Cryobiology **41,** 251–255 (2000)
doi:10.1006/cryo.2000.2282, available online at http://www.idealibrary.com on IDEAL®

BRIEF COMMUNICATION

Survival Rate of Microbes after Freeze-Drying and Long-Term Storage

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The survival rates of 10 species of microorganisms were investigated after freeze-drying and preserving in a vacuum at 5°C. The survival rates varied with species. The survival rates immediately after freeze-drying were different among yeast, gram-positive bacteria, and gram-negative bacteria, and the change in the 10-year survival rate was species-specific. The survival rate of yeast, *Saccharomyces cerevisiae*, was about 10% immediately after drying, and the rate did not decrease significantly during the 10-year storage period. Survival rates after the drying of gram-positive bacteria, i.e., *Brevibacterium flavum, B. lactofermentum, Corynebacterium acetoacidophilum, C. gultamicum,* and *Streptococcus mutans*, were around 80%. The survival rate of *Brevibacterium* and *Corynebacterium* did not decrease greatly during the storage period, whereas the rate of *S. mutans* decreased to about 20% after 10 years. Survival rates after the drying of gram-negative bacteria, i.e., *Escherichia coli, Pseudomonas putida, Serratia marcescens,* and *Alcaligenes faecalis,* were around 50%. The survival rate decreased for the first 5 years and then stabilized to around 10% thereafter.

Key Words: freeze-drying; storage; survival; yeast; gram-positive bacteria; gram-negative bacteria.

Freeze-drying is a popular method of preserving microorganisms because the long-term viability is excellent in most cases and the storage and distribution requirements are simple. Most strains of microorganisms in our laboratory are stored in freeze-drying ampoules. The viability of a strain can be maintained for more than 20 years if the cell concentrations are 10^6–10^{10} cell/ml before freeze-drying and the cells survive the drying process. Freeze-dried bacteria have been reported to survive for as many as 35 years (7). The survival rates differ according to genus, but it is unknown whether they differ among species.

The aim of this study was to clarify differences in survival rates among species of microorganisms. The survival rates after drying and during the first 10–16 years of storage were analyzed for one yeast and nine bacteria species. Table 1 shows the strains analyzed. Recombinant strains were not used to avoid the possibility of treating the same host strains. All strains were furnished by the Patent Microorganism Depository. Medium and incubation conditions were as indicated in the depositor's instructions.

The method used for freeze-drying the yeast and bacteria species has been described previously (3). Each strain was cultured on a 1.5% agar (010-08725; Wako Pure Chemical Industries, Osaka, Japan) slant in the beginning of the stationary phase. Five milliliters of suspension medium (10% skimmed milk and 1% sodium glutamate) was added to the slant culture and the cells were scraped to form a uniform cell suspension. The cell suspension was dispensed in 0.2-ml aliquots into an ampoule. The ampoules were immersed in cold ethanol at −60°C, quickly stirred for the first 10 s, and left to freeze for 2–10 min. The ampoules were then connected to a FREEZVAC-4C (Tozai Tsusho, Tokyo, Japan) manifold-type freeze-dryer or a FREEZEMOBILE (Virtis, Gardiner, NY, U.S.A.) freeze-dryer and dried for 4–20 h under a vacuum at <0.1 Pa. This method satisfied most of the conditions for good freeze-drying

Received January 11, 2000; accepted September 26, 2000; published online December 7, 2000.

This work was funded by institutional sources.

0011-2240/00 $35.00

TABLE 1
Numbers of Strains Tested

Species	Storage period (years)		
	<5	5–10	>10
Saccharomyces cerevisiae	25	18	52
Brevibacterium flavum	2	12	45
Brevibacterium lactofermentum	2	13	46
Corynebacterium acetoacidophilum	1	4	17
Corynebacterium gultamicum	1	17	100
Streptococcus mutans	0	8	13
Escherichia coli	25	27	75
Serratia marcescens	3	4	16
Pseudomonas putida	18	15	6
Alcaligenes faecalis	0	1	8

survival rates (i.e., preservation of fluids, culture age, etc.) (2, 4, 5). Drying ampoules were sealed with a gas burner, and the vacuum was checked with a high-frequency spark tester. The ampoules were stored at the recommended temperature of 5°C (9, 10).

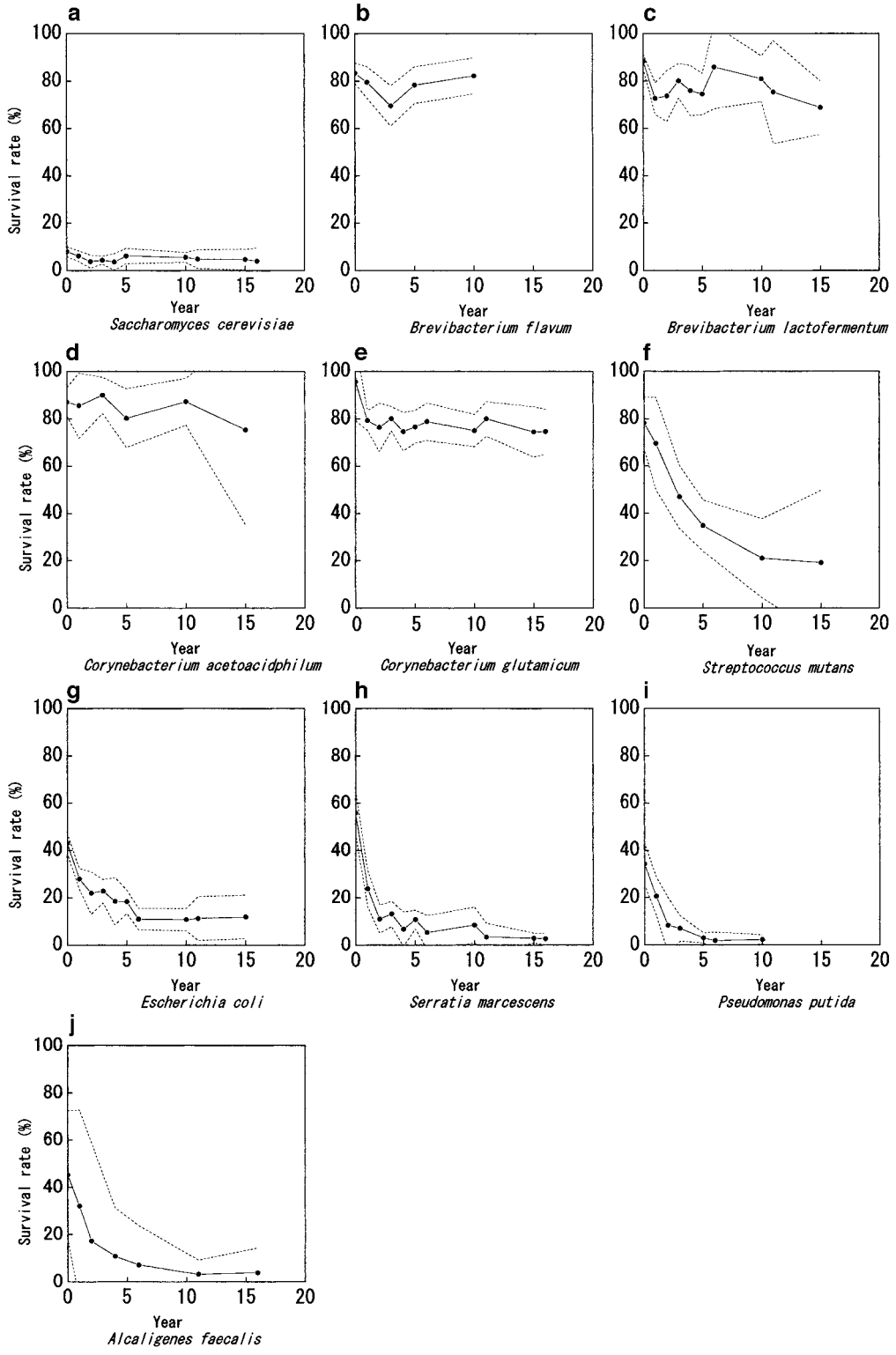
To test the survival of microorganisms, an ampoule was opened, the contents were diluted with sterilized water to 10^2 times, 10^4 times, or 10^6 times, and then 0.05 ml of the dilution was inoculated onto an agar plate culture medium using a spiral platter on a Model D (Spiral System Instrument, Bethesda, MD, U.S.A.) or an AUTOPLATE Model 3000 inoculater (Spiral Biotech, Bethesda, MD, U.S.A.). The colonies formed on the plate were counted to calculate the concentration of surviving cells. The concentration was taken as the average of more than three plates for each ampoule. The survival rate was calculated relative to the cell density before freeze-drying, which was considered to be 100%. This survival test was usually carried out after 1, 3, 5, 10, and 15 years.

Figures 1a–1j show the survival rates of each species after drying. The survival rate immediately after drying is shown at the start point (year 0), followed by that on each successive year. All strains showed concentrations of more then 10^4/ml after 10 years, which were sufficient for revival, whereas the start cell concentration varied from 10^6 to 10^{10}/ml. Each strain had equal aliquots of cell concentration in the ampoules before freeze-drying, but some strains had ampoules with unequal aliquots due to viscosity. Different technicians performed the experiments in different years, and the spiral platters also varied from year to year. Some data varied widely for these reasons, but each species had similar survival patterns. The 10 species were divided into three groups by the survival rate after freeze-drying: yeast, gram-positive bacteria, and gram-negative bacteria. The survival rate of yeast was 10% or less, that of the gram-positive bacteria was around 80%, and that of the gram-negative bacteria was around 50%.

The survival rate of *Saccharomyces cerevisiae* is shown in Fig. 1a. The confidence interval was 5.9–10.1% at year 0 after drying (95 strains), which was significantly lower than that of bacteria. Thereafter, survival rates did not decrease significantly during storage. The confidence interval at year 10 was 3.6–7.7% (43 strains), and all strains of *S. cerevisiae* were viable and maintained this confidence interval for 16 years or more.

Brevibacterium flavum, B. lactofermentum, Corynebacterium acetoacidophilum, C. glutamicum, and *Streptococcus mutans* were analyzed and their survival rates are shown in Figs. 1b–1f. The survival rates of these species immediately after drying (year 0) were about 80%, but the rates after the various storage periods differed by genus. *Brevibacterium* and *Corynebacterium* maintained an average survival rate of around 80% after 10 years. There was a slight but insignificant difference between the confi-

FIG. 1. (a)–(j) The change in the survival rate after freeze-drying. The continuous line shows the mean value, and the broken lines show the 95% confidence intervals. Survival rates before freeze-drying were assumed to be 100% and the start point was year 0 (immediately after drying).


a
b
c
d
e
f
g
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i
j
Survival rate (%)
Year
Saccharomyces cerevisiae
Brevibacterium flavum
Brevibacterium lactofermentum
Corynebacterium acetoacidphilum
Corynebacterium glutamicum
Streptococcus mutans
Escherichia coli
Serratia marcescens
Pseudomonas putida
Alcaligenes faecalis

dence interval of *B. flavum* after drying and that at year 10. The confidence interval after drying was 79.0–87.7% (59 strains) and that after 10 years was 74.6–89.8% (37 strains) (Fig. 1b). The mean value of *B. lactofermentum* over 10 years varied widely between 70 and 90%. The confidence interval after drying was 85.1–91.4% (58 strains) and that after 10 years was 71.3–90.6% (33 strains) (Fig. 1c). The confidence interval of *C. acetoacidophilum* also varied widely, but the average rate of survival was around 80% over 10 years. The confidence interval after drying was 81.1–93.1% (21 strains) and that after 10 years was 77.3–97.1% (17 strains) (Fig. 1d). The confidence interval of *C. glutamicum* dropped by about 10% after 1 year, but then was maintained for the following 10 years. The confidence interval after drying was 79.2–112.0% (112 strains) and that after 10 years was 68.1–81.8% (63 strains) (Fig. 1e). The survival rate of *Streptococcus mutans* tended to decrease during storage, whereas those of *Brevibacterium* and *Corynebacterium* remained the same. The decrease in the survival rate was great for the first 5 years, but it stabilized thereafter. The confidence interval after drying was 67.5–89.2% (20 strains) and 4.3–37.7% after 10 years (9 strains) (Fig. 1f).

Escherichia coli, Serratia marcescens, Pseudomonas putida, and *Alcaligenes faecalis* were analyzed and the results are shown in Figs. 1g–1j. The survival rates after drying were around 50% and decreased over the first 5 years and then stabilized thereafter. The pattern of decrease differed by species. The survival rate of *E. coli* decreased significantly in the first year, decreased slightly more over the next 5 years, and then stabilized. The confidence interval after drying was 38.9–47.3% (126 strains), that after 1 year was 23.5–32.4% (111 strains), and that after 10 years was 6.1–15.5% (61 strains) (Fig. 1g). The survival rate of *S. marcescens* also decreased significantly for the first year and then stabilized over the next 4 to 6 years. The confidence interval after drying was 46.8–65.0% (23 strains), 16.1–31.6% after 1 year (22 strains), and 0.8–16.0% after 10 years (12 strains) (Fig. 1h). The survival rate of *P. putida* decreased gradually over the first 2 years, decreased slightly between years 2 and 5, and stabilized thereafter. The confidence interval was 25.5–43.1% after drying (38 strains), 12.6–28.7% after 1 year (35 strains), and 0.4–4.3% after 10 years (5 strains) (Fig. 1i). The strain number of *A. faecalis* used for the analysis was low, and the width of the confidence interval was greater than that for other species. The survival rate tended to decrease over the first 5 years, but stabilized thereafter. The confidence interval was 18.3–72.3% after drying (10 strains) and after 11 years was 2.9–9.2% (5 strains) (Fig. 1j).

The survival rate of gram-positive bacteria immediately after freeze-drying tended to be higher than that of gram-negative bacteria at the same time point, which agrees with the results of Rudge (7). The same results have been reported in a study on L-drying (9), a method which dries without freezing (1). It has been suggested that gram-positive bacteria have greater resistance to drying than gram-negative bacteria and that the difference is due to the structure of the cell surface (6).

The survival rates of some species were fixed while those of other species decreased. Those that decreased dropped within the first 5 years but tended to stabilize within 15 years.

ACKNOWLEDGMENTS

The authors express their thanks to Mr. J. Someya, Ms. F. Nozawa, Ms. Y. Tsukamoto, Ms. H. Numata, Ms. Y. Okamura, Ms. K. Ishikawa, and the technicians of the Patent Microorganism Depository for their technical assistance. We also thank Mr. K. Kimura for his guidance with the freeze-drying method.

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