Storage of Brewing Yeasts by Liquid Nitrogen Refrigeration

A. M. WELLMAN AND G. G. STEWART

Department of Plant Sciences, University of Western Ontario, and Beverage Science Department, Labatt Breweries of Canada Ltd., London, Ontario, Canada

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Many yeast strains are difficult to maintain in culture in a stable state, and long-term preservation by lyophilization, which has proved useful for other fungi, has given poor results with brewing yeasts. As an alternative to continuous subculture, which maximizes strain variability, various methods of cryogenic storage were investigated. Yeast strains were frozen with or without cryoprotectants (such as glycerol or inositol) and stored at −196 C. Recovery after warming was estimated from plate counts, and survivors were screened to detect changes in the frequency of morphological types, respiratory-deficient mutants, and glycerol-sensitive mutants. Strains varied in their sensitivity to freezing, and survival was modified by the growth medium, the freezing munstrua, and the freezing conditions. Suspension of cells in 10% (vol/vol) glycerol, cooled at 1 C/min, warmed rapidly and plated on malt-yeast extract-glucose-peptone agar produced the highest percentage of viable colonies with a minimal change in metabolic characteristics. In two of the strains tested, no significant increase in mutation rate was detected under any of the treatments; the strains were maintained in a stable state and were metabolically comparable to unfrozen strains. In one strain of Saccharomyces uvarum after some freezing treatments, the percentage of respiratory-deficient mutants increased markedly, the fermentation rate declined, and a loss of flocculation occurred. The freezing parameters which increased the level of respiratory-deficient cells should be avoided in maintaining this strain. Maintenance of cultures of brewing yeasts by cryogenic storage has several advantages over other preservation techniques: the method is simple and reproducible, the cultures have remained stable over a 3-year test period, and the viability is high.

In the brewing industry, an important aspect of quality control is the maintenance of stock cultures with a minimum of strain variability. The necessity to return periodically to the stock culture arises from the accumulation of spontaneous mutants in the rapidly growing populations of yeast cells during fermentation, which after a time may noticeably alter the characteristics of a brew (5, 18). It is also a precautionary measure against possible contamination with wild-type yeasts or bacteria. Changes such as partial loss of ability to grow in wort and to ferment fructose, glucose, sucrose, maltose, or maltotriose and poor settling of bottom-growing yeasts are relatively common. Consequently, a batch of yeast is only used about 12 times before it is discarded, and a new starter culture is prepared from the stock cultures (14). The simplest, and still most extensively used method, of maintaining yeast stock cultures is on slants of brewer's wort (Wo), malt extract (M), or malt-yeast extract-glucose-peptone (MYGP) agar, stored at 5 to 10 C (15) or at 20 to 25 C under sterile medicinal-grade liquid paraffin (11). Alternatively cultures may be kept in MYGP broth at 0 C (12). Subculturing at regular intervals, usually less than 4 months, is necessary. With repeated serial subculture, genetic variants occur (1, 22), although these are reduced in liquid broth at 0 C, where recombination during spore formation is inhibited. Odintsovo (20) compared the morphological and physiological characteristics of strains of Saccharomyces ellipsoideus stored for periods of 3 and 10 years in the sporing states or by other methods and reported no change in any parameter measured in cultures stored as spores, whereas variation in one or more parameters was found in cultures stored on agar slants, in liquid media, or in the lyophilized state.
To reduce the labor of repeated subculturing, in large collections more cultures are being stored in the freeze-dried state (4, 10, 13, 19, 22, 23, 25, 31, 32). The major disadvantages of this technique are low viability and the large number of petite variants in revitalized cultures (1, 33). Considerable loss may occur during the freeze-drying process, with little further loss during storage, provided the cultures are held at 5 C. Survival is greatly influenced by the suspending medium during freezing (9). High survivals immediately after drying have been reported with glucose, sucrose plus peptone, or sucrose plus sodium glutamate and PVP. Changes in nutritional requirements, e.g., vitamin deficiencies, have been found in 50% of the revitalized cultures (1), but significant alterations in more basic physiological characters such as fermentation, oxidation rate, flocculating ability have not been reported (33). In a recent review, Beech and Davenport (3) comment "while lyophilisation offers a convenient method for storing large numbers of cultures, it is by no means the perfect method for storing yeasts with completely unchanged characteristics."

There have been reports on the use of liquid nitrogen refrigeration as an alternative method of preservation of yeasts (6, 7, 16, 17, 26, 28), but this method has not been applied to a wide range of yeast strains, and its application for preservation of brewing yeasts required further investigation. The purpose of this study was to assess the viability and morphological and physiological stability of strains of S. uvarum and S. cerevisiae after various conditions of freezing, storage in liquid nitrogen, and warming. Particular attention was paid to strain composition in mixed cultures, to levels of respiratory-deficient mutants and glycerol-sensitive mutants, and to fermentation after freezing and warming.

MATERIALS AND METHODS

**Strains.** A number of strains of S. cerevisiae and S. uvarum (S. carlsbergensis) were studied; they are referred to in the text by their number in the Labatt Culture Collection. The purity of the cultures was investigated by using the giant colony technique (24). Yeast strain BB17 was found to consist of two distinct colony morphologies in a proportion of 75:25. Species identification was performed with the usual sugar fermentation tests (15).

All cultures were maintained on MYGP (30) slopes at 4 C until required, at which time fresh slopes were inoculated 5 days before use.

**Freezing and warming.** Cultures were grown on M, MYGP, or Wo agar slants for 5 to 7 days at 25 C either in test tubes (18 by 150 mm) or in 1.2-ml cryogenic ampoules. From cultures in test tubes, suspensions of stationary-phase cells (approximately 10⁶ cells per ml) in either 10% (vol/vol) glycerol or 5% (vol/vol) myo-inositol were prepared, and 0.2 ml of the cell suspension was pipetted into each cryogenic ampule. The ampoules were sealed and submerged in dye solution at 4 C to check for defective seals. The samples were either frozen slowly at a controlled rate of 1 C/min (17) or rapidly by immersion into liquid nitrogen, 9 to 17 C/s (29). Ampoules were stored in liquid nitrogen refrigerators (Linde LR-35-9) at −169 to −196 C, for various periods of time up to 3 years. To recover the cultures, the ampoules were warmed rapidly (at 12 to 23 C/s) in a water bath at 37 to 40 C for 2 min and then removed to room temperature. Frozen and warmed cultures were compared with replicate ampoules prior to freezing and with 5- to 7-day-old cultures maintained by serial transfer at 25 C.

**Viability.** Treated and control samples were serially diluted in sterile water, and 0.5 ml of each dilution was spread on each of three plates of M, MYGP, and Wo agar. Colonies were scored after 95 h at 30 C, in the dark, and compared with estimates of the total number of cells plated, as determined from hemocytometer counts.

**Strain composition of S. uvarum (BB17).** The giant colony morphology method of Richards (24) was used to distinguish two colonial forms in this mixed culture. Inoculum from revitalized and control cultures on MYGP agar plates was grown in MYGP liquid medium at 20 C for 3 days, and after suitable dilution with Ringers solution plated on Wo gelatin agar and incubated at 18 C for 3 to 4 weeks before being scored for colony morphology.

**Respiratory-deficient mutants.** Dilutions of treated and untreated cells were plated onto glucose-peptone-yeast extract agar, incubated at 25 C for 7 days, and then overlayed with triphenyl tetrazolium chloride (TTC) as described by Ogur et al. (21). Respiratory-deficient mutants retain their original color, whereas respiratory-sufficient colonies turned red within 3 h at 30 C.

**Glycerol-sensitive mutants.** The scoring of petites by the TTC test was compared with detection of cytoplasmic respiratory mutants by their failure to grow on glycerol. MYGP-grown colonies were replicated onto a comparable medium in which glycerol was substituted for glucose.

**Fermentation rate in hopped Wo.** Liquid nitrogen was stored and control cultures were propagated at 21 C in 10 liters of hopped Wo (13.8 Plato [1 Plato grams dry weight] of material in 100 g of solution at 20 C 30% corn adjunct) in a New Brunswick triple fermenter, with stirring (400 rpm) and aeration (1 liter of air/min) for 72 h. The yeast was harvested, washed with chilled distilled water, and pressed into a yeast cake on a membrane filter.

The pressed yeast was then inoculated into 2 liters of Wo (13.8; Plato; 30% corn adjunct) in an unsterilized glass cylinder. An inoculation rate of 0.3% (wet weight) cells (1.35 × 10⁶ viable cells/ml) and an
incubation temperature of 21 C were used. Immediately after the yeast had been inoculated, the Wo was oxygenated to a concentration of approximately 8 μg/ml.

Sequential Wo samples were taken throughout the course of the fermentation, and the total carbohydrate (8) level and concentration of yeast in suspension were determined on each sample.

RESULTS AND DISCUSSION

The growth media employed in this study were those routinely used for culturing brewing yeasts and were chosen to avoid additional selective pressures on the strains. When cells were grown on MYPG prior to and after freezing, although there was no obvious change in growth or morphological characteristics compared to other media, the loss after the freeze-thaw treatment was minimized, especially at the more rapid rate of cooling. Similar effects have been reported by Davies (6) for S. cerevisiae.

The viability of two strains of S. uvarum and one of S. cerevisiae after various treatments is summarized in Table 1. A four-way factorial design (ANOVA) of the transformed data (Table 2), coupled with Wilcoxon’s signed rank tests, showed that strains of S. uvarum BB17 and BB32 were significantly different from S. cerevisiae 169A. The freezing rates, 1 C/min or 9 to 17 C/s, were significantly different from each other and from the prefreeze control. Highest survivals were obtained with a freezing rate of 1 C/min. Of the freezing menstrua, Wo agar was significantly different from M, MYGP agar, and 10% (vol/vol) glycerol. Recovery expressed as a percentage of the prefreeze control was highest in cells frozen in 10% (vol/vol) glycerol and poorest in cells frozen on Wo agar. Inositol (5%, vol/vol) had little or no effect as a cryoprotectant. The unexpectedly high recovery of cells of S. uvarum and S. cerevisiae suspended in water during the freezing process has also been reported by Davies (6) and may be due to freeze resistance of cells grown in the presence of peptone and yeast extract. The low recovery of S. cerevisiae suspended in water during freezing and later plated on Wo or M agar is consistent with results obtained by Mazur (17) for a strain of S. cerevisiae. The improved recovery of S. cerevisiae cells plated onto MYGP after warming is significant and was noted in several treatments, although recovery medium had little or no effect on the S. uvarum strains. The results re-emphasize the complex interactions between previous growth conditions and freezing and warming response previously noted by Tsuji (28). For the strains of brewing yeasts investigated, maximal recovery has been obtained with MYGP-grown cells frozen in 10% glycerol or water at 1 C/min, warmed rapidly, and plated on MYGP medium. Over a 3-year test period, cultures have remained stable and the viability remained high.

The numbers of respiratory-deficient colonies, determined by the TTC test or glycerol-sensitive test, was slightly higher in postfreeze than in prefreeze treatments in S. cerevisiae 169A and S. uvarum BB32. However, the maximal percentage of respiratory-deficient colonies did not exceed 3.5% in prefreeze controls, 6.3% after freezing at 1 C/min, or 7.6% after freezing at 9 to 17 C/s. The small increase in respiratory deficient indicates some irreversible freeze injury. In S. uvarum BB17, which spontaneously produces a higher proportion of respiratory-deficient mutants than other S. uvarum strains (G. Stewart, unpublished data), there was a marked increase in respiratory-deficient colonies recovered after freezing and warming, especially with cells grown on a prefreeze MYGP medium and subsequently plated on M recovery medium (Table 3). Since after the freezing and warming treatments the same suspension was plated on all three recovery media, the effect of M recovery medium is highly significant and appears to be independent of freezing rate or the numbers of cells surviving each treatment.

Liquid nitrogen treatment had little or no effect upon the fermentation characteristics of S. cerevisiae 169A or S. uvarum BB32 (Fig. 1), but the fermentation rate of BB17 liquid nitrogen stored cultures was found to be decreased (due to the high level of respiratory-deficient mutants) when compared to untreated cultures (Fig. 2). This decreased rate of fermentation was also accompanied by a decreased concentration of yeast in suspension (Fig. 1); loss of the flocculation property in respiratory-deficient cultures, when compared to the wild type of this yeast strain, has previously been reported (27).

To retain maximal fermentative ability of strain BB17, it is necessary to use MYGP as the recovery medium and to select only wild-type colonies from the survivors after freezing and warming. Storage under liquid nitrogen did not effect other morphological characteristics such as colony form. The proportion of the two forms of colony in this mixed strain, as measured by the giant colony technique, were not affected by the freezing treatments.

In lyophilized cultures, the large numbers of respiratory deficient in revitalized cultures is a major disadvantage of the technique (1, 33).
### Table 1. Viability of brewing yeasts before and after freezing and warming

| Prefreeze medium (agar) | Freezing menstruum | Recovery medium | S. uvarum BB17 | BB32 | S. cerevisiae 169A |
|------------------------|--------------------|-----------------|----------------|------|------------------|
|                        |                    |                 | C  | LN₁  | LN₂  | %LN₁/C | %LN₂/C | C  | LN₁  | LN₂  | %LN₁/C | %LN₂/C |
| Wo                     | Wo                 | Wo              | 79.1 | 0.0  | 13.1 | 0.0  | 16.6  | 63.0 | 2.2  | 12.3 | 3.5  | 19.5  | 46.1  | 0.1  | 11.0 | 0.5  | 23.9 |
|                        | M                  | 72.6            | 1.9  | 10.1 | 2.6  | 13.9  | 38.4  | 1.3  | 16.2 | 3.4  | 42.2  | 63.9  | 0.9  | 11.9 | 1.3  | 18.6 |
| MYGP                   | 67.1              | 1.3             | 12.8 | 1.9  | 19.1 | 45.1  | 1.9  | 16.2 | 4.2  | 35.9  | 59.3  | 0.1  | 12.1 | 0.2  | 20.4 |
|                        | M                  | 79.0            | 3.0  | 53.2 | 3.8  | 67.3  | 75.7  | 1.0  | 37.2 | 1.3  | 49.1  | 66.8  | 0.1  | 25.7 | 0.2  | 38.5 |
| MYGP                   | 58.8              | 3.1             | 51.9 | 5.3  | 88.3 | 79.4  | 0.6  | 53.2 | 0.8  | 67.0  | 65.1  | 0.1  | 26.1 | 0.1  | 40.1 |
|                        | MYGP               | 57.7            | 0.1  | 49.0 | 0.2  | 84.9  | 72.3  | 0.2  | 58.0 | 0.3  | 80.2  | 50.9  | 0.1  | 25.9 | 0.2  | 50.9 |
|                        | M                  | 97.7            | 0.0  | 30.1 | 0.0  | 30.8  | 79.7  | 8.6  | 38.4 | 10.8 | 48.2  | 35.7  | 0.3  | 1.3  | 0.8  | 3.6  |
| MYGP                   | 87.6              | 2.2             | 31.1 | 2.5  | 35.5 | 60.4  | 12.9 | 37.8 | 21.4 | 62.6  | 40.6  | 0.2  | 4.5  | 4.9  | 11.1 |
|                        | MYGP               | 78.6            | 10.5 | 29.0 | 13.4 | 36.9  | 66.6  | 15.7 | 34.5 | 23.6 | 51.8  | 42.9  | 2.5  | 8.8  | 5.8  | 20.6 |
| MYGP                   | H₂O               | Wo              | 84.7 | 23.5 | 56.8 | 27.7 | 67.1  | 81.3 | 37.7 | 40.1 | 46.4  | 49.3  | 55.5 | 1.4  | 2.4  | 2.6  | 4.2  |
| MYGP                   | M                  | 79.3            | 23.0 | 58.3 | 29.0 | 73.5  | 69.0 | 34.1 | 59.2 | 48.4  | 85.8  | 60.6 | 2.6  | 8.6  | 4.3  | 14.2 |
| MYGP                   | 65.3              | 28.7            | 66.2 | 44.0 | 101.4 | 64.9 | 35.7 | 51.0 | 55.0 | 78.6  | 69.0  | 33.3 | 48.1 | 48.3 | 69.8 |
| MYGP                   | 10% v/v Glycerol   | Wo              | 43.5 | 0.9  | 34.9 | 2.1  | 80.2  | 49.6 | 26.1 | 49.2 | 52.7 | 99.1  | 42.8  | 2.1  | 11.4 | 4.9  | 26.6 |
| MYGP                   | M                  | 36.4            | 5.4  | 37.2 | 14.8 | 102.2 | 45.8 | 20.3 | 46.9 | 44.3 | 102.5 | 41.3  | 4.6  | 35.0 | 11.1 | 84.7 |
| MYGP                   | 38.0              | 10.0            | 32.6 | 26.3 | 85.8 | 46.3 | 20.5 | 49.4 | 44.3 | 106.7 | 37.6  | 4.2  | 41.9 | 11.2 | 111.4|
| MYGP                   | 5% v/v Inositol    | Wo              | 80.0 | 8.5  | 4.7  | 10.6 | 5.9  | 57.8 | 6.5  | 26.6 | 11.2 | 46.0  | 38.5  | 2.8  | 1.3  | 7.3  | 3.4  |
| MYGP                   | M                  | 57.1            | 6.6  | 5.7  | 11.6 | 9.9  | 62.1 | 4.8  | 28.8 | 7.7  | 46.4  | 55.2  | 4.7  | 2.0  | 8.5  | 3.6  |
|                        | MYGP               | 56.2            | 7.1  | 8.1  | 12.6 | 14.4 | 59.6 | 3.5  | 28.8 | 5.8  | 45.0  | 54.3  | 3.0  | 11.8 | 5.5  | 21.7 |

*Average of six replicates. Abbreviations: C, prefreeze (control); LN₁, freezing rate 9 to 17 C/s; LN₂, freezing rate 1 C/min.
TABLE 2. ANOV of data presented in Table 1*

| Factor | df | BB17 F  | BB32 F  | 169A F  |
|--------|----|---------|---------|---------|
| Freezing menstruum | 5  | 49.48, *** | 29.33, *** | 7.96, *** |
| Freezing rate | 2  | 668.28, *** | 351.93, *** | 287.78, *** |
| Interaction freezing menstruum and freezing rate | 10 | 26.39, *** | 9.88, *** | 4.96, ** |
| Recovery medium | 2  | 5.23, * | 0.49, NS | 9.20, ** |
| Interaction recovery medium and freezing menstruum | 10 | 1.20, NS | 0.66, NS | 3.58, * |
| Interaction freezing rate and recovery medium | 4  | 10.31, *** | 3.50, * | 2.44, NS |

*Significance; ***, 0.001; **, 0.005; *, 0.01. NS, not significant.

TABLE 3. Effect of liquid nitrogen refrigeration on mutation in S. warum BB17

| Prefreeze medium | Freezing menstruum | Recovery medium | Prefreeze control | Respiratory-deficient colonies (%) |
|------------------|--------------------|----------------|------------------|-----------------------------------|
| Wo               | Wo                 | Wo             | 3.3              | 0.7                               |
| Wo               | M                  | 1.0            | 2.2              | 11.0                              |
| M                | M                  | 1.0            | 2.2              | 10.0                              |
| MYGP             | MYGP               | Wo             | 0.0              | 7.0                               |
| MYGP             | Water              | 7.0            | 3.0              | 100.0                             |
| MYGP             | 10% (vol/vol) Glycerol | 7.0 | 3.0              | 6.0                               |
| MYGP             | 5% (vol/vol) Inositol | 7.0 | 3.0              | 5.0                               |

* 100-200 colonies scored.

Fig. 1. Effect of liquid nitrogen storage on the growth and flocculation characteristics of yeast strains in static culture.
two of the three strains tested, this factor was not a problem in liquid nitrogen preservation. With the optimal parameters as outlined in this paper, cryogenic storage is recommended for the maintenance of stock cultures of brewing yeasts—the method is simple and reproducible, the cultures have remained stable over a 3-year test period, and the viability is high.

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