SURVIVAL AND METABOLIC ACTIVITY OF MESOPHILIC AND PSYCHROPHILIC YEASTS SUBJECTED TO FREEZE-THAW STRESS

by

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TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	v
LIST OF TABLES	vi
ABSTRACT	vii
INTRODUCTION	1
MATERIALS AND METHODS	7
Organisms	7
Growth Media	7
Growth Curves	8
Freeze-Thaw Procedures	8
Survival versus Time Held at -60 C	10
Survival versus Freezing Rate	10
Survival versus Freeze-Thaw Cycling	10
Changes in Population Parameters as a Function of Cooling Rate	12
RESULTS	14
Growth Curves	14
Survival versus Time Held at -60 C	14
Percentage Survival versus Cooling Rate	15
Survival, Respiration, and Fermentation in Serially Frozen cells	16
Microscopic Examination of Freeze-Stressed Cells	17
DISCUSSION	27
REFERENCES	32

LIST OF ILLUSTRATIONS

Figure		Page
1.	Growth curves of *Candida utilis* and *Leucosporidium stokesii* in shake culture	21
2.	Effect of holding time at -60 C on the viability of *Candida utilis* and *Leucosporidium stokesii* exponential phase cells	22
3.	Effect of various cooling rates on survival of the psychrophile - *Leucosporidium stokesii*	23
4.	Effect of various cooling rates on survival of the mesophile - *Candida utilis*	24
5.	Effect of repeated freeze-thaw cycling on survival and metabolic activity of exponential and stationary phase cells of *Leucosporidium stokesii*	25
6.	Effect of repeated freeze-thaw cycling on survival and metabolic activity of exponential and stationary phase cells of *Candida utilis*	26

LIST OF TABLES

Table		Page
1.	Combinations of test tubes used to achieve an approximate cooling rate	9
2.	Cell dimensions of exponential and stationary phase cells of Leucosporidium stokesii measured prior to and after slow-freezing and fast-freezing	18
3.	Cell dimensions of exponential and stationary phase cells of Candida utilis measured prior to and after slow-freezing and fast-freezing	19

ABSTRACT

A mesophilic yeast, Candida utilis, and a psychrophilic yeast, Leucosporidium stokesii, were subjected to freeze-thaw cycling over the range of 25 C to -60 C. Optimum cooling rates were determined for exponential and stationary phase populations of each strain. Exponential and stationary phase populations were serially frozen at their optimum cooling rates and assayed for survival, fermentative activity, and respiratory activity after one, two, and three freeze-thaw cycles. The psychrophile was found to be more labile to freeze-thaw stress than was the mesophile under the conditions tested. In addition, for both strains the stationary phase population was more stable to freeze-thaw stresses than was the exponential phase population.

INTRODUCTION

Microorganisms are noted for their ability to survive under environmental conditions which preclude or severely limit the existence of higher forms of life. Brock (1) has shown that bacteria alone exist in thermal springs above 73 C. Likewise, at the lower end of the temperature scale as temperatures become more extreme, the number of species declines. In the Antarctic, flowering plants are extremely rare; only two species have been identified to date. A few species of mosses and algae are able to maintain a precarious existence (32). Cameron and Merck (4) report viable bacterial counts as low as 25 per gram of soil for samples taken in the McKelvey Valley of Antarctica. This ecosystem is too harsh to support algae, fungi, lichens, or mosses.

Studies of cryogenic (below zero) environments assume new importance as man prepares to seek an answer to the question of whether or not life exists elsewhere in the Universe (20, 28). Since an exobiota has yet to be discovered, one can only speculate on the forms such a biology might take. Geocentrism dominates such speculations because it is difficult to conceive of a biology which is not based on organic compounds in a water solvent (36). Mars will be the first planet to be explored after the earth's moon. As currently envisioned, at some time in the second half of this decade, an unmanned Automated Biological Lander will be positioned on the martian surface. This lander will be equipped with a number of experimental modules designed

to test for the existence of life (35). Such tests will depend on measurements of the metabolic activities of an indigenous biota free from terrestrial contaminants. Among the many questions raised in preparation for these experiments is whether or not terrestrial microorganisms can survive the extremely low temperatures characteristic of Mars and of space in general.

Based on the data obtained by Mariners 6 and 7, the following conditions are believed to prevail on the martian surface at the equator: a predominantly CO_2 atmosphere with a trace of nitrogen, nitrogen oxides, and oxygen; barometric pressure of 7-10 mm Hg. The diurnal temperature range is 12 C to -70 C with a maximum of 4.5 hours at temperatures above 0 C. Due to the thin atmosphere and the lack of an ozone layer, the ultraviolet (UV) flux at the surface is quite high (25). Water is extremely scarce and has been assumed to be a critical factor. Based on the results of spectroscopic analyses of the martian atmosphere and surface, Owen and Mason (27) have suggested that at the martian surface the water component varies from 15 to 50 microns of precipitable water. Many students of martian "biology", however, assume the presence of ample water in microenvironments (31).

Laboratory studies involving exposure of terrestrial microorganisms to extremes simulating parameters believed to be present in the martian and space environments fulfill two purposes. They serve to indicate those features of terrestrial biology most likely to characterize a biology adapted to extreme environments, assuming this biology to be similar to ours in other respects. In addition, they serve to

underline the importance of excluding terrestrial microorganisms from the test vehicle since the metabolic activities of such contaminants could lead to equivocal results, particularly if the indigenous biota were present only in relatively low numbers.

Among others, Brueschke, Suess, and Willard (2) and Portner et al. (29) have demonstrated that common terrestrial microbes can survive exposure to ultra-high vacuums on the order of 10^{-6} mm Hg. In 1936, Lipmann (16) reported the ability of bacterial spores and seeds of higher plants to survive 44 hours exposure to temperatures as low as 1.35 degrees Kelvin with prior desiccation. These studies and similar experiments (5, 37) suggest the possibility of terrestrial microorganisms successfully "hitching" rides on inadequately sterilized space vehicles if such contaminants are protected from the UV flux.

Serious attempts to assess the effect(s) of a martian temperature regime on survival of terrestrial microorganisms date from the late 1950's. Kooistra, Mitchell, and Strughold (12) exposed soil samples to simulated martian parameters (nitrogen atmosphere at 54.1 mm Hg, 1% or lower moisture and temperature cycling: 9 hours at room temperature and 15 hours at -22 C) and noted a trend for the microbial population to accommodate to the extremes. Gram-negative bacteria predominated while yeasts and yeast-like forms gradually disappeared.

Young et al. (37) subjected an Aerobacter aerogenes strain to freeze-thaw cycling (4.5 hours above 0 C, 19.5 hours at -75 C) under a nitrogen atmosphere. Cells suspended in growth medium experienced a constant rate of killing over each freeze-thaw cycle but due to the

short generation time of the bacterium there was a net 4000-fold increase in the number of viable organisms during the four-day experiment.

Curtis (5) reported the response of 18 strains of fungi to diurnal temperature extremes of -93 C (19.5 hours) and 23 C (4.5 hours). After 35 cycles, nine strains showed mycelial growth of 1 mm or more. Cooling rates for this study were approximately 1-2 C per minute from 0 C to -40 C comparing favorably with the data reported by Mazur (19) and discussed below.

In a continuing series of investigations, Mazur and his coworkers (15, 17, 21) have described the quantitative and subcellular aspects of injury to yeast cells which have been exposed to freeze-thaw stress. Current cryobiotic theory postulates that each cell type has an optimum cooling rate at which cells achieve their highest levels of survival. In a cellular system undergoing freezing, extracellular water freezes before intracellular water. Intracellular water vapor then "diffuses out of the cell in response to the vapor pressure differential between the external medium and the supercooled internal medium" (15, p. 447). Cells cooled at rates less than the optimum lose too much water, dehydrate, and the resulting concentration of intracellular contents leads to a solute effect, intracellular damage due to prolonged exposure to solutions with changed physical and chemical properties. On the other hand, cells cooled at rates above the optimum are affected by a second factor. They are unable to equilibrate fast enough and consequently undergo formation of internal ice crystals

(either large or small, depending on still other factors). Large crystals are disruptive in themselves while smaller crystals can serve as nuclei for the formation of larger crystals if the rewarming stage is too slow (20). With regard to rewarming, Mazur has demonstrated that cells warmed slowly are less likely to survive than cells warmed rapidly, assuming that both populations were previously cooled at the same rate (19, 21).

Mazur and Schmidt (21) have reported maximum survival of 55% for Saccharomyces cerevisiae cells suspended in M/15 KH_2PO_4. Nei (26) obtained similar results with another unidentified yeast strain suspended in distilled water. Survival was 80% at cooling rates of 5-10 C per minute with rapid rewarming. In addition to strain differences, differences in percentage survival may be owing to the techniques used for assessing viability. Mazur and Schmidt (21) assessed viability on the basis of colony-forming ability while Nei (26) estimated viability using methylene blue staining techniques.

As a corollary to the above, numerous reports in the literature (6, 23, 30) stress the importance of the suspending medium itself as a factor in cryoprotection. Generally the more hydrogen-bonding sites possessed by a molecule, the greater its cryoprotective properties. Compounds such as monosaccharides, glycerol, and dimethyl sulfoxide afford cryoprotection while alcohols, phenols, urea, and inorganic salts offer little or no protection (6).

The purpose of the present study was to determine the effect(s) of multiple freeze-thaw cycling on survival of a psychrophilic yeast,

Leucosporidium stokesii, as compared to survival of a mesophilic yeast, Candida utilis, exposed to similar stress conditions. Survival was assessed on the basis of colony-forming ability, respiratory activity, and fermentative activity. Cells were suspended in 0.3 M dextrose solution to simulate a "substrate" which might conceivably be selected for inclusion in an extraterrestrial lander. Both exponential and stationary phase populations were examined to determine the possible effect of physiological age as a factor in survival. In addition, each population was examined microscopically to determine the size distribution of untreated, slow frozen and fast frozen cells. Based on these data, surface-to-volume ratios were calculated in accordance with the suggestion of Mazur (20) that this ratio is an important factor in determining the optimum cooling rate of a population of cells.

MATERIALS AND METHODS

Organisms

Leucosporidium stokesii, CBS 5917, a psychrophilic yeast originally isolated by Sinclair and Stokes (33) from an Antarctic snow core, was maintained on yeast extract and dextrose agar (YAD) slants (see below) with incubation at 15 C until use. Subcultures were made at three- to five-week intervals. *Leucosporidium stokesii* is a typically self-sporulating heterobasidiomycetous yeast which ferments glucose slowly (7).

Candida utilis, ATCC 8205, an asporogenous yeast commonly found as a contaminant in food industries, was maintained on YAD slants (see below) by incubation at 15 C until use. Subcultures were made at three- to five-week intervals. Kreger-van Rij (13) has suggested that some *Candida* species may be imperfect forms of *Leucosporidium* or related heterobasidiomycetous genera.

Growth Media

Cells were grown in YAD broth which contained yeast extract, 10 g; dextrose, 10 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g in one liter of distilled water. To insure adequate aeration, a medium to Ehrlenmeyer flask volume ratio of 1:5 was used. Cultures were incubated at 15 C while shaking at 160 oscillations per minute. For solid medium YAD broth was supplemented with 1.5% agar.

Growth Curves

A four-day-old YAD agar slant culture was washed with 5 ml of distilled water. One ml of this cell suspension was then used to seed 100 ml of YAD broth and the culture was incubated as noted above. Periodic turbidometric readings were taken using the Klett-Summerson photoelectric colorimeter, Model 800-3 (red filter). At selected intervals, 10-ml portions of the culture were filtered into preweighed 47-mm Millipore Metricel filters (pore size 0.45 μ) and dried to constant weight.

Freeze-Thaw Procedures

Cells to be frozen were centrifuged three times at 4340 x gravity for 10 minutes at 10 C, washed with sterile distilled water and resuspended in 0.3 M dextrose solution to 200 to 450 Klett units turbidity. Three-ml portions were dispensed to 16 mm x 150 mm glass Bellco tubes. To approximate a desired cooling rate, various combinations of different-sized pyrex culture tubes were nestled inside each other with the sample tube in the center of the next. The complete system was then immersed in an ethanol:dry ice bath. Table 1 lists the various combinations of tubes utilized, the average specimen cooling rate achieved, and the 95% confidence intervals for that combination. Note that much variation was present, especially at the higher cooling rates. However, the specific cooling rate for each experiment was measured with a Yellow Springs Instrument tele-thermometer, Model 42SL, using a Model 423 thermister probe positioned in the center of the cell suspension. Depending on the protocol involved, the frozen cultures were either

Table 1. Combinations of test tubes used to achieve an approximate cooling rate.

Tube combination	Cooling rate[a]
Culture tube only	60.9 ± 41.08 C per minute
Culture tube in small[b] tube plus 95% ethanol (1 ml)	30.4 ± 5.29 C per minute
Culture tube in large[c] tube plus 95% ethanol (2 ml)	19.1 ± 6.91 C per minute
Culture tube in dry small tube	7.4 ± 2.14 C per minute
Culture tube in dry large tube	4.1 ± 0.45 C per minute
Culture tube in dry small tube in dry large tube	2.0 ± 1.60 C per minute

a. Cooling rate ± 1.96 standard deviations.

b. Small tube = 18 mm x 150 mm.

c. Large tube = 22 mm x 200 mm.

stored at -60 C or held for one hour at -60 C and then rapidly thawed (at approximately 100 C per minute) by immersion in cold tap water (12-15 C) with constant agitation of the culture tube. Cooling rates and rewarming rates were calculated over the range -5 C to -50 C (22).

Survival versus Time Held at -60 C

Samples of cell suspensions, prepared as above (Freeze-Thaw Procedures section), were frozen at 4-5 C per minute to -60 C, then stored at that temperature. At 0, ½, 1, 2, 4, 8, 16, and 32 hours, tubes were selected at random and thawed rapidly to room temperature. Viability was determined by plate counts of serial dilutions of each sample.

Survival versus Freezing Rate

Samples of cell suspensions, prepared as above (Freeze-Thaw Procedures section), were frozen at controlled rates to -60 C, held at that temperature for one hour, and thawed rapidly to room temperature. Serial dilutions were made of the initial cell suspension and of the treated cell suspensions in sterile distilled water and plated on YAD agar plates with incubation at 15 C. Colonies were counted after four- and six-day incubations; the plates were reincubated and counted again after six- and eight-day incubations for *L. stokesii* and *C. utilis*, respectively.

Survival versus Freeze-Thaw Cycling

Samples of cell suspensions, prepared as above (Freeze-Thaw Procedures section), were frozen at 4-5 C per minute to -60 C, then

stored at that temperature. After 19.5 hours, the cultures were rapidly thawed to room temperature and incubated at 15 C for 4.5 hours. This cycle was repeated to provide a total of three freeze-thaw cycles for each culture. Survival was determined by plate counts of serial dilutions of untreated, once-frozen, twice-frozen, and thrice-frozen cells. In some cases, viable counts were made of samples taken at the end of the 4.5 hour, 15 C incubation period immediately prior to freezing.

In order to determine metabolic activity, a portion of the untreated, once-, twice-, and thrice-frozen cell suspensions were centrifuged twice, washed in distilled water and resuspended to the original sample volume in M/15 KH_2PO_4. These suspensions were incubated for 2-3 hours at 15 C with constant shaking. Following this starvation period, standard manometric techniques were employed (34). Respiration was measured at 25 C on either the Gilson Differential Respirometer or the GME Circular Warburg Apparatus. Fermentative activity was measured at 25 C using a nitrogen atmosphere and the GME Circular Warburg Apparatus. Turbidometric readings (Klett-Summerson, red filter) were made on the starved cell suspensions immediately prior to the manometric determinations. Dry weights were determined by reference to a previously prepared dry weight versus turbidity curve. In some cases, plate counts of serial dilutions of the phosphate-suspended cells were made both prior to and at the end of the starvation period.

Each Warburg vessel contained 2.0 ml of cells suspended in M/15 KH_2PO_4 with the total dry weight varying from 7-10 mg depending on the treatment involved. Four micromoles of glucose were added to the

sidearm of some vessels. For respiration studies 0.2 ml of 20% KOH was included in the center well along with an accordian-folded strip of filter paper to increase surface area. All vessels contained a total liquid volume of 2.3 ml.

Changes in Population Parameters as a Function of Cooling Rate

Cell suspensions, prepared as above (Freeze-Thaw Procedures section), were frozen at slow (4-5 C per minute) or fast (greater than 50 C per minute) rates to -60 C, held at that temperature for one hour, then rapidly thawed to room temperature. Samples of these treated suspensions as well as samples of untreated suspensions were treated as follows: a loopful of a 1/1000 dilution of neutral red suspension and a loopful of the cell suspension were mixed and observed under oil immersion optics. Cells showing uptake of stain into the cytoplasm were considered nonviable or at the very least injured. Cells showing uptake of stain in the vacuoles only were considered to be viable. The latter cells were selected at random and length and width measurements were made using a calibrated ocular micrometer. These data were then evaluated to determine the mean parameters for each population including surface, volume, and surface/volume ratios. The following formulae of Muller (24) were used to determine the surface and volume:

$$\text{Surface} = 2\pi b\left(b + a\,\frac{\arcsin \epsilon}{\epsilon}\right), \quad \epsilon = \frac{1}{a}\sqrt{a^2 - b^2}$$

$$\text{Volume} = \frac{4}{3}\pi ab^2$$

$$\text{Length} = 2a, \quad \text{Width} = 2b$$

Data obtained in this experiment and in those listed above were tested for statistical significance by analysis of variance or other appropriate procedures. Statements of significance are based on the 0.05 level of probability.

RESULTS

Growth Curves

Growth curves were plotted for Leucosporidium stokesii and Candida utilis to determine harvesting times for cells to be used in the experiments described below. Figure 1 depicts growth curves for L. stokesii and C. utilis in YAD. The growth curves at 25 C have been included for comparison. It should be noted that the psychrophile does not grow at 25 C. The calculated generation time for the psychrophile at 15 C was approximately 5.5 hours while that of the mesophile was approximately 4.5 hours at 15 C and 1.5 hours at 25 C. On solid media at 15 C, however, C. utilis took longer (5 days) to form visible colonies than did L. stokesii (3 days). For the experiments reported below, exponential phase cells were harvested at 80-100 Klett units turbidity while stationary phase cells were harvested from cultures after 48 hours incubation. (See end of Results chapter for Figures 1 through 6.)

Survival versus Time Held at -60 C

In order to determine the effect(s) of holding time at -60 C on survival of exponential phase populations, populations of both the mesophile and the psychrophile in the exponential phase were frozen to -60 C at 4.1 ± 0.45 C per minute and held at that temperature for varying periods of time. Figure 2 depicts numbers of viable cells versus time held at -60 C. The mesophile showed approximately 15% survival. The level for the mesophile was much lower for this experiment than in the

experiments reported below. Both populations showed a slight but steady decline in cell numbers over the 32-hour period. Based on these data, a holding time of 60 minutes was selected for the experiments cited below.

Percentage Survival versus Cooling Rate

Survival versus cooling rate was studied in order to determine the optimum cooling rates for exponential and stationary phase populations of both the mesophile and the psychrophile. The data presented in Figure 3 show percentage survival versus rate of cooling for exponential and stationary phase populations of Leucosporidium stokesii. The optimum cooling rate was 4.5 C to 6.5 C per minute. The maximum percentage survival obtained, however, at this optimum cooling rate was less than 12% for exponential phase cells compared to 45% for stationary phase cells. At rates above or below the optimum, survival dropped markedly.

The data obtained for the mesophile are shown in Figure 4. The optimum cooling rate for exponential phase cells was approximately 4.5 C to 6.5 C per minutes with steep shoulders as the cooling rate was increased or decreased. At this optimum cooling rate, percentage survival was 40%. However, percentage survival for the stationary phase population was approximately 90% and remained relatively constant up to a cooling rate of 70 C per minute.

Survival, Respiration, and Fermentation in Serially Frozen Cells

Leucosporidium stokesii and C. utilis cells were exposed to multiple freeze-thaw stress conditions. Survival, respiration, and fermentation were measured. The data are shown in Figures 5 and 6. Percentage survival, respiratory activity, and fermentative activity of L. stokesii and C. utilis exponential and stationary phase populations frozen once, twice, and thrice are shown.

Cooling exponential phase cells of the psychrophile, L. stokesii, at a rate of 4.1 to 0.45 C per minute (Figure 5) markedly affected survival, fermentation, and respiration. For example, after one freeze-thaw cycle only 5% of the initial activities remained. After two and three cycles, survival dropped to 0.1% and 0.04%, respectively. Fermentative and respiratory activities were too low to be measured by standard manometric techniques. Stationary phase cells of the psychrophile were less affected by multiple freeze-thaw cycle, survival was 76%, fermentative activity was 43%, and respiratory activity was 68%. Even after three freeze-thaw cycles, 18% survival, 20% fermentative activity, and 20% respiratory activity remained.

The mesophile, C. utilis, was less affected by multiple freeze-thaw cycling than the psychrophile, L. stokesii (Figure 6). After one freeze-thaw cycle, exponential phase cells showed 32% survival, 44% fermentative activity, and 36% respiratory activity remaining. After three freeze-thaw cycles, survival was 4%, fermentative activity was 13%, and respiratory activity was 5%. Stationary phase cells showed levels after one freeze-thaw cycle of 77% survival, 57% fermentative

activity, and 84% respiratory activity remaining. After three freeze-thaw cycles these levels were 37% survival, 29% fermentative activity, and 48% respiratory activity.

Irrespective of the physiological age of the culture, decreases in metabolic activities paralleled decreases in viable cell numbers for both organisms. However, respiration of exponential phase cells of both organisms appeared to be more labile to multiple freeze-thaw stress than did fermentation. For stationary phase cells the converse appeared to be true. Fermentation was more labile than respiration.

Microscopic Examination of Freeze-Stressed Cells

In order to obtain data on the physical changes, particularly in cell dimensions, occurring in cells exposed to freeze-thaw stress, untreated, slow-frozen (4.1 to 0.45 C per minute) and fast-frozen (rates greater than 50 C per minute) exponential and stationary phase cells of both organisms were observed microscopically for uptake of neutral red dye. Only cells showing no uptake, or uptake only into the vacuoles, were measured using a calibrated ocular micrometer. Cell dimension data collected for exponential and stationary phase cells of *L. stokesii* and *C. utilis*, respectively, are shown in Tables 2 and 3. Volume and surface data were calculated from the length and width measurements according to the formulae of Muller (24). The two organisms differed from each other in overall dimensions. In general, exponential and stationary phase cells of the psychrophile were larger than exponential and stationary phase cells of the mesophile. For example, untreated exponential phase cells of *L. stokesii* (Table 2) had a mean

Table 2. Cell dimensions of exponential and stationary phase cells of Leucosporidium stokesii measured prior to and after slow-freezing and fast-freezing.*

Treatment	Untreated	Slow-freeze	Fast-freeze
		Exponential phase cells	
Length (μ)	9.55 ± 1.728[a]	9.21 ± 1.788	8.92 ± 2.074
Width (μ)	4.61 ± 0.448	4.70 ± 0.821	4.16 ± 0.838
Surface (μ^2)	119.18 ± 26.639	119.51 ± 36.739	102.11 ± 38.967
Volume (μ^3)	109.69 ± 34.335	114.23 ± 52.801	89.19 ± 51.658
Surface/volume	1.12 ± 0.124	1.13 ± 0.212	1.27 ± 0.241
Population size	99 cells	100 cells	101 cells
		Stationary phase cells	
Length (μ)	10.32 ± 2.116	9.60 ± 2.118	9.94 ± 1.560
Width (μ)	4.52 ± 0.693	4.04 ± 0.699	4.05 ± 0.679
Surface (μ^2)	126.49 ± 38.117	104.97 ± 35.056	107.38 ± 27.916
Volume (μ^3)	118.33 ± 50.162	88.97 ± 43.670	90.12 ± 36.007
Surface/volume	1.15 ± 0.199	1.28 ± 0.224	1.27 ± 0.206
Population size	100 cells	100 cells	100 cells

*. Cells measured showed exclusion of a 1:1000 dilution of neutral dye from their cytoplasm.

a. Parameter ± standard deviation.

Table 3. Cell dimensions of exponential and stationary phase cells of Candida utilis measured prior to and after slow-freezing and fast-freezing.*

Treatment	Untreated	Slow-freeze	Fast-freeze
		Exponential phase cells	
Length (μ)	6.46 ± 1.028[a]	6.68 ± 0.990	5.63 ± 0.913
Width (μ)	4.53 ± 0.658	4.66 ± 0.610	3.66 ± 0.486
Surface (μ^2)	85.39 ± 22.763	90.49 ± 22.215	59.34 ± 13.938
Volume (μ^3)	73.72 ± 28.498	80.12 ± 27.094	41.84 ± 13.530
Surface/volume	1.23 ± 0.196	1.19 ± 0.188	1.49 ± 0.251
Population size	100 cells	100 cells	40 cells
		Stationary phase cells	
Length (μ)	6.45 ± 1.135	6.52 ± 1.097	5.76 ± 0.928
Width (μ)	3.97 ± 1.090	3.88 ± 0.972	3.35 ± 0.772
Surface (μ^2)	74.42 ± 33.850	72.93 ± 30.144	54.81 ± 21.253
Volume (μ^3)	61.63 ± 43.986	58.65 ± 37.124	37.65 ± 23.031
Surface/volume	1.43 ± 0.347	1.44 ± 0.370	1.65 ± 0.346
Population size	100 cells	100 cells	100 cells

*. Cells measured showed exclusion of a 1:1000 dilution of neutral dye from their cytoplasm.

a. Parameter ± standard deviation.

length of 9.55 microns (μ) and a mean width of 4.61 μ whereas untreated exponential phase cells of C. utilis (Table 3) had a mean length of 6.45 μ and a mean width of 4.53 μ. It is obvious that the strains differ also with respect to surface and volume and surface-to-volume ratio since these parameters were calculated from the length and width measurements. With the exception of slow-frozen stationary phase L. stokesii cells, surface-to-volume ratios did not change significantly for slow-frozen cells of both organisms irrespective of culture age. However, this ratio increased 10 to 20% for exponential and stationary phase cells of both organisms subjected to fast-freezing.

In addition to overall changes in cell dimensions, fast freezing resulted also in an alteration of the intracellular structure. Untreated stationary phase cells of C. utilis had at least one and often two large vacuoles while cells which had survived the fast-freeze treatment were devoid of vacuoles. The same was true for stationary phase cells of L. stokesii. A few of the surviving cells of this strain, however, retained one vacuole.

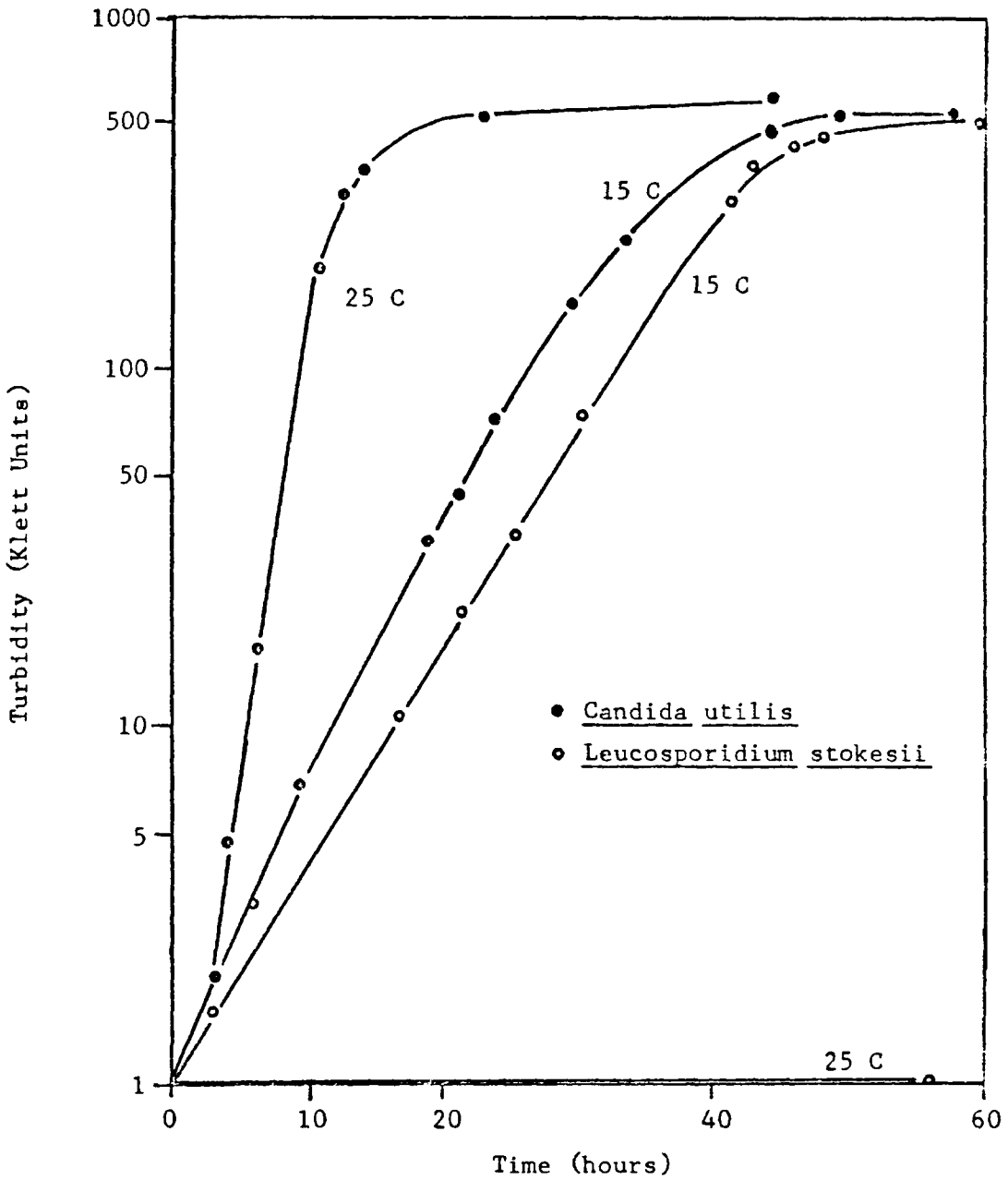

Figure 1. Growth curves of Candida utilis and Leucosporidium stokesii in shake culture.

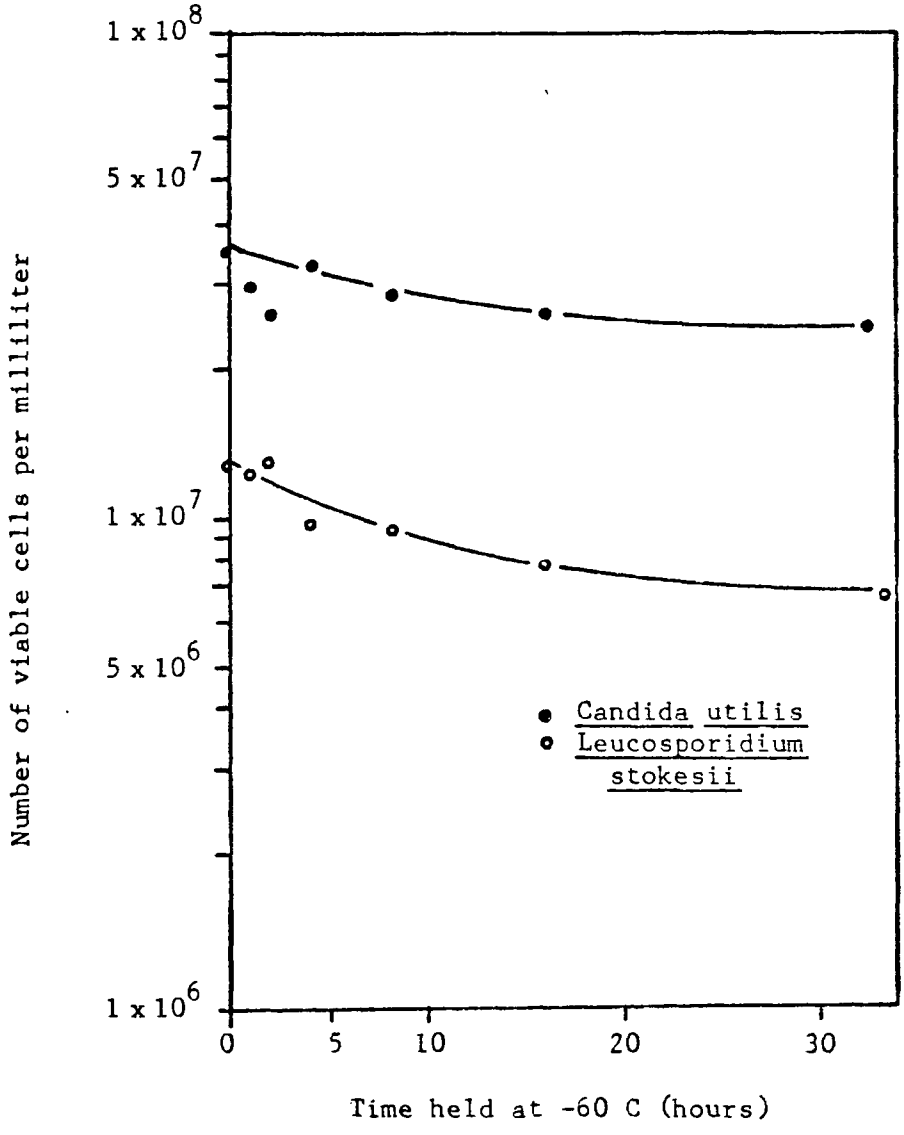

Figure 2. Effect of holding time at -60 C on the viability of Candida utilis and Leucosporidium stokesii exponential phase cells.

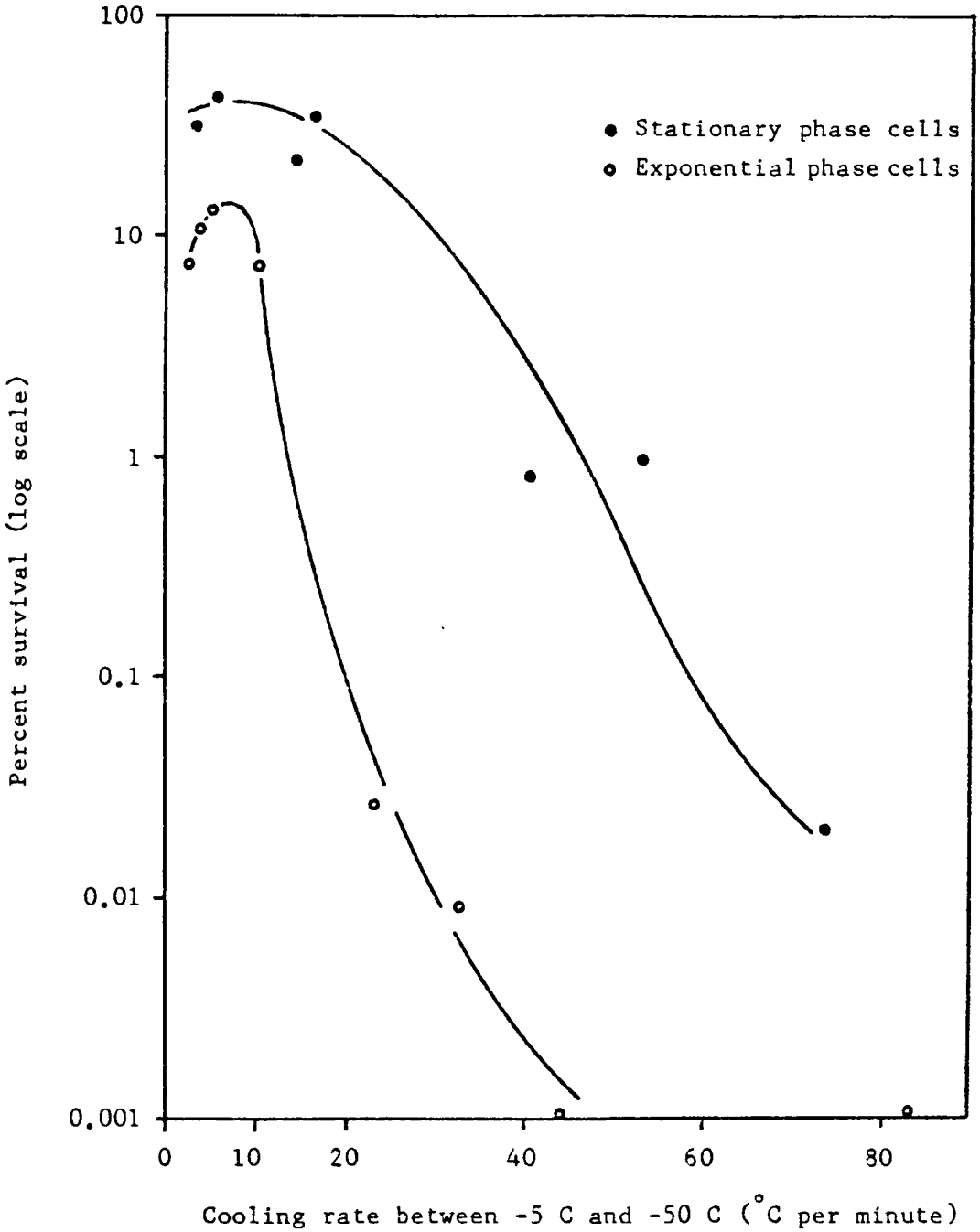

Figure 3. Effect of various cooling rates on survival of the psychrophile - Leucosporidium stokesii.

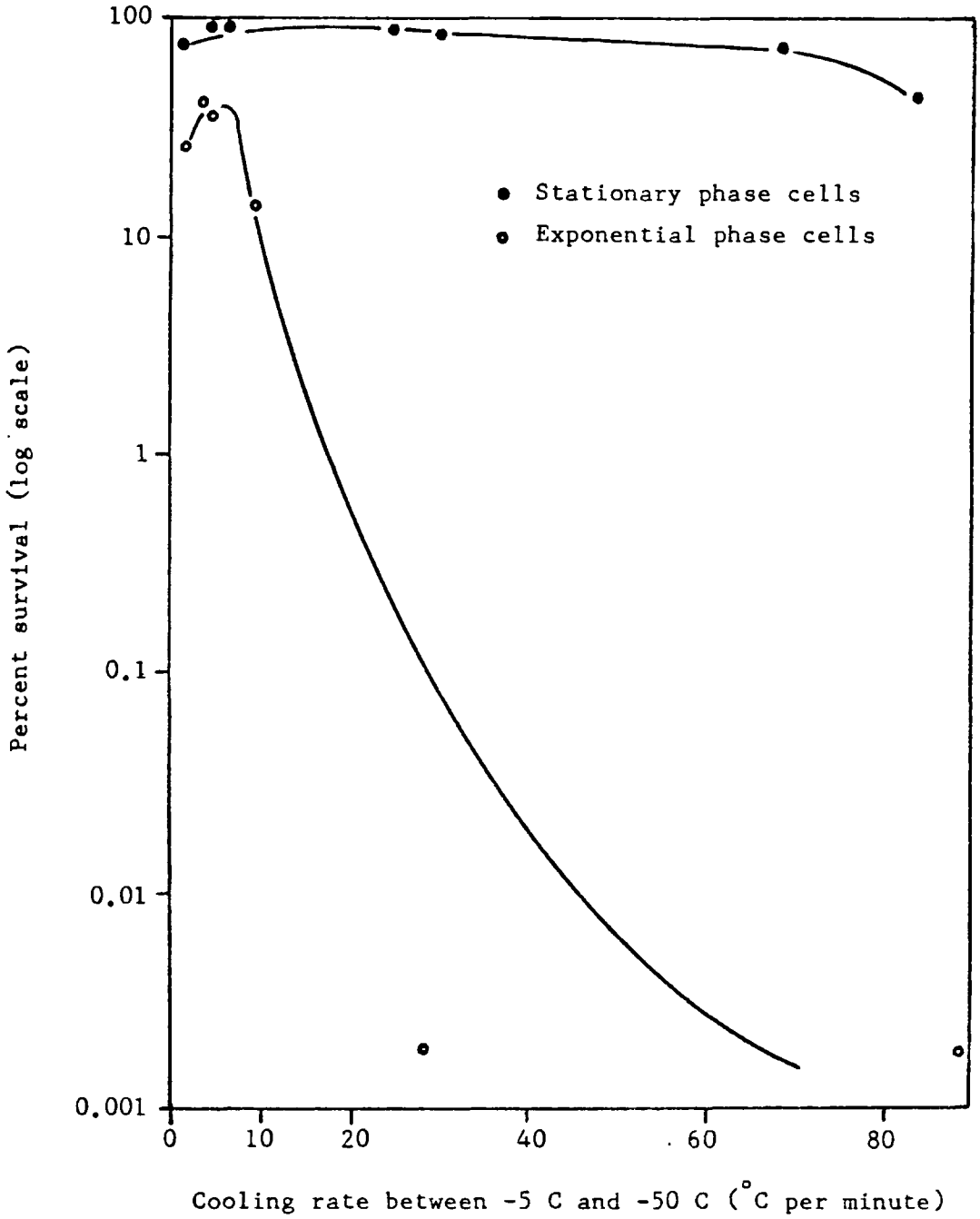

Figure 4. Effect of various cooling rates on survival of the mesophile - Candida utilis.

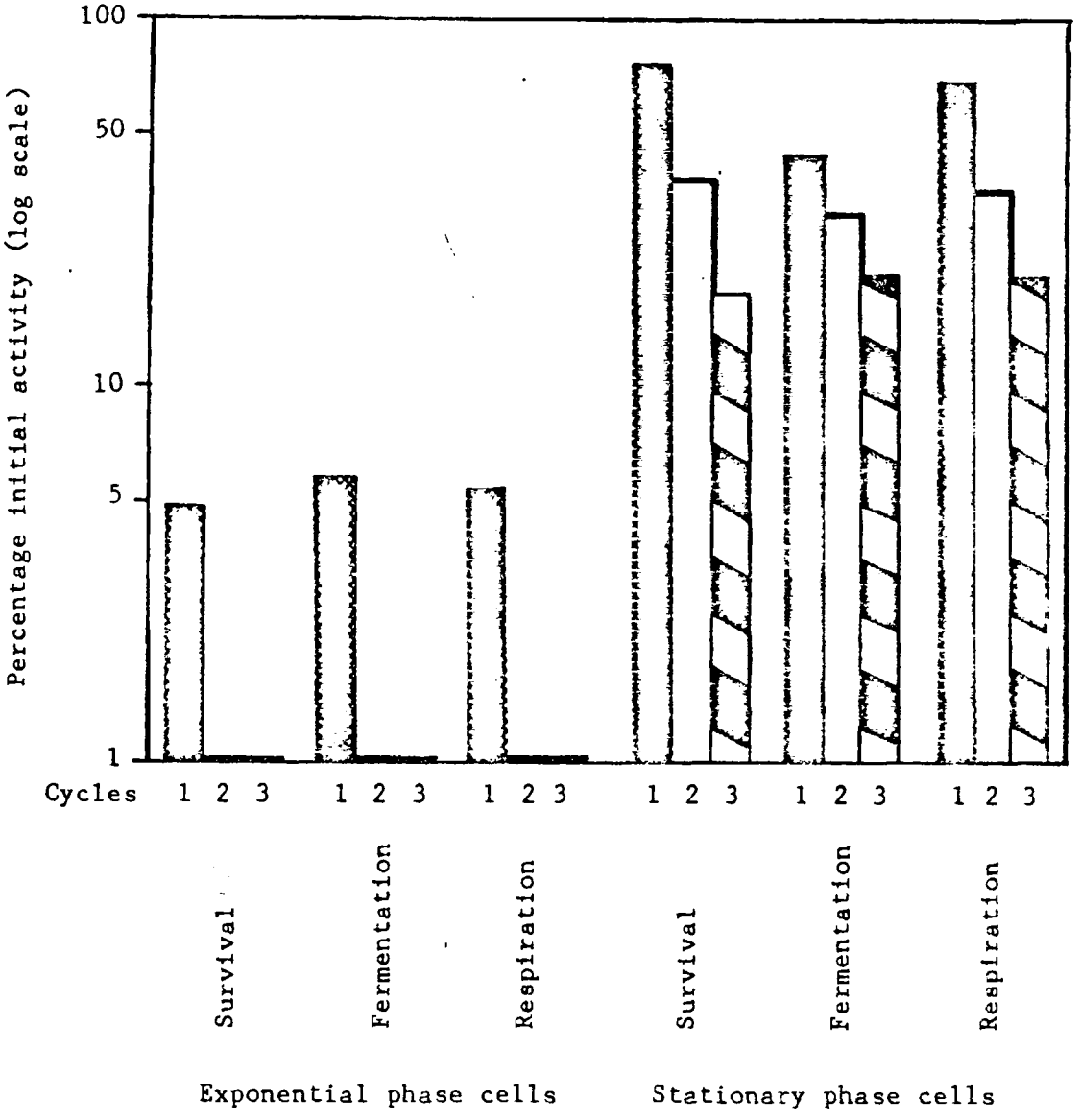

Figure 5. Effect of repeated freeze-thaw cycling on survival and metabolic activity of exponential and stationary phase cells of Leucosporidium stokesii.

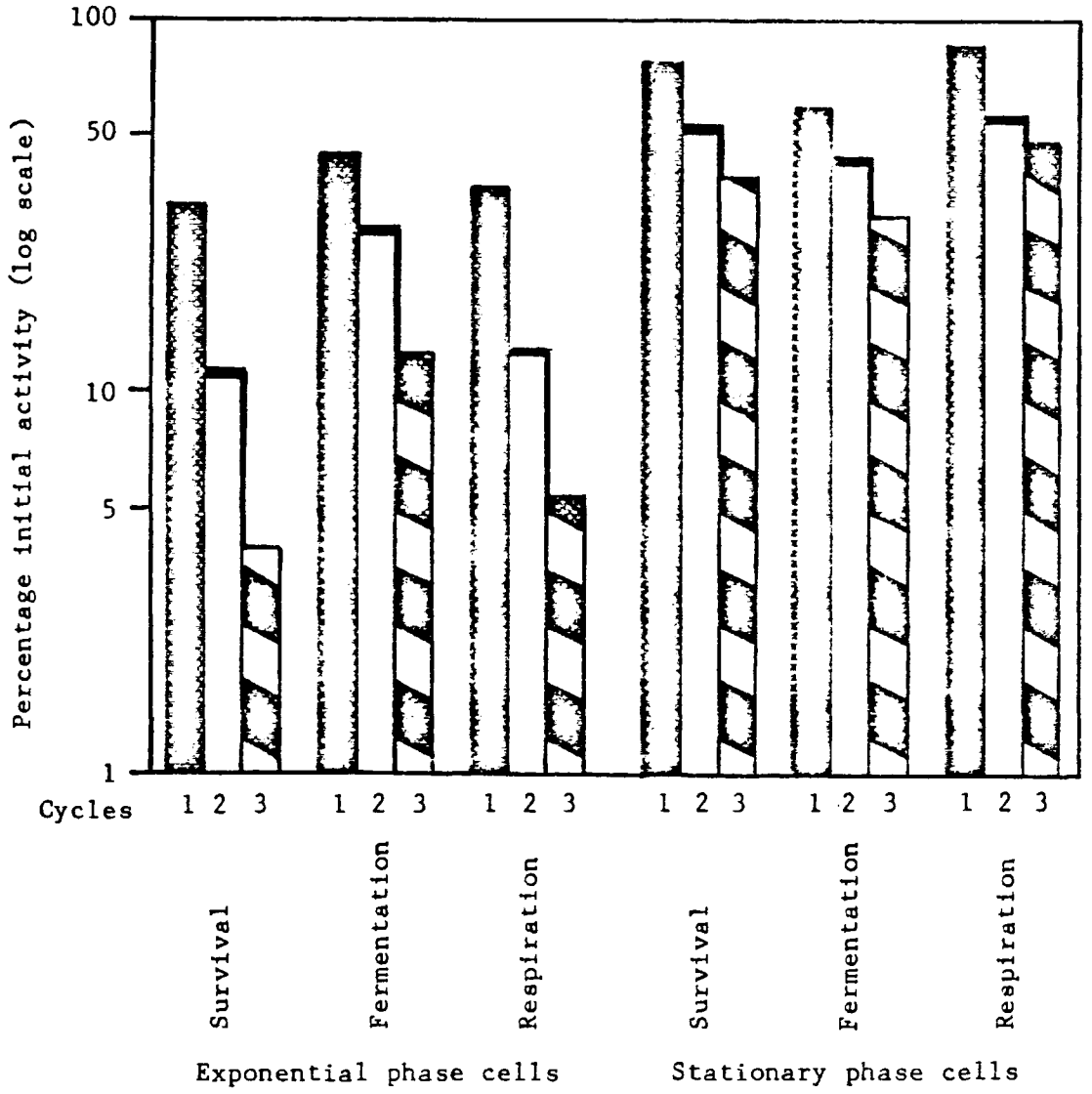

Figure 6. Effect of repeated freeze-thaw cycling on survival and metabolic activity of exponential and stationary phase cells of Candida utilis.

DISCUSSION

As noted above, Kreger-van Rij (13) has suggested that some species of Candida may be imperfect forms of Leucosporidium or related heterobasidiomycetous genera. It was for this reason that this pair of strains was selected for this study. However, as the data indicate, the two strains selected differ markedly both with respect to overall dimensions as well as in their responses to freeze-thaw stress. Although optimum cooling rates for both organisms are similar (in the range 4.5 C to 6.5 C per minute), the psychrophile is much more sensitive to freeze-thaw cycling over the entire range of cooling rates tested. In spite of these differences, the possibility of a close genetic relationship between the two genera is not precluded since the strains selected do not necessarily correspond one-to-one.

Generation times of both organisms are equal to or slightly longer than the 4.5 hour period of incubation during temperature cycling studies. Perhaps because of this, viable cell numbers did not increase significantly at either physiological age during any of the 4.5 hour incubation periods. This suggests that the slightly shorter generation time of C. utilis was not a contributing factor in its measured ability to better undergo the stresses of freeze-thaw cycling.

Mazur (17, p. 663) has stated that "percentage survival figures are a measure of the lethality of the overall sequence of cooling and warming but tell nothing of the degree and nature of injuries that lead

to death." With the notable exception of the studies of Hansen and Nossel (8) and Mazur (17), most reports on freeze-thaw injury of microorganisms have concentrated on survival as measured by colony-forming ability or uptake of vital dyes. For this reason, experiments were undertaken to examine not only viability but also the effects of subzero incubation and freeze-thaw cycling on overall metabolic activity. Percentage remaining respiratory and fermentative activities were usually somewhat higher following freezing than was percentage survival. However, the differences obtained were for the most part not significant. Nevertheless, it is readily apparent that within a given strain, stationary phase cells are much more stable to freeze-thaw stress than are exponential phase cells. These results are in agreement with those reported for other microorganisms exposed to freeze-thaw stress (3). Respiration of exponential phase cells of both strains appears to be more affected than fermentation. Conversely, fermentation of stationary phase cells of both strains appears to be more affected than respiration.

Microscopic data shed further light on the cellular events occurring during the freeze-thaw process. Neutral red was used as early as 1894 by Paul Ehrlich for vital staining of cells (11). Knaysi (11) distinguished dead from living bacteria by observing the differences in their uptake of the dye. Living cells did not take up the dye while dead cells were stained throughout their cytoplasm. Heucke and Henneberg (9) made similar observations with entorula yeast. Katznelson

(10), furthermore, used neutral red to measure the toxicity of an antibiotic on cells of Schizosaccharomyces pombe.

Microscopic fields of Leucosporidium stokesii and Candida utilis fast-frozen cells contained considerable amounts of cellular debris. These observations are in agreement with the findings of Mazur and Schmidt (21) that cells cooled at rates above the optimum are injured by internally formed ice crystals while cells cooled at rates below the optimum are injured by nondisruptive internal events. Lysis was so extensive in fast-frozen exponential phase cells of Candida utilis that only 40 viable cells could be found in over 100 fields observed. As noted above, when stationary phase cells were exposed to fast-freezing, those which survived (as indicated by neutral red exclusion from the cytoplasm) lost their vacuoles and their surface-to-volume ratios increased. These changes were not noted for slow-frozen cells. Hansen and Nossel (8) and Mazur (17) have also reported distortion and shrinkage of the cell outline and disappearance of vacuoles in fast-frozen yeast. In their reports, however, rupture of the external membrane was not noted under the same conditions. In addition, Mazur (17), who observed Saccharomyces cerevisiae cells freeze-substituted with ethanol, reported that slow-frozen cells underwent a decrease in volume. In the data presented here only stationary phase cells of L. stokesii decreased in volume when slow-frozen (Table 2). The other three cell populations studied underwent no significant change in volume when slow-frozen (Tables 2 and 3).

The data reported above derive from several approaches to the same problem--to determine how various strains of yeast react to freeze-thaw stress. These data, when considered together with similar studies such as those of Hansen and Nossel (8) and Mazur and his co-workers (15, 17, 18, 21), suggest that the critical events which occur during freezing are multiple. While surface-to-volume ratios are probably important in determining the optimum cooling rate as suggested by Mazur (20), additional factors such as cell age, cellular composition, suspending medium, warming rates, precultural history, etc. contribute to determining the extent and nature of injury.

Several additional questions arise. In particular, considering their cardinal growth temperatures, why is the psychrophile more labile to freeze-thaw stress than the mesophile and why are the stationary phase cells less susceptible to freeze-thaw stress than exponential phase cells? It is possible that the data reported above and the answers to these questions are all related to the character of the cell walls and/or cell membranes of the different cell populations, that the mesophile has, in general, a more flexible integument system than the psychrophile and that as a yeast cell ages the minor defects in structural integrity serve as an outlet for the stress resulting from the freeze-thaw process.

It is also possible that the stability of stationary phase cells to freeze-thaw stress is in part related to the physiological age of the culture. Numerous reports in the literature support the conclusion that exponential phase cells are more labile to the deleterious

effects of numerous chemical and physical agents (14). The data obtained in this study, in part, support this conclusion.

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