Appl Microbiol Biotechnol (1992) 37:647-651

Applied and Microbiology Biotechnology

Lipid content of *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance

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Received 5 November 1991/Accepted 14 April 1992

Summary. We analysed the fatty acid and sterol compositions of various *Saccharomyces cerevisiae* strains with ethanol tolerance varying from 4% to 12% (v/v) ethanol and at different concentrations of ethanol. The results we obtained agree with the existence of a relationship between membrane fluidity and ethanol tolerance but they do not support a direct role of unsaturated fatty acids in this tolerance. On the other hand, they support the importance of ergosterol in this phenomenon.

Introduction

Yeasts, such as *Saccharomyces cerevisiae*, are widely used for industrial purposes. One of their principal applications is the fermentation of sugars to produce alcoholic beverages or industrial alcohol. Tolerance to ethanol is an important constraint for industrial strains, limiting growth and fermentation rates and consequently ethanol yield. For this reason, important efforts have been made to understand the physiological effects of ethanol in yeast cells.

Ethanol tolerance for growth or fermentation capability seems to depend on different mechanisms. Some strains that can grow in the presence of 12% (by vol.) of ethanol, are not able to produce more than 5% (by vol.) from 24% (w/v) glucose (del Castillo Agudo 1985), whereas any strain able to ferment completely high concentrations of sugar show good ethanol tolerance for growth. Inhibition of cell growth seems to act in any case as an important limiting factor for fermentation (Benitez et al. 1983; del Castillo Agudo 1985).

Tolerance to ethanol depends on complex genotypic determination (del Castillo Agudo 1985). Efforts to understand this phenomenon have been centred on the importance of the cell plasma membrane as the main target for ethanol. A relationship between plasma membrane fluidity and ethanol tolerance has been proposed by many authors (Rose 1980, 1987; Thomas et al. 1978; Thomas and Rose 1979; Hayashida and Ohta 1980; Beavan et al. 1982; Jones and Greenfield 1987; Mishra and Prasad 1988, 1989). An increase in membrane fluidity caused by a higher amount of unsaturated fatty acids is supposed to increase ethanol tolerance (Thomas et al. 1978; Thomas and Rose 1979; Beavan et al. 1982; Mishra and Prasad 1989).

However, membrane fluidity is not solely due to levels of unsaturated fatty acids; Oura et al. (1959) found that membranes from cells grown under anaerobiosis, were 4.5 times more fluid than membranes from aerobically growing cells, which displayed a higher unsaturation index. Sterols represent another category of lipid components in the yeast plasma membrane that could play a role in ethanol tolerance (Thomas et al. 1978; Hayashida and Ohta 1980; Jones and Greenfield 1987). A relationship between the sterol type present in the membranes and ethanol tolerance (Thomas et al. 1978), as is the case for resistance to freeze-thawing (Calcott and Rose 1982), has been proposed. Ergosterol been preferred to other sterols for increasing tolerance.

In this work were analysed the fatty acyl residues and sterol composition of cells from strains with different degrees of ethanol tolerance, based on their growth ability in the presence of ethanol (del Castillo Agudo 1985).

Materials and methods

Strains. The yeast strains used in this work were obtained previously (del Castillo Agudo 1985); they are listed in Table 1.

Media and growth conditions. Media and growth conditions were carried out as described by Beavan et al. (1982). Ethanol was added to growing cultures (0.5 l) when absorbance at 660 nm (A_{660}) reached a value between 0.5 and 0.6. The final volume was adjusted to 1 l and cells harvested by centrifugation 10 h later.

Lipid analysis. After harvesting, cells were washed with 100 ml water, and disrupted with 35 g glass beads (Sigma type V, 0.45–0.50 mm diam.) in a Braun homogeniser, and the total lipids extracted as described by Beavan et al. (1982). The phospholipid and sterol fractions were separated by thin layer chromatography on 0.5-mm thick silica gel H plates (Merck), with a solvent system of hexane/diethyl-ether/glacial acetic acid (70:30:2 by vol.). Lipid

Table 1. Ethanol tolerance of different strains of *Saccharomyces cerevisiae*

Strain	Origin	Genotype	Ethanol tolerance Phenotype[a]	
			[Ethanol] (% v/v)	Growth: generation time (h)
LS1	(5)	*MATα ade2-1 CYH^R*	4	13
LS3-2B	LS3 spore (5)	*MATα CYH^R*	7	15
LS2	(5)	*MATα ade2-1 CYH^R*	8	8
LS3-1D[b]	LS3 spore (5)	*MATa/α HO*	9	14
LS3-2D[b]	LS3 spore (5)	*MATa/α HO CYH^R*	10	13
LS3-3D[b]	LS3 spore (5)	*MATa/α HO ade2-1*	11	25
LA1[b]	ACA7 spore (5)	*MATa/α HO*	12	24
LS3	LS1*LA1 (5)	*MATa/α HO/ho ADE2/ade2-1 CYH^R/cyh^s*	12	26
ACA7	Wine isolate (5)	*MATa/α HO*	12	ND

[a] Maximal ethanol concentration (% v/v) observed that allowed growth, and generation time at this concentration

[b] Homothallic and homozygous diploid yeast strain
ND, not determined; (5), del Castillo Agudo (1985)

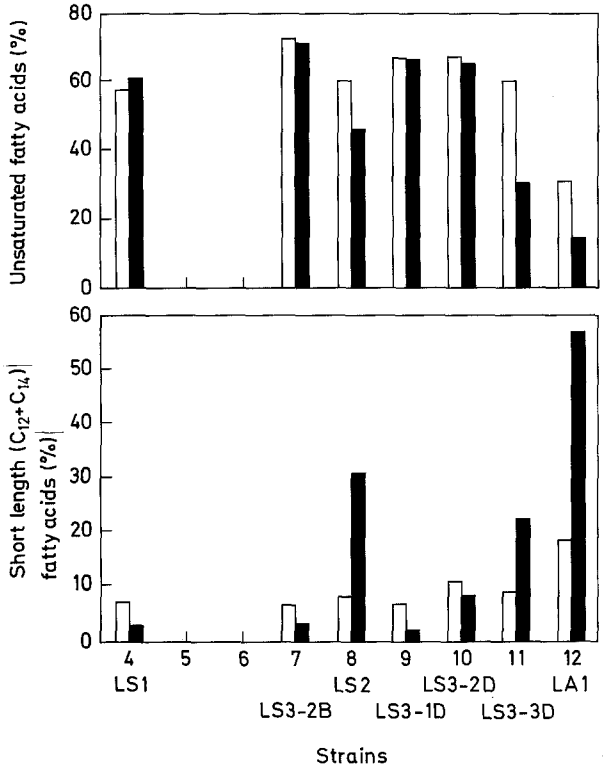

Fig. 1. Relationship between the levels of unsaturated and short-length fatty acyl groups with strain tolerance to ethanol in *Saccharomyces cerevisiae*. Fatty acyl composition was determined for each strain after growth in the absence of ethanol (□) and with the maximal concentration of ethanol that permits growth (■), to each particular strain tolerance

bands were identified using phosphatidyl choline and cholesterol as standards run in parallel after visualisation with iodine vapour. Bands were scraped from the plates and lipids extracted from the silica gel by reflux with methanol.

Fatty acyl residues from the phospholipid fraction were analysed by gas liquid chromatography (GLC) and the unsaturation index determined as described by Beavan et al. (1982), except that short-chains (C_{12}) were included in unsaturation index calculations. Sterol analysis was carried out, after saponification, by gas chromatography as performed by Astin et al. (1977). The column was calibrated with a mixture of squalene, ergosterol, 24(28)-dehydroergosterol and lanosterol. Sterols were identified by their retention time on the column according to the data from Astin et al. (1977).

Results

Fatty acyl composition from phospholipids present in strains with different degrees of ethanol tolerance

Fatty acyl composition was determined in the phospholipid fraction extracted from *S. cerevisiae* strains, covering a wide range of tolerance to ethanol and the results obtained are presented in Fig. 1. It can be observed that more tolerant strains did not show an increase in fatty acid unsaturation when growing at the limits of ethanol concentration for growth. On the contrary, they exhibited a marked decrease in this parameter, in contrast to the importance attributed to unsaturated fatty acids for increasing ethanol tolerance (Thomas et al. 1978; Thomas and Rose 1979; Beavan et al. 1982; Mishra and Prasad 1989). On the other hand, tolerance seems to correlate with a significant increase in the proportion of short-length fatty acids (mainly C_{12}).

Modification of fatty acyl groups by growth with different ethanol concentrations and strain tolerance

Strains LS1, LS2 and LA1 showing ethanol tolerance (determined by growth ability in the presence of ethanol) to 4, 8, and 12% (v/v) respectively, were selected in order to analyse variations in their fatty acyl composition when grown in the presence of variable ethanol concentrations.

In agreement with data presented in Fig. 1, the more tolerant strain (LA1) showed a clear reduction in the fatty acid unsaturation index, concomitant with an increase in C_{12} fatty acid content when the highest ethanol concentrations were reached (Fig. 2). Strain LS2 displayed similar behaviour (Fig. 2), with a constant fatty acids

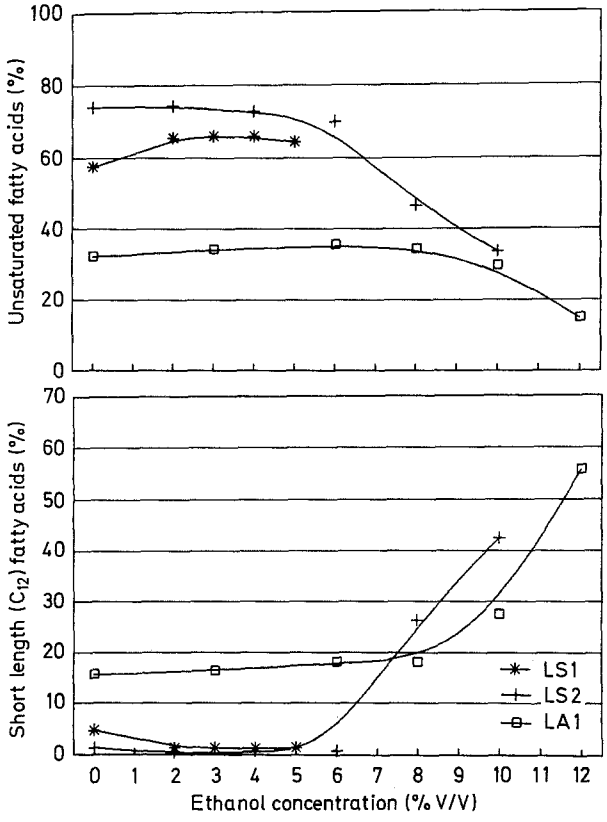

Fig. 2. Variation in fatty acyl unsaturation index and the proportion of short-length fatty acyl groups in *S. cerevisiae* strains according to the concentration of ethanol. Strains with different ethanol tolerance, such as LS1 (4%; *), LS2 (8%; +) and LA1 (12%; □) were grown in the presence or absence of different concentrations of ethanol. Similar profiles were observed when the unsaturation index was calculated computing only long-chain ($>C_{16}$) fatty acids (not shown)

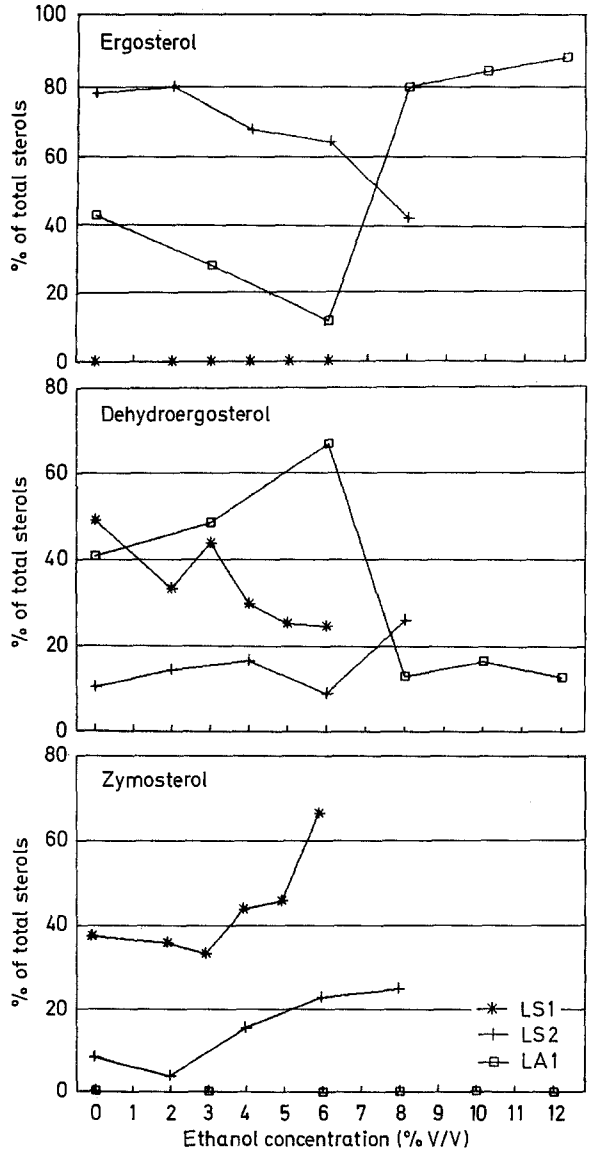

Fig. 3. Changes in the content of sterols: ergosterol, 24(28)dehydroergosterol and zymosterol in *S. cerevisiae* strains with different ethanol tolerance. Strains LS1 (*), LS2 (+) and LA1 (□), resistant to 4%, 8% or 12% ethanol, respectively, were grown in the presence or absence of different concentrations of ethanol and the content of the different sterols was measured as described in the text

unsaturation index at low ethanol concentration. After a critical concentration of ethanol was present, there occurred a decrease in this index, in parallel with a spectacular increase in C_{12} residues.

Strain LS1 could not grow at ethanol concentrations higher than 4% (v/v). In this range of ethanol concentrations there were no variations in the fatty acid unsaturation index or that of the short-length (C_{12}) fatty-acid fraction.

Modification in sterol contents depending on ethanol concentration and their relationship to strain alcohol tolerance

The sterol content and its variations when strains LS1, LS2 and LA1 were grown in different concentrations of ethanol are shown in Fig. 3. The following significant observations can be drawn:

1. Strain LS1, the more sensitive strain, lacked detectable amounts of ergosterol independently of ethanol concentration.
2. Ergosterol was the main sterol of strain LS2, the middle tolerant strain, at any ethanol concentration.
3. Ergosterol contents showed a decrease when ethanol in the medium increased up to 6% (v/v), followed by a marked increase at the highest ethanol concentrations tested. In the case of LA1, the most tolerant strain, ergosterol was almost 90% of the total sterol detected, when growing in the presence of 12% (v/v) ethanol in the medium.
4. Zymosterol was totally absent at any ethanol concentration in strain LA1, but it was present in significant amounts in strain LS2, even showing an increase with ethanol concentration, and becoming the predominant sterol in strain LS1 at the limiting ethanol concentration.

5. 24(28)Dehydroergosterol showed a symmetrical behaviour to ergosterol in strains LA1 and LS2, and with zymosterol in the case of strain LS1.

Discussion

Yeast tolerance to ethanol has been correlated with membrane fluidity, and both phenomena have been associated with membrane lipid composition. Work developed by Rose and co-workers (Thomas et al. 1978; Beavan et al. 1982; Rose 1980, 1987) and supported by observations from other groups (Hayashida and Ohta 1978; Jin et al. 1981; Janssens et al. 1983; Mishra and Prasad 1989), has established that an increase in the fatty acid unsaturation index represents an adaptation mechanism to the increase in ethanol concentration in the culture. The increase in unsaturation leads to greater membrane fluidity.

Our results do not accord with the role attributed to fatty acid unsaturation as an adaptative response to ethanol. Tolerant strains show an unsaturation index smaller than less tolerant strains, and even a decrease in unsaturation with increasing ethanol concentration was observed. We also observed a relationship between ethanol tolerance and short-length (C_{12}) fatty-acid content, with higher amounts of this type of fatty acid associated with both ethanol tolerance and ethanol concentration in the medium. It is likely that an increase in the proportion of short-length fatty acids derives from higher membrane fluidity, as does unsaturation. We cannot conclude, however, that variation in the content of C_{12} fatty acids is a direct effect of adaptation to ethanol, since it may be the result of the sensitivity of the fatty-acid-synthesis protein complex to ethanol. Such is the case in *Escherichia coli*, in which inhibition of enzymes implicated in fatty-acid-chain elongation has been reported, both in vivo an in vitro (Buttke and Ingram 1978, 1980), the presence of short-length fatty-acyl groups being the manifestation of a poor membranes state, rather that an adaptive mechanism to ethanol. Nevertheless the fact that the most tolerant strain contains a significantly higher proportion of C_{12} fatty acids may be relevant.

The role of ergosterol in ethanol tolerance has also been reported by Lees et al. (1980). These authors pointed out that cells carrying mutations affecting genes *ERG2* and *ERG6* accumulated zymosterol and displayed ethanol sensitivity. It is also known that supplementing cultures with ergosterol results in an increased resistance of yeast to ethanol (Thomas et al. 1978; Hayashida and Ohta 1980; Larue et al. 1980; Lees et al. 1980; Janssens et al. 1983).

A role of sterols in ethanol tolerance was clearly supported by the present work. Thus the total absence of any detectable amount of ergosterol in a sensitive strain (LS1), together with the spectacular increase in ergosterol at the highest ethanol concentrations in a tolerant strain, suggest an important role of this lipid in the response of yeast to ethanol. The absence of ergosterol is compensated in the sensitive strains LS1 and LS2 by zymosterol, although apparently this sterol cannot replace ergosterol for ethanol tolerance. In fact, the more sensitive strain LS1 behaved like strains bearing a genotype *erg2+erg6*, in accumulating zymosterol and sensitivity to ethanol, although we do not know whether phenotypic similarities correspond with equivalent genotypes.

The role of lipids in ethanol tolerance has been mainly analysed by evaluating the tolerance of phenocopies, created by supplementing cultures with a specific lipid. The results presented in this work were based on strains with defined ethanol tolerance. Genetic segregation of this tolerance, showed the implication of a minimum of two genes for increased tolerance from 4% to 8% (v/v), and a minimum of two more genes to reach tolerance to 12% (v/v) ethanol (del Castillo Agudo 1985). This difference in approach could be responsible for the discrepancies observed for the role of unsaturation of fatty acids in previous reports. Nevertheless they point out that the cell may adopt different strategies to alter membrane fluidity (such as increasing ergosterol content) and, although it is clearly established that unsaturated fatty acids improve yeast cell tolerance to ethanol, this does not mean that cells were able to respond in this way for adaptation to the presence of increasing concentrations of ethanol in the growth medium.

Acknowledgements. We are indebted to Dr. Jose Ruiz Herrera for critical reading of the manuscript.

References

Astin AM, Haslam JM, Woods RA (1977) The manipulation of cellular cytochrome and lipid composition in haem mutant of *Saccharomyces cerevisiae*. Biochem J 166:275–285

Beavan MJ, Charpentier C, Rose AH (1982) Production and tolerance of ethanol in relation to phospholipid fatty acyl composition in *Saccharomyces cerevisiae*. J Gen Microbiol 128:1447–1455

Benitez T, del Castillo L, Aguilera A, Conde J, Cerdá Olmedo E (1983) Characterization of *Saccharomyces* strains with respect to their ability to grow and ferment in the presence of ethanol and sucrose. Appl Environ Microbiol 45:1429–1436

Butkke TM, Ingram LO (1978) Mechanism of ethanol induced changes in lipid composition of *Escherichia coli*: Inhibition of fatty acid synthesis in vivo. Biochemistry 17:637–644

Butkke TM, Ingram LO (1980) Ethanol-induced changes in lipid composition of *Escherichia coli*: inhibition of saturated fatty acid synthesis in vitro. Arch Biochem Biophys 203:465–471

Calcott PH, Rose AH (1982) Freeze-thaw and cold shock resistance of *Saccharomyces cerevisiae* as affected by plasma membrane lipid composition. J Gen Microbiol 128:549–555

Castillo Agudo L del (1985) Genetic aspects of ethanol tolerance and production by *Saccharomyces cerevisiae*. Curr Microbiol 12:41–44

Hayashida O, Ohta K (1978) Cell structure of yeast grown anaerobically in *Aspergillus oryzae* proteolipid supplemented media. Agric Biol Chem 42:1139–1145

Hayashida S, Ohta K (1980) Effects of phosphatidylcholine or ergosteryloleate on physiological properties of *Saccharomyces sake*. Agric Biol Chem 44:2561–2567

Janssens JM, Burris N, Woodward A, Bailey RB (1983) Lipid-enhanced ethanol production by *Kluyveromyces fragilis*. Appl Environ Microbiol 45:598–602

Jin CK, Chiang HL, Wang SS (1981) Steady state analysis of the enhancement in ethanol productivity of a continuous fermen-

tation process employing a protein-phospholipid complex as protecting agent. Enzyme Microb Technol 3:249–257

Jones RP, Greenfield PF (1987) Ethanol and the fluidity of the yeast plasma membrane. Yeast 3:223–232

Larue F, Lafon-Lafourcade S, Ribereau-Gayon P (1980) Relationship between the sterol content of yeast cells and their fermentation activity in grape must. Appl Environ Microbiol 39:808–811

Lees ND, Lofton SL, Woods RA, Bard M (1980) The effects of varied energy source and detergent on the growth of sterol mutants of *Saccharomyces cerevisiae*. J Gen Microbiol 118:209–214

Mishra P, Prasad R (1988) Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. J Gen Microbiol 134:3205–3211

Mishra P, Prasad R (1989) Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 30:294–298

Oura E, Soumalainen H, Collander R (1959) The permeability of yeast to acids. Physiol Plantarum 12:534–544

Rose AH (1980) Recent research on industrial important strains of *Saccharomyces* strains. In: Skinner FA, Passmore SM, Davenport RR (eds) Academic Press, London, p 103

Rose AH (1987) Responses to the chemical environment. In: Rose AH, Harrison JS (eds) Academic Press, London, pp 5–40

Thomas SD, Rose AH (1979) Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma membrane composition. Arch Microbiol 122:49–55

Thomas SD, Hossack JA, Rose AH (1978) Plasma membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. Arch Microbiol 117:239–245