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Ethanol tolerance of five non-*Saccharomyces* wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*—influence of different culture conditions

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Abstract

The kinetics of cell inactivation at high concentrations of ethanol (22.5% and 25% v v^{-1}) was studied with particular reference to the influence of different cultivation conditions (aerobiosis, semi-aerobiosis, anaerobiosis, addition of survival factors) prior to ethanol challenge. The levels of fatty acids and sterols in cells grown under these different conditions were analysed in order to derive a potential relationship between the ethanol tolerance and the lipid composition of the different strains studied. Under the conditions tested, *Hanseniaspora guilliermondii* showed an ethanol tolerance very similar to that of *Saccharomyces cerevisiae*, and very different to that of the other apiculate yeast, *H. uvarum*. The survival of *S. cerevisiae* and *H. guilliermondii* at 25% (v v^{-1}) ethanol was strongly influenced by the conditions of cultivation prior to the ethanol challenge. A small increase in survival was observed for *H. uvarum* and *Torulaspora delbrueckii* in the cultures grown in aerobiosis. Growth of these yeasts in anaerobiosis in the presence of added ergosterol and Tween 80 (as a source of oleic acid) did not induce a considerable tolerance to the ethanol challenge, in spite of the incorporation of these compounds by the cells. In these growth conditions *Debaryomyces hansenii* was not capable of incorporating the survival factors and did not increase the ethanol tolerance. It is shown in this work that, besides *S. cerevisiae*, the presence of oxygen and the addition of survival factors to the culture conditions and the subsequent increase in the proportion of cell sterols and unsaturated fatty acids may play an important role in the ethanol tolerance of some non-*Saccharomyces* yeasts. This effect was not, however, observed in all of the yeasts studied.

Keywords: Ethanol; Wine yeast; Lipid composition; *Saccharomyces cerevisiae*; *Candida stellata*; *Debaryomyces hansenii*; *Hanseniaspora* spp.

1. Introduction

In the production of wine, natural grape juice fermentation is carried out by a succession of different yeast populations. Several authors report that the early stages of the alcoholic fermentation are characterized by the activity of mostly apiculate, non-*Saccharomyces* yeasts from *Kloeckera* and *Hanseniaspora* genera (Martinez et al., 1989; Fleet, 1990; Cianni and Picciotti, 1995; Gil et al., 1996; Lema et al., 1996; Romano et al., 1997; Charoenchai et al., 1998; Gutiérrez et al., 1999). The growth of these yeasts is generally limited to the first two or three days of fermentation, after which they die off, giving way to the more tolerant strains of *Saccharomyces cerevisiae* (Martinez et al., 1989; Fleet, 1990; Fleet and Heard, 1993; Cianni and Picciotti, 1995; Gil et al., 1996). Many physiological parameters allow *Saccharomyces* to dominate grape juice fermentations, but its tolerance to high concentrations of ethanol is the principal feature of this yeast that allows its survival in this specific environment (Boulton et al., 1995). The progressive disappearance of non-*Saccharomyces* fermentation species is generally attributed to their inability to survive the increasing concentrations of ethanol produced in the fermentation, with the maximum tolerance of 6% v v^{-1} being often cited (Fleet, 1990; Gil et al., 1996). The persistence of these non-*Saccharomyces* fermentation species during fermentation may depend, however, upon many factors, such as the temperature of fermentation, nutrient availability,

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inoculum strength of *Saccharomyces*, use and levels of sulphur dioxide, the numbers and kinds of organisms present on the grapes and the vinification technology (Fleet, 1990; Romano et al., 1997; Charoenchai et al., 1998; Gutiérrez et al., 1999). However, the common spoilage yeasts of wine, *Brettanomyces* and *Zygossacharomyces*, are as tolerant of ethanol as the *Saccharomyces* species (Boulton et al., 1995).

The accumulation of ethanol in the microbial environment represents a form of chemical stress on the organisms living there and several studies have shown that the plasma membrane is the prime target of ethanol action in such situations (Thomas et al., 1978; Beavan et al., 1982; Ingram and Buttke, 1984). Furthermore, yeast ethanol tolerance has been correlated with the capacity of cells to modify their lipid composition to respond to the disrupting action of ethanol. This is part of a regulatory system, which ensures the adjustment of the physicochemical properties of the membrane lipid matrix in a physiologically optimal range (Ingram, 1986; Sajbidor, 1997). The high ethanol tolerance of *S. cerevisiae* has received widespread attention throughout the last five decades, although the mechanisms underlying it and the way membrane lipids modulate membrane fluidity are not yet fully understood (Mishra and Kaur, 1991; Sajbidor, 1997; Chi and Arneborg, 2000). However, it is well known that yeasts supplemented with various sterols and unsaturated fatty acids (UFA) exhibit increased ethanol tolerance as a result of the incorporation of some of these compounds, as has been demonstrated by several studies on *S. cerevisiae* (Thomas et al., 1978; Thomas and Rose, 1979; Walker-Caprioglio et al., 1990). These substances can act as ''survival factors'' (allowing cells to maintain their viability and fermentative activity) when added to the grape must (Larue et al., 1980). However, similar studies concerning non-*Saccharomyces* yeasts are very scarce, and the recent resurgence in interest in the role of these organisms in the early stages of fermentation (Hansen et al., 2001) has highlighted the necessity of obtaining more information concerning their physiological and metabolic features.

Some of the most frequently employed methods to measure ethanol tolerance include growth and cellular viability in the presence of ethanol; fermentative capacity, i.e. the maximal amount of ethanol produced during fermentation; and fermentative ability, i.e. the ability to ferment glucose as measured by CO_2 production (Mishra and Kaur, 1991). In this study, the kinetics of inactivation in the presence of high concentrations of ethanol (22.5% and 25% $v\,v^{-1}$) was used as an inference of the ethanol resistance status of five non-*Saccharomyces* strains and one strain of *S. cerevisiae*. Cultivation conditions prior to ethanol challenge were of particular interest—anaerobiosis, semi-aerobiosis, active oxygenation, addition of ergosterol and Tween 80 (as a source of oleic acid). Changes in certain cell fatty acids and sterols were examined in order to derive a potential relationship between ethanol tolerance and the lipid composition of the different strains.

2. Materials and methods

2.1. Yeast strains

The strains used were *S. cerevisiae* PYCC (Portuguese Yeast Culture Collection, Institute Gulbenkian de Ciência, Oeiras, Portugal) 3507, *Candida stellata* PYCC 3044, *Debaryomyces hansenii* PYCC 2968T, *Hanseniaspora uvarum* PYCC 4193T, *Torulaspora delbrueckii* PYCC 2477T and *H. guilliermondii* NCYC (National Collection of Yeast Cultures, Norwich, UK) 2380 (isolated from grape must used for Port wine production in the Douro region of Portugal).

2.2. Culture medium

The base culture medium used was YM broth (Difco), with the initial pH set at 4.5 with lactic acid (Merck). Cells (preinoculum) were grown semi-aerobically (flasks under static incubation and not firmly closed) in 100 ml Erlenmeyer flasks containing 50 ml of medium at 25°C until mid-exponential growth phase. An inoculum of 2% ($v\,v^{-1}$) was used.

2.3. Growth conditions prior to ethanol challenge

Six cultivation regimes were used: (i) active oxygenation, achieved by orbital shaking (150 rpm) in 100 ml flasks with 50 ml of culture medium; (ii) semi-aerobic conditions, achieved by leaving the flasks under static incubation and not closed firmly; (iii) semi-aerobic conditions supplemented with 25 mg l^{-1} of ergosterol (Sigma) and 1 g l^{-1} of Tween 80 (Merck) (as a source of oleic acid); (iv) strict anaerobic conditions, achieved by sparging the culture medium with nitrogen gas for 15 min in 100 ml serum flasks, which were then firmly closed with 4 mm thick butyl rubber septa. Resazurin (0.1 g l^{-1}) (Sigma) was added to indicate the redox potential. After sterilizations, cystein (Sigma) was added (from a sterile stock solution of 5 g l^{-1}) at a final concentration of 50 mg l^{-1} to guarantee an anaerobic atmosphere; (v) strict anaerobic conditions supplemented with 25 mg l^{-1} of ergosterol (Sigma); and (vi) strict anaerobic conditions supplemented with 1 g l^{-1} of Tween 80 (Merck). Prior to their addition, ergosterol and Tween 80 were dissolved in 1 ml of absolute ethanol with gentle heating for 5 min and at room temperature, respectively. The inoculum (2% $v\,v^{-1}$) was introduced via a 1 ml sterile syringe. The cultures were grown at 25°C until the mid-exponential growth phase.

2.4. Ethanol challenge (viability assays)

A concentration of 25% ($v\,v^{-1}$) ethanol in YM broth was used as a stress challenge. This concentration was determined in preliminary studies as being that which permits the determination of inactivation kinetics over a relatively short period of time. Cells grown under the above-mentioned conditions were harvested by centrifugation at 3000*g* for 10 min (JP SELECTA, Spain) and resuspended in a solution of 25% ($v\,v^{-1}$) ethanol in YM broth (experiment time point 0). Cells were exposed to this level of ethanol for 4 min at 25°C. At the time points 0.5, 1.0, 1.5, 2.0, and 4.0 min, samples were removed, appropriately diluted in sterile Ringer solution (9 ml), and plated in duplicate on YM agar (Difco) using the Drop Count Technique (Miles and Misra, 1938) for the determination of viability. Counts were done after incubation at 25°C for 72 h. Similar experiments were done with 22.5% $v\,v^{-1}$ ethanol for some of the more sensitive strains.

2.5. Extraction and simultaneous analysis of fatty acids and sterols by GC

The content of the fatty acids (caprylic, capric, lauric, palmitic, linolenic, linoleic, oleic, and stearic acids) and sterol (ergosterol, campesterol, lanosterol) content of the yeast cells grown in each of the culture regimes was analysed. The analytical method (essentially a direct extraction from a paste of harvested cells, followed by a saponification in aqueous/ethanolic potassium hydroxide solution, before chloroform extraction and silanization for GC determination of free and combined fatty acids and sterols) was performed according to the technique described by Cocito and Delfini (1994). Quantitative analysis was carried out with the use of three different internal standards (nonanoic acid, Fluka; nonadecanoic acid, Fluka and cholesterol, Sigma). The gas chromatographic analysis was performed with a Hewlett Packard 5890A (Avondale, USA) equipped with a capillary column of fused silica, phase DB1, of length 30 m and 0.25 mm i.d., film thickness 0.25 μm (J & W. Scientific, Folson, CA, USA). The repeatability of the methods is illustrated in Cocito and Delfini (1994). The average coefficient of variation was 5,63%, since coefficients of variation were less than 15%.

3. Results and discussion

3.1. Influence of different culture conditions on the ethanol tolerance

The effect of ethanol stress (25% or 22.5% $v\,v^{-1}$) on the survival of five non-*Saccharomyces* yeasts grown in different cultures conditions in comparison to a strain of *S. cerevisiae* is shown in Figs. 1 and 2. These levels of ethanol were used because it represents an ethanol stress, which generates kinetics of inactivation measurable over a reasonable period of time ($\leqslant 5$ min). The results indicate that, according to this method, the resistance of *H. guilliermondii* and *C. stellata* to ethanol was comparable to that of *S. cerevisiae* (Figs. 1a–c), while *D. hansenii* behaved differently (Fig. 1d). *H. uvarum* and *T. delbrueckii* were the most sensitive yeast strains. Under the stress condition of 25% $v\,v^{-1}$ of ethanol, the kinetics of inactivation was too rapid to note any induced effect of the cultivation regimes in these strains (data not shown). These results suggest that certain non-*Saccharomyces* yeasts (certainly *H. guilliermondii* and *C. stellata*) could have a higher tolerance to ethanol than previously reported in the literature (Boulton et al., 1995; Gil et al., 1996). The growth of *H. guilliermondii* and *S. cerevisiae* under active oxygenation increased cell viability to subsequent ethanol stress in comparison to growth under anaerobiosis (Figs. 1a and b). The same result was obtained when cultures were grown under anaerobiosis with the addition of ergosterol and Tween 80. These results are in agreement with what was found for *S. cerevisiae,* for which the presence of oxygen or of certain compounds added exogenously to the growth medium could enhance the tolerance to ethanol (Larue et al., 1980; Walker-Caprioglio et al., 1990; Mauricio et al., 1997). The supplementation of *S. cerevisiae, H. guilliermondii,* and *C. stellata* cultures with ergosterol and Tween 80 in semi-aerobic conditions did not increase the ethanol resistance in comparison to non-supplemented semi-aerobic cultures (Figs. 1a–c). When comparing these data with those from anaerobically cultivated cells, it would appear that the effect of oxygen on the ethanol resistance was sufficient in itself to raise the ethanol resistance. *C. stellata* showed a high resistance to ethanol under all the growth conditions tested. The cell viability of this strain was apparently not influenced by any of the different cultivation regimes (Fig. 1c).

As mentioned above, 25% $v\,v^{-1}$ of ethanol was too high to investigate ethanol-induced inactivation of *H. uvarum* and *T. delbrueckii*. However, a slight increase in cell viability was observed when cells where submitted to a lower ethanol challenge (22.5% $v\,v^{-1}$ ethanol) after having grown under active aerobiosis (Fig. 2). The addition of ergosterol and Tween 80 under anaerobiosis did not increase the ethanol tolerance of these non-*Saccharomyces* yeasts and also of *D. hansenii*. It is worth noting that *H. uvarum* showed a different behaviour to that of the other apiculate yeast—*H. guilliermondii*. These results suggest that the addition of certain survival factors to the medium did not have the same effect on the ethanol tolerance of all the yeasts studied.

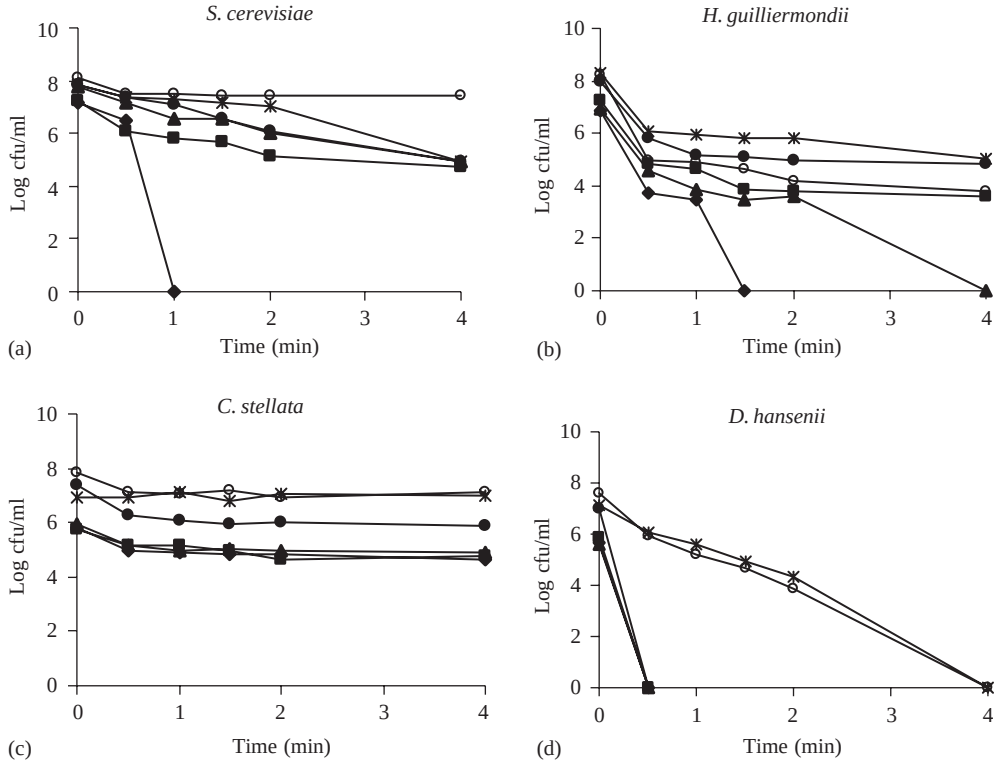

Fig. 1. Effect of ethanol stress (25% v v^{-1}) lasting 4 min on the survival of *S. cerevisiae* (a), *H. guilliermondii* (b), *C. stellata* (c), and *D. hansenii* (d). Prior to exposure, cultures were cultivated under different culture conditions: with oxygenation (○); semi-aerobiose (∗); semi-aerobiose with ergosterol + Tween 80 (●); anaerobiose (♦); anaerobiose with Tween 80 (■); and anaerobiose with ergosterol (▲). The values are means of three independent experiments. The log cfu ml^{-1} = 0 represents values lower than 3.5×10^2.

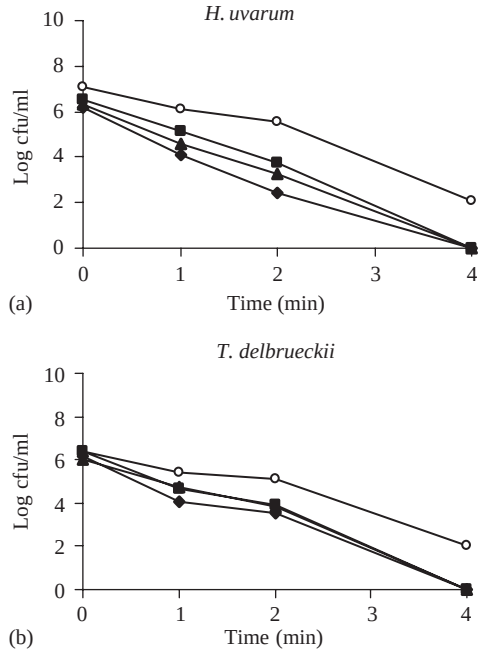

Fig. 2. Effect of ethanol stress (22,5% v v^{-1}) lasting 4 min on the survival of *H. uvarum* (a) and *T. delbrueckii* (b). Prior to exposure, the cultures were cultivated under different culture conditions: with oxygenation (○); anaerobiose (♦); anaerobiose with Tween 80 (■); and anaerobiose with ergosterol (▲). The values are means of three independent experiments. The log cfu ml^{-1} = 0 represents values lower than 3.5×10^2.

3.2. Relationship between ethanol tolerance and lipid composition in yeasts grown in different culture conditions

Tables 1 and 2 show the cellular composition in fatty acids and sterols (as % of total) of the various yeast strains grown under different cultures conditions. The most abundant compounds found in all of the yeasts studied where palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and ergosterol. The proportion of ergosterol differed according to the yeast strain and the culture conditions, as was also reported by Larue et al. (1980), *H. guilliermondii* showing the highest content of this sterol under aerobic conditions (Tables 1 and 2). Oleic acid was the most abundant UFA analysed in the yeasts strains studied. *C. stellata* showed the highest value (48%) (Table 2) of this fatty acid under aerobic conditions, whereas *H. guilliermondii* showed the lowest content (14%) (Table 1). Among the saturated fatty acids (SFA), the content of palmitic acid was the most representative, with the highest values being found in anaerobic culture conditions in most of the yeasts studied (Tables 1 and 2). This result is in accordance with the studies on *S. cerevisiae* by Steels et al. (1994), who reported that, in cells grown anaerobically, the phospholipids were tipically enriched in SFA rather than in mono-UFA. It is worthy of note that, for each yeast strain, the lipids that showed most variation under different culture conditions were

Table 1
Cellular contents of fatty acids and sterols (as % of total) in *S. cerevisiae*, *H. guilliermondii*, and *H. uvarum* grown under different culture conditions

	Anaerobic			Aerobic	Semi-aerobic	
	No addition	E	TW		No addition	E + TW
S. cerevisiae						
Caprylic acid	2.44	0.79	ND	ND	1.40	0.33
Capric acid	11.95	4.49	1.64	1.10	7.33	0.49
Lauric acid	10.23	3.29	3.49	2.77	9.29	1.38
Palmitic acid	27.09	13.34	18.70	14.71	32.72	11.76
Stearic acid	6.52	3.90	5.02	5.18	ND	3.19
Linolenic acid	ND	ND	ND	ND	ND	ND
Linoleic acid	1.49	0.53	ND	ND	ND	0.72
Oleic acid	6.33	6.70	60.63	27.82	12.68	31.99
Ergosterol	28.77	66.18	9.06	47.91	36.59	49.91
Campesterol	ND	ND	ND	0.18	ND	0.06
Lanosterol	5.17	0.78	1.45	0.33	ND	0.16
H. guilliermondii						
Caprylic acid	1.09	0.21	1.14	ND	0.21	ND
Capric acid	1.97	0.17	0.45	0.24	0.87	0.82
Lauric acid	8.76	1.89	9.04	0.78	7.43	7.45
Palmitic acid	20.36	3.57	9.06	11.92	15.41	15.91
Stearic acid	4.81	2.76	2.33	1.11	1.68	1.56
Linolenic acid	ND	ND	ND	ND	ND	ND
Linoleic acid	ND	7.73	1.72	ND	ND	ND
Oleic acid	10.77	10.26	31.43	14.21	11.81	11.85
Ergosterol	46.34	82.40	43.68	71.74	62.59	62.40
Campesterol	ND	ND	ND	ND	ND	ND
Lanosterol	5.90	ND	1.15	ND	ND	ND
H. uvarum						
Caprylic acid	1.12	0.17	0.76	0.16	0.22	0.17
Capric acid	1.56	0.25	1.64	0.12	0.76	0.23
Lauric acid	14.19	1.47	9.73	0.16	4.77	0.54
Palmitic acid	37.42	ND	23.81	19.74	26.26	10.24
Stearic acid	23.24	ND	3.16	2.99	2.02	4.94
Linolenic acid	ND	ND	ND	ND	ND	ND
Linoleic acid	4.28	ND	ND	5.76	4.34	12.60
Oleic acid	2.68	ND	59.69	21.50	6.98	33.61
Ergosterol	14.34	73.76	ND	49.57	51.80	33.88
Campesterol	ND	ND	ND	ND	ND	ND
Lanosterol	1.16	ND	1.22	ND	2.84	3.79

E—Ergosterol (25 mg l^{-1}); TW—Tween 80 (1 g l^{-1}), as a source of oleic acid; ND—not detected.
Results are the mean of three independent experiments (coefficient of variation was less than 10%, except for caprylic, capric, and lauric were less than 23%).

ergosterol and oleic acid. The largest difference was found between cells grown in the presence of oxygen and those grown in anaerobiosis, with the levels being considerably higher in the former case (Tables 1 and 2). These results corroborate the fact that oxygen plays an important role in the synthesis of sterols and UFA (Pfisterer et al., 1976; Larue et al., 1980; Strydom et al., 1982; Eglinton and Henschke, 1991; Mauricio et al., 1997).

Fig. 3 displays the effect of aerobic, semi-aerobic, and anaerobic (without supplements) growth conditions on the sterol and fatty acid (saturated and unsaturated) composition of yeast cells. As expected, the content of sterols and UFA in all of the yeasts studied is markedly higher in cells grown under active oxygenation than under semi-aerobic or anaerobic conditions. A higher percentage of SFA was formed in cells grown under anaerobiosis. These findings have also been reported by other workers in their studies mainly with *S. cerevisiae*. Steels et al. (1994) reported that aerobic *S. cerevisiae* cells contained about 80% UFA, while in cells grown anaerobically, the phospholipids were typically enriched in SFA. Similar behaviour is displayed by non-*Saccharomyces* yeasts in this study. In an aerobic environment, a healthy *Saccharomyces* culture is able to synthesize its full component of both saturated and UFA, but, in an anaerobic environment, such as in the wine must during fermentation, the synthesis of

Table 2
Cellular contents of fatty acids and sterols (as % of total) in *C. stellata*, *T. delbruekii,* and *D. hansenii* grown under different culture conditions

	Anaerobic			Aerobic	Semi-aerobic	
	No addition	E	TW		No addition	E + TW
C. stellata						
Caprylic acid	2.62	0.09	0.46	0.14	0.15	ND
Capric acid	2.09	ND	ND	0.18	ND	ND
Lauric acid	3.87	0.19	0.35	0.37	0.22	ND
Palmitic acid	53.49	1.79	18.35	4.40	7.19	4.55
Stearic acid	0.55	1.32	11.53	3.46	5.27	2.95
Linolenic acid	ND	ND	ND	ND	ND	ND
Linoleic acid	ND	2.24	34.87	2.16	2.65	ND
Oleic acid	32.46	4.61	11.63	48.10	32.21	24.22
Ergosterol	ND	88.66	19.98	40.63	52.31	68.27
Campesterol	ND	ND	ND	ND	ND	ND
Lanosterol	ND	1.11	2.84	0.55	ND	ND
T. delbrueckii						
Caprylic acid	5.09	0.07	0.00	0.00	0.00	2.29
Capric acid	3.63	0.06	1.19	0.00	0.00	0.85
Lauric acid	7.06	0.13	14.08	0.07	3.22	0.91
Palmitic acid	48.44	1.58	15.45	9.69	69.40	7.82
Stearic acid	0.00	0.00	7.15	1.55	8.30	4.08
Linolenic acid	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic acid	22.45	0.00	18.79	24.68	6.18	2.09
Oleic acid	13.31	0.16	24.24	26.06	5.47	13.74
Ergosterol	0.00	97.99	17.55	37.55	4.92	33.10
Campesterol	0.00	0.00	0.00	0.39	1.35	0.00
Lanosterol	0.00	0.00	1.54	0.00	1.15	0.00
D. hansenii						
Caprylic acid	1.52	1.50	0.55	0.03	0.23	0.10
Capric acid	0.00	0.00	0.83	0.01	0.21	0.00
Lauric acid	7.49	0.58	6.94	0.12	0.54	0.20
Palmitic acid	12.02	0.00	12.57	15.85	17.06	4.95
Stearic acid	78.97	97.92	68.95	5.04	8.82	1.41
Linolenic acid	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic acid	0.00	0.00	4.44	25.23	51.44	0.00
Oleic acid	0.00	0.00	5.73	30.54	21.70	8.25
Ergosterol	0.00	0.00	0.00	23.10	0.00	85.09
Campesterol	0.00	0.00	0.00	0.05	0.00	0.00
Lanosterol	0.00	0.00	0.00	0.03	0.00	0.00

E—Ergosterol (25 mg l^{-1}); TW—Tween 80 (1 g l^{-1}), as a source of oleic acid; ND—not detected.
Results are the mean of three independent experiment (coefficient of variation was less than 10%, except for caprylic, capric, and lauric were less than 23%).

unsaturated fatty acid and sterols is not possible (Morrisey et al., 1999).

Fig. 4 shows the effect of lipid supplements (ergosterol and Tween 80) under anaerobic and semi-aerobic growth conditions on the total fatty acid and sterol yeast composition. The yeasts that were studied display a range of sterol and UFA uptake capacities. It seems that the ergosterol and oleic acid uptake is higher in cultures grown under anaerobiosis than in those grown under semi-aerobic conditions for all yeasts except *D. hansenii* (cf. Figs. 3b and c with Figs. 4a–c). As reported for *S. cerevisiae*, the biosynthesis of sterols and UFA is an aerobic process (Pfisterer et al., 1976; Eglinton and Henschke, 1991; Mauricio et al., 1997). The addition of ergosterol and oleic acid (from Tween 80) to anaerobic cultures, and the consequent incorporation of these lipids by cells, increased the ethanol tolerance of *S. cerevisiae* and *H. guilliermondii* (cf. Fig. 1a and b with Fig. 4c). However, in the case of the most sensitive ethanol yeast strains, *H. uvarum* and *T. delbrueckii*, the addition of these compounds under anaerobiosis did not increase their ethanol tolerance, in spite of the incorporation of these lipids in their cell membranes (cf. Fig. 2a and b with Fig. 4c). On the contrary, *D. hansenii* seems not to incorporate these lipids under this cultivation regime (Tables 1 and 2).

From the results obtained, it seems that, only for *S. cerevisiae* and *H. guilliermondii*, there is a relationship

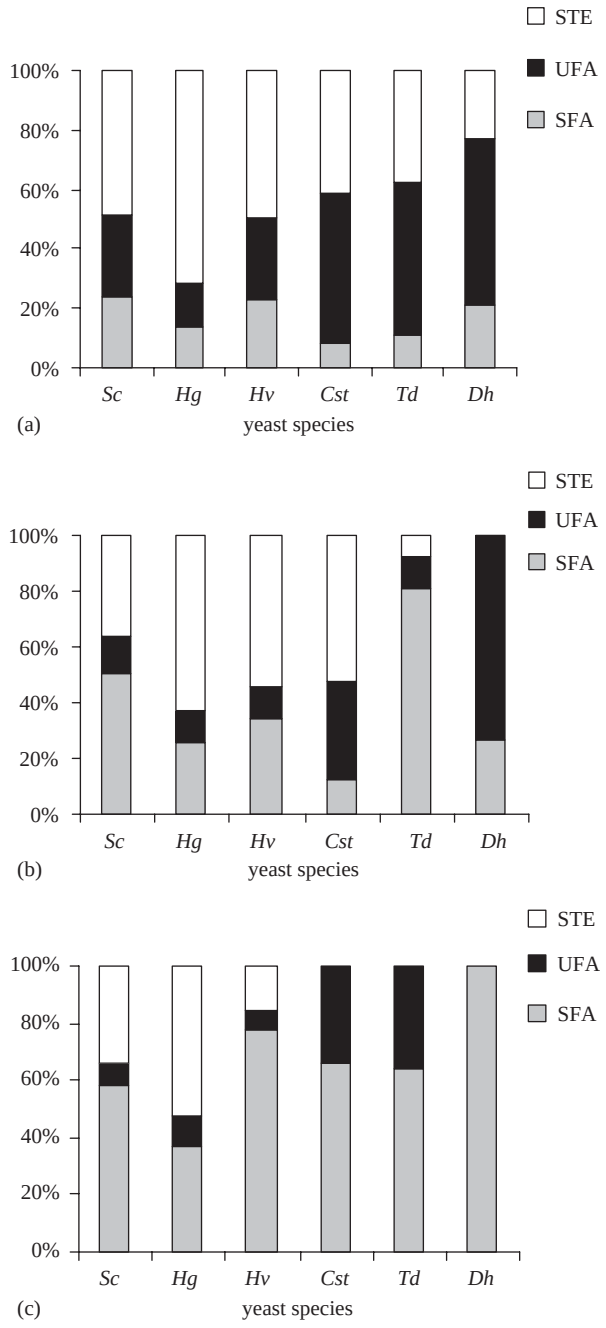

Fig. 3. Distribution of sterols (STE), (SFA) and UFA content (as % of total) in the different yeast species: *Sc*—*S. cerevisiae*, *Hg*—*H. guilliermondii*, *Hv*—*H. uvarum*, *Cst*—*C. stellata*, *Td*—*T. delbrueckii,* and *Dh*—*D. hansenii*, grown in aerobic (a), semi-aerobic (b), and anaerobic (c) culture conditions without supplements.

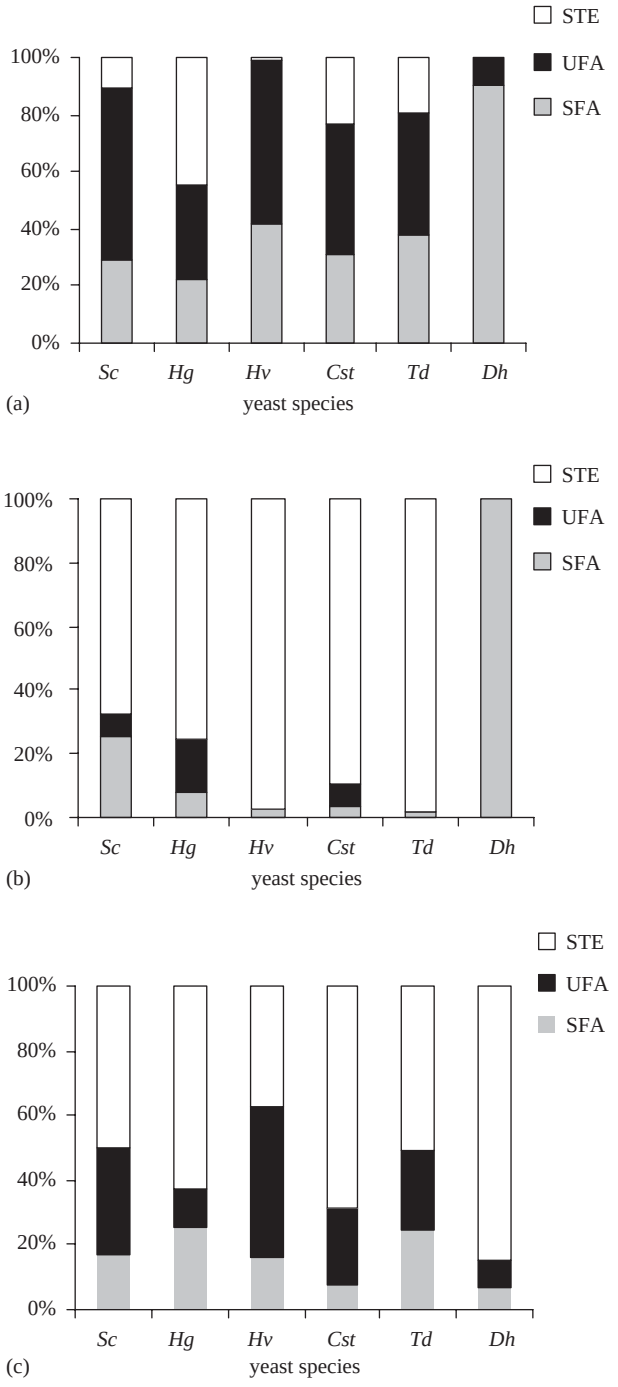

Fig. 4. Distribution of sterol (STE), SFA, and UFA content (as % of total) in the different yeast species: *Sc*—*S. cerevisiae*, *Hg*—*H. guilliermondii*, *Hv*—*H. uvarum*, *Cst*—*C. stellata*, *Td*—*T. delbrueckii,* and *Dh*—*D. hansenii*, grown under anaerobic culture conditions with Tween 80 (a); anaerobic culture conditions with ergosterol (b); and semi-aerobic culture conditions with Tween 80 and ergosterol (c).

between sterol and fatty acid cell composition and ethanol tolerance. An increase in the proportion of either oleic acid or ergosterol or of both compounds in these yeast cells provide organisms with a greater adaptation to high environmental ethanol levels. Several authors reported that the addition of fatty acids and/or ergosterol to *S. cerevisiae* cultures elicits a greater ethanol yield and increased ethanol tolerance by adjusting the membrane lipid composition (Thomas et al., 1978; Ghareib et al., 1988; Mishra and Prasad, 1989) and that the requirement for oxygen can be eliminated by adding ergosterol and/or oleic acid to the

culture medium (Larue et al., 1980; Mauricio et al., 1997). It is shown in this work that UFA and sterols also seem to play an equivalent role as modulators of ethanol tolerance in *H. guilliermondii*, but not in all non-*Saccharomyces* yeasts studied. The presence of the survival factors in the cells did no produce the same physiological state in all of the yeasts analysed. These results corroborate the fact the ethanol tolerance mechanism is complex and not yet fully understood.

The role of non-*Saccharomyces* yeasts in wine must fermentation is usually limited by their inability to tolerate the fermenting must environment; this is most often considered to be due to their intolerance to ethanol. The results of this study show that, by manipulating the cultivation conditions, the ethanol tolerance of some non-*Saccharomyces* strains can be increased. Thus, the capacity of these species to participate in the alcoholic fermentation as part of more complex starter cultures systems than merely *S. cerevisiae* can be enhanced. Another important finding is the high ethanol resistance exhibited by the *H. guilliermondii* strain studied in this work, which is not in accordance with previous studies, which report and assume that apiculate yeasts (*Kloeckera* and *Hanseniaspora*), are low ethanol tolerant species. However, some recent quantitative studies on winemaking ecology have shown that non-*Saccharomyces* species survived at significant levels for longer periods during fermentation than previously thought (Fleet, 1990; Cianni and Picciotti, 1995). It is possible that other non-*Saccharomyces* strains isolated from the wine environment (*H. guilliermondii* NCYC 2380 was isolated from grape must used for Port wine production in the Douro region of Portugal) may show similar behaviour. Cell lipid changes induced by certain growth culture conditions play an important role in the adaptation of yeasts to ethanol containing environments. However, a better understanding of the intrinsic resistance of yeasts to ethanol is still required.

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