Liquid Nitrogen Storage of Yeast Cultures Compared to More Traditional Storage Methods[1]

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ABSTRACT

Twelve brewing yeast strains were stored for a two-year period under a variety of conditions in order that an ideal method for the storage of such cultures could be determined. Liquid nitrogen storage was found to be the method of choice if cost and availability of liquid nitrogen were not significant factors. Cultures stored in liquid nitrogen had the lowest death rate and were the easiest to revitalize; the degree of flocculation, the fermentative rate, sporulation ability, and giant colony plate morphology were unaffected. The proportion of respiratory-deficient and glycogen-deficient mutants did not increase. Storage at 4° C on nutrient agar slopes subcultured every six months was the next method of preference. Lyophilization and other storage methods revealed yeast instability that varied from strain to strain.

Key words: *Brewer's yeast, Culture collection, Liquid nitrogen, Storage, Yeast*

The most important consideration in the maintenance of a culture collection of brewing yeasts is that the stored cultures and their subsequent progeny continue to accurately represent the strains initially deposited. A plethora of storage methods is available for yeast cultures. The current literature on the preservation of microorganisms has been recently reviewed by Heckly (9), who very succinctly states that

> the microbiologist needs to have a convenient method for maintaining organisms, for without such tools he or she is out of business. Similarly, a number of industries must maintain the culture used in the manufacture of their product, whether it be beer, wine, antibiotics, bread or milk products such as cheese or buttermilk.

Many yeast strains are difficult to maintain in a stable state, and long-term preservation by lyophilization, which has proved useful for other fungi, has been found to give poor results with some brewing yeasts (17,27). Barney and Helbert (3) have reported satisfactory lyophilization of a single brewing strain of *Saccharomyces uvarum (carlsbergensis)* by using a suspending medium of double strength skim milk or Mist. dessicans (12). A number of culture collections, for example the National Collection of Type Cultures in England maintains most of its collection of yeasts in the lyophilized state (14). However, as a probable reflection of some dissatisfaction with lyophilization, the collection is also maintained in liquid media at 4° C. The American Type Culture Collection maintains most of its cultures in the lyophilized state (2); however, it uses liquid nitrogen to store virus and phage cultures and all those cultures that survive lyophilization poorly. Indeed, Beech and Davenport (4) have commented,

> While lyophilization 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.

The objective of this study was to examine whether a single method could be recommended for the storage of production brewing yeast strains.

EXPERIMENTAL

Yeast Strains

Twelve polyploid brewing strains of undefined genotype were studied, including eight strains of *Saccharomyces cerevisiae*, three of *S. uvarum (carlsbergensis)*, and one of *S. diastaticus*. The purity of the cultures was ascertained using the giant colony technique of Richards (18). The strains were numbered according to the code in the Labatt culture collection (Table I). Further, six laboratory strains of defined genotype were tested using only the liquid nitrogen method with repeated freeze-thaw cycles (Table II).

Preservation Methods

All yeast cultures were grown for 72 hr at 21° C in 100 ml of malt-yeast extract-glucose-peptone (MYGP) broth (28); aliquots were then taken for the seven storage methods. Cell concentrations were determined by hemocytometer counts. To revive the cultures after storage, they were inoculated into 10 ml of MYGP broth overnight and then further subcultured to 100 ml of MYGP broth and shaken at 21° C for 72 hr.

Liquid Nitrogen Storage. Suspensions of stationary phase cells

[1] Presented at the 46th Annual Meeting, Minneapolis, MN, May 1980.

0361-0470/81/01001906/$03.00/0

(10^6 cells per milliliter) in MYGP containing 10% (v/v) glycerol were prepared and 0.5 ml of the suspension pipetted into each cryogenic ampoule. The ampoules were sealed and submerged in dye solution at 4° C to check for defective seals. The samples were frozen at a controlled rate of 1° C/min (27) and then stored in liquid nitrogen refrigerators (Linde LR-35-9) at −196° C. To recover the cultures, the ampoules were warmed rapidly (at 12–23° C/sec) in a 40° C water bath for 2 min and then removed to room temperature.

Lyophilization. MYGP slopes were inoculated and incubated at 21° C for one week. Aliquots of a suspension containing 2×10^{10} cells per milliliter in cryoprotective medium (7.5% glucose, 0.2% nutrient broth, 25 ml of water, 75 ml of horse serum) were lyophilized under vacuum on a Virtis dryer for 18 hr; the vials were sealed, checked for vacuum, and stored at 4° C.

Storage in Distilled Water. Stationary phase cells pregrown in MYGP were washed three times with glass-distilled water and 1 ml, containing about 1×10^6 cells, was pipetted into each vial (13). The ampoules were sealed by fusing the neck with a flame and were stored at 4° C.

Storage Under Oil. MYGP agar slopes were inoculated, incubated for one week at 21° C, layered with 4 ml of sterile mineral oil, and stored at 21° C.

Repeated Direct Transfers on Culture Media. MYGP slopes were inoculated, incubated at 21° C for one week, stored at 4° C for one week, then resubcultured to a fresh MYGP slope every two weeks. The slopes thus were subcultured a total of 52 times over the two-year period.

Long-Term Storage at 21° C—No Subcultures. MYGP slopes were inoculated, incubated at 21° C for one week, sealed with paraffin to prevent desiccation, and then stored at 21° C with no further subcultures for two years.

Long-Term Storage at 4° C— No Subcultures. Treatment was the same as above but storage was at 4° C.

Viability Stain

The fluorescent stain 0.01% aqueous primuline (7) was used to assess the viability of the cells.

TABLE I
Brewing Yeast Strains

Yeast Code Number[a]	Species	Wort Flocculation
1	*S. cerevisiae*	Coflocculent
2	*S. cerevisiae*	Coflocculent
3	*S. cerevisiae*	Nonflocculent
15	*S. uvarum (carlsbergensis)*	Flocculent
21	*S. uvarum (carlsbergensis)*	Slightly flocculent
24	*S. cerevisiae*	Flocculent
27	*S. uvarum (carlsbergensis)*	Nonflocculent
30	*S. cerevisiae*	Flocculent
125	*S. cerevisiae*	Flocculent
126	*S. cerevisiae*	Slightly flocculent
156	*S. cerevisiae*	Flocculent
164	*S. diastaticus*	Nonflocculent

[a] Labatt culture collection.

TABLE II
Laboratory Yeast Strains

Yeast Code Number[a]	Genotype
3,762	*a/α, MAL/MAL, FLO,/flo, mel/mel*
3,767	*a/α, MAL/MAL, FLO,/flo, MEL/mel*
3,773	*a, DEX*
3,778	*α, MAL* I, *FLO* I, *MEL*
1,206	*α, ade* 6, *trp* 1, *gal* 7, *arg* 4, *tyr* 1, *lys* 2, *MAL* 2
1,210	*a, ade* 1, *ura* 3, *trp* 1, *his* 2, *leu* 1, *FLO* 1

[a] Labatt culture collection.

Fermentation Tests—Shaken

Wort fermentability of each yeast culture was determined by incubating 100 ml of an 11.8° P wort (30% corn adjunct) with 2% (w/v) pressed yeast in a 300-ml conical flask on a New Brunswick gyratory shaker at 160 rpm for 96 hr at 21° C. Dextrin utilization was determined using the same fermentation method but with yeast pregrown in wort for 96 hr and shaking at 21° C for 120 hr. Fermentability levels of greater than 85% indicate the yeast's ability to utilize wort dextrins.

Flocculation Tests

Flocculation tests were carried out after growth of yeast strains in 1-L volumes of 11.8° P wort (30% corn adjunct) and 1-L volumes of defined medium (25) in 2-L flasks on a New Brunswick gyratory shaker at 160 rpm for 72 hr at 21° C. The cells were harvested by centrifugation, washed with deionized water, treated twice with 10*mM* ethylenediamine tetraacetic acid to remove adsorbed divalent ions from the cell surface, and rewashed four times with deionized water. The flocculation state of each culture was determined in deionized water at pH 4.0 and also in deionized water, pH 4.0, containing 80 μg of calcium ion per milliliter.

Respiratory-Deficient Mutants

Dilutions of cells were plated onto glucose peptone-yeast extract agar, incubated at 25° C for five days (500 colonies counted per sample), and then overlaid with triphenyl tetrazolium chloride as described by Ogur et al (16) and recommended by the subcommittee on microbiological controls of the ASBC (1). Respiratory-deficient (RD) mutants retain their original color, whereas respiratory-sufficient colonies turn red within 3 hr. All questionable colonies were tested for their ability to grow on lactate peptone-yeast extract agar. Inability to grow on media where lactate is the sole carbon source confirms the RD nature of the mutants.

Glycogen-Deficient Mutants

Dilutions of cells were plated onto glucose peptone-yeast extract agar, incubated at 25° C for five days and then overlaid with a solution of 0.2% iodine in 0.4% potassium iodide. Glycogen-deficient (GD) colonies retain their original white color, whereas glycogen-sufficient colonies turn brown (5).

Sporulation

Sporulating ability was determined by inoculating the yeast onto sodium acetate sporulation agar (11) for 12 days at 25° C. A malachite green-safranin spore stain (21) was used and the culture scored for percentage sporulation and number of spores per ascus.

RESULTS

Survival Ability

Any change in the strain characteristics of the brewing strains is important; however, the most important factor is that they survive

TABLE III
Number of Brewing Yeast Strains
That Failed to Survive Storage Treatments[a]

Storage Treatment	Year 1	Year 2
Liquid nitrogen	0	0
Lyophilization	3	5
Distilled water (sealed vial)	2	4
MYGP[b] slope		
Under oil	2	3
21° C	1	3
4° C	0	0
Strain subcultured biweekly	0	0

[a] Twelve brewing strains were studied.
[b] Malt-yeast extract-glucose-peptone.

the storage treatment. Table III shows the number of strains that failed to survive the various storage methods. All 12 strains survived two years of storage when liquid nitrogen was the storage method. However, with lyophilization, three strains failed to survive after one year's storage and, surprisingly, after two years, two more strains failed to survive, indicating that once lyophilized, the cells were not stable and that death was still occurring. The yeast stored in distilled water in a sealed glass vial also exhibited low viability but not lower than that of the lyophilized cultures. The MYGP slope stored at 21° C with mineral oil covering the slope and the MYGP slope stored at 21° C with no mineral oil showed the same result after two years of storage. With both methods, three of the 12 strains failed to survive. The MYGP slope stored at 4° C gave excellent results, with all 12 cultures surviving the two-year storage period. The slopes that were subcultured every two weeks also survived, as was expected. Thus, strictly on the basis of storage methods that give the best survival, liquid nitrogen and MYGP slope storage at 4° C would appear to be the methods of choice.

TABLE IV
Effect of Two-Year Storage on Viability[a] (%)

Strain	In Liquid Nitrogen	On MYGP[b] Slope Subcultured Biweekly[c]
1	98	99
2	98	99
3	98	80
15	94	97
21	96	98
24	85	99
27	98	99
30	98	99
125	98	80
126	99	99
156	98	98
164	99	96

[a] Using fluorochrome primuline yellow.
[b] Malt-yeast extract-glucose-peptone.
[c] Yeasts subcultured to a fresh slope every two weeks, thus undergoing a total of 52 subcultures. Viability determined 72 hr after the final subculture.

Viability Stain

The cells taken from storage were suspended in water and a dead cell count determined using the fluorochrome primuline. As reported by Graham (7), a high correlation was found between nonfluorescence and viability, but the correlation decreased with increasing age of cell cultures. After one year, the yeast cultures that were nonviable (as determined by their inability to grow in MYGP) also did not stain with primuline and therefore did not fluoresce, yielding erroneous viability counts. Therefore, this method of determining viability after storage cannot be used after long-term storage, with the exception of long-term storage under liquid nitrogen. As can be seen in Table IV, the percentage of viable cells after two-year storage in liquid nitrogen was very similar to the percentage of viable cells on the slopes subcultured every two weeks, and the accuracy of the primuline stain was confirmed by observing the stored yeast's ability to form buds in MYGP.

Ease of Regeneration After Storage

Because in some cases the viability stains on the two-year-old cells gave questionable results, we decided to use a more subjective method of scoring the ease with which a culture could be revitalized after storage.

Upon removal from storage, all cultures were inoculated into 10 ml of MYGP broth overnight and then further subcultured to 100 ml of MYGP broth for 72 hr.

TABLE V
Effect of Two-Year Storage on Ease of Revival[a]

Saccharomyces Yeast		Storage Treatment						
					MYGP[b] Slope			
Species	Code Number	Liquid Nitrogen	Lyophilization	Distilled Water	Under Oil	21° C	4° C	Subcultured Biweekly
cerevisiae	1	3	0	1	0	0	2	3
	2	3	0	0	1	1	1	3
	3	3	1–2	1–2	1–2	1–2	3	3
	24	3	1	0	1	0	1	3
	30	3	0	1	2	1–2	2	3
	125	3	0	0	0	0	1–2	3
	126	2	0	1–2	0	1	1	2
	156	2	1–2	0	1	1	2	2
uvarum (carlsbergensis)	15	3	1–2	1–2	1–2	1	1–2	2
	21	2	1	1	1	1	1–2	3
	27	3	1–2	1–2	2	1	1–2	3
diastaticus	164	2	1–2	2	2	1–2	2	2

[a] 0 = Nonviable, 1 = poor growth, 2 = moderate growth, 3 = good growth.
[b] Malt-yeast extract-glucose-peptone.

TABLE VI
Effect of Two-Year Storage of Yeast[a] on Flocculation Intensity

Yeast Code No.	Storage Treatment						
				MYGP Slope			
	Liquid Nitrogen	Lyophilization	Distilled Water	Under Oil	21° C	4° C	Subcultured Biweekly
15	n.c.[b]	n.c.	Decreased	n.c.	Decreased	n.c.	n.c.
30	n.c.	n.c.	Decreased	n.c.	n.c.	n.c.	n.c.
156	n.c.	Decreased	Nonviable	Decreased	Decreased	Decreased	Decreased

[a] Strains cultured in 12° P wort at 21° C.
[b] No change.

The flasks of MYGP were checked for growth at 24, 48, and 72 hr. Table V summarizes the ease of revitalization of the stored cultures after two years of storage. Yeasts stored under liquid nitrogen were the easiest to revitalize, and at 24 hr, good growth was always found in the MYGP flasks. Yeasts subcultured every two weeks did equally well, but subculturing every two weeks is not a true storage method. The yeasts stored at 4° C on MYGP slopes were also relatively easy to revive, but after two years, a large decline in ease of revival could be seen, which was not found with liquid nitrogen storage. The four remaining storage methods showed great strain-to-strain variation, high death rates, and poor to moderate ease of regeneration.

Effect of Storage on Fermentative Ability

In the batch fermentation of brewer's wort individual sugars are removed in a distinct order. The precise sequence may vary from one strain of yeast to another, and, depending on the relative concentrations of individual sugars in the wort, some degree of overlap may occur; nevertheless, for most strains of *S. cerevisiae* and *S. uvarum (carlsbergensis)*, the general order is sucrose, glucose, fructose, maltose, and maltotriose. In addition, one of the brewing strains tested, cc No. 164, a *S. diastaticus*, produces an extracellular amyloglucosidase that can hydrolyze wort dextrins to glucose (6). A shaken fermentation system was employed to test whether the various storage methods affected sugar fermentative ability. A progressive volume scale-up in wort was made. The yeast was pitched into 100-ml shake flasks, and wort gravities were determined after 96 and 120 hr of incubation. In all cases, where viable cells could be recovered after storage, both the extent and rate of fermentation were unaltered, indicating that the yeast could still utilize the five major fermentable wort sugars, and that the *S. diastaticus* strain could still utilize the dextrins. Thus, sugar fermentative capability is apparently an extremely stable characteristic in brewing strains of yeast.

Effect of Storage on Flocculation and Coflocculation Ability

The technical importance of the flocculation characteristics of brewery yeasts needs no accentuation. Such yeasts have long been grouped into two types, flocculent (to varying degrees) and nonflocculent, one of which will be preferred according to the individual requirements of the brewery concerned. Numerous publications have dealt with investigations of flocculation (23,24,26); flocculation genes have been identified and mapped and specific suppressor genes of flocculation have been identified (10,19). One of the least understood aspects of flocculation is the phenomenon by which the flocculation characteristics of a production yeast strain spontaneously change. Numerous studies have dealt with this complex subject but none has led to a universally accepted explanation. We were thus greatly interested in determining whether the storage methods exerted any mutagenic pressure not only upon flocculation but also upon coflocculation.

Except that all 12 strains studied showed no flocculation changes when stored in liquid nitrogen, susceptibility to flocculation changes showed great strain-to-strain variation as well as variation due to storage method. Table VI shows the variation in three strains. In addition, coflocculation was also studied. Coflocculation is the type of behavior in which two yeast strains are nonflocculent alone but flocculent when mixed together. Strains 1 and 2 of the 12 strains studied had good coflocculation before storage (Table VII). Again liquid nitrogen preserved this ability, as did the MYGP slope with the mineral oil overlay. However, yeasts from both the MYGP slope stored at 4° C and the MYGP slope that had undergone repeated subcultures exhibited downgraded coflocculation.

TABLE VII
Effect of Two-Year Storage on Coflocculation of Strains 1 and 2

Storage Treatment	Coflocculation
Prestorage	Good
Liquid nitrogen	Good
Lyophilization	Nonviable
Distilled water	Nonviable
MYGP[a] slope	
Under oil	Good
21° C	Nonviable
4° C	Downgraded
Subcultured biweekly	Downgraded

[a] Malt-yeast extract-glucose-peptone.

RD Mutants

RD mutants, characterized by defects of aerobic metabolism, are among the most frequently occurring mutants of yeast (20). This mutation is found 10^6–10^8 times more frequently than other mutational types; approximately 1% of a brewing yeast culture consists of RD colonies (22). Reduced wort attenuation rates have been attributed to the selection of RD mutants, which exhibit an increased sedimentation rate and an inability to ferment maltotriose (8). Silhánková (22) found RD mutants less flocculent than their parents that produced a beer with flavor modified from the original type. These factors indicate that an ideal storage method should not increase the level of RD colonies present.

The RD levels for all the cultures were determined before storage.

TABLE VIII
Formation (%) of Respiratory-Deficient (RD) Colonies

Saccharomyces Yeast			Storage Treatment						
						MYGP[a] Slope			
Species	Code Number	Starting RD Level	Liquid Nitrogen	Lyophilization	Distilled Water	Under Oil	21° C	4° C	Subcultured Biweekly
cerevisiae	1	< 1	< 1	nv[b]	< 1	nv	nv	< 1	< 1
	2	< 1	< 1	nv	nv	< 1	< 1	< 1	< 1
	3	< 1	< 1	12	6.5	3	< 1	< 1	< 1
	24	< 1	< 2	31	nv	6.5	nv	< 1	< 1
	30	< 2	< 1	nv	< 1	9	6	17	< 1
	125	1	< 1	nv	nv	nv	nv	< 1	< 1
	126	< 1	< 1	nv	6	nv	< 1	< 1	< 1
	156	< 1	< 1	2	nv	4	1	1	1
uvarum (carlsbergensis)	15	< 3	3	4.5	< 2	6.5	< 2	4	3
	21	< 3	< 2	13	14	3.4	< 2	18	< 2
	27	< 2	< 1	5	< 2	7	16	< 2	< 1
diastaticus	164	< 2	2	9	2	4	< 1	< 1	< 2

[a] Malt-yeast extract-glucose-peptone.
[b] Nonviable.

The starting levels were all low (Table VIII), with the lager strains exhibiting slightly higher RD starting levels than did the ale strains. Storage in liquid nitrogen had no effect upon the RD level. Lyophilization yielded extremely high levels of RD colonies with some strains studied and quite acceptable levels with others. This great strain-to-strain variation has probably led to some of the conflicting reports in the literature about whether or not lyophilization leads to increased RD formation. The MYGP slopes stored at 4° C gave relatively low RD levels, perhaps because RD mutants generally survive poorly on slopes. However, two of the cultures gave very high RD levels of 18 and 17%, suggesting that caution should be exercised when using a sloped yeast for production purposes to ensure that a high number of RD colonies are not present.

GD Mutants

GD cells are yeast mutants deficient in the accumulation of the storage polysaccharide glycogen (5). We were interested to see whether the various storage methods influenced the formation of GD mutants. Yeast stored in liquid nitrogen showed no increase in the level of such mutants. With the other storage methods tested, strain-to-strain variation was greater than was variation between individual storage methods, and no definitive conclusions regarding glycogen deficiency and storage methods could be drawn.

Sporulating Ability

Genetic work involving brewer's yeast is fraught with difficulties because of the yeast's frequent triploid, polyploid, or aneuploid nature (11,29); its low degree of sporulation; and, especially, its low spore viability. These same characteristics, however, give the brewing yeast great stability and we wished to determine whether the method of storage influenced sporulation.

No increase in sporulation ability was detected; however, some strains showed evidence of a slight downgrading in the percentage of cells sporulating. Only one culture completely lost its ability to sporulate (cc No. 15), and this occurred only at the 21° C storage temperature. Thus storage methods appear to have little influence upon a brewing yeast's sporulation ability.

Giant Colony Plate Morphology

The giant colony plate morphology of the strains was monitored over the two-year interval by comparing the colonial morphology after storage with the giant colony plate pictures taken of the strains before storage. No change in colonial morphology due to storage methods was observed.

Stability of Genetically Defined Strains After Liquid Nitrogen Storage

Because the brewing strains studied were all polyploids and thus probably inherently more stable than haploids or diploids, six genetically defined laboratory strains were selected to undergo liquid nitrogen storage. Two diploids and four haploids with various markers (Table II) were subjected to repeated freeze-thaw cycles. Because any damage that occurs in the liquid nitrogen storage method probably occurs in the freezing or thawing of the culture rather than while the culture is being held at the low temperature of storage, repeated freeze-thaw cycles of heavily marked haploids and diploids were used to demonstrate whether the freezing and thawing were exerting mutagenic pressure. Controlled rapid thawing (12–23° C/sec) was found to be preferable because this resulted in a lower death rate (15,27).

The six genetically defined strains were frozen and thawed three times, with a sample removed after each thaw for testing. The controls and samples were then tested to see if any change had occurred in their nutritional requirements or sugar fermentative ability. Repeated liquid nitrogen freeze-thaw cycles had no detectable effect on the genetically defined strains.

DISCUSSION

At the end of the two-year period, liquid nitrogen storage appeared from an experimental viewpoint to be the method of choice. The advantages of liquid nitrogen storage included superior viability, with the storage time period having little or no influence. The cultures stored in liquid nitrogen were the easiest to revitalize, and the degree of flocculation, the fermentative rate, sporulation ability, and giant colony plate morphology were unaffected. The proportion of RD and GD mutants showed no increase. Liquid nitrogen storage yielded the least alteration in strain characteristics, with repeated freeze-thaw cycles having no effect on the nutritional requirements of extensively "marked" haploid and diploid strains. Storage in liquid nitrogen also yielded culture security. Currently a substantial debate is going on concerning the patentability of microorganisms, with a case being considered by the U.S. Supreme Court regarding this matter. Cultures stored in liquid nitrogen can be held in a much more secure and safe manner than would be the case, for example, on an agar slope. Further, in these days of possible industrial labor disputes, industrial strains can be maintained with a considerable degree of confidence in liquid nitrogen. A similar degree of stability is not apparent with the other storage methods. However, disadvantages to liquid nitrogen storage also exist. This type of storage requires a constant and guaranteed source of liquid nitrogen, which can be an expensive continuing commitment. For example, the 1980 price of liquid nitrogen in London, Ontario, is currently $2/L. Another disadvantage is the requirement for specialized cryogenic equipment and techniques. Finally, the casual availability of stored cultures is limited. Although this can be a security advantage, for routine day-to-day use in the laboratory, access is limited when compared to the easy access of cultures stored on agar slopes.

Routine subculturing every two weeks on slopes also gave acceptable results with little variation from the zero time cultures after two years. However, this maintenance technique cannot be considered a storage method. The 4° C storage on nutrient agar slopes subcultured every six months is the next method of preference. The other storage methods revealed degrees of instability that varied from strain to strain.

ACKNOWLEDGMENTS

We wish to acknowledge with gratitude the support and assistance of T. E. Goring (Labatt Brewing Company) and A. M. Wellman and S. R. Legeza (Department of Plant Sciences, University of Western Ontario, London, Ontario).

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[Received June 13, 1980]