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Magnesium Limitation and Its Role in Apparent Toxicity of Ethanol during Yeast Fermentation†

K. M. DOMBEK AND L. O. INGRAM*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

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The rate of ethanol production per milligram of cell protein begins to decline in the early stage of batch fermentation before high concentrations of ethanol have accumulated. In yeast extract-peptone medium (20% glucose), this initial decline appears to be related to growth and to result in part from a nutrient deficiency. The addition of yeast extract, peptone, and ashed preparations of these restored the ability of glucose-reconstituted medium (in which cells had been previously grown) to support vigorous growth. Magnesium was identified as the active component. Supplementing fermentations with 0.5 mM magnesium prolonged exponential growth, resulting in increased yeast cell mass. The addition of magnesium also reduced the decline in fermentative activity (micromoles of CO_2 evolved per hour per milligram of protein) during the completion of batch fermentations. These two effects reduced the time required for the conversion of 20% glucose into ethanol by ⅓ with no measurable loss in ethanol yield (98% of theoretical maximum yield). It is possible that some of the reported beneficial effects of complex nutrients (soy flour and yeast extract) for ethanol production also result from the correction of a simple inorganic ion deficiency, such as magnesium.

The rate of ethanol production by *Saccharomyces* spp. decreases in batch fermentations as alcohol accumulates in the medium (26, 35, 36). The onset of this decline in fermentative activity occurs at very low ethanol concentrations, often less than 3% (vol/vol). Since ethanol has been shown to accumulate to a level sufficient to inhibit fermentation (2, 4, 11), it has been generally accepted that this accumulation of ethanol is responsible for the progressive decline in fermentative activity (1, 9, 23). However, the extent of inhibition by exogenously added ethanol is less than would be predicted by the decline in fermentation rate which normally occurs during the fermentative accumulation of ethanol (30; Dombek and Ingram, J. Indust. Microbiol., in press).

Further studies have attempted to define the mechanism(s) of ethanol inhibition of fermentation and to reconcile the failure of added ethanol to inhibit fermentation to the extent observed during the fermentative accumulation of ethanol. Early studies provided evidence that the intracellular concentration of ethanol was much higher than that of the surrounding medium during fermentation (27, 28, 33), a condition not readily duplicated by the exogenous addition of ethanol. However, these early data can be explained by problems in the measurement of internal ethanol concentrations (7). Several research groups have developed independent methods which demonstrated that ethanol is freely permeable in *Saccharomyces* spp. and that the intracellular concentration of this metabolic product is essentially the same as that in the surrounding fermentation broth (6, 8, 12).

Additional studies have investigated the sensitivity of glycolytic enzymes and alcohologenic enzymes in vitro to inhibition by ethanol. Millar et al. (24) have shown that these enzymes are stable in ethanol concentrations higher than 20% (vol/vol). The two enzymes most sensitive to inhibition by ethanol were pyruvate decarboxylase and phosphoglycerate kinase. Both, however, retained 50% of maximal activity in the presence of over 12% (vol/vol) ethanol, the final alcohol concentration achieved by the complete fermentation of 200 g of glucose per liter. Similarly, Larue et al. (20) concluded that the cessation of alcohol production during stuck fermentations was not due to ethanol inhibition of alcohol dehydrogenase or hexokinase activities.

Casey et al. (3) have reported that nutrient limitation is a major factor in ethanol production during high-gravity fermentations. Anaerobically cultured yeasts are known to have a nutritional requirement for ergosterol and unsaturated lipids (14, 29, 34). Unsaturated lipids have been shown to increase cell yield, alcohol production, and ethanol durability of yeast cells during anaerobic fermentation (15, 16, 19, 37). A variety of lipid-protein complexes and nutrient supplements, ranging from albumin-ergosterol-monoolein to soy flour and yeast extract, have also been shown to yield increased rates of alcohol production and higher final ethanol concentrations (5, 13, 19, 31).

Recent studies in our laboratory have shown that the initial decline in fermentative activity during batch fermentation is not caused by the presence of ethanol or by growth in the presence of 5% (wt/vol) ethanol (Dombek and Ingram, in press). These studies indicated that a component(s) of yeast extract and peptone was limiting growth and contributing to the early loss of fermentative activity. The results presented in this article identify magnesium as the active component of these complex nutrients.

MATERIALS AND METHODS

Organism and growth conditions. The principal organism used in these studies was *Saccharomyces cerevisiae* KD2 (8), a petite mutant of strain CC3 (G. G. Stewart, Labatts Brewery, London, Canada). In addition, *S. cerevisiae* CC3, *S. cerevisiae* A10 (NRRL Y-12707), and *S. sake* (NRRL Y-11572) were also used for comparison in some experiments. The latter two strains were generously provided by N. J. Alexander (Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill.). All organisms were grown on YEPD medium, described by Leo and van Uden (22), which contained yeast extract (5 g/liter), peptone (10 g/liter), and glucose (200 g/liter). The medium was adjusted

* Corresponding author.
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to pH 5.0 with 2.0 N HCl prior to autoclaving. Solid medium for culture maintenance consisted of YEPD broth containing 1.5% agar.

Batch fermentations were carried out in 250-ml tissue culture spinner bottles (Bellco Glass, Inc., Vineland, N.J.), immersed in a 30°C water bath, and agitated at 150 rpm. Culture bottles were fitted with water-trapped exit ports for the escape of carbon dioxide and sampling ports for the removal of culture by syringe. Growth was allowed to proceed under conditions of self-induced anaerobiosis. Under these conditions, the addition of ergosterol and fatty acids as supplements did not affect the rate of fermentation (data not presented). Inocula were prepared by transferring cells from a slant to a test tube containing 10 ml of YEPD broth. Cells were incubated at 30°C for 36 h without agitation and diluted 1:40 into a 300-ml spinner bottle culture. The spinner culture was incubated for approximately 12 h until an optical density at 550 nm of 3.5 (1.3 mg of cell protein per ml) was reached. Fermentations were started by diluting this 12-h culture 1:100 into 300 ml of growth medium.

Preparation of fermentation samples for analysis. Fermentation samples were centrifuged at 10,000 × *g* for 0.5 min. The supernatant was removed and saved by freezing at −20°C. Cells were washed once in 50 mM KH_2PO_4 buffer (pH 5.0), and the pellets were saved for further analysis by freezing at −20°C.

Preparation of glucose-reconstituted medium for growth experiments. Batch fermentations were allowed to reach an optical density at 550 nm of 3.5. Cells were removed by centrifugation in a Sorvall RC-2B centrifuge at 10,000 × *g* for 2 min. The amount of ethanol in the supernatant was determined and used to estimate the amount of glucose needed to reconstitute the medium to a concentration of 20% (wt/vol). This glucose-reconstituted medium was sterilized by vacuum filtration with 0.45-μm Metricel membrane filters (Gelman Sciences Inc., Ann Arbor, Mich.).

Preparation of ashed medium components. Yeast extract (20 g) and peptone (30 g) were each burned over a gas burner for 5 h in a porcelain crucible. The crucibles were then transferred to a muffle furnace and ashed at 600°C for 72 h. The yeast extract ash was suspended in 40 ml of deionized water, and the peptone ash was suspended in 30 ml of deionized water. These aqueous suspensions of ash were adjusted to pH 5.0 with concentrated HCl and sterilized by autoclaving.

Nutrient supplementation growth experiments. Nutrient supplements were added to culture tubes (13 by 100 mm) containing 5 ml of fresh YEPD medium or glucose-reconstituted medium and a 1% inoculum (initial optical density at 550 nm of 0.035). Culture tubes were incubated at 30°C and agitated (30 rpm) in a Rototorque culture rotator (Cole-Parmer, Chicago, Ill.).

Medium analyses. Ethanol was measured by gas-liquid chromatography as described by Goel and Pamment (10) with 2% (vol/vol) acetone as an internal standard. Glucose determinations were done with a YSI model 27 glucose analyzer (YSI, Yellow Springs, Ohio). The magnesium concentration of the medium was measured with the 60 Second Magnesium reagents purchased from American Monitor Corporation, Indianapolis, Ind., as previously described (32).

Cellular analyses. Cell mass was measured as optical density at 550 nm with a Bausch and Lomb Spectronic 70 spectrophotometer and as total cell protein by the method of Layne (21). To determine the amount of intracellular magnesium, yeast cell pellets containing 1 to 3 mg of cell protein were permeabilized by incubation in a boiling-water bath for 1.5 min. The resulting debris was washed once with 1 ml of 50 mM KH_2PO_4 buffer (pH 5), and this wash was analyzed for magnesium.

TABLE 1. Effect of nutrient supplementation on growth of *S. cerevisiae* KD2

Medium	Supplement	OD_{550} after 48 h (SD)	% of control (SD)
YEPD	None	13.8 (1.5)	100
12-h[a]	None	1.25 (0.48)	9.1 (4.0)
12-h	Yeast extract (5 g/liter)	9.77 (0.75)	71 (9)
12-h	Peptone (10 g/liter)	7.43 (0.35)	54 (6)
12-h	Ashed yeast extract[b]	9.70 (0.40)	70 (8)
12-h	Ashed peptone[b]	3.47 (0.20)	25 (3)
12-h	Trace minerals[c]	1.88 (0.20)	14 (2)
12-h	KH_2PO_4 (7.3 mM)	1.49 (0.17)	11 (2)
12-h	$(NH_4)_2SO_4$ (7.6 mM)	1.60 (0.35)	12 (3)
12-h	$MgSO_4$ (2 mM)	12.7 (0.1)	92 (10)
12-h	$MgCl_2$ (2 mM)	12.1 (0.5)	88 (10)
12-h	$CaCl_2$ (2 mM)	1.55 (0.14)	11 (2)
12-h	Na_2SO_2 (2 mM)	1.62 (0.20)	12 (2)

[a] Medium isolated from a batch fermentation after 12 h of yeast growth and supplemented to 20% (wt/vol) glucose.
[b] An amount of ashed yeast extract equivalent to 5 g of whole yeast extract per liter or an amount of ashed peptone equivalent to 10 g of whole peptone per liter.
[c] As described by Wickersham (40).

Viable-cell determinations. Cell numbers were determined microscopically with a Petroff-Hausser counting chamber. Viable-cell counts were determined by the methylene blue staining procedure of Mills (25).

Respirometry measurements. Samples were loaded into Warburg flasks and equilibrated for 10 min at 30°C. During the first 5 min of the equilibration period, the flasks were flushed with nitrogen gas. Rates of CO_2 production were measured with a differential respirometer (Gilson, Middleton, Wis.). These values were used to calculate fermentation rates as micromoles of CO_2 evolved per hour per milligram of cell protein.

Chemicals. Yeast extract, peptone, and agar were obtained from Difco Laboratories, Detroit, Mich. Glucose and other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Magnesium sulfate and other inorganic salts were purchased from Fisher Scientific Company, Orlando, Fla. Absolute ethanol was supplied by AAPER Alcohol and Chemical Co., Shelbyville, Ky. Gas chromatography supplies were obtained from Supelco, Bellefonte, Pa.

RESULTS

Effect of nutrient supplements on growth in glucose-reconstituted medium. As shown in Table 1, fermentation broth in which strain KD2 had grown for 12 h supported very little further growth even after resupplementation with glucose (glucose-reconstituted medium). At this stage of fermentation (1.2% [wt/vol] accumulated ethanol), ethanol production was at its maximum (50 μmol/h per mg of protein). This time point also marked the end of exponential growth (data not shown), indicating either nutrient limitation or the presence of an inhibitor.

The addition of yeast extract and of peptone at the original medium concentration restored the ability of the used medium to support growth at 71 and 54% of the control level,

respectively (Table 1). These results indicate that nutrient limitation rather than the presence of an inhibitor was responsible for the inability of the used medium to support further yeast growth. Vitamin supplements were also tested and did not promote growth in this glucose-reconstituted medium (data not shown).

The organic components of yeast extract and peptone are both diverse and complex. Before embarking on a fractionation of these, the inorganic constituents were tested after ashing. Supplementation with ashed yeast extract was as effective as with whole yeast extract, while ashed peptone was only half as effective as whole peptone. These results suggested that an inorganic component of YEPD medium was the principal factor limiting growth.

The inorganic constituents of a mineral-based minimal medium were tested to determine which ions were limiting (Table 1). The addition of potassium, ammonium, sodium, calcium, phosphate, sulfate, and a trace mineral mixture described by Wickersham (40) did not promote growth in glucose-reconstituted medium. Only magnesium salts were effective as nutrient supplements, allowing growth equivalent to 90% of the control in fresh YEPD medium.

The dose response of growth to added magnesium, yeast extract, and ashed yeast extract is shown in Fig. 1. Yeast extract contained 27 μmol of magnesium per g. This value was used to calculate the appropriate amount of whole and ashed yeast extract to be added. Whole yeast extract, ashed yeast extract, and $MgSO_4$ gave similar dose responses. However, at concentrations below 0.2 mM, $MgSO_4$ appeared to be a better supplement. $MgSO_4$-supplemented fresh YEPD medium was also plotted for comparison. Maximum growth occurred at added magnesium concentrations above 0.2 mM. A $MgSO_4$ concentration of 0.5 mM was chosen for subsequent fermentation studies because growth at this concentration was no longer limited by an inadequate supply of magnesium.

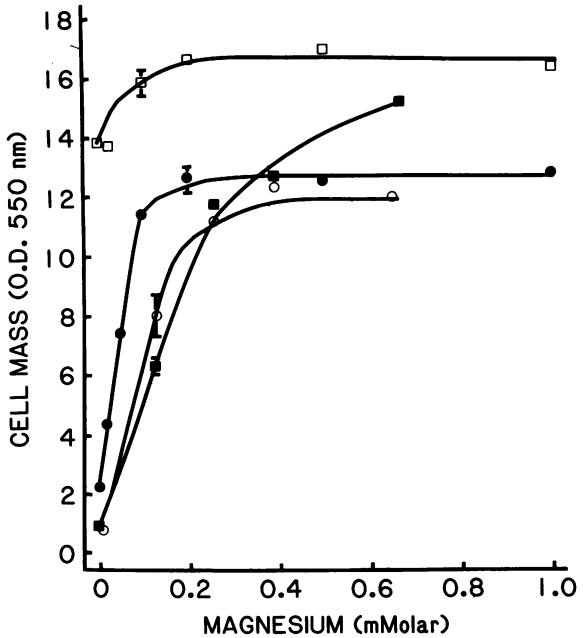

FIG. 1. Dose response of cell growth to added magnesium. Magnesium values represent the amount of magnesium contained in the added nutrient supplement. Error bars represent the average SD for each experiment. Symbols: ■, whole yeast extract added to glucose-reconstituted, used medium; ○, ashed yeast extract added to glucose-reconstituted, used medium; ●, $MgSO_4$ added to glucose-reconstituted, used medium; □, $MgSO_4$ added to fresh YEPD broth.

To confirm that magnesium was indeed limiting in YEPD medium, the magnesium content of cells and the surrounding broth was determined at various times during batch fermentation (Fig. 2). The magnesium content of the cells reached a maximum of 130 nmol/mg of cell protein at 12 h, rapidly declining to 48 nmol/mg of cell protein by 24 h and remaining at this lower level throughout the final period of fermentation. In the medium, the magnesium content fell to less than 0.05 mM by 24 h and remained constant until fermentation had been completed. Thus, the decline in magnesium content per milligram of cell protein observed after 12 h appears to result from continued cell growth after near depletion of the magnesium in the surrounding broth. Supplementing the broth with 0.5 mM magnesium resulted in the peak accumulation of higher levels of magnesium (200 nmol/mg of cell protein) at 12 h, followed by a decline to about 130 nmol of magnesium per mg of cell protein after 24 h. Magnesium-supplemented cultures maintained a higher level of cellular magnesium throughout fermentation than cultures grown in unsupplemented YEPD medium.

Magnesium limitation of growth of other strains. Three other strains were investigated to determine whether magnesium also limited their growth: *S. cerevisiae* CC3 (parent organism), *S. cerevisiae* A10, and *S. sake*. Glucose-reconstituted medium was prepared from each of these cultures. Cultures were inoculated into their respective glucose-reconstituted medium with and without added magnesium (0.05 mM) and incubated for 48 h on a rotator. *S. cerevisiae* CC3 and the strain of *S. sake* exhibited magnesium-dependent growth almost identical to that reported for strain KD2. The optical density at 550 nm after 48 h with added magnesium was 8.3 to 9.4, and 0.5 without. *S. cerevisiae* A10 grew poorly in its glucose-reconstituted medium, with an optical density at 550 nm after 48 h of 1.0 to 1.2 for both the control and supplemented cultures. All three strains reached a similar cell density in fresh YEPD medium (optical density at 550 nm of 14.2 to 15.2). These results indicate that the magnesium limitation observed in strain KD2 was not caused by the petite mutation and was not limited to strain CC3 and its derivatives. However, additional factors were clearly involved with *S. cerevisiae* A10.

Effect of magnesium supplementation on batch fermentation. The effects of supplementing YEPD medium with 0.5 mM $MgSO_4$ on batch fermentation are illustrated in Fig. 3. The production of cell mass as measured by cellular protein is shown in Fig. 3A. Supplementation with magnesium prolonged the exponential rise in cellular protein, allowing a 53% increase in cell mass over that of the control within 18 h after inoculation. The addition of magnesium also increased the rate at which glucose was consumed and ethanol was produced (Fig. 3B and C). After 30 h of incubation, magnesium-supplemented cultures had produced ⅓ more ethanol than the controls. The conversion of glucose to ethanol was complete after 48 h in magnesium-supplemented cultures, but required 72 h in control YEPD broth. The final yield of ethanol was essentially identical in both magnesium-supplemented and control cultures, 12.7% (vol/vol) (98% of theoretical maximum yield).

Effect of magnesium supplementation on rate of fermentation. Samples were removed from magnesium-supplemented and control fermentations at various times during batch fermentation. Ethanol concentration, cell protein, and CO_2

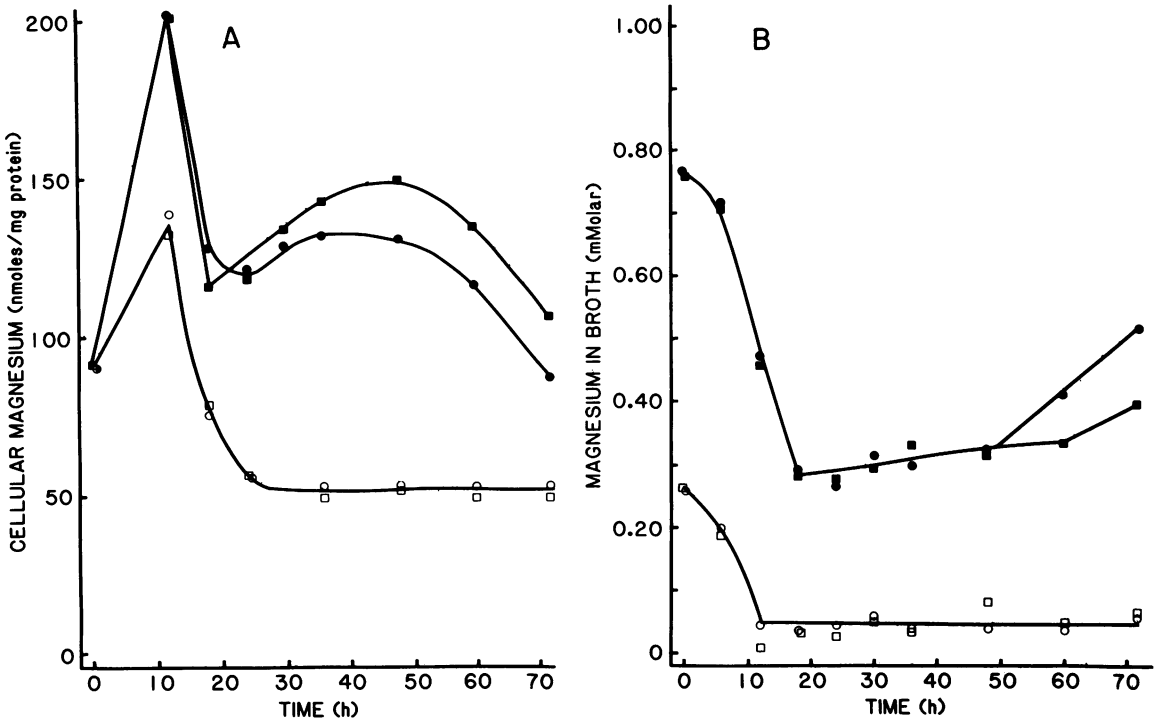

FIG. 2. Magnesium levels in broth and cells during the course of batch fermentation. (A) Intracellular magnesium; (B) magnesium concentration in the culture broth. Results have been plotted for four separate batch fermentations. Closed symbols represent fermentations supplemented with 0.5 mM $MgSO_4$, and open symbols represent control fermentations in YEPD broth alone.

evolution (unwashed) were measured. Figure 4 shows the fermentation rate (micromoles of CO_2 evolved per hour per milligram of cell protein) as a function of accumulated ethanol. Both control and magnesium-supplemented cultures exhibited the same maximum rate of fermentation at about 1% (vol/vol) ethanol. However, magnesium-supplemented cultures maintained a higher rate of fermentation as ethanol accumulated during the completion of the batch fermentation. This rate was 40% higher than that of control cells after the accumulation of 8% (vol/vol) ethanol. The fermentation rate of both supplemented and unsupplemented cultures fell precipitously at about 12.5% ethanol, coincident with the exhaustion of glucose.

Effect of magnesium addition on cell viability. The percentage of viable cells in both magnesium-supplemented and unsupplemented batches remained greater than 90% for the first 48 h of the fermentation. Glucose was exhausted at this time in supplemented cultures, and the percentage of viable cells began to decrease, reaching 58% by 72 h. The unsupplemented batches consumed glucose more slowly and maintained high viability (>90%) until between 60 and 70 h, the time at which glucose was exhausted.

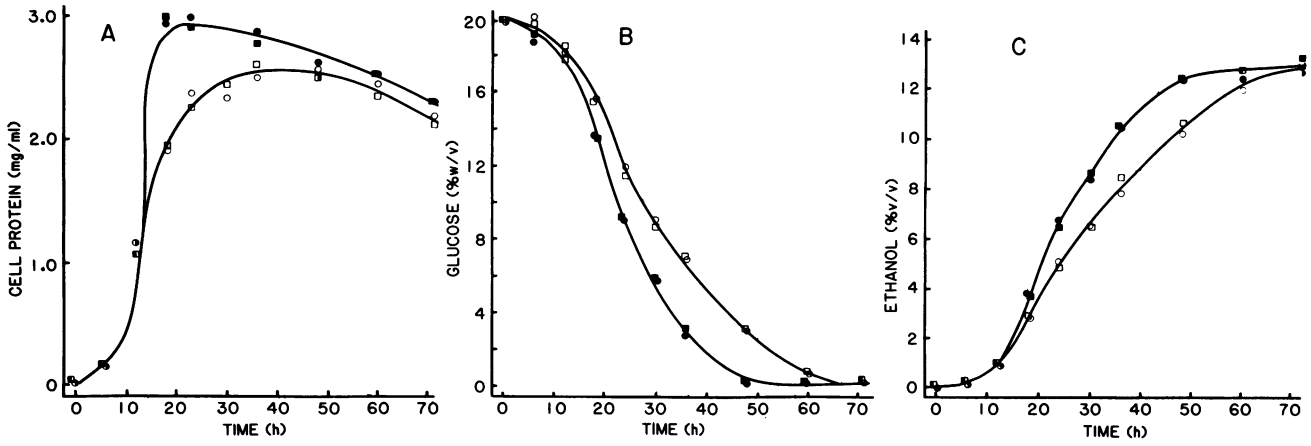

FIG. 3. Effect of magnesium addition on cell growth and fermentation. Results have been plotted for four separate batch fermentations. Closed symbols represent fermentations supplemented with 0.5 mM $MgSO_4$, and open symbols represent control fermentations in YEPD broth alone. (A) Growth; (B) glucose utilization; (C) ethanol production.

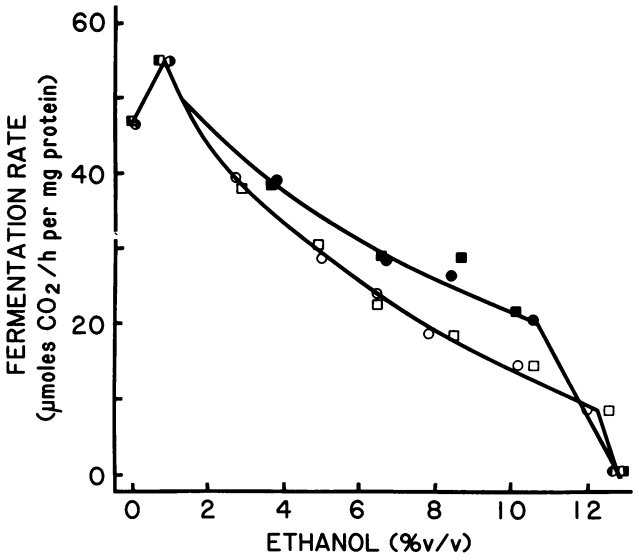

FIG. 4. Effect of added magnesium on the rate of fermentation. Fermentation rates were measured by respirometry of unwashed cells immediately after sampling and are plotted as a function of accumulated ethanol for four separate batch fermentations. Closed symbols represent cultures supplemented with 0.5 mM $MgSO_4$, and open symbols represent control fermentations in YEPD broth alone.

Effect of ethanol on the fermentation rate of magnesium-supplemented cultures. Two points during fermentation were chosen at which to compare cells grown with and without added magnesium. The first set of samples was taken from fermentations which had been allowed to accumulate 1.2% (vol/vol) ethanol (approximately 12 h). Cells from these samples were still undergoing exponential growth. The second set of samples was taken from fermentations which had been allowed to accumulate 5.5% (vol/vol) ethanol (approximately 24 h). At this point, cells were in early stationary phase.

Figure 5A shows the dose response of fermentative activity of the younger cells plotted as a function of total ethanol concentration (endogenous plus added). Magnesium-supplemented and unsupplemented cells had a similar initial fermentation rate, about 57 μmol of CO_2 evolved per h per mg of cell protein, and exhibited identical dose-response curves. A concentration of 7.6% (vol/vol) ethanol resulted in 50% inhibition of fermentative activity.

Figure 5B shows the effect of ethanol on the fermentation rate of the older cells. Supplemented cells had an initial fermentation rate of 27.4 μmol of CO_2 evolved per h per mg of cell protein, with a standard deviation (SD) of 0.5, while unsupplemented cells exhibited a significantly lower rate, 22.9 μmol of CO_2 evolved per h per mg of cell protein with an SD of 1.2. The fermentation rate of cells from supplemented cultures was always higher than that of control cells. The amount of ethanol present in the supplemented culture was 3.0% (vol/vol) higher than in the unsupplemented culture when compared at equal fermentation rates. The fermentation rate of magnesium-supplemented and control fermentations exhibited linear dose responses to ethanol. A measure of the sensitivity of fermentation rate to ethanol is the slope of the dose-response curve. The slope for supplemented batches was −0.17 with an SD of 0.02, while that of the controls was −0.14 with an SD of 0.01. The differences

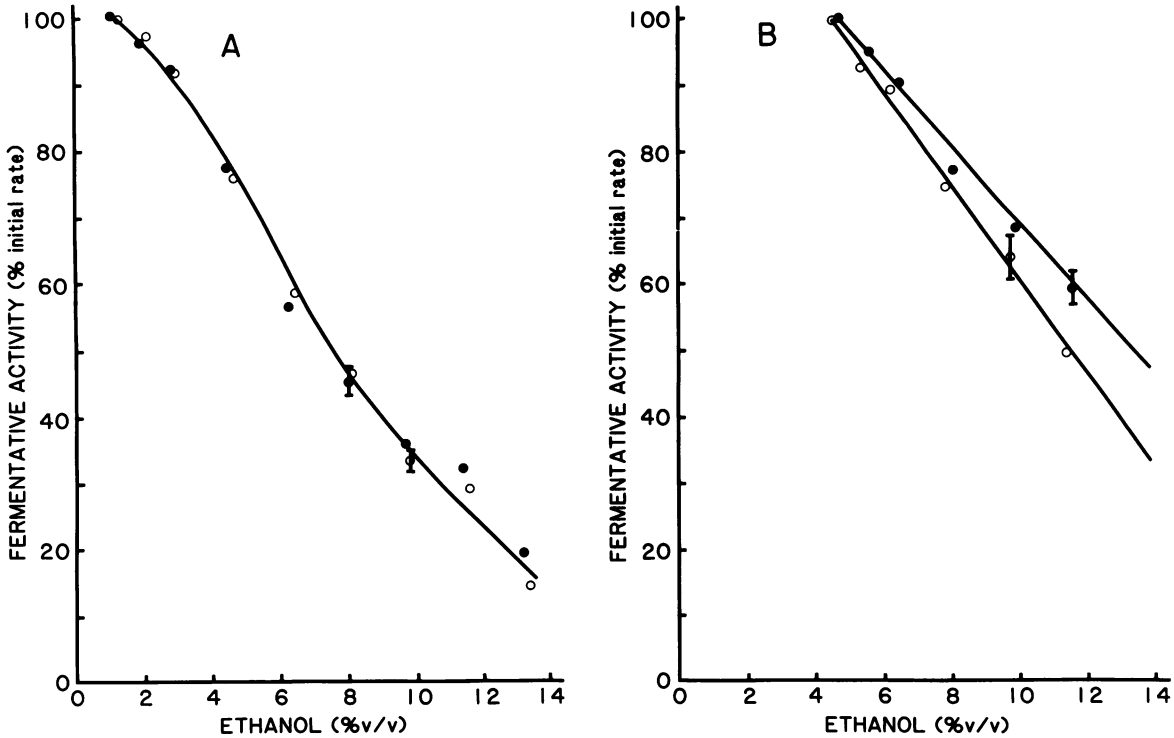

FIG. 5. Effect of magnesium supplementation on the inhibition of fermentation rate by added ethanol. Error bars represent average SDs for three separate determinations. (A) Samples from batch fermentations that had accumulated 1.2% (vol/vol) ethanol. (B) Samples from batch fermentations that had accumulated 5.5% (vol/vol) ethanol. Symbols: ●, 0.5 mM magnesium-supplemented fermentations; ○, control fermentations in YEPD broth alone.

in these slopes (Fig. 5A) are suggestive but do not conclusively demonstrate that supplementation with magnesium reduced the sensitivity of fermentation in older cells to inhibition by ethanol.

DISCUSSION

The decline in fermentation rate that begins at low alcohol concentrations does not seem to be caused by the acute presence of ethanol, by growth in the presence of ethanol, or by cell death (Dombek and Ingram, in press). This decline appears to be related in part to a magnesium deficiency, although other factors are also involved. In yeast extract-peptone-based medium, a magnesium deficiency is developed which limits cellular growth and the rate of carbohydrate conversion into ethanol. The addition of magnesium to batch fermentations prolonged exponential growth, allowing greater accumulation of cell mass without affecting cell viability. In addition, cells in magnesium-supplemented cultures maintained a higher fermentation rate as ethanol accumulated. These two factors, increased cell mass plus increased fermentation rate, combined to reduce the time required for the conversion of 20% glucose to ethanol by ⅓ in magnesium-supplemented cultures.

During batch fermentation, yeast cells concentrated magnesium from the medium. In unsupplemented cultures, magnesium uptake stopped at the end of exponential growth. At this point, the concentration of magnesium in the medium was 48 μM, within the range of K_m values reported for magnesium transport by microorganisms (17). The end of exponential growth also coincided with the beginning of the decline in fermentative activity. In magnesium-supplemented cultures, higher levels of intracellular magnesium were achieved early in fermentation and decreased to a lesser extent than observed in unsupplemented cultures. Magnesium-supplemented cultures had 6.5 times more magnesium in the medium at the end of exponential growth than did unsupplemented cultures. Thus, the magnesium supply of the supplemented culture appears to be adequate for growth, and other factors are limiting the fermentative ability of these yeasts under these conditions.

The ubiquitous role of magnesium in cellular processes is well documented (17). Magnesium constitutes a major portion of the cellular cations, mostly bound in structures such as ribosomes and the cell envelope. The free cation concentration, however, may play a more direct role in regulating overall cellular metabolism and cell division (39). Many of the enzymes that function in DNA replication, transcription, and translation require magnesium for activity. In fermentation pathways, magnesium is a required cofactor and nucleotide counterion in many reactions. Magnesium levels are typically maintained at millimolar intracellular concentrations, and it is not surprising that this cation is a limiting nutrient during high-gravity fermentations.

Previous studies in our laboratory (32) have demonstrated that the inhibition of fermentation by added ethanol in *Zymomonas mobilis* is primarily due to ethanol-induced leakage, particularly of magnesium, the cation required in the active form of the nucleotide cofactors for glycolytic enzymes. The addition of magnesium salts at 0.5 mM substantially reversed the inhibitory effects of up to 13% (vol/vol) ethanol. Although analogous studies have not been performed with *S. cerevisiae*, it is likely that ethanol also increases the leakage of small molecules in this organism.

Casey et al. (3) have also reported that nutrient limitation is an important factor in fermentation. Supplementation of high-gravity brewing wort (containing up to 31% dissolved solids) with yeast extract, ergosterol, and oleic acid allowed the production of 16.2% (vol/vol) ethanol by brewers' yeast. Higher rates of alcohol production resulted primarily from an increase in cell mass associated with nutrient-supplemented fermentations and did not appear to include an increase in the resistance of fermentation rate to ethanol. Viegas et al. (38) reported that nutrient addition to a yeast extract-based medium containing 30 to 40% glucose enhanced the rate of ethanol production by *Saccharomyces bayanus*. Their nutrient source was soy flour, which had previously been shown to increase the fermentative productivity of *S. cerevisiae* (5) and *Z. mobilis* (18). Again, supplementation led to an increase in cell concentration. Viegas et al. further demonstrated that the aqueous fraction of soy flour rather than the lipid fraction contained the components beneficial for fermentation. This aqueous fraction would have included inorganic ions such as magnesium. Indeed, it is possible that many of the complex nutrient additives used to increase ethanol production are also correcting an inorganic-ion deficiency.

The causes of the progressive decline in fermentative activity which are observed as ethanol accumulates during batch fermentation appear to be much more complicated than expected. Our results indicate that direct ethanol inhibition is only partially responsible. A nutrient limitation for magnesium also appears to be partly responsible. With abundant magnesium, only a 50% further increase in cell mass was observed, indicating that another factor(s) becomes limiting for growth and fermentation at this point. Indeed, a complete understanding of the biochemical basis for the decline in fermentation rate in yeasts may require determination of the factors responsible for the termination of exponential growth and the associated physiological and enzymatic changes.

ACKNOWLEDGMENTS

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