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Ethanol tolerance and membrane fatty acid adaptation in *adh* multiple and null mutants of *Kluyveromyces lactis*

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Abstract – The effects of ethanol and 1-octanol on growth and fatty acid composition of different strains of *Kluyveromyces lactis* containing a mutation in the four different alcohol dehydrogenase (KlADH) genes were investigated. In the presence of ethanol and 1-octanol *K. lactis* reduced the fluidity of its lipids by decreasing the unsaturation index (UI) of its membrane fatty acids. In this way, a direct correlation between nonlethal ethanol concentrations and the decrease in the UI could be observed. At concentrations which totally inhibited cell growth no reaction occurred. These adaptive modifications of the fatty acid pattern of *K. lactis* to ethanol contrasted with those reported for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Whereas these two yeasts increased the fluidity of their membrane lipids in the presence of ethanol, *K. lactis* reduced the fluidity (UI) of its lipids. Among the different isogenic *adh* negative strains tested, the strain containing no ADH (*adh*^{0}) and that containing only *KlADH1* were the most alcohol-sensitive. The strain with only *KlADH2* showed nearly the same tolerance as reference strain CBS 2359/152 containing all four ADH genes. This suggests that the *KlADH2* product could play an important role in the adaptation/detoxification reactions of *K. lactis* to high ethanol concentrations. © 2000 Éditions scientifiques et médicales Elsevier SAS

***Kluyveromyces lactis* / *adh* mutants / ethanol tolerance / membrane adaptation / fatty acid unsaturation**

1. Introduction

In *Kluyveromyces lactis* four ADH genes have been identified which encode cytosolic (KlADHI and KlADHII) and mitochondrial isozymes (KlADHIII and KlADHIV) [31, 32, 34]. Like *ADH1* in *Saccharomyces cerevisiae*, both cytosolic activities seem to play a fermentative role. They are essentially constitutive and are mainly expressed in glucose-grown cells [8, 10]. On the contrary, the genes encoding the mitochondrial isozymes are tightly regulated by the carbon source: *KlADH4* is specifically induced by ethanol and is not sensitive to glucose repression [26], while *KlADH3* is repressed by ethanol and induced by the other respiratory carbon sources [30]. But the physiological function of these two mitochondrial genes is not clear.

Kinetic studies of all four ADH isozymes, carried out by Bozzi et al. [5], showed that the K_m values of the two cytosolic enzymes (KLADHI and KLADHII) are about 10-fold higher than those of the inducible mitochondrial systems (KLADHIII and KLADHIV). Therefore, it is still unclear which ADH isozyme is the most important in the degradation (detoxification) of externally added ethanol.

Recently, it has been shown that this compound can play a direct role in gene regulation.

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Table I. List of *K. lactis* strains.

Strain	Genotype	Reference
CBS 2359/152	*MATa, metA, KlADH1-4*	Wesolowski-Louvel et al. [36]
MS7-62	*MATα, lysA, argA, kladh1::URA3, kladh2::URA3, kladh3::URA3, kladh4::KlURA3*	Saliola et al. [29]*
MS7-62/KlADH1	*MATα, lysA, argA, kladh1::URA3, kladh2::URA3, kladh3::URA3, kladh4::KlURA3, KlADH1*	this work
MS7-62/KlADH2	*MATα, lysA, argA, kladh1::URA3, kladh3::URA3, kladh4::KlURA3, KlADH2*	this work
MS7-62/KlADH3	*MATα, lysA, argA, kladh1::URA3, kladh2::URA3, kladh3::URA3, kladh4::KlURA3, KlADH3*	this work
MW98-8C	*MATα, ura3, argA1, lysA1, rag1, rag2, kladh3*	Bianchi et al. [3]

* In the text, also called adh^0.

In fact, ethanol can repress not only genes directly involved in its metabolism, like *KlADH3* [30], but also *PDC1* of *S. cerevisiae* [25], one of the genes that code for the pyruvate decarboxylase [15, 22, 33]. A *cis*-acting element contained in the promoter of *PDC1* is responsible for the ethanol repression [25]. This element, named ERA (ethanol repression and autoregulation), is also present in the promoters of many glycolytic genes, suggesting a general mechanism of repression (and/or inhibition) modulated by ethanol.

The main target for the toxicity of alcohols is the membrane. Like many other organic solvents they inhibit cell growth because they partition preferentially in membranes, disturbing the integrity of this cellular barrier leading to nonspecific permeabilization [14, 21]. However, it is well known that yeasts in particular can adapt to alcohol toxicity by different mechanisms, one of the most important being the modification of membrane lipids [9, 18].

In the present paper, we investigated the growth inhibition of different ADH strains, lacking three of the four ADH genes, in response to externally added ethanol and its effect on the fatty acid composition of the membrane phospholipids.

2. Materials and methods

2.1. Microorganisms

The *K. lactis* strains used in this work are listed in *table I*. The strains containing only one ADH gene reintegrated into the genome were constructed transforming the adh^0 strain MS7-62 with pBR322-based plasmids, unable to stably replicate in yeast.

The plasmids used were, in the case of *KlADH1*, a derivative of p23-V-55 [32] harboring a 7.5-kb *Bam*HI fragment encompassing the *KlADH1* gene (1), and pDS100A (a gift from C. Denis) containing the *KlADH2* gene as a 4.5-kb *Bgl*II segment cloned in the *Bam*HI site of YRP7, while *KlADH3* was included in the 4.5-kb *Bam*HI fragment of plasmid 12-V-14 [32]. The transformations were carried out using a Biorad electroporation apparatus. Transformants were selected on minimal medium plus ethanol due to the inability of MS7-62 to grow on this compound as sole carbon source. Transformants that retained the ability to grow on ethanol for many generations were further analyzed for reintegration of the ADH genes into the genome. DNA prepared from these clones was digested with restriction enzymes and compared in a Southern analysis with the MS7-62 *adh* null strain digested in the same way [29]. In *figure 1* are reported the analyses of some of these clones digested with *Bam*HI.

The coding region of *KlADH2* was used as a probe to detect for integration of the ADH genes (*figure 1A*), while the *URA3* gene of *S. cerevisiae* was used to verify whether the integrations had occurred in the corresponding *ADH* loci (*figure 1B*).

The analysis revealed that the two *KlADH1* (*figure 1A* and *B*; lanes 7 and 8) and the three

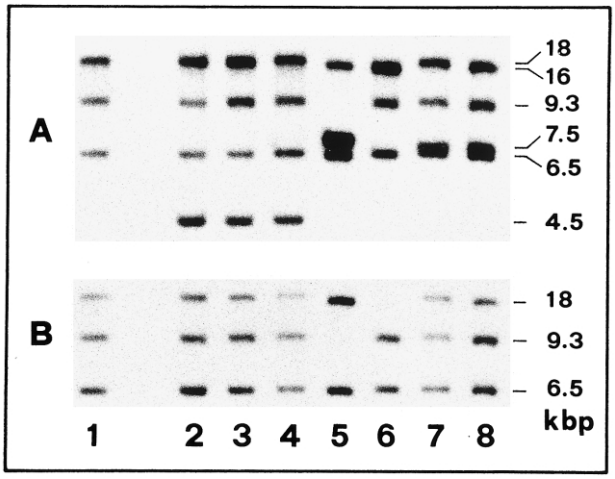

Figure 1. Southern analysis of the *KlADH1*, *KlADH2* and *KlADH3* integrants. DNA from the *adh* null *K. lactis* MS7-62 strain (lane 1), the *KlADH1* (lanes 7 and 8), *KlADH2* (lanes 5 and 6) and *KlADH3* (lanes 2–4) strains was digested with *Bam*HI, separated by an agarose electrophoresis and transferred onto a nylon filter. Hybridizations were carried out, using as probe the coding region of the *KlADH2* gene (*figure 1A*) and of the *URA3* gene of *S. cerevisiae* (*figure 1B*).

KlADH3 (*figure 1A* and *B*; lanes 2–4) integrations reported in *figure 1* were due to a single crossing-over event of the respective plasmid in the corresponding ADH DNA region. As a result we obtained a duplication of each locus: a disrupted and a functional copy for both *KLADH1* (band of 9.3 and 7.5 kb, respectively) and *KlADH3* (band of 6.5 and 4.5, respectively). The expected new 4.5-kb *Bam*HI band of hybridization in the case of *KlADH3* (*figure 1A*; lanes 2–4) (1) and the 7.5 kb (1) obtained in the case *KlADH1* (*figure 1A*; lanes 7 and 8) confirmed the correct integrations of the two genes in the respective loci and were further verified with other digestions (not shown). The disappearance of the *URA3* marker in the18-kb *KlADH2* locus (*figure 1B*; lane 6) and the appearance of a new *Bam*HI band of 16 kb instead of 18 kb (*figure 1A*; lane 6) revealed that one of the integrants obtained with this gene was the result of a double crossing-over between the *KlADH2* plasmid in its own locus. While, with the other *KlADH2* integrant obtained the result was not clear, in fact the double crossing-over had occurred in the *KlADH1* locus (*figure 1A* and *B*; lane 5) producing probably a fusion between *KlADH1* and *-2*. This was shown by the disappearance of the 9.3-kb band in *figure 1A* and *B*, lane 5.

2.2. Culture conditions

The strains were grown at 28 °C in a magnetic stirrer (Brown) at 175 rpm in YP medium (1% yeast extract, 2% Bactopeptone, Difco) with 2% glucose as carbon and energy source (medium YPD2).

2.3. Growth experiments with *K. lactis*

Five hundred-milliliter Erlenmeyer flasks containing 100 mL YPD2 medium were inoculated with fresh inoculum from an overnight culture to a titer of 1×10^5 mL^{-1}. When the cells reached a titer between 1–3 $\times 10^7$ mL^{-1}, the cultures were supplied with ethanol or 1-octanol at the selected concentrations and further incubated. Cell growth was measured once an hour by monitoring the cell density. This was done: a) by measuring the turbidity (OD_{600nm}) of cell suspensions by using a spectrophotometer, b) by counting the cells with a Thoma chamber, and c) by determining wet and dry weights of the cultures. All experiments were carried out in triplicate. The average standard deviation was lower than 5%.

2.4. Determination of growth inhibition

Growth inhibition caused by the two alcohols was calculated by comparing the differences in growth rates μ (h^{-1}) between intoxicated cultures with that of a control culture as described by Heipieper et al. [12]. The growth inhibition of different concentrations of toxic compounds was defined as the percentages of the growth rates μ (h^{-1}) of cultures grown with the toxins and that of a control culture without toxin:

inhibited growth (%) = μ_1 (+ toxin) / μ_0 (control) × 100

This method has already been demonstrated to show the growth inhibition of toxic compounds very precisely, even in physiological situations were the differences between two

toxin concentrations and / or cultures were small [12, 13, 20].

2.5. Lipid extraction and transesterification

Cells of 100-mL suspensions of *K. lactis* were harvested 4 h after addition of the toxic agents by centrifugation and washed with phosphate-buffer (50 mM, pH 7.0). The lipids were extracted with chloroform / methanol / water as described by Bligh and Dyer [4]. Fatty acid methyl esters were prepared by a 15-min incubation at 95 °C in boron trifluoride / methanol using the method of Morrison and Smith [27]. The fatty acid methyl esters were extracted with hexane.

2.6. Determination of fatty acid composition

Fatty acid analysis was performed using gas chromatography (GC) (capillary column: CP-Sil 88; 50 m; temperature program from 160–220 °C; flame ionization detector). The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard). The fatty acids were identified with the aid of standards. The relative amounts of the fatty acids were determined from the peak areas of the methyl esters using a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). Replicate determinations indicated that the relative error (standard deviation / mean) × 100% of the analytical system was 2–5%.

The fluidity of the membrane lipids was expressed as the fatty acid unsaturation index (UI). This was calculated as follows:

fatty acid unsaturation index (UI) = (% C16:1 + C18:1) + (% C18:2 · 2) + (% C18:3 · 3) / 100

3. Results

3.1. Effect of ethanol on growth of *K. lactis* strains containing different ADH isozymes

Cells of *K. lactis* were grown aerobically in stirred cultures on glucose and during the exponential growth phase (growth rate μ of the cells was about 0.46 h^{-1}) ethanol was added at different concentrations. The cultures continued to grow exponentially, but at reduced growth rates (*figure 2*). A direct correlation between ethanol concentration and growth inhibition could be observed. Strain CBS 2359 / 152 containing all ADH genes showed the highest tolerance towards ethanol. Among the different isozymic mutants tested the most tolerant strain was the one containing *KlADH2*, whereas the adh null strain (adh^0) was the most sensitive one followed by the *KlADH1* strain.

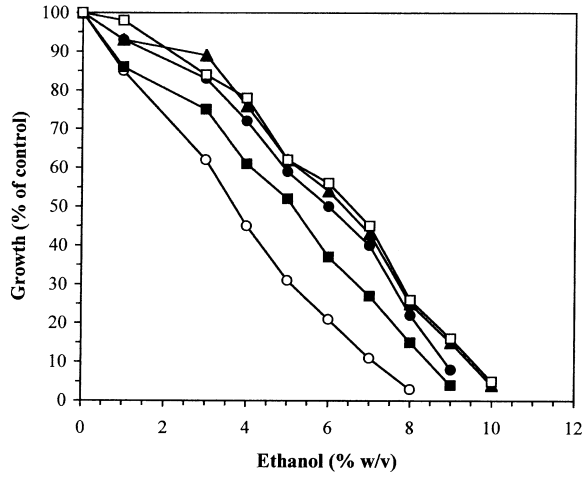

Figure 2. Effect of ethanol on growth of different *K. lactis* strains. *KlADH1-4* (□), adh^0 (○),*KlADH1* (■),*KlADH2* (▲), and *KlADH3* (●). All experiments were carried out in triplicate. Thereby, the average standard deviation was lower than 5%.

As a comparison, the behavior of CBS 2359 / 152, a reference strain, containing all ADH, was also included in the analysis and showed the highest tolerance towards ethanol. The behavior of MW98-8C (data not shown), another reference strain containing only *KlADH1*, *-2* and *-4*, did not diverge significantly from CBS2359 / 152, indicating that, in addition to the genetic context, the ethanol tolerance may also depend on the number of *adh* genes present.

Total inhibition of growth (minimal inhibitory concentration, or MIC) occurred at 10% (w / v) ethanol for strains CBS 2359 / 152 and *KlADH2*; at 9.5% (w / v) for *KlADH3*; at 9% (w / v) for *KlADH1*; and at 8% (w / v) for the adh^0 strain, respectively.

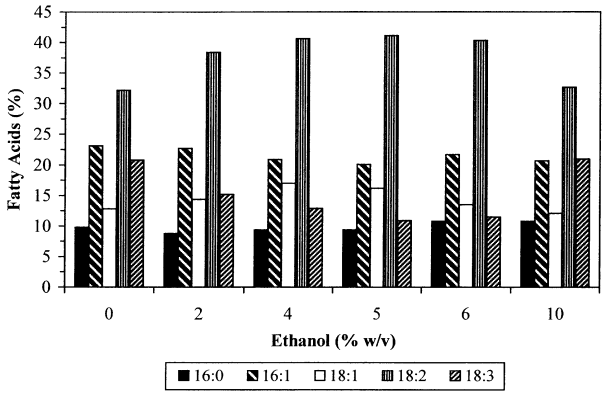

Figure 3. Effect of ethanol on the composition of the major fatty acids of *K. lactis* CBS 2359/152 (*KlADH1-4*).

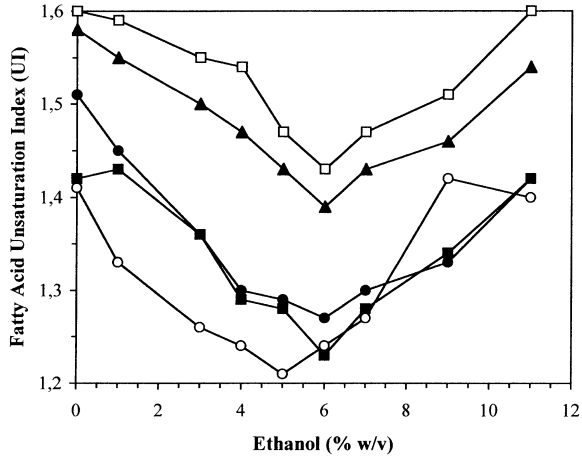

Figure 4. Effect of ethanol on the UI of membrane fatty acids of different *K. lactis* strains. *KlADH1-4* (□), *adh*^{0} (○),*KlADH 1* (■), *KlADH2* (▲) and *KlADH3* (●).

3.2. Modifications of the fatty acid composition of *K. lactis* in the presence of ethanol

One major adaptive response to the addition of toxins, like ethanol are changes in the fatty acid composition of the cells. The cells contained the following fatty acids: C16:0 (palmitic acid); C16:1 Δ9*cis* (palmitoleic acid); C18:1 Δ9*cis* (oleic acid); C18:2 Δ9*cis*,Δ12*cis* (linoleic acid); C18:3 Δ9*cis*,Δ12*cis*,Δ15*cis* (linolenic acid). C17*cyclo* and C18:0 (stearic acid) were present in lower amounts of approximately 5%.

At increasing ethanol concentrations, strain CBS 2359/152 reacted by a reduction in the content of C18:3 followed by an increase in the contents of C18:2 and C18:1 (*figure 3*). The other fatty acids were more or less constant at all ethanol concentrations. This adaptive reaction showed the major variations in fatty acid composition at a 6% (w/v) ethanol concentration which led to an inhibition of cell growth of about 50%. At an ethanol concentration (10% w/v) which caused full inhibition of cell growth and also cell death, the cells no longer reacted; all fatty acids showed nearly the same percentages as the control cells without ethanol addition.

3.3. Ethanol-induced variations in the fatty acid unsaturation of *K. lactis* strains containing different ADH isozymes

The changes in the fatty acid composition caused by ethanol intoxication in all strains investigated were nearly the same as shown for the wild type (see *figure 3*). Another indirect membrane fluidity parameter often used is the UI, evaluated from the analysis of the fatty acid composition. This factor is a value for the average number of double bonds per fatty acid and gives an indication of the fluidity of the membrane lipids. *Figure 4* shows the pattern of the UI for all the investigated strains in the presence of ethanol. A significant decrease in this value could be observed at increasing ethanol concentrations. Hence, the lowest UI values correlated with the measured ethanol concentration for a 50% growth inhibition determined for each strain which was at about 6% (w/v) for all strains except *adh*^{0} at about 5% (w/v). At higher ethanol concentrations a reduced decrease in the UI values could be observed up until values which were similar to those of the control cells.

A remarkable finding was the direct relationship between the UI values and the tolerance towards ethanol measured for the different strains containing the various ADH genes. As for growth inhibition (*figure 2*), the strain containing only *KlADH2* showed the highest ethanol tolerance and, at the same time, displayed the highest UI value, whereas the most ethanol sensitive strains (*adh*^{0} and *KlADH1*) showed the lowest ones. The UI value obtained for the

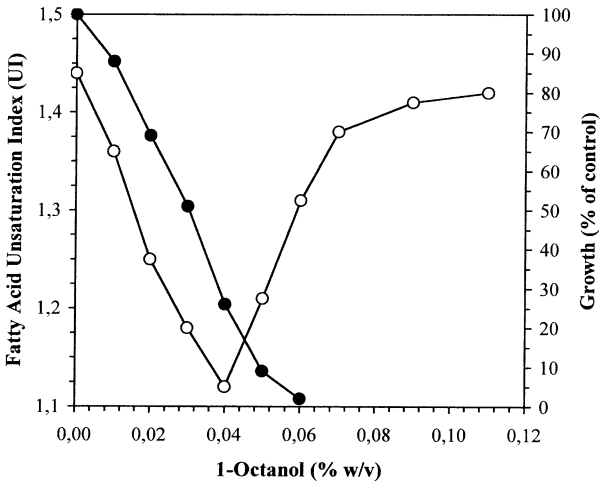

Figure 5. Effect of 1-octanol on growth (○) and the UI of membrane fatty acids (●) of *K. lactis adh*^{0}.

KlADH2 strain was very similar to that of the reference strains CBS2359/152 (*figure 3*) and MW98-8C (data not shown). This could indicate an important role of *KlADH2* in the adaptation to ethanol.

3.4. Effect of 1-octanol on growth and fatty acid composition of the *adh*^{0} strain

For other microorganisms a potential difference between the adaptive reactions towards short-chain aliphatic alcohols, like e.g. ethanol, and long-chain aliphatic alcohols, like e.g. 1-octanol has been observed [17, 18]. Therefore, we also tested the effect of 1-octanol on *K. lactis*. *Figure 5* shows both growth inhibition and UI values of the *adh*^{0} strain. It is obvious that 1-octanol is a stronger toxin than ethanol: whereas the cells tolerated ethanol concentrations of up to 8%, their growth was totally inhibited at 1-octanol concentrations of about 0.05%. In addition, in the presence of growth-inhibiting concentrations of 1-octanol, the cells reacted with a decrease in their UI similar to that of ethanol.

4. Discussion

The adaptive modifications of the fatty acid composition observed in *K. lactis* to externally added ethanol are in contrast to those reported for *S. cerevisiae* and *Schizosaccharomyces pombe* [1, 23, 24]. In fact, *S. cerevisiae* and aerobically grown *S. pombe* in the presence of ethanol increase the unsaturation (fluidity) of their membrane lipids by decreasing their content of palmitic acid (C16:0) and increasing the palmitoleic acid (C16:1*cis*) [1, 21], while *K. lactis* reacts in the opposite way by reducing the unsaturation of its lipids (this paper).

It is widely reported that ethanol increases the fluidity and permeability of yeast cell membranes [1, 6, 21]. Therefore, the behavior of *K. lactis* is much more in agreement with the general adaptive mechanisms of microorganisms to membrane active toxins, like organic solvents, which increase membrane fluidity [14, 19]. The cells try to maintain their membrane in the same viscosity condition and therefore counteract changes in the environment such as an increase in temperature or the presence of solvents, by decreasing the fluidity of their membranes [13]. This reaction has been defined as 'homeoviscous adaptation' [35].

The opposite response, an increase in membrane fluidity in the presence of ethanol observed in *S. cerevisiae* and *S. pombe*, is difficult to understand. In bacteria where this reaction to ethanol has also been observed [11], it was explained by the inhibition of cytosolic enzymes involved in the biosynthesis of (saturated) fatty acid caused by ethanol [18]. *K. lactis* displays a more complex fatty acid composition as compared to *S. cerevisiae* and *S. pombe*, also containing polyunsaturated fatty acids C18:2 (linoleic acid) and C18:3 (linolenic acid), and therefore also consists of a different pathway of fatty acid biosynthesis which may not be influenced by ethanol. Other authors investigated the toxicity of copper on membrane fluidity and composition in *S. cerevisiae* [2, 16]. Cells with a higher UI value induced by supplementation with linolenic acid showed an increased susceptibility towards lethal concentrations of copper with respect to nonsupplemented cells. Therefore, the reaction observed in *K. lactis* is the more logical response to the toxic action of membrane active compounds.

Among the ADH strains, the one containing only *KlADH2* showed nearly the same ethanol tolerance as the wild type containing all four ADH genes. Interestingly, sensitivity / tolerance towards ethanol is related to the UI values found in these strains. There is a correlation between the presence of an ADH gene, the amount of lipid fatty acid unsaturation of these strains and its ability to tolerate externally added ethanol. The fact that the more tolerant strains show higher UI value indicates that in these strains mechanisms other than the fatty acid composition are already induced which increase the rigidity of the membrane [20]. This allows the cells with their high content of unsaturated fatty acids to react more quickly to nonlethal concentrations of ethanol.

Therefore, it can be postulated from these results that *KlADH2* may be involved in the adaptation to high ethanol concentrations. This hypothesis was also verified by the kinetic parameters of the ADH isozymes of *K. lactis* reported by Bozzi et al. [5], where the two cytoplasmic ADH isozymes, KLADHI and KLADHII, showed about 10-fold higher K_m values for ethanol as compared to the two mitochondrial isozymes, KLADHIII and KLADHIV. KLADHI seems to be the enzyme involved in ethanol production, whereas KLADHII may be necessary for ethanol metabolism. An explanation of these observations may be that KlADHII is also involved in the general detoxification apparatus of *K. lactis*, leading to better 'fitness' of the cells towards stress. This has also been demonstrated by Chatterjee et al. [7] who found a strong correlation between the amount of unsaturated fatty acids and the heat shock response of these cells which can also be induced by solvents like, for example, ethanol [28]. Further investigations are in progress in our laboratories to clarify the detailed role of *KlADH2* in ethanol adaptation.

Acknowledgments

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