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Nutrient limitation as a basis for the apparent toxicity of low levels of ethanol during fermentation

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SUMMARY

The rate of alcohol production (per mg cell protein) by *Saccharomyces cerevisiae* declines as ethanol accumulates during fermentation. The results of these studies indicate that this initial decline in activity is not due to the presence of ethanol or to growth in its presence. Nutrient limitation is proposed as a major factor responsible for the decline in fermentative activity during the early stages of fermentation.

INTRODUCTION

The rate of alcohol production per unit cell mass decreases substantially during fermentations by yeasts as ethanol accumulates [8,16,19]. This decrease has in most cases been attributed to the inhibitory effects of ethanol [1,2,6,12,13,15,18]. However, recent studies by Casey et al. [3,4] have provided evidence that nutrient limitation rather than ethanol accumulation is a major factor limiting the rate of fermentation during high gravity brewing. Our studies extend this hypothesis as a possible explanation for the initial decline in fermentative activity observed during the accumulation of low levels of ethanol in batch fermentations.

MATERIALS AND METHODS

Organism and growth conditions. The organism used in these studies was *Saccharomyces cerevisiae* strain KD2, a petite derivative of strain CC3 (generously supplied by G.G. Stewart, Labatt's Brewery, London, Canada). This organism was grown in complex medium (grams per liter): glucose, 200; peptone, 10; yeast extract, 5; adjusted to pH 5.0 with 2 M HCl. Cultures were maintained on solid medium containing 1.5% agar.

Fermentations were carried out at 30°C in spinner bottles designed for tissue culture and were vented with a water trap to allow the escape of carbon dioxide. Cultures (300 ml) were agitated at 150 rpm during fermentation. To prepare inocula, cells were transferred from a slant and incubated for 36 h at 30°C without agitation, diluted 1:40 into a spinner culture and grown for 12 h to an absorbance at 550 nm of 3.5 (1.3 mg/ml cell protein). This 12-h

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culture was diluted 1:100 as inoculum for batch fermentations. 'Conditioned broth' refers to broth in which cells have been allowed to grow for 12 or 24 h and removed by centrifugation.

Analytical methods. Cell mass was measured as absorbance at 550 nm using a Bausch and Lomb Spectronic 70 spectrophotometer. Total cell protein was determined using the method of Lowry et al. [11] as described by Layne [10]. Glucose was measured by the glucose oxidase procedure [17] using the Glucostat reagents supplied by the Sigma Chemical Company (St. Louis, MO). Ethanol was determined by gas chromatography as described by Goel and Pamment [7] and is expressed as percentage by volume. Fermentation rates were measured as carbon dioxide production at 30°C under a nitrogen atmosphere using a Gilson differential respirometer. The intracellular concentration of ethanol was measured as previously described [5].

Chemicals. Agar and complex medium components were obtained from Difco Laboratories (Detroit, MI). Glucose and biochemicals were purchased from the Sigma Chemical Company (St. Louis, MO). Gas chromatography supplies were obtained from Supelco (Bellefonte, PA).

RESULTS

Fig. 1 shows a typical fermentation profile of strain KD2. Nearly identical profiles were obtained in medium supplemented with Tween 80 (5 g/l), linoleate (45 mg/l) and ergosterol (30 mg/l), and using a pH stat to maintain pH at 5.0. Glucose conversion was essentially completed after 60 h under these conditions with the production of 13% ethanol. Cell protein stopped increasing after 24 h, although absorbance continued to rise for an additional 12 h period (not shown).

Using data from batch fermentations, we have computed the rate of alcohol production per mg cell protein over 1.6-h time intervals and have plotted these as a function of average accumulated ethanol (Fig. 2). The validity of these calculated fermentation rates was confirmed by manometric determinations using samples from batch fermentations with excellent agreement. The trends observed were similar for cells grown with and without lipid supplements and during growth in a pH stat (pH 5). The fermentative activity of cells exhibited a biphasic decline as a function of accumulated ethanol. An initial decline in fermentation rate occurred during the accumulation of 3.7% ethanol with a 50% loss of activity. This was followed by a more

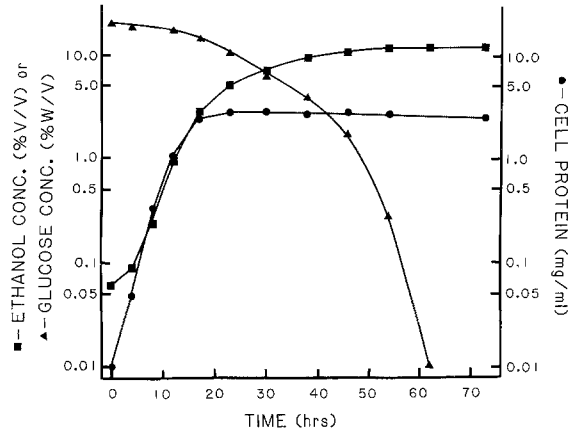

Fig. 1. Alcohol production by strain KD2 during batch fermentation with 20% glucose. ▲, glucose; ■, ethanol; ●, cell protein.

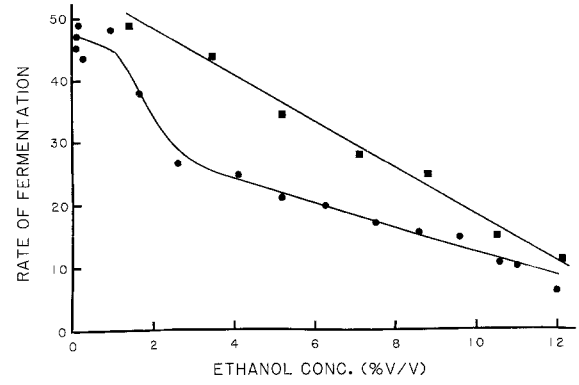

Fig. 2. Inhibition of fermentation by ethanol. Fermentative activity was determined from batch culture experiments and is plotted as a function of ethanol accumulated in the growth media. The rate of fermentation was calculated as the increase in ethanol over 1.6-h time intervals divided by the average cell protein concentration and is expressed as μmol ethanol produced/h per mg cell protein. The effects of added ethanol on the activity of cells from early in fermentation (12-h) are included for comparison, expressed as μmol of carbon dioxide produced/h per mg cell protein. ■, effect of added ethanol on 12-h cells; ●, batch fermentation with 20% glucose.

gradual decline in fermentation rate with approximately 20% of the original activity remaining in 12% ethanol.

Unlike ethanol accumulated during fermentation, the addition of low concentrations of ethanol to rapidly fermenting cells from 12-h old batch fermentations did not result in a large decline in fermentation activity (Fig. 2). No inhibition was observed up to 2% ethanol. Higher concentrations of ethanol caused a dose-dependent decline in activity. Fermentation was inhibited only 12% by the addition of 3.7% ethanol; 8.5% added ethanol was required to cause 50% inhibition.

We have focussed on two time points in batch fermentation to investigate the possible reasons for the initial drop in fermentative activity: 12-h and 24-h. To minimize possible variability arising from inocula, autoclaving, etc., we have operationally defined 12-h cells as those which have increased in cell mass 100-fold (as measured by absorbance at 550 nm). Typically, these have produced 1.2–1.3% ethanol and contain 1.3 mg cell protein/ml. Cells which have produced 5.0–5.6% ethanol (in addition to any ethanol which may have been present in the original medium) were operationally defined as 24-h cells. Typically, these contained 2.6 mg cell protein/ml.

Table 1 shows the effects of ethanol removal on the fermentative activities of 12-h and 24-h cells. The activity of 12-h cells was much higher than that of 24-h cells. Ethanol removal by resuspension in fresh broth had little effect on the activity of 12-h cells and did not result in an increase in the activity of the 24-h cells. Similarly, resuspension in conditioned broth from 12-h fermentations (containing 1.1% ethanol) did not affect activity. Resuspension of cells in the 24-h conditioned broth (containing 5.6% ethanol) reduced the fermentative activity of 12-h cells but appeared to have much less effect on the activity of 24-h cells. Removal of volatiles from the 24-h conditioned broth eliminated its inhibitory effect on fermentation in 12-h cells but did not result in a significant increase in activity of the 24-h cells. The addition of ethanol to the 24-h conditioned broth restored its ability to inhibit the activity of 12-h cells, indicating that ethanol was the

Table 1

Effects of ethanol and fermentation medium on the rate of fermentation

Cells from 12-h and 24-h batch fermentations were harvested by centrifugation at ambient temperature and resuspended to their original volume in various broths. Where indicated, volatiles were removed from conditioned broth by vacuum distillation at 55°C, reducing the volume by 2/3 and the broth was then reconstituted with distilled water or distilled water plus ethanol. Fermentation rates were measured with a Gilson differential respirometer. Averages and standard deviations (S.D.) represent the results from three separate batch fermentations.

Assay medium	Fermentation rate (μmol CO_2/mg protein per h (S.D.))	
	12-h cells	24-h cells
Original broth	36.3 (2.4)	16.5 (2.6)
Fresh broth	39.5 (2.3)	20.3 (2.5)
Conditioned broth (12-h, 1.1% ethanol)	38.9 (1.9)	22.7 (5.7)
Conditioned broth (24-h, 5.6% ethanol)	22.8 (0.6)	16.1 (2.0)
Conditioned broth (24-h, volatiles removed under vacuum)	34.9 (0.8)	17.6 (2.0)
Conditioned broth (24-h, volatiles removed under vacuum, reconstituted to give 5.6% ethanol)	21.9 (0.2)	15.7 (1.0)

principal volatile component responsible for this inhibition.

A trivial possibility for the failure of 24-h cells to recover activity after resuspension in broth lacking ethanol would be the presence of large numbers of dead cells. However, over 90% of these cells appeared active and intact based upon the exclusion of dye using the method of Trevors et al. [20]. Another trivial possibility for the failure of 24-h cells to recover after resuspension in fresh medium is that the internal ethanol was not effectively removed by this procedure. Prior to resuspension in fresh broth, the internal ethanol concentration of 24-h cells was 3.4% (S.D. 0.7) ethanol. After resuspension in fresh broth lacking ethanol, the internal concentration was 0.7% (S.D. 0.1) ethanol. This washed value was somewhat higher than expected based upon dilution and appears to have resulted from ethanol production during the brief resuspension and sampling period. The inclusion of 50 mM KF (a potent inhibitor of glycolysis) during resuspension in broth lacking ethanol resulted in an intracellular ethanol concentration of 0.06% (S.D. 0.01) ethanol, consistent with dilution.

In an analogous fashion, the failure of exogenously supplied ethanol to raise internal ethanol concentrations in 24-h cells to a level equivalent to that of cells during fermentative alcohol production could provide an explanation for the apparent resistance of 24-h cells to the inhibitory effects of added ethanol. After 48 h, batch fermentations contained approximately 11.5% ethanol. The intracellular ethanol concentration of these cells was 8.5% (S.D. 2.0). Resuspension of 24-h cells in broth containing 10% ethanol resulted in an intracellular ethanol concentration of 9.7% (S.D. 0.8). Although these values are not identical, they do indicate that the addition of ethanol to 24-h cells results in an increase in intracellular ethanol concentration similar to that of cells during batch fermentation.

We have examined the sensitivity of 12-h cells and 24-h cells to inhibition of fermentative activity by added ethanol (Fig. 3). The activity of 24-h cells was approximately one-half that of 12-h cells when assayed in fresh broth lacking ethanol. Both types of cells were resistant to ethanol concentrations up

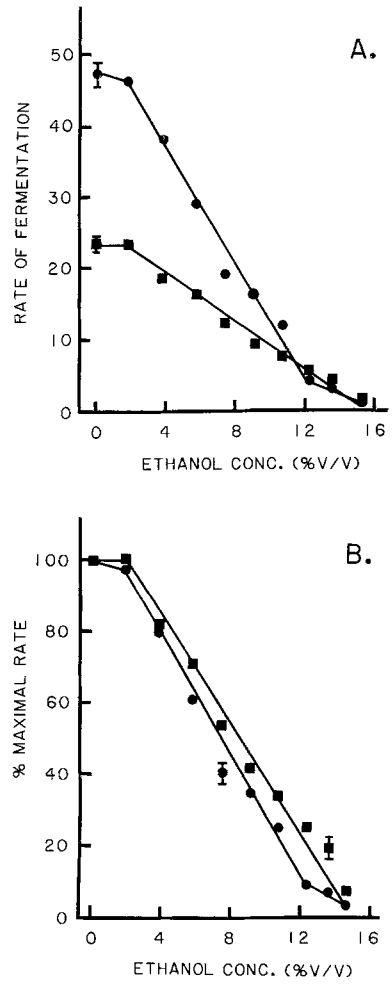

Fig. 3. Comparison of the inhibition of fermentation by added ethanol between 12-h and 24-h cells. Cells were harvested by centrifugation and resuspended in fresh medium containing various concentrations of ethanol. Fermentation rates are expressed as μmol of carbon dioxide produced/h per mg cell protein in A and as a percentage of the control rate lacking ethanol in B. The average standard deviation is indicated on each graph. ●, 12-h cells; ■, 24-h cells.

to 2% and exhibited a dose-dependent decline in activity to around 12% ethanol. When plotted as a percentage of maximal activity, 24-h cells appeared slightly more ethanol resistant, 50% inhibition at 8.3% ethanol as compared to 7.4% for 12-h cells.

The differences in sensitivity to inhibition by ethanol and the failure of ethanol removal to re-

store fermentative activity indicate that the reduced activity of 24-h cells is due primarily to physiological changes in the cells rather than a direct effect of ethanol. We have carried out several experiments to identify possible causes of the physiological changes which may be involved, summarized in Table 2. In these experiments, cells were grown under a variety of conditions, harvested by centrifugation at ambient temperature and resuspended in fresh medium lacking ethanol to measure fermentative activity under standard conditions. In all experiments, inoculation into fresh broth was included as a control.

Experiment 1 examined the possibility that the physiological changes in 24-h cells were due to growth in the presence of ethanol. Fermentations in which 5% ethanol was added prior to inoculation were examined. These were allowed to grow to the same cell mass as 12-h control cells. The fermentative activity of these cells grown in the presence of added ethanol was similar to that of control cells grown for 12 h in the absence of added ethanol, indicating that exposure to 5% ethanol during growth was not sufficient to cause the reduction in fermentative activity.

Experiment 2 was performed to examine the possibility that growth in the presence of ethanol along with other fermentation products is responsible for the reduction in fermentative activity. Cultures were inoculated into bottles containing filter-sterilized conditioned broth (from 12-h cultures, 1.2% ethanol; from 24-h culture, 4.5% ethanol) which had been supplemented with yeast extract (5 g/l) and glucose (to make 20%). These fermenta-

Table 2

Effects of growth in different broths on the fermentative activity of strain KD2

Experimental designs are described in the text. Fermentations were carried out in various media. Cells were harvested by centrifugation at ambient temperature and resuspended to original volume in fresh broth (20% glucose) immediately prior to the measurement of fermentative activity using a Gilson differential respirometer. N.D., not determined.

Experiment	Fermentation rate (μmol CO_2 evolved/mg protein per h (S.D.))		
	inoculum	12-h cells	24-h cells
1. Growth with added ethanol			
Control	48.5	50.7 (1.0)	N.D.
5% ethanol	—	44.5 (2.0)	N.D.
2. Inhibitor production			
Control	48.1	41.8 (0.1)	12.5 (0.1)
Conditioned broth (12-h, 1.2% ethanol, resupplemented with yeast extract and glucose)	—	40.7 (1.6)	14.8 (0.3)
Conditioned broth (24-h, 4.5% ethanol, resupplemented with yeast extract and glucose)	—	33.5 (1.2)	13.6 (0.2)
3. Nutrient limitation I			
Control	48.1	41.8 (0.1)	12.5 (0.1)
Conditioned broth (12-h, 1.2% ethanol, resupplemented with glucose alone)	—	Less than 5 doublings after 71 h	
Conditioned broth (24-h, 4.5% ethanol, resupplemented with glucose alone)	—	Less than 5 doublings after 71 h	
4. Nutrient limitation II			
Control	48.8	46.1 (0.6)	13.5 (0.2)
5X yeast extract	—	44.6 (1.3)	28.2 (0.4)

tions were allowed to proceed until the cells had undergone an increase in mass equivalent to the control. Cells grown in the supplemented 12-h conditioned broth were equivalent in activity to control cells. The activity of cells grown in the supplemented 24-h broth was lower but was at least twice that of the 24-h control. After allowing these fermentations to continue until 5% ethanol was produced (in addition to that present at the time of inoculation), the fermentative activities of both types of '24-h' cells were similar to that of control cells. Thus the decline in fermentative activity observed after approximately 24 h of active fermentation (production of 5% ethanol) is not due to the accumulation of ethanol and/or other stable inhibitors in the fermentation broth.

Next we examined the possibility that the decline in fermentative activity of cells which we observed after 24 h was due to nutrient limitation. Neither 12-h conditioned broth nor 24-h conditioned broth (resupplemented with glucose) supported the vigorous growth of strain KD2 following reinoculation (experiment 3). The addition of yeast extract restored the ability of condtioned broth to support growth with fermentative activity equivalent to the control (compare with experiment 2). Cells grown in broth containing 25 g/l of yeast extract (5-fold that of control broth) were equivalent in activity to control, cells after 12 h and were twice as active as control cells after the production of 5% ethanol, after approximately 24 h (experiment 4).

DISCUSSION

Previous studies have shown that the rate of alcohol production by yeast per unit cell mass decreases rapidly during the accumulation of ethanol ([8,16,19]; Fig. 2). Most of these studies have attributed this reduction in fermentative activity to adverse effects of ethanol [9,13–15]. Casey et al. [3,4] have recently shown that yeast nutritional requirements are a major limitation of fermentative activity in high gravity brewing and that supplementing worts with yeast extract and lipids substantially improves fermentation rates and reduces the times required to complete fermentation. Our studies using a yeast extract/peptone-based fermentation broth also illustrate this point and provide further support for the hypothesis that nutritional deficiencies rather than accumulated ethanol are primarily responsible for the initial decline in fermentation activity during the accumulation of low levels of ethanol.

The reduced activity of cells after the production of approximately 5% ethanol (24-h cells) appears to result from a combination of a small inhibitory effect of ethanol and physiological changes in the cells. These physiological changes in the cells were not induced by growth in the presence of 5% added ethanol or by growth in the presence of ethanol along with the other natural fermentation products. Conditioned broth was deficient in nutrients provided by yeast extract and supported very little yeast growth. The addition of 5 g/l of yeast extract restored the ability of this spent broth to support vigorous growth and fermentation. Further increasing the concentration of yeast extract (25 g/l) in growth medium partially prevented the decline in fermentative activity associated with the initial production of 5% ethanol. These results support the hypothesis that physiological changes in the cells as a result of nutrient limitation are major factors in the initial 50% decline in fermentative activity.

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REFERENCES

1 Aiba, S., M. Shoda and M. Nagatani. 1968. Kinetics of product inhibition in alcohol fermentation. Biotechnol. Bioeng. 10: 845–864.

2 Bazua, C.D. and C.R. Wilke. 1977. Ethanol effects on the kinetics of a continuous fermentation with *Saccharomyces cerevisiae*. Biotechnol. Bioeng. Symp. 7: 105–118.

3 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. Env. Microbiol. 48: 639–646.

4 Casey, G.P., C.A. Magnus and W.M. Ingledew. 1983. High-gravity brewing: Nutrient enhanced production of high concentrations of ethanol by brewing yeast. Biotechnol. Lett. 6: 429–434.

5 Dombek, K.M. and L.O. Ingram. 1986. Determination of the intracellular concentration of ethanol in *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. 51: 197–200.

6 Ghose, T.K. and R.D. Tyagi. 1979. Rapid ethanol fermentation of cellulose hydrolysate II. Product and substrate inhibition and optimization of fermentor design. Biotechnol. Bioeng. 21: 1401–1420.

7 Goel, S.C. and N.B. Pamment. 1984. Direct injection technique for gas chromatographic determination of ethanol and other volatiles in concentrated cell suspensions. Biotechnol. Lett. 3: 177–182.

8 Holzberg, I., R.K. Finn and K.H. Steinkraus. 1967. A kinetic study of the alcoholic fermentation of grape juice. Biotechnol. Bioeng. 9: 413–427.

9 Ingram, L.O. and T.M. Buttke. 1984. Effects of alcohols on microorganisms. Adv. Microb. Physiol. 25: 256–300.

10 Layne, E. 1957. Spectrophotometric and turbidometric methods for measuring proteins. Methods Enzymol. 3: 447–454.

11 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.

12 Luong, J.H.T. 1985. Kinetics of ethanol inhibition in alcohol fermentation. Biotechnol. Bioeng. 27: 280–285.

13 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. 4: 601–606.

14 Maiorella, B., H.W. Blanch and C.R. Wilke. 1983. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 25: 103–121.

15 Moulin, G., H. Boze and P. Galzy. 1984. Inhibition of alcoholic fermentation. Biotechnol. Bioeng. Rev. 2: 365–382.

16 Navarro, J.M. and G. Durand. 1978. Fermentation alcoolique: Influence de la temperature sur l'accumulation d'alcool dans les cellules de levure. Ann. Microbiol. Inst. Pasteur 129B: 215–224.

17 Raabo, E. and T.C. Terkildsen. 1960. On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12: 402–407.

18 Rahn, O. 1929. The decreasing rate of fermentation. J. Bacteriol. 18: 207–226.

19 Strehaiano, P. and G. Goma., 1983. Effects of initial substrate concentration on two wine yeasts: Relation between glucose sensitivity and ethanol inhibition. Am. J. Enol. Vitic. 34: 1–5.

20 Trevors, J.T., R.L. Merrick, I. Russell and G.G. Stewart. 1983. A comparison of methods for assessing yeast viability. Biotechnol. Lett. 2: 131–134.