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Multiplication and Fermentation of *Saccharomyces cerevisiae* Under Carbon Dioxide Pressure in Wine

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ABSTRACT

KUNKEE, RALPH E. (University of California, Davis), AND C. S. OUGH. Multiplication and fermentation of *Saccharomyces cerevisiae* under carbon dioxide pressure in wine. Applied Microbiol. **14**:643–648. 1966.—Conditions for rapid fermentation of sugar in wine under pressure were sought for use in continuous production of naturally fermented sparkling wine. Wine yeast growth and fermentation were measured under CO_2 pressure. The medium was white wine with added glucose. Pressure was very inhibitory to growth, especially at low *p*H or high alcohol concentration. Use of various strains of wine yeast, cultures of various ages, or cells adapted to wine did not give more rapid growth. Addition of nutrients increased growth, but under no conditions was growth rapid enough to bring about sufficiently rapid fermentation rates. Conditions for rapid fermentation were sought by use of high levels of cells as inocula. Fermentation rates in wine also were inhibited by pressure, and were dependent on *p*H and alcohol levels. Addition of nutrients did not increase the fermentation rate, but rapid fermentation rates were obtained, under pressure, by inoculation with high levels of cells adapted several weeks to the base wine. Thus, continuous sparkling-wine production might be practical with proper amounts of adapted cells used as inocula, or perhaps with reuse of the fermentation culture.

Production of naturally fermented sparkling wines by continuous processes have been reported (1, 2, 5, 6). These processes, in general, require the rapid fermentation under pressure (several atmospheres) of sugar that has been added to wine. In bottle or Charmat production of sparkling wine, the CO_2 pressure in the bottle or tank builds up during fermentation; however, in a continuous process, the fermentation must begin at a high pressure. Attempts in this laboratory to produce, on a small scale, sparkling wines by a continuous process have not thus far been successful (Ough, Amerine, and Kunkee, *unpublished data*). We found fermentation to be so greatly inhibited by high pressure that the continuous process was not practical. Some microorganisms can withstand very high pressures (15), but about 7 atm of pressure inhibits yeast metabolism, and 30 atm will kill yeast (4, 8, 13). The inhibitory effects have been used for the preservation of grape juice by use of the Böhi process (11). However, we have not found any examination of the effect of pressure on yeast added to wine. To obtain rapid fermentation under CO_2 pressure of sugar in wine, we examined yeast growth and fermentation under a variety of conditions under pressure.

MATERIALS AND METHODS

Cultures. Unless otherwise indicated, the yeast strain used was Montrachet strain (UCD Enology no. 522) of *Saccharomyces cerevisiae* var. *ellipsoideus*. Also used was the Champagne strain (no. 505) of the same variety.

Propagation of yeast. Yeast cells were usually propagated in grape juice. The juice was prepared from crushed and pressed 1964 Sémillon grapes grown in Davis [23° Brix, total acidity (as grams of tartaric acid per 100 ml) of 0.49]. The juice was stored at 0 C with the addition of 125 ppm of SO_2. Before use, the juice was filtered with Hyflo Super-Cel (Johns-Manville, New York, N.Y.) as filter aid, glucose was added to increase the degrees Brix from 22 to 24, and the *p*H was adjusted from 3.5 to 3.0 with HCl. The *p*H and degrees Brix were altered so that the yeast would be adapted to the least favorable conditions used in the wine experiments. To the autoclaved juice was added 150 ppm of SO_2 at least 1 hr before yeast inoculation. Starter cultures of yeast adapted to SO_2 were prepared by inoculation of 10 ml of the grape juice with yeast from a slant. About 4 days of shaking were required for turbid growth of starter cultures.

For yeast propagation, grape juice was inoculated with 1% yeast starter. Unless otherwise indicated in the text, for the multiplication experiments, yeast was propagated in shake flasks for 1 day and, for the fermentation experiments, for 2 weeks in stationary cultures. Yeasts were also propagated in base wine (see below) adjusted to *p*H 3.0, to which 2% (w/v) glucose, 1% (w/v) alcohol, and 75 ppm of SO_2 had been added. Propagation of cultures, unless otherwise indicated, was done at 25 C.

Base wine. White wine suitable as sparkling wine stock was prepared by standard methods of this laboratory from 1964 Folle Blanche grapes grown at Oakville. The juice was 20.7° Brix and had a total acidity (as grams of tartaric acid per 100 ml) of 0.84. The chemical analysis of the base wine was as follows: total acidity (as grams of tartaric acid per 100 ml), 0.63; volatile acidity (as grams of acetic acid per 100 ml), 0.021; *p*H, 3.15; reducing sugar (grams per 100 ml), 0.06; extract (grams of solids per 100 g), 2.6; alcohol (milliliters per 100 ml), 10.6; tannin (grams per 100 ml), 0.03; total SO_2, 75 ppm. The *p*H of the wine was lowered or raised by addition of either HCl or KOH. Alcohol concentration was lowered by addition of distilled water (6% original volume), or raised by addition of 95% ethyl alcohol (0.47 or 1.67% of original volume). Generally, no attempt was made to maintain a low oxidation-reduction potential of the base wine. When anaerobic conditions were desired, O_2 was removed from the base wine by N_2 stripping, and the wine was placed in flasks fitted with ground-glass stoppers connected to water traps. When indicated, 0.5% (w/v) yeast extract (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the base wine. Unless otherwise stated, all experiments with the base wine were made at 21 C. For all experiments, the base wine was supplemented with 2.5 to 2.8% (w/v) glucose and 75 ppm of SO_2.

Pressure. Samples to be fermented under pressure were contained in heavy-walled ("Champagne") bottles, and sealed with rubber stoppers fitted with 316 stainless-steel pressure valves. The rubber stoppers were wired into the neck of the bottles. The bottles were pressurized with gas from cylinders of commercial CO_2. It generally took several days to saturate completely the wines with gas. The pressure valves also accommodated gauges for measurement of pressure. Bottle pressures were measured daily and adjusted when necessary. Pressures were maintained at about 2.5 or 5 atm (about 37.5 or 75 psi above atmospheric pressure).

Yeast counts. Concentration of yeast cells was determined microscopically with the use of a Levy-Hausser counting chamber. Budding cells were counted as two cells whenever the daughter cell was nearly the same size as the mother cell. Concentration of viable cells was determined by colony formation on solid medium of 10% (w/v) Fleischmann's Diamalt and 2% (w/v) agar.

Glucose. Glucose concentration was determined enzymatically with Glucostat (Worthington Biochemical Corp., Freehold, N.J.). Wine samples were diluted 100-fold in distilled water for assay. Concentions are given in per cent (w/v).

Other analyses. Density, alcohol, reducing sugar, *p*H, total acidity, volatile acidity, and tannin content were determined according to the methods of Amerine (3).

RESULTS

Yeast multiplication. The effect of *p*H and alcohol on multiplication of yeast was determined at various pressures (0, 2.5, and 5 atm). The base wine was lowered to *p*H 3.0 and raised to *p*H 3.3 and 3.7 (see Materials and Methods). Yeast cells were added to give a concentration of 5×10^4 cells per milliliter, and the cell concentration was determined over a period of about 1 month. The results are given in Table 1. Both the multiplication rate and maximal cell concentration obtained were inhibited at low *p*H at atmospheric pressure; more than 2 weeks were required for 100-fold increase in cells as compared with 10 days at the high *p*H. At increased pressures, the effects of *p*H were also evident, but the pressure, itself, had a greater inhibitory effect. At 2.5 atm, at low *p*H, there was only about a 10-fold increase in the number of yeast cells, and, at 5 atm, cell concentration never reached this amount. At high *p*H and intermediate pressure, the growth response was about the same as at low *p*H and atmospheric pressure. Anaerobic conditions (see Materials and Meth-

TABLE 1. *Effect of pH and pressure on yeast multiplication*[a]

Pressure	Maximal cell concn obtained (cells/ml)		
	*p*H 3.0	*p*H 3.3	*p*H 3.7
Atmospheric pressure	7×10^6 (17)[b]	1×10^7 (12)	1.1×10^7 (10)
2.5 atm	7×10^5	4×10^6	7×10^6 (15)
5 atm	2×10^5	3×10^5	5×10^5
Anaerobic at atmospheric pressure	8×10^6	5×10^6	5×10^6

[a] All samples at 11% (v/v) alcohol.

[b] Numbers in parentheses refer to days required for 100-fold increase in cell concentration (from 5×10^4 to 5×10^6 cells per milliliter).

ods) at atmospheric pressure were inhibitory to yeast multiplication, except at the lowest *p*H level (as compared with controls).

Table 2 shows the effects on yeast growth of alcohol concentration [adjusted as given in Materials and Methods to give 10, 11, and 12% (v/v)]. These samples were maintained at 5 atm of pressure. All samples were inhibited at this pressure, as compared with multiplication at atmospheric pressure (see Table 1). The extent of inhibition was dependent on alcohol concentration and *p*H.

The results in Tables 1 and 2 show that pressure is so inhibitory that adjustments in *p*H and alcohol concentration do not allow yeast multiplication to be as rapid as at atmospheric pressure. Even at the lowest alcohol and highest *p*H level, yeast multiplication under pressure never reached 100-fold increase.

The rate of adaptation of yeast to the base wine was undoubtedly an important factor. To find conditions for more rapid adaptation of yeast in wine under pressure, cells were propagated in a variety of ways. Table 3 gives the effects of pressure on yeast multiplication in wine inoculated with yeast propagated for various lengths of time in grape juice or base wine media. One might expect either that the younger cultures might be more vigorous and able to adapt more quickly to the base wine or that the medium of the older cultures might be more like the composition of the base wine to which the yeast cells were transferred. The results in Table 3 show very little differences in yeast multiplication, even when wine was used as a propagation medium for the yeast. The addition of yeast extract, as a nutrient, was effective; nearly a 100-fold multiplication of yeast was observed, but this multiplication was still not as high as that at atmospheric pressure (see Table 1).

Champagne yeast strains are usually used in sparkling-wine production. These are highly flocculent strains, and probably should be avoided in a continuous process. When the Champagne strain was tested, its multiplication was very much like that of the Montrachet strain (Table 3).

Fermentation. It was apparent from the above experiments that pressure was so inhibitory to yeast growth that high concentrations of yeast in the base wine under pressure could be obtained only by heavy inoculation rather than by cell growth. In the following experiments, high concentrations of yeast were added to the base wine, and the rates of fermentation of glucose were measured. In Table 4 are given the inhibitory effects of pressure on fermentation rate, at three different values of *p*H, with 3×10^6 cells per milliliter as initial yeast concentration. Strong inhibition of fermentation at low *p*H and high

TABLE 2. *Effect of alcohol on yeast multiplication under pressure*[a]

Alcohol (v/v)	Maximal cell concn obtained (cells/ml)		
	*p*H 3.0	*p*H 3.3	*p*H 3.7
%			
10	6×10^5	3×10^5	3×10^6
11	2×10^5	3×10^5	5×10^5
12	1.5×10^5	3×10^5	3×10^5

[a] All samples were maintained at 5 atm; initial inoculum of 5×10^4 cells per milliliter.

TABLE 3. *Effect of method of propagation and nutrients on yeast multiplication under pressure*[a]

Propagation		Maximal multiplication under pressure[b] (cells/ml)	
		Yeast extract	
Method	Final count (cells/ml)	0	0.5%
Montrachet strain			
Shake culture, 1 day	8.0×10^7	6×10^5	
Shake culture[c], 1 day	2.0×10^8	3×10^5	
Stationary culture, 7 days	9.2×10^7	5×10^5	3×10^6
Stationary culture in wine[d], 3 days	3.4×10^6	5×10^5	3×10^6
Champagne strain			
Shake culture, 1 day	1.4×10^8	5×10^5	

[a] Yeast cells were propagated in grape juice, unless otherwise indicated, with an initial inoculum of 2×10^7 cells per milliliter.

[b] All samples at 11% (v/v) alcohol, *p*H 3.3, and maintained at 5 atm. Initial inoculum was 5×10^4 cells per milliliter.

[c] Inoculated with 2×10^5 cells per milliliter.

[d] Propagated in base wine with added glucose.

TABLE 4. *Effect of pressure, pH, and nutrients on fermentation*

Initial count (cells/ml)	pH	Alcohol (%, v/v)	Per cent glucose remaining after 3 weeks		
			0 atm	5 atm	
				Yeast extract	
				0	0.5%
3×10^6	3.0	11	1.4	2.7	
3×10^6	3.3	11	0.5	2.5	
3×10^6	3.7	11	0.3	2.2	
3×10^6	3.3	12	2.7	2.3	
1×10^7	3.3	11		1.5	1.5

TABLE 5. *Effect of temperature on fermentation*[a]

Pressure maintained	Per cent glucose remaining after 3 weeks at		
	10 C	16 C	21 C
atm			
0	2.5	1.5	<0.5
2.5	2.4	2.0	1.3
5	2.5	2.2	1.9

[a] All samples at pH 3.3, alcohol concentration 11%, and initial inoculum of 10^7 cells per milliliter.

alcohol, in wine, was apparent even at atmospheric pressure. At higher levels of added yeast (an initial concentration of 10^7 cells per milliliter), some fermentation did take place under pressure. Addition of nutrients (yeast extract) had no effect on fermentation rate under these conditions.

The above experiments were all done at 21 C. At lower temperatures, the solubility of CO_2 is greater, and the pressure resulting from the same concentrations of CO_2 would be correspondingly lower (7, 13). Fermentation rates also would be lower at lower temperature, but at lower temperature the inhibitory effect of pressure might be less than the inhibitory effect of temperature. In Table 5 are given the fermentation rates at three different pressures and temperatures. Large inocula of yeast were used (10^7 cells per milliliter). At the end of 3 weeks, the only samples showing a large drop in glucose concentration were those at the highest temperature or at atmospheric pressure.

The effect of adaptation of the inoculum to the base wine was determined. The cells were propagated in the base wine (see Materials and Methods) under 5 atm of pressure for 5 weeks at 21 C. Figure 1 shows the fermentation rates with these cells as compared with the rates obtained from cells propagated in grape juice. The initial cell concentration was the same for both (10^7 cells per milliliter). With cells adapted to wine, the glucose was nearly gone by 11 days, but in the control there was very little loss of glucose.

In another experiment, it was determined whether pressure was required for the adaptation to the base wine. Cells were propagated in wine

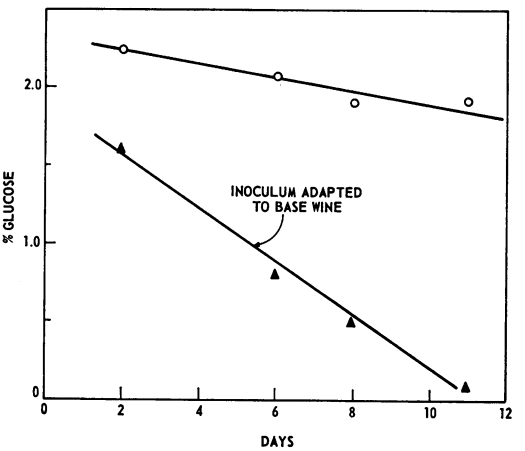

FIG. 1. *Fermentation of glucose in wine under CO_2 pressure by yeast propagated in base wine and propagated under 5 atm of CO_2 pressure in grape juice. Inoculum from grape juice (○) was 10^7 cells per milliliter (5×10^6 viable cells per milliliter), and inoculum from base wine (▲) was 6×10^6 cells per milliliter (2×10^6 viable cells per milliliter).*

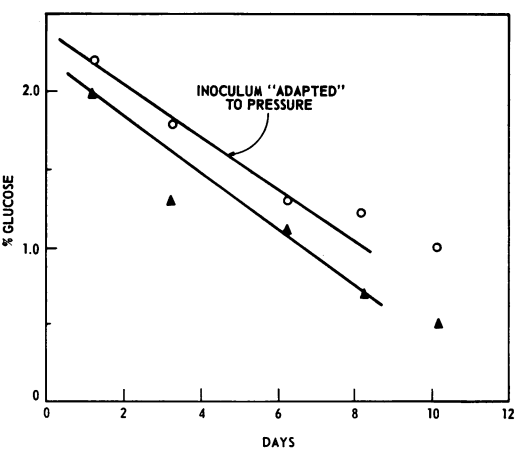

FIG. 2. *Fermentation of glucose in wine under CO_2 pressure by yeast propagated in wine with and without 5 atm of CO_2 pressure. Inoculum from propagation under pressure (○) was 5×10^6 cells per milliliter, and inoculum from propagation at atmospheric pressure (▲) was 1.5×10^6 cells per milliliter.*

for 2 weeks, either at 0 or 5 atm of pressure. About equally rapid fermentation rates were observed with inocula of cells propagated under pressure and those propagated without pressure (Fig. 2).

DISCUSSION

In these experiments, it was shown that the inhibitory effects of pressure on yeast multiplication were so great that even under the most favorable conditions, i.e., with low alcohol, high *p*H, and added nutrients, yeast multiplication was very slow. In the reported continuous production of naturally fermented sparkling wine (6), the concentration of cells in the first fermentation tank was 3×10^7. At 5 atm of pressure, maximal cell concentration would never reach this high concentration if an inoculum of, for example, 1% of this were used (that is, with 3×10^5 cells per milliliter as initial inoculum). For rapid fermentation, large amounts of yeast must be added to the pressure tanks. The Russian workers report (6, 7) the use of freshly propagated yeast grown under aerobic conditions and apparently not adapted to the base wine. In our experiments, rapid fermentation rates under pressure were obtained only by use of yeast adapted to the base wine; adjustment of *p*H and alcohol concentration or addition of nutrients did not bring about as rapid a fermentation rate when unadapted yeast cells were used. The most rapid fermentation rate obtained in our experiments produced a drop in glucose concentration from 2.5 to about 0.1% in 11 days (Fig. 1), or a fermentation rate of about 0.009% per hour. This seems to be rapid enough to be practical for a continuous process. In any tank, the concentration of glucose is dependent on both the rate of decrease of glucose, because of fermentation, and the rate of increase of glucose, because of inflow of medium:

$$\frac{dG}{dt} = (G_i - G_e)D - f$$

where dG/dt is the instantaneous rate of change in glucose concentration in the tank, G_i is the concentration of glucose entering the tank, G_e is the concentration of glucose leaving the tank, D is the dilution rate ($D = F/V$, where F is the flow rate of liquid through the system and V is the volume of the tank), and f is the fermentation rate (*see* 10). Under steady-state conditions, where the inflow of glucose is exactly balanced by the fermentation of glucose, $dG/dt = 0$ and G_e is the concentration of glucose in the tank. Thus:

$$f = \frac{F}{V}(G_i - G_e)$$

From the Russian example (1, 6), a drop of 0.5% sugar was obtained in the first tank, where F = 7 dkl/hr and V = 500 dkl. Solving for f, a fermentation rate of 0.007% per hour is obtained.

We are considering methods by which it might be practical to propagate large amounts of yeast adapted to the base wine. Reutilization of cultures in this case may be practical, as has been demonstrated in some continuous processes (14). Lüthi et al. (12) found under high alcohol, 13%, that cells used in submerged flor sherry production could be recovered and reused.

The rapid fermentation rates under pressure obtained by the Russian workers (1, 6) with aerobically propagated yeasts may be explained on the basis of concentration of sugar used. In our experiments, we desired rapid fermentation of a low concentration of glucose to produce sparkling wine of maximal dryness. In the cited procedure (1, 6), rapid fermentation of part of high concentrations of sugar was obtained, resulting in a comparatively sweet product. Rates of fermentation of low concentrations of sugar have been shown to be much slower than those of moderately higher concentrations (9).

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LITERATURE CITED

1. AGABALIANZ, G. G., A. A. MERZHANIAN, AND S. A. BROOSILOVSKI. 1963. Continuous champagne production method patented. Wines and Vines **44**(6):49–50. (U.S. Patent 3062656, Nov. 6, 1962.)
2. AMERINE, M. A. 1963. Continuous fermentation of wines. Wines and Vines **44**(8):27–29.
3. AMERINE, M. A. 1965. Laboratory procedures for enologists. Univ. California, Davis.
4. AMERINE, M. A., AND C. S. OUGH. 1957. Studies on controlled fermentations. III. Am. J. Enol. **8**:18–30.
5. BROUSSILOVKY, S. A. 1963. Essai industriel du procédé de la champagnisation du vin en continu. Bull. Office Intern. Vin. **36**:206–217.
6. BROUSSILOVSKY, S. A. 1963. Amélioration du procédé de la champagnisation du vin en continu. Bull. Office Intern. Vin. **36**:319–340.
7. DEINHARDT, H. 1961. Zur Löslichkeit von Kohlensäure in Wein. Deut. Wein-Ztg. **97**: 68–72.
8. GEISS, W. 1950. Gezügelte Gärung im Hochdruck-Tank. Deut. Wein-Ztg. **86**:425–435.
9. HOPKINS, R. H., AND R. H. ROBERTS. 1935. The kinetics of alcoholic fermentation of sugars by

brewer's yeasts. II. The relative rates of fermentation of glucose and fructose. Biochem. J. **29**:931–936.

10. JAMES, T. W. 1961. Continuous culture of microorganisms. Ann. Rev. Microbiol. **15**:27–46.
11. JENNY, J. 1952. Les bases scientifiques de la conservation des jus sans alcool de raisin et la fruits à pépins sous pression d'acid carbonique. Absorption, pression, concentration. Ind. Agr. Aliment. (Paris) **69**:687–700.
12. LÜTHI, H. R., B. STOYLA, AND J. C. MOYER. 1965. Continuous production of flor sherry from New York State wines. Appl. Microbiol. **13**:511–514.
13. SCHMITTHENNER, F. 1949. Die Wirkung der Kohlensäure auf Hefen und Bakterien. Wein-Wiss. Beil. Fachz. Deut. Weinbau **3**:147–187.
14. SHICHIJI, S., AND N. FUTAI. 1964. Continuous fermentation by a yeast reuse method using sedimentary yeast. III. Physiological characteristics of yeast cells during long periods of continuous fermentation. Kogyo Gijutsuin, Hakko Kenkyusho Kenkyu Hokoku **26**:1–27.
15. STANIER, R. Y., M. DOUDOROFF, AND E. A. ADELBERG. 1963. The microbial world, 2nd ed., p. 359. Prentice-Hall, Inc., Englewood Cliffs, N.J.