

RESEARCH ARTICLE

Resistance of *Neosartorya fischeri* to Wet and Dry Heat

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ABSTRACT: Dry heat resistance parameters for *Neosartorya fischeri* ascospores were obtained at 90°C and 95°C under 30%, 40%, 50%, 60%, and 75% relative humidity (RH) conditions. The dry heat treated spores were exposed to saturated water vapor (for 20–24 h at 4°C) prior to recovery in buffer held at two temperatures (0°C and 80°C). Approximately the same level of recovery was obtained at the two buffer temperatures except at the shortest heating times for the heat treatment carried at 30% and 40% RH, where the number of survivors was significantly higher for spores placed in the buffer held at 80°C. The effect of this high temperature was attributed to heat activation of the ascospores that remained dormant during the dry heat treatment conditions mentioned above. The wet heat resistance of *N. fischeri* ascospores was also determined at temperatures ranging from 82.5°C to 95°C. The results indicate that as the RH decreased, the heat resistance of the ascospores increased. There were about four orders of magnitude difference in the heat resistance between wet heat (100% RH) and the lowest dry heat treatment condition (30% RH).

Introduction

Much of the knowledge of the resistance of fungi to dry heat has come from studies where the water activity of the heating medium was controlled by the addition of solutes and was confined to the upper range of water activity, usually above 0.8 (1, 2, 3, 4, 5). However, with the development of aseptic packaging systems using hot air sterilization, the need exists to quantitatively study the dry heat resistance of fungi since these organisms have the potential to spoil acid food products (6, 7). From these, as well as other studies (8), it has become apparent that the heat resistance of several different fungal structures increases as the water activity or relative humidity during the heat treatment decreases.

The present study was conducted to investigate the dry heat resistance parameters of *Neosartorya fischeri* ascospores which are among the fungal spores possessing a considerably high heat resistance (9).

As previously reported (10), the temperature of the buffer in which the spores were placed after the dry heat treatment (DHT) had a profound influence on the number of spores recovered. After a DHT at 95°C, 50% relative humidity (RH) for 60 minutes, there was an exponential increase in the number of survivors as the initial temperature of the recovery buffer increased from 0°C to 60°C, leveling off between 60°C and 80°C. It was also shown that if the DHT spores were first exposed to

an atmosphere saturated with water vapor (100% RH, 4°C), the number of spores recovered was independent of the initial temperature of the buffer.

The present study was undertaken to: (i) evaluate the effect of recovery medium and cooling temperature on the enumeration of wet heated spores, (ii) investigate if the temperature-dependent recovery of the spores is observed under different DHT regimes, (iii) find a recovery procedure that enumerates the highest number of spores following a DHT, and (iv) determine the heat resistance parameters of *N. fischeri* ascospores using the procedure in which the maximum number of survivors is recovered.

Materials and Methods

Spores

N. fischeri Morton Grove strain M-51 (Baxter Healthcare Corporation, Round Lake, IL) was inoculated onto potato dextrose agar (Difco) and incubated for 21 days at 25 ± 2°C as previously described (10). The spores were suspended in 0.01% Tween 80 in Water for Irrigation (11). In this final spore suspension, conidia were present at a concentration of ca. 0.1%.

The spores were placed on paper disks (#740E, Schleicher and Schuell, Keene, NH). To control the moisture content of the spores prior to the heat treatment, the inoculated disks were placed in jars containing dry silica gel (≈ 0% RH). The jars were kept in a 4°C refrigerator for at least 7 days.

Wet Heat Treatment (WHT)

The inoculated disks were placed directly in 10 ml of 0.02% Tween 80 in Butterfield's buffer (T80-BB) pre-equilibrated at 82.5, 85.0, 87.5, 90.0, or 95.0 ± 0.2°C.

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Heating was ended by transferring the tubes either to an ice water bath (0–0.5°C) or to a 20°C water bath. Three disks (replicate units) were heated for each particular condition and the experiment was repeated twice.

The initial number of ascospores (N_0) was determined by heating the samples at 80°C for 15 minutes (heat activation treatment). A direct microscopic count of the spore suspension was also performed.

Dry Heat Treatment (DHT)

The DHT was carried out in a temperature and humidity controlled environmental chamber (model 1247, Hotpack Corporation, Philadelphia, PA).

In the first series of experiments, where the objective was to investigate the effect of the temperature of the recovery buffer, the DHT spores were either: (i) transferred directly to 10 ml of T80-BB pre-equilibrated at 0–0.5°C (ice water) and 35, 60, and 80 ± 0.5°C. The disks placed at 35°C, 60°C, and 80°C were left at the specified temperature for 1 minute, after which they were transferred to an ice water bath, or (ii) transferred to a jar containing silica gel–water slush (100% RH). The jar was placed at 4 ± 2°C for 20–24 hours until recovery in 10 ml of T80-BB held at the same temperatures and time as specified above. This treatment will also be referred to as conditioning in an atmosphere saturated with water vapor.

In the second series of experiments, heat resistance data were obtained at two temperatures, 90 and 95 ± 0.2°C, and five conditions of RH: 30%, 40%, 50%, 60%, and 75% ± 2%. For these experiments, the DHT samples were placed in a jar containing silica gel–water slush (100% RH) at 4 ± 2°C for 20–24 hours. After this conditioning period, the disks were transferred to 10 ml of T80-BB held at 0–0.5°C or 80°C. The exposure time at 80°C was 1 minute. Four disks were used for each heating time and the experiment was repeated at least twice.

Recovery Procedures

The wet heat treated and DHT spores were recovered by macerating the disks with a pestle and plating the appropriate dilutions using Sabouraud dextrose agar (BBL, Cockeysville, MD) containing 8 µg of dichloran (Aldrich Chemical Co, Milwaukee, WI) per ml of medium (SDAd). Two different concentrations of this medium were evaluated: full-strength (1.0X SDAd) which is that recommended by the manufacturer, 65 grams of dry medium/liter, and one-tenth strength (0.1X SDAd) which was prepared by using one-tenth of the manufacturer's recommended amount of dry medium, i.e., 6.5 grams/liter, to which 13.5 grams of Bacto-Agar (Difco, Detroit, MI) were added to obtain the same level of agar as that present in the full strength medium. The plates were incubated at 35 ± 2°C for up to 8 days.

Analysis of the Heat Resistance Data

Semilogarithmic survivor curves were plotted and the D_T -values (time for a 90% reduction in the population at

temperature T) with their 95% confidence intervals, as well as the intercept ratio ($IR = \log N_{y_0}/\log N_0$) were calculated as described by Pflug and Holcomb (12). In addition, the time for a specified level of reduction in the population was calculated.

Thermal resistance curves and thermal death time curves were obtained by plotting, respectively, the D_T -value and the time for a specified level of reduction in the population as a function of the temperature. The temperature coefficient of inactivation, or z -value, was calculated as previously described (12).

RESULTS

Wet Heat Treatment (WHT)

The recovery capacity of two media, 1.0X SDAd and 0.1X SDAd, can be compared from data presented in Figure 1a. For the samples heated for 15, 20, and 25 minutes at 85.0°C, the lower strength medium recovered a significantly ($\alpha = 0.05$; paired t-test) higher number of survivors. On 0.1X SDAd the colonies were smaller, permitting longer incubation with less overcrowding in the plates. In Figure 1b, the effect of cooling the samples heated at 85.0°C in an ice water bath (0–0.5°C) was compared to cooling in a water bath held at 20°C. No difference was observed between the two treatments.

The value of N_0 shown in Figure 1 was obtained from plate counts of samples heat activated at 80°C for 15 min. This value was 20–30% lower than the direct microscopic count.

Survivor curves were obtained at 82.5°C, 85.0°C, and 87.5°C using 0.1X SDAd as the recovery medium and cooling in an ice water bath. At the three temperatures the survivor curves were not linear, presenting a concave downward shape, as seen in Figure 1 for the samples heated at 85.0°C, and as evidenced by the $IR > 1$ (Table I). The D -values were obtained from the linear portion of the curves, and the results are presented in Figure 2 as a thermal resistance curve. In the same figure the time for a 4-log reduction in the initial spore population versus temperature is shown. Survivor curves were not obtained at 90.0°C and 95.0°C due to the rapid wet heat inactivation of the ascospores at these temperatures, but at least a 4-log reduction in the population was observed after a heating time of 3 minutes and 1 minute, respectively. The heat resistance parameters at these temperatures were extrapolated from the thermal resistance and thermal death time curves for later comparison with the dry heat resistance.

The calculated temperature coefficient parameter, z value, was 5.1°C (from the D -values) and 5.0°C (from the time for a 4-log reduction).

Dry Heat Treatment

The effect of the temperature of the recovery buffer on the number of survivors obtained following a DHT at 95°C, 50% RH for several heating times, is shown in Figure 3. Data are shown for spores placed directly in the buffer after the DHT, and for DHT spores which were first conditioned at 100% RH before being placed

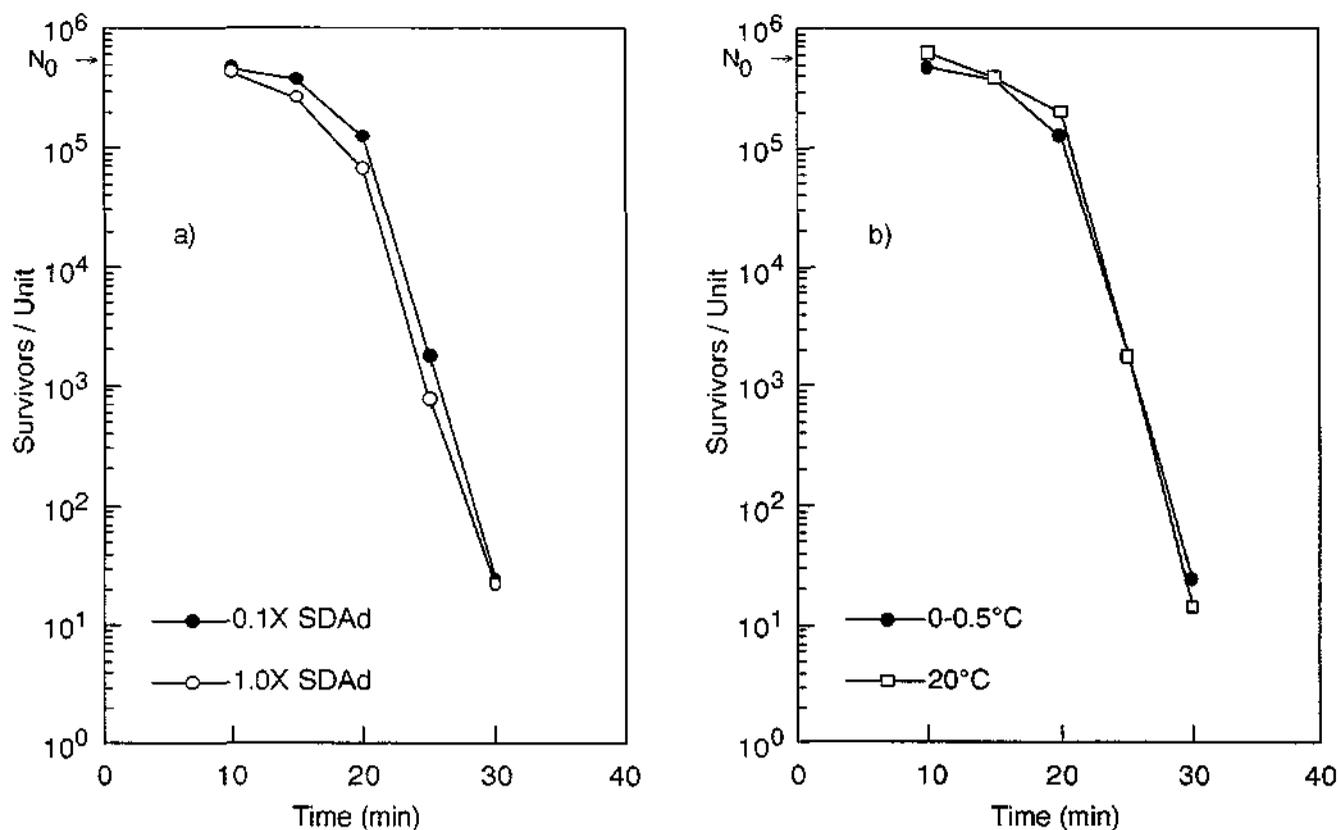


Figure 1—(a) Effect of the relative concentration of Sabouraud dextrose agar containing 8 μ g dichloran per ml medium (SDAd) on the recovery of *N. fischeri* ascospores heated in T80-BB at 85.0°C. Full strength medium (1.0X SDAd); one-tenth strength medium (0.1X SDAd). (b) Effect of the cooling temperature on the recovery of *N. fischeri* ascospores heated in T80-BB at 85.0°C and recovered using 0.1X SDAd. 0–0.5°C (ice water bath) vs. 20°C water bath.

in the buffer. With the DHT spores placed directly in the buffer, a higher number of survivors was obtained as the initial temperature of the buffer increased. With those spores which were first exposed to 100% RH, the same level of recovery was obtained at the buffer temperatures tested. Similar results were obtained with spores heated at 95°C, 75% RH (13).

Dry heat resistance data were obtained at 90°C and 95°C under five different conditions of RH: 30%, 40%, 50%, 60%, and 75%. The DHT spores were first conditioned at 100% RH and then recovered in buffer held at either of two temperatures (0°C or 80°C). The D-values and IR values are summarized in Table II and Table III. Since a direct comparison among the five survivor curves at each of the temperatures cannot be made due to the large differences in the heating times, the log of the number of survivors was plotted as a

function of the log of the heating time (Fig. 4). Similar curves were obtained at 95°C (13).

From these data the following observations are made: (i) the semilogarithmic survivor curves are not linear; (ii) the resistance of the spores at both temperatures increased with decreasing RH; (iii) after a dry heat treatment at 75%, 60%, and 50% RH at 90°C (or 95°C (13)), equivalent recovery was observed at the two buffer temperatures; and (iv) after a dry heat treatment at 30% and 40% RH at 90°C (or 95°C (13)), the number of survivors at the shortest heating times was significantly lower in the 0–0.5°C buffer than in the 80°C buffer. As the heating times were increased, recovery in the cold buffer increased and reached a level similar to that obtained when the spores were placed in the 80°C buffer. As the heating time was further increased, a decrease in the number of survivors was observed at both recovery buffer temperatures.

This last observation was further investigated. The DHT samples recovered in buffer held in ice water were transferred to a water bath at 80°C for 5 minutes. It was observed that the number of survivors increased to levels equal to those obtained for samples placed at 80°C (see Table IV for the results obtained with spores exposed to 90°C, 30% RH). In the same table are shown results obtained with spores directly placed in the buffer after the DHT (dry heated spores not exposed to water vapor). With these spores the number of survivors initially rehydrated at 0–0.5°C did not increase by

TABLE I

Wet Heat Resistance Parameters for *N. fischeri* Ascospores

Temp (°C)	D-value (min)	95% C.I. ^a	IR ^b
82.5	10.3	9.18–11.7	1.85
85.0	2.69	2.45–2.98	2.17
87.5	1.07	0.97–1.18	1.79

^a 95% Confidence Interval of the D-value.

^b Intercept Ratio: IR = $(\log N_{0.1} - \log N_0)$ where $N_{0.1}$ is the antilog of the y-intercept based on the linear portion of the survivor curve, and N_0 is the initial number of spores.

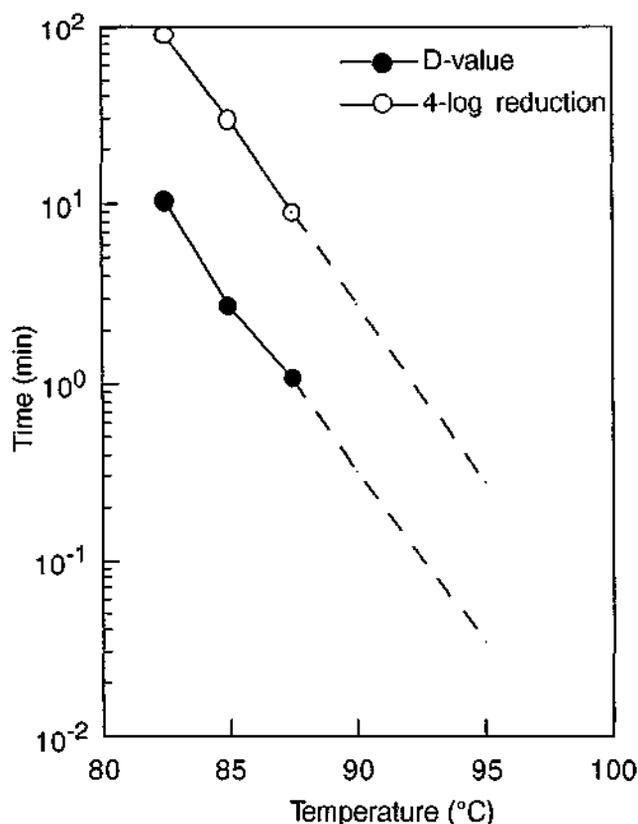


Figure 2—Effect of the temperature on the rate of wet heat inactivation of *N. fischeri* ascospores as measured by the D-values (thermal resistance curve) and time for a 4-log reduction in the population (thermal death time curve). The values at 90°C and 95°C were extrapolated from the linear regression of the experimental values (shown as dashed lines).

subsequent heating to 80°C. In supporting experiments where the ascospores were activated before the dry heat treatment (the disks were inoculated with a wet-heat activated spore suspension), the number of survivors obtained at both buffer temperatures after exposure to water vapor was the same.

Comparison of the dry heat resistance of the spores under the various conditions of RH can be made from Figure 4, by examining the differences in time (which was plotted on a logarithmic scale) to reach a certain level of reduction in the number of survivors.

The increase in heat resistance with a decrease in RH during heating can also be observed by plotting the logarithm of the D-value as a function of the RH. In Figure 5 this type of curve is presented for spores heated at 90°C and recovered in 0–0.5°C buffer (similar curves were obtained for the other treatments). Since the survivor curves were not linear, the time to reduce the original spore population by 1, 2, 3, and 4 logs is shown in the same graph to be compared with the D-value, which was calculated from the linear portion of the curves. The curves obtained are parallel. By fitting a line through the five points at each condition of RH, a “relative humidity coefficient of inactivation” (analogous to the z-value) could be obtained for this particular range of RH (30% to 75% RH). At 90°C this coefficient was 25 units of RH (the heat resistance changes by an

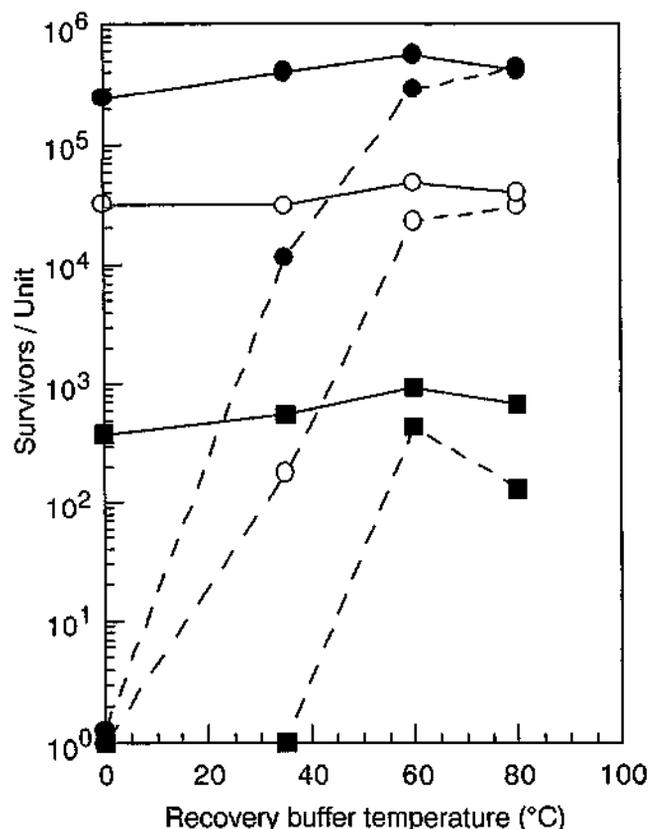


Figure 3—Effect of the recovery buffer temperature on the apparent number of survivors after a dry heat treatment at 95°C, 50% RH for: 1 hour (●), 3 hours (○), and 5 hours (■). DHT spores placed directly in the buffer (---). DHT spores exposed to water vapor prior to recovery in the buffer (—).

order of magnitude with a 25 unit change in RH), and at 95°C it was 20 units. However, extrapolations outside this range of RH should not be made since the curves might not be linear, as is the case for bacterial spores (14).

The D-values at 90°C and 95°C as a function of the RH during heating are shown in Figure 6. The wet heat resistance data shown in this figure were obtained by extrapolation of the values experimentally obtained at

TABLE II
Dry Heat Resistance Parameters for *N. fischeri* Ascospores Heated at 90°C and Recovered after Exposure to Water Vapor

% RH	Recovery Buffer Temp (°C)	D-value (min)	95% CI ^a	IR ^b
30	0–0.5	1,100	990–1,260	1.55
	80	1,040	887–1,260	1.56
40	0–0.5	709	650–780	1.21
	80	653	607–706	1.23
50	0–0.5	230	205–262	1.32
	80	209	190–231	1.39
60	0–0.5	53.9	48.9–59.9	1.56
	80	50.7	46.0–56.5	1.63
75	0–0.5	21.6	19.9–23.7	1.58
	80	20.7	19.0–22.7	1.64

^a 95% Confidence Interval of the D-value.

^b Intercept Ratio: IR = $(\log N_{y_0} / \log N_0)$ where N_{y_0} is the antilog of the y-intercept based on the linear portion of the survivor curve, and N_0 is the initial number of spores.

TABLE III
Dry Heat Resistance Parameters for *N. fischeri* Ascospores
Heated at 95°C and Recovered
after Exposure to Water Vapor

% RH	Recovery Buffer Temp (°C)	D-value (min)	95% CI ^a	IR ^b
30	0-0.5	433	376-510	1.65
	80	430	348-563	1.58
40	0-0.5	275	246-312	1.08
	80	256	232-286	1.08
50	0-0.5	66.5	60.7-73.5	1.24
	80	66.6	57.9-78.2	1.26
60	0-0.5	12.9	11.0-15.5	1.34
	80	11.8	10.5-13.5	1.42
75	0-0.5	2.74	2.49-3.05	1.52
	80	2.70	2.57-2.85	1.57

^a 95% Confidence Interval of the D-value.
^b Intercept Ratio: IR = $(\log N_{vi} / \log N_0)$ where N_{vi} is the antilog of the τ -intercept based on the linear portion of the survivor curve, and N_0 is the initial number of spores.

lower temperatures. They are presented for purposes of comparison.

Discussion

Wet Heat Treatment

Although a number of studies have addressed the heat resistance of the ascospores of *N. fischeri*, direct comparisons of these results with published data are not possible due to differences in the strain used, sporulation conditions, age of the spores, heating medium, and recovery procedures, all of which will have an effect on the number of survivors recovered after a heat treatment (9, 15, 16, 17, 18, 19, 20, 21). Nevertheless, the data presented here confirm the relatively high heat resistance of these ascospores as compared to other fungal structures.

Initially, two of the factors that could affect recovery of wet-heated ascospores were investigated. These were the type of recovery media and cooling temperature. The higher recovery obtained with the one-tenth strength media is attributed, in part, to differences in the pH of the media. Preliminary observations have indicated that the lower pH of 1.0X SDA as compared to 0.1X (there is a difference of 0.7 pH units) accounts, in part, for this difference. This observation is supported by previous reports of the increased sensitivity of stressed fungi to an acid environment in the recovery medium (22). Since it has been reported that recovery of heat-stressed fungi is enhanced in media containing a decreased concentration of glucose (23, 24), the still-unaccounted-for increase in recovery may be due to the lower concentration of glucose in the one-tenth strength medium (0.4% as compared to 4%). The value of 0.4% is in the range of that reported by Koburger and Rodgers (25) to be the optimal for recovery.

The possibility of chilling injury occurring during rapid cooling to low temperatures was investigated. Equivalent recovery was observed when the wet heated

spores were cooled in an ice water bath or in a 20°C bath.

Since the survivor curves obtained at the three temperatures presented a shoulder or concave downward shape, the D-values were calculated from the linear portion of the curves. The time for a 4-log reduction in the population was also determined. Both values are shown in Figure 2 so that a comparison can be made. The two curves are parallel; therefore, the calculated temperature coefficient is approximately the same.

Dry Heat Treatment

The previously reported effect of the temperature on the recovery of spores following a DHT at 95°C, 50% RH for 60 minutes (10), was extended to more severely stressed spores and to spores subjected to a different set of RH conditions.

The above observations were taken into consideration for obtaining dry heat resistance parameters. The method employed for this purpose consisted of exposing the DHT spores to an atmosphere saturated with water vapor prior to placing them in the buffer. Instead of choosing one buffer temperature for recovery, two temperatures corresponding to the lower and the higher of the range previously tested, 0-0.5°C and 80°C, were used. This provided an additional control for all of the other DHT regimes.

The choice of two temperatures also provided a means for observing surviving ascospores that had remained dormant during the shortest heating times at the lower conditions of RH, i.e., 30% and 40%. This was evident in the lower recovery obtained with the 0°C buffer as compared to 80°C buffer. The difference in recovery was indeed due to the lack of activation of the ascospores because: (i) the number of survivors obtained when the samples were initially placed in the cold buffer could be increased by exposing the spores to an additional higher temperature treatment; (ii) the same level of recovery was obtained at both buffer temperatures for spores activated prior to the DHT; and (iii) the number of survivors obtained with the cold buffer increased with increasing heating time under the particular DHT conditions.

It has been reported that *Phycomyces blakesleeianus* sporangiospores require an increase in the activation temperature with a decrease in water potential of the heating medium (26). *Neosartorya fischeri* ascospores can be wet-heat activated at 80°C for a period as short as 1 minute. When the ascospores are heated at 30% and 40% RH, they require for activation an increase in temperature to 90°C or 95°C, and an increase in the heating time. At 40% RH it takes > 3 hours at 95°C and > 10 hours at 90°C; and at 30% RH > 24 hours at 95°C and > 67 hours at 90°C, respectively (Fig. 4, (13)).

Comparison of the results obtained with dry heated samples placed directly in 0-0.5°C buffer and subsequently heated to 80°C, or conditioned at 100% RH before being placed in 0-0.5°C buffer and subsequently heated to 80°C, emphasizes the different effect of the low temperature in the two cases. For spores placed

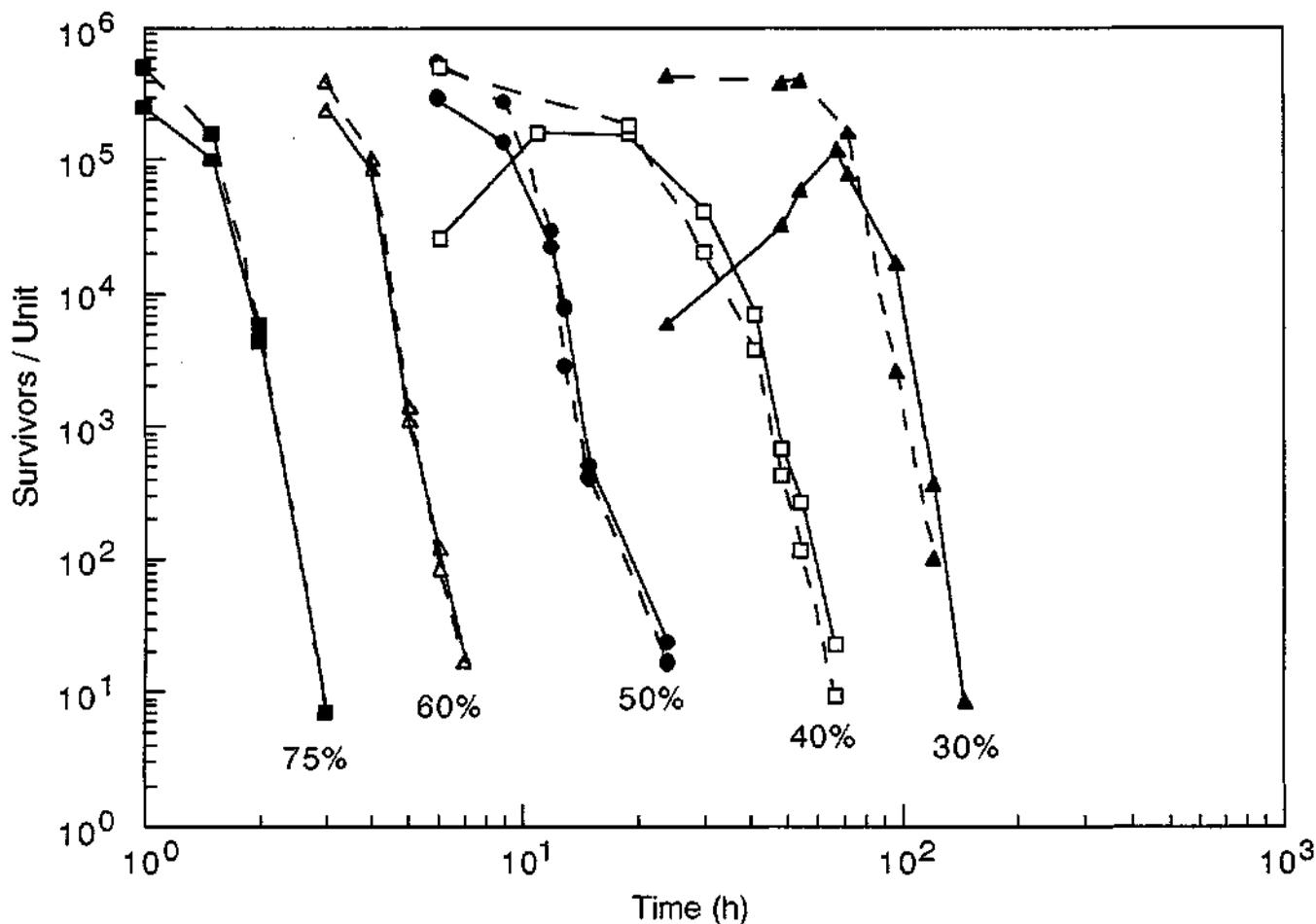


Figure 4—Dry heat resistance of *N. fischeri* at 90°C and five different relative humidity conditions. The DHT spores were exposed to water vapor (20–24 h, 4°C) prior to recovery in the buffer held at: 0–0.5°C (—), and 80°C (---). In the interest of clarity, some data points at the shortest heating times were omitted.

directly in the buffer, rehydration at the low temperature injured the spores. When the spores were rehydrated by taking up moisture from the surrounding atmosphere, the low temperature of the buffer was not lethal. In this case, the low recovery obtained can be attributed to dormancy of the ascospores. These spores

can be recovered by the subsequent wet heat activation treatment at 80°C.

The shoulders observed in the initial portion of the semilogarithmic survivor curves might be an indication that an activation phase is preceding the inactivation phase as suggested by Put (4). For the heat treatments at

TABLE IV
Effect of the Temperature of the Recovery Buffer on the Number of Survivors Obtained after a DHT at 90°C, 30% RH

Heating Time (h)	Initial Buffer Temp (°C)	No. Survivors/Unit Conditioned (100% RH) ^a		No. Survivors/Unit Recovered Directly ^b	
		→ 80°C, 5 min ^c	→ 80°C, 5 min ^c	→ 80°C, 5 min ^c	→ 80°C, 5 min ^c
1	0–0.5	3.38×10^3	4.11×10^5	7.76×10^2	5.87×10^2
	80	4.07×10^5	4.65×10^5	5.20×10^5	6.56×10^5
6	0–0.5	3.86×10^3	5.12×10^5	<10 est.	<10 est.
	80	4.82×10^5	6.20×10^5	5.05×10^5	5.13×10^5
24	0–0.5	5.94×10^3	4.06×10^5		
	80	4.32×10^5	4.37×10^5		
48	0–0.5	3.22×10^4	2.27×10^5		
	80	3.91×10^5	2.92×10^5		
54	0–0.5	6.09×10^4	2.33×10^5		
	80	4.02×10^5	2.41×10^5		
72	0–0.5	7.72×10^4	5.12×10^4		
	80	1.61×10^5	4.08×10^4		

^a The DHT spores were conditioned at 100% RH, 4°C, 20–24 hours prior to recovery in the buffer.

^b The DHT spores were placed directly in the buffer (data available only for 1-hour and 6-hour samples).

^c The DHT samples were initially placed in the buffer at the specified initial temperature after which they were transferred to an 80°C water bath for 5 min.

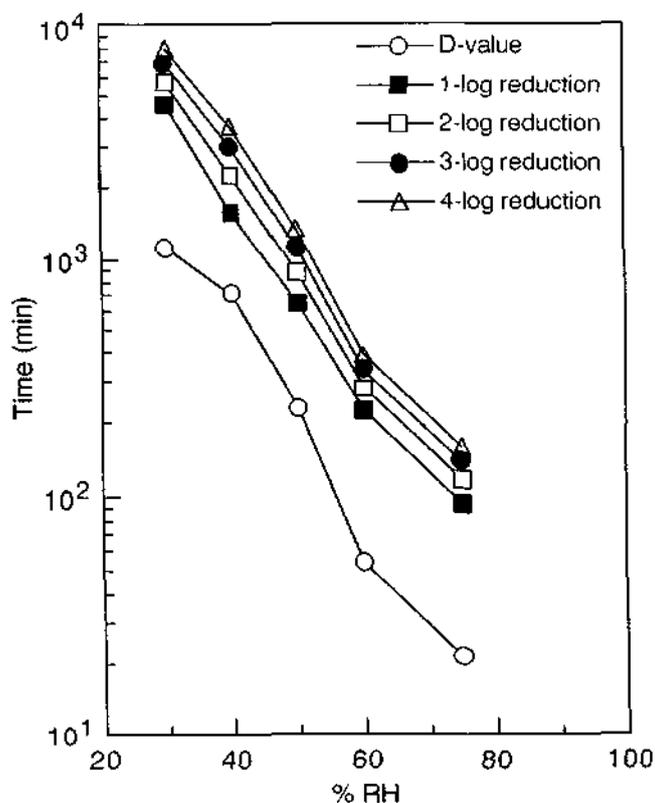


Figure 5—Dry heat resistance of *N. fischeri* at 90°C under different conditions of relative humidity as measured by the D-value and the time to reduce the population to a fraction of the original number. The data were obtained with DHT spores exposed to water vapor prior to recovery in the buffer held at 0–0.5°C.

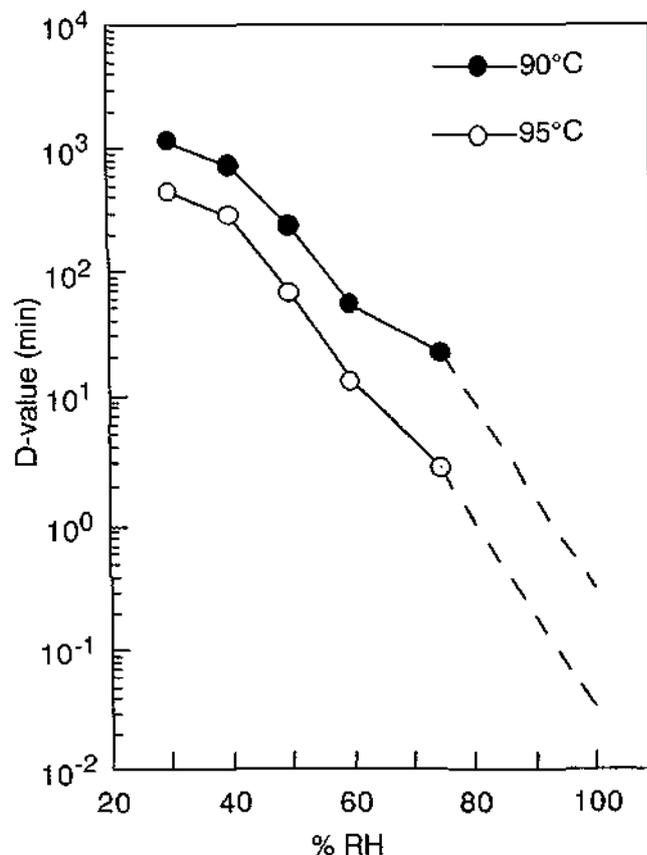


Figure 6—Heat resistance of *N. fischeri* at 90°C and 95°C at different conditions of relative humidity. The 100% RH values were extrapolated from experimental data gathered at lower temperatures.

30% and 40% RH, the end of the shoulder for the samples recovered at 80°C is coincident with the end of the activation curve for the samples recovered at 0°C.

It is important to mention that the heating times shown are the actual heating times. No attempt was made to correct for the lag in attaining the specified conditions of temperature and RH. This might have an effect in the DHT conditions at the higher RH. However, at the lower RH conditions this factor is negligible due to the much longer heating times. It is known that water is free to move rapidly in and out of the spores and equilibrate with the RH of the environment (27).

From the results presented here it is evident that the heat resistance of the ascospores increases with a decrease in RH during heating. Even though wet heat inactivation parameters could not be obtained experimentally at 90°C and 95°C, the extrapolated values are compared. As is seen in Figure 6 there were about four orders of magnitude difference between wet heat resistance and the resistance at 30% RH, the lowest RH tested.

The recovery procedure in which the spores were exposed to an atmosphere saturated with water vapor prior to recovery in the buffer proved to be a useful one since, not only was injury to the spores avoided during rehydration at the low temperatures, but dormancy of the ascospores could be detected. Water plays an important role in the heat activation of the ascospores as evidenced by the longer times and higher temperatures

necessary to activate the spores as the RH during heating decreases.

Acknowledgments

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