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Influence of Medium Buffering Capacity on Inhibition of *Saccharomyces cerevisiae* Growth by Acetic and Lactic Acids

K. C. Thomas, S. H. Hynes, and W. M. Ingledew*

Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8

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Acetic acid (167 mM) and lactic acid (548 mM) completely inhibited growth of *Saccharomyces cerevisiae* both in minimal medium and in media which contained supplements, such as yeast extract, corn steep powder, or a mixture of amino acids. However, the yeast grew when the pH of the medium containing acetic acid or lactic acid was adjusted to 4.5, even though the medium still contained the undissociated form of either acid at a concentration of 102 mM. The results indicated that the buffer pair formed when the pH was adjusted to 4.5 stabilized the pH of the medium by sequestering protons and by lessening the negative impact of the pH drop on yeast growth, and it also decreased the difference between the extracellular and intracellular pH values (ΔpH), the driving force for the intracellular accumulation of acid. Increasing the undissociated acetic acid concentration at pH 4.5 to 163 mM by raising the concentration of the total acid to 267 mM did not increase inhibition. It is suggested that this may be the direct result of decreased acidification of the cytosol because of the intracellular buffering by the buffer pair formed from the acid already accumulated. At a concentration of 102 mM undissociated acetic acid, the yeast grew to higher cell density at pH 3.0 than at pH 4.5, suggesting that it is the total concentration of acetic acid (104 mM at pH 3.0 and 167 mM at pH 4.5) that determines the extent of growth inhibition, not the concentration of undissociated acid alone.

The yeast *Saccharomyces cerevisiae* under aerobic conditions can use short-chain organic acids, such as acetic acid and lactic acid, as carbon sources. The process involves induction of certain anabolic pathways, enzymes, and specific transport mechanisms (2, 4, 5, 6, 17). If glucose is available in the growth medium, these pathways and permeases are repressed. Glucose-repressed yeast cells are unable to take up the anions of these acids (6), but the undissociated acids diffuse freely into the cells. Once inside, these acids dissociate because of the higher intracellular pH and cause acidification of the cytoplasm. Generally, eucaryotic cells maintain their intracellular pH within a narrow range despite wide variations that may occur in the extracellular pH (7). Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 (9) or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0 (8). To maintain the intracellular pH within a physiological range optimum for metabolism, the cells pump out protons at the expense of metabolic energy (ATP). Increased diversion of energy (ATP) to pump out protons results in decreased molar growth yield with respect to glucose ($Y_{glucose}$) (19, 24). It has also been reported that the Y_{ATP} (grams of biomass produced per mole of ATP generated) decreased from 14 to 4 when the concentration of acetic acid was increased from 0 to 170 mM (19). As the gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast.

Growth of the yeast is faster and the biomass produced is greater in media containing complex ingredients than is seen in minimal medium (15). Improved growth in complex media has been thought to be entirely due to the availability of greater amounts of nutrients, although it is now recognized that ingredients of complex media also play nonnutritional roles in promoting the growth and survival of yeast (1, 12, 22). Therefore, it is reasonable to expect that complex ingredients in media will continue to stimulate yeast growth, albeit to a lesser degree, even in the presence of organic acids, such as acetic acid or lactic acid. In other work, it was suggested that components such as yeast extract in yeast extract-peptone-dextrose broth offer some protection against stress conditions (15). No systematic study, however, has been carried out to prove whether complex-medium ingredients can protect yeast against the inhibitory effects of organic acids.

In the absence of good buffering, the pH of a growth medium is lowered when organic acids are added. The inhibitory effect of low pH on yeast growth is compounded by the presence of organic acids in the medium. First, lowering the pH increases the concentration of undissociated acid and thus enhances the inhibitory effect for a given (total) amount of acid. Second, since accumulation of organic acids is a function of the difference between the extracellular and the intracellular pHs (ΔpH) (2, 14), greater inhibition would be expected as the pH is decreased. It is not clear how much of the growth inhibition observed is due to low pH and how much is due to the level of undissociated acid. In this study, we report that the inhibition of yeast growth by acetic acid and lactic acid is a function of the pH and the buffering capacity of the medium and of the total amount of the organic acid added.

* Corresponding author. Mailing address: Department of Applied Microbiology and Food Science, Agriculture 3E08, University of Saskatchewan, 51 Campus Dr., Saskatoon, Saskatchewan, Canada S7N 5A8. Phone: (306) 966-5028. Fax: (306) 966-8898. E-mail: ingledew@sask.usask.ca.

MATERIALS AND METHODS

Yeast. An industrial strain of *S. cerevisiae* (obtained from Alltech Biotechnology Center, Nicholasville, Ky.) was used throughout the study. This strain is widely used for fuel alcohol production and is available as active dry yeast (superstart yeast). However, we obtained a slant culture of the yeast and ensured its purity by selection on yeast-peptone-dextrose agar. The yeast was kept at −70°C as a suspension in 20% glycerol.

Chemicals and growth media. Common chemicals were of reagent grade and were purchased locally. Yeast extract (AYE 2200; Gilette Foods Inc., Union, N.J.), corn steep powder (Marcor Development Corp., Hackensack, N.J.), and amino acids (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were used as nutrient supplements for yeast cultivation.

The minimal medium described by Wickerham (25) was used with minor modifications. The modifications included raising the concentrations of glucose and ammonium sulfate to 556 and 10 mmol per liter, respectively. The effect of nutrient supplementation on yeast growth was studied by adding yeast extract (10 g per liter), corn steep powder (10 g per liter), or a mixture of 18 amino acids. The following amino acids were included in the mixture: aspartic acid, glutamic acid, glycine, alanine, methionine, proline, arginine, cysteine, valine, threonine, leucine, isoleucine, tryptophan, phenylalanine, lysine, histidine, serine, and tyrosine. Concentrated solutions of these ingredients were sterilized separately by autoclaving them at 121°C for 15 min, and each was added to sterile minimal medium to give a final concentration of 1.8 mM. Where necessary, heat-sterilized solutions of acetic acid or lactic acid were added to sterile media to give the final concentrations described in the text.

Growth conditions. The yeast was grown by either batch or continuous cultivation. In the batch method, 100-ml quantities of medium in sidearm 250-ml Erlenmeyer flasks (Klett flasks) were inoculated with 0.5 ml of broth culture. The inoculum was grown for 24 h at 30°C in the minimal medium described above. The inoculated flasks were incubated at 30°C with shaking (200 rpm). Growth was followed turbidometrically using a Klett-Summerson colorimeter fitted with a red filter. In some cases, the pH of the sterile medium before inoculation was aseptically adjusted to 4.5 with 2 M KOH.

Continuous cultivation was carried out at 30°C and at a dilution rate of 0.15 h^{-1}. For this, a 1-liter Omni-culture fermentor (The Virtis Company Inc., Gardiner, N.Y.) was converted to operate in a continuous mode. The growth medium was pumped into the fermentor with a variable-speed peristaltic pump (model Piper-31; Fred A. Dungey Inc., Agincourt, Ontario, Canada). The yeast was grown in 600 ml of the minimal medium (the working volume) with or without added acetic acid. The pH of the culture was continuously monitored with a pH controller (model 169136; The Virtis Company Inc.) and maintained at predetermined values by automatic addition of 2 M KOH or 2 M HCl.

Analysis. Glucose, ethanol, acetic acid, and lactic acid were measured by high-performance liquid chromatography using a Waters (Milford, Mass.) chromatographic system. Supernatant portions of cultures obtained by centrifugation (10,300 × *g*; 15 min) were filtered through a Millipore membrane (0.22-μm pore size) and diluted with distilled water, and a sample (5 μl) was injected into an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, Calif.) maintained at 40°C. Deionized water (Milli-Q) containing sulfuric acid (5 mM) was used as the eluant. The elution rate was 0.7 ml per min, and boric acid was used as the internal standard. The separated components were detected with a Waters differential refractometer (model 410) and quantified with a Millennium^{32} Chromatography Manager computer program supplied by Waters Corp.

RESULTS

Yeast growth without pH adjustment. The yeast grew to only a limited extent in the control minimal medium (Fig. 1). On supplementation of the medium with yeast extract, corn steep powder, or the amino acid mixture, growth improved considerably. The growth response to corn steep powder was identical to that shown for yeast extract and therefore is not included in Fig. 1. The results seemed to indicate that the poor growth in the minimal medium was the result of nutritional deficiency. Although the supplements used here are excellent sources of nutrients, the buffering provided by these ingredients (see "Buffering capacity of growth medium" below) played an important role in stimulating yeast growth. The yeast failed to grow when acetic acid (167 mM) was incorporated into the medium (Fig. 1). None of the three nutrient supplements alleviated the growth inhibition caused by acetic acid. Lactic acid at a concentration of 548 mmol per liter inhibited yeast growth in a similar fashion (data not shown). As will be shown below, the amounts of acetic and lactic acid added to the medium were such that, at pH 4.5, they would provide identical concentrations (102 mM) of undissociated acid.

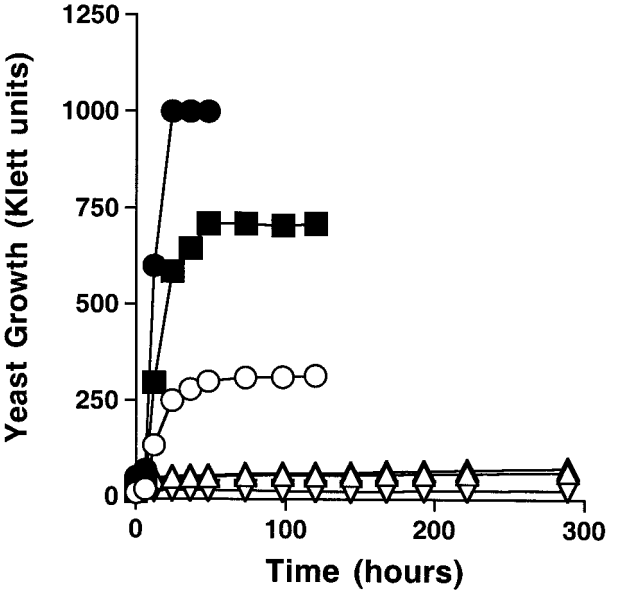

FIG. 1. Growth of *S. cerevisiae* in minimal medium with or without nutrient supplements and in the presence or absence of 167 mM acetic acid. ○, control; ●, 10 g of yeast extract per liter; ■, mixture of 18 amino acids, each at a final concentration of 1.8 mM; △, 10 g of yeast extract plus 167 mmol of acetic acid per liter; ▲, 10 g of corn steep powder plus 167 mmol of acetic acid per liter; ▽, amino acid mixture plus 167 mmol of acetic acid per liter. (Note that the solid triangles are almost hidden by the open triangles.)

The addition of acetic acid and lactic acid lowered the pH of the minimal medium to 2.76 and 2.12, respectively (Table 1). If

TABLE 1. Effects of various additives and initial pH values of media on final pH at the end of incubation with *S. cerevisiae*

Medium[a]	Initial pH not adjusted		Initial pH adjusted	
	pH at start	Final pH	pH at start	Final pH
Minimal medium	4.44	2.30	4.50	2.30
+ YE	4.99	2.55	4.50	2.30
+ CSP	4.05	2.52	4.50	2.69
+ AA	3.20	2.35	4.50	2.13
+ Acetic acid	2.76	2.80	4.50	4.32
+ Lactic acid	2.12	2.20	4.50	4.97
+ YE + acetic acid	3.39	3.50	4.50	4.32
+ CSP + acetic acid	3.39	3.50	4.50	4.42
+ AA + acetic acid	2.94	2.97	4.50	4.39
+ YE + lactic acid	2.45	2.48	4.50	4.92
+ CSP + lactic acid	2.46	2.46	4.50	4.89
+ AA + lactic acid	2.31	2.30	4.50	4.92

[a] YE, yeast extract (10 g/liter); CSP, corn steep powder (10 g/liter); AA, 18 amino acids, each at a final concentration of 1.8 mM. Corn steep powder contained 3.5% (wt/wt) lactic acid, and at the level used, it contributed 3.9 mmol of lactic acid per liter of medium. The added concentrations of acetic acid and lactic acid were 167 and 548 mM, respectively.

the medium contained nutrient supplements, such as yeast extract, corn steep powder, or a mixture of amino acids, the pH did not decrease to the same extent on addition of the acids. Growth inhibition was, however, still observed. The final pH values of media which contained either acetic acid or lactic acid were slightly elevated. This may be related to the fact that there was no yeast growth in these media (Fig. 1), and some loss of acids may have occurred through evaporation during the 12 days of incubation or through lysis of the yeast cells used for inoculation.

Yeast growth with pH adjustment. The initial pH of the medium and the presence or absence of either of the two acids seemed to determine whether the yeast grew or failed to grow. As shown in Table 1 and Fig. 1, the yeast grew if the initial pH of the medium was 3.2 or higher and if the medium did not contain either acetic acid or lactic acid. To study the effect of the initial pH on yeast growth, the pH of each medium listed in Table 1 was adjusted aseptically to 4.5, and then the medium was inoculated with a 24-h yeast culture prepared in minimal medium. Once again, the yeast grew to only a limited extent in the minimal medium (Fig. 2A). The nutrient supplements stimulated yeast growth. The addition of yeast extract reduced the lag period and increased the production of biomass. The unexpected observation was that if the initial pH of the medium was adjusted to 4.5, the yeast grew to a greater extent in the presence of 167 mM acetic acid or 548 mM lactic acid than in the absence of either acid. The lag phase in each case, however, was extended by about 6 h by these acids. Similar extended lag periods have been reported by Pampulha and Loureiro-Dias (19). Growth of the yeast in yeast extract-supplemented medium was marginally reduced by the presence of acetic acid or lactic acid in the medium. Similar results were obtained when corn steep powder was used as the nutrient supplement (Fig. 2B). Yeast growth in the medium supplemented with a mixture of amino acids was about the same as that in the medium that contained lactic acid (Fig. 2).

High-performance liquid chromatography analysis confirmed that the yeast used neither acetic acid nor lactic acid as a carbon source while glucose was still in the medium. However, within 48 h, 95 to 98% of the glucose was consumed in all media which contained supplements (results not shown). Moreover, as long as glucose remained in the medium, the yeast did not produce detectable quantities of acetic acid. This is in agreement with the observation that production of acetic acid by *S. cerevisiae* occurs only after the exhaustion of glucose from the medium (23), although other workers have reported "consecutive" production and reassimilation of acetate in media containing low concentrations of glucose (<0.35%) (20). In the present study, only 38% of the glucose was used in media which did not contain supplements. A greater proportion of glucose was converted to ethanol if the medium contained acetic acid and if the pH was adjusted to 4.5. This increased conversion was independent of the presence of nutrient supplements in the medium. On average, 19.8% more ethanol was produced when acetic acid was present in the medium than when it was absent (Fig. 3). The average conversion efficiency of glucose to ethanol in the absence of acetic acid was 72.6%, while in its presence the efficiency increased to 87%. It appears that in the presence of acetic acid, a greater proportion of glucose was diverted for the production of energy (ATP), and

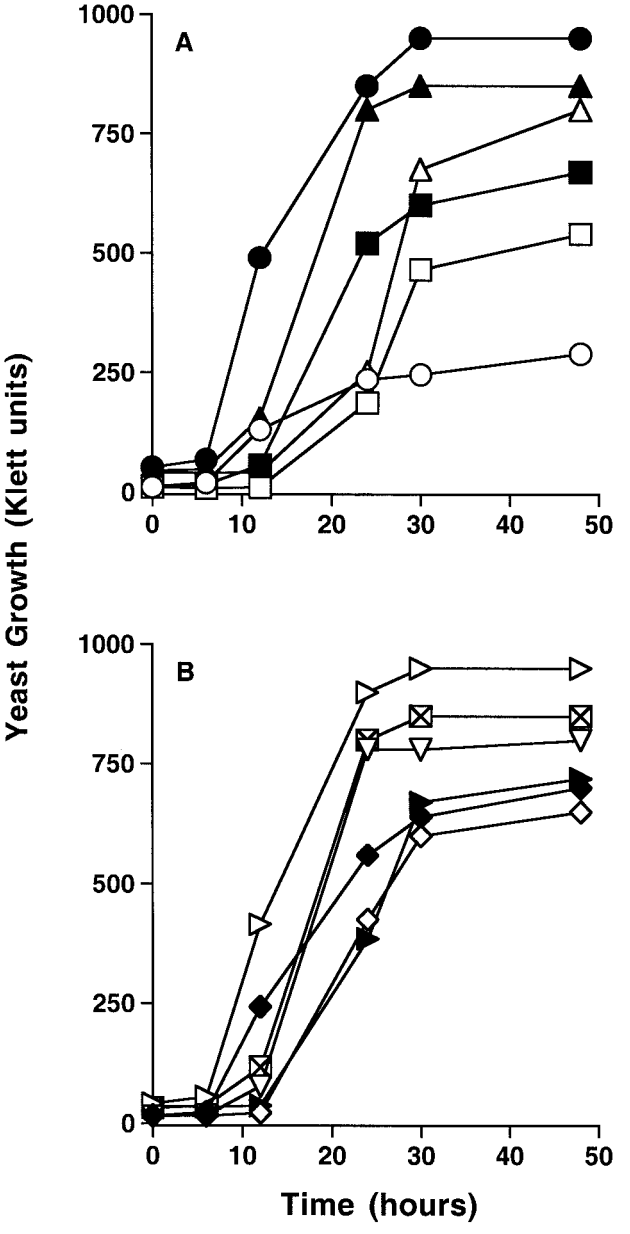

FIG. 2. Effect of adjusting pH to 4.5 on the growth of *S. cerevisiae* in media with or without 10 g of yeast extract per liter, acetic acid (167 mM), or lactic acid (548 mM) (A) and with or without a mixture of 18 amino acids (each at a final concentration of 1.8 mM), 10 g of corn steep powder per liter, acetic acid (167 mM), or lactic acid (548 mM) (B). ○, control; ●, yeast extract; □, acetic acid; ■, lactic acid; △, yeast extract plus acetic acid; ▲, yeast extract plus lactic acid; ◆, mixture of amino acids; ◇, mixture of amino acids plus acetic acid; ▽, mixture of amino acids plus lactic acid; ▷, corn steep powder; ▶, corn steep powder plus acetic acid; ☒, corn steep powder plus lactic acid.

this resulted in increasing the ethanol yield. Lactic acid did not have similar stimulatory effects on the conversion of glucose to ethanol. This suggested that the mechanisms by which these two acids inhibit yeast growth may be different, as reported by Narendranath et al. (16).

Buffering capacity of growth medium. Stimulation of yeast growth by acetic and lactic acids at pH 4.5 appeared most likely to be through the buffering action of these acids. The final pH

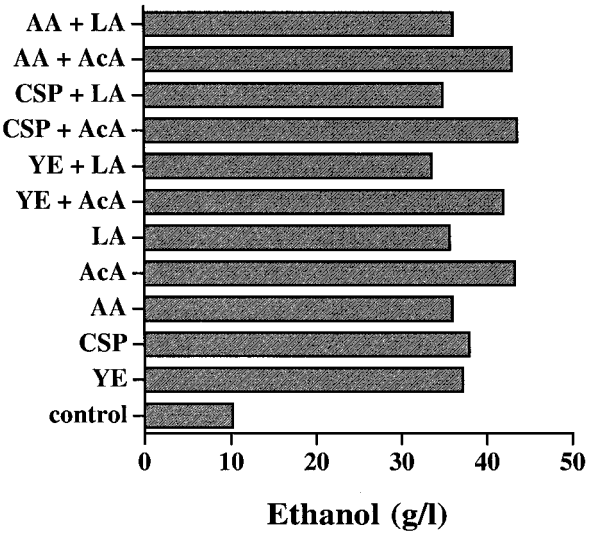

FIG. 3. Effects of various additions to minimal medium on ethanol production by *S. cerevisiae.* The minimal medium contained 100 g of glucose per liter. AA, 18 amino acids (each at a final concentration of 1.8 mM); LA, lactic acid (548 mM); AcA, acetic acid (167 mM); CSP, corn steep powder (10 g per liter); YE, yeast extract (10 g per liter).

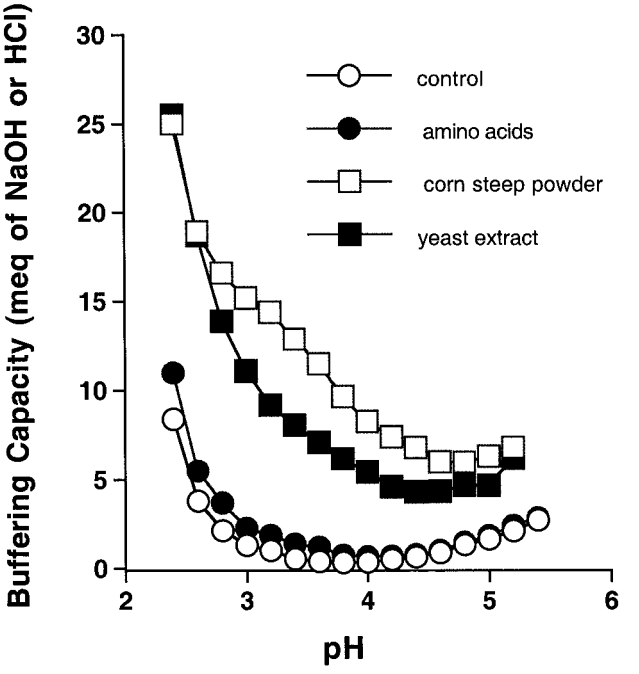

FIG. 4. Buffering capacities of minimal medium (control) and minimal medium with a mixture of 18 amino acids (each at a final concentration of 1.8 mM), with yeast extract (10 g per liter), or with corn steep powder (10 g per liter).

values of the fermented media that contained these acids did not drop to the same extent as in the controls (Table 1). In such cases, the final pH remained at 4.3 or higher. The final pHs of the cultures that received nutrient supplements, but not the acids, also decreased to relatively low values. Considering the amount of biomass produced in the presence of these nutrients, the yeast would have pumped large quantities of protons into the medium. Nutrient supplements likely sequestered some of these protons (buffering) before the pH became too low for the yeast to grow. This was verified by measuring the buffering capacities of the media. A 50-ml aliquot of each medium was titrated with 0.1 M NaOH or 0.1 M HCl, and the buffering capacity was calculated. Buffering capacity is defined here as the number of milliequivalents of NaOH or HCl required to shift the pH of 1 liter of the medium by 1 unit. As shown in Fig. 4, the minimal medium demonstrated very poor buffering in the pH range that was optimal for yeast growth. The buffering capacity of the minimal medium at pH 2.8 was 2.14 meq of NaOH, while that of the medium with amino acids was 3.73 meq (1.74 times more buffering capacity than that shown by the control). This increased buffering by amino acids was sufficient to stimulate growth significantly.

A reference point of pH 2.8 was chosen to compare the buffering capacities because below this value yeast growth begins to decline drastically. Yeast extract and corn steep powder, each at a concentration of 10 g per liter, increased the buffering capacity of the medium at pH 2.8 to 13.9 and 16.6 meq of NaOH, respectively (Fig. 4). The greatly improved growth with these nutrients in the medium (Fig. 1) is most likely related to these increased buffering capacities.

To test whether the reduced growth of the yeast in minimal medium was caused by the rapid lowering of the pH (lack of buffering), the yeast was grown under pH control. The pH of the batch culture during growth was continuously monitored and was controlled at 4.6 by automatic addition of 1 M KOH. The yeast grew to a greater extent (2.3-fold) in the minimal medium with pH control than in medium without it (Fig. 5). This suggested that it was the low pH itself and not the exhaustion of nutrients or the accumulation of toxic compounds that reduced the growth of the yeast. This was further confirmed by another approach. The yeast was grown in minimal medium without pH control until the culture was well into the stationary phase (96 h), and then the pH of the culture was adjusted to 4.6 aseptically with 1 M KOH. The culture was further incubated. As shown in Fig. 5, the culture grew to a much higher cell density during the second phase of growth. This suggested that the original medium was not deficient in nutrients and that toxic metabolic products did not accumulate to force the culture to enter the stationary phase. The sole reason for the reduced growth during the primary phase (no pH control) appeared to be the rapid drop in pH. The lack of a similar lowering of pH during the second phase (after the pH of the stationary-phase culture was adjusted to 4.6) was unexpected. As shown in Fig. 5, the pH during the second phase of growth did not decrease as fast as it did during the first phase. In addition, greater amounts of biomass were produced during the second phase. The improved growth and reduced drop in pH seem again to be related to the improved buffering provided by the culture. Since the original medium itself did not have appreciable buffering, the resistance to change in pH during the second phase must have been caused by some substance produced by the yeast during the first phase of growth and excreted into the medium. To test this, the yeast was grown in minimal medium without pH control for 96 h until the culture was well into the stationary phase. The culture was filtered through membrane filters (pore diameter, 0.45 μm).

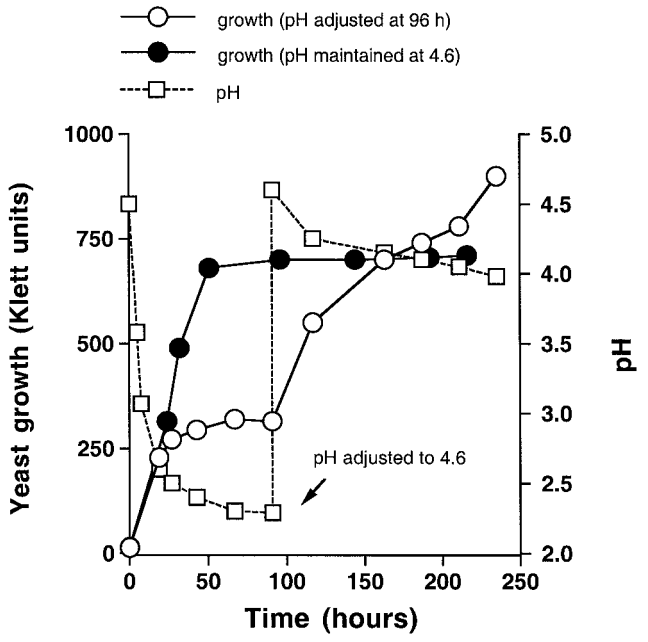

FIG. 5. Growth of *S. cerevisiae* in minimal medium during batch cultivation with pH controlled at 4.6 and without pH control until the culture reached stationary phase (96 h), at which time the pH of the culture was adjusted to 4.6 but not controlled. Changes in pH during growth of the yeast in minimal medium without pH control (pH) are also shown.

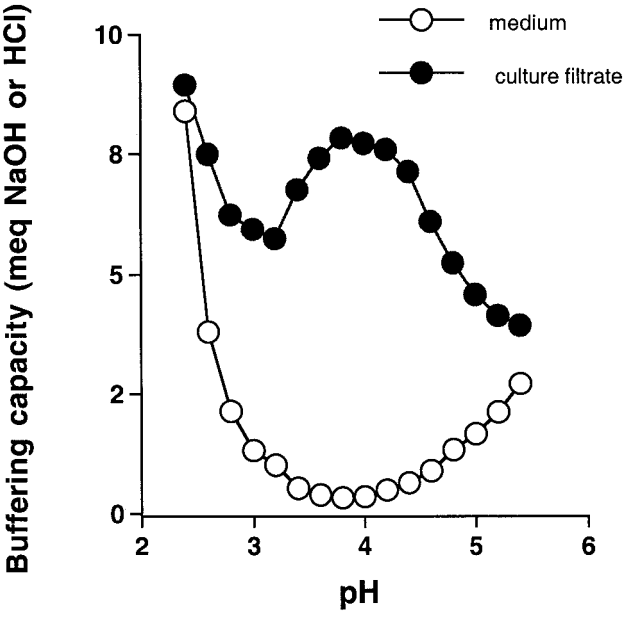

FIG. 6. Buffering capacities of the minimal medium and a culture filtrate recovered after *S. cerevisiae* was grown in minimal medium without pH control until the culture was in stationary phase.

Fifty milliliters of the filtrate was titrated with 0.1 M NaOH, and the buffering capacity was calculated. The culture filtrate had greater buffering capacity at all pH values than did the original minimal medium (Fig. 6). The pH of the culture had decreased to 2.32 during yeast growth, and at this pH both the culture filtrate and the minimal medium had relatively strong buffering. The culture filtrate contained a substance, or substances, which had a higher buffering capacity than did the minimal medium at pH values between 3.2 and 5.0. At present, the nature of this substance or substances is not known. Once the pH of the yeast culture (grown without pH control) was adjusted to a value in this range, the yeast was able to grow to a higher cell density. The reduced decrease in pH during the second phase of growth (Fig. 5) also seems to be related to the presence of the buffering substance(s) in the medium.

pH controlled growth in continuous culture. Although the mechanism of the inhibition of yeast growth by organic acids is well studied, not much is known about how that inhibition is affected by the pH of the medium itself. The yeast grew quite well at pH 4.5 and 4.0, but the growth declined gradually as the pH was lowered below 4.0 (Fig. 7A). The greatest reduction in growth occurred when the pH was lowered to 2.0 from 2.5. The presence of 167 mM acetic acid in the medium reduced the growth of the yeast at all pH values, and there was no growth when the pH was maintained below 3.0. With acetic acid in the medium, the cell density attained at pH 4.5 was only about half of that observed in the medium which did not contain the acid.

The volumetric productivity of ethanol in the absence of acetic acid in the medium was unchanged between pH 2.5 and 3.5, but at higher pH values, the productivity increased (Fig. 7B). In the presence of acetic acid, the productivity at pH 4.0 and 4.5 was about the same as that observed in media which did not contain the acid.

Undissociated acetic acid concentration and inhibition of yeast growth. Since only undissociated acid diffuses into glucose-repressed cells, growth inhibition is a function of the concentration of the undissociated acid in the medium. The pH of the medium directly influences the concentration of undissociated acid. To study the effects of pH and the concentration of undissociated acid on yeast growth, the yeast was grown at 30°C by continuous culture methods at a dilution rate of 0.15 h^{-1} and with pH control. The pH was held at 4.5 or at 3.0. As shown in Table 2, when the total concentration of the acid was 167 mM, the calculated (using a pK_a of 4.7 for acetic acid) concentration of undissociated acid was 102 mM at pH 4.5 and 164 mM at pH 3.0. The decreased growth at pH 3.0 was, therefore, the direct result of low pH and an elevated concentration of the undissociated acid (Table 2). However, when the concentration of the undissociated acid was lowered to the same level as at pH 4.5 (102 mM) by decreasing the total acid concentration to 104 mM, the yeast was not inhibited at pH 3.0 to the same extent as it was at pH 4.5. The expected result was that at equal concentrations of undissociated acid in the medium, the yeast growth would be inhibited to a greater extent at pH 3.0 than at pH 4.5. The only difference between the treatments (other than the respective pH values) was in the total concentration of the acid used. This suggests that the inhibition of yeast growth by acetic acid is not only a function of the concentration of the undissociated acid but also of the total concentration of the acid.

DISCUSSION

The results clearly show that the poor growth of *S. cerevisiae* in minimal medium was the result of a very rapid decrease of

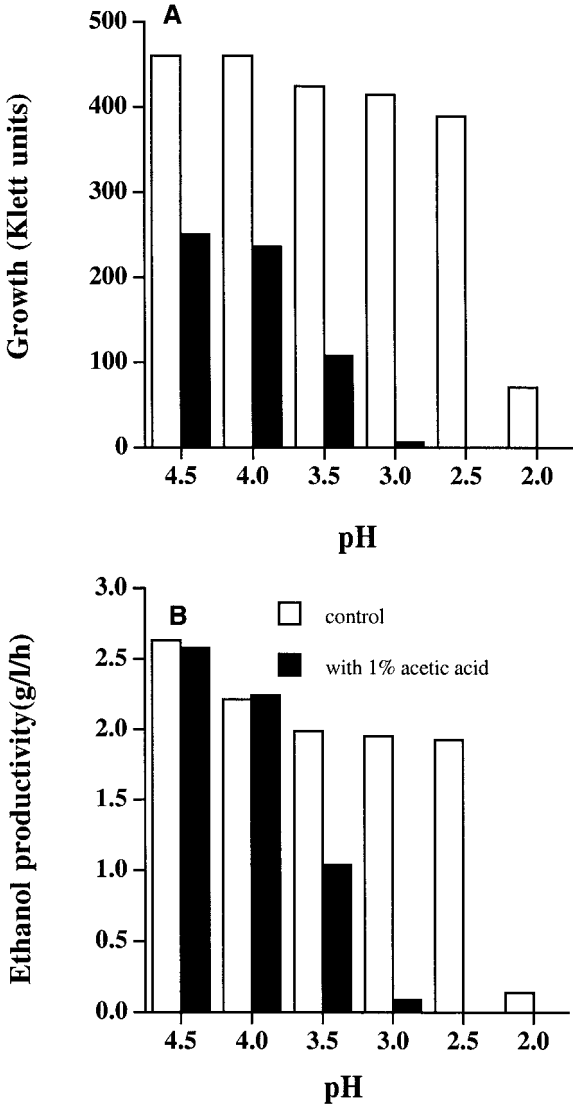

FIG. 7. Changes in growth (A) and ethanol productivity (B) at different pH values during continuous cultivation of *S. cerevisiae* at a dilution rate of 0.15 per h in minimal medium with and without (control) 167 mM (1%) acetic acid.

pH to values below 2.5, and it did not appear to be due to nutritional insufficiency. This was indicated by the fact that the yeast grew to much higher cell density in the same minimal medium if the pH was maintained at 2.5 or higher. The observed improvement of yeast growth in media supplemented with yeast extract, corn steep powder, or a mixture of amino acids also appeared to be due to maintaining the pH (buffering) above certain critical values below which growth was drastically reduced. It is possible that the greatly improved growth and fermentation performance seen on incorporation of soy flour (26) or other "yeast foods" (10) is due more to buffering than to yeast nutrition. The ability of the yeast to completely ferment wheat mashes in spite of their low free amino nitrogen content may also be related to buffering—in this case provided by soluble and insoluble wheat proteins (21). Apparently, the critical pH at which growth inhibition occurs is increased if

TABLE 2. Growth of *S. cerevisiae* during continuous cultivation ($D = 0.15\ h^{-1}$) in minimal medium at two pH values and at two different concentrations of added acetic acid

pH	Total acetic acid added (mM)	Undissociated acetic acid (calculated) (mM)	Steady-state biomass (Klett units)
4.5	0	0	460
4.5	167	102	250
4.5	267	164	312
3.0	0	0	410
3.0	104	102	340
3.0	167	164	6

carboxylic acids, such as acetic acid or lactic acid, are added to the medium (Fig. 7).

Growth of the yeast was completely inhibited if 167 mM acetic acid or 548 mM lactic acid was incorporated into the minimal medium. Neither the nutrients contained in yeast extract or corn steep powder nor the buffering these preparations provided was sufficient to protect the yeast against inhibition by the weak acids. This lack of protection may be related to the relatively high concentrations of the acids used. Inhibition of yeast growth by weak acids in media containing glucose is a function of the concentration of the undissociated acid (2, 5), which in turn is a function of the concentration of total acid (dissociated plus undissociated) and the pH of the medium. Acidification of cytoplasm, the primary cause of growth inhibition by weak acids, increases (i) with increasing concentrations of undissociated acid (18), (ii) through increased permeation of the acids at higher pH (3), and (iii) in proportion to the amount of ethanol in the culture (3).

The inhibition of yeast growth by acetic acid and lactic acid was alleviated considerably if the pH of the medium after addition of the acids was adjusted to 4.5. When the pH was adjusted, the yeast grew to a greater extent than those observed in minimal media which did not contain either of these acids (controls). This was unexpected. The likely reason for this improved growth was that in each case, the buffer pair formed by poising the medium at pH 4.5 buffered the medium and prevented the pH from dropping to inhibitory levels (Table 1). Such buffering was not available in the controls because the acids were not added to them. The yeast also grew in complex media containing these acids if the pH was adjusted to 4.5, although the maximum biomass produced was less than that observed in the medium that did not contain the acids. The total concentrations of acetic and lactic acids were such that the concentration of both undissociated acids at pH 4.5 was 102 mM. Even at this concentration, inhibition of yeast growth was not as severe as expected, although it is reported that diffusion of undissociated acetic and lactic acids into yeast cells increases with increasing pH and with increasing concentrations of undissociated acid (2, 3, 5). Inhibition of growth at pH 4.5 did not increase when the concentration of undissociated acetic acid was raised to 164 mM by increasing the total concentration of the acid to 267 mM (1.6% [wt/vol]). It has been reported by Pampulha and Loureiro-Dias (18) that the decrease in internal pH with increasing concentration of total acid in the external medium was less at high than at low pH values. Thus, the reduced inhibition of growth at increased total acetic acid concentration at pH 4.5 (Table 2) may be

related to a decreased acidification of cytoplasm. We suggest that the buffer pair formed internally resisted the decrease in pH (acidification of cytosol) and that this allowed the cells to survive and grow.

Raising the external pH has a number of effects on yeast metabolism. First, an external pH closer to the intracellular pH places less stress on cells, and less energy is wasted in maintaining the internal pH within a range optimal for growth. Second, if the media contain organic acids, raising the pH to a higher value would reduce the concentration of undissociated acid for a given amount of total acid and thus decrease its inhibitory effect on yeast growth. It has been suggested, however, that passive diffusion of undissociated acetic acid or lactic acid across the plasma membrane of *S. cerevisiae* is subject to opposing influences and that with increasing pH, the permeability of undissociated acids increases while their concentrations decrease (2, 5). Third, although undissociated acids enter cells by passive diffusion, their accumulation inside cells is still a function of the difference between external and internal pH values (ΔpH) (2, 14). One reason for the reduced inhibition by weak acids at higher pH values may be the smaller ΔpH. Fourth, the buffer pairs formed from organic acids when the pH is adjusted protect the yeast against a rapid decrease in pH. This explains why the acidification of cytoplasm with increasing concentrations of acetic acid is much less in adapted cells than in nonadapted cells (19). Adaptation may involve building up a sufficient concentration of the acid to create a buffer pair internally to resist pH change. It has been reported that anions formed through dissociation of carboxylic acids are retained within the cells (18) while protons are ejected through an energy (ATP)-coupled reaction involving plasma membrane ATPase. Potassium ions are taken up by yeast cells in exchange for hydrogen ions to maintain ionic stasis (11, 13). Depending on the ΔpH and the external concentration of the acid, the molarity of the buffer pair inside the cells at equilibrium can be very large (16), and this would enhance the internal buffering capacity. Reduced inhibition by acetic acid may therefore be related to maintenance of intracellular pH by internal buffering at a level that is close to optimum for growth. Less than expected growth inhibition at low total acetic acid concentration (in spite of the low pH) may be attributed to the depletion of undissociated acid or to a reduction in its concentration through its continued diffusion into yeast cells and subsequent dissociation inside the cells. As protons formed through dissociation of the acid are pumped out, more of the undissociated acid from the medium diffuses into the cells. This process will continue until the undissociated acid in the medium is exhausted or until the cells can no longer pump out protons and thereby prevent acidification of the cytoplasm. As more and more undissociated acid from the medium diffuses into the cells, more of the dissociated acid external to the cells is converted to undissociated acid to reestablish the equilibrium. These observations can be summarized as follows. First, the amount of undissociated acid that diffuses into cells and its equilibrium concentration inside the cells are functions of the external concentration of undissociated acid and the pH of the medium. Second, the equilibrium between the external and internal concentrations of undissociated acid will be reestablished if the undissociated acid inside the cells dissociates as a result of higher intracellular pH. If the dissociation continues, more and more undissociated acid will diffuse into the cells, and the amount taken up will be a function of the external concentration of the undissociated acid, which is, in turn, a function of the total acid concentration and the pH. The implication is that the magnitude of growth inhibition is a function of the total concentration of the acid even though only undissociated acid diffuses into the cells. Because of the dynamic equilibrium between undissociated and dissociated forms both outside and inside the cells and the passage of undissociated acids across the membrane, continued removal of one component of the equilibrium reaction (protons in this case) would shift the reaction to further dissociation, and the removal of undissociated acid from the medium would continue.

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