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Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes

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SUMMARY

Very high gravity wheat mashes containing 300 g or more sugars per liter were prepared by enzymatic hydrolysis of starch and fermented with a commercial preparation of active dry yeast. The active dry yeast used in this study was a blend of several strains of *Saccharomyces cerevisiae*. The fermentation was carried out at 20 °C at different pitching rates (inoculation levels) with and without the addition of yeast extract as nutrient supplement. At a pitching rate of 76 million cells per g of mash an ethanol yield of 20.4% (v/v) was obtained. To achieve this yeast extract must be added to the wheat mash as nutrient supplement. When the pitching rate was raised to 750 million cells per g of mash, the ethanol yield increased to 21.5% (v/v) and no nutrient supplement was required. The efficiency of conversion of sugar to ethanol was 97.6% at the highest pitching rate. This declined slightly with decreasing pitching rate. A high proportion of yeast cells lost viability at high pitching rates. It is suggested that nutrients released from yeast cells that lost viability and lysed, contributed to the high yield of ethanol in the absence of any added nutrients.

INTRODUCTION

It is generally accepted that growth of *Saccharomyces cerevisiae* under anaerobic conditions and alcoholic fermentation are tightly coupled. In fact nearly all of the energy (ATP) required for growth and maintenance of yeast cells is derived through the catabolism of glucose in glycolytic and fermentative pathways. Mitochondrial oxidative phosphorylation is either absent or contributes only small amounts of energy to the overall cellular processes [7,8]. *S. cerevisiae* does not seem to have many 'energy spilling reactions' (a term introduced by Neijssel and Tempest [13] to denote reactions that waste energy when organic compounds are oxidized). The implication of this statement is that unless there is a need for energy, cells do not take up and ferment sugars. Resting or nongrowing cells ferment sugars only to produce energy for maintenance purpose. Usually the amount of energy required for maintenance is so low that when cells cease to grow and enter into the maintenance phase, fermentation rates decrease greatly. Searle and Kirsop [17] calculated that under brewing conditions, a consumption rate of 0.06 g of glucose per g of yeast per h was sufficient to meet the maintenance requirement. Under growing conditions, the sugar consumption rate was considerably faster (30-times or more) than that observed in resting cells. There have been attempts to increase the rate of fermentation by resting and by growing-cells through genetic manipulation of yeast cells by incorporating 'futile cycles' so that these cells continue to produce (and partially waste) greater amounts of energy (D. Rogers and J.W. Szostak, International Patent Publication No. WO 87/03006, 1987). This would result in partial decoupling of fermentation from growth.

An alternate method to increase fermentation rate is by increasing the pitching rate (inoculation level). Here the large number of cells, although not growing, consume great amounts of sugar for maintenance with the result that the fermentation is accelerated. Very high pitching rates have been used in 'rapid fermentation' of honey, although the yeast cells lost viability to a great extent within a short time [11]. Pitching rates as high as 3.0×10^9 cells per ml have been successfully used for the production of fuel alcohol [4]. Many aspects of the effects of pitching rates on yeast growth and fermentation have been studied. Among them are the effects of pitching rate on rate of fermentation, growth rate of the yeast, viability of yeast cells, ethanol yield and productivity [5,6,11,18,22]. Recently a report from our laboratory showed that high pitching rates can be used to compensate for sluggish or stuck fermentations of wort [14]. Such fermentations normally result from an

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inadequate supply of nutrients for yeast growth. When addition of extra nutrients to fermentation media is not allowed, as in the case of wine fermentation, increasing the pitching rate may be the only legal means of overcoming stuck or sluggish fermentations.

In a previous report [19], we showed that sugars (> 300 g/l) derived by enzymatic hydrolysis of wheat starch were completely fermented in spite of the fact that these mashes contained low amounts of assimilable nitrogen. The reason for the very efficient utilization by yeast of available nitrogen in wheat mash is discussed in a separate communication [20]. In this report we show that very high gravity (VHG) wheat mashes can be fermented to produce up to 21.5% (v/v) ethanol by increasing the pitching rate to very high levels. Results also show that nitrogen supplementation can compensate for low inoculation rates when the gravity of the mash is normal.

MATERIALS AND METHODS

Materials

Active dry yeast, High-T™ (high-temperature α-amylase) and Allcoholase™ (glucoamylase) were supplied by Alltech Biotechnology Center, Nicholasville, KY. According to the supplier the 'active dry yeast' was a blend of several strains of *S. cerevisiae*. The high-temperature α-amylase preparation contained 10 mg protein per ml and had a specific activity of 1.14 g starch (hydrolyzed) min^{-1} mg^{-1} protein at 80 °C. The glucoamylase preparation contained 98.5 mg protein per ml and had a specific activity of 1 mg glucose (produced) min^{-1} mg^{-1} protein (5.6 μmol glucose min^{-1} mg^{-1} protein). Amino acids, and chemicals required for ethanol assay were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals were obtained through local suppliers and were of reagent grade. Yeast extract (type AYE 2200) was purchased from Gillette Foods Inc., Union, NJ. Red hard spring wheat obtained from a local supplier (Schimdt Farm, Fox Valley, Saskatchewan) was used throughout the study.

Grinding and mashing of wheat

Wheat was ground with a plate grinder (Type KT-30, Falling Number AB, Stockholm, Sweden) at setting number 2. Eight-five percent of the ground wheat had a particle size distribution between 12–100 mesh, while the rest of the flour was finer than 100 mesh size. This ground wheat was gelatinized and liquefied as described previously [19]. The water soluble portion of a mash thus prepared contained 19 to 20 g dissolved solids (mostly sugars and dextrins) per 100 ml. This mash could be saccharified without any further treatment and then fermented, or most of the particulate matter from the mash could be removed by straining through a stainless steel food strainer (20 mesh) and then saccharified and fermented. Removal of the particulate material facilitates easy stirring during fermentation; this was especially important when the dissolved solid content of the mash was very high. When the dissolved solid content of the mash was to be raised for VHG fermentation, freeze dried 'wheat hydrolysate' (wheat mash treated with HT α-amylase and clarified) [19] was added to the mash described above.

Fermentation

In these experiments 500 g samples of wheat mashes were transferred to sterile jacketed Celstir fermentors (Wheaton Scientific, Millville, NJ) and the fermentors then connected to a D3-G water bath circulator (Haake Inc., Saddle Brook, NJ) maintained at 30 °C. To each fermentor 1 ml of Allcoholase II was added to saccharify the dextrins to fermentable sugars. Thirty minutes after the addition of the enzyme, the temperature was lowered to 20 °C for fermentation. Fermentors were then inoculated with active dry yeast at the predetermined pitching rate.

Determination of dissolved solids

The mash was centrifuged at 10 300 × *g* for 15 min and the specific gravity of clear supernatant was determined at 20 °C with a digital density meter (DMA-45, Anton Paar KG, Graz, Austria). With the aid of appropriate tables, the specific gravities were converted to grams of dissolved solids (expressed as g of sucrose) per 100 ml.

Cell number, cell size and percentage of viability

Total cell counts and viable cell counts were determined by the direct microscopic method described previously [19]. Cell sizes were determined by the microscopic difference imagery technique of Caldwell and Germida [1].

Cell dry weights

In experiments where changes in cell dry weights were to be determined, wheat mashes were clarified by centrifugation (10 300 × *g* for 15 min) and the clear supernatant liquid thus obtained was saccharified by treating with glucoamylase and fermented as described for unclarified mash. For dry weight determination, 20 ml samples withdrawn at regular intervals were centrifuged (10 300 × *g* for 15 min) and the yeast pellets washed twice with ice-cold water and resuspended in 5 ml water. Four ml of this suspension was transferred to preweighed aluminum pans and dried to constant weight at 105 °C.

Ethanol

Ethanol was measured enzymatically by the alcohol dehydrogenase assay as reported previously [19]. Known concentrations of ethanol were used as standards.

Reducing sugars

Reducing sugars present in the clear supernatant of wheat mashes were determined by the dinitrosalicylic acid method [10].

RESULTS

The concentration of fermentable sugar in the wheat mash, the amount of assimilable nitrogen available and the pitching rate all play important roles in determining rate of fermentation of wheat mash and the final yield of ethanol. It is not known how these factors interact during the fermentation of VHG wheat mashes because mashes which contain more than 300 g fermentable sugars per liter have not been used for fermentation. It is known, however, from the studies with VHG brewing worts, that as sugar content is increased the pitching rate and the amount of assimilable nitrogen also have to be increased [2].

To study the effects of these three factors and their interactions, a factorial experiment with eight treatment combinations (each factor at two levels) was designed. The factors and their levels are given in Table 1.

The experiment was conducted in duplicate and the results were analyzed statistically. Since the rate of fermentation and growth of the yeast are tightly coupled [17], data on cell number (cell multiplication) was used for statistical analysis. As expected the main effects of all three factors were highly significant ($\alpha = 0.005$). Interactions of these factors with the exception of gravity × nitrogen were also highly significant ($\alpha = 0.005$). At both sugar concentrations (gravities), fermentation was stimulated to a greater extent with nitrogen supplemention than with increased pitching rate (Fig. 1). Increasing the pitching rate from 26 million cells to 76 million cells per g of mash reduced the fermentation time of low gravity wheat mash from 120 h to 96 h, but increasing the level of assimilable nitrogen reduced the fermentation time even further (120 h to 48 h). It was the assimilable nitrogen present in the yeast extract and not other nutrients such as minerals and vitamins that stimulated fermentation. This has been well established in reports published previously [3,15,16,19]. There was no appreciable difference between the rates of fermentation of low gravity wheat mashes pitched at low and high rates if these mashes were supplemented with yeast extract. At higher gravities, increasing the pitching levels, however, did make a difference between the rates of fermentation. Supplementing VHG mash with yeast extract stimulated fermentation as it did with low gravity mash.

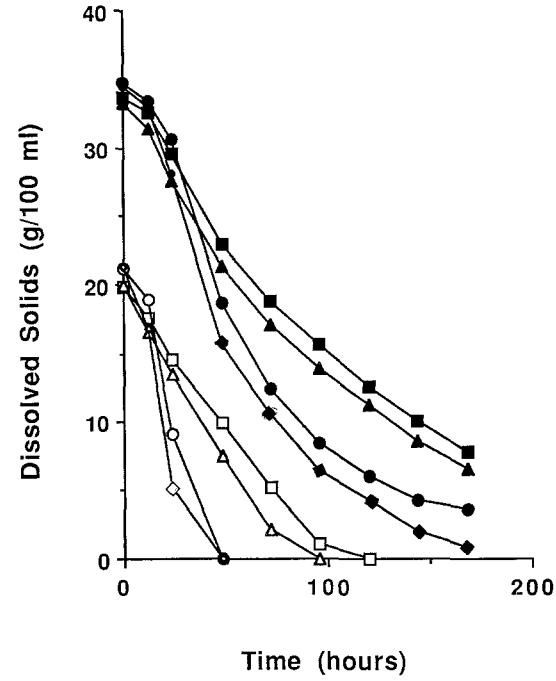

Fig. 1. Rate of disappearance of dissolved solids (sugars) during the fermentation of wheat mashes by active dry yeast in the presence or absence of added nutrient supplement. The main effects of three factors and their interactions were studied in the same experiment. Open and closed symbols represent low and high gravity wheat mashes, respectively. Symbols: □, ■, 26 million cells per g of mash; △, ▲, 76 million cells per g mash; ○, ●, 26 million cells per g mash and 2% (w/w) yeast extract; and ◇, ◆, 76 million cells per g mash and 2% (w/w) yeast extract.

The rate of fermentation was clearly reflected by the increase in cell number. A maximum of 400 million yeast cells/g of mash were produced when the mash contained low amounts of sugar and no nitrogen supplement was added. Raising the gravity of the mash from 20 g/100 ml

TABLE 1

Factors affecting fermentation

Factor	Low level	High level
Dissolved solids (gravity)	20% (w/v)	34% (w/v)
Nitrogen supplement	no supplement	2% (w/v) yeast extract
Pitching rate	26 million cells/g	76 million cells/g

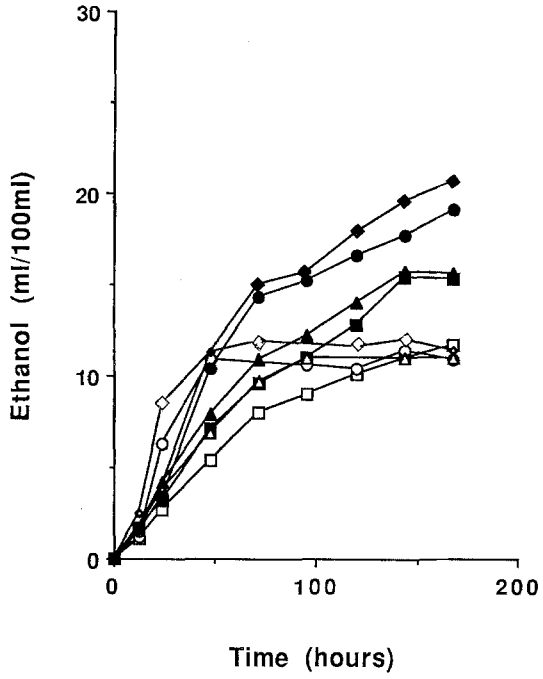

Fig. 2. Rate of production of ethanol during the fermentation of wheat mashes by active dry yeasts in the presence or absence of added nutrient supplement. Concentration of ethanol in the clear supernatant liquid of the fermentation fluid was determined by the enzymatic method cited in the text. Open and closed symbols represent low and high gravity wheat mashes, respectively. Symbols: □, ■, 26 million cells per g of mash; △, ▲, 76 million cells per g mash; ○, ●, 26 million cells per g mash and 2% (w/w) yeast extract; and ◇, ◆, 76 million cells per g mash and 2% (w/w) yeast extract.

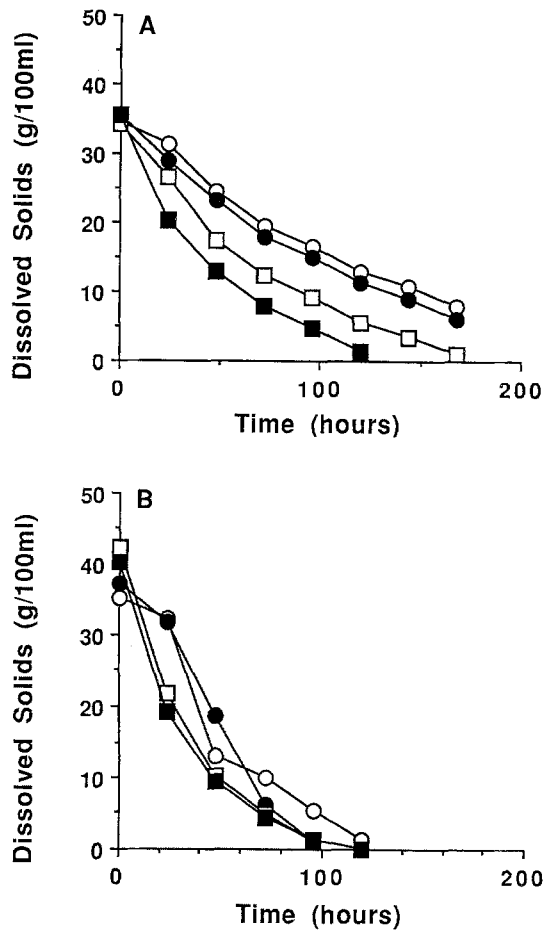

Fig. 3. Rate of fermentation of very high gravity wheat mashes without nutrient supplement (A), and with 2% yeast extract as nutrient supplement (B) at different pitching rates. Number of millions of active dry yeast cells pitched per g of mash were: ○, 15; ●, 30; □, 150; and ■, 300.

to 34.0 g/100 ml resulted in a decrease in maximum cell number attained (150 million cells/g). Nitrogen supplementation partially compensated for this, but the cell number observed in the lower gravity mash was never reached. Increasing the pitching rate to 76 million cells per g restored the maximum cell number attainable during the fermentation of VHG mash. Then yeast extract was without any significant effect on maximum number of cells produced.

It can be concluded from these experiments that a low pitching rate with nitrogen supplementation stimulates fermentation more than a high pitching rate without nitrogen supplement. At both gravity levels the fastest fermentation occurred when wheat mashes were pitched at high levels and also supplemented with yeast extract. The same result could be obtained at the lower gravity with yeast extract supplement alone (i.e., without raising the pitching level), but it could not be achieved at high gravity levels. Here high pitching along with yeast extract supplement was essential to achieve the fastest rate of fermentation and maximum yield of ethanol. The rate of production and final yield of ethanol paralleled the rate of disappearance of dissolved solids from the mashes (Fig. 2). A remarkably high concentration of ethanol (20.4% v/v) was produced when VHG mash (35% dissolved solids w/v) was fermented with nitrogen supplementation and with high pitching rate (all three factors at high levels). Even in the presence of this high concentration of ethanol there was no major loss of yeast cell viability. The viability at the end of fermentation was never less than 90%.

Although we have never experienced stuck fermentation with wheat mash, fermentation was often somewhat slow because of the low levels of assimilable nitrogen of wheat mashes. As shown above, fermentation could be accelerated by supplying external sources of assimilable nitrogen and increasing the pitching rate. The high pitch-

ing rates used here were not sufficient to depress cell proliferation. Studies conducted in this laboratory [14] have shown that high gravity worts could be fermented without a net increase in cell number if the pitching rate was raised to very high levels. Use of immobilized yeast cells or cell recycle technology also implicitly assumes that there is no need for cell proliferation in order to achieve a fast rate of fermentation. To explore the possibility of using very high pitching rate to increase the fermentation and maximize the conversion of sugars to ethanol, pitching rates of 15, 30, 150, 300, and 750 million cells per g of mash were studied. The two lower levels corresponded to the pitching rates normally employed in high gravity and VHG fermentations. The object of this study was to explore whether extremely high pitching rates (150, 300, or 750 million cells per g mash) could accelerate fermentation without any nutrient supplement and cell proliferation. Experiments with the highest pitching rate (750 million cells per g mash) were conducted separately because the heavy inoculum (19.5 g of active dry yeast per kg of mash) required several hours for homogeneous dispersion and this did not permit sampling in the early stages of fermentation.

Very high gravity mashes containing 37 to 42 g of dissolved solids per 100 ml were used in these studies. Yeast extract (2% w/w) was added to some fermentors as a nutrient supplement. More than 168 h were required to complete the fermentation if wheat mashes were pitched at 15 or 30 million cells per g and no nutrient supplement was provided (Fig. 3A). At very high pitching rates of 150 and 300 million cells per g, the fermentations were completed by 168 and 120 h, respectively. Addition of yeast extract stimulated the rate of fermentation at all pitching rates (Fig. 3B). Completion of fermentation appeared to be independent of pitching rate if the mash was supplemented with yeast extract, although the rate of fermentation was still a function of the pitching rate. The differences in the time taken to complete the fermentation at different pitching rates, however, became smaller in the presence of added yeast extract. For example, at pitching rates of 15 or 30 million cells per g of mash, fermentation was completed within 120 h, while at higher pitching rates it took only 96 h. Thus, when the mash was supplemented with yeast extract, there was only marginal advantage in using high pitching rates.

The greatest advantage of high pitching rate appears to be that fermentation gets an early start. For example, during the first 24 h, about 5-times as much sugar was consumed at a pitching rate of 300 million cells per g of mash, compared to that taken up when the pitching rate was only 15 million cells per g (Table 2). With added nutrient supplement, the amount of sugar used in the same time interval was seven times greater at the higher pitching rate. In addition, with a high pitching rate no lag was observed before the fermentation initiated.

At all pitching rates both biomass and cell number increased (Figs. 4 and 5). At low pitching rates (15 or 30 million cells per g mash) the increase in biomass was considerably greater in yeast extract supplemented mashes than in the unsupplemented controls. The total number of cells increased several fold at low pitching rates with and without yeast extract supplement (Fig. 5). In these sam-

TABLE 2

Effect of pitching rates (inoculation levels) and nutrient supplement (2% yeast extract) on sugar consumption during very high gravity (VHG) fermentation of wheat mash

Pitching rate (million cells/g mash)		Sugar consumed (g/100 ml)[a]		
		Total[b]	0 to 24 h	24 to 48 h
15	control	26.7 (>168)	3.2	6.9
	+Y.E.	35.4 (120)	2.9	19.1
30	control	29.5 (>168)	6.5	5.9
	+Y.E.	37.2 (96)	5.3	13.0
150	control	34.2 (144)	7.5	9.4
	+Y.E.	42.4 (96)	20.5	11.7
300	control	38.4 (120)	15.2	7.2
	+Y.E.	40.1 (96)	20.8	9.8

[a] Dissolved solids were calculated from specific gravity measurements.
[b] Figures in parentheses indicate the number of hours required to complete the fermentation.

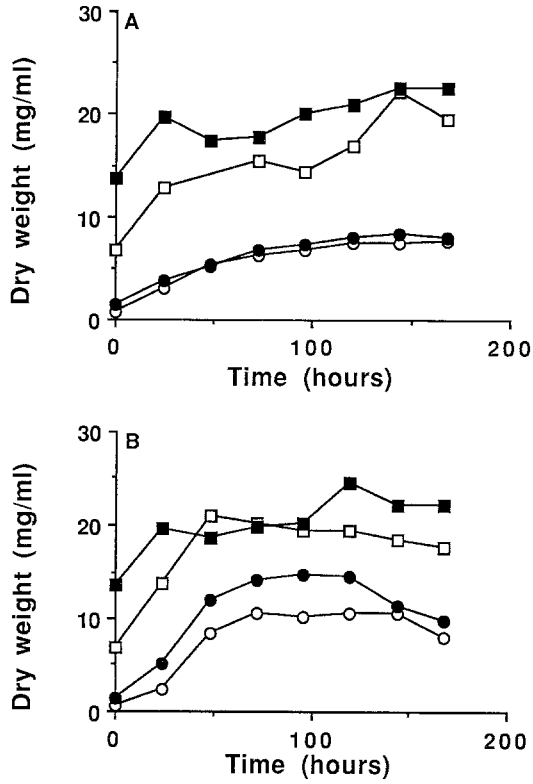

Fig. 4. Changes in the biomass of the yeast cells pitched at different rates during the fermentation of very high gravity wheat mashes without nutrient supplement (A), and with 2% yeast extract as nutrient supplement (B) at different pitching rates. Number of millions of active dry yeast cells pitched per g of mash were: ○, 15; ●, 30; □, 150; and ■, 300.

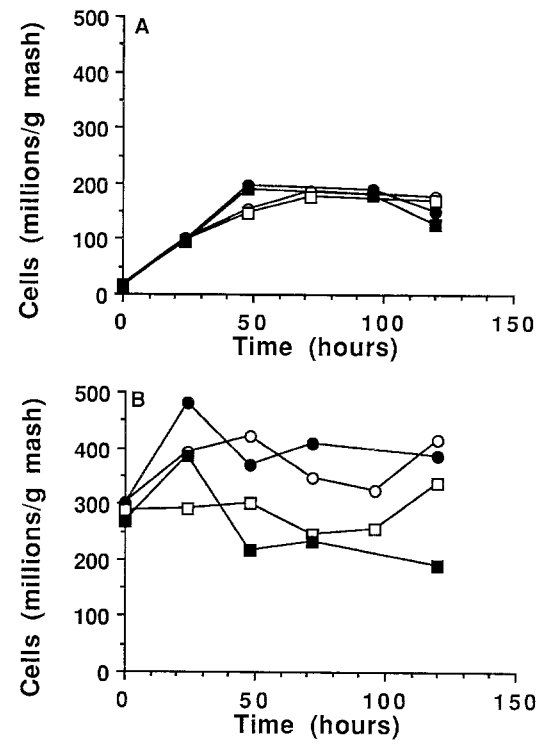

Fig. 5. Rate of multiplication and viability of yeast cells during the fermentation of very high gravity wheat mashes pitched at 15 million cells per g of mash (A), and 300 million cells per g of mash (B). Total and viable counts were determined by the microscopic method using methylene blue as the vital stain as described in the text. Symbols: ○, total and □, viable number of cells without any nutrient supplement; ●, total and ■, viable number of cells with 2% (w/w) yeast extract as nutrient supplement.

ples there was no appreciable loss of cell viability. At high pitching rates the level of cell multiplication was small. At a pitching rate of 300 million cells per g, the cells multiplied 1.4-times in the absence of yeast extract supplement, while the cell number increased 1.6-times in its presence.

Irrespective of the pitching rates, about 180 million new cells were produced in yeast extract supplemented mash. This is in agreement with the concept that the mash will support a certain number or mass of new yeasts depending on the composition. The assumption that cells do not grow or multiply at very high pitching rates is not substantiated by our results. Even at the pitching rate of 750 million cells per g of mash, there was a small degree of cell multiplication (data not shown). At low pitching rates viability of the cells was maintained at high levels (Fig. 5A). In contrast considerable loss of viability was observed at high pitching rates (Fig. 5B).

Although about the same number of new cells were produced at low and high pitching rates the average sizes of the cells in the population examined at 72 h were different. The mean sizes and the corresponding standard deviations of the populations pitched at 15 and 300 million cells per g of mash were $124.4 \pm 17.5\ \mu m^3$ and $109.3 \pm 24.7\ \mu m^3$, respectively. Results obtained with biomass determination are in agreement with this observation (Fig. 4). For example, at the low pitching rate of 15 million cells per g mash, the biomass increased by 9.9 mg (dry wt) per ml, while at high pitching rate of 300 million cells/ml, the increase was only 6.3 mg per ml, although in both cases the number of newly formed cells was about the same. At low pitching rate the cell population had a normal size distribution, while most of the cells in the samples pitched at high rates were small and the size distribution of the cells was skewed to the left.

Pitching VHG wheat mashes (400 g reducing sugar per liter) at high rates resulted not only in rapid fermentation but yielded greater amounts of ethanol (Table 3). Supplementing the mash with 2% (w/v) yeast extract did not make a difference in the rate or final yield of ethanol at the

TABLE 3

Relationship between pitching rates and ethanol yield during fermentation of very high gravity (VHG) wheat mashes

Pitching rate (million cells/g mash)	Sugar consumed (g reducing sugar/100 ml)	Ethanol (% v/v)	Theoretical yield (%)
30	30.2	17.4	89.2
300	31.9	19.0	92.2
750	34.1	21.5	97.6

highest rate of 750 million cells per ml. In these experiments, active dry yeast was added in the dry form (19.5 g per l) rather than as a dilute suspension to prevent excessive dilution of the mash. At this pitching rate an ethanol yield of 21.5% (v/v) was obtained. Assuming that all the sugars in the mash were monosaccharides it can be calculated that this concentration of ethanol is equal to a theoretical yield of 97.6% of the sugar consumed.

DISCUSSION

Under the experimental conditions used in this study the yield of ethanol appeared to approach the maximum possible with respect to sugar concentration and ethanol tolerance. All but 60 g of the 400 g of the reducing sugar present per l of wheat mash was consumed at the pitching rate of 750 million cells per g of mash. Ethanol produced accounted for 97.6% of the sugar consumed. In contrast to this, the efficiency of conversion of sugar to ethanol at lower pitching rates varied from 89.2% at 30 million cells per g of mash to 92.2% at a pitching rate of 300 million cells per g of mash. This is in agreement with the results reported by O'Connor-Cox and Ingledew [14] who observed that during the fermentation of a 16°P brewer's wort, the ethanol yield was increased up to 0.8% (w/v) when the inoculation rate was raised 5-fold. It is known for some time that ethanol productivity can be improved by increasing the cell density (pitching rate) [2,9], although several workers reported that at low pitching rates, ethanol productivity was independent of cell density [6,18]. The highest inoculation rates used by these workers, however, were considerably less than that used in this study and those of other workers [4,11].

Many reasons may be attributed for the increased production of ethanol at high pitching rates. Among them is the observation that the severity of inhibition of yeast growth by ethanol decreases as the inoculation rate is increased [21]. It is also known that as the inoculum level is increased both the specific growth rate of the yeast [18] and the biomass yield decrease [22]. Under such conditions, lesser amounts of carbohydrates would be diverted for the production of biomass and more would be available for conversion to ethanol.

We have consistently observed that at very high pitching rates such as 750 million cells per g of mash there was considerable loss of cell viability. Similar observations have been made during the rapid fermentation of 25° Brix honey [12]. In one instance these workers observed that only 2.1% of cells in the population remained viable at the end of a fermentation period of 2.5 to 3.0 h. Our studies showed that 30 to 40% of the cells in the population lost viability at the highest pitching rate. Nagodawithana and Steinkraus [12] reported that the loss of viability could be prevented by increasing the supply of oxygen to the fermentor, but for some unexplained reason this resulted in prolonging the fermentation time. This suggested that the loss of viability somehow facilitated the rate of fermentation. Our observations that at a pitching rate of 750 million cells per g of mash no nutrient supplement was needed (or rather nutrient supplement in the form of yeast extract did not seem to have an effect) to produce maximum amounts of ethanol, seems to suggest that the cells that lost viability soon lysed and liberated enough nutrients for the viable yeast cells to grow and continue their metabolic activity. This conclusion is further strengthened by the fact that when there was no loss of viability as in the case of low pitching rates, nutrient supplement in the form of yeast extract was required in the wheat mash to obtain maximum amounts of ethanol.

ACKNOWLEDGEMENTS

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