

## Effect of Dissolved Oxygen, Temperature, Initial Cell Count, and Sugar Concentration on the Viability of *Saccharomyces cerevisiae* in Rapid Fermentations<sup>1</sup>

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By using  $7 \times 10^8$  cells of *Saccharomyces cerevisiae* per ml with which 25° Brix honey solutions were fermented to 9.5% (wt/vol; 12% vol/vol) ethanol in 2.5 to 3 h at 30 C, i.e., rapid fermentation, the death rate was found to be high, with only 2.1% of the yeast cells surviving at the end of 3 h under anaerobic conditions. As the dissolved oxygen in the medium was increased from 0 to 13 to 20 to 100% in rapid fermentations at 30 C, there was a progressive increase in the percentage of cells surviving. The ethanol production rate and total were not seriously affected by a dissolved oxygen concentration of 13%, but fermentation was retarded by 20% dissolved oxygen and still further decreased as the dissolved oxygen content reached 100%. When the fermentation temperature was decreased to 15 C (at 13% dissolved oxygen), the rate of fermentation decreased, and the fermentation time to 9.5% ethanol (wt/vol) increased to 6 h. It was found that the higher the temperature between 15 and 30 C, the greater the rate of death as initial cell counts were increased from  $1.1 \times 10^7$  to  $7.8 \times 10^8$  cells per ml. At the lowest level of inoculum,  $1.1 \times 10^7$  cells per ml, there was actual multiplication, even at 30 C; however, the fermentation was no longer rapid. The addition of 15% sugar, initially followed after an hour by the remaining 10%, or addition of the sugar in increments of 2.5 or 5% yielded a better survival rate of yeast cells than when the fermentation was initiated with 25% sugar.

"Rapid fermentations" are defined as fermentations in which the ethanol content increases from 0 to 9.5% (wt/vol; 12% vol/vol) in 6 h or less. For example, Steinkraus (11) reported that at a temperature of 25 C, honey solution or grape juice (25° Brix sugar concentration) was fermented to 9.5% (wt/vol) ethanol within a 4-h period. The high rate of ethanol production was achieved primarily by using a yeast concentration of  $3 \times 10^8$  cells per ml. A fermentation time of 6 h or less makes continuous fermentation feasible. However, a problem that severely limits the possibility of applying rapid fermentation to continuous fermentation has been the high death rate of yeast cells.

For continuous fermentation, it is essential that yeast viability (as a percentage of the initial yeast charge) remains at 100% in rapid fermentation, and it is desirable that some low level multiplication occurs continuously to replace dead cells and to replace those lost during centrifuging of the product and during the recycling of cells to the fermentor.

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Although there have been numerous studies on the effect of temperature on growth and death of cells, some having dealt with the effect of temperature on viability of yeast suspensions (14, 15), specific information regarding the effect of temperature on viability during rapid alcoholic fermentation appears to be lacking.

At incubation temperatures of 32 C and higher, *Saccharomyces cerevisiae*, Steinberg strain 618, failed to achieve as high alcohol concentrations in a 25° Brix clover honey medium as it did at lower fermentation temperatures (11).

The role of oxygen in alcoholic fermentations has been reviewed (7). Experience has demonstrated that air or oxygen is essential for good brewery fermentation. Oxygen acts primarily as a terminal acceptor of electrons from the respiratory chain. It also acts as a yeast growth factor. Oxygen appears to be involved in the synthesis of oleic acid and ergosterol, which stimulate yeast growth under anaerobic conditions (1).

In brewery fermentation, 20% oxygen saturation of the wort prior to pitching resulted in the maximal production of yeast cells (7). Above

that level, increases of cell mass were small, and below that level production of yeast cells was directly related to oxygen supplied. It may be that the yeast cells have the ability to retain the effect of oxygen within the cell and use it anaerobically later (2). The amount of ethanol produced during fermentation is independent of the amount of oxygen supplied prior to pitching.

A high degree of aeration results in an increase of biomass, i.e., multiplication and less ethanol production (8). *Brettanomyces claussenii* showed a higher rate of ethanol production in air than in nitrogen (M. T. J. Custers, Ph.D. thesis, Delft Univ. of Technology, Delft, The Netherlands, 1940). The term "negative Pasteur effect" was introduced for the phenomenon to emphasize the striking contrast to the well-known Pasteur effect. A similar phenomenon was reported in intact cells of *S. cerevisiae* (16).

Generally, when yeast multiplication is desired, as with bakers' yeast, aeration is used and sugar is added in small increments. This eliminates repression of cell growth by high sugar concentration and insures oxygen availability to the cell. Yeast in a glucose medium consumed oxygen at a very slow rate until glucose was nearly all used (13). Thus, growth in the presence of glucose took place as though the yeast was under anaerobic conditions. Bakers' yeast ferments more than 80% of the sugar to ethanol in spite of vigorous aeration (5). Thus, the classical Pasteur effect does not appear to operate under conditions of yeast growth. Słonimski (9) called this the counter-Pasteur effect. It describes the relationship between the rate of synthesis of respiring enzymes and the rate of fermentation. Yeast growing aerobically in glucose medium does not rapidly synthesize its respiratory enzymes (3). Most of the energy for growth comes from glycolysis.

The counter-Pasteur effect is one aspect of what used to be called the glucose effect and is now described as catabolite repression (6). This is the inhibitory effect of glucose on the synthesis of enzymes, which do not immediately contribute to the growth of the cell. At glucose concentrations above 3%, cellular mechanisms for the formation of nucleic acids and proteins are saturated. Consequently, the cell becomes rich in adenosine 5'-triphosphate and intermediates such as pyruvate. The cells, therefore, have no need to tap any extra energy from the citric acid cycle, and to prevent unnecessary tapping of energy the biosynthesis of respiratory enzymes is repressed.

The level of aeration supplied to the yeast before or during fermentation is related to the

viability of the yeast. There appears to be no information in the literature dealing specifically with the effect of oxygen on the viability of yeasts and on the rates of fermentation under conditions of rapid fermentation.

Both batch and continuous fermentation data (F. F. Pironti, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1971), with an 18% (wt/vol) glucose concentration at 30 C, have shown that air does have a protective effect against product inhibition. By vacuum fermentation, it has also been shown that alcohol is not the only factor contributing to inhibition of growth or fermentation. Substrate inhibition has been shown to have a relatively minor effect compared to the product inhibition at high concentrations of sugar. Pironti's calculations indicated that rates of sugar accumulation intracellularly were higher than the corresponding rate of sugar consumption from batch data.

This study was undertaken to determine the effects of controlled oxygen levels, temperature, number of yeast cells inoculated, and addition of sugar in increments rather than in total on the percentage of initial cells inoculated remaining viable during rapid fermentation.

#### MATERIALS AND METHODS

**Yeast strain.** All experiments were conducted with a beer strain of *S. cerevisiae* kindly supplied by Mario Frati, Genesee Brewing Co., N.Y. The yeast was obtained as a solid paste containing from  $3.85 \times 10^9$  to  $4.04 \times 10^9$  cells per g. The yeast was suspended in citrate-phosphate buffer (pH 6.8) and centrifuged to recover the cells used in the experiments.

**Yeast counts.** Based upon the number of yeast cells desired in the inoculated medium, a given weight, generally 1 kg, of yeast paste was slurried with 1 liter of the medium. After thorough dispersion, a 1-ml sample was serially diluted and plated (two plates per dilution) to obtain the initial viable count. The culture medium used for pour plates contained 1.5% maltose, 1.5% malt extract (Difco), and 1.5% agar (Difco). The plates were incubated at 30 C for 48 h, and the final colony count was taken as the average of the two plates for the dilution containing 30 to 300 colonies per plate. Calculations involving cell populations were based upon viable counts. The percentage of viability was determined by dividing the viable count at the desired fermentation time by the initial viable count.

**Fermentation medium.** Clover honey stored at 1 C was diluted with water to provide the 25° Brix sugar substrate. A basal level of 0.25 g of Actiferm per liter (Budde and Westermann, New York, N.Y.), a yeast vitamin mixture, was added to the fermentation medium, and this level is referred to as X. The 0.25 g of Actiferm per liter contained: biotin, 12.5  $\mu$ g; pyridoxine, 250  $\mu$ g; meso-inositol, 1.87 mg; calcium pantothenate, 2.5 mg; thiamine, 5 mg; peptone, 25 mg; and ammonium sulfate, 215 mg. The medium

was also supplemented with 1.0 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g of  $\text{K}_2\text{PO}_4$ , 0.2 g of  $\text{MgCl}_2$ , 0.05 g of  $\text{NaHSO}_4$ , and 5.0 g of citric acid per liter as per formula I of Steinkraus and Morse (11). The basic level of mineral salts-citric acid supplement was referred to as Y. Certain fermentations in this study were conducted using a 2Y level of mineral salt-citric acid supplement and 4X concentration (1 g/liter) of vitamins (Actiferm). The pH of the fermentation medium was adjusted to 4.2, and it was pasteurized at 76.5 C for 30 min and cooled to fermentation temperature before inoculation.

**Apparatus.** (i) Microferm laboratory fermentor model 214 (New Brunswick Scientific Co., New Brunswick, N.J.) was used. A fermentor sampler (New Brunswick model S 21) was used to sample medium during fermentation.

(ii) Dissolved oxygen (DO) controller model DO-60 (New Brunswick Co.) was used in conjunction with the above to measure and control the oxygen level.

(iii) The fermenting medium was sampled at desired intervals, and cell-free supernatants were obtained for ethanol determinations by centrifuging at  $14,000 \times g$  for 10 min in a Sorvall centrifuge model SS-3. All batch fermentations were continued until essentially complete.

(iv) A Carle gas chromatograph, model 9000 equipped with a flame-ionization detector, was employed for the separation and quantitation of ethanol. A stainless-steel column (3 feet  $\times$   $\frac{1}{8}$  inch [91.44  $\times$  0.32 cm] outer diameter and 0.085 inch [0.214 cm] inner diameter) fitted into the instrument to provide on-column injection. The column packing was Poropak Q-S, 100/120 mesh. The column oven was operated isothermally at 150 C. The carrier gas was nitrogen at a flow rate of 20 ml/min. The combustion gases were hydrogen and air, at flow rates of 21 and 300 ml/min, respectively. Sensitivity of the instrument for the analysis was maintained at  $2 \times 10^{-9}$  A full scale. A 2- $\mu$ liter sample was injected into the instrument with a no. 701 Hamilton syringe.

The gas chromatograph was connected to a Hewlett-Packard integrator, model 3370A, to determine the area of the ethanol and internal standard curves. Visual display of the chromatogram was accomplished by means of a Houston "Omniscribe" strip chart recorder with a sensitivity of 1 mV full scale and a chart speed of 1 inch/min (2.54 cm/min).

Standard solutions of ethanol were prepared. The standards contained 0.5, 1.0, 1.5, and 2.0% ethanol (wt/vol). A 2% (wt/vol) acetone solution was used as the internal standard. The areas of the peaks were determined by the electronic integrator. The area ratio of the ethanol to the internal standard was calculated as follows: area ratio = area of the ethanol peak/area of internal standard peak. The standard curve was prepared by using the method of least squares. The following equation resulted in: percent ethanol =  $a + b$  (peak area ratio of unknown), where  $a$  = intercept of standard curve and  $b$  = slope of standard curve. Knowing the intercept and the slope, a simple calculation gave the percentage of ethanol in the unknown sample injected. When the ethanol concentrations were high, the samples were diluted 1:5 or 1:10 depending on the concentration, and 1 ml

was added to 1 ml of the internal standard. The resulting mixture was analyzed and the percentage of ethanol obtained was multiplied by the dilution factor (L. R. Mattick, Agr. Exp. Station, Geneva, N.Y., and A. C. Rice, Taylor Wine Co., Hammondsport, N.Y., unpublished data).

(v) A temperature-compensated refractometer (American Optical Co., Buffalo, N.Y.) was used to determine the percentage of sugar and other soluble solids in degrees Brix.

**Method of calculating ethanol molecules produced per cell per second.** The number of viable cells at different time intervals during the course of the fermentation was determined by serial dilution and plating, as described above, and the results were plotted.

Also, the ethanol concentrations, in grams per 100 ml, were determined at the same time intervals by centrifuging the yeast cells and analyzing the ethanol content of the supernatant in the gas chromatograph. The grams of ethanol produced during a specific fermentation time were changed to ethanol molecules by multiplying the moles of ethanol produced times Avogadro's number ( $6.02 \times 10^{23}$  molecules per mol).

The grams of ethanol produced per 100 ml was plotted against time. By drawing a tangent at the desired point on the curve, it was possible to get the slope or instantaneous rate of ethanol production at that time. Dividing the rate of ethanol molecules produced by the number of viable cells at the same time yielded the number of ethanol molecules being produced per cell per unit of time.

## RESULTS AND DISCUSSION

**Effect of DO in the substrate and temperature on yeast viability during rapid fermentation.** The percentage of DO in the medium was found to have a considerable influence upon the percentage of cells remaining viable under conditions of rapid fermentation at 30 C. The percentage of yeast cells surviving increased from 2 to 13 to 34 to 60% as the DO content in the substrate was increased from 0 to 13 to 20 to 100% (Fig. 1). The ethanol concentration reached the desired 9.5% (wt/vol; 12% vol/vol) in the substrates containing 0 and 13% DO after 3 h, but reached 8.6 and 7.3% ethanol (wt/vol) in the fermentations in which the DO was maintained at 20 and 100%, respectively, after 5.5 h (Fig. 2).

At 15 C, nearly all the yeast cells survived rapid fermentation at 13 and 20% DO concentration in the substrate (Fig. 3), and, at 15 C and 100% DO, there was actually multiplication of the yeast with the final count reaching 195% of the initial population. The ethanol concentration at 0 and 13% DO content reached 9.5% (wt/vol) ethanol after 6 h at 15 C; but, similar to experiments conducted at 30 C, the ethanol content reached only 5.7 and 4.0% (wt/vol) after

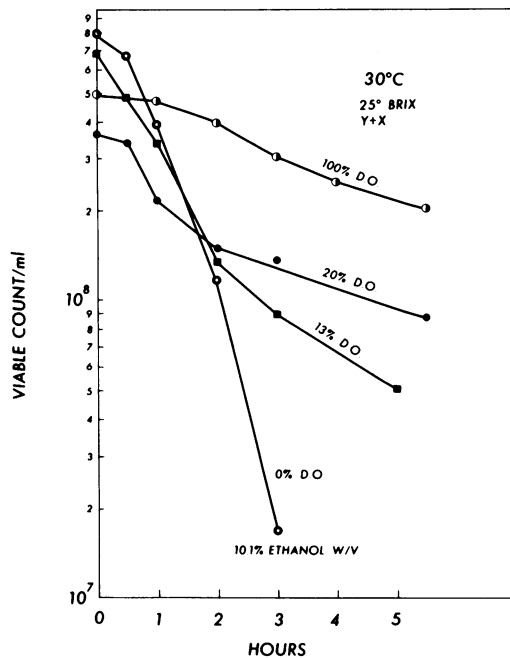


FIG. 1. Effect of the percentage of DO in the medium on the viability of yeast cells during rapid fermentation at 30 C.

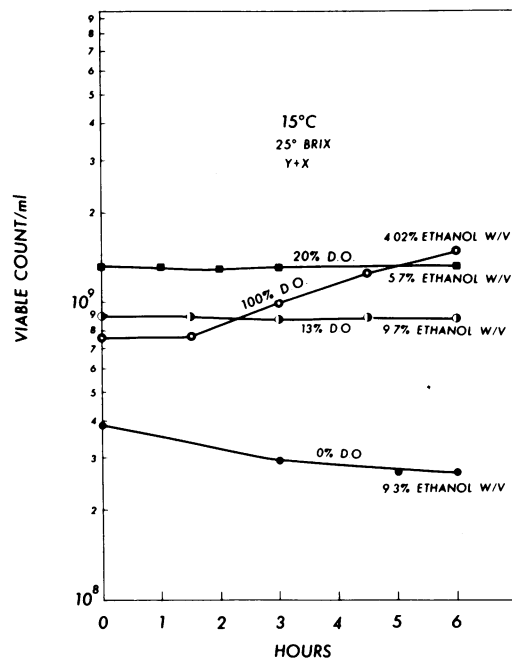


FIG. 3. Effect of the percentage of DO in the medium on the viability of yeast cells during rapid fermentation at 15 C.

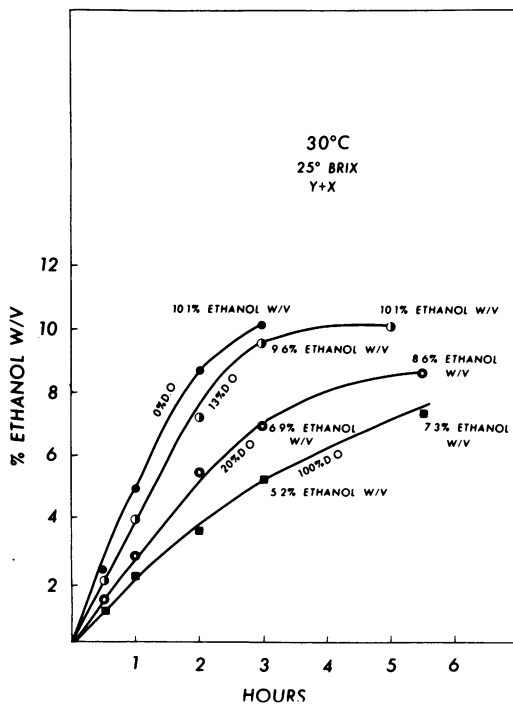


FIG. 2. Effect of the percentage of DO in the medium on the percentage of ethanol produced during rapid fermentation at 30 C.

6 h at 15 C in fermentations where DO was maintained at 20 and 100%, respectively.

Anaerobically (0% DO), only 2.1% of the cells survived rapid fermentation to 9.5% ethanol (wt/vol) at 30 C, whereas 84% of the cells survived anaerobic fermentation at 15 C. Whereas 13% of the cells survived rapid fermentation to 9.5% ethanol (wt/vol) at 30 C when the DO was increased to 13%, 94% of the cells survived under similar conditions at 15 C.

Rapid fermentations carried out at 30 C with a cell concentration of  $7 \times 10^8$  cells of *S. cerevisiae* per ml and a sugar substrate of 25° Brix honey solution, with nutrients added at the X + Y concentration with a DO content of 13%, reached 9.5% (wt/vol; 12% vol/vol) ethanol concentration in approximately 3 h (Fig. 4). Corresponding time intervals taken for similar fermentations at 13% DO carried out at 25, 20, and 15 C were approximately 4, 5, and 6 h, respectively. Whereas 13% of the initial cells survived rapid fermentation to 9.5% ethanol (wt/vol) after 3 h at 30 C (13% DO), 48, 80, and 94% of the initial cells survived the fermentation to 9.5% ethanol (wt/vol) at 25, 20, and 15 C (13% DO), respectively (Fig. 5). Thus, survival of yeast cells in rapid fermentation was greatly improved by increasing the DO content of the substrate to 13% and by lowering the fermentation temperature to 15 C.

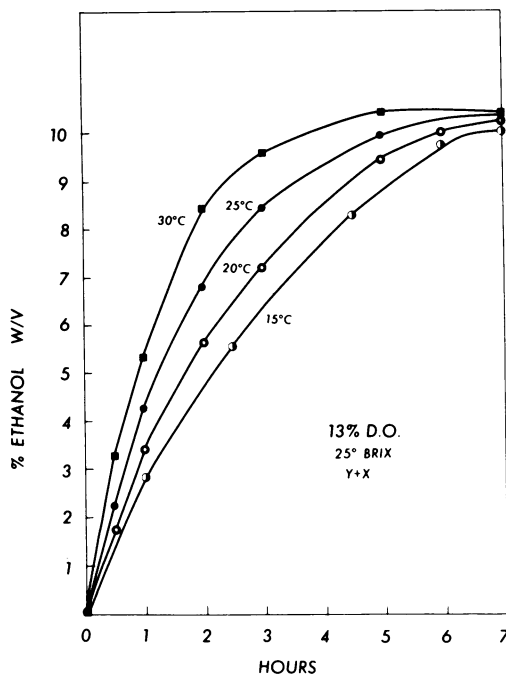


FIG. 4. Effect of temperature of the fermenting medium on the rate of ethanol production during rapid fermentation at 13% DO.

These initial studies demonstrated that the lower the fermentation temperature, the better the retention of viability of the yeast. It also was noted that the greater the aeration, the better the retention of cell viability; and, as DO content approached 100% at 15 C, a good rate of cell multiplication was reached with the yeast count nearly doubling after a 6-h fermentation. However, as DO content increased above 13%, the rate of fermentation was inhibited, and fermentation time was extended beyond 6 h, the time arbitrarily set as a maximum for rapid fermentation. The best conditions for rapid fermentation on the basis of the initial studies were fermentation at 15 C and 13% DO, under which 94% of the cells inoculated remained viable after the ethanol concentration reached 9.5% (wt/vol) (6 h). This survival rate would not be acceptable for continuous fermentations in which it is necessary to maintain 100% viability and, preferably, a slight rate of multiplication to offset losses of yeast which must be recovered from the output of the fermentor.

**Effect of initial cell count on viability during rapid fermentation.** To insure that rapid death was not due to the deficiency of any nutrients required for growth or fermentation, the medium was supplemented with twice the usual quantity of ammonium salts, phosphate,

and magnesium, along with four times the usual quantity of vitamins generally required in a regular fermentation. The DO was maintained at 13% to insure that oxygen was not limiting. At 15 C, the cells, even at the high initial cell population of  $8.3 \times 10^8$  cells per ml, showed slight multiplication with the added nutrients (Fig. 6). Thus, the initial cell population by itself was not responsible for the rapid death of cells. The rate of multiplication was progressively increased as the initial cell count was decreased from  $1.61 \times 10^8$  to  $5.9 \times 10^7$  to  $1.03 \times 10^7$  cells per ml at 15 C. However, the fermentations at the two lower levels were no longer rapid. At 30 C, the higher the initial yeast count, the greater the rate of death as the initial cell counts were increased from  $1.1 \times 10^7$  to  $8.0 \times 10^8$  cells per ml (Fig. 7). The cells inoculated at the high level of  $7.8 \times 10^8$  cells per ml died rapidly at 30 C, even though the nutrients and oxygen were not apparently limiting. The loss of the viability of cells inoculated at an intermediate population ( $1.52 \times 10^8$  cells per ml) was not nearly as prominent at 30 C as it was in the fermentation with high cell population. Using an inoculum in the range of  $1.1 \times 10^7$  cells per ml, it was found that a high level multiplication occurred both at 15 C and 30 C, with the highest maximum population at 15 C (Fig. 6

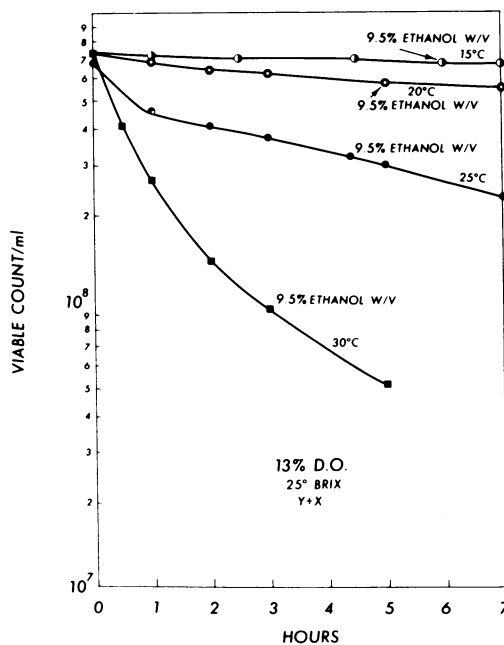


FIG. 5. Effect of temperature of the fermenting medium on the percentage of cells surviving during rapid fermentation at 13% DO.

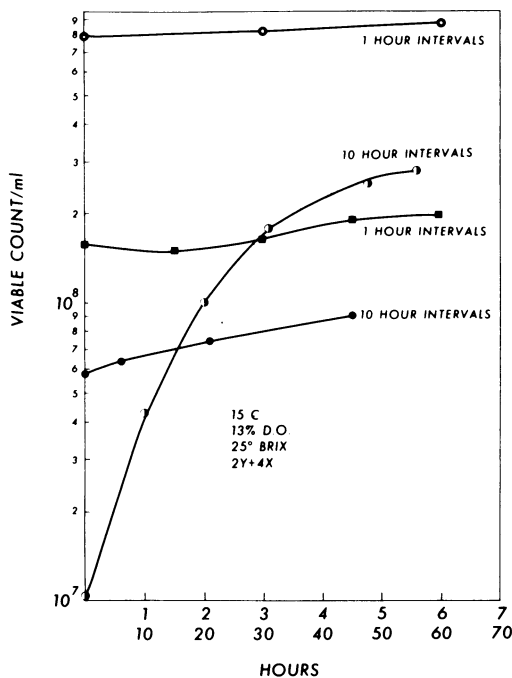


FIG. 6. Effect of initial cell count of yeast cells on the percentage of cells surviving during rapid fermentation at 13% DO and 15 C.

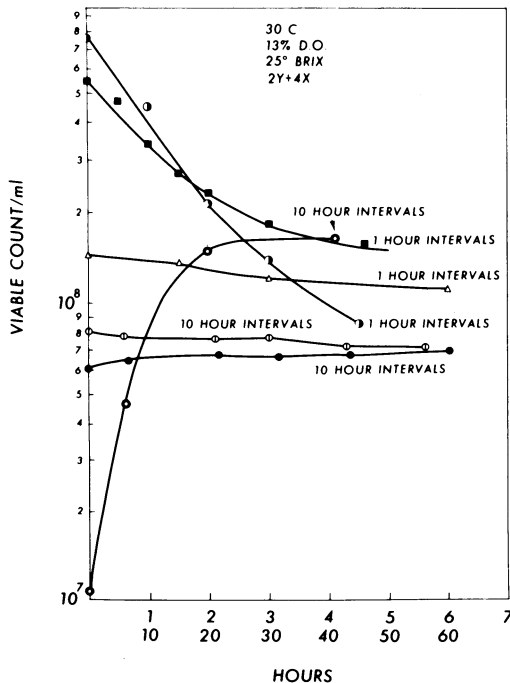


FIG. 7. Effect of initial cell count of yeast cells on the percentage of cells surviving during rapid fermentation at 13% DO and 30 C.

and 7). Thus, the initial cell population was found to be an important factor in determining whether or not multiplication, population leveling, or moderate or high death rates occurs at 30 C. It should be noted, however, that below  $1.5 \times 10^8$  cells per ml, the fermentation was no longer rapid, even at 30 C. The above results suggested that it might be possible to get a slight multiplication of yeast cells in a fermentation at 30 C by selecting an initial yeast count between  $1.1 \times 10^7$  cells per ml, where considerable multiplication occurred, and  $1.52 \times 10^8$  cells per ml, where there was slight death at 30 C. The value of the initial cell count, which would result in a slight degree of multiplication of yeast cells in a fermentation at 30 C, was calculated graphically, and the cell levels calculated were confirmed experimentally.

**Method of calculation.** It was observed that the percentage of viability at 30 C increased from 54 to 62 to 92 to 135% (after 1 h) and 13 to 29 to 74 to 262% (after 4 h) as the initial cell counts for the fermentations were decreased from  $7.8 \times 10^8$  to  $5.8 \times 10^8$  to  $1.52 \times 10^8$  to  $1.1 \times 10^7$  cells per ml.

When the initial cell count of these fermentations was plotted against the percentage of viability at the end of 1 and 4 h, a relationship was observed between the initial cell count and the percentage of viability at the particular hour. These two lines intersected at the same point where there should be neither death nor multiplication at an initial cell count of  $7.3 \times 10^7$  cells per ml (Fig. 8). The percentages of viability of 262%, obtained after 4 h for the fermentation initiated with  $1.1 \times 10^7$  cells per ml, was higher than the value expected from the graph. However, it should be noted that this was no longer a rapid fermentation. The fermentation to 7.0% (wt/vol) ethanol using  $1.1 \times 10^7$  cells of inoculum per ml required 74 h, at which time 1,536% viability (15-fold increase) of cells was obtained.

Fermentations were run with  $6.3 \times 10^7$  and  $8.2 \times 10^7$  cells per ml initial cell count to verify the validity of the calculated value of  $7.3 \times 10^7$  cells per ml, which was expected to maintain a stable population at 30 C. Viability figures of 100.8% and 99.2% at the end of 1 h and 103% and 98.8% at the end of 4 h were observed for the two respective fermentations (Fig. 8). This confirms the calculations, which were very close to what was observed experimentally. However, again these were not rapid fermentations, and 58 and 60 h were required to reach an ethanol concentration of 9.2% (wt/vol) and 9.4% (wt/vol), at which time the viabilities were 114% and 90.3%, respectively. This part of the study

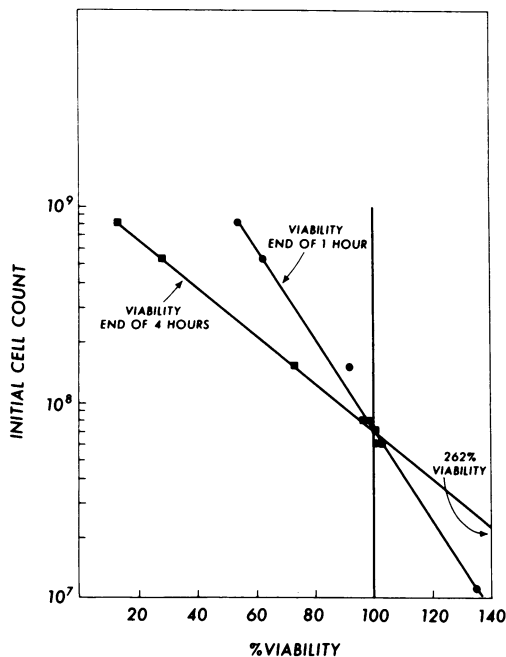


FIG. 8. Effect of initial cell count on the percentage of viability after 1 and 4 h of fermentation at 30 C.

demonstrates that, if fermentation time is not of prime significance, conditions of fermentation can be selected which will enable the fermentation to be conducted at 30 C while maintaining a stable yeast population. However rapid fermentations, i.e., a fermentation time less than 6 h, could not be carried out at 30 C with maintenance of yeast viability.

**The effect of sugar concentration on yeast viability.** Adding the 25% sugar in increments of 2.5, 5, or even 15% initially, with 10% sugar added after 1 h, resulted in improved viabilities of 83, 70, and 41%, respectively, compared with 16% viability for anaerobic (0% DO) fermentation started with 25° Brix sugar and continued for 3 h at 30 C, at which time the ethanol content had reached 9.5% (wt/vol; 12% vol/vol; Table 1).

The use of 13% DO resulted in a considerable improvement in viability (41 to 79%) at 30 C when the sugar was added 15% at the start with 10% added after 1 h (Table 1). Using an initial concentration of 25° Brix sugar, there was no improvement in viability with an increase of DO from 0 to 13%. With 5% sugar increments, the viability was 70%, even in the absence of oxygen, and it improved moderately (79%) with use of 13% DO. Similarly, the use of 2.5% sugar increments resulted in still higher viability (83%) anaerobically and the use of 13% DO

increased viability to 88%. The improvement in viability, with lower levels of sugar added incrementally, can be explained on the basis that the yeast under conditions of aeration at 30 C in a high-sugar medium does not rapidly synthesize its respiratory enzymes due to catabolite repression and, hence, in spite of aeration, has to depend on the glycolytic pathway for its energy requirements. In a rapidly fermenting cell, a further feed-back control on the glycolytic pathway may be expected due to product inhibition. This could cause a depletion of the energy level in the system, which could ultimately bring about a curtailing of the metabolic activity causing death to the cell.

By use of incremental feeding, the concentration of sugar in the medium has been maintained at a low level permitting respiratory enzymes to function whenever energy is in short supply. This would lower the rate of ethanol production in the cell and may contribute to the higher percentage of viability maintained during incremental feeding.

At 15 C, under anaerobic conditions (0% DO), the addition of sugar in increments of 2.5, 5, or 15% initially, followed by 10% after 2 h, resulted in viabilities of 95, 91, and 80%, respectively, compared with 71% viability for the fermentation started with 25° Brix sugar concentration and continued for 6 h, at which time the ethanol content had reached 9.5% (wt/vol). The improved viability, following the addition of sugar incrementally at 15 C anaerobically, also can be accounted for by a decrease in substrate inhibition. Furthermore, at 15 C a lower rate of metabolic activity can be expected. Thus, the

TABLE 1. Effect of adding sugar incrementally on yeast viability under anaerobic and aerobic conditions

Increments <sup>a</sup>	Viability <sup>b</sup> (%)			
	Anaerobic (0% DO)		Aerobic (13% DO)	
	15 C, 6 h	30 C, 3 h	15 C, 6 h	30 C, 3 h
25% at zero time . . . . .	71	16	109	16
15% at zero time, 10% after 1 h . . . . .	80	41	110	79
5% at 90-min intervals (15 C) and 45-min intervals (30 C) . . . . .	91	79	119	79
2.5% at 10-min intervals (15 C) and 20-min intervals (30 C) . . . . .	95	83	122	88

<sup>a</sup> Mineral salts added at level 2Y; Actiferm (vitamins) at 4X.

<sup>b</sup> At 9.5% (wt/vol) ethanol.

energy requirements also may be much less than at 30 C, and the energy derived from glycolysis could be sufficient to keep the cells alive. At higher concentrations of sugar, substrate inhibition may affect the rate of glycolysis restricting the energy supply for the survival of yeast cells. This deficiency with respect to energy may ultimately cause death to the cell even though the death rate was lower than that observed at 30 C.

At 15 C, under aerobic conditions (13% DO), the addition of sugar in increments of 2.5, 5, or 15% initially, followed by 10% after 2 h, resulted in viabilities of 122, 119 and 110%, respectively, compared with 109% viability for the fermentation started with 25° Brix sugar concentration and continued for 6 h, at which time the ethanol content reached 9.5% (wt/vol) (Table 1). The higher degree of cell multiplication at lower incremental rates may again be due to the influence of oxygen on viability when catabolite repression is minimal at low sugar concentrations.

**Rates of ethanol produced per cell per second.** The rates of ethanol molecules produced in batch fermentations per yeast cell per second, at different temperatures, are presented in Table 2. Rates of ethanol production ranged from about  $10^7$  to nearly  $3 \times 10^8$  molecules of ethanol produced per cell per s. The higher the temperature in the range of 15 to 30 C, the faster the rate of ethanol production. The rates of ethanol production gradually decreased as ethanol concentration increased to 9.5% (wt/vol; 12% vol/vol).

An anomaly was observed in the rates of ethanol produced per cell per second at 30 C. The rate of ethanol production per cell per second appeared to reach its highest point at 2

h, although the highest rate of death was encountered after 30 min at 30 C. We have no explanation of this anomaly at this time.

The rates of ethanol molecules produced per cell per second have proven very useful. For example, at 15 C the overall average rate was  $4.7 \times 10^7$  ethanol molecules produced per cell per s. If the desired ethanol concentration was 9.5% (wt/vol; 12% vol/vol), the yeast cell must produce 9.5 g of ethanol per 100 ml in the time interval specified. The 9.5 g of ethanol is 9.5/46 mol or  $1.24 \times 10^{23}$  molecules. The number of cells required to produce that many molecules in 6 h ( $2.16 \times 10^4$  per s) at 15 C can be calculated from the following formula: (number of ethanol molecules desired/100 ml)/(time in seconds  $\times$  ethanol molecules produced per cell per second) = number of cells to inoculate per 100 ml, i.e.,  $(1.24 \times 10^{23}) / (2.16 \times 10^4 \times 4.7 \times 10^7) = 1.22 \times 10^{11}$  cells per 100 ml or  $1.22 \times 10^9$  cells per ml.

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TABLE 2. Ethanol molecules produced per viable cell per second in fermentations at several temperatures at different stages of fermentation

Fermentation time (h)	Ethanol molecules produced per cell per second			
	30 C <sup>a</sup>	25 C	20 C	15 C
0.5	$2.1 \times 10^8$	$1.4 \times 10^8$	$8.8 \times 10^7$	$7.3 \times 10^7$
1	$2.4 \times 10^8$	$1.2 \times 10^8$	$7.1 \times 10^7$	$5.7 \times 10^7$
2	$2.9 \times 10^8$	$9.2 \times 10^7$	$5.2 \times 10^7$	$4.2 \times 10^7$
3	$1.2 \times 10^8$	$5.8 \times 10^7$	$4.1 \times 10^7$	$3.8 \times 10^7$
4	$1.1 \times 10^8$	$4.1 \times 10^7$	$3.3 \times 10^7$	$3.3 \times 10^7$
5	$4.6 \times 10^7$	$2.5 \times 10^7$	$2.3 \times 10^7$	$2.6 \times 10^7$
6		$1.4 \times 10^7$	$6.4 \times 10^6$	$1.3 \times 10^7$

<sup>a</sup> Fermentation temperature.



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