


AN ABSTRACT OF THE THESIS OF

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Title: Recovery of *Saccharomyces cerevisiae* from Ethanol-Induced Growth Inhibition

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Ethanol caused altered mobility of the lipophylic probe, 1,6-diphenyl-1,3,5-hexatriene in plasma membrane preparations of *Saccharomyces cerevisiae*. Because lipids had been shown to protect yeast against ethanol toxicity, sterols, fatty acids, proteins, and combinations of these were tested, but protection from growth inhibition was not seen. Ethanol-induced prolonged lag periods and diminished growth rates in *Saccharomyces cerevisiae* were reduced by an auto-conditioning of the medium by the inoculum. The auto-conditioning agent is water soluble and moderately heat labile, with a molecular weight below 6000-8000.

RECOVERY OF SACCHAROMYCES CEREVISIAE FROM
ETHANOL-INDUCED GROWTH INHIBITION

by

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RECOVERY OF SACCHAROMYCES CEREVISIAE FROM ETHANOL-INDUCED
GROWTH INHIBITION

INTRODUCTION

The yield of ethanol in industrial fermentations is limited by sensitivity of the organism to its product. Although this problem has been the topic of much speculation (3,43,44), no direct relationship between the effect and a specific target site has been established. A further understanding of the effects of ethanol on Saccharomyces growth will aid in developing more tolerant strains to allow for greater ethanol production. This would be especially useful in fuel production from biological wastes. Studies of the effects of ethanol on yeast may also aid in understanding the cellular mechanism of central nervous system depression, thereby providing information on ethanol dependence, intoxication and tolerance in man (21).

In laboratory culture, both produced and added ethanol limited the growth of Saccharomyces in a concentration-dependent manner (33,35,45). Produced ethanol was found more toxic than added ethanol (33), as was intracellular more toxic than extracellular (35). Care must be taken in interpretation of these results since the accuracy of intracellular ethanol measurements depends on the method of sampling, because ethanol, like other metabolites, rapidly exits the cell during manipulations (39). The mechanism of this inhibition is believed to be lipid related, since the membrane is the first cellular component to contact ethanol in culture. Therefore a study of how ethanol affects the membrane could provide valuable information.

LITERATURE REVIEW

Ethanol stress has been shown to produce a change in the lipid composition of the yeast plasma membrane (3). An increase in the percentage of unsaturated fatty acids has been observed following growth in ethanol. Alterations in the lipid composition of the membrane also produce changes in ethanol tolerance. Supplementation with unsaturated fatty acids (as opposed to saturated fatty acids) has been noted to increase ethanol tolerance (43,44). Changes in lipids can also alter the lipid-to-protein ratio of the plasma membrane. Similar changes in phospholipid fatty acid composition, as well as lipid-to-protein ratio, have been noted in Escherichia coli (10,20,21,22), Clostridium thermocellum (8), and mammalian systems (24,31). In C. thermohydrosulfuricum, which has a unique C₃₀ dicarboxylic acid in its membrane, greater ethanol tolerance and less membrane-mediated effects were observed (29).

These lipid alterations would result in changes in membrane order, and could compensate for the fluidizing effects of ethanol on the membrane. When a variety of organisms were grown in ethanol-containing medium, physical studies of their membranes have shown changes in fluidity and appearance or alteration of phase transitions in membrane lipids (8,10,22). Studies of lipids extracted from an ethanol-resistant mutant of C. thermocellum showed a lower T_m than the parental strain, as measured by differential scanning calorimetry (DSC) (8). Transition temperatures were also lowered in the presence

of ethanol in model membranes studied by spectrometry (38). A reduction of the disorder caused by ethanol (as measured by electron paramagnetic resonance) has been noted upon in vivo and in vitro addition of cholesterol to mammalian membranes (7).

Since the membrane lipid composition and fluidity are changed, transport properties of the membrane are affected. Ethanol has been observed to noncompetitively inhibit the transport of maltose and glucose across the membrane (26,28). Alcohol also enhances passive proton influx (25) and increases model membrane permeability and fusion (18). A higher osmotic pressure decreased the efflux of ethanol from the cells (35), but the literature is contradictory on whether or not cells will accumulate ethanol against a concentration gradient (3,13,33,34). This ambiguity is most likely due to the different methods of sampling and measurement.

Because the major emphasis of ethanol research has been in the area of membrane lipids, attempts to identify a lipid compound which would offer the cell some degree of protection from ethanol toxicity have been made. It was rationalized that since lipid composition changes during ethanol stress, these lipids could be a target, and the deleterious effects of ethanol are possibly compensatable (3,44). In sake yeasts (Saccharomyces sake) which typically ferment to higher ethanol concentrations (up to 18%) it was found that the presence of Aspergillus oryzae in the brewing malt allowed for greater ethanol production (15,17). It was found that this was due to the presence of a proteolipid from Aspergillus oryzae which "protected" the cells.

Addition of extracted Aspergillus proteolipid to Saccharomyces cultures produced this "protective" effect (15,17). This protection was also obtained by adding a combination of lipid, sterol and albumin to anaerobic cultures with ethanol, suggesting that nutritional supplementation of certain lipids or lipid-protein complexes could increase ethanol tolerance and thus allow elevated production of alcohol (6,14,16,34). Lipid-enhanced ethanol production was also observed in Kluyveromyces fragilis cultures (23).

In order to probe the reported protective effect of nutritional supplementation on alcohol-stressed cultures of yeast, I have studied the effect of alcohol addition on a wild-type and lipid auxotrophic mutant of Saccharomyces cerevisiae. Fluorescence anisotropy measurements with the lipophilic probe diphenylhexatriene provide a means to study effects of ethanol on membrane lipid transitions.

METHODS

A. Organisms and Culture Conditions

Saccharomyces cerevisiae strain X2180-1A (a, SUC2, mal, mel, gal2, CUP1) and sterol auxotroph mutant RD5-R (a, hem1, erg3, erg7), were grown at 28°C in aerobic culture. Rich medium (YTD) consisted of 0.5% yeast extract, 1.0% tryptone and 2.0% dextrose (w/v). Defined medium was prepared as described previously (42). Growth in liquid cultures was measured by the use of a Klett-Summerson model 900-3 photoelectric colorimeter. Fifty klett units correspond to 2×10^6 cells-ml⁻¹. Cultures were inoculated to 10^4 cells-ml⁻¹, with numbers being determined by a Coulter counter model B.

B. Nutritional Supplementation

By fortifying growth media with lipids and protein, the effect of these components on ethanol tolerance was studied. Media were supplemented with one or more of the following: ergosterol or cholesterol (5-30 µg/ml), 4:1 oleic:palmitic acid (50-100 µg/ml), and bovine serum albumin (0.1-1.0% w/v). Growth response was measured as described above.

C. Fluorescence Analyses

A facile, highly reproducible, presumptive test was needed to assess the physical effect of ethanol on the plasma membrane. For this, the steady-state fluorescence anisotropy (r_s) of the lipophilic

probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used (30). While such measurements have been reported to measure lipid layer microviscosity (η_0) (32,46), inaccuracies with precise determination have been described (37). Since our intent was to use the probe as a diagnostic of the effect of different concentrations of ethanol on membrane preparations, we have chosen to report our data simply as r_s values.

Steady-state fluorescence anisotropy changes were measured with a computerized anisotropy spectrophotometer as described by Ayres et al. (2). Anisotropy was determined from the equation:

$$r_s = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$$

where $I_{||}$ and I_{\perp} are the intensities of parallel and perpendicular polarized light respectively (5). At each temperature point, more than 1,000 individual anisotropy measurements were taken. The standard deviation of each anisotropy value plotted is 0.001. Temperature was determined within $\pm 0.5^\circ\text{C}$, and absorbance (460 nm) was less than 0.15 for all samples, which contained DPH at a concentration of 1 μM in 10 mM Tris, 1 mM EDTA buffer, pH 7.4.

Plasma membranes were prepared from cells by lyticase digestion and osmotic lysis followed by gradient fractionation, using the methods of Bottema, et al. (5). Anisotropy measurements were made over the physiological temperature range of 15 to 40°C.

D. Ethanol Quantitation

Ethanol concentrations were measured by enzymatic assay with alcohol dehydrogenase (4) and by gas chromatography on Chromosorb 101

(150°C), using a flame-ionization detector (oven 300°C) (3). Injection oven was kept at 250°C and carrier gas flow rate was 40 ml-min⁻¹.

Butanol was used as external standard.

E. Medium Conditioning and Treatment

For conditioning of medium, cultures were grown to early log phase (2×10^6 cells-ml⁻¹) and medium was filtered through sterile 0.45 micron Millipore filters to remove cells. This conditioned medium was then inoculated with fresh cells grown without ethanol. Controls were included of non-conditioned medium and medium without ethanol.

Culture dialysis was performed in the apparatus diagrammed in Fig. 1, adapted from Stuart and Street (40). Membranes were autoclaved in distilled water before use to remove any impurities. The flask contained 300 mls fresh 6% ethanol medium and the dialysis membrane contained 50 mls medium as indicated in Fig. 8. Spectrapor 1 membrane was used, with a molecular weight cutoff of 6000-8000.

Treatment of media included heat, lyophilization, hexane extraction, and distillation. Ethanol concentrations were measured after each medium treatment and adjusted to 6% (v/v) before inoculation. Controls of fresh 6% ethanol medium were treated similarly. Conditioned medium was heat treated by incubating 20 minutes in a steam chamber or autoclaving for 20 minutes. Lyophilization was performed with a FTS Systems Flexi-Dry model FDY-1-54. Hexane extraction was performed by extracting 10 mls of

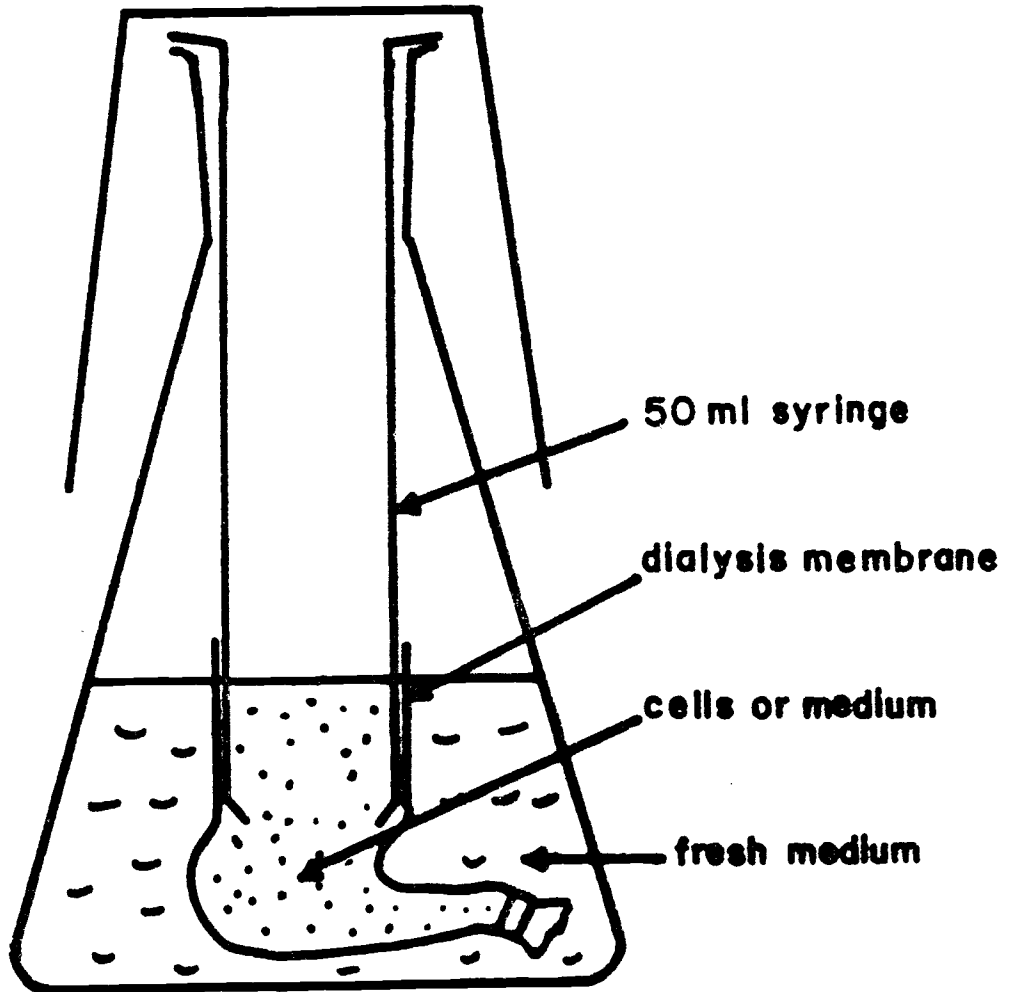


FIG. 1. Apparatus used in conditioning dialysis experiments.

medium with 5 mls of n-hexane, three times. Culture medium (100 mls) was distilled in a Buchi Rotovapor-R and distillate collected (15 mls). Medium and distillate both contained ethanol. Distillate was then added to fresh and distilled medium ($4-80 \mu\text{s}\cdot\text{ml}^{-1}$).

F. Materials

Solvents were reagent grade and were redistilled prior to use. Media components were from Difco. DPH and other chemicals were obtained from Sigma. Chromosorb 101 was from Supelco, and dialysis membranes were from Spectrum.

RESULTS AND DISCUSSION

A. Fluorescence Studies

The behavior of the lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene, was used to determine if ethanol did in fact have an effect on the lipid domains of the membrane. Plots of steady state fluorescence anisotropy of plasma membranes from cells grown on YTD (Fig. 2) showed changes in slope for preparations in which two and six percent ethanol were added. For one percent ethanol addition, no change in slope was observed. Such discontinuities were not evident in preparations without added ethanol. These data indicate a change in probe mobility in the membrane due to the presence of ethanol.

B. Inhibition of Growth

Strain X2180-1A was then grown in ethanol stress to measure the effect of ethanol on growth kinetics in the aerobic system. Fig. 3 shows growth curves for cultures grown on YTD with a range of ethanol concentrations. The cultures showed increased lag times and decreased growth rates with increasing ethanol concentration. The use of defined medium in place of rich medium (YTD) resulted in even longer lag periods prior to growth (Fig. 4). Altering the concentrations of yeast nitrogen base and casamino acids in the defined media did not decrease the lag period. When a range of cell densities (10^3 - 10^6 cells-ml⁻¹) was used as inoculum, although growth rates remained the same, a decrease in lag time was seen with increasing inoculum size (Fig. 5).

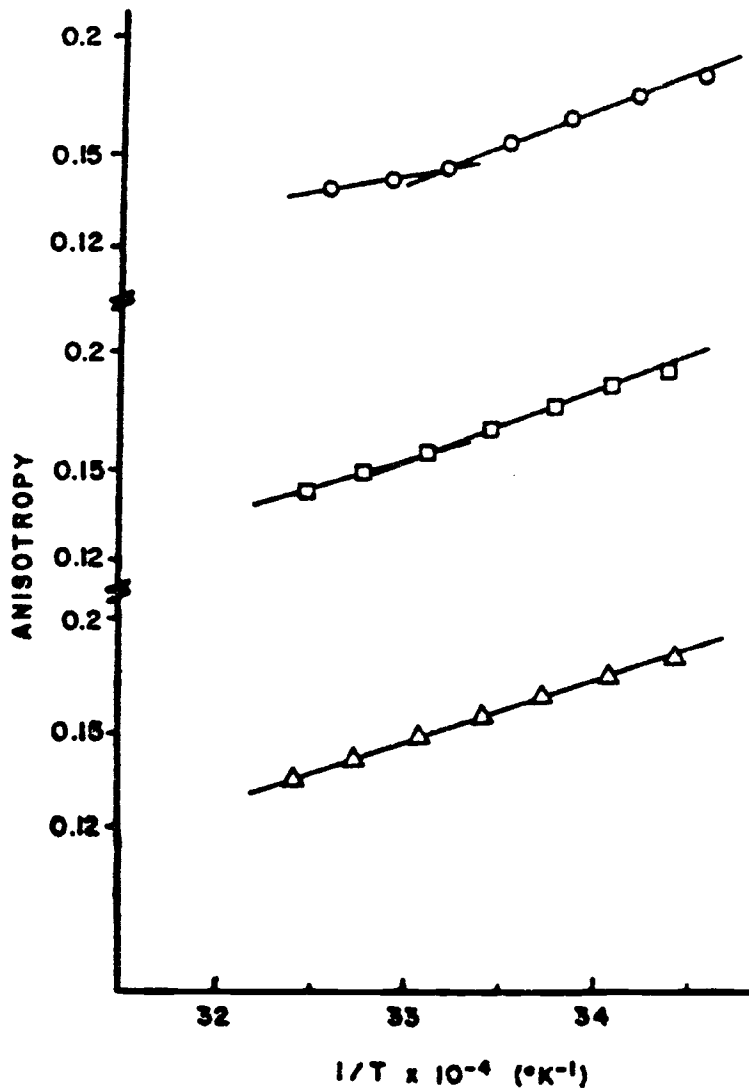


FIG. 2. Fluorescence anisotropy of DPH embedded in plasma membranes prepared from strain X2180-1A as described in text. Anisotropy was measured over temperature range of 15 to 40°C with ethanol added to reaction mix: (Δ) 1% ethanol; (□) 2% ethanol; (O) 6% ethanol.

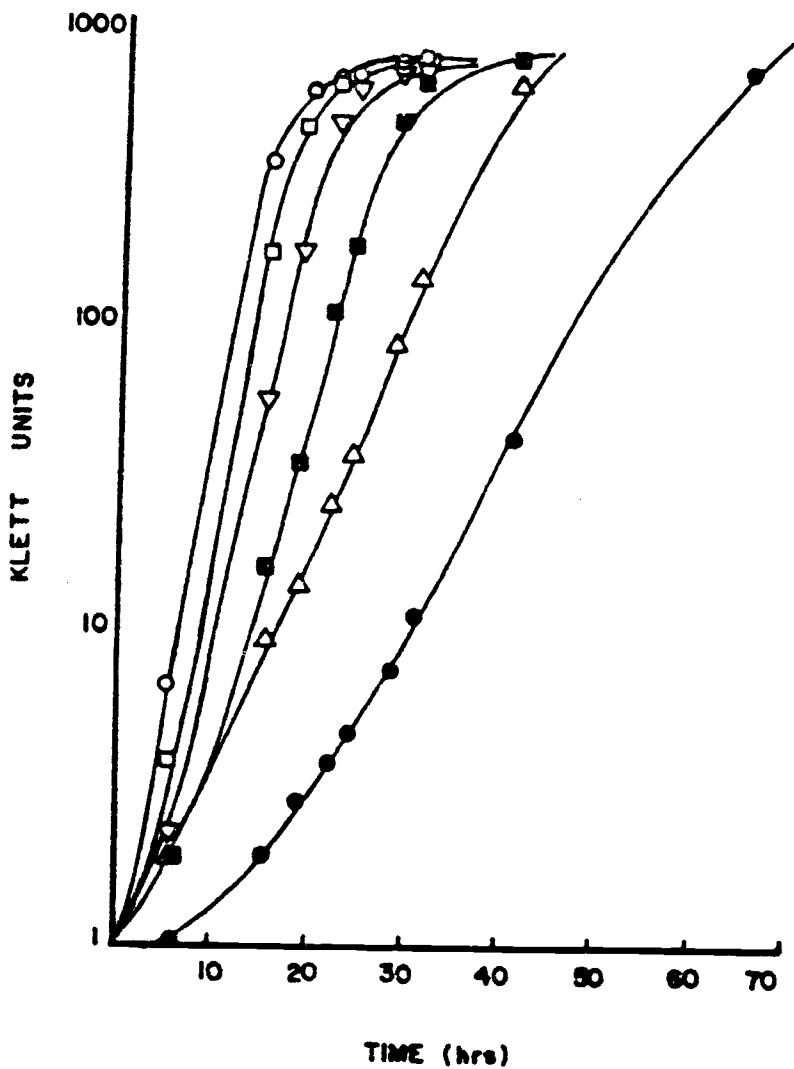


FIG. 3. Effect of ethanol on the growth of strain X2180-1A in YTD medium: (○) no ethanol; (□) 1% ethanol; (▽) 2% ethanol; (■) 4% ethanol; (△) 6% ethanol; (●) 8% ethanol.

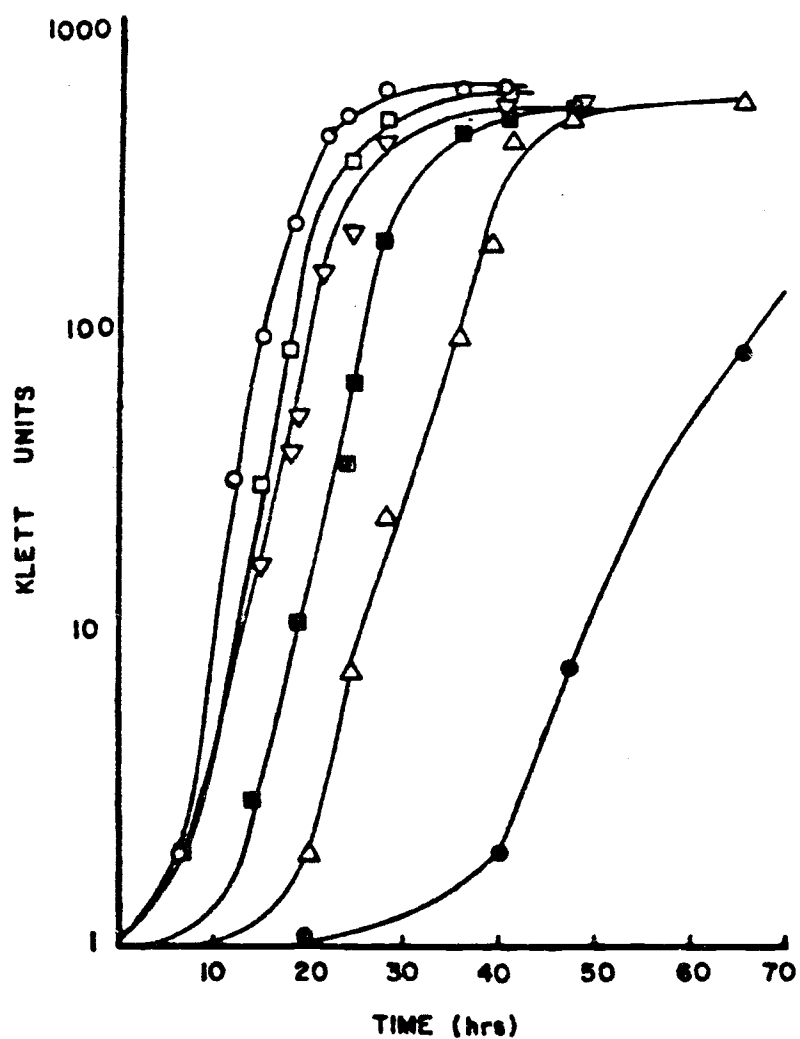


FIG. 4. Effect of ethanol on the growth of strain X2180-1A in defined medium: (○) no ethanol; (□) 1% ethanol; (▽) 2% ethanol; (■) 4% ethanol; (△) 6% ethanol; (●) 8% ethanol.

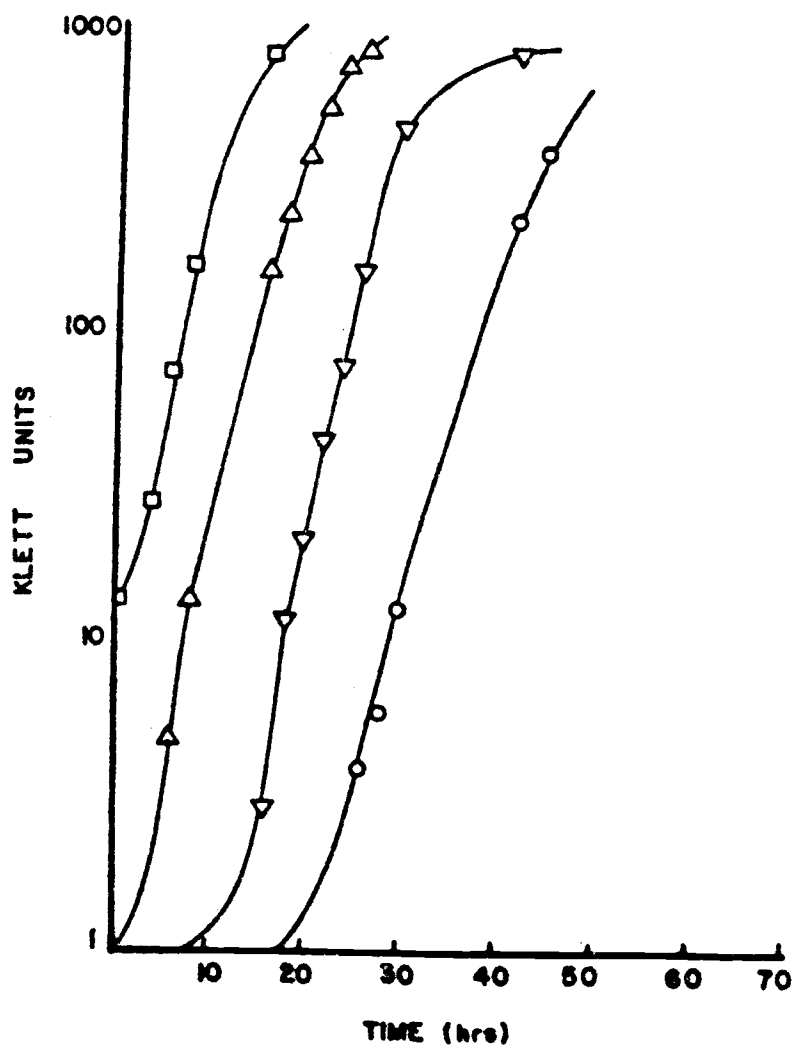


FIG. 5. Effect of inoculation cell number on the growth of strain X2180-1A in YTD with 4% ethanol: (□) 10^6 /ml; (△) 10^5 /ml; (▽) 10^4 /ml; (○) 10^3 /ml.

Lag times should be independent of inoculum size unless growth-enhancing substances are being contributed to the medium along with the inoculum. Appropriate controls in our experiments ruled out nutritional supplementation via the inoculum.

C. Lipid Supplementation

Since ethanol is affecting membrane lipids and inhibiting growth, nutritional supplementation of lipid components was applied in an attempt to reverse the growth-inhibiting effect of ethanol. Sterol auxotroph RD5-R provided a system whereby a specific plasma membrane lipid composition could be obtained. By adjusting the concentration of sterol in the growth medium, the amount of sterol in yeast membranes can be manipulated (T. A. Lewis, R. J. Rodriguez, and L. W. Parks, in preparation). This allows the "loading" of membranes with a specific sterol in order to study the effects of varying sterol composition on membrane structure and function. When protein, fatty acid, sterol, or a combination of these was added to cultures of both X2180-1A and the sterol auxotroph RD5-R, no decrease in growth lag was seen (results not shown). No protection from ethanol inhibition was seen with increased levels of ergosterol or cholesterol in RD5-R membranes. This result does not appear to support the finding that an elevated level of sterol decreases the disordering effect of ethanol on the membrane (7).

D. Cell Studies

Physiological adaptation or genetic selection of an ethanol-tolerant organism could result during the long adaptation periods. Such cells should no longer express prolonged lag phases when subcultured on ethanol. Ethanol-grown cells from log and stationary phase cultures were inoculated into fresh ethanol-containing media. These cultures gave similar profiles to original growth data, in that the same long lag period was observed (data not shown--see Fig. 3), suggesting there was no genetic selection or stable physiological adaptation. Whatever factor is allowing the cells to grow in high concentrations of ethanol is not retained and/or expressed following growth on ethanol and reinoculation of the cells into fresh ethanol-containing medium.

Growth initiation following a prolonged lag period requires the presence of cells in the medium during the lag. When uninoculated ethanol-containing medium was incubated for time intervals equivalent to observed lag times and subsequently inoculated with yeast, the same delayed growth was noted.

E. Ethanol Levels

Because of the long onset of growth, the ethanol could have been disappearing through evaporation or by chemical or enzymatic modification. Alcohol dehydrogenase (ADH) was used to assay the ethanol level of the medium over the growth curve; no detectable loss of ethanol was seen. The pH of media with or without ethanol, before

and during growth, was not appreciably different (± 0.1 pH units), and no alteration was seen during the lag period.

A conversion of ethanol to ethyl acetate has been noted in several yeast species (1,47). In order to investigate this phenomenon, ethyl acetate was added to rich medium and the growth of strain X2180-1A was monitored. Ethyl acetate was found to be more growth inhibitory than ethanol at similar concentrations (data not shown). ADH assays were also run with medium containing ethyl acetate. No reaction was observed with ethyl acetate at concentrations similar to those used for ethanol. Gas chromatographic data supported enzymatic data in the stability of the ethanol concentration over lag and growth ($\pm 0.1\%$ v/v).

Thus, following long adaptation times, the yeast cells were able to overcome the ethanol-enforced growth inhibition. The lag period did not cause the selection of more resistant forms and the lag interval was affected by inoculum size. Lipid supplementation did not reduce inhibitory effects of ethanol. Ethanol concentrations were not being reduced and pH was not altered.

F. Medium Conditioning Studies

We hypothesized that the cells could be auto-conditioning the media by producing a substance or mixture of components which increased the cellular resistance to ethanol. A study was therefore initiated to determine if such a medium conditioning effect was occurring.

Medium was conditioned and inoculated as explained in methods. Controls were included of non-conditioned medium and medium without ethanol. A shortened lag time was seen in 6% ethanol conditioned medium compared to both fresh medium (6% ethanol) and conditioned medium (0% ethanol) with 6% added ethanol (Fig. 6). Controls (0% ethanol) of conditioned and fresh medium gave identical curves. This lag reduction was not due to a loss of ethanol in the medium since the ethanol concentration in the medium did not change over the course of conditioning, as measured by both enzymatic and chromatographic methods. Reinoculation with ethanol-grown or control cells showed no difference in growth profiles. Conditioning results were similar in rich and defined medium. With YTD faster auto-conditioning of the medium was observed, probably a result of higher metabolic rates in that medium.

Samples of media conditioned to different extents were obtained by filtering at increasingly later times over the course of growth. These media were then inoculated and growth was measured. Lag periods were seen to lessen with increasing amounts of conditioning (Fig. 7), but this effect reached a maximum. Whatever substance(s) account(s) for lag reduction reach(es) a concentration beyond which additional conditioning has little or no added effect.

G. Conditioning Treatment Studies

Studies were then implemented in an attempt to characterize the nature of the conditioning agent(s). Properties examined included

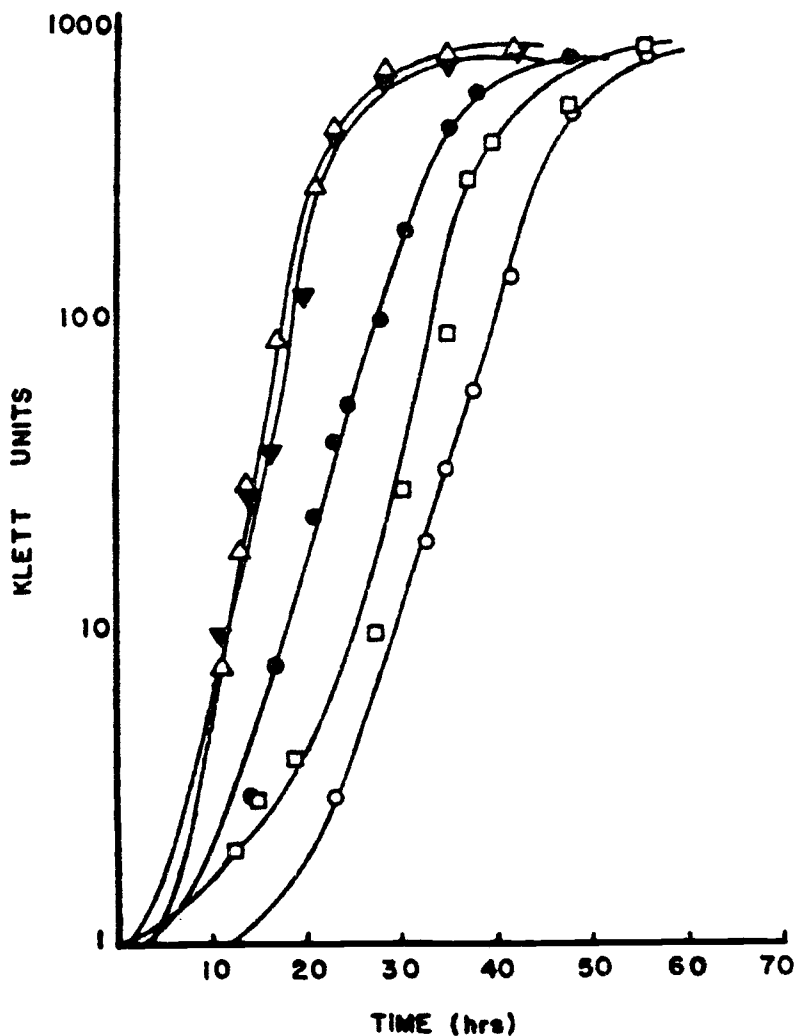


FIG. 6. Effect of medium conditioning on growth of strain X2180-1A in YTD medium. Medium was conditioned by growth to a density of 2×10^6 /ml, then filtering and reinoculating. Symbols: (●) 6% ethanol-conditioned medium; (○) fresh 6% ethanol medium; (□) 0% ethanol-conditioned medium plus 6% ethanol; (▼) 0% ethanol-conditioned medium; (△) fresh 0% ethanol medium.

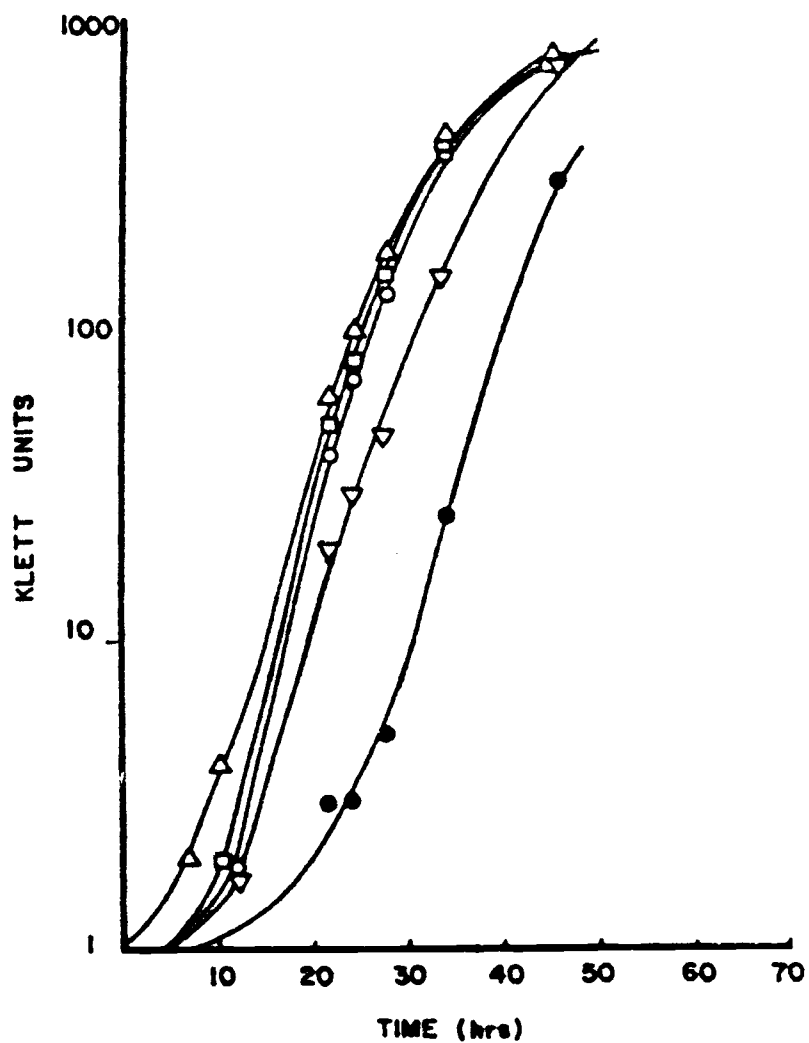


FIG. 7. Growth of strain 2180-1A on YTD with varied extents of conditioning by growth to the indicated cell density: (●) fresh; (▽) 2×10^6 /ml; (○) 4×10^6 /ml; (□) 8×10^6 /ml; (△) 1.6×10^7 /ml.

size, stability and limits of solubility.

Conditioned medium was allowed to dialyze against fresh medium across a dialysis membrane. The substance was observed to cross the membrane, as exhibited by enhanced growth with both conditioned medium and medium plus cells (see Fig. 8). Continued growth of cells, and presumably production of conditioning, inside the membrane allows for a greater lag reduction than is seen with sterile conditioned medium alone.

Upon heating or autoclaving, the conditioning agent was partially or completely lost. (see Fig. 9) This indicates a heat lability of the conditioning substance(s). Lyophilization and reconstitution of the conditioned medium did not increase lag time any more than in fresh medium (see Fig. 10).

Distilling the medium reduced conditioning (see Fig. 11), most likely due to the heating involved, since the distillate did not reduce lags upon addition to fresh medium. Hexane extraction of medium did not remove conditioning from the medium.

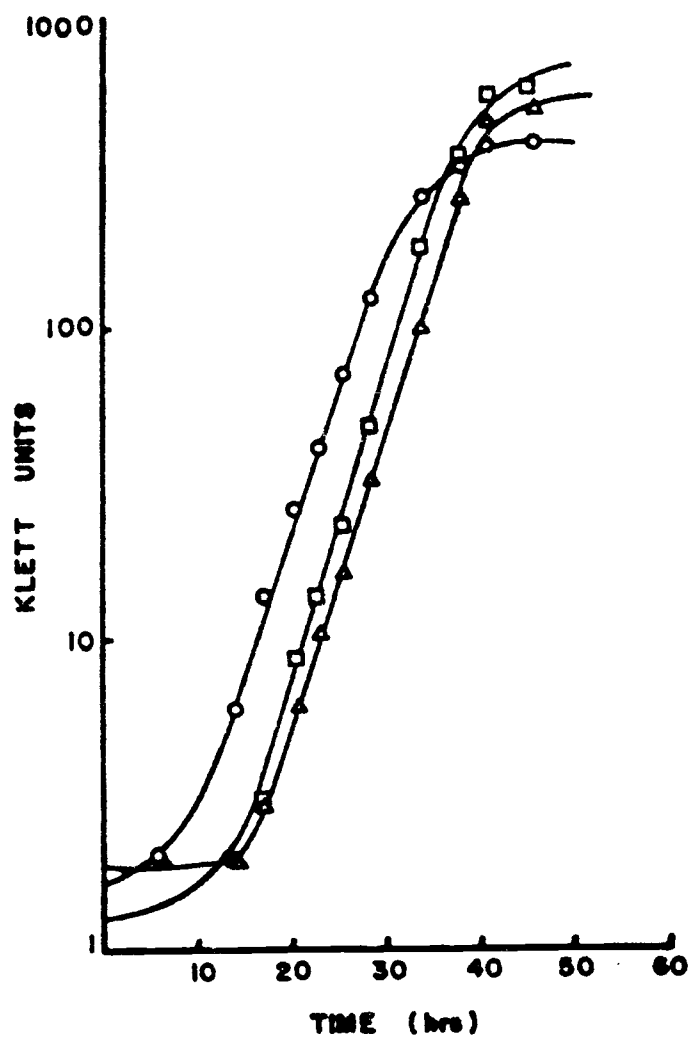


FIG. 8. Growth of cells in exterior of dialysis apparatus. Dialysis membrane contained: (O) conditioned cells and medium, (□) conditioned medium, (Δ) fresh medium.

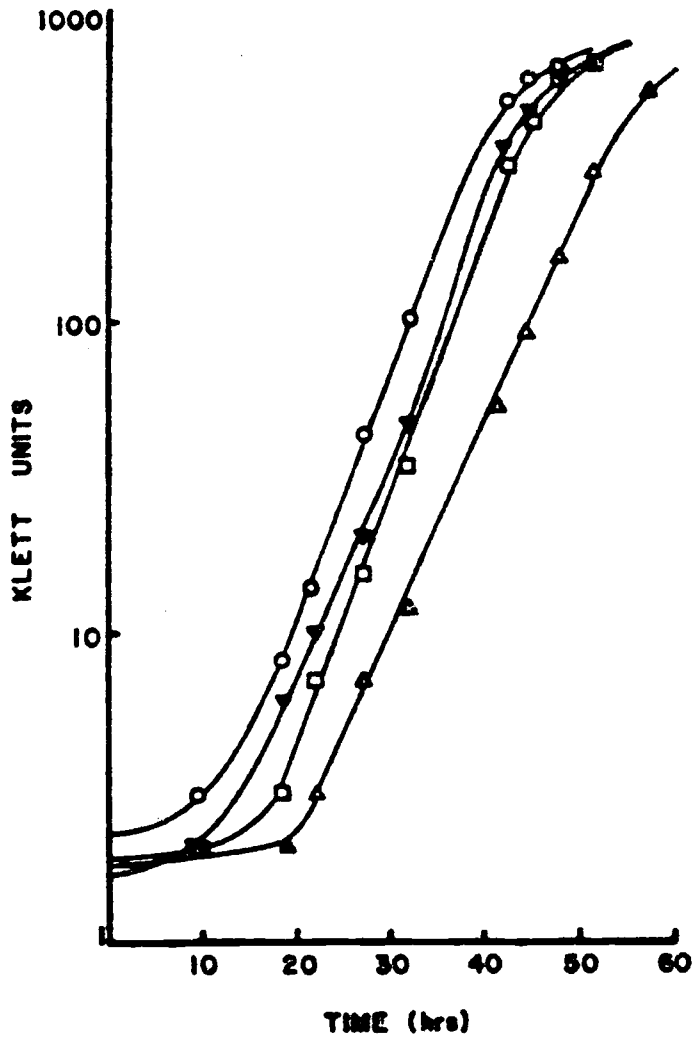


FIG. 9. Effect of heat on medium conditioning: (O) conditioned medium, (▼) autoclaved conditioned medium, (□) steamed conditioned medium, and (Δ) fresh medium.

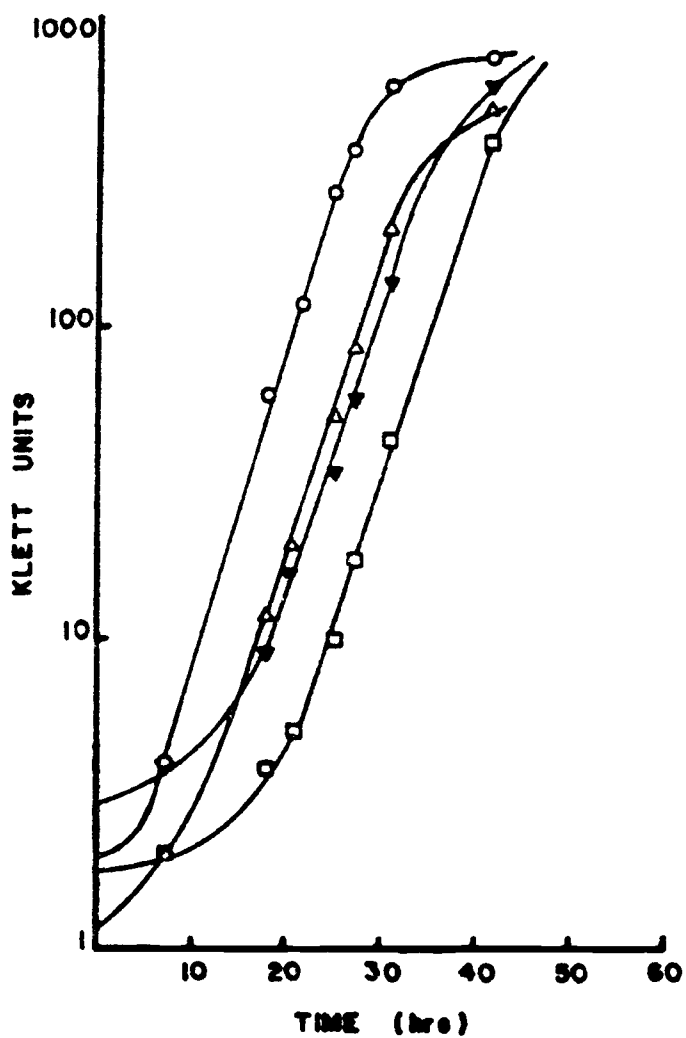


FIG. 10. Effect of lyophilization on medium conditioning: (O) conditioned medium, (Δ) lyophilized conditioned medium, (\blacktriangledown) fresh medium, (\square) and lyophilized fresh medium.

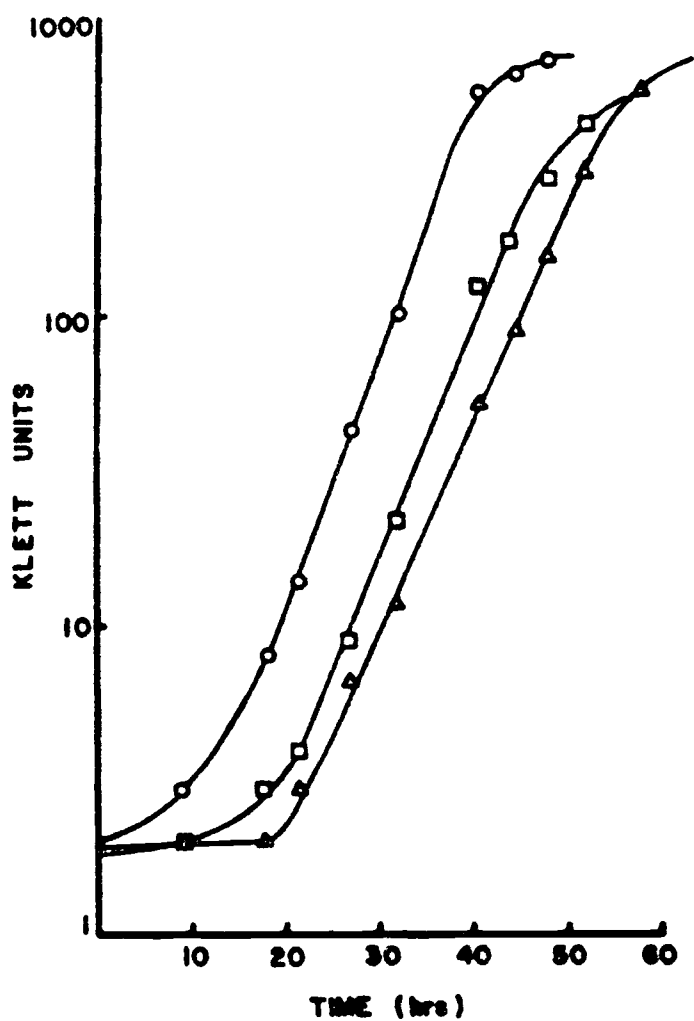


FIG. 11. Effect of distillation on medium conditioning: (O) conditioned medium, (□) distilled conditioned medium, (Δ) fresh medium.

CONCLUSION

Ethanol causes an alteration in the mobility of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene in Saccharomyces cerevisiae plasma membranes. It also inhibits the growth of this yeast in a concentration-dependent manner. Supplementation of lipids does not reduce the ethanol-induced lag period, even when performed in the fatty acid and sterol auxotroph, RD5-R. This is in opposition to previous studies, in anaerobic culture, which found increased fermentative activity (as measured by CO₂ output and shortened fermentation periods) and higher ethanol production (≥ 20% v/v) with the addition of a lipid-albumin or lipid-methylcellulose complex (11,12,13,19).

No stable physiological adaptation or genetic selection for more resistant organisms is occurring during prolonged lag periods caused by ethanol. Ethanol concentration and pH are not involved in the eventual growth of cells in ethanol, as these factors remained stable during lag and growth.

Conditioning of ethanol-containing medium by pregrowth with Saccharomyces cells was noted to reduce long lag periods. This effect is analogous to that observed in various eukaryotic systems in a number of different cultural environments (9,11,12,40,41). Cultured Drosophila cells grew as well or better in conditioned medium (9). Acer pseudoplatanus L. cells were found to condition the medium and to produce a volatile factor, the combination of which allows culture

growth from smaller inocula (40,41). Bovine endothelial cells produced two conditioning factors. One of high molecular weight and one of low molecular weight as determined by dialysis (12). Human malignant melanoma cell cultures produced a conditioning substance of low molecular weight, moderate heat stability and nonlipophilicity (11). It is possible that the response seen with Saccharomyces is a universal one that occurs in many other fungi and bacteria. The present study indicates a water soluble compound(s), moderately heat labile, which is produced during growth. Its chemical identity is as yet unknown. Further studies with mutants may help elucidate the nature of this factor.

During growth of X2180-1A on ethanol-containing medium, the cells are not observed to obtain a lasting adjustment which permits alcohol tolerance upon subinoculation into fresh media. The response to ethanol might somehow cause a block in transport and/or diffusion of the ethanol into the cell, or might intracellularly alter the ethanol to allow cell growth. Current evidence to support or disprove these alternatives is inconclusive. We have observed a direct effect of ethanol on the membrane lipids as measured by fluorescence anisotropy.

Ethanol stress has been shown to induce synthesis of proteins similar to those which are produced during heat shock in a number of cell types, from yeast to mammalian systems (27,36). Treatment with ethanol has also been shown to result in acquisition of thermotolerance (27,36). It is possible that upon further research, this protein synthesis and thermotolerance may be related to ethanol production or tolerance in Saccharomyces strains.

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