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Comparison of the Survival and Metabolic Activity of Psychrophilic and Mesophilic Yeasts Subjected to Freeze-Thaw Stress

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A mesophilic yeast, *Candida utilis*, and a psychrophilic yeast, *Leucosporidium stokesii*, were subjected to freeze-thaw cycling over the range 25 to −60 C. Viability after freeze-thaw stress was directly correlated with the rate of cooling and the physiological age of the cultures. Rates of glucose fermentation and oxidation could be directly correlated with viability. The optimal cooling rate for both yeast strains was 4.5 to 6.5 C/min; however, their levels of survival obtained at this optimal cooling rate varied considerably. In addition, the psychrophile was less resistant to freeze-thaw stress than was the mesophile.

Studies on the effects of freeze-thaw stress on terrestrial microorganisms have been stimulated by increased scientific activities in the Arctic and Antarctic regions and by the preparations for detecting possible evidence for life in extraterrestrial (Martian) environments. Although it would seem natural to include psychrophilic microorganisms, i.e., those which reproduce at 0 C, investigations on the effects of freeze-thaw stress have for the most part involved mesophilic forms. Thus, despite the large volume of data available on the effects of freeze-thaw stress on microbial cells in general, relatively little is known about the effects of such stress on those microorganisms whose most decisive characteristic is their ability to grow at low temperatures.

The purpose of the present study was to investigate the effects of multiple freeze-thaw cycling on survival, metabolic activity, and overall cell morphology of the obligately psychrophilic yeast *Leucosporidium stokesii* and the mesophilic yeast *Candida utilis*. Yeasts were selected for this study for the reasons previously cited by Mazur (10). (i) Their viability can be assayed relatively unambiguously by plate count methods, and (ii) they are large enough to permit detailed morphological observations.

Survival of both yeasts during freeze-thaw stress depended on the physiological age of the cells and the rate of cooling below −5 C. However, under all conditions tested the psychrophile was significantly more sensitive to freeze-thaw stress than the mesophile.

MATERIALS AND METHODS

Organisms and cultural conditions. *L. stokesii* CBS 5917 is an obligately psychrophilic, self-sporulating, heterobasidiomycetous yeast (4) originally isolated from an Antarctic snow-core sample (16). *L. stokesii* has a maximum growth temperature of 20 C and an optimum of 15 C and has been grown as low as -7 C (9). *Candida utilis* ATCC 8205 is a mesophilic, asporogenous yeast which grows over the range 5 to 43 C. The organisms were maintained at 15 C with repeated subcultures on YED medium containing yeast extract (10 g), dextrose (10 g), KH_2PO_4 (1 g), and $MgSO_4 \cdot 7H_2O$ (0.5 g) in 1 liter of distilled water. Cultures were aerated with shaking at 160 oscillations/min and a medium-to-flask volume ratio of 1:5. Cells for freeze-thaw studies were harvested in early exponential and mid-stationary phase, washed twice with sterile distilled water, and suspended in 0.3 M dextrose solution to a turbidity of 200 to 400 Klett units (Klett-Summerson photoelectric colorimeter, model 800-3, red filter, no. 64).

Freeze-thaw procedures. Replicate 3-ml suspensions were dispensed into glass test tubes (16 by 150 mm). To achieve a desired cooling rate the sample tubes were inserted into various sizes of larger glass test tubes. For example, to achieve a cooling rate of 4.1 ± 0.45 C/min, the sample tube was placed in a 22- by 200-mm glass test tube. The combination of sample and test tubes was then immersed in an ethanol-dry ice bath. Temperature changes were measured using a Yellow Springs Instrument telethermometer, model 42 SL with a model 423 thermister probe, positioned in the center of the cell suspension. Freezing and warming rates were calculated over the range −5 to −50 C. Frozen suspensions were rapidly thawed (100 C/min) by immersion and shaking of the sample tube in cold (12 C) tap water.

The freeze-thaw cycle involved freezing cultures either at 4.1 C/min (slow freeze) or 70+ C/min (fast freeze) to −60 C, storage at the latter temperature for 19.5 h followed by rapid thawing to 15 C, and incubation at 15 C for 4.5 h. The sequence was repeated for a total of three cycles.

Measurements. Viable cell counts were determined by spread plate technique using YED medium plus 1.5% agar. Plates were incubated at 15 C, and colonies were counted after 6 and 8 days for *L. stokesii* and *C. utilis*, respectively. Data for the calculation of volume and surface area values of viable cells, as indicated by neutral red exclusion, were obtained as follows. A standard loopful of cell suspension was mixed with a loopful of a 0.1% (wt/vol) neutral red solution and observed microscopically. Only unstained cells or cells showing uptake of stain into vacuoles exclusively were measured using a calibrated occular micrometer. The formulas of Müller (13) were used to determine the volume and surface area values. All data were evaluated for statistical significance at the 95% level of confidence by analysis of variance.

Metabolic activity. Treated and untreated cell suspensions were washed twice with distilled water, resuspended to the original volume in 0.067 M KH_2PO_4, and starved 2.5 h at 15 C with shaking. Dry weights were determined by reference to a standard dry weight-turbidity curve. Conventional manometric techniques were used to measure the oxidation and fermentation of glucose (18). For oxidation studies each Warburg vessel contained 7 to 10 mg of cell (dry weight) in 2.0 ml of 0.067 M KH_2PO_4 and 0.1 ml of M/25 glucose (2mM) in the main compartment and 0.2 ml of 20% KOH in the center well. The gas phase was air and the bath temperature was 25 C. For fermentation studies the KOH was omitted from the center well and the gas phase was nitrogen. $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ were calculated using standard formulas (18).

RESULTS

Effect of cooling rate on survival. Exponential- and stationary-phase cells of *C. utilis* and *L. stokesii* were cooled at various controlled rates to −60 C, held at that temperature for 1 h, and then rapidly thawed to 15 C. Percentage of survival versus cooling rate for the four cell populations is shown in Fig. 1. In all cases the optimum cooling rate was 4.5 to 6.5 C/min. However, percentage of survival obtained varied considerably between populations. For example, only 5% of the exponential-phase cells of *L. stokesii* survived at the optimum cooling rate as compared to 71% of the stationary-phase cells. Percentage of survival of exponential- and stationary-phase cells of *C. utilis* was 47 and 71% at the optimum cooling rate, respectively. Percentage of survival of stationary-phase cells of *C. utilis* was relatively constant over the range 4.1 to 70 C/min in contrast to survival of the other three cell populations.

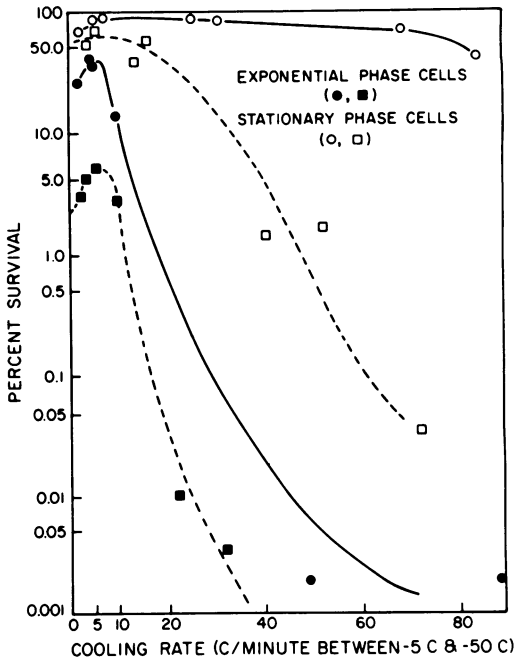

FIG. 1. *Effect of cooling rate on survival of C. utilis* (●, ○) *and L. stokesii* (■, □).

Effect of holding time at −60 C on survival. Exponential-phase cells of *L. stokesii* and *C. utilis* were cooled to −60 C at 4.1 C/min and held at that temperature for 32 h. During this experiment replicate tubes were removed at various time intervals, thawed, and plated as described. Viability of both yeasts declined during the 32-h holding period (Fig. 2). The decrease in viability over the 32-h holding period was negligible compared to the loss in viability which resulted from the freezing process itself.

Effect of freeze-thaw cycling on survival and metabolic activity. Exponential- and stationary-phase cells of both strains were subjected to three freeze-thaw cycles as described. Oxidation and fermentation of glucose as well as survival were determined on samples taken at the end of each 19.5-h holding period at −60 C. Percentage of survival and oxidation and fermentation rates of cells exposed to one, two, and three freeze-thaw cycles are shown in Table 1. Exponential-phase *L. stokesii* cells were least resistant to freeze-thaw cycling. After three cycles viability decreased to less than 0.1%. After two cycles neither oxidation nor fermentation could be detected. Percentage of survival of *L. stokesii* stationary-phase cells after three

cycles was 16%; 18.8% of the initial oxidative and 11.1% of the initial fermentative activities remained.

C. utilis cells were generally more resistant to freeze-thaw stress. After three cycles 5% of the exponential-phase cells remained viable. Moreover, 11.1% of the initial fermentative activity and 4.3% of the initial oxidative activity remained. By comparison, 31% of the stationary-phase cells remained viable after three cycles, whereas 24.6% of the initial fermentative and 31.0% initial oxidative activities remained.

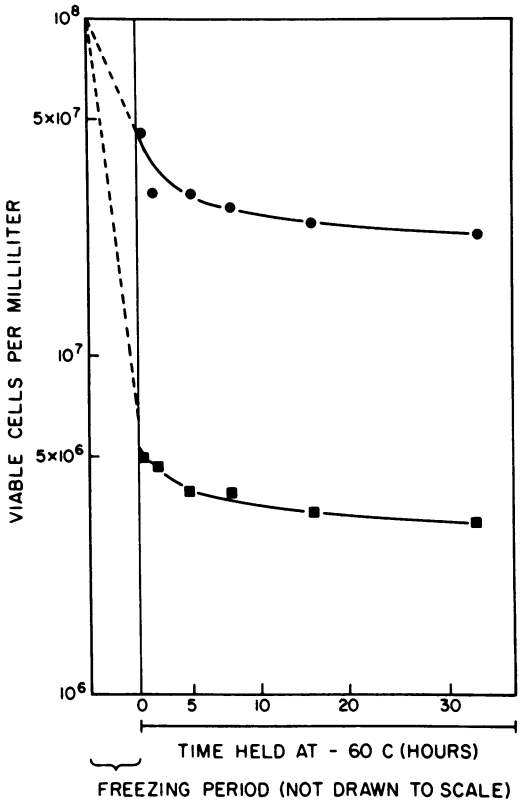

FIG. 2. *Effect of holding time at −60 C on viability of C. utilis (●) and L. stokesii (■) exponential-phase cells.*

Changes in morphology and viability in freeze-thaw stressed cells. Untreated, slow-frozen (4.1 C/min) and fast-frozen (70+ C/min), exponential- and stationary-phase cells of both yeast strains were observed microscopically for exclusion of neutral red dye. Cells showing uptake of the dye into the cytoplasm were assumed nonviable, whereas cells excluding the dye or taking dye into the vacuoles only were assumed viable. Viable cells were measured and cell volume and surface area values were calculated as described. The data are shown in Table 2. The two yeast strains differed considerably in overall dimensions; i.e., cells of the psychrophile were significantly larger than cells of the mesophile. However, the volume-to-surface ratios of cells of the psychrophile were significantly less than of the cells at the mesophile, with the exception of fast-frozen, stationary-phase cells. Decreases in viability in both slow- and fast-frozen cells did not correlate with changes in volume, surface, or volume-to-surface ratios. The volume distribution of viable cells within all four populations decreased significantly as a result of fast freezing (Fig. 3). However, when samples of these same four populations were slow frozen, only the stationary-phase cells of the psychrophile showed a significant decrease in volume distribution. In addition to changes in their cell dimensions, stationary-phase cells of both strains lost their

TABLE 1. *Effect of freeze-thaw cycling on survival and metabolic activity of exponential- and stationary-phase cells of C. utilis and L. stokesii*

Organism	Culture age	Viability (%)			Oxidation ($Q_{O_2}^{air}$)[a]				Fermentation ($Q_{CO_2}^{N_2}$)[b]			
		1[c]	2	3	0	1	2	3	0	1	2	3
C. utilis	Exponential	47.0	21.5	5.0	116 (100.0)[d]	56 (48.3)	21 (18.1)	5 (4.3)	81 (100.0)	34 (42.0)	17 (21.0)	9 (11.1)
	Stationary	71.5	52.0	31.0	58 (100.0)	43 (74.1)	29 (50.0)	18 (31.0)	69 (100.0)	50 (72.5)	28 (40.6)	17 (24.6)
L. stokesii	Exponential	5.3	-[e]	-	49 (100.0)	3 (6.1)	-	-	10 (100.0)	1 (10.0)	-	-
	Stationary	71.0	40.5	16.0	32 (100.0)	21 (65.6)	11 (34.4)	6 (18.8)	27 (100.0)	10 (37.0)	5 (18.5)	3 (11.1)

[a] $Q_{O_2}^{air}$, Microliters of O_2 taken up per milligram of cell (dry weight) per hour.
[b] $Q_{CO_2}^{N_2}$, Microliters of CO_2 produced per milligram of cell (dry weight) per hour.
[c] Number of freeze-thaw cycles.
[d] Numbers in parentheses indicate percent.
[e] Too low for measurement (<0.1%).

TABLE 2. *Volume, surface, volume-to-surface ratio, and viability of untreated, slow-frozen and fast-frozen, exponential- and stationary-phase cells of C. utilis and L. stokesii*

Culture age	Treatment	*C. utilis*				*L. stokesii*			
		Vol (μm^3)	Surface area (μm^2)	Vol/ surface area[a]	Viability (%)	Vol (μm^3)	Surface area (μm^2)	Vol/ surface area[a]	Viability (%)
Exponential	Untreated	85.39 ± 4.462	73.72 ± 5.586	1.23 ± 0.038	100.0	130.83 ± 6.307	121.69 ± 8.907	1.15 ± 0.034	100.0
	Slow frozen (4.1 C/min)	90.49 ± 4.354	80.12 ± 5.469	1.19 ± 0.037	47.0	122.05 ± 9.963	117.12 ± 15.310	1.11 ± 0.051	5.3
	Fast frozen (70+ C/min)	59.34 ± 4.319	41.84 ± 4.193	1.49 ± 0.078	<0.1	106.05 ± 7.543	94.04 ± 10.057	1.14 ± 0.039	<0.1
Stationary	Untreated	74.42 ± 6.635	61.63 ± 8.621	1.43 ± 0.067	100.0	129.40 ± 7.879	120.57 ± 10.604	1.13 ± 0.037	100.0
	Slow frozen (4.1 C/min)	72.93 ± 5.908	58.65 ± 7.276	1.44 ± 0.073	71.5	106.75 ± 7.865	90.69 ± 9.567	1.27 ± 0.049	71.0
	Fast frozen (70+ C/min)	54.81 ± 4.166	37.54 ± 4.514	1.64 ± 0.068	64.0	106.96 ± 6.958	88.22 ± 8.935	1.79 ± 0.043	0.1

[a] $\bar{Y} \pm 1.96$ (standard deviation).

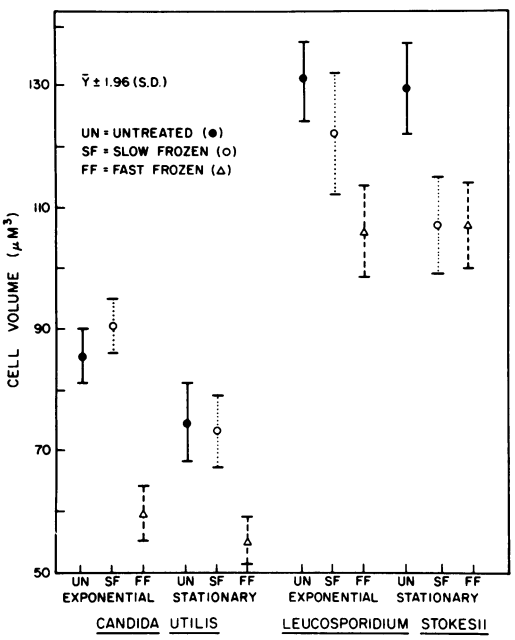

FIG. 3. *Volumes of untreated, slow-frozen and fast-frozen, exponential- and stationary-phase cells of C. utilis and L. stokesii.*

characteristically large vacuoles while undergoing fast-freeze stress.

DISCUSSION

Survival of *L. stokesii* and *C. utilis* cells after exposure to freeze-thaw stress is dependent on the physiological age of the culture and the rate of cooling. Moreover, after exposure to freeze-thaw stress, rates of glucose oxidation and fermentation could be correlated with viable cell numbers. Storage time at −60 C and changes in cell volume had little effect on survival.

Mazur (10, 11) has described various aspects of freeze-thaw injury to microorganisms. Each cell type has an optimum cooling rate at which survival is maximum. For example, with *Saccharomyces cerevisiae* the optimum cooling rate lies within the range 1 to 10 C/min (11). Likewise, in our studies with *L. stokesii* and *C. utilis* the optimum cooling rate was within the range 4.5 to 6.5 C/min irrespective of physiological age. The level of survival obtained at the optimum cooling rate, however, depended upon the physiological age of the cells. Exponential-phase cells of both strains were more susceptible to freeze-thaw injury than were stationary-phase cells. Similar physiological age-dependent differences in susceptibility have been reported for yeasts and other microorganisms exposed to freeze-thaw stress (2, 14), as well as to other forms of lethal and sublethal stress (8).

Nei (14) observed that *Escherichia coli* cells exposed to from one to three freeze-thaw cycles had an increased respiratory rate as compared to untreated cells. He suggested that leakage products from lethally damaged cells served as metabolic substrates for the nonlethally damaged cells. To preclude this effect, in our study the treated cells were washed prior to manometric determinations. Under these conditions the rates of both oxidation and fermentation of glucose could be correlated with percentage of survival. Likewise, Bradley (1) re-

ported that the rate of fermentation of glucose and sucrose by *Saccharomyces pastorianus* after freeze-thaw stress could be correlated with percentage of survival.

The literature is replete with reports on the responses of microorganisms to freeze-thaw cycling (3, 5, 7, 19). Many of the studies reported involved temperature regimes approximating those on Mars. For example, Curtis (3) exposed 18 strains of fungi to diurnal temperature extremes of −93 C (19.5 h) and 23 C (4.5 h). Nine strains showed mycelial growth of 1 mm or more after 35 cycles. In this study the cooling rates were approximately 1 to 2 C/min. Kooistra et al. (7) exposed soil samples to the following simulated Martian environmental parameters: nitrogen atmosphere at 54.1 mm of Hg, 1% or lower moisture and temperature cycling, 15 h at −22 C, and 9 h at room temperature. Gram-negative bacteria predominated, whereas yeasts and yeastlike forms gradually disappeared. Young et al. (19) subjected an *Aerobacter aerogenes* strain to freeze-thaw cycling (4.5 h at 24 C, 19.5 h at −75 C) under a nitrogen atmosphere. Cells suspended in a growth medium were killed at a constant rate during each freeze-thaw cycle. However, owing to the short generation time there was a net 4,000-fold increase in viable numbers during the 4-day experiment. Our studies compliment the findings of Young et al. (19). Both yeast strains used in our study, however, have generation times at 15 C slightly longer than the 4.5-h incubation period. Furthermore, the cells were suspended in a "nongrowth" medium. Under these conditions it is not surprising that viable cell numbers did not increase significantly for either strain during the 4.5-h incubation period.

Suspensions of fast-frozen *L. stokesii* and *C. utilis* cells contained considerable amounts of cellular debris. For example, lysis was so extensive in one fast-frozen suspension of *C. utilis* exponential-phase cells that only 40 viable cells could be found in over 100 fields observed. In addition, stationary-phase cells of both strains which survived the fast-freezing process lost their vacuoles and decreased significantly in volume. With the exception of slow-frozen *L. stokesii* stationary-phase cells which underwent a significant decrease in volume, these changes were not observed for the slow-frozen cell population. Our observations are in agreement with those of Hansen and Nossel (6) and Mazur (11). These investigators reported distortion and shrinkage of the cell outline and disappearance of vacuoles in fast-frozen yeast. In their reports, however, no mention was made of extensive lysis. Mazur and Schmidt (12) have shown that yeast cells cooled at rates greater than the optimum are injured by internally formed ice crystals, whereas cells cooled at rates less than the optimum are injured by other, nondisruptive internal events.

Our data suggest that the inability of *L. stokesii* to withstand freeze-thaw stress is unrelated to its ability to grow at subzero temperatures (9). The underlying basis for the observed differences in response of the psychrophilic and mesophilic yeasts, however, remain unknown, although Siegel et al. (15) suggested the possibility that organisms living at extremely low temperatures are less pressed for survival than are those exposed to periodic temperature fluctuations. The former situation may apply to the Antarctic snow habitat of the psychrophile, since the ability of the psychrophile to grow at subzero temperatures is unrelated to its inability to survive freeze-thaw stress.

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