerance of a *S. cerevisiae* laboratory strain could be enhanced by introducing the mitochondria from a 'flor' wine yeast (Ibeas and Jiménez, 1997; Jiménez and Benitez, 1988). In addition, Kubota *et al.* (2004) reported 22 genes involved in mitochondrial function that were important for growth on 11% ethanol, indicating that certain mitochondrial functions are important to resist high levels of ethanol. The fact that *C. tropicalis* C0-7 exhibited higher ethanol tolerance than certain *S. cerevisiae* strains was not surprising, as there are increasing studies reporting wine isolates of non-*Saccharomyces* species, which exhibit ethanol tolerances similar to those of *S. cerevisiae* (Pina *et al*., 2004; Nisiotou *et al.*, 2007).

It is known that *S. cerevisiae* possesses diverse strategies to counteract the stress produced by high ethanol concentrations, such as: (i) changing the membrane composition to antagonize membrane fluidization (by increasing levels of UFA and ergosterol); (ii) expression of factors that stabilize and/or repair denatured proteins; (iii) synthesis of proteins involved in the expression of stress-related genes; and (iv) increase in plasma membrane ATPase activity which counteracts the ethanol-induced proton influx (Ding *et al*., 2009). So the FA, neutral lipid and phospholipid cell compositions of all the tested strains were analyzed. The results showed that the most abundant UFA were oleic and linolenic acids while the most SFA was palmitic acid. On contrary to Torija *et al.* (2003), our results revealed that cell UFA increased with ethanol concentration except for *S. cerevisiae* A12-1 and D12-10. These results are consistent with previous evidences obtained by You *et al*. (2003) who proved that the increasing proportion of UFA (C18: 1) is also accompanied by a decrease in the proportions of the FA C16: 0 and C16: 1 in response to increasing ethanol concentrations. These authors also demonstrated that oleic acid was the most efficient UFA in overcoming the toxic effects of ethanol in growing yeast cells, whilst palmitoleic acid did not confer any ethanol tolerance. Our results were in the same direction, and the yeasts with the highest oleic relative proportions (*S. cerevisiae* F12-7 and *C. tropicalis* C0-7) were also the most ethanol resistant. Four neutral lipids were detected in this study. In absence of ethanol, ergosterol was found as the most abundant compound except for *S. cerevisiae* F12-7 and A12-1. According to Parks and Casey (1995), it is involved in membrane integrity and fluidity and the activity of membrane bound enzymes. Shobayashi *et al.* (2005) showed that its biosynthesis is reduced in the presence of ethanol and under oxygen depletion. In contrast, Daum *et al.* (1998) reported that its level increase in response to the high concentration of ethanol. In the same way, Walker-Caprioglio *et*

al. (1990) and del Castillo Agudo (1992) found that ethanol tolerant *S. cerevisiae* strains grown in batch culture increase the proportion of ergosterol when grown in the presence of ethanol. Our experiments resulted in similar changes in sterol composition, indicating that the results found by the above-mentioned authors are attributable to a true effect of ethanol. It may be deduced from these results that ergosterol is the predominant sterol in yeast under ethanol stress conditions.

The change in phospholipid contents was investigated during the imposed stress process by determining PC and PE relative compositions. Our observation that yeast strains adapt to increased concentrations of ethanol by increasing the proportion of PC at the expense of PE has been reported by several authors (Arneborg *et al*., 1995; Shin *et al.*, 1995). It suggests that PC may play a major role in the adaptive response of yeast cells to ethanol. Hunter and Rose (1972) found, contrary to our observation, that the proportion of PE increased at the expense of PC by increasing the specific growth rate in chemostat-cultivated *S. cerevisiae* cells. One reason for the contradictory results may be that these authors grew their cells under oxygen-sufficient conditions, whereas we did not.

Conclusion

The ability of yeast to resist and adapt to ethanol stress remains an important property in the production of foods and fermented beverages. In this investigation, *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 were the most resistant yeasts to high ethanol concentration. They counteracted the stress produced by high ethanol concentrations by increasing their levels of UFA (mainly oleic and linolenic acids) and ergosterol. They also increased the proportion of PC at the expense of PE. Therefore, these strains could be used for sorghum beer production purpose but another studies should be conducted on their ability to produce volatile compounds which intervene in the beer flavour before a definitive conclusion.

Acknowledgments

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