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Yeast membrane adaptation during fermentation

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Abstract

Adaptation of yeasts to changing environments experienced during biomass propagation and ethanolic fermentation for biofuel requires rapid modulation of membrane fluidity. We applied fluorescence spectroscopy and microscopy to investigate yeast cell membrane and physiological responses to such changing environments. Our investigations included development and adaptation of methodology to assess membrane fluidity of yeasts, and has shown that membrane fluidity relates to growth phase, cellular physiology and nutrition as well as to membrane lipid and protein composition. We found that membrane responses to glucose depend on particular membrane proteins. Furthermore our studies have shown that rather than the commonly held view of membrane fluidization, heat damage of membranes leads to lower membrane fluidity, likely due to membrane protein denaturation. In addition, we investigated membrane responses to fluidization by high ethanol concentrations. We found that the fluidizing effects of ethanol were minimized by decreased membrane fluidity, leading to higher ethanol tolerance. The roles of various cellular factors in these adaptive responses are considered.

Keywords: yeast, ethanol, fermentation, adaptation, stress, membrane, fluidity

Introduction

Yeast fermentations and byproducts have many applications in production of foods, beverages and bioethanol. The yeasts experience constantly changing and often stressful environments during their growth, fermentation and processing. The changes encountered include high or low temperatures, acidic conditions, high osmotic pressures, low availability of nutrients, anaerobic conditions and accumulation of ethanol to toxic levels. To be useful in an industrial process, the yeasts must be able to adapt to changing environments and stresses and to continue their production of biomass, ethanol or other product.

Cell membrane systems are critical to an organism's capacity to adapt to changing environments, and better knowledge of the roles of membranes in adaptation will provide insights to improve commercial fermentations for biomass or ethanol production. This paper summarizes a number of research projects, outlining progress towards elucidating yeast responses to environmental change, particularly in respect to cell physiology and membrane fluidity. The research on yeast stress tolerance and adaptation has a biological focus on the role of membranes in adaptation to changing conditions during bioprocesses, and a technological focus on assessing membrane fluidity during adaptation by fluorescence spectroscopy and microscopy, and precise definition of growth stages.

Although there are examples of continuous and fed-batch fermentations, many industrial processes involve batch fermentation, i.e. nutrients and cofactors are only supplied at the start of the process. During batch fermentation yeasts progress sequentially through several growth stages, and capacity to adapt to changing conditions as well as to survive stresses is strongly related to the growth phase (Lewis *et al*. 1993a). Aerobically grown diauxic yeasts such as *Saccharomyces* species have distinct phases that include initial lag, respiro-fermentative, diauxic lag, respiratory, stationary and death phases (Lewis *et al.* 1993b). The lag phases are periods of metabolic adjustment with limited growth (initial lag in response to dilution in a new growth medium usually containing glucose, and diauxic when glucose supplies are exhausted and cells adapt to respiration of ethanol). We chose the term respiro-fermentative to describe the mainly fermentative metabolism and avoid the terms "exponential" or "logarithmic" growth, as the second, slower respiratory growth phase can also be described by the same terms. When glucose is available at above about 0.2 % (w/v), glycolysis and fermentation to ethanol are stimulated and many other metabolic pathways are repressed, including respiration. As respiratory growth on the accumulated ethanol is generally much slower than respiro-fermentative growth, it has been wrongly classified as stationary phase in many studies (Lewis *et al.* 1993b). Another important point to note is that in the presence of oxygen, the yeasts may progress to a second, slower growth phase via respiration of

ethanol. In cases where oxygen is not available then yeasts proceed directly from respiro-fermentative to stationary phase. Anaerobic growth requires major adaptation as synthesis of unsaturated fatty acids and sterols can not occur in the absence of oxygen, so the yeasts must use other ways to modulate membrane fluidity in the absence of these key components.

Membrane fluidity moderates many aspects of membrane function, such as permeability and activity of receptors, enzymes or transporters. An excellent example is moderation of activity of the yeast plasma membrane H^+-ATPase (Learmonth & Carlin 1997), thereby affecting the plasma membrane electrochemical gradient (Portillo 2000). Membrane fluidity can be transiently or permanently modified by environmental stresses (Learmonth & Carlin 1996, 1997, Shah & Learmonth 1998). To firstly survive, and then adapt to a stress, yeasts must modulate their membrane fluidity to compensate. We believe that fluidity modulation is a key part of adaptation and is related to signal transduction responses (Thevelein 1994, Banuett 1998) which lead to changes in cell physiology. For example we observed membrane fluidity modulation on a similar time-scale to induction of cAMP (Learmonth & Carlin 1997, Ma *et al.* 1997). During growth in batch culture membrane lipid composition of *S. cerevisiae* also varies (Watson & Rose 1980, Steels *et al.* 1994), leading to inferences of membrane fluidity from composition in many publications. However these inferences do not include the impacts of minor lipid components, membrane proteins, cell metabolic status or environmental factors. Hence we preferred to measure fluidity in viable cells by fluorescence-based techniques, during, rather than after adaptation.

Materials and Methods

In the research described herein several strains of *Saccharomyces cerevisiae* were investigated, including baker's yeast strains A9 and A12 (Lewis *et al.* 1997), brewing yeast C1 (Learmonth & Gratton 2002), a commercial wine yeast, a sake yeast K7 (ATCC 26422), and in studies of membrane proteins FY1679-28c and its deletion mutants of the Ole1 lipid desaturase and Hsp30 (Braley & Piper 1997). Yeasts were maintained on slopes at 4°C. Cells were inoculated into YNB broth containing 0.67% (w/v)Yeast Nitrogen Base (Difco) with 1% (w/v) glucose and incubated overnight on an orbital shaker (30 ± 1°C, 180 opm). These starter cultures were used to inoculate fresh YNB broth with 1% glucose to a cell density of 0.1 OD600nm. These experimental cultures were incubated under the same conditions and analyzed at specific growth phases, as noted in the results section. Where relevant stresses were applied using standard methods (Lewis *et al.* 1997).

To study membrane fluidity during yeast adaptation, we needed to fine-tune existing approaches and methods to make certain that the measurements were made under the environmental conditions of a particular culture phase (Learmonth 2012). This included elucidating optimal procedures for sampling, labeling and analysis of the labeled cells. At first we assessed membrane fluidity by fluorescence spectroscopy by the standard practice of measuring polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). While this technique had previously been applied to yeasts, methodology needed modification as outlined below. Particular problems to overcome included cell density-dependent scattering of the polarized light, as well as minimizing fluorescence background from cells and growth media. To prevent problems from scatter we included an alternative approach much less susceptible to light scattering; spectroscopic measurement of Generalized Polarization (GP) of laurdan (Weber & Farris 1979). This also allowed us to extend the studies further by 2-photon microscopy of laurdan-labeled cells (Learmonth & Gratton 2002). These methods have been applied to observe membrane changes during the processes of adaptation and stress. To minimize background noise from cells and media, we investigated their fluorescence. At the wavelengths used for excitation (360 nm for DPH and 340 nm for laurdan) and emission (~430 nm for DPH and 440 and 490 nm for laurdan) the background fluorescence from unlabelled cells was negligible, however the culture media exhibited high levels of fluorescence (Figure 1). The fluorescence background from rich media such as yeast extract peptone (YEP) was so high that measurements of DPH or laurdan fluorescence were not possible. We were able to use the defined medium YNB, which has lower fluorescence, and optimizing fluorescent label concentration maximized the signal to noise ratio (Learmonth 2012). Labeled cell fluorescence is generally higher than that of YNB but lower than the YEP emission.

Steady State Fluorescence Spectroscopy was carried out in stirred, temperature controlled cuvettes with a SLM Aminco Bowman Series 2 Luminescence Spectrometer or an ISS PC1 Photon Counting Spectrofluorimeter (Learmonth 2012). Two-photon fluorescence scanning microscopy was performed as described previously (Learmonth & Gratton 2002). Anisotropy of DPH labeled cells was calculated as detailed in Learmonth *et al.* (2009), and Generalized Polarization (GP) of laurdan-labeled cells was calculated as described previously (Parasassi *et al.* 1990, Learmonth & Gratton 2002). It is important to note that the anisotropy or GP parameters relate inversely to membrane fluidity, i.e. lower anisotropy or GP indicate higher fluidity and *vice versa*.

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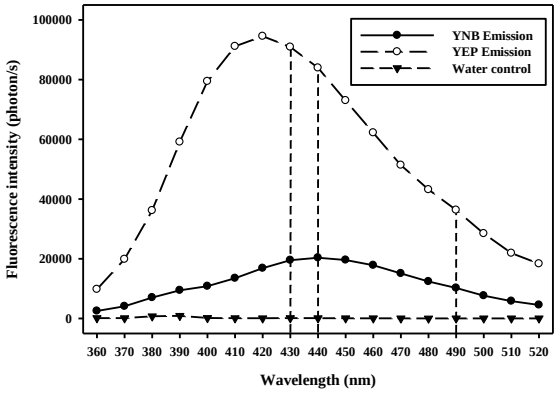

Figure 1 Fluorescence emission spectra of YEP and YNB media, both with 1% (w/v) glucose. Critical emission wavelengths are marked by vertical lines: 430 nm for DPH emission, and 440 and 490 nm for laurdan emission. Excitation was at 340 nm.

Results and Discussion

Membrane fluidity and growth phase

Throughout aerobic batch culture, yeasts progress through several phases, as outlined above. These growth phases are followed by measuring multiple parameters such as cell number or density, division rate, viability, glucose and ethanol concentrations (Lewis *et al.* 1993b). Data from a representative culture of baker's yeast strain A9 is shown in Figure 2. As evidenced by OD_{600nm} glucose and ethanol concentrations, the culture was in respiro-fermentative growth on glucose to 12 h, followed after diauxic lag by respiratory growth on ethanol to 72 h, and thereafter in stationary phase. DPH anisotropy and laurdan GP were measured by spectroscopy, and laurdan GP was also measured by 2-photon microscopy. During respiro-fermentative growth phase, values for DPH anisotropy and laurdan GP by spectroscopy or microscopy were comparatively low, indicating higher membrane fluidity. Upon depletion of glucose and progression through diauxic lag to respiratory growth phase, the DPH anisotropy and laurdan GP increased, reaching steady values by stationary phase. These results signify that rapidly dividing, glucose repressed respiro-fermentative phase cells had higher membrane fluidity, while de-repressed slowly or non-dividing cells in respiratory or stationary phases had less fluid membranes. A similar age related GP rise has been reported in cultured mammalian cells (Parasassi *et al.* 1992).

This suggests that structural changes lower membrane fluidity as the cells in the population age.

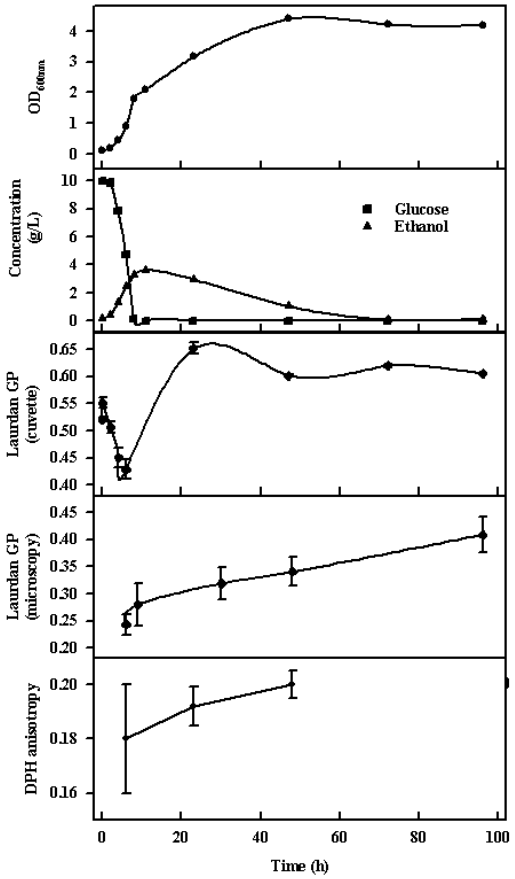

Figure 2 Growth and membrane fluidity parameters of *S. cerevisiae* A9 in aerobic batch culture in YNB with 1% (w/v) glucose. Error bars represent standard deviation. Adapted from Learmonth (2012).

It is possible that during rapid growth and cell division greater water penetration associated with membrane biogenesis leads to relatively higher laurdan fluorescence at 490 nm, consequently lower GP. Areas of membrane biogenesis also tend to have higher fluidity and less constraint on probe mobility, leading to lower DPH anisotropy values. Increased GP values in the later growth phase could also be due to reduced flux of water molecules across the membrane as a consequence of reduction in the nutrient uptake rate. Another explanation, consistent with the rising level of ethanol over time (causing fluidization of membranes), is that membrane composition and organization is altered. Our findings are consistent with the generally accepted notion that yeast cell membranes are more fluid when growing rapidly on glucose, as well as having higher sensitivity to a variety of stresses (Lewis *et al.* 1993a). Thus it has been assumed that higher sensitivity (lower tolerance) to stresses is caused by higher membrane fluidity. Decreased membrane fluidity at the end of respiro-fermentative growth may be correlated with marked changes in cell physiology and membrane structure. Specific

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changes that may affect membrane fluidity include increased levels of trehalose (Lewis *et al.* 1993a), appearance of the protein Hsp30 (Panaretou & Piper 2002) that regulates the plasma membrane H^+-ATPase (Braley & Piper 1997) and induction of membrane assembly proteins (Deshaies *et al.* 1988).

The increase in GP as a cell population ages could also reflect an increasing proportion of dead cells. We found that heat-killed cell populations had higher GP values (Learmonth 2012). Thus a combination of factors including changes in membrane structure and increase in dead cell proportion could lead to increased GP. Approaches to differentiate live and dead cells include flow cytometry or microscopy. We utilized 2-photon laser scanning microscopy to assess laurdan GP in individual yeast cells (Learmonth & Gratton 2002). Figure 3 shows GP images at three stages of batch culture of *S. cerevisiae* strain A9. In respiro-fermentative (6 h) and to a lesser extent respiratory phase cells (24 h) the typical morphology of growing yeasts and division by budding can be seen. Such images supported our prior conclusions that on average GP increased (fluidity decreased) after respiro-fermentative phase. Figure 2 shows similar trends for GP measured by microscopy or spectroscopy (note that absolute values of GP vary between instruments: Learmonth & Gratton 2002).

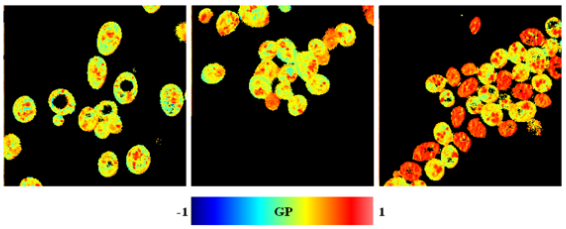

Figure 3 Laurdan GP images of *S. cerevisiae* strain A9, 23 x 23 µm images of depth 1 µm. Left - respiro-fermentative cells (6 h, mean GP 0.25); middle - respiratory cells (30 h, mean GP 0.30); right - stationary phase cells (4 d, mean GP 0.43). Adapted from Learmonth (2012)

The resolution is insufficient to precisely define intracellular membranes in such small organisms; however clustering of high GP values is obvious. We consider that this may reflect membrane lipid rafts. Studies on much larger mammalian cells found remarkably similar clustering of high-GP patches and concluded they were rafts (Gaus *et al.* 2003). We also observed that individual cells in images had different GP distributions and average values. In sequential images from cultures, particularly after 24 h, the increased average GP values could be correlated with an increase in uniformly high GP cells, while other cells continued to show generally low and variable GP. Figure 4 shows a typical GP histogram associated with an image containing a significant proportion of high GP cells. Numerical analysis of histograms showed that cells could be effectively assigned into two classes, in this example one centered at GP 0.32 representing 57% of the image data and another centered at GP 0.64 representing 43% of the values.

By double labeling with methylene blue, we ascertained that the cells with uniform high GP were dead. As cultures aged, live cells evidenced a typical "healthy" GP pattern while dead cells had uniformly high GP, with no intermediate form identified.

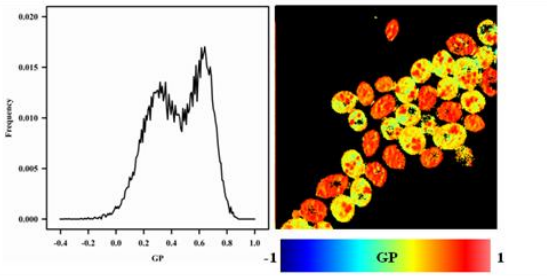

Figure 4 GP frequency histogram and image of *S. cerevisiae* A9 cells in stationary phase at 4 d, with mean GP of 0.43 and two subpopulations with GP means of 0.32 and 0.64. Adapted from Learmonth and Gratton (2002).

Numerical analysis of Figure 4 compared well with counting of low GP live cells (56%) and high GP dead cells (44 %). Finding that a rising proportion of dead cells leads to higher average GP indicates that care must be taken in interpreting cuvette-based studies; viability must be closely monitored to enable discrimination between GP variation due to membrane structural change or to cell death.

Membrane fluidity and response to glucose

Glucose availability is a critical factor that varies over batch culture. In addition to providing energy and carbon for growth, glucose is a major regulator of metabolism, stimulating its own fermentation while repressing fermentation of other sugars as well as respiration in general (Klein *et al.* 1998), with resultant impact on many cellular functions (Thevelein 1994). This should influence membrane structure and function; however possible effects have not been elucidated. In the previous section we noted that respiro-fermentative cells growing on glucose had higher membrane fluidity than respiratory or stationary phase cells. In other experiments utilizing DPH anisotropy, we found that membrane fluidity increased immediately upon addition of glucose to respiratory or stationary phase cells (Carlin & Learmonth 1995, Learmonth & Carlin 1997). The fluidity changes may relate to other effects of glucose addition to yeasts, such as induction of cleavage of a cAMP receptor protein from the

membrane (Muller & Brandlow 1993), alteration of membrane potential and K^+ flux (Pena & Ramirez 1991) and activation of the H^+-ATPase causing acidification (Eraso & Portillo 1994).

We observed rapid glucose-induced acidification and K^+ uptake associated with fluidity changes (Learmonth & Carlin 1997), indicating modified membrane function associated with the structural changes inferred from altered fluidity. cAMP-signaling may play a role, since glucose also induces rapid transient increases in cAMP (Ma *et al.* 1997), which may directly affect membrane fluidity (Morril *et al.* 1993). Furthermore, artificially high cAMP levels interfere with gene expression on glucose depletion (Boy-Marcotte *et al.* 1996). Glucose addition to yeast rapidly increased cellular cAMP and decreased internal pH (Eilam *et al.* 1992), on a similar time-scale to our observed changes in membrane fluidity and external pH. In our experiments membrane fluidity responses to glucose seemed too rapid to reflect marked changes in lipid composition by desaturases, phospholipases or *de novo* synthesis. The responses were probably due to proteins which could associate or dissociate with membranes, change conformation, or cross-link with other proteins. Glucose affects membrane association of a cAMP receptor protein (Muller & Brandlow 1993), a glycolytic enzyme (Dey *et al.* 1994) and Hsp30 which regulates the H^+-ATPase (Braley & Piper 1997). The small proteolipids Pmp1 and Pmp2 (Navarre *et al.* 1994) and an uncharacterized membrane protein (76) may also contribute to glucose induced activation of the ATPase.

To illuminate possible processes underlying this modulation of membrane fluidity we compared responses in *S. cerevisiae* strains which were deficient in important membrane functions (Learmonth & Butcher 2007), including deletion mutants lacking the Δ9 acyl-CoA lipid desaturase (Ole1) or the heat shock protein Hsp30, comparing their responses to the parent strain FY1679-28c (Braley & Piper 1997). We assessed growth, physiological parameters and laurdan GP during aerobic and anaerobic batch cultures, as well as response to glucose addition at levels above (0.5% w/v) and below (0.1%) the threshold for glucose repression. During batch growth the parent strain showed characteristic, balanced patterns of membrane fluidity, membrane phospholipid and fatty acyl composition, growth rate, glucose or ethanol consumption and viability. However the Ole1 and Hsp30 mutants seemed unbalanced, exhibiting less efficient metabolism with higher glucose requirements for equivalent growth or ethanol production, lower cell division rates and lower viability. The Ole1 mutant performed similarly to the parent strain grown under anaerobic conditions, as would be expected. In addition, when this mutant was supplemented with unsaturated fatty acids the defect was rectified and it performed similarly to aerobically grown parent cells. The Hsp30 defect could not be addressed by supplementation and was particularly prominent in the diauxic lag phase. After extended incubation in the absence of glucose, the parent strain showed negligible membrane fluidity responses to glucose addition, in contrast to previous studies utilizing DPH anisotropy. However as shown in Figure 5 the Hsp30 and Ole1 mutants had strikingly different behavior, with increased laurdan GP after addition of glucose either above or below the catabolite repression threshold. Thus the membrane-associated protein deficient mutants had compromised fluidity regulation mechanisms. We hope to further elucidate these defects in future studies.

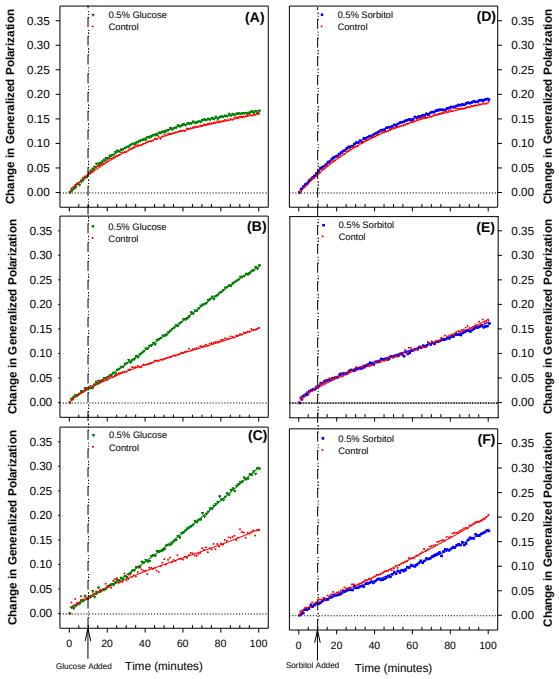

Figure 5 Changes in GP in aerobic stationary phase cells in response to addition of 0.5% (w/v) glucose (green lines) or 0.5% (w/v) sorbitol as a control for osmotic changes (blue lines), or no addition (red lines). (A, D) FY1679-28c; (B, E) FY1679-28c Δole1; (C, F) FY1679-28c Δhsp30. Adapted from Butcher (2008).

Membrane fluidity and response to heat stress

While cell membranes are considered primary sites for heat damage, the underlying mechanisms are controversial. Intrinsic membrane fluidity correlates with heat sensitivity (Carlin & Learmonth 1994, Learmonth & Carlin 1995, 1996), and assessment of DPH anisotropy (Learmonth & Carlin 1996) and laurdan GP (Shah & Learmonth 1998) showed that fluidity of yeast membrane increases as

temperature rises. While these observations are consistent with the theory that heat damages membranes by increasing fluidity to unstable levels (van Uden 1984), we believe that impairment of membrane proteins may be the major mechanism of heat damage, a view supported by other researchers (Lepock 1982, Konings & Ruifrok 1985, Gille *et al.* 1993). It is generally accepted that cellular responses to heat are triggered by damage to cytosolic proteins. Membrane proteins may be equally or even more vulnerable, since heat damage may involve oxygen-derived free radicals (Steels *et al.* 1994, Davidson *et al.* 1996, Guerzoni *et al.* 1997) which tend to concentrate in membranes (Steels *et al.* 1994). Our experiments (Learmonth & Carlin 1996) revealed that during heat stress membrane fluidity progressively and irreversibly decreased, as seen in Figure 6. The damage was most prominent in respiro-fermentative cultures. Thus the initially high fluidity of respiro-fermentative membranes is counteracted by heat. Further experiments showed that cultures became progressively more resistant to heat damage as they approached diauxic lag, and that addition of glucose to cells caused a rapid reversion to the sensitive phenotype. The membrane changes correlated with cell viability; survival of *S. cerevisiae* A12 cells after 10 min at 52°C was 4% in respiro-fermentative phase, increasing to 30% in respiratory phase.

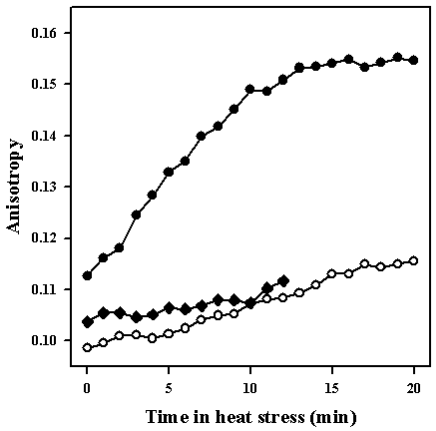

Figure 6 DPH anisotropy of *S. cerevisiae* strain A12 during heat stress (52°C) at three culture ages. Closed circles - respiro-fermentative cells (6 h); open circles - respiratory cells (24 h); closed diamonds - stationary phase cells (7 d). Similar responses were found in other strains including A9. Adapted from Learmonth (2012).

To investigate whether all cells were affected by heat stress to a similar degree or whether the spectroscopy data might represent an increasing proportion of dead cells, we examined laurdan GP in heat stressed cultures by 2-photon microscopy (Learmonth & Gratton 2002). Figure 7 shows that the microscopic analysis confirmed spectroscopy data and indicated that membrane fluidity was affected to a similar degree in all cells of a population. The mean GP of respiro-fermentative cultures increased consistently across all cells from 0.20 to 0.36. Respiratory phase cells had higher intrinsic mean GP (0.38) and were more resistant to heat stress, which only increased the mean GP marginally to 0.39. Thus our studies have shown that rather than the commonly held view of membrane fluidization, heat damage of membranes leads to lower membrane fluidity, likely due to membrane protein denaturation.

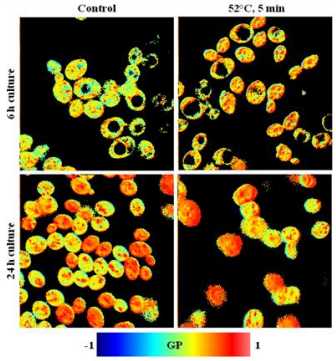

Figure 7 GP images of *S. cerevisiae* A9 cells growing on glucose (respiro-fermentative, 6h) and ethanol (respiratory, 24h) before and after heat stress (52 °C, 5 min). Adapted from Learmonth and Gratton (2002).

Membrane fluidity and response to ethanol stress

Most industrially important applications of yeasts involve fermentation of high sugar concentrations to produce toxic concentrations of ethanol. Survival, tolerance and adaptation to ethanol are thus important criteria for the commercial use of yeasts. Like heat, ethanol stress causes accumulation of damaged proteins and the responses induced are similar (Piper 1995). Ethanol can drastically increase membrane fluidity due to its solvent action. An initial survey of several *S. cerevisiae* strains (Lewis *et al.* 1997) indicated that tolerance to 20% (v/v) ethanol varied markedly. Further experiments showed that ethanol tolerance was generally greater when intrinsic membrane fluidity was lower and that the fluidity slightly decreased despite slightly increased unsaturation indices (Table 1).

When we followed cells of respiratory phase *S. cerevisiae* A9 during exposure to 20% (v/v) ethanol (Figure 8, Learmonth & Carlin 1995) the yeasts were rapidly killed and an immediate increase in fluidity was noted. This was followed by a partial recovery in DPH anisotropy values although most cells were dead (viability was 0.08% after 15 min and 0.04% after 30 min).

Table 1 Tolerance to 30 min exposure to 20% (v/v) ethanol, DPH anisotropy and unsaturation index (UI) of four *S. cerevisiae* strains.

Strain	Tolerance to 20% ethanol (% survivors)	DPH Anisotropy	UI
A2	0.3 ± 0.1	0.19	87
A9	12.3 ± 10.9	0.20	87
A12	65.1 ± 1.8	0.20	88
A16	17.6 ± 8.7	0.22	90

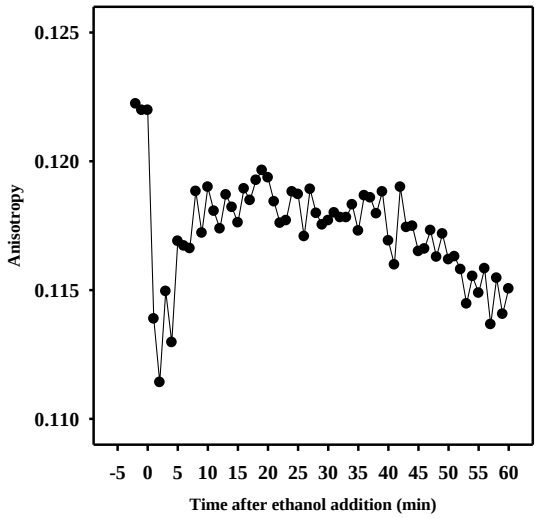

Figure 8 DPH anisotropy of respiratory phase (48 h) *S. cerevisiae* A9 cells during stress with 20% (v/v) ethanol. Adapted from Learmonth (2012).

We obtained similar results when laurdan GP was measured by spectroscopy (Figure 9, Shah & Learmonth 1998) and microscopy (Figures 10 and 11, Learmonth & Gratton 2002). As with heat stress, the GP microscopy images showed that membrane fluidity of all cells in a population were affected by the high levels of ethanol. However these experiments were subject to technical difficulties with cell immobilization, especially when ethanol concentrations were high, leading to further refinement of methodology, as discussed elsewhere (Learmonth & Gratton 2002, Learmonth 2012).

The variable recovery of membrane fluidity is worthy of note. Figure 8 indicates reasonable recovery of DPH anisotropy. However Figure 9 indicates poor recovery of laurdan GP, possibly due to greater solvent relaxation of laurdan in ethanolic solution. Furthermore Figure 11 indicates variable but generally good recovery when GP data is assessed in cells only. Future experiments will further determine the extent and elucidate the nature of the apparent recovery of membrane fluidity.

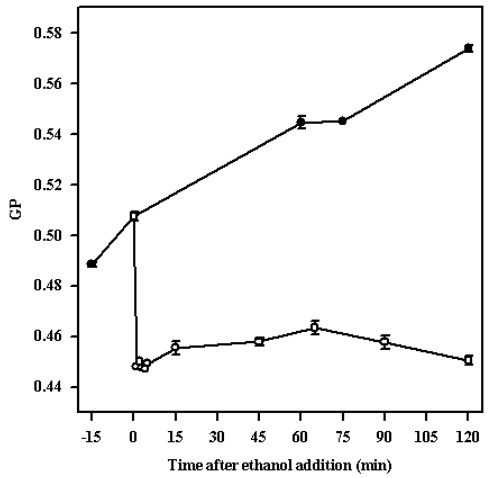

Figure 9 Laurdan GP of respiratory phase (24 h) *S. cerevisiae* A9 cells during stress with 20% (v/v) ethanol. Closed circles – control cells; open circles – ethanol stress. Adapted from Learmonth (2012).

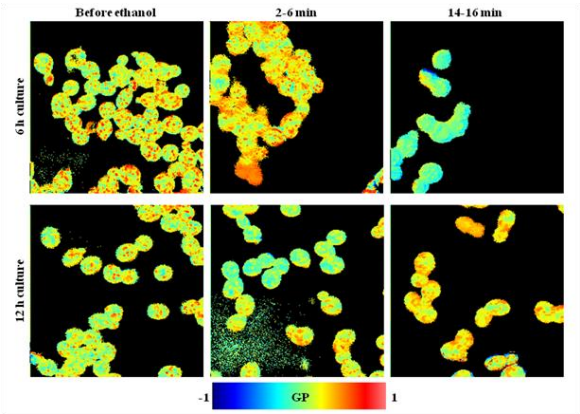

Figure 10 Laurdan GP of respiratory phase (24 h) *S. cerevisiae* GP images of a brewing strain of S. cerevisiae (C1) cells during (6 h) and at the end (12 h) of respiro-fermentative growth on glucose, and during respiratory growth on ethanol (29 h) before and after stressing with 20% (v/v) ethanol. Adapted from Learmonth and Gratton (2002).

More recent studies focused on ethanol tolerance and membrane fluidity as part of assessment of novel yeasts for bioethanol production (Ishmayana *et al.* 2011). In Figure 12 it can be seen that respiratory phase cells have higher GP, and better viability when exposed to 20% (v/v) ethanol, consistent with our previous experiments.

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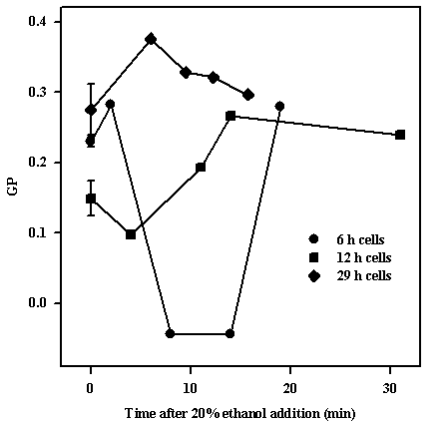

Figure 11 Mean GP values from images of *S. cerevisiae* C1 in absence and presence of 20% (v/v) ethanol. Data represent mean values from several GP images. Adapted from Learmonth (2012).

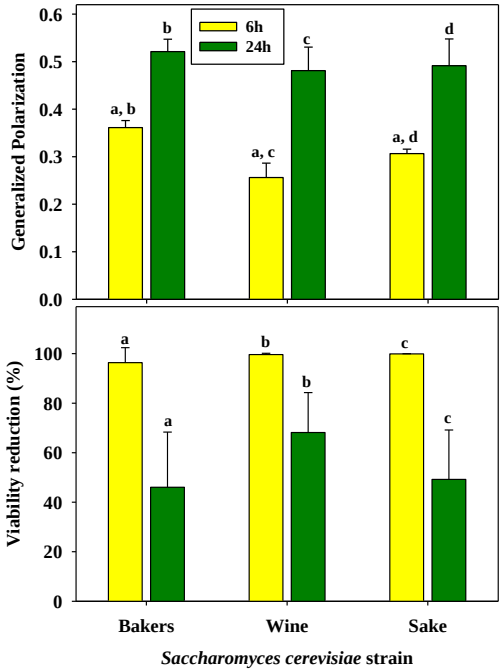

Figure 12 *S. cerevisiae* strains grown under aerobic conditions in YNB with 2% (w/v) glucose in respiro-fermentative (6 h) and respiratory phase (24 h) cultures. Top: GP of cultures. Bottom: % reduction in viability after ethanol stress(18% v/v ethanol for 60 min). Error bars represent standard deviation. Statistically significant differences are indicated by the same letters above the bars.

In summary, our studies indicated that the fluidizing effects of ethanol were minimized by decreased membrane fluidity, leading to higher ethanol tolerance.

Conclusions

The studies summarized in this paper illustrate how determination of membrane fluidity by fluorescence spectroscopy and microscopy has aided the elucidation of contributions of yeast membranes to adaptation to stressful environmental change.

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