*RESEARCH ARTICLE*

# Factors Affecting Recovery of *Neosartorya fischeri* Ascospores after Exposure to Dry Heat*

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*ABSTRACT: Recovery of* Neosartorya fischeri *ascospores subjected to a dry heat treatment (DHT) at 95°C, 50% relative humidity (RH) for 60 minutes increased exponentially as the initial temperature of the recovery buffer increased. Different diluents were evaluated and the same recovery pattern was obtained when water or dilute buffers were used to recover the DHT spores. However, when glycerol was added to the buffer, the number of spores recovered in solutions held in ice water increased with increasing glycerol concentration. When the DHT spores were exposed to an atmosphere saturated with water vapor (100% RH) before being placed in the buffer, the recovery was independent of the initial temperature of the buffer. This occurred even if the spores were subsequently dried before being introduced into the buffer. It is hypothesized that the temperature-dependent recovery was due to injury of the DHT spores during the sudden rehydration in dilute solutions at low temperatures.*

## Introduction

The relatively high heat resistance of *Neosartorya fischeri* ascospores has been the subject of several studies due to their implication in the spoilage of heat-processed fruit products (1, 2). However, little is known about their resistance to dry heat and the factors affecting it.

We have previously reported (3) that the temperature of the buffer in which the spores are placed following a dry heat treatment (DHT) has a profound influence on the number of spores recovered. As the initial temperature of the buffer was increased from 0–0.5°C (buffer held in ice water) up to 80°C, there was an exponential increase in the number of survivors.

The study reported here was conducted to investigate some of the factors affecting the recovery of ascospores after exposure to dry heat. The objectives were to determine the effect on the temperature-dependent recovery of the ascospores of (i) time of exposure at the various recovery buffer temperatures, (ii) type of buffer or diluent, and (iii) conditioning treatment (placing the DHT spores under different conditions of relative humidity).

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## Materials and Methods

### *Spores*

Two spore crops of *N. fischeri* Morton Grove strain M-51 (Baxter Healthcare Corporation, Round Lake, IL) were produced on potato dextrose agar (Difco, Detroit, MI) incubated for 21 days at 25 ± 2°C (spore crop H) or 35 ± 2°C (spore crop E). The spores were harvested by flooding the plates with sterile water for irrigation (USP) (4) and rubbing the surface with a bent glass rod. After the suspension was collected, asci and cleistothecia were disrupted by sonification for 5 to 7 min at 70 Watts, 20 KHz (Sonifer model 450 or S-12, Branson Ultrasonics Co., Danbury, CT). To remove hyphal fragments and any remaining cleistothecia, the sonicated spore suspension was filtered through 54 and 25 μm nylon mesh. The spore suspensions were washed by repeated centrifugations for 10 min at 3,000 × g. Due to the presence of clumps of ascospores in crop H, the final suspension was prepared in 0.01% Tween 80 in water for irrigation (USP). Since clumping was not observed in spore crop E, the final spore suspension was prepared in water for irrigation (USP). In the final spore suspensions conidia were present at a concentration of ca. 0.1% (spore crop H) and 2% (spore crop E).

### *Dry Heat Treatment (DHT)*

The spores were inoculated on paper disks (#740E, Schleicher and Schuell, Keene, NH). To control the moisture content of the spores, the inoculated disks were placed in jars containing dry silica (≈ 0% RH). The silica had been dried in a forced air oven at 177°C for 8 hours. The jars were kept in a 4°C refrigerator for 7 days.

The DHT was carried out in an environmental chamber (model 1247, Hotpack Corporation, Philadelphia, PA). The spores were subjected to a DHT at 95°C, 50% RH for 60 min.

In a set of experiments (with spores from crop H), a post-DHT conditioning step was performed, which consisted of exposing the spores to either 0% RH (by placing the disks in a jar containing dry silica) or 100% RH (by placing the disks in a jar containing silica gel-water slush). The following post-DHT conditioning treatments were performed:

(A) 100% RH, 4°C, 1 day
(B) 0% RH, 4°C, 1 day
(C) 100% RH, 4°C, 1 day → 0% RH, 4°C, 1 day (or 7 days)
(D) 0% RH, 4°C, 1 day → 100% RH, 4°C, 1 day

*Recovery Procedures*

After the DHT, or post-DHT conditioning step, the disks were transferred to test tubes containing 10 ml of the diluent which, unless otherwise indicated, was 0.02% Tween 80 in Butterfield's buffer (T80-BB). Butterfield's buffer is 0.3 mM potassium phosphate buffer, pH 7.2. The buffer was pre-equilibrated at temperatures ranging from 0–0.5°C (ice water) to 80°C. After an exposure time of 1, 10, and 30 minutes at the buffer temperature, the tubes were transferred to an ice water bath. The paper disks were then macerated with a pestle and appropriate dilutions were plated using Sabouraud dextrose agar (BBL, Cockeysville, MD) containing 8 µg dichloran (Aldrich Chemical Company, Milwaukee, WI) per ml of medium. The plates were incubated at 35 ± 2°C for up to 5 days.

Non-DHT disks, subjected to the same preparatory and recovery steps, were used as controls. The steps from inoculation to recovery are presented in Figure 1.

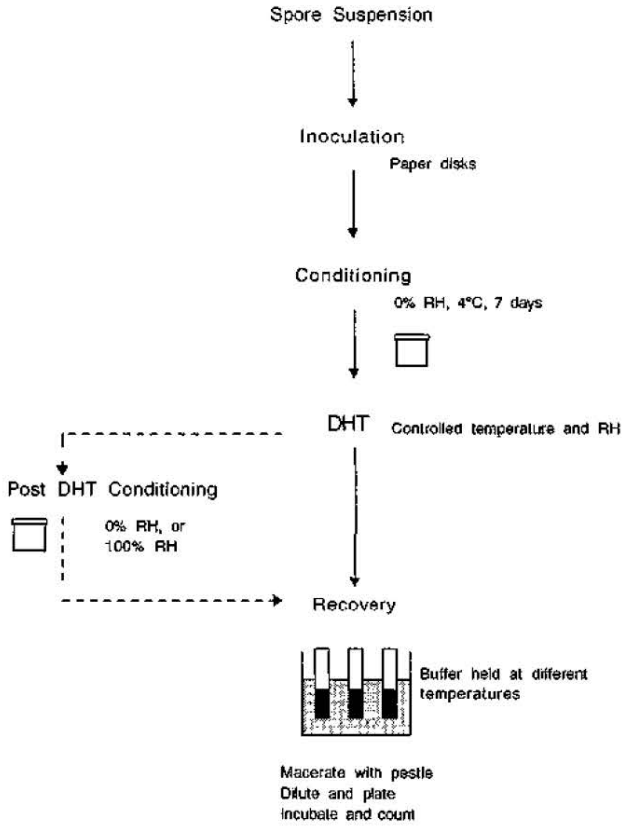


Figure 1—Dry heat treatment (DHT) protocol.

## Results

The effect of the temperature of the recovery buffer (T80-BB) on the apparent number of surviving ascospores following DHT at 95°C, 50% RH for 60 min is presented in Figure 2. The linear portion of the semilogarithmic plot indicates that the number of survivors increased exponentially with increasing temperature of the buffer. This increase eventually leveled off at the higher temperatures. At 80°C the number of survivors decreased as the exposure time increased from 1 to 10 and 30 minutes. At the other temperatures no difference in the number of survivors was observed between the three exposure times in the buffer.

The effect of the type of diluent was evaluated. Equivalent recovery was obtained in T80-BB, water for irrigation (USP), 0.1 M potassium phosphate buffer pH 7.2, 1.0 M potassium phosphate pH 7.2, 0.1% peptone, and 0.5 M sucrose (Fig. 3). However, when the spores were placed at 0–0.5°C in 0.1 M potassium phosphate buffer pH 7.2 containing different concentrations of glycerol, the number of survivors recovered increased with increasing glycerol concentration (Fig. 4). This increased recovery was only observed when the DHT spores were initially placed in the glycerol solution.

Results in Figure 5 demonstrate the effect of the various post-DHT conditioning steps. When the DHT spores were placed in an atmosphere of 100% RH prior to recovery (A), the initial temperature effect of the buffer was no longer observed. This occurred even if these spores were subsequently dried over silica for 1 or 7 days (C).

When the DHT spores were kept dry by storing the disks at 0% RH (B), the temperature of the buffer influenced the number of survivors in the same way as it had when the spores were recovered immediately. If following this conditioning at 0% RH the disks were placed at 100% RH (D), the recovery was unaffected by the initial temperature of the buffer.

The effect of the recovery buffer (T80-BB) temperature on the non-DHT spores is shown in Figure 6. Since both conidia and ascospores were present in the spore suspension, the following facts are pertinent: (i) conidia are not heat resistant; their inactivation begins at temperatures above 60°C, and (ii) ascospores are dormant and require an activation treatment (e.g., the application of sublethal heat) to facilitate germination (5). Therefore, it is expected that at the lower temperatures the number of survivors recovered represents the population of conidia (which are present at a concentration of ca. 0.1% in spore crop H and 2% in spore crop E) and, at the higher temperatures and longer heating times the

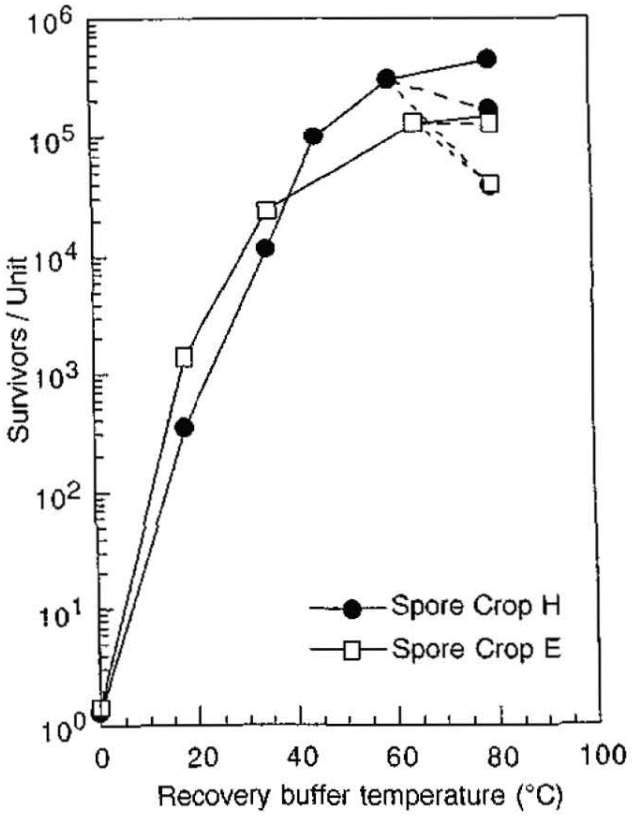


Figure 2—Effect of recovery buffer (0.02% Tween 80 in Butterfield's buffer) temperature on the apparent number of survivors following a DHT at 95°C, 50% RH for 60 min. Exposure times at the buffer temperature were: 1 min (—), 10 min (—), 30 min (----). At temperatures < 80°C, recovery was the same for all exposure times. The points represent the geometric mean of six replicates. The average standard deviation—viewed as a fraction of a log cycle—was 0.17.

number of survivors recovered represents the ascospore population being activated by the heat treatment. It can be seen that for spore crop H an unexpectedly high number of survivors was observed when the dry spores were placed in buffer at 0–0.5°C. This effect was not observed if the disks were placed at 100% RH prior to recovery.

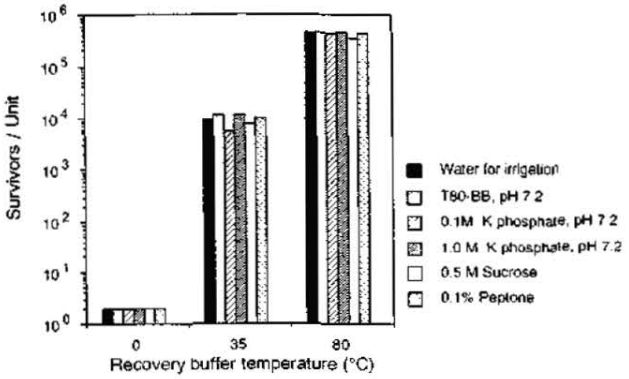


Figure 3—Effect of different diluents on the recovery of *N. fischeri* ascospores following a DHT at 95°C, 50% RH for 60 minutes. The DHT spores were placed in diluents pre-equilibrated at 0–0.5°C, 35°C and 80°C. Exposure times in the diluents at 0–0.5°C and 35°C were 1, 10, and 30 minutes (recovery was the same for these exposure times), and at 80°C, 1 minute.

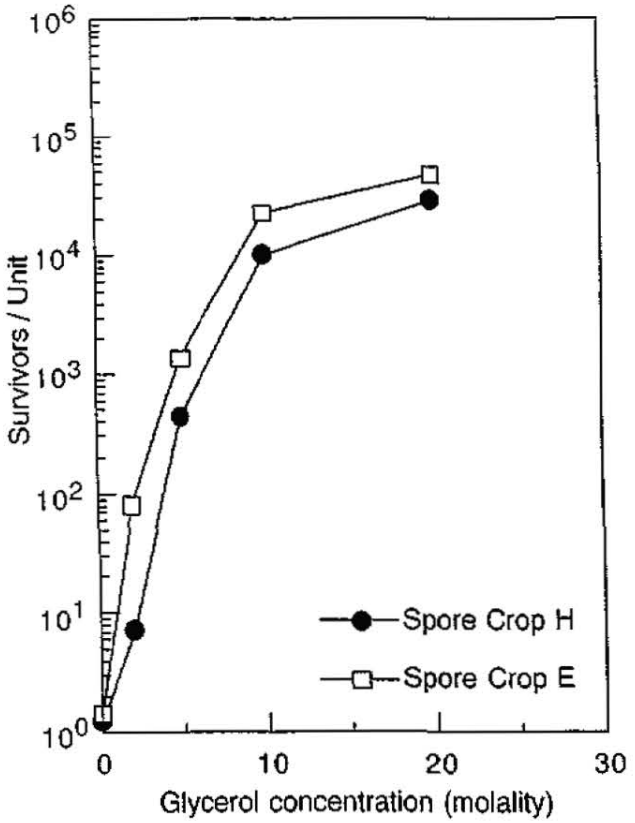


Figure 4—Recovery of DHT (95°C, 50% RH, 60 min) *N. fischeri* ascospores in glycerol solutions of different molalities held at 0–0.5°C. The points represent the geometric mean of six replicates (spore crop E) and three replicates (spore crop H). The average standard deviation—viewed as a fraction of a log cycle—was 0.20.

## Discussion

*N. fischeri* ascospores were able to survive a DHT at 95°C, 50% RH for 60 min. However, the apparent number of survivors observed was greatly influenced by the temperature of the recovery buffer. The exposure time at the specified buffer temperature did not affect recovery, except for the samples held at 80°C, where a decrease in the number of survivors was observed as the exposure time increased. This decreased recovery may be attributed to wet heat inactivation of the DHT spores. As compared to the non-DHT spores (controls), the DHT spores were more sensitive to the effects of wet heat; the non-DHT spores were not inactivated by the longer exposure times at 80°C.

When the effect of the type of buffer or diluent was investigated, no difference in recovery was observed in water or dilute solutions. In glycerol solutions held at 0–0.5°C, a higher recovery was obtained as the glycerol concentration increased. Preliminary experiments with sucrose indicated that even though an increased recovery was not obtained with a concentration of 0.5 M (Fig. 3), the use of a higher concentration (4.4 m) in the 0–0.5°C diluent resulted in a higher number of survivors.

The temperature-dependent recovery of the DHT spores was not observed when the spores were placed at 100% RH prior to recovery (Fig. 5a and 5b). Under this condition, rehydration of the spores took place at a low

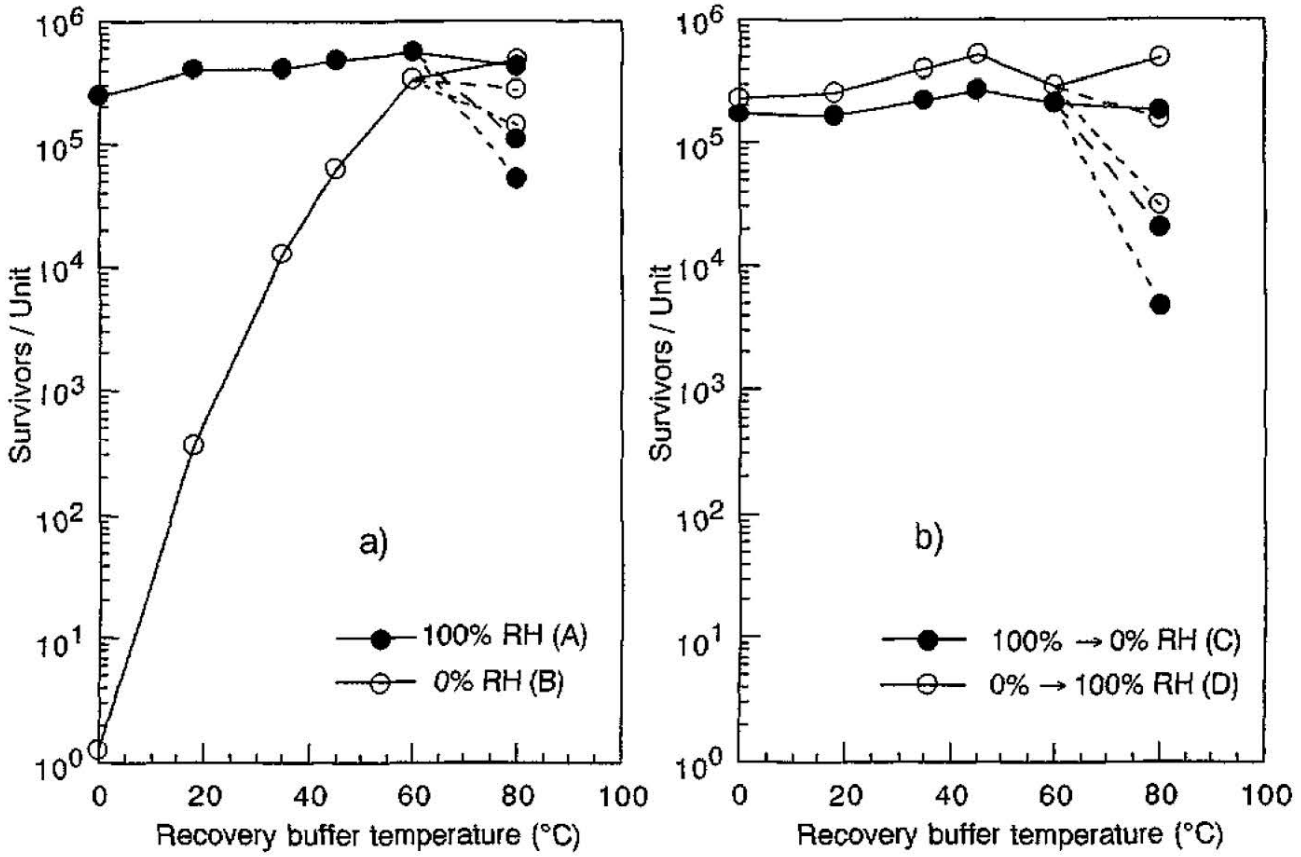


Figure 5—Effect of recovery buffer temperature and post-DHT conditioning treatment on the number of survivors following DHT (95°C, 50% RH for 60 min). Exposure times at the buffer temperature were: 1 min (—), 10 min (—), 30 min (----). At temperatures <80°C, recovery was the same for all exposure times. The points represent the geometric mean of six replicates, the average standard deviation—viewed as a fraction of a log cycle—was 0.15.

temperature (4°C), but in this case the spores slowly took up moisture from the water vapor in the surrounding air. After this conditioning period at 100% RH, even when the spores were dried again by placing over dry silica, the subsequent rehydration temperature no longer affected their recovery. This observation suggests that, after exposure to water vapor, a change takes place in the spores which is not reversed by subsequent drying without heat.

There are at least two possible explanations for the temperature-dependent recovery of the DHT ascospores: (i) temperature activation of the DHT ascospores or (ii) inactivation of the DHT spores during the sudden rehydration at low temperatures.

The activation explanation was proposed due to the observed increase in the number of survivors with temperature, and the fact that for heat activation a certain amount of available water must be present in the spores (6). The possibility existed that during the DHT the water content was not sufficient to allow for the activation of the spores even at the high temperature used (95°C). If dormancy of the ascospores was responsible for the decreased recovery at the low buffer temperatures, then after rehydration at the low temperatures overall recovery could be increased by heating in the buffer. As previously reported (3), the number of survivors rehydrated at the low temperatures was not increased by the subsequent application of heat. This was different than that observed with spores not exposed to DHT. After rehydration in buffer held at low temperatures, the non-DHT spores could be activated by exposure to higher temperatures.

The heat activation (or heat shock) hypothesis has been proposed to explain the higher recovery of bacteria when samples are rehydrated in a warm diluent as compared to a cold diluent (7, 8, 9). According to this hypothesis, the dry cells required the application of a sudden and direct heat treatment in order to resume growth.

Alternatively, it has been postulated that dry cells are inactivated during rehydration at a low temperature due to the loss of cell constituents (10, 11). This loss of cell constituents was largely diminished by placing the dry organisms in a 'humid' atmosphere before reconstitution (12, 13, 14). Strange and Cox (15) attributed this effect to a decreased and controlled rate of rehydration which could also be achieved by placing the dry organisms in an environment of high osmotic pressure (16, 17, 18, 19).

At this point, it could be hypothesized that DHT spores are injured as a consequence of the sudden rehydration in dilute solutions. However, attributing injury to the rapid rate of rehydration and to osmotic shock does not explain the large effect of the temperature of rehydration.

A hypothesis that explains the effect of the tempera-

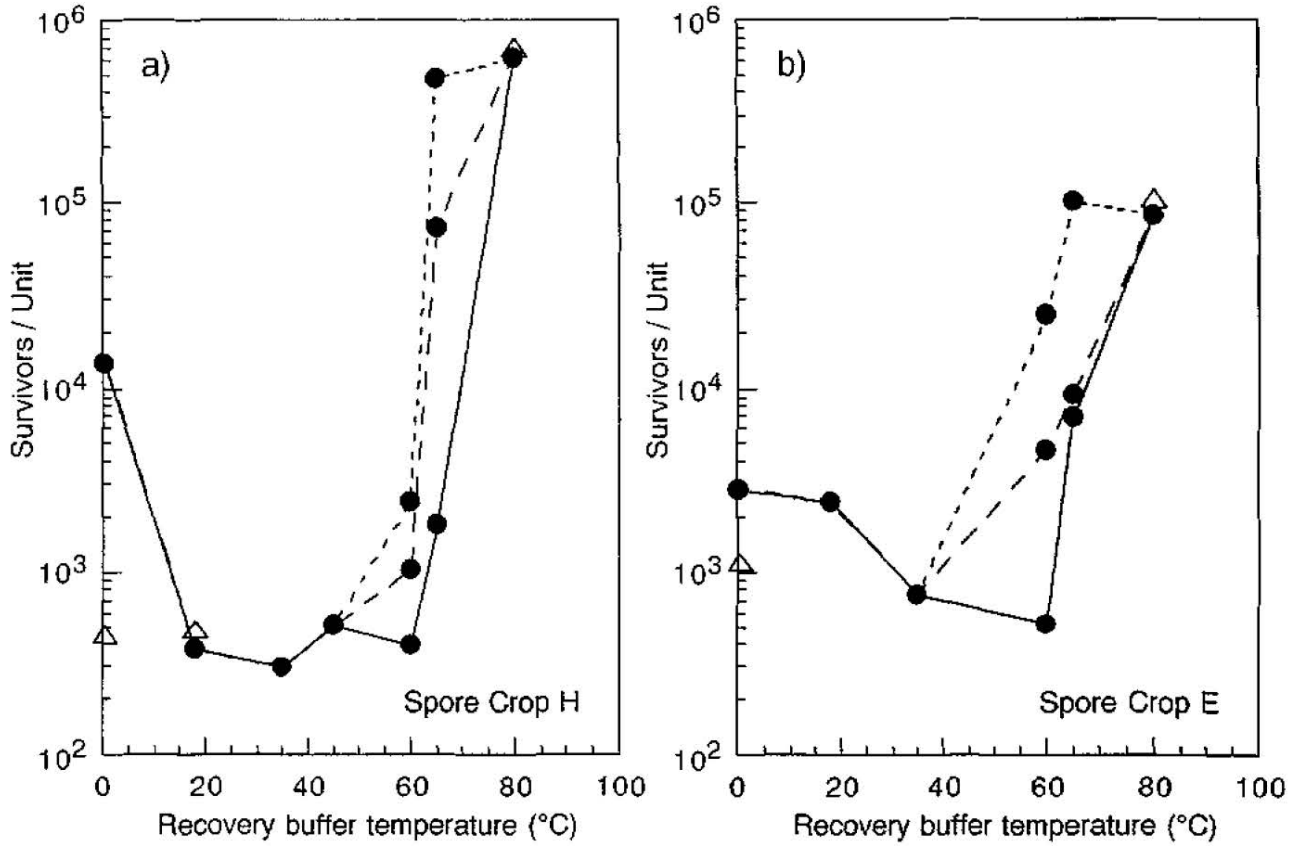


Figure 6—Effect of the recovery buffer temperature on non-DHT spores (controls). Exposure times at the buffer temperature were: 1 min (—), 10 min (——), 30 min (----). Controls additionally exposed to 100% RH, 4°C, 1 day prior to recovery (△). The points represent the geometric mean of at least four replicates. The average standard deviation—viewed as a fraction of a log cycle—was 0.13.

ture of rehydration is that proposed by Crowe and co-workers (20). Leakage of cell constituents is attributed to transient changes in membrane permeability arising as a result of dehydration-induced phase transitions in the membrane phospholipids. If the phase transition during rehydration is prevented by exposing the organisms to water vapor before being placed in bulk water, or if the organisms are heated (or rehydrated) to above the temperature where this transition takes place, no leakage is observed.

If Crowe's hypothesis explains the low recovery after cold rehydration of the DHT spores, then an explanation must be sought for the observation that the non-DHT spores (controls) were not inactivated by rehydration in cold diluents. It could be hypothesized that this is a result of the protective effect of trehalose, which during the DHT is lost, rendering the DHT spores susceptible to the damaging effects of cold rehydration (21).

It must be realized that due to the nature of the very complex system under study, the ascospore, and the high temperature used, changes might very well occur in other macromolecules. Therefore, other phenomena such as protein denaturation and renaturation could be the explanation for the observed effect of rehydration on the recovery of DHT spores.

The observation of the unexpectedly high number of survivors recovered when the non-DHT spores from crop H were rehydrated at 0–0.5°C warrants further discussion. When the spores were exposed to saturated water vapor before they were placed in the 0–0.5°C buffer, this high recovery was no longer observed (Fig. 6). The higher number of survivors obtained when the non-DHT spores were rehydrated at 0°C is interpreted on the basis of activation of a fraction of the ascospore population as a result of the sudden rehydration at a low temperature. It is possible that this phenomenon also occurs with spores from crop E, but that conidia are masking this effect. The mechanism of this "cold-rehydration" activation is, at the moment, unknown.

In studies dealing with the dry heat resistance of microorganisms, the effect of the temperature of the recovery buffer has often been overlooked and, as can be concluded from the data presented here, this is a major factor affecting the recovery of dry-heated *N. fischeri* ascospores. For the particular conditions under study the difference in survival was found to be as high as 100,000 fold between the lowest (0–0.5°C) and the highest (80°C) buffer temperatures. By exposing the DHT spores to an atmosphere saturated with water vapor or by increasing the osmotic pressure of the diluent used to rehydrate the spores, an increased recovery at the low temperatures was observed. These factors should be considered when evaluating and reporting the dry heat resistance of microorganisms.

## Acknowledgment

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