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Survival of yeasts stored after freeze-drying or liquid-drying

Yukie Miyamoto-Shinohara,* Fumie Nozawa, Junji Sukenobe, and Takashi Imaizumi

International Patent Organism Depository (IPOD), National Institute of Advanced Industrial Science and Technology (AIST),
Tsukuba, Ibaraki 305-8566, Japan

(Received August 11, 2009; Accepted October 16, 2009)

We investigated the survival mechanisms of freeze-dried or liquid-dried (L-dried) yeast cells in ampoules. Type strains of various yeasts were freeze-dried or L-dried and sealed in ampoules under high vacuum (< 1 Pa) or low vacuum (4.8×10^4 Pa), then stored at 37°C (accelerated storage test) for up to 17 weeks. Among strains in each of the genera *Saccharomyces*, *Saccharomycopsis*, *Debaryomyces*, and *Pichia*, survival rates immediately after freeze-drying varied more widely than those after L-drying. Freeze-dried cells stored at 4.8×10^4 Pa had lower survival rates than those stored at < 1 Pa. L-dried cells stored at 4.8×10^4 Pa also had lower survival rates than those stored at < 1 Pa, but the decrease in survival was not as marked as in freeze-dried cells. Strains that had high survival rates immediately after freeze-drying tended to have small cells, to be osmotolerant, and to be able to utilize many kinds of carbohydrates. L-dried cells of most *Candida* strains had stable survival rates regardless of the vacuum pressure. In basidiomycetous yeasts, strains forming extracellular polysaccharides had markedly lower survival.

Key Words—accelerated storage test; cell size; freeze-dry; L-dry; survival curve; yeast

Introduction

Freeze-drying and L-drying are popular methods of preserving microorganisms, because long-term viability is excellent in most cases and the storage and distribution requirements are simple. By plotting the survival curves of freeze-dried species sealed under high vacuum (< 1 Pa) and stored at 5°C in the dark, we previously found that the survival rates of Gram-positive bacteria immediately after freeze-drying and during storage were higher than those of Gram-negative bacteria (Miyamoto-Shinohara et al., 2008). In addition, the survival rate of the yeast *Saccharomyces cerevisiae* immediately after freeze-drying was lower than

those of bacterial species, although *S. cerevisiae* survival rates were stable during storage (Miyamoto-Shinohara et al., 2000, 2006).

To increase the survival rates of *S. cerevisiae* strains, we tested the applicability of L-drying, in which cells are desiccated by vacuum evaporation at room temperature. In contrast, during freeze-drying, cells are desiccated by sublimation. L-drying is effective for the long-term preservation of cultures sensitive to freeze-drying (Malik, 1990, 1999). In this research, yeast strains were L-dried according to the method of the Institute for Fermentation, Osaka (Banno et al., 1979), in which the cell suspension was dispensed into a glass ampoule (in the same way as for freeze-drying) and a cotton wool plug was inserted to prevent the temperature in the ampoule from falling to the freezing point (Iijima and Sakane, 1973).

To clarify the mechanisms underlying tolerance to L-drying and freeze-drying, we investigated the survival of type strains of various yeast species. The survival

* Address reprint requests to: Dr. Yukie Miyamoto-Shinohara, Institute for Biological Resources and Functions, AIST, Tsukuba Central 6 1-1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan.
Tel: 81-29-861-9461 Fax: 81-29-861-6587
E-mail: y.miyamoto@aist.go.jp

rates of these strains were compared by the accelerated storage test, in which desiccated cells are stored at a higher temperature than usual to allow us to predict their long-term storage stability over a shorter period than usual (Mitic et al., 1974). For L-dried yeast strains, survival rates after storage for 30 days at 37°C roughly correspond to those after storage for 5 years at 5°C (Mikata and Banno, 1989). Survival rates of L-dried bacterial strains stored for 2 weeks at 37°C correspond well to those after storage for 10 years at 5°C (Sakane and Kuroshima, 1997). The ampoules in these previous studies were sealed under a high vacuum. We described how our freeze-dried bacteria sealed in ampoules at < 1 Pa lived longer than those sealed at approximately 7 Pa (Antheunisse, 1973); survival duration was positively correlated with vacuum pressure (Antheunisse, 1973; Miyamoto-Shinohara et al., 2006). In this study, we investigated the effect of vacuum pressure and storage temperature on the survival rates of various yeast strains.

Materials and Methods

Strains and culture conditions. We studied the following NITE Biological Resource Center (NBRC) type strains and type species, some of which had been reclassified into other genera by 1995 (Barnet et al., 2000): *Lodderomyces elongisporus* NBRC 1676, *Saccharomyces cerevisiae* NBRC 10217, *Saccharomyces transvaalensis* NBRC 1625, *Saccharomycodes ludwigii* NBRC 798, *Saccharomycopsis selenospora* NBRC 1850, *Saccharomycopsis javanensis* NBRC 1848, *Debaryomyces robertsiae* NBRC 1277, *Debaryomyces hansenii* NBRC 15, *Debaryomyces occidentalis* NBRC 1841, *Pichia anomala* NBRC 10213, *Pichia membranifaciens* NBRC 10215, *Ambrosiozyma monospora* NBRC 1965, *Candida tropicalis* NBRC 1400, *Clavispora lusitaniae* NBRC 10059, *Wickerhamiella domercqiae* NBRC 1857, *Issatchenkia orientalis* NBRC 1279, *Citeromyces matritensis* NBRC 954, *Torulaspora delbrueckii* NBRC 955, *Sterigmatomyces halophilus* NBRC 1488, *Trichosporon inkin* NBRC 10131, *Rhodotorula glutinis* NBRC 1125, *Bulleromyces albus* NBRC 1192, and *Xanthophyllomyces dendrorhous* NBRC 10129. The strains were cultured on YM agar at 24°C until the beginning of the stationary phase.

Freeze-drying and recovery. Cells on slant culture were homogenously suspended in 5 ml of sterilized suspension medium (10% skim milk, 1% sodium glu-

tamate), and 0.2 ml of the suspension was dispensed into a glass ampoule (7 × 150 mm, Takizawa Syouten, Tokyo, Japan) with a long needle (1.5 × 160 mm, Takizawa Syouten). Ampoules were frozen in -60°C ethanol for 2 min; the suspension was stirred for the first few seconds to freeze the cells equally. The ampoules were then connected to a manifold-type freeze-dryer (FreezeMobile 12EL, Virtis, Gardiner, NY, USA) for 4 h under a vacuum (< 1 Pa), the gauge pressure of which indicated -76 mm Hg. Half of the ampoules were sealed by heating with a gas burner to maintain the high vacuum (< 1 Pa), which was checked by vacuum discharge with a Tesla coil (TSL-50, Kaburagi-kagakukikaikogyo, Tokyo, Japan). Then the lever of the freeze-dryer was opened slightly to introduce air into the freeze-dryer until the gauge pressure indicated -40 mm Hg, which corresponded to 4.8×10^4 Pa. The rest of the ampoules were sealed at this low vacuum (4.8×10^4 Pa), which was only slightly below normal atmospheric pressure (1.0×10^5 Pa). The use of a low vacuum during sealing of the heated ampoules helped to stop the ampoules from bursting. To recover the cells, each ampoule was opened with an ampoule cutter and 0.2 ml of physiological saline added.

L-drying and recovery. Cells on slant culture were suspended in 5 ml sterilized suspension medium (5% sodium glutamate, 5% lactose, 6% polyvinylpyrrolidone [PVP] in phosphate buffer; pH 7.0), and then 0.1 ml of the suspension was dispensed into a glass ampoule and a cotton plug was inserted (Banno et al., 1979). Ampoules were connected to a freeze-dryer at room temperature at normal pressure (1.0×10^5 Pa), and then the pressure was gently reduced to < 1 Pa. Cells in these ampoules were dried for 4 h and sealed under the same conditions as used for the freeze-dried cells, that is, at < 1 Pa (high vacuum) or 4.8×10^4 Pa (low vacuum). To recover the cells, each ampoule was opened and 0.1 ml of physiological saline was added.

Survival rates measured by the accelerated storage test. Ampoules were stored at 37°C for up to 17 weeks, and each ampoule prepared at low or high vacuum was used for one survival test. Cells before drying (control) and rehydrated cells in an opened ampoule were diluted with sterilized water to $\times 10^2$ and $\times 10^4$ per the rehydrated cells, respectively. A 0.05-ml volume of the dilution was inoculated onto a plate of YM agar (90 mm in diameter) by using a spiral plater (Autoplate Model 3000, Spiral Biotech, Bethesda, MD, USA). Colony forming units (CFUs) were measured as the mean

of three plates for each dilution. CFU values were determined for cells before drying, just after drying, and at weeks 1, 2, 4, 9, and 17 of storage. Survival rates immediately after freeze-drying (FD) or L-drying (LD) are expressed as percentages. The survival rates were calculated from the mean survival under high-vacuum conditions immediately after drying and the mean survival before drying.

Cell size. Images of cells before freeze-drying were acquired through a differential interference microscope with Axioplan 2 (Carl Zeiss Vision, Hallbergmoos, Germany). The major and minor axes of the cells on the image (*S*, *L*) were measured with a computerized image analysis system (KS 300 Ver. 2.00, Carl Zeiss Vision).

Characteristics of strains. The ability to grow on various carbon sources in an agar medium was tested in tubes. The basal agar medium was 0.67% YNB (Difco, Sparks, USA) with 1.5% agar supplied with one of the following compounds at 0.5%: glucose, galactose, sorbose, D-glucosamine, ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α,α -trehalose, raffinose, or glycerol. The ability to grow in high concentrations of sugar was tested by growth on YM agar media (Difco) containing 50% or 60% (w/v) glucose or 10% or 16% (w/v) NaCl; the control medium was YM agar. Each strain was inoculated on a slant of the medium and cultured at 25°C. Strains that had formed colonies on the slant by day 3 are marked with a '+' symbol in the tables. Strains that had formed colonies by day 7 but not by day 3 are marked with a "d", which stands for "delayed". Strains with insufficient growth even by day 7 are marked with a '-' symbol.

To examine the formation of extracellular polysaccharide, strains were inoculated onto ammonium sulfate-glucose agar medium on plates or onto YNB (Difco) agar medium with 1% glucose on plates. The cultures were incubated at 25°C for 3 days and then flooded with diluted Lugol's iodine and inspected for the formation of a blue to green shading (Barnet et al., 2000; Kurtzman and Fell, 1998).

Results

Cells of each type strain were freeze-dried or L-dried and sealed in ampoules under high vacuum (< 1 Pa) or low vacuum (4.8×10^4 Pa), then stored at 37°C (accelerated storage test) for up to 17 weeks. We com-

pared the survival rates of strains in the same genus as well as among basidiomycetous yeast strains. To determine the causes of the different survival rates among strains in the same genus, we compared the physiological characteristics of each species, as described in *Yeasts: Characteristics and Identification* (Barnet et al., 2000) and *The Yeast: A Taxonomic Study* (Kurtzman and Fell, 1998). In these two texts, species in the same genus are described as having about the same characteristics in terms of semi-anaerobic fermentation ability; vitamin requirements; ability to utilize particular compounds as sole sources of nitrogen for aerobic growth; growth in the presence of cycloheximide or acetic acid; acetic acid production; urea hydrolysis; and response to the diazonium blue B test. We tested several characteristics that differed, according to these reference tests among the species that we used: ability to utilize particular compounds as sole sources of carbon for aerobic growth; growth in media with a high concentration of sugar or NaCl (i.e., osmotolerance); and production of extracellular starch-like compounds.

Survival of Saccharomyces, Saccharomycopsis, and Debaryomyces

Type strains of *Saccharomyces*, *Saccharomycopsis*, and *Debaryomyces* showed similar survival rates. Survival rates after freeze-drying varied more widely among strains in the same genus than the rates after L-drying.

Figure 1 illustrates the survival curves of two *Saccharomyces* type strains (*Saccharomyces transvaalensis* and *Saccharomyces cerevisiae*) and two type strains formerly classified as *Saccharomyces* (*Lodderomyces elongisporus* and *Saccharomycodes ludwigii*). Table 1 lists the strains in descending order of survival rate immediately after freeze-drying, along with the survival rates immediately after L-drying; it also shows the cell sizes, which were calculated from differential interface micrographs before the cells were freeze-dried, and the physiological characteristics of the cells. Most characteristics were coincident with the data in the above-mentioned works by Barnet et al. (2000) and Kurtzman and Fell (1998), although we found that *S. ludwigii* had positive osmotolerance in 50% and 60% glucose, whereas the species was listed as negative for osmotolerance in the references (Barnet et al., 2000; Kurtzman and Fell, 1998). Comparison of the survival of each strain immediately after drying

Fig. 1. Survival curves at 37°C of type strains presently classified as *Saccharomyces*.

(a) *Lodderomyces elongisporus*, (b) *Saccharomyces transvaalensis*, (c) *Saccharomyces cerevisiae*, (d) *Saccharomyces ludwigii*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

(Table 1) revealed that survival rate after freeze-drying decreased as cell size increased: *L. elongisporus* had the highest survival rate immediately after freeze-drying (24.3%) and the smallest cell size ($3.8 \times 5.2 \mu\text{m}$), whereas *S. ludwigii* did not survive after freeze-drying (0%) and its cell size was largest ($5.6 \times 10.2 \mu\text{m}$). Survival rates immediately after L-drying were not as different among the strains as those immediately after freeze-drying. The strain with the highest survival rate, *L. elongisporus*, can grow at high osmotic pressures (50% and 60% glucose, 10% NaCl). Our analysis of the assimilation of carbon compounds revealed that *S. transvaalensis* was the only strain in this group not to use sucrose and raffinose. *Saccharomyces ludwigii* was the only strain not to use galactose or trehalose; it showed no survival immediately after freeze-drying. Comparison of the survival curves of freeze-dried cells stored at high vacuum showed that *L. elongisporus* had a high survival rate (Fig. 1 (a)), *S. transvaalensis* and *S. cerevisiae* (Fig. 1 (b), (c)) had lower survival rates, and *S. ludwigii* did not survive after freeze-drying (Fig. 1 (d)). The first three strains showed decreased survival of freeze-dried cells stored at low vacuum compared to that at high vacuum. The three strains also showed decreased survival of L-dried cells stored at low vacuum compared to that at high vacuum, but the differences were smaller than those of the freeze-

Fig. 2. Survival curves at 37°C of *Saccharomycopsis* type strains.

(a) *Saccharomycopsis selenospora*, (b) *Saccharomycopsis javanensis*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

dried cells. The difference in survival rate between the two vacuum conditions in *L. elongisporus* was larger than that in *S. transvaalensis* or *S. cerevisiae*.

Figure 2 illustrates the survival curves of two *Saccharomycopsis* type strains. Table 2 lists the strains in descending order of survival immediately after freeze-drying, along with survival rates immediately after L-drying and the cell sizes, which were calculated from micrographs before the cells were freeze-dried, and physiological characteristics. *Saccharomycopsis selenospora* had a higher survival rate immediately after freeze-drying (0.20%) and a smaller cell size ($3.0 \times 7.4 \mu\text{m}$) than *S. javanensis* (0.0011%, $3.0 \times 18.2 \mu\text{m}$). Survival rates immediately after L-drying did not differ greatly between the two strains (3.2% and 8.5%, respectively). The characteristic difference between the two strains lay in the assimilation of carbon: *S. selenospora* could utilize galactose as a sole source of carbon, whereas *S. javanensis* could utilize sorbose and trehalose. Freeze-dried cells of both strains stored at low vacuum could not grow after week 4 or week 1 respectively, though those stored at high vacuum had stable curves (Fig. 2). L-dried cells stored at low vacuum had lower survival rates than those stored at high vacuum; the difference was larger in *S. selenospora* (Fig. 2 (a)) than in *S. javanensis* (Fig. 2 (b)).

Figure 3 illustrates the survival curves of three *Debaryomyces* type strains. Table 3 lists the strains in descending order of survival rate immediately after freeze-drying, along with their survival rates immediately after L-drying, the cell sizes which were calculated from micrographs before the cells were freeze-dried, and physiological characteristics. Most of the

Table 1. Characteristics of strains presently classified as *Saccharomyces*.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
1676	<i>Lodderomyces elongisporus</i>	24.3	65.3	3.8±0.9	5.2±1.2	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-
1625	<i>Saccharomyces transvaalensis</i>	0.62	21.1	4.6±0.9	5.9±1.4	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
10217	<i>Saccharomyces cerevisiae</i>	0.0037	35.7	5.4±1.0	6.6±1.3	+	+	-	-	-	-	-	-	-	+	+	+	+	-	d	d	-	-	-
798	<i>Saccharomycodes ludwigii</i>	0	17.4	5.6±0.9	10.2±2.6	+	-	-	+	+	-	-	-	-	+	-	-	+	+	+	+	-	-	-

FD and LD are the survival rates immediately after freeze-drying and L-drying. S and L (µm) are lengths of the minor axis and major axis of cells prepared for freeze-drying, each of which were calculated from measurements of 38 to 276 cells on differential interference micrographs. Symbols: +, positive; -, negative; d, delayed within 7 days.

Table 2. Characteristics of strains of *Saccharomycopsis*.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
1850	<i>S. selenospora</i>	0.20	3.2	3.0±0.7	7.4±4.1	+	+	-	+	+	d	d	-	-	+	-	-	+	+	-	-	-	-	-
1848	<i>S. javaensis</i>	0.0011	8.5	3.0±0.7	18.2±7.1	+	-	+	+	+	d	-	-	-	+	d	+	+	+	-	-	-	-	-

See Table 1 for details. S and L were calculated on the basis of measurements of 134 to 383 cells on differential interference micrographs.

Fig. 3. Survival curves at 37°C of *Debaryomyces* type strains. (a) *Debaryomyces robertsiae*, (b) *Debaryomyces hansenii*, (c) *Debaryomyces occidentalis*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

physiological characteristics shown in Table 3 corresponded to the data in the two references (Barnet et al., 2000; Kurtzman and Fell, 1998), although we found that *D. occidentalis* showed positive assimilation of rhamnose and *D. robertsiae* showed negative growth at 16% NaCl, results opposite to those in the references. *Debaryomyces occidentalis* had the lowest survival rate immediately after freeze-drying and had a larger cell size (0.012%, $3.9 \times 5.3 \mu\text{m}$) than *D. robertsiae* (52.4%, $3.5 \times 3.9 \mu\text{m}$) and *D. hansenii* (41.4%, $3.8 \times 4.3 \mu\text{m}$). *Debaryomyces occidentalis* also had by far the lowest survival rate immediately after L-drying (0.49%) compared to *D. robertsiae* (78.5%) and *D. hansenii* (89.2%). This strain was the only one of the three that could utilize D-arabinose as a sole source of carbon, and it showed limited osmotolerance (negative for 60% glucose and 10% NaCl). For each strain, the survival of freeze-dried cells stored at low vacuum was much lower than that of cells stored at high vacuum (Fig. 3). L-dried cells stored at low vacuum showed lower survival than those stored at high vacuum, but the differences were less than those in freeze-dried cells. The differences between the two survival curves were large in *D. robertsiae* and small in *D. occidentalis*.

Survival of *Pichia*

Figure 4 illustrates the survival curves of two *Pichia* type strains and a type strain formerly classified as *Pichia*. Table 4 lists the strains in descending order of survival rate immediately after freeze-drying, along with survival rates immediately after L-drying, the cell sizes which were calculated from micrographs before the cells were freeze-dried, and physiological characteristics. Most characteristics were coincident with the

Fig. 4. Survival curves at 37°C of type strains presently classified as *Pichia*.

(a) *Pichia anomala*, (b) *Ambrosiozyma monospora*, (c) *Pichia membranifaciens*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

data in the two references (Barnet et al., 2000; Kurtzman and Fell, 1998), although we found that *P. membranifaciens* showed positive osmotolerance at 60% glucose but it was listed as negative for this feature in the references. Of the three strains, *P. anomala* had the smallest cell size ($4.0 \times 4.3 \mu\text{m}$), compared with those of *A. monospora* ($6.6 \times 7.8 \mu\text{m}$) and *P. membranifaciens* ($3.6 \times 6.1 \mu\text{m}$). *Pichia anomala* had by far the highest survival rates immediately after both freeze-drying and L-drying (48.2%, 62.6%), and the survival rate was stable during storage except when the strain was freeze-dried and sealed at $4.8 \times 10^4 \text{ Pa}$. *Ambrosiozyma monospora* showed low survival rates immediately after freeze-drying and L-drying (0.93%, 0.54%). Freeze-dried or L-dried cells stored at low vacuum (Fig. 4 (b)) did not grow after week 7 or week 17, respectively; the cells of *A. monospora* did not have greater osmotolerance than those of the other strains (negative for glucose and NaCl). *Pichia membranifaciens* had a much higher survival rate immediately after L-drying than after freeze-drying (0.012%, 29.9%). Unlike the other two strains, this strain was unable to use many kinds of carbohydrates as sole carbon sources (negative for ribose, sucrose, maltose, trehalose, and raffinose) and was not osmotolerant (negative for NaCl); these results were in accord with those for strains of *Saccharomyces*, *Saccharomycopsis*, and *Debaryomyces* that had low survival rates immediately after freeze-drying.

Survival of *Candida*

Figure 5 illustrates the survival curves of a *Candida* type strain and five type strains asexually classified as *Candida*. Table 5 lists the strains in descending order

Table 3. Characteristics of strains of *Debaryomyces*.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
1277	<i>D. robertsiae</i>	52.4	78.5	3.5±0.8	3.9±0.9	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-
15	<i>D. hansenii</i>	41.4	89.2	3.8±0.9	4.3±1.0	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-
1841	<i>D. occidentalis</i>	0.012	0.49	3.9±1.0	5.3±1.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

See Table 1 for details. S and L were calculated on the basis of measurements of 222 to 315 cells on differential interference micrographs.

Table 4. Characteristics of strains presently classified as *Pichia*.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
10213	<i>P. anomala</i>	48.2	62.6	4.0±0.8	4.3±0.8	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-
1965	<i>Ambrosiozyma monospora</i>	0.93	0.54	6.6±1.6	7.8±1.4	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
10215	<i>P. membranifaciens</i>	0.012	29.9	3.6±0.8	6.1±1.9	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-

See Table 1 for details. S and L were calculated on the basis of measurements of 74 to 284 cells on differential interference micrographs.

Fig. 5. Survival curves at 37°C of type strains presently classified as *Candida*.

(a) *Candida tropicalis*, (b) *Clavispora lusitanae*, (c) *Wickerhamiella domercqiae*, (d) *Torulaspora delbrueckii*, (e) *Citeromyces matritensis*, (f) *Issatchenkia orientalis*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

of survival rate immediately after freeze-drying, along with the survival rates immediately after L-drying; it also lists the cell sizes, which were calculated from micrographs before the cells were freeze-dried, and physiological characteristics. Most characteristics were coincident with the data in the two references (Barnet et al., 2000; Kurtzman and Fell, 1998), except we found that *C. lusitanae* and *I. orientalis* showed positive osmotolerance at 60% glucose. The survival rates among the strains immediately after freeze-drying ranged from 8.6% to 46.9% and immediately after L-drying from 28.2% to 100%. The differences in survival rates among the strains were less than those within the genera of *Saccharomyces*, *Saccharomycopsis*, *Debaryomyces*, and *Pichia*. Unlike these four genera, all strains had osmotolerance in 60% glucose. *Issatchenkia orientalis* had the lowest survival rate immediately after freeze-drying and after L-drying (8.6%, 28.2%) and the largest cell size ($4.7 \times 8.0 \mu\text{m}$) in this group. L-dried cells of this group showed stable survival rates whether stored under high or low vacuum (Fig. 5). However, the survival rates of freeze-dried cells stored at low vacuum were lower than for those stored at high vacuum.

Survival of basidiomycetous yeasts

Figure 6 illustrates the survival curves of five type

Fig. 6. Survival curves at 37°C of basidiomycetous yeast type strains.

(a) *Sterigmatomyces halophilus*, (b) *Trichosporon inkin*, (c) *Rhodotorula glutinis*, (d) *Xanthophyllomyces dendrorhous*, (e) *Bulleromyces albus*. ●, freeze-dried, high vacuum; ○, freeze-dried, low vacuum; ▲, L-dried, high vacuum; △, L-dried, low vacuum. Each data point represents the mean of three replicates.

strains classified as basidiomycetous yeasts. Table 6 lists the strains in descending order of survival immediately after freeze-drying, along with the survival rates immediately after L-drying, the cell sizes, which were calculated from micrographs, and physiological characteristics. In Table 6, most of the characteristics corresponded to the data in the two references (Barnet et al., 2000; Kurtzman and Fell, 1998), except we found that *T. inkin* was able to assimilate rhamnose. L-dried and freeze-dried cells of *S. halophilus* and *T. inkin* had stable survival rates during storage (Fig. 6 (a), (b)). L-dried cells of *R. glutinis* and *B. albus* (Fig. 6 (c), (e)) could not grow after week 17, whereas freeze-dried cells sealed at high vacuum showed stable survival rates. The contents of L-dried ampoules of these strains turned brown when the strains died although they were colorless immediately after L-drying, as were the other strains. L-dried and freeze-dried *X. dendrorhous* cells could not grow after week 17 although the cells did not change color (Fig. 6 (d)). The three strains with the lowest survival rates after L-drying had the ability to use raffinose as a sole carbon source and showed negative osmotolerance at 10% NaCl. *Xanthophyllomyces dendrorhous* and *B. albus* showed starch formation, whereas the others in this group did not.

Table 5. Characteristics of strains presently classified as *Candida*.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
1400	<i>Candida tropicalis</i>	46.9	100.0	4.3±1.0	5.6±1.8	+	+	d	+	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
10059	<i>Clavispora lusitanae</i>	46.1	66.3	2.7±0.6	4.0±0.8	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
1857	<i>Wickerhamiella domercqiae</i>	45.5	81.2	2.4±0.4	3.4±0.6	+	-	+	-	d	-	-	-	-	-	-	-	-	+	+	d	-	-	-
955	<i>Torulasporea delbrueckii</i>	20.3	51.2	3.0±1.0	3.5±1.1	+	+	d	-	-	-	-	-	-	+	-	d	+	+	+	+	+	-	-
954	<i>Citeromyces matritensis</i>	16.0	100.0	4.0±1.0	4.3±1.0	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-
1279	<i>Issatchenkia orientalis</i>	8.6	28.2	4.7±0.8	8.0±2.3	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-

See Table 1 for details. S and L were calculated on the basis of measurements of 143 to 763 cells on differential interference micrographs.

Table 6. Characteristics of basidiomycetous yeast.

NBRC No.	Strain	FD	LD	S±SD	L±SD	Glucose	Galactose	Sorbose	D-Glucosamine	Ribose	D-Xylose	L-Arabinose	D-Arabinose	L-Rhamnose	Sucrose	Maltose	Trehalose	Raffinose	Glycerol	50% Glucose	60% Glucose	10% NaCl	16% NaCl	Polysaccharide formation
1488	<i>Sterigmatomyces halophilus</i>	8.7	69.3	3.9±0.8	4.6±0.8	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	d	+	-	-
10131	<i>Trichosporon inkin</i>	6.9	83.5	4.9±1.0	7.1±2.1	+	+	+	+	+	+	d	-	+	+	+	+	d	+	-	-	+	-	-
1125	<i>Rhodotorula glutinis</i>	2.2	44.3	5.2±1.0	6.7±1.3	+	+	+	+	+	-	-	d	+	+	+	+	+	+	-	-	-	-	-
10129	<i>Xanthophyllomyces dendrorhous</i>	0.53	34.5	6.3±0.1	9.8±0.3*	+	-	-	-	-	d	+	-	-	+	+	+	+	-	-	-	-	-	+
1192	<i>Bulleromyces albus</i>	0.078	40.0	3.9±0.8	6.1±1.3	+	+	d	+	+	+	+	+	+	+	+	+	+	d	-	-	-	-	+

See Table 1 for details. *Data are from cells stored for a week after freeze-drying. S and L were calculated on the basis of measurements of 86 to 261 cells on differential interference micrographs.

Discussion

Survival immediately after freeze-drying depends on tolerance to rapid freezing (osmotolerance)

Freeze-dried cells were rapidly frozen and the water they contained was sublimated. Most strains showed lower survival rates after freeze-drying than after L-drying. In each genus, the strains more tolerant to freeze-drying tended to have small cells, to be osmotolerant, and to be able to utilize many kinds of carbohydrates.

For the freeze-drying process, a 0.2-ml cell suspension in a glass ampoule was frozen in -60°C ethanol. The cooling rate of the ampoules was rapid—nearly $1,800^{\circ}\text{C min}^{-1}$, according to Dumont et al. (2003), who showed the influence of cooling rates from 5 to $30,000^{\circ}\text{C min}^{-1}$ on *S. cerevisiae* in liquid nitrogen. At the rapid rate, the external water is frozen before the cell contents, thus increasing the osmotic pressure. Under such a rapid cooling rate, the freeze-tolerant *T. delbrueckii* is more tolerant to osmotic stress than the freeze-sensitive *S. cerevisiae* (Hernandez-Lopez et al., 2003). Rapid freezing results in a lower survival rate after freeze-drying, and the survival rates of cells seem to be related to cell size, the water permeability of the cell membrane, and the presence or absence of a cell wall (Dumont et al., 2003).

Survival rates depend on cell wall structure

The structure of the cell wall appears to be related to survival after rapid freezing. Our previous research showed that Gram-positive bacteria, which have thick cell walls, generally had higher survival rates after freeze-drying than Gram-negative bacteria, which have thin cell walls (Miyamoto-Shinohara et al., 2008). In *S. cerevisiae*, hyperosmotic shock induces a change in the organization of the cell wall, apparently resulting from the displacement of periplasmic and cell-wall-matrix material into invaginations of the plasma membrane created by the plasmolysis (Slaninova et al., 2000).

The major constituents of yeast cell walls are carbohydrates, although the composition and the structure of the cell wall differ between ascomycetous and basidiomycetous yeasts (Bastide et al., 1979; Depree et al., 1993; McGinnis and Tying, 1996; Nguyen et al., 1998; Prillinger et al., 1993). Cell wall synthesis is induced by the incorporation of glucose and threonine into the cytoplasm and cell wall of yeast (Sentandreu and Northcote, 1969). In this research, all the strains

were cultured under the same conditions. Each strain, however, has different structure of cell wall, which may account for the survival difference among the strains.

Strains of *Saccharomyces*, *Saccharomycopsis*, and *Debaryomyces*, which had high survival rates after freeze-drying, showed lower survival rates when they were sealed in low-vacuum ampoules than in high-vacuum ampoules. In contrast, when these strains were L-dried, the survival rates were similar regardless of whether low- or high-vacuum conditions were used for storage. In *S. cerevisiae* the β 1,3-glucan-chitin complex is the major constituent of the inner cell wall, and β 1,6-glucan links the components of the inner and outer cell walls (Lipke and Ovalle, 1998; Smits et al., 1999). Synthesis of β 1,3-glucan follows the extrusion of polysaccharides inside the cytoplasm into the extracellular space (Cabib et al., 1998; Shematek and Cabib, 1980).

Low-vacuum ampoules contain more moisture than high-vacuum ampoules. This residual moisture may become trapped in the glucan of the cell wall and injure freeze-dried cells. Thus, although a strain with more glucan in the cell wall has osmotolerance and a good survival rate during the freeze-drying process, this glucan can trap moisture and decrease the survival rate during storage. In L-dried cells in a low-vacuum ampoule, however, most residual moisture may be trapped in the cotton plug, which may bind water molecules more strongly than glucan in the cell wall. The possibility that most of the water is trapped in the cotton and not in the glucan in the cell wall needs to be investigated in the future.

Freeze-dried *Candida* strains in high-vacuum ampoules had stable survival rates, and L-dried cells showed stable survival rates regardless of the degree of vacuum during storage. The cell walls of *Candida* species are composed of mannan with fatty acids (Kappeli and Fiechter, 1977; Kappeli et al., 1978; Kobayashi et al., 2003; Shinoda et al., 1981). Adsorption of moisture on the cell walls of *Candida* may be weaker than that of *D. hansenii* and *S. cerevisiae*, whose cell walls are composed of glucan and mannoprotein (Nguyen et al., 1998). Thus, when *Candida* strains were stored, more moisture would be trapped in the cotton plug.

Compared with those of the ascomycetous yeasts, the survival rates of basidiomycetous yeasts were less affected by the degree of vacuum during storage. Among the basidiomycetous yeasts, *B. albus* and *X.*

dendrorhous showed markedly decreased survival curves of L-dried cells even at high vacuum; extracellular starch-like polysaccharides were formed. The extracellular polysaccharides of *B. albus* and *X. dendrorhous* probably bind water molecules so tightly that they cannot be removed by L-drying or freeze-drying; this tight binding may thus injure the dried cell during storage and reduce survival rates. The basidiomycetous yeast *Cryptococcus neoformans* produces extracellular polysaccharides composed of glucuronoxylomannan and galactoxylomannan, the predominant forms of which are composed of multiple long fibers entangled with one or more similar fibers, as shown by scanning transmission electron microscopy (McFadden et al., 2006). The fibers of these extracellular polysaccharide may trap water molecules. Glucuronoxylomannan is synthesized in the cytoplasm of *C. neoformans* (Feldmesser et al., 2001); it has urease activity and the ability to assimilate raffinose, a trisaccharide composed of galactose, glucose, and fructose (Garcia-Rivera et al., 2004). The results for *C. neoformans* agree with those of this research: although species of basidiomycetous yeast have urease activity, those species producing extracellular polysaccharides (such as *Cryptococcus aquaticus*, *Filobasidium globisporum*, and *Tremella aurantia*) are able to assimilate raffinose (Barnet et al., 2000). Species able to assimilate raffinose may synthesize extracellular polysaccharide, which may trap water so tightly that it cannot be segregated by freeze-drying or L-drying. The affinity of cotton or various polysaccharides for water molecules should be investigated in the future.

Survival during storage depends on the amount of moisture in the ampoule

In the accelerated storage test, desiccated cells in high- or low-vacuum ampoules were stored at 37°C. To maintain survival during storage, it is recommended that desiccated microorganisms be vacuum-encapsulated and stored in the dark at low temperature (Rudge, 1991; Tsvetkov and Brankova, 1983; Wolff et al., 1990). Oxygen in residual air is thought to decrease the survival of freeze-dried bacteria (Heckly and Quay, 1983; Israeli et al., 1974, 1975), whereas humidity or the presence of residual water is thought to result in damage to the freeze-dried cells under storage conditions (Gu et al., 2001). By trapping water, extracellular polysaccharides are important for bacterial survival in desiccated natural environments (Billi and Potts, 2002;

Potts, 1994). However, our previous study indicated that extracellular polysaccharides of freeze-dried bacteria may trap residual water in the ampoule and decrease the survival rates of the desiccated cells (Miyamoto-Shinohara et al., 2008).

For most strains in this research, freeze-dried cells stored in low-vacuum ampoules showed lower survival rates than those stored in high-vacuum ampoules, whereas the survival rates of L-dried cells were similar, regardless of the vacuum pressure at which they were stored. A freeze-dried ampoule contains dried cells with suspension medium, whereas in a L-dried ampoule there are dried cells, the suspension medium, and a cotton plug, which acts as a desiccant to decrease the moisture level in the residual air during storage (Iijima and Sakane, 1973).

Several L-dried strains turned brown during storage and did not survive, whereas no freeze-dried strains turned brown. This difference may be due to differences in the components of the suspending media. L-drying medium contains 6% PVP, a cryoprotective additive that is adsorbed onto the microbial surface, where it forms a viscous layer that can retain water (Hubalek, 2003). The presence of this retained water may have contributed to the cells turning brown. According to Mattern et al. (1999) and Sharma and Kalonia (2004), cell proteins show different forms and activities after freeze-drying and L-drying. For instance, in *Schizosaccharomyces pombe*, proteins are required for cell wall integrity, cell morphology, and resistance to high KCl concentration (Sengar et al., 1997). The morphology of desiccated cells should be investigated in the future.

Acknowledgments

We thank Dr. T. Sakane of the Institute for Fermentation, Osaka, for instructing us in L-drying methods and Dr. T. Nakahara of IPOD for his comments on this manuscript. We are deeply thankful to Prof. K. Furukawa, Prof. T. Ohsima, and Prof. K. Sonomoto of the Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, for their valuable suggestions and guidance.

References

- Antheunisse, J. (1973) Viability of lyophilized microorganisms after storage. *Antonie van Leeuwenhoek*, **39**, 243–248.
- Banno, I., Mikata, K., and Sakane, T. (1979) Viability of various yeasts after L-drying. *IFO Res. Commun.*, **9**, 27–34.

- Barnet, J. A., Payne, R. W., and Yarrow, D. (2000) Yeasts: Characteristics and Identification, Cambridge University Press, Cambridge, 1150 pp.
- Bastide, J. M., Hadibi, E. H., and Bastide, M. (1979) Taxonomic significance of yeast sphaeroplast release after enzymatic treatment of intact cells. *J. Gen. Microbiol.*, **113**, 147–153.
- Billi, D. and Potts, M. (2002) Life and death of dried prokaryotes. *Res. Microbiol.*, **153**, 7–12.
- Cabib, E., Drgonova, J., and Drgon, T. (1998) Role of small G proteins in yeast cell polarization and wall biosynthesis. *Annu. Rev. Biochem.*, **67**, 307–333.
- Depree, J., Emerson, G. W., and Sullivan, P. A. (1993) The cell wall of the oleaginous yeast *Trichosporon cutaneum*. *J. Gen. Microbiol.*, **139**, 2123–2133.
- Dumont, F., Marechal, P. A., and Gervais, P. (2003) Influence of cooling rate on *Saccharomyces cerevisiae* destruction during freezing: Unexpected viability at ultra-rapid cooling rates. *Cryobiology*, **46**, 33–42.
- Feldmesser, M., Kress, Y., and Casadevall, A. (2001) Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. *Microbiology*, **147**, 2355–2365.
- Garcia-Rivera, J., Chang, Y. C., Kwon-Chung, K. J., and Casadevall, A. (2004) *Cryptococcus neoformans* CAP59 (or Cap59p) is involved in the extracellular trafficking of capsular glucuronoxylomannan. *Eukaryot. Cell*, **3**, 385–392.
- Gu, M. B., Choi, S. H., and Kim, S. W. (2001) Some observations in freeze-drying of recombinant bioluminescent *Escherichia coli* for toxicity monitoring. *J. Biotechnol.*, **88**, 95–105.
- Heckly, R. J. and Quay, J. (1983) Adventitious chemistry at reduced water activities: Free radicals and polyhydroxy agents. *Cryobiology*, **20**, 613–624.
- Hernandez-Lopez, M. J., Prieto, J. A., and Randez-Gil, F. (2003) Osmotolerance and leavening ability in sweet and frozen sweet dough: Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. *Antonie van Leeuwenhoek*, **84**, 125–134.
- Hubalek, Z. (2003) Protectants used in the cryopreservation of microorganisms. *Cryobiology*, **46**, 205–229.
- Iijima, T. and Sakane, T. (1973) A method for preservation of bacteria and bacteriophages by drying in vacuo. *Cryobiology*, **10**, 379–385.
- Israeli, E., Giberman, E., and Kohn, A. (1974) Membrane malfunctions in freeze-dried *Escherichia coli*. *Cryobiology*, **11**, 473–477.
- Israeli, E., Kohn, A., and Gitelman, J. (1975) The molecular nature of damage by oxygen to freeze-dried *Escherichia coli*. *Cryobiology*, **12**, 15–25.
- Kappeli, O. and Fiechter, A. (1977) Component from the cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis* with possible relation to hydrocarbon transport. *J. Bacteriol.*, **131**, 917–921.
- Kappeli, O., Muller, M., and Fiechter, A. (1978) Chemical and structural alterations at the cell surface of *Candida tropicalis*, induced by hydrocarbon substrate. *J. Bacteriol.*, **133**, 952–958.
- Kobayashi, H., Oyamada, H., Matsuda, K., Shibata, N., and Suzuki, S. (2003) Distribution of antigenic oligomannosyl side chains in the cell wall mannans of several strains of *Candida tropicalis*. *Arch. Microbiol.*, **180**, 76–80.
- Kurtzman, C. P. and Fell, J. W. (1998) The Yeast: A Taxonomic Study, Vol. 4, Elsevier, Amsterdam, 1074 pp.
- Lipke, P. N. and Ovalle, R. (1998) Cell wall architecture in yeast: New structure and new challenges. *J. Bacteriol.*, **180**, 3735–3740.
- Malik, K. A. (1990) A simplified liquid-drying method for the preservation of microorganisms sensitive to freezing and freeze-drying. *J. Microbiol. Methods*, **12**, 125–132.
- Malik, K. A. (1999) Preservation of some extremely thermophilic chemolithoautotrophic bacteria by deep-freezing and liquid-drying methods. *J. Microbiol. Methods*, **35**, 177–182.
- Mattern, M., Winter, G., Kohnert, U., and Lee, G. (1999) Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. *Pharm Dev Technol*, **4**, 199–208.
- McFadden, D. C., De Jesus, M., and Casadevall, A. (2006) The physical properties of the capsular polysaccharides from *Cryptococcus neoformans* suggest features for capsule construction. *J. Biol. Chem.*, **281**, 1868–1875.
- McGinnis, M. R. and Tyring, S. K. (1996) Introduction to mycology. In Medical Microbiology, ed. by Schuenke, S., University of Texas Medical Branch, Galveston, pp. 889–902.
- Mikata, K. and Banno, I. (1989) Preservation of yeast cultures by L-drying: Viability after 5 years of storage at 5 °C. *IFO Res. Commun.*, **14**, 80–103.
- Mitic, S., Otenhajmer, I., and Damjanovic, V. (1974) Predicting the stabilities of freeze-dried suspensions of *Lactobacillus acidophilus* by the accelerated storage test. *Cryobiology*, **11**, 116–120.
- Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, Y., Kawamura, S., and Komatsu, Y. (2000) Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology*, **41**, 251–255.
- Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., and Nakahara, T. (2006) Survival curves for microbial species stored by freeze-drying. *Cryobiology*, **52**, 27–32.
- Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., and Nakahara, T. (2008) Survival of freeze-dried bacteria. *J. Gen. Appl. Microbiol.*, **54**, 9–24.
- Nguyen, T. H., Fleet, G. H., and Rogers, P. L. (1998) Composition of the cell walls of several yeast species. *Appl. Microbiol. Biotechnol.*, **50**, 206–212.
- Potts, M. (1994) Desiccation tolerance of prokaryotes. *Microbiol. Rev.*, **58**, 755–805.
- Prillinger, H., Oberwinkler, F., Umile, C., Tlachac, K., Bauer, R., Dorfler, C., and Taufratzhofer, E. (1993) Analysis of cell wall carbohydrates (neutral sugars) from ascomycetous and

- basidiomycetous yeasts with and without derivatization. *J. Gen. Appl. Microbiol.*, **39**, 1–34.
- Rudge, R. H. (1991) Maintenance of bacteria by freeze-drying. In *Maintenance of Microorganisms*, ed. by Kirsop, B. E. and Doyle, A., Academic Press, London, pp. 31–43.
- Sakane, T. and Kuroshima, K. (1997) Viabilities of dried cultures of various bacteria after preservation for 20 years and their prediction by the accelerated storage test. *Microbiol. Cult. Coll.*, **13**, 1–7.
- Sengar, A. S., Markley, N. A., Marini, N. J., and Young, D. (1997) Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.*, **17**, 3508–3519.
- Sentandreu, R. and Northcote, D. H. (1969) Yeast cell-wall synthesis. *Biochem. J.*, **115**, 231–240.
- Sharma, V. K. and Kalonia, D. S. (2004) Effect of vacuum drying on protein-mannitol interactions: The physical state of mannitol and protein structure in the dried state. *AAPS Pharm Sci Tech*, **5**, E10.
- Shematek, E. M. and Cabib, E. (1980) Biosynthesis of the yeast cell wall. II. Regulation of beta-(1 leads to 3) glucan synthetase by ATP and GTP. *J. Biol. Chem.*, **255**, 895–902.
- Shinoda, T., Kaufman, L., and Padhye, A. A. (1981) Comparative evaluation of the latron serological *Candida* check kit and the API 20C kit for identification of medically important *Candida* species. *J. Clin. Microbiol.*, **13**, 513–518.
- Slaninova, I., Sestak, S., Svoboda, A., and Farkas, V. (2000) Cell wall and cytoskeleton reorganization as the response to hyperosmotic shock in *Saccharomyces cerevisiae*. *Arch. Microbiol.*, **173**, 245–252.
- Smits, G. J., Kapteyn, J. C., van den Ende, H., and Klis, F. M. (1999) Cell wall dynamics in yeast. *Curr. Opin. Microbiol.*, **2**, 348–352.
- Tsvetkov, T. and Brankova, R. (1983) Viability of micrococci and lactobacilli upon freezing and freeze-drying in the presence of different cryoprotectants. *Cryobiology*, **20**, 318–323.
- Wolff, E., Delisle, B., Corrieu, G., and Gibert, H. (1990) Freeze-drying of *Streptococcus thermophilus*: A comparison between the vacuum and the atmospheric method. *Cryobiology*, **27**, 569–575.