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Ethanol Production during Batch Fermentation with *Saccharomyces cerevisiae*: Changes in Glycolytic Enzymes and Internal pH

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During batch fermentation, the rate of ethanol production per milligram of cell protein is maximal for a brief period early in this process and declines progressively as ethanol accumulates in the surrounding broth. Our studies demonstrate that the removal of this accumulated ethanol does not immediately restore fermentative activity, and they provide evidence that the decline in metabolic rate is due to physiological changes (including possible ethanol damage) rather than to the presence of ethanol. Several potential causes for the decline in fermentative activity have been investigated. Viability remained at or above 90%, internal pH remained near neutrality, and the specific activities of the glycolytic and alcohologenic enzymes (measured in vitro) remained high throughout batch fermentation. None of these factors appears to be causally related to the fall in fermentative activity during batch fermentation.

Saccharomyces cerevisiae is used extensively in batch fermentations to convert sugars to ethanol for the production of beverages and biofuels. Despite the obvious importance of this process, the physiological constraints which limit the rate of glycolysis and ethanol production are not fully understood (7, 15, 23). Identification of these constraints represents an important step toward the development of improved organisms and process conditions for more rapid ethanol production. Such improvements could result in an increase in the ethanol production capacity of existing fermentation plants and a reduction in the cost of future facilities.

S. cerevisiae is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, producing over 50 mmol of ethanol per h per g of cell protein (11, 12). However, this high rate is maintained for only a brief period during batch fermentation and declines progressively as ethanol accumulates in the surrounding broth (7, 15, 23). Earlier studies have identified a requirement for lipids (2, 8, 26) or molecular oxygen for lipid biosynthesis (1, 4, 5) in many fermentation broths as being essential for the maintenance of high fermentative activity. Magnesium is an essential cofactor for many of the glycolytic enzymes and has also been identified as a limiting nutrient in fermentation broth containing peptone and yeast extract (11, 12). Supplying these nutritional needs reduces but does not eliminate the decline in fermentative activity during batch fermentation.

The basis for the decline in fermentation rate is not fully understood. Since the addition of ethanol to cells in batch cultures and in chemostats causes a dose-dependent inhibition of ethanol production (7, 11, 12), most investigations have focused on ethanol as an inhibitor (7, 15, 22). Ethanol is known to alter membrane permeability and disrupt membrane function in a variety of biological systems (7, 15). In yeasts, ethanol causes an increase in hydrogen ion flux across the plasma membrane of cells suspended in water (6). This increased hydrogen ion flux has been proposed as being responsible for the ethanol-induced decline in transport rates observed under similar conditions (2, 17–19).

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Evidence has been accumulating which indicates that the presence of ethanol may not be the only factor responsible for the decline in fermentative activity. The replacement of fermentative broth containing ethanol with fresh medium lacking ethanol did not immediately restore fermentative activity (11). In a comprehensive study, Millar et al. (22) demonstrated that concentrations of ethanol below 12% (vol/vol) do not denature glycolytic enzymes or cause appreciable inhibition of activity in vitro under substrate-saturating conditions. Since ethanol does not accumulate within yeast cells but rapidly diffuses across the cell membrane (10, 13), direct inhibition of glycolytic enzymes by intracellular ethanol is unlikely during fermentations which produce 12% (vol/vol) ethanol or less.

In this paper, we have examined three physiological factors (glycolytic and alcohologenic enzymes, internal pH, and viability) as possible causes for the decline in fermentative activity during batch fermentation.

MATERIALS AND METHODS

Organism and growth condition. *S. cerevisiae* KD2 (petite strain) was used in this study and was grown in complex medium supplemented with 0.5 mM magnesium sulfate as previously described (10, 12). Batch fermentations (initial optical density at 550 nm of 0.035; 0.01 mg of cell protein per ml) with 20% glucose were carried out in 300-ml Spinner bottles at 30°C with a 1% inoculum from a 12-h culture.

Analyses of fermentation broth. Cell protein was determined by the method of Lowry et al. (20). Glucose, ethanol, and cell mass were measured as described (12).

Respirometry measurements. The rate of glycolysis and ethanol production was estimated by measuring carbon dioxide evolution with a Gilson differential respirometer (Gilson, Middleton, Wis.). This rate is taken as equivalent to glycolytic flux, assuming the production of 2 mol of ethanol and carbon dioxide per mol of glucose consumed. Results are expressed as micromoles of carbon dioxide evolved per hour per milligram of cell protein (12).

Enzyme analyses. Activities of glycolytic and alcohologenic enzymes were determined in 2-ml samples removed at various times during batch fermentation. Cells were har-

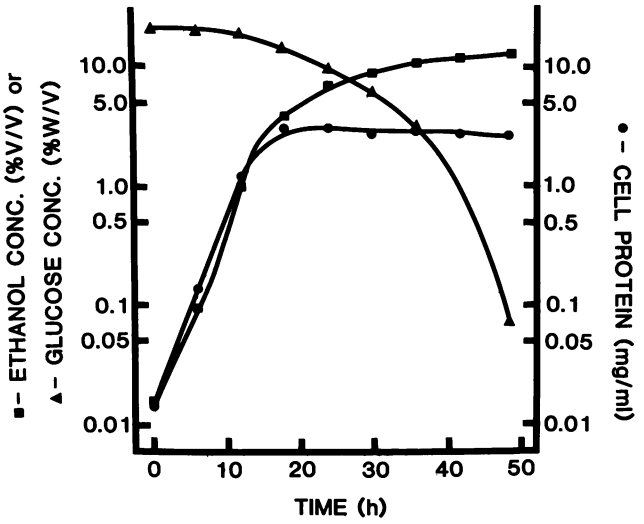

FIG. 1. Alcohol production by strain KD2 during batch fermentation with 20% glucose and 0.5 mM magnesium sulfate.

vested by centrifugation at 10,000 × *g* for 30 s at 4°C and washed in an equal volume of 50 mM potassium phosphate buffer (pH 7.4). All subsequent steps were carried out at 4°C. The pellet was suspended in the same buffer containing 2 mM mercaptoethanol and 2 mM EDTA and disrupted with 0.1-mm glass beads using a Mini-Bead Beater (Biospec Products, Bartlesville, Okla.; five periods of disruption, 1 min each, with cooling on ice between treatments). Cell debris was removed by centrifugation at 10,000 × *g* for 5 min, and the supernatant was assayed immediately for enzymatic activities. Only two enzymes at a time were assayed in each batch fermentation experiment to avoid potential problems which could result from storage of cells or extracts.

Pyruvate decarboxylase and all glycolytic enzymes were assayed spectrophotometrically by the methods of Maitra and Lobo (21) as modified by Clifton et al. (9). All enzymes were assayed under substrate-saturating conditions except triose phosphate isomerase, which was assayed with 1 mM substrate. The amounts of coupling enzymes were adjusted as needed to ensure a linear reaction rate. Alcohol dehydrogenase was assayed by measuring the oxidation of ethanol as described by Maitra and Lobo (21) but using a buffer at pH 8.7 containing 75 mM sodium pyrophosphate, 75 mM semicarbazide hydrochloride, and 21 mM glycine (3).

Determination of internal pH and membrane energization. The measurements of internal pH and $\Delta\Psi$ were performed using 7-[^{14}C]benzoic acid and [^{3}H-phenyl]tetraphenyl phosphonium bromide, respectively. Protocols were similar to those described by Cartwright et al. (6) except that cells were incubated in their native growth medium rather than distilled water and 0.4-μm-pore-size polycarbonate filters were used instead of mixed cellulose ester filters. Cell volumes were determined as previously described (10). These ranged from 2.23 μl/mg of cell protein for cells removed from the 12-h stage of batch fermentation to 0.86 μl/mg of cell protein for cells removed from the 48-h stage. As a control for adventitious binding of radioactive compounds, cells were permeabilized with a combination of ethanol, toluene, and Triton X-100 as described by Salmon (25), washed with 50 mM phosphate buffer, resuspended in native broth, and processed. This treatment resulted in a complete collapse of ΔpH and loss of membrane potential. Calculations were performed as described by Rottenberg (24).

Materials. Yeast extract, peptone, and agar were obtained from Difco Laboratories, Detroit, Mich. Glucose, coupling enzymes, coenzymes, and substrates were purchased from Sigma Chemical Co., St. Louis, Mo. Inorganic salts were obtained from Fisher Scientific Co., Orlando, Fla. Absolute ethanol was supplied by AAPER Alcohol and Chemical Co., Shelbyville, Ky. Radioactive compounds were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Reversibility of the decline in fermentative activity. Figure 1 shows a representative batch fermentation with 20% glucose beginning with a low inoculum. Growth as measured by cell protein was exponential for the first 12 h and became stationary between 18 and 24 h. During these growth periods, relatively low concentrations of ethanol had accumulated (<5% [vol/vol]), well below the minimum inhibitory concentration of added ethanol for growth (8% [vol/vol]). Ethanol production proceeded exponentially for the initial 12 h (1% [vol/vol] accumulated ethanol).

Cells were removed at various times during batch fermentation, and the rate of ethanol production per milligram of cell protein was determined (Fig. 2). Cells were most active at the earliest times measured, 12 h, and declined by 50% after 24 h (6.5% [vol/vol] accumulated ethanol). Approximately 40% of the fermentative activity remained after the accumulation of 10% (vol/vol) ethanol (30 g of remaining glucose per liter). The abrupt, final decline in activity reflects the near-complete exhaustion of glucose, the substrate. Removal of ethanol from cells by washing and suspending in fresh medium resulted in only a modest increase in fermentative activity in all but the highest level of accumulated ethanol. The apparent increase in activity in the cells which had accumulated 12.1% (vol/vol) ethanol was primarily due to the restoration of fermentable substrate glucose.

Loss of viability was examined as a possible cause for the failure of washing to restore full fermentative activity (data not shown). Cell number paralleled the increase in cell protein (Fig. 1) and increased exponentially for the initial 12 h, reaching a maximum of 3 × 10^8 cells per ml after 18 h. Cell number remained constant for the remaining period of fer-

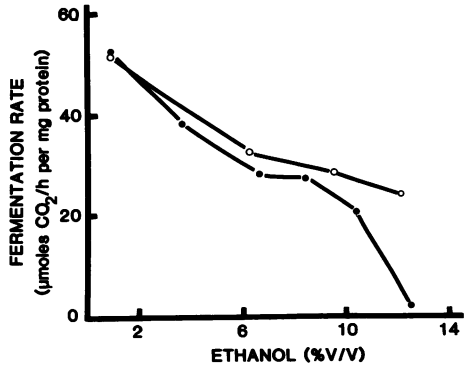

FIG. 2. Changes in fermentative activity of cells during batch fermentation. Fermentative activity is expressed as micromoles of carbon dioxide evolved per hour per milligram of cell protein. Symbols: ●, activity measured in native broth; ○, activity measured after cells were suspended in fresh medium containing 20% glucose.

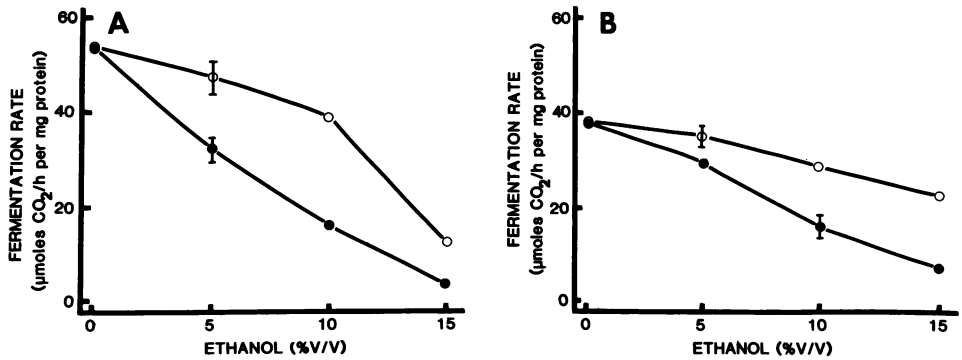

FIG. 3. Effects of ethanol exposure on the fermentative activities of 12- and 24-h cells. Fermentative activity is expressed as micromoles of evolved carbon dioxide per hour per milligram of cell protein. Cells were harvested after 12 h (A) or 24 h (B) and suspended in fresh medium containing various concentrations of ethanol, and their fermentative activity was measured after 10 min at 30°C. A parallel set of samples was exposed to ethanol for 10 min, harvested by centrifugation, washed once, and suspended in fresh medium lacking ethanol. Bars denote a representative standard deviation for an average of three determinations. Symbols: ●, cells in the presence of added ethanol; ○, cells exposed to ethanol and suspended in fresh medium.

mentation, with 90% viability after 48 h as measured by the exclusion of methylene blue dye (12).

In a control experiment, we investigated the inhibition of ethanol production by added ethanol and its reversibility after washing (Fig. 3). Cells were harvested and suspended in fresh medium containing various concentrations of ethanol. Cells from the 12-h period (Fig. 3A) were more active and more sensitive to inhibition by added ethanol than cells from the 24-h period (Fig. 3B). Ethanol caused a progressive, dose-dependent inhibition of fermentation in both, with 12-h cells being more sensitive. Inhibition by ethanol was immediate and appeared complete within the first 10 min. No further decline in activity was observed during a subsequent 2 h of incubation (30°C) with 10% added ethanol (data not shown). The concentrations of ethanol required to inhibit 50% of fermentative activity were 6.5 and 9% (vol/vol), respectively, for 12- and 24-h cells.

The inhibition of fermentation caused by exposure to concentrations of ethanol above 5% (vol/vol) for 10 min was only partially reversed by resuspension in fresh medium lacking ethanol (Fig. 3), indicating that exposure to ethanol damaged the cells in some way. Again, 12-h cells appeared more sensitive to ethanol damage than 24-h cells. Longer incubation periods with 10% ethanol before washing resulted in further loss of activity, with only 40% of the original activity remaining after 2 h.

TABLE 1. Specific activities of glycolytic enzymes at the peak of fermentative activity (12 h) and after a 50% decline (24 h)

Enzyme	Sp act (μmol/min per mg of protein) (SD)[a]	
	12-h cells	24-h cells
Glycolytic flux (hexose)[b]	1.0	0.5
Hexokinase	0.84 (0.05)	1.1 (0.1)
Phosphoglucose isomerase	4.2 (0.6)	3.3 (0.1)
Phosphofructokinase	0.64 (0.03)	0.53 (0.05)
Fructose diphosphate aldolase	1.4 (0.1)	1.2 (0.1)
Glycolytic flux (triose)[b]	2.0	1.0
Triose phosphate isomerase	110 (2)	97 (2)
Glyceraldehyde-3-phosphate dehydrogenase	16 (1)	18 (1)
Phosphoglycerate kinase	11 (1)	12 (1)
Phosphoglycerate mutase	6.2 (0.5)	9.0 (0.8)
Enolase	3.0 (0.2)	3.2 (0.4)
Pyruvate kinase	10 (3)	8.4 (1.4)
Pyruvate decarboxylase	1.1 (0.2)	0.92 (0.04)
Alcohol dehydrogenase	4.8 (0.4)	3.8 (0.4)

[a] Cells were removed from batch fermentations after 12 and 24 h. Standard deviations are based upon determinations from three separate batch fermentations.

[b] Glycolytic flux for hexose and triose intermediates was estimated from measurements of fermentation rate.

Changes in the levels of glycolytic and alcohologenic enzymes during batch fermentation. The specific activities of the enzymes involved in alcohol production (under substrate saturating conditions) are listed in Table 1 for cells harvested after 12 h (the most active stage of fermentation) and 24 h (50% maximal activity). For comparison, the rates of glycolytic flux for hexose and triose intermediates have been included (based on rates of carbon dioxide evolution). The activities of all but three of these are clearly in excess of that required to support the measured rates of glycolytic flux. The exceptions were hexokinase at 12 h and phosphofructokinase and pyruvate decarboxylase at both 12 and 24 h. However, the true in vivo activities must be sufficient to support the measured rates of carbon dioxide evolution except for a small contribution from anabolic processes.

It is of interest to compare the relative activities of each enzyme at these two times. After 24 h, glycolytic flux had declined by approximately half while the specific activities of six enzymes remained unchanged, four had declined by approximately 20%, and two had increased. Figure 4A illustrates the changes in the specific activities of these enzymes throughout batch fermentation, relative to that of 12-h cells (100%). An analogous plot of fermentation rate is included for comparison. None of the specific activities declined dramatically during batch fermentation. At times beyond 12 h, with the exceptions noted above, all enzymes were in excess of the measured fermentation rates. Phosphofructokinase declined to the greatest extent with a 20% drop in activity after 48 h. The specific activities of three enzymes increased by more than 20%: phosphoglucomutase (100% increase), hexokinase (50% increase; not shown), and enolase (50% increase; not shown). All other enzymes exhibited a similar increase of up to 20%.

Figure 4B illustrates the changes in the amounts of these enzymes present per milliliter of broth relative to that at 12 h. Analogous plots of soluble cell protein and fermentative activity per milliliter are included for comparison. The peak

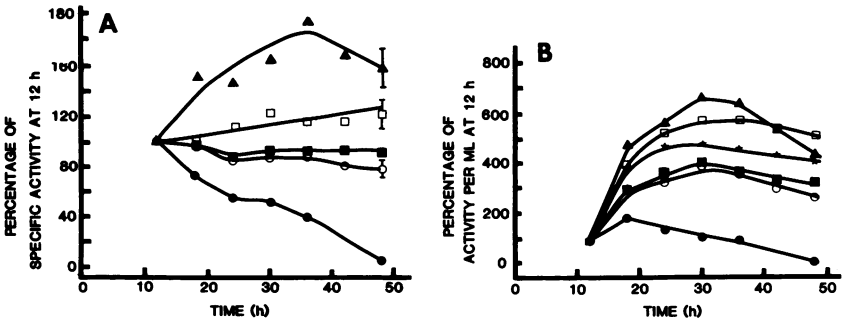

FIG. 4. Changes in the levels of glycolytic and alcohologenic enzymes during batch fermentation with 20% glucose. Cells were removed from various stages of fermentation and disrupted, and the activities of individual enzymes were determined under substrate-saturating conditions. Values are expressed relative to 12-h cells, the time at which the highest activity per milligram of cell protein was observed. Bars denote a representative standard deviation for an average of three determinations. (A) Changes in the specific activities of representative enzymes. (B) Changes in the activities per milliliter of culture of representative enzymes. For comparison, analogous plots of the changes in fermentation rate (A and B) and the changes in the amount of soluble cell protein (B only) have been included. Symbols: ▲, phosphoglucomutase; □, glyceraldehyde-3-phosphate dehydrogenase; ■, triose phosphate isomerase; ○, phosphofructokinase; ●, glycolysis; ★, soluble cell protein.

of fermentative activity on a volumetric basis occurred after 18 h. Although the rate of fermentation declined beyond 18 h, the activities of all of the glycolytic enzymes continued to increase until 30 h, the peak of soluble proteins. These increases in activities roughly paralleled the increases in soluble proteins. The activities of phosphoglucomutase, enolase (not shown), hexokinase (not shown), and glyceraldehyde-3-phosphate dehydrogenase increased more rapidly than soluble cell protein, consistent with the observed increases in specific activities of these enzymes during fermentation. With the exception of phosphoglucomutase, which declined more rapidly, the rates of decline of the glycolytic enzyme activities per milliliter paralleled that of the bulk soluble cell proteins, indicating neither a preferential retention nor degradation of these central catabolic activities.

Changes in internal pH and membrane energization during batch fermentation. Although ethanol is the principal, reduced fermentation product from the metabolism of glucose by *S. cerevisiae*, organic acids are also produced which lower the external pH of the fermentation broth to pH 3.5 (Fig. 5A). Since the pH optima for glycolytic enzymes are near neutrality or above (9), the failure of *S. cerevisiae* to maintain a large ΔpH during the accumulation of ethanol could explain the rapid decline in the fermentative activities of cells despite the abundance of glycolytic and alcohologenic enzymes. However, this does not appear to be the case. The ΔpH of yeast cells increases coincident with the decrease in the external pH, maintaining a relatively constant internal pH of between 6.7 and 7.0 throughout batch fermentation (Fig. 5A). Similarly, ΔΨ also increased during batch fermentation, resulting in an overall increase in proton motive force (Fig. 5B).

These results were somewhat surprising since previous workers have shown that ethanol increases the permeability of yeast suspended in water to hydrogen ions (6). To further examine this point, we determined the effect of added ethanol on the internal pH of cells from various stages of fermentation (Fig. 6). Ethanol concentrations of 15% (vol/vol) or above were required to cause a measurable decline in internal pH. The addition of 20% (vol/vol) ethanol to 12- and 24-h cells caused a complete collapse of ΔpH. The ΔpH of cells from 36- and 48-h cultures was considerably more resistant to 20% (vol/vol) added ethanol, consistent with an adaptation of older cells.

DISCUSSION

The fermentation of glucose to ethanol represents a series of coordinated enzymatic reactions. This process is internally balancing and thermodynamically favorable provided that cellular enzymes consume the net ATP generated from substrate-level phosphorylation. The requirements for this process include glucose, functional enzymes, coenzymes (NAD^+, thiamine pyrophosphate, ADP, ATP), cofactors (Mg^{2+}, Zn^{2+}), appropriate internal pH, a functional membrane to maintain the concentration of reactants and enzymes, and a glucose uptake system. Indeed, fermentation can proceed well in concentrated preparations of disrupted cells (14, 27).

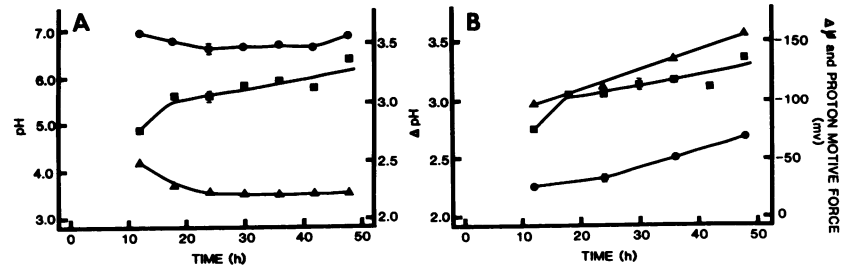

FIG. 5. Changes in internal pH and membrane energization during batch fermentation on 20% glucose. Bars denote a representative standard deviation for an average of three determinations. (A) Internal pH (●), external pH (■), and ΔpH (▲). (B) Membrane energization. Symbols: ●, proton motive force; ■, ΔpH; △, ΔΨ.

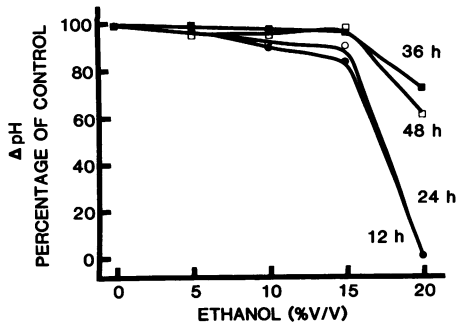

FIG. 6. Effects of added ethanol on ΔpH. Cells were removed from various stages of batch fermentation (indicated on graph), harvested, and suspended in fresh medium containing various concentrations of ethanol at 30°C for 10 min, and ΔpH was determined.

Why then does the rate of glycolysis in viable yeast cells decline during batch fermentation? Two nutritional factors have been identified previously which reduced but did not eliminate the ethanol-associated decline in activity (7, 11, 12). Our results with added and accumulated ethanol indicate that physiological changes such as ethanol damage, rather than an immediately reversible effect of ethanol, appear responsible. Added ethanol inhibited fermentation, but washing did not restore full activity. Similarly, the replacement of ethanol-containing broth from the middle to later stages of fermentation with fresh medium did not immediately restore fermentative activity. The exposure of cells to ethanol in some way damaged their ability to produce ethanol. The extent of this damage appears related to both ethanol concentration and the duration of exposure.

We have examined cell viability, internal pH, and individual enzymes involved in glycolysis and alcohologenesis as sites for changes (including ethanol damage) which could be responsible for the loss of ethanol productivity during batch fermentation. No appreciable loss of cell viability was observed during 48-h batch fermentations. The activities of glycolytic and alcohologenic enzymes measured in vitro remained high and did not appear limiting, consistent with earlier reports of the persistence of hexokinase and alcohol dehydrogenase activity (16). The specific activities of many of these continued to increase even after increases in total cell protein had ended, suggesting that they may be preferentially synthesized. Only a modest loss of total activity (per milliliter) was observed during the latter stages of fermentation, consistent with a low rate of turnover of these enzymes.

The internal pH of the cell was maintained near neutrality despite acidification of the broth and the accumulation of over 12% ethanol. This latter observation was contrary to expectation based upon earlier studies with cells suspended in water (6). These earlier studies had demonstrated that ethanol enhanced the leakage of protons (6), with an acidification of the cytoplasm below the optimal pH for glycolytic and alcohologenic enzymes. Although such enhanced leakage may also occur in fermentation broth, the maintenance of a high internal pH in broth containing ethanol indicates that such leakage must be offset by the action of hydrogen ion pumps such as ATPase.

Cells from the later stages of fermentation were more resistant to inhibition by ethanol and to the disruptive effects of ethanol on membrane integrity (as measured by proton leakage). During batch fermentation, cells may be undergoing progressive adaptations to accumulated ethanol. Changes in the lipid composition of yeast cell membranes have been observed in response to accumulated ethanol and have been proposed as an important factor involved in such adaptation (2, 7, 15).

The results of our investigations do not identify the cause for the decline in fermentative activity during batch fermentation but rather narrow the range of remaining factors. Although the activities of glycolytic and alcohologenic enzymes assayed in vitro under substrate-saturating conditions remained high during batch fermentation, the in vivo activities of these enzymes within the cell cannot be accurately predicted. The activities of some of these are subject to modulation by allosteric effectors in addition to constraints imposed by the availability of individual substrates, cofactors, and coenzymes. Further studies are now under way to explore the levels of these low-molecular-weight intracellular constituents.

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LITERATURE CITED

1. **Andreason, A. A., and T. J. B. Stier.** 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in defined medium. J. Cell. Comp. Physiol. **43**:271–281.
2. **Beavan, M. J., C. Charpentier, and A. H. Rose.** 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. J. Gen. Microbiol. **128**:1447–1455.
3. **Bernt, E., and I. Gutman.** 1971. Ethanol determination with alcohol dehydrogenase, p. 1499–1502. *In* H. U. Bergermeyer (ed.), Methods of enzymatic analysis, vol. 3. Academic Press, Inc., New York.
4. **Buttke, T. M., S. D. Jones, and K. Bloch.** 1980. Effect of sterol side chains on growth and membrane fatty acid composition of *Saccharomyces cerevisiae*. J. Bacteriol. **144**:124–130.
5. **Buttke, T. M., and A. L. Pyle.** 1982. Effects of unsaturated fatty acid deprivation on neutral lipid synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **152**:747–756.
6. **Cartwright, C. P., J.-R. Juroszek, M. J. Beavan, F. M. S. Ruby, S. M. F. De Morais, and A. H. Rose.** 1986. Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132**:369–377.
7. **Casey, G. P., and W. M. Ingledew.** 1986. Ethanol tolerance in yeasts. Crit. Rev. Microbiol. **13**:219–290.
8. **Casey, G. P., C. A. Magnus, and W. M. Ingledew.** 1984. High-gravity brewing: effects of nutrition on yeast composition, fermentative ability, and alcohol production. Appl. Environ. Microbiol. **48**:639–646.
9. **Clifton, D., S. B. Weinstock, and D. G. Fraenkel.** 1978. Glycolysis mutants in *Saccharomyces cerevisiae*. Genetics **88**: 1–11.
10. **Dombek, K. M., and L. O. Ingram.** 1985. Determination of intracellular concentration of ethanol in *Saccharomyes cerevisiae* during fermentation. Appl. Environ. Microbiol. **51**:197–200.
11. **Dombek, K. M., and L. O. Ingram.** 1986. Nutrient limitation as a basis for the apparent toxicity of low levels of ethanol during fermentation. J. Ind. Microbiol. **1**:219–225.
12. **Dombek, K. M., and L. O. Ingram.** 1986. Magnesium limitation and its role in the apparent toxicity of ethanol during yeast fermentation. Appl. Environ. Microbiol. **52**:975–981.
13. **Guijarro, J. M., and R. Lagunas.** 1984. *Saccharomyces cerevi-*

siae does not accumulate ethanol against a concentration gradient. J. Bacteriol. **160**:874–878.
14. **Harden, A.** 1923. Alcoholic fermentation. Longmans, Green and Co., New York.
15. **Ingram, L. O., and T. M. Buttke.** 1984. Effects of ethanol on micro-organisms. Adv. Microb. Physiol. **25**:253–300.
16. **Larue, F., S. Lafon-Lafourcade, and P. Ribereau-Gayon.** 1984. Relationship between the inhibition of alcoholic fermentation by *Saccharomyces cerevisiae* and the activities of hexokinase and alcohol dehydrogenase. Biotechnol. Lett. **6**:687–692.
17. **Leão, C., and N. van Uden.** 1982. Effects of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **24**:2601–2604.
18. **Leão, C., and N. van Uden.** 1984. Effects of ethanol and other alkanols on the general amino acid permease of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **26**:403–405.
19. **Leão, C., and N. van Uden.** 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **774**:43–48.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. **Maitra, P. K., and Z. Lobo.** 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246**:475–488.
22. **Millar, D. G., K. Griffiths-Smith, E. Algar, and R. K. Scopes.** 1982. Activity and stability of glycolytic enzymes in the presence of ethanol. Biotechnol. Lett. **9**:601–606.
23. **Moulin, G., H. Boze, and P. Galzy.** 1984. Inhibition of alcoholic fermentation. Biotechnol. Bioeng. **25**:365–382.
24. **Rottenberg, H.** 1979. The measurement of membrane potential and ΔpH in cells, organelles and vesicles. Methods Enzymol. **55**:547–569.
25. **Salmon, M.** 1984. Application of the technique of cellular permeabilization to the study of the enzymatic activities of *Saccharomyces cerevisiae* in continuous alcoholic fermentation. Biotechnol. Lett. **6**:43–48.
26. **Thomas, D. S., J. A. Hossak, and A. H. Rose.** 1978. Plasma membrane composition and ethanol tolerance in *Saccharomyces cerevisiae*. Arch. Microbiol. **117**:239–245.
27. **Welch, P., and R. K. Scopes.** 1985. Studies on cell-free metabolism: ethanol production by a yeast glycolytic system reconstituted from purified enzymes. J. Biotechnol. **2**:257–273.