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Osmotic pressure effects and intracellular accumulation of ethanol in yeast during fermentation

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SUMMARY

The intracellular accumulation of ethanol in yeast and its potential effects on growth and fermentation have been topics of controversy for the past several years. The determination of intracellular ethanol based on the exclusion of [^{14}C]sorbitol to estimate aqueous cell volume was used to examine the question of intracellular ethanol accumulation. An intracellular accumulation of ethanol in *Saccharomyces cerevisiae* was observed during the early stages of fermentation. However, as fermentation continued, the intracellular and extracellular concentrations of ethanol became similar. Increasing the osmotic pressure of the medium with glucose or sorbitol was observed to cause an increase in the intracellular ethanol concentration. Associated with this was a decrease in yeast growth and fermentation rates. In addition, increasing the osmotic pressure of the medium was observed to cause an increase in glycerol production. Supplementation of the media with excess peptone, yeast extract, magnesium sulfate and potassium phosphate was found to relieve the detrimental effects of high osmotic pressure. Under these conditions, though, no effect on the intracellular and extracellular ethanol distribution was observed. These results indicate that nutrient limitation, and not necessarily intracellular ethanol accumulation, plays a key role during yeast fermentations in media of high osmolarity.

INTRODUCTION

The addition of ethanol to *Saccharomyces cerevisiae* cultures has been shown to be less toxic to the yeast cells than endogenous ethanol produced by the yeast [9,18]. The reasons for this have been proposed to be due to the build-up of toxic by-products [23], depletion of nutrients [5,9] and/or the intracellular accumulation of ethanol during fermentation [1,18,19]. Various research groups have reported the intracellular accumulation of ethanol in yeast during fermentation [1,16–18,20–22]. On the other hand, other reports suggest that the intracellular concentrations of ethanol in fermenting suspensions of yeast are less than or equal to those in the extracellular environment [8,10,12].

This laboratory [7] has recently reported the intracellular accumulation of ethanol in *S. cerevisiae*

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during the early stages of brewer's wort fermentation (< 6 h). As fermentation proceeds, the intracellular and extracellular ethanol concentrations become similar. Furthermore, the ratio of intracellular to extracellular ethanol was observed to increase with increasing osmotic pressure, although no adverse effect was observed on cell growth and fermentation rates. Similar results were observed when fermentations were conducted in synthetic media [16,21,22]. However, a decrease in growth and fermentation rates was associated with the increases in intracellular ethanol concentration observed with increasing osmotic pressure.

Preliminary results in this laboratory suggest that nutrient supplementation of synthetic media prevented the decrease in growth and fermentation rates observed at higher osmotic pressures. Recent studies have provided evidence that nutrient limitation rather than ethanol accumulation may be the major factor limiting the rates of fermentation at higher osmotic pressures [4,5,9]. This article examines the effect of osmotic pressure on intracellular ethanol accumulation and the role of nutrient limitation in fermentation.

MATERIALS AND METHODS

Chemicals

D-[U-^{14}C]Sorbitol (304 mCi/mmol) and tritiated water (5 mCi/ml) were obtained from Amersham (Amersham, U.K.). All other chemicals were obtained from commercial sources and were of the highest available purity.

Yeast strain

The yeast strain used in this study was a polyploid *S. cerevisiae* brewing ale strain with Labatt Culture Collection No. 3001.

Growth medium

The yeast cells were subcultured in PYN medium which consisted of: peptone, 3.5 g; yeast extract, 3.0 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; glucose, 100 g; all dissolved in 1 litre of distilled water and adjusted to pH 5.6. Fermentations were conducted in PYN medium containing varying concentrations of sugar and PYN components and were carried out at 30°C in a BioFlow Model C30 bench top fermentor (New Brunswick Scientific, NJ) containing 1.3 l of medium and with constant agitation of 150 rpm and constant aeration. Alternatively, fermentations were carried out at 30°C in 300 ml Erlenmeyer shake flasks containing 100 ml of medium. The inoculum used in all studies was 3.5 g wet weight of cells/l.

Cell sampling and determination of ethanol

At specified times during fermentation, 10 ml of cell suspension were withdrawn. Intracellular ethanol was determined using a modification of the perchloric acid extraction method described previously [7,21]. In this procedure, 1 ml samples of cell suspension were placed into Eppendorf tubes, in triplicate. A 0.1 ml sample from each suspension was withdrawn and added directly to a sample vial containing 0.1 ml ice-cold 0.58 M perchloric acid. The remaining suspensions were immediately centrifuged at 10000 × *g* for 1 min in a microcentrifuge. A 0.1 ml sample from each supernatant fluid was added directly to a sample vial containing 0.1 ml of 0.58 M perchloric acid. To the pellets was also added 0.1 ml of 0.58 M perchloric acid to liberate the intracellular ethanol. All samples were incubated at 4°C for 12 h. Perchlorate ions were precipitated by addition of 0.4 ml of 4 M KOH and incubation at room temperature for 15 min. The precipitates were removed by centrifuging at 10000 × *g* for 5 min. The extracts were quickly frozen for subsequent ethanol determination. As a control, known volumes of ethanol were subjected to perchloric acid treatment. Five millilitres of the remaining cell suspension were immediately centrifuged at 10000 × *g* for 10 min and the supernatant fluid was frozen for subsequent analysis.

Determination of intracellular aqueous volume

The yeast intracellular aqueous volume was determined by two methods. The first method was based on the procedures developed by Dombek and Ingram [10] and Guijarro and Lagunas [12]. The total aqueous volume of packed cells (interstitial

plus true cellular volume) was measured with tritiated water (^3H_2O). The interstitial volume of packed cells was measured with [^{14}C]sorbitol. To a 1 ml sample of cell suspension were added 20 μl of [^{14}C]sorbitol and 10 μl of ^3H_2O at a final concentration of 60.8 nCi/ml and 0.5 μCi/ml, respectively. The suspension was incubated at room temperature for 5 min, and 0.1 ml samples were pipetted directly into 10 ml of scintillation fluid. The remaining suspension was centrifuged and 0.1 ml samples of the supernatant fluid were pipetted directly into 10 ml of scintillation fluid. The cell pellet was solubilized by addition of 1.0 ml of 1% Triton X-100 and incubated at 30°C for 15 min. A 0.1 ml sample of this suspension was then pipetted directly into 10 ml of scintillation fluid. The intracellular concentration of ethanol was computed based on the aqueous cell volume, which was calculated by subtracting the interstitial volume (sorbitol-excluded volume) from the total aqueous volume, as described elsewhere [10,21].

In the second method, the total aqueous volume in the pellet was determined from the difference in wet and dry weights as described elsewhere [17]. The interstitial volume of the pellet was determined with [^{14}C]sorbitol as described above. The aqueous cell volume was calculated by subtracting the interstitial volume from the total aqueous volume. Both methods yielded similar results.

Biomass

Biomass during fermentation was determined by withdrawing 5 ml of suspension at various times, washing the cells twice with distilled water and weighing the pellet after drying at 100°C for 4 h.

Analytical methods

Ethanol was determined using a Carle AGC Series 100 gas chromatograph operating at 180°C having a 2.4 m long column packed with Poropak Q (81000 mesh) and a Hewlett Packard Integrator 3380A. Glucose, sorbitol and glycerol were determined using a Spectra-Physics SP8100 high performance liquid chromatograph (HPLC) incorporating a Bio-Rad oligosaccharide column (Aminex HPX-42A; 300 × 7.8 mm), a Micromeritics Model 771 refractive index detector and a Spectra-Physics SP4270 computing integrator.

RESULTS

Increases in osmotic pressure have been correlated with decreases in yeast growth and fermentation [2,13,14]. Thus, the effects of increasing sugar concentration on *S. cerevisiae* growth and fermentation were investigated. Table 1 summarizes the results obtained from fermentations conducted in the presence of various concentrations of glucose. These results indicate that there is a decrease in growth and fermentation rates, as well as the percent theoretical yield of ethanol, with increasing glucose concentration. Similar results were observed when the osmotic pressure of the media was increased with a non-fermentable sugar, such as sorbitol (Fig. 1). As in the case with glucose, a de-

Table 1

Effect of glucose concentration on *S. cerevisiae* growth and fermentation

Growth and fermentation rates were determined from the linear portion (exponential phase) of their respective plots. The percent theoretical ethanol yield was determined by dividing the actual amount of ethanol produced at the end of fermentation by the theoretical amount of ethanol expected based on the starting glucose concentration.

Glucose (g/l)	Growth rate (mg dry weight/ml per h)	Fermentation (mol ethanol/ml per h)	% Theoretical ethanol yield
100	0.33	54.0	93.5
200	0.24	52.7	66.4
300	0.11	42.5	59.0
400	0.03	14.2	23.6

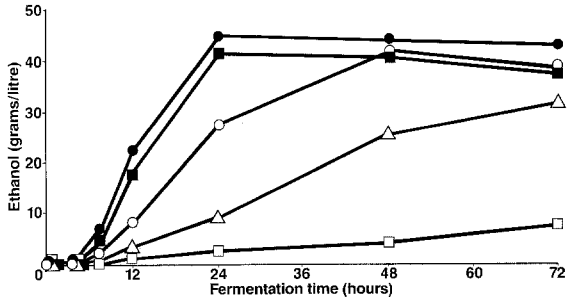

Fig. 1. Effect of sorbitol concentration on glucose fermentation. *S. cerevisiae* strain 3001 was grown on 10% glucose in the presence of 0% (●), 10% (■), 20% (○), 30% (△) and 40% (□) sorbitol. At various times during fermentation, 5 ml of cell suspension were withdrawn and immediately centrifuged at 10000 × *g* per min. Ethanol values were determined from the supernatant fluids as described in the Materials and Methods section.

crease in growth rates with increasing sorbitol concentration was also observed (data not shown).

It has been shown that increases in osmotic pressure result in increases in glycerol production by yeasts [3,15,21,24]. Fig. 2 illustrates such a response by *S. cerevisiae* to increasing glucose and sorbitol concentrations. Thus, a linear response of glycerol production was observed with increasing osmotic pressure. Similar observations were made when fermentations were conducted in wort [7].

The decrease in growth and fermentation rates observed at higher osmotic pressure have been attributed to the intracellular accumulation of ethanol [18,21]. To determine whether yeast cells accumulate ethanol under these conditions, the intracellular and extracellular concentrations of ethanol were determined during the course of fermentation. Furthermore, various concentrations of sugar in the media were employed to observe any effects due to increasing osmotic pressure. Table 2 demonstrates that ethanol does accumulate in the cells during the early stages of fermentation of 20 and 30% glucose. The highest ratio of intracellular to extracellular ethanol was observed after 3 h of fermentation and this value decreased during fermentation until the intracellular and extracellular concentrations were similar. Similar results were also observed with 10% glucose (data not shown). In addition, the results indicate that under higher osmotic pressure (higher sugar concentration), the ratio of intracellular to extracellular ethanol was greater than at lower osmotic pressure.

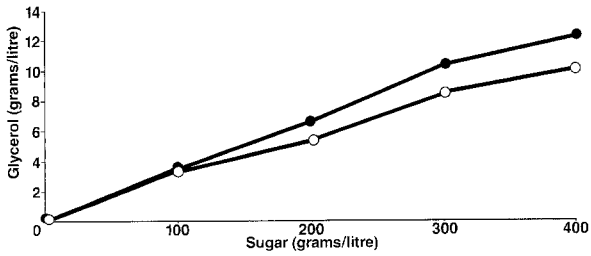

Fig. 2. Effect of sugar concentration on glycerol production. *S. cerevisiae* was grown on various concentrations of glucose (●) or on 10% glucose in the presence of various concentrations of sorbitol (○). Sugar concentration refers to total sugar (glucose plus sorbitol) added. After 96 h of fermentation, 5 ml of cell suspension were withdrawn and centrifuged at 10000 × *g* for 5 min. Glycerol concentrations were determined as described in the Materials and Methods section.

Table 2 also indicates that nutrient supplementation of the fermentation media results in an increase in ethanol production. However, no significant effect on the intracellular and extracellular ethanol distribution during fermentation could be observed. Furthermore, nutrient supplementation of the media results in an increase in biomass by the end of fermentation (Table 3), supporting the increase in ethanol production observed. These results suggest that nutrient limitation, not intracellular ethanol accumulation, is responsible for the decrease in fermentation activity at higher glucose concentrations.

Fig. 3A and B illustrates the fermentation of 20 and 30% glucose, respectively, by *S. cerevisiae* in the presence of increasing concentrations of peptone-yeast extract media components. An increase in ethanol production is observed with increasing nutrient supplementation. Increases in ethanol production from 40% glucose were also observed with increasing nutrient supplementation (data not shown).

In an effort to identify the active components in the peptone–yeast extract media responsible for increased fermentation activity, the various components were added individually. Fig. 4 demonstrates

Table 2

Distribution of intracellular and extracellular ethanol during fermentation

S. cerevisiae strain 3001 was grown on 20% and 30% glucose with increasing amounts of peptone–yeast extract media. After incubation for the indicated times, the intracellular (int) and extracellular (ext) ethanol concentrations were determined as described in the Materials and Methods section. Results represent the average of at least three trials.

Time (h)	% Ethanol (w/v)							
	20% glucose		20% glucose + 2× PYN		30% glucose		30% glucose + 3× PYN	
	int	ext	int	ext	int	ext	int	ext
3	2.03	0.09	2.49	0.12	1.5	0.06	1.58	0.06
6	1.15	0.41	1.68	0.38	0.62	0.16	0.54	0.15
12	1.87	1.78	3.02	2.69	0.7	0.48	0.53	0.41
24	3.58	3.45	8.1	6.8	3.18	3.14	2.28	2.55
48	n.d.[a]	n.d.	10.2	10.8	6.9	6.68	8.02	8.90
72	n.d.	n.d.	n.d.	n.d.	7.21	7.51	9.81	10.64

[a] n.d. = not determined.

that increasing the magnesium sulfate and/or the potassium phosphate concentration in the media resulted in a significant increase in ethanol production during the fermentation of 20% glucose. No effect was observed upon increasing the ammonium sulfate concentration. Supplementation with additional yeast extract or peptone resulted in the largest increase in yeast growth and fermentation activity (data not shown). In fact, complete fermentation of glucose was achieved under these conditions.

Table 3

Effect of glucose concentration on biomass

S. cerevisiae was grown on 20% and 30% glucose with increasing amounts of peptone–yeast extract media. After incubation for the indicated times, the biomass was determined as described in the Materials and Methods section. The results are the average of at least three trials.

Time (h)	Biomass (mg dry weight of cells/ml)			
	20% glucose	20% glucose + 2× PYN	30% glucose	30% glucose + 3× PYN
0	1.0	1.0	1.0	1.0
3	1.3	0.9	1.5	1.0
6	2.7	1.0	2.1	1.2
12	4.4	2.4	2.9	1.5
24	4.7	4.6	4.2	3.6
48	4.3	7.3	4.6	6.5
72	4.1	8.7	4.4	5.8

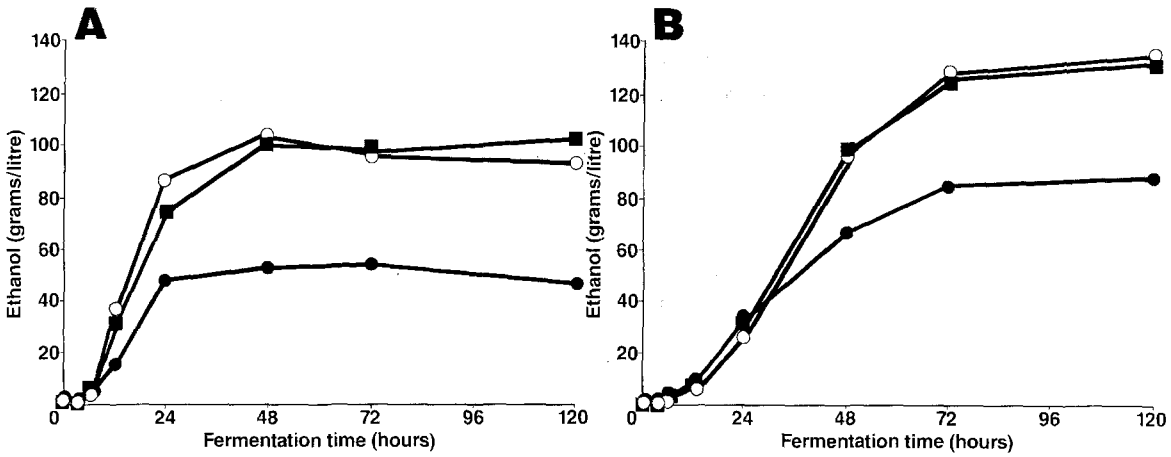

Fig. 3. Effect of increasing peptone–yeast extract media components on glucose fermentation. *S. cerevisiae* was grown on (A) 20% glucose, and (B) 30% glucose in the presence of 1× (●), 2× (■) and 3× (○) PYN. Ethanol was determined as described in the Materials and Methods section.

DISCUSSION

During the alcoholic fermentation by brewer's yeast strains, an increase in substrate concentration generally results in increased metabolic activity. However, at very high concentrations of solute, the effect of osmotic pressure on the cells becomes more pronounced, resulting in decreases in yeast cell growth and fermentation [2,13,14,21]. Such observations were confirmed when fermentations were conducted with *S. cerevisiae* in the presence of increasing concentrations of glucose (Table 1) or sorbitol (Fig. 1). However, with increasing concentrations of carbohydrate in wort no effect was observed [7]. It should be noted, though, that 20 degree Plato wort, which is approximately equivalent to 20% sugar, was the highest concentration employed and therefore the osmotic pressure may not have been great enough to affect fermentation. Increases in osmotic pressure by addition of a sugar adjunct to wort resulting in original gravities in excess of 30 degree Plato would most likely result in decreases in cell growth and fermentation.

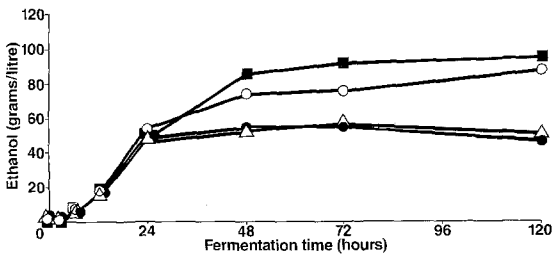

Fig. 4. Effect of nutrient supplementation on glucose fermentation. *S. cerevisiae* was grown on 20% glucose in the presence of 1× PYN (●) and 1× PYN plus 3× magnesium sulfate (■), 1× PYN plus 3× potassium phosphate (○) and 1× PYN plus 3× ammonium sulfate (△).

An intracellular accumulation of ethanol in *S. cerevisiae* was observed during the early stages of fermentation (3 h). As fermentation proceeded, the ethanol diffused out through the yeast cell membrane so that by 12 h of fermentation the intracellular and extracellular concentrations of ethanol were similar (Table 2). The intracellular accumulation of ethanol in yeast cells has been the topic of considerable controversy for the past several years [6]. It has been proposed by several research groups that ethanol does accumulate in yeast cells during fermentation and that this may be a factor contributing to the toxic effects of ethanol [1,7,16,18,19,21]. Other reports suggest that the intracellular concentration of ethanol in fermenting suspensions of yeast are less than or equal to those in the extracellular environment [8,10,12]. These differences in the results from the various studies have been attributed to inaccuracies in the techniques employed to measure

intracellular ethanol concentration. However, in this and previous investigations from this laboratory [7], the method of Dombek and Ingram [10] for the determination of intracellular ethanol has been employed. This method avoided many of the technical problems encountered in other studies, such as washing the cells or breaking them open. Furthermore, full recovery of known standard volumes of ethanol were obtained with the use of this method (data not shown).

Associated with the increase in osmotic pressure and decrease in cell growth and fermentation is an increase in the ratio of intracellular to extracellular ethanol (Table 2). This is in agreement with the previous observations from this laboratory in which sorbitol was employed to increase the osmotic pressure [21]. Similarly, we have recently demonstrated that an increase in wort carbohydrate content resulted in an increase in intracellular ethanol accumulation, although no effect on cell growth and fermentation was observed [7]. Increases in osmotic pressure have also been correlated with increased glycerol production to counteract the effect of the higher osmotic pressure [15,24]. In support of this observation, increasing the glucose or sorbitol concentration in the media resulted in an increase in glycerol production (Fig. 2). A similar response was previously observed to increasing the carbohydrate concentration in wort [7].

The results indicate that the increase in intracellular ethanol at higher osmotic pressures may contribute to the decreased yeast growth and fermentation activities. However, other factors such as nutritional requirements and accumulation of toxic by-products (such as octanoic and decanoic acids) at higher osmotic pressures may also have a contributing role [5,9,23]. The data presented in Fig. 3 demonstrate that supplementation of the media with increasing amounts of peptone–yeast extract media components results in an increase in fermentation activity of both 20 and 30% glucose. In fact, virtually complete fermentation was achieved. Associated with this increase in fermentation activity was also an increase in cell growth (Table 3). However, under these conditions of nutrient supplementation, no effect on intracellular and extracellular ethanol distribution was observed compared to standard growth conditions (Table 2). The increase in biomass observed at the same glucose concentration after nutrient supplementation was not evident until 48 h of fermentation. This would indicate that nutrient limitation has a critical role in influencing the growth and fermentation rates of yeast. Furthermore, this provides strong evidence that nutrient limitation, and not necessarily the intracellular accumulation of ethanol or by-product accumulation, is responsible for the observed decreases in growth and fermentation activities of yeast at higher osmotic pressures. In support of this, it has recently been shown that nutrient limitation is the major factor responsible for the decline in fermentative activity during the early stages of fermentation in yeast [9]. Studies on the effect of nutrient supplementation on glucose fermentation in the presence of increasing sorbitol concentrations are currently in progress.

When some of the peptone–yeast extract media components were tested separately, it was found that addition of increasing amounts of magnesium sulfate and potassium phosphate resulted in increased fermentation activity (Fig. 4). Peptone and yeast extract were observed to give the maximum effect (data not shown). No effect was observed with increasing concentrations of ammonium sulfate. Magnesium has recently been identified as playing a key role in relieving ethanol toxicity during yeast fermentation [11]. This may be related to the requirement of some of the glycolytic enzymes for magnesium. The increase in fermentation activity observed with potassium phosphate may be related to the increased buffering capacity of the media, since replacement with increasing concentrations of sodium phosphate also increased fermentation activity (data not shown). We are currently in the process of identifying the active component(s) in peptone and yeast extract which is responsible for increasing fermentation activity, as well as determining its mode of action.

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