Effects of Particulate Materials and Osmoprotectants on Very-High-Gravity Ethanol Fermentation by Saccharomyces cerevisiae

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The effects of osmoprotectants (such as glycine betaine and proline) and particulate materials on the fermentation of very high concentrations of glucose by the brewing strain Saccharomyces cerevisiae (varum) NCYC 1324 were studied. The yeast growing at 20°C consumed only 15 g of the sugar per 100 ml from a minimal medium which initially contained 35% (wt/vol) glucose. Supplementing the medium with a mixture of glycine betaine, glycine, and proline increased the amount of sugar fermented to 30.5 g/100 ml. With such supplementation, the viability of the yeast cells was maintained above 80% throughout the fermentation, while it dropped to less than 12% in the unsupplemented controls. Among single additives, glycine was more effective than proline or glycine betaine. On incubating the cultures for 10 days, the viability decreased to only 55% with glycine, while it dropped to 36 and 27%, respectively, with glycine betaine and proline. It is suggested that glycine and proline, known to be poor nitrogen sources for growth, may serve directly or indirectly as osmoprotectants. Nutrients such as tryptone, yeast extract, and a mixture of purine and pyrimidine bases increased the sugar uptake and ethanol production but did not allow the population to maintain the high level of cell viability. While only 43% of the sugar was fermented in unsupplemented medium, the presence of particulate materials such as wheat bran, wheat mash insolubles, alumina, and soy flour increased sugar utilization to 68, 75, 81, and 82%, respectively.

Research in recent years has shown that the application of very-high-gravity (VHG) fermentation technology for industrial scale production of fuel alcohol is a distinct possibility (19). The term “gravity” (actually specific gravity) is commonly used in the fermentation industry to indicate the dissolved solids content of the fermentation medium. The progress of fermentation is usually monitored by measuring the specific gravity of the medium. VHG technology for fuel alcohol production is defined as “the preparation and fermentation to completion of mashes containing 27 or more grams of dissolved solids per 100 g mash” (38). Saccharomyces cerevisiae, the yeast commonly used for ethanolic fermentation, is not very tolerant to diminished water activity (13), yet at 20°C it can completely ferment wheat mash containing 38 to 39% (wt/vol) dissolved solids, yielding over 23% (vol/vol) ethanol (38). The amount of sugar this yeast can ferment in a defined medium, however, is considerably less, even though all required nutrients are provided in adequate amounts. It has been suggested that yeasts grown in synthetic media produce a substance which inhibits their growth (17). However, the identity of this substance has not been established. It is also possible that an unknown nonnutritional factor or factors available in complex media such as grain mashes, brewer’s wort, or grape juice play a role in stimulating yeast growth and fermentation in VHG media. The effect of such factors may not be as apparent in low- or normal-gravity media since all of the sugars from these media are consumed by the yeast irrespective of the presence or absence of the growth-promoting particulate materials.

It has been reported that the addition of insolubles such as grape solids, bentonite, or diatomaceous earth to clarified grape juice caused the fermentation to finish more rapidly and to a greater degree of completion (16). Alcoholic fermentation of grape juice has also been shown to be stimulated by microcrystalline cellulose and yeast cell wall preparations (27). Ethanol productivity from molasses was increased by the addition of γ-alumina (18) or soy flour (43). Various particulate materials such as trub, diatomaceous earth, activated carbon, pea flour, and Chromosorb W have been reported to stimulate yeast growth and increase the rate of sugar utilization from brewer’s worts (34).

An important consideration in VHG fermentation is that the yeast is subjected to considerable osmotic stress which reduces the growth and increases the loss of cell viability (8, 28, 40). Successful VHG fermentation is therefore dependent on the yeast’s ability to withstand increased osmotic stress and to tolerate high concentrations of ethanol. The mechanism of osmotolerance in S. cerevisiae has not been studied in detail. It is known, however, that this nonsmotolerant yeast (1) synthesizes glycerol in response to increased salt concentrations in the medium, whereas one or more polyols are synthesized as compatible solutes by osmotolerant yeasts (7).

Certain components of complex media appear to alleviate the effects of osmotic stress on yeast growth and viability. For example, by increasing the concentration of peptone and yeast extract in the medium, a yeast strain’s tolerance to osmotic stress and elevated temperature can be improved (36). In yeast extract, the active ingredient that plays a part in the osmoprotection appears to be betaine (12). Dulaney et al. (12) isolated and purified glycine betaine from yeast extract and showed that it contained about 110 μg of betaine per g of yeast extract.

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They demonstrated that the amount of betaine present in yeast extract was sufficient to relieve inhibition of bacterial growth in defined medium of high osmolality, although it had no effect on the performance of some osmotolerant yeasts (12).

Several low-molecular-weight organic compounds are known to function in the osmoregulation of bacteria, fungi, and plants. These compounds include polyols for yeasts (1, 7, 32, 42; glutamate (26), glutamine (2), N-acetlyglutamylglutamine (11), and N-acetlyglutamylglutamine (11), and N-acetlyglutamylglutamine amide (30) for bacteria; and betaine (10–12, 24, 30) and proline (2, 10, 24) for plants and bacteria.

In this report, we show that the addition of certain insoluble materials to a modified Wickerham's medium increased the rate of sugar fermentation and the maximum amount of sugar fermented by a brewing strain of yeast. In addition, we show that amendment of glycine betaine, proline, glycine, or a combination of these three compounds resulted in maintaining the viability of cells at high levels throughout the course of fermentation.

**MATERIALS AND METHODS**

An industrial strain of lager yeast, *S. cerevisiae* (varum) NCYC 1324, was grown at 20°C with shaking in 250-ml sidearm flasks containing 100 ml of modified Wickerham's medium (44). In some cases, where particulate materials were not added, the experiments were conducted in duplicate with 15 ml of medium contained in test tubes and incubated with shaking (30 oscillations per min) in a temperature gradient incubator (Scientific Industries, Inc., Mineola, N.Y.) set to run isothermally at 20°C. Various analyses were done in duplicate, and the results were analyzed statistically. The medium contained 1.94 M (35% [wt/vol]) glucose, 10 mM (NH₄)₂SO₄, 50 mM KH₂PO₄, 50 mM MgSO₄, and other minerals and vitamins at three and five times the normal concentrations, respectively. The initial pH of the medium was adjusted to 5.2. The inoculum was prepared by growing the yeast to mid-exponential phase at 20°C in 100 ml of the medium described above. The cells were harvested by centrifugation and resuspended in 10 ml of fresh medium. The experimental flasks were then inoculated to give 10⁷ cells per ml. Growth was monitored by measuring absorbance with a Klett-Summerson colorimeter equipped with a red filter or by counting the cells. Sugar utilization was monitored by measuring, at 20°C, the decrease in specific gravity of the supernatant liquid (10,300 × g for 15 min) with a digital density meter (DMA-45; Anton Paar KG, Graz, Austria). Total numbers of cells were determined microscopically, and viabilities were estimated by the methylene blue technique (39). Where necessary, the initial dissolved solids concentrations were adjusted to the same osmolarity by adding the nonmetabolizable sugar alcohol sorbitol to the medium. Glycerol and ethanol levels were measured by analyzing the supernatant portion of the medium by high-performance liquid chromatography as described previously (38).

All routinely used chemicals and microbiological medium ingredients were purchased from local suppliers. Particulate materials used in this study were either purchased or prepared in the laboratory. Wheat bran (Quaker) and defatted soy flour (Vita, Winnipeg, Manitoba, Canada) were purchased from local stores. Alumina (80/200 mesh) and sea sand (primarily quartz) were obtained from Fisher Scientific Co. Mash solids were prepared by freeze-drying washed insoluble materials obtained during the preparation of wheat mash as described previously (39). Ash was obtained by incinerating yeast extract.

### TABLE 1. Effect of various particulate materials on glucose utilization, cell growth, and cell viability of *S. cerevisiae* (varum) NCYC 1324*

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Amt of dissolved solids (g/100 ml)</th>
<th>Maximum no. of cells (10⁷) ± SE</th>
<th>% Viability ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>36.4</td>
<td>194 ± 1</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>37.2</td>
<td>266 ± 5</td>
<td>14.2 ± 2.4</td>
</tr>
<tr>
<td>Mash solids</td>
<td>36.4</td>
<td>189 ± 1</td>
<td>69.7 ± 4.1</td>
</tr>
<tr>
<td>Alumina</td>
<td>36.1</td>
<td>218 ± 9</td>
<td>56.6 ± 0.5</td>
</tr>
<tr>
<td>Sea sand</td>
<td>34.8</td>
<td>178 ± 6</td>
<td>21.4 ± 2.1</td>
</tr>
<tr>
<td>Soy flour</td>
<td>36.9</td>
<td>231 ± 6</td>
<td>74.1 ± 2.7</td>
</tr>
</tbody>
</table>

* Yeast was grown at 20°C in modified Wickerham's medium containing 35 to 36% (wt/vol) glucose.

RESULTS

Particulate materials stimulate yeast growth and fermentation. Although the total amount of glucose used by the yeast with a particular additive in the medium remained about the same, the times taken to reach the end of fermentation varied slightly between experiments, and therefore the results from different experiments could not be averaged. The results reported here are averages from single experiments. Various particulate materials added to the defined VHG medium stimulated fermentation, as indicated by the greater disappearance of dissolved solids (Table 1). While only 44.5% of the total sugar was fermented in 9 days in the unsupplemented control, addition of 2% soy flour or 2% alumina increased the amount of sugar consumed to 82%. Other additives had intermediate effects on fermentation. Even sea sand had a moderate stimulatory effect. Part of the stimulatory effect, it appears, is not mediated through the provision of nutrients. With all additives except sea sand, cell viability was maintained at high levels for longer periods of time (Table 1).

Soluble nutrients provide or act as osmoprotectants for the yeast. Nutrients such as tryptone, yeast extract, and a mixture of purine and pyrimidine bases also stimulated yeast growth and fermentation (Table 2). The normal-gravity minimal medium (<12% sugar) contained enough nutrients to allow the production of greater amounts of biomass than that observed in a medium which contained 35% sugar (data not shown). This suggested that the stimulatory effect of the supplements described above was probably mediated, not through provision of nutrients, but by providing something which protected the yeast from the deleterious osmotic effect. Ergosterol and Tween 80, known to stimulate yeast growth under oxygen-deficient conditions, were without effect. In these studies, we did not exclude oxygen from fermentors since the presence of oxygen did not adversely affect the ethanol yield. This clearly suggested that, under VHG fermentation conditions, synthesis of sterols and unsaturated fatty acids by the yeast continued unimpaired. Ash obtained from yeast extract had no effect on fermentation—probably because the medium already contained an excess supply of all required minerals.

Proline, glycine betaine, and glycine promote yeast growth and improve cell viability in VHG medium. The effects of proline and glycine betaine, two proven osmoprotectants in plants and bacteria (24), on growth and metabolism of yeast in VHG medium were studied. Glycine, a precursor of glycine betaine (41), was also included in this study. Glycine and proline are not used as sources of nitrogen by *Saccharomyces*...
spp. under fermentation conditions (33), although with low concentrations of glucose (high concentrations of glucose repress aerobic metabolism in yeasts) and under aerobic conditions, proline may serve as a nitrogen source for yeast growth. Addition of these three compounds singly or together stimulated yeast growth (Fig. 1A) and fermentation (Fig. 1B). Glycine was more effective than proline and glycine betaine. In the control, only 43% of the sugar was fermented by the yeast, but when all three compounds were present, 87% of the sugar was consumed. Sugar consumption was about 65% when glycine was the only additive. Ethanol production was proportional to the amount of glucose removed from the medium by the yeast. With the control, the ethanol yield was 8.8% ± 0.3% (vol/vol), while in the presence of all three additives, 18.0% ± 0.6% (vol/vol) ethanol was realized. With glycine as the only additive, an ethanol yield of 13.1% ± 0.7% (vol/vol) was obtained.

A mixture of glycine betaine, glycine, and proline (30 mM each) increased the viability of the yeast and maintained it at high levels throughout the course of fermentation (Fig. 2). The osmolality of the medium was maintained at the same level by adding sorbitol, a sugar alcohol not used by the yeast. Each supplement, when added alone, was less effective in maintaining the cell viability than a mixture of all three. Glycine was the best single additive.

**Osmoprotection by glycine is concentration dependent.** The stimulatory effect of glycine on yeast growth increased with increasing concentration up to 40 mM (Table 3). Further increase in the concentration did not improve growth or fermentation. The amount of sugar consumed (from a medium which initially contained 35 g of glucose per 100 ml) in 13 days at 20°C was 49% in unsupplemented controls, while it increased to 92% with glycine supplementation at concentrations of 40 mM. The viability of yeast cells also increased with increasing glycine concentration and was maintained at 80% or higher at a concentration of 40 mM or higher (Table 3).

**DISCUSSION**

The rate and extent of utilization of sugar by *S. cerevisiae* (uvarum) NCYC 1324 from a defined VHG medium were influenced by the two factors studied here. First, the presence of insoluble materials such as wheat bran, wheat mash in-

### TABLE 2. Effect of various soluble supplements on glucose utilization, cell growth, and cell viability of *S. cerevisiae* (uvarum) NCYC 1324a

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Amt of dissolved solids (g/100 ml)</th>
<th>Maximum growth (Klett units) ± SE</th>
<th>% Viability ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>35.1 20.2</td>
<td>340 ± 7</td>
<td>11.6 ± 2.1</td>
</tr>
<tr>
<td>Tryptone</td>
<td>35.2 0.6</td>
<td>483 ± 6</td>
<td>10.8 ± 1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>35.1 1.0</td>
<td>488 ± 9</td>
<td>31.2 ± 0.9</td>
</tr>
<tr>
<td>Adenine-uracil-cytosine</td>
<td>35.1 5.9</td>
<td>477 ± 2</td>
<td>55.3 ± 0.9</td>
</tr>
<tr>
<td>Ash</td>
<td>35.1 19.0</td>
<td>390 ± 4</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Ergosterol-Tween 80</td>
<td>35.4 20.9</td>
<td>360 ± 7</td>
<td>6.8 ± 0.1</td>
</tr>
</tbody>
</table>

a Yeast was grown at 20°C in modified Wickerham’s medium containing 34 to 35% (wt/vol) glucose.

b Tryptone and yeast extract were added at 1% (wt/vol). The adenine-uracil-cytosine supplement contained a 10 mM concentration of each component. The amount of ash added was equivalent to 1% (wt/vol) yeast extract. The ergosterol-Tween 80 (a source of oleic acid) supplement contained 0.1 and 3% (wt/vol) ergosterol and Tween 80, respectively.

c Viability was determined by the methylene blue technique at the end of 9 days of fermentation.

![Fig. 1. Effects of glycine betaine (▲), glycine (○), and proline (■) on the growth (A) of *S. cerevisiae* (uvarum) NCYC 1324 in minimal media containing 35% (wt/vol) glucose and their effects on sugar utilization (B). Each compound was added at a concentration of 30 mM. Osmolality in all cases was adjusted to the same level by adding sorbitol. ○, control; △, betaine plus glycine plus proline.](http://aem.asm.org/)
Inhibition of yeast growth by metabolic CO₂ may become a serious problem under VHG fermentation conditions. First, the VHG medium because of its high carbohydrate content is considerably more viscous than normal-gravity media. For example, a 35% glucose solution at 20°C is about 3.4 times more viscous than a 5% glucose solution (η = 1.14 and 3.94 cP for 5 and 35% glucose solutions, respectively). Because of their high protein and, in some cases, high β-glucan contents, viscosities of VHG grain mashes are considerably higher than those of simple sugar media with equivalent sugar contents. Carbon dioxide may not escape from a viscous medium as readily as from a normal-gravity fermentation broth. Second, for maximum productivity, VHG fermentation is carried out at temperatures close to 20°C rather than at 30 to 35°C as normally employed in fuel alcohol production. Low fermentation temperature increases the solubility of CO₂ and the viscosity of the medium. Both increased viscosity and low fermentation temperature may cause retention of greater amounts of CO₂ in the medium, and this may enhance inhibition of yeast growth. It is known that addition of peptone and yeast extract can improve the stress and temperature tolerance of yeast (36), although the mechanism of this protection is not understood. The improvement in fermentation observed with yeast extract did not result from an increased supply of minerals, since addition of ash obtained from yeast extract or addition of increased concentrations of minerals or trace elements had no effect on the fermentation. Yeast extract (and possibly other complex ingredients such as tryptone and peptone) may play a dual role: it may supply growth factors to nutritionally inadequate media, and it may stimulate microbial growth that could occur in its absence (35). It is apparent that it is the second role that was important in the stimulation of fermentation observed in the present study. This role may be through the supply of preformed molecules (amino acids, nucleic acids, vitamins, etc.), or it may be through the provision of compounds that protect the yeast cells. The role of soy flour is difficult to assess, since it contains both soluble and insoluble components and both may have contributed to the stimulation.

While tryptone, yeast extract, and nucleic acids stimulated yeast growth and fermentation, they did not prevent loss of cell viability (Table 2). This suggested that the mechanisms of stimulation of fermentation under VHG conditions need not be coupled to the protection of yeast cells from osmotic stress. Much work in the past has been concerned with the physiological response of the yeast to osmotic stress, and very little is known about how yeast cells can be protected by the exogenous addition of osmoprotectants or by stimulating the synthesis of osmoprotectants. Increased synthesis of glycerol has been cited as a means of osmoregulation in S. cerevisiae (5, 13, 21, 32). In the present study, glycerol production varied between 1 and 1.2% (wt/vol), irrespective of the nature or amount of various additives (data not shown). It is not clear whether the synthesis of glycerol is a response to osmotic stress or whether it is a means of protecting the yeast cells. In general, compatible solutes are synthesized to counteract the effects of osmotic stress and to maintain cell turgidity (6). Since most of the glycerol produced by S. cerevisiae under stress is excreted into the medium (6, 23), it is likely that glycerol production is not a means of protecting the yeast but is a response to eliminate the excessive reducing power generated in glycolysis. Reduction of dihydroxyacetone to glycerol provides a convenient means of disposing of reducing equivalents (31). Since anabolic activities are decreased in S. cerevisiae under osmotic stress (25), reducing equivalents generated are not used to a great extent for growth but are channelled for the reduction of dihydroxyacetone to glycerol. There is some evidence to suggest a stimulation of synthesis of glycolytic enzymes in S. cerevisiae under osmotic stress (13, 36a) while there is a reduction in the activities of enzymes of the pentose phosphate pathway (36a).

A number of factors affect the osmotic tolerance of S. cerevisiae. An important factor appears to be the physiological state of the cells (15). Generally, nongrowing cells are more stress tolerant than their actively growing counterparts (14), and it has been suggested that respiratory metabolism confers greater stress tolerance to yeast cells (25). The mechanism of this increased stress tolerance is unclear. Exogenously added trehalose has been shown to offer cryoprotection to yeast (4) and to increase the dehydration resistance of stationary-phase cells (14). In the present study, exogenously added trehalose did not affect growth, cell viability, or fermentation (data not shown). We are in agreement with the suggestion that trehalose is not an osmoregulator in growing yeasts (29), although it is synthesized in response to nutrient limitation, desiccation, heat shock, and other forms of stress (9, 14, 25).

The results presented here show that unrecognized protectants may be involved in the osmoregulation of S. cerevisiae. In a previous report (37), we suggested that proline, produced

### TABLE 3. Effect of various concentrations of glycine on glucose utilization, cell growth, and viability of S. cerevisiae (uvatum) NCYC 1324

<table>
<thead>
<tr>
<th>Conc of glycine (mM)</th>
<th>Maximum amt of glucose consumed (g/100 ml)</th>
<th>Maximum growth (Klett units) ± SE</th>
<th>% Viability ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.3</td>
<td>365 ± 5</td>
<td>13 ± 2.3</td>
</tr>
<tr>
<td>5</td>
<td>20.0</td>
<td>415 ± 4</td>
<td>28 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>23.7</td>
<td>435 ± 7</td>
<td>40 ± 1.1</td>
</tr>
<tr>
<td>20</td>
<td>25.9</td>
<td>465 ± 7</td>
<td>53 ± 2.3</td>
</tr>
<tr>
<td>40</td>
<td>32.4</td>
<td>475 ± 5</td>
<td>78 ± 1.2</td>
</tr>
<tr>
<td>80</td>
<td>32.1</td>
<td>475 ± 7</td>
<td>85 ± 0.3</td>
</tr>
</tbody>
</table>

* Yeast was grown at 20°C in modified Wickerham's medium which initially contained 35% (wt/vol) glucose.

b Glucose consumption was determined at the end of 13 days of fermentation.

Viability was determined by the methylene blue technique at the end of 10 days of fermentation.
through the catabolism of arginine, may confer osmoprotection to *S. cerevisiae* during fermentation of VHG wheat mash. The results in this report show that exogenously added proline stimulated yeast growth and increased cell viability. About the same degree of response was obtained when glycine betaine was added. Both these compounds have been extensively studied as osmoprotectants in bacteria. Very little information is available, however, regarding their osmoprotective effect on *S. cerevisiae*, although proline has been shown to have some cryoprotective effect (4).

It was surprising to see that exogenously added glycine betaine, a compound actively synthesized by yeast (12) (it is a component of yeast extract), had only a moderate effect in protecting the yeast from osmotic stress. This compound is an effective osmoprotectant in several bacteria (10), but no noticeable effect was observed when it was added to growing osmotolerant yeasts (12). It is possible that the reduced effectiveness of exogenously added glycine betaine in *S. cerevisiae* may be related to the difficulty of transporting this positively charged molecule into yeast cells. Glycine, on the other hand, was the most effective single additive in combating the osmotic stress in *S. cerevisiae*. Glycine has been used as a nutrient supplement during wine fermentation (20), although its effect on osmoregulation was not recognized. It is interesting that where exogenously added glycine betaine served as an effective osmoprotectant, glycine was without effect, as, for example, in the case of *Pseudomonas aeruginosa* (11). Since glycine and proline are not used by *S. cerevisiae* as sources of nitrogen (33), it is likely that these amino acids may have other biological roles. We suggest that one of these roles is to serve directly or indirectly as osmoprotectants. As reported in the case of some bacteria (41), glycine in yeast may serve as the precursor of glycine betaine, which may be the actual intracellular osmoprotectant. This aspect is currently under investigation.

ACKNOWLEDGMENTS

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REFERENCES