Preservation of *Serratia marcescens* by High-Vacuum Lyophilization

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**ABSTRACT**

DEWALD, ROBERT R. (Fort Detrick, Frederick, Md.). Preservation of *Serratia marcescens* by high-vacuum lyophilization. Appl. Microbiol. 14:561–567. 1966.—Water-washed *Serratia marcescens* (ATCC strain 14041) cells were lyophilized in an all-glass system capable of evacuation to pressures of less than 5 × 10⁻⁶ torr. Lyophilization at the lowest pressures resulted in 50 to 65% survival for unstabilized washed organisms compared with 10 to 20% when the cells were lyophilized at pressures of about 2.5 × 10⁻² torr. At the latter pressures, 45 to 65% survival was obtained when NaCl or Naylor-Smith stabilizer was added to the cell suspensions before lyophilization. However, the stabilizers failed to increase significantly the levels of survival compared with water suspension when cells were lyophilized at pressures less than 10⁻⁴ torr. The high survival rates obtained by the high-vacuum technique may be attributed to the reduction of traces of molecular oxygen which has been reported to be destructive of the dried bacteria. Survival of unstabilized dried *S. marcescens* after 1-day storage increased markedly with decreasing sealing pressure. Under the highest vacuum attained, survival of the dried bacteria was not impaired by storage for up to 1 month at Dry Ice temperatures; at higher temperatures, viability losses occurred. Exposure of the dried unstabilized bacteria to dry air resulted in rapid viability loss. The inactivation could be stopped almost immediately by evacuation to pressures of less than 10⁻⁵ torr, but the evacuation failed to reverse the viability losses that occurred during exposure.

Lyophilization or vacuum freeze-drying is the most commonly employed method for the preservation of bacteria by desiccation (7). This method can be considered to be a two-stage process involving first freezing and then sublimation of water from the frozen material. Death of organisms may occur during the freezing, drying, storage after drying, or reconstituting processes.

Generally, no serious problems are encountered during freezing of bacteria (18). Satisfactory recoveries have been obtained, even with bacteria which are sensitive to freezing, by control of temperature, rates of cooling, and suspending media (14). Most viability losses are considered to occur during the drying phase of the lyophilization process (19). A number of factors affecting survival after lyophilization also have been reported (6, 7). The suspending medium has been considered by many investigators as the most important single factor in determining the survival of an organism after lyophilization. Searches for protective substances or stabilizers that improve the survival of organisms have been highly successful, and numerous substances have been reported to have protective action (6, 7). However, Wagman and Weneck (19) pointed out that occasionally contradictory claims are made for protective effects observed for certain stabilizers. Moreover, stabilizers that afford good protection to one organism are, at times, ineffective in protecting other organisms. The mode of action of the stabilizers is unknown, but attempts to answer this question have been recently reviewed by Heckly (7). The addition of stabilizers limits the usefulness of the dried material for subsequent studies, but freeze-drying from distilled water suspensions results in dried organisms that are essentially pure and free from the components of any stabilizing medium, allowing more valid studies. Equally important in studying the effects of various stresses on dried organisms is that the viability following drying should be at a high level, so that the dried organisms when rehydrated are characteristic of the original bacterial population.

Limited information has been reported concerning lyophilization of washed cells from water suspensions. Naylor and Smith (15) and Zimmer-
man (20) reported that poor recoveries (<5%) resulted when unfortified suspensions of Serratia marcescens were lyophilized. However, Lion and Bergmann (10) reported high recoveries (50%) for Escherichia coli dried from distilled water suspensions. Lion and Bergmann also reported that the dried bacteria died rapidly upon exposure to oxygen, and they listed substances which afford protection against the lethal effects of oxygen (11). Electron paramagnetic resonance (EPR) studies by Lion, Kirby-Smith, and Randolph (12) showed that free radical formation occurred during the period of rapid killing due to oxygen exposure. Similar EPR experiments by Dimmick, Heckly, and Hollis (5) and Heckly, Dimmick, and Windle (8) showed that free radical formation occurred for several species of dried microorganisms, including S. marcescens, when exposed to oxygen. Benedict et al. (3) reported that lyophilized S. marcescens cells were killed rapidly when exposed to atmospheric oxygen, but certain reducing agents minimized the extent of inactivation. They also pointed out that rehydration under vacuum is necessary when certain stabilizers are employed. More recently, Wagman and Weneck (19) reported a study on the effect of the residual moisture level upon the survival of bacteria dried in the absence of stabilizers. They reported low levels of survival after vacuum freeze-drying, but they failed to mention whether their rehydration was performed under vacuum or after exposing the dried bacteria to air.

A number of investigators (10, 11, 13, 15, 17) compared survival during storage in vacuo with storage in atmospheres of various gases. They all found that the survival was highest for dried organisms stored under vacuum and lowest for those stored in air or oxygen. Christian and Stockton (4) studied the influence of sealing pressure on the recovery of S. marcescens and Staphylococcus aureus, and noted on increase in survival when the sealing pressure was decreased.

The information reported thus far suggests that the degree of vacuum employed in vacuum freeze-drying is an important factor affecting recovery, especially when washed bacteria are dried in the absence of protective additives. The primary objective of this study was to determine the importance of the degree of vacuum employed during freeze-drying S. marcescens in the absence and presence of protective substances. Other objectives were to determine (i) the effect of cell concentrations on the survival level after high vacuum lyophilization, (ii) the temperature dependence during storage under pressures less than $10^{-4}$ torr, (iii) the effect of sealing pressure upon the storage of S. marcescens dried from distilled water suspensions, and (iv) the effects of exposure of the dried organisms to dry air, followed by evacuation.

**Materials and Methods**

**Preparation of organisms.** S. marcescens strains (Fort Detrick strain 8UK, ATCC strain 14041) used in this study were prepared by two different methods. In one method, cells were grown and stored as frozen pellets by a process already described (20). Other organisms were grown for 18 hr at 25 C in 3% Trypticase Soy Broth (BBL) fortified with 2% glucose. The harvested organisms were washed twice by alternate suspension and centrifugation in distilled water and were shaken aerobically at 25 C for 3 hr. The cells were then centrifuged and suspended in distilled water to yield suspensions containing the desired number of viable cells per milliliter. The washed cell suspensions were refrigerated between experiments. Viable-cell populations were determined by the standard surface plating technique, with the use of nutrient agar (Diffco) and incubation at 37 C for 24 hr.

**Assembly for high-vacuum lyophilization.** Figure 1 illustrates the vacuum assembly used in these experiments. A three-stage oil diffusion pump permitted evacuation of the system to pressures of less than $5 	imes 10^{-4}$ torr as determined by an ionization gauge. The high-vacuum stopcocks were lubricated with Apiezon N vacuum grease, and the standard tapers were sealed with Apiezon W wax. The condensable vapors were collected in liquid nitrogen traps. The freeze-drying ampoules were connected to the high-vacuum manifold via standard taper joints. The ampoules during lyophilization were immersed in a bath containing an ethylene glycol-water mixture held at selected temperatures within ±0.5 C. During the final 2 hr of the 16- to 18-hr lyophilization cycle, the cold-temperature bath was replaced with a water bath that was thermostatically controlled at 25 C (±0.1).

**Lyophilization procedure.** Cell suspensions (1 ml) were introduced into 20-ml ampoules containing glass beads (4-mm diameter). The ampoules were then connected to the high-vacuum manifold and degassed by repeatedly pumping the liquid suspensions until the noncondensable gas pressure between the two liquid nitrogen traps was less than $10^{-2}$ torr.

![Fig. 1. Assembly for high-vacuum lyophilization.](http://aem.asm.org/Downloaded from http://aem.asm.org)
Next, the suspensions were frozen as a solid plug containing the glass beads by immersing them into the cold bath at -30 to -40°C. The suspensions were held at that temperature until the pressure between the liquid nitrogen traps was less than 10^-4 torr. The frozen suspensions were then warmed to -12 to -8°C and held at those temperatures with continual evacuation at noncondensible gas pressures of less than 10^-1 torr (approximately 14 to 16 hr). The cold-temperature bath was then replaced by a 25°C bath, and evacuation was continued for another 2 hr. Next, ampoules were either sealed off under vacuum or kept on the high-vacuum manifold through the experiments. Rehydration was accomplished by a method designed to prevent exposure of the dried organisms to the atmosphere. A piece of rubber tubing, fitted to the three-way stopcock as shown in Fig. 1 or to the neck of the sealed-off ampoule, was completely filled with water and clamped-off from the atmosphere. Rehydration was then achieved by either opening the stopcock or breaking the flame-sealed tip of glass ampoules with a hammer. The amount of water added was determined by the difference in weight between the ampoule containing the rehydrated cell suspension and the empty dried ampoule. The weight of the dried cells (approximately 4 to 6 mg) was considered negligible, compared with the 2 ml of distilled water used for hydration.

**Influence of dry air on survival after storage.** In these experiments, dried air at various pressures was introduced into the ampoules before sealing. The pressures were determined with a McLeod gauge immediately before sealing. The air was dried by passing it through a trap containing activated silica gel at Dry Ice temperatures.

**Storage experiments.** Samples were sealed at pressures less than 10^-4 torr and stored at room temperature, at 4 to 5°C in a refrigerator, and at Dry Ice temperatures. Samples were reconstituted after storage for various time intervals up to 1 month. The samples held under vacuum at various temperatures (-198°C to 100°C) for 1 hr under high vacuum were sealed under vacuum or left on the vacuum line during the experiment. These samples were warmed or cooled to 25°C before rehydration.

**Residual moisture measurements.** High-vacuum lyophilized samples (approximately 0.1 g) were held overnight under vacuum (2 torr) at 80°C. The moisture contents were estimated by weight difference.

**RESULTS**

To determine the optimal bath temperature for maximal survival of *S. marcescens* subjected to high-vacuum drying, cell suspensions at two different initial cell concentrations were dried at various temperatures for the periods and at the pressures already described in the lyophilization procedure. The results of this experiment with triply washed cells are plotted in Fig. 2. The temperatures correspond to those of the bath in which the ampoules were immersed during the vacuum drying process. At temperatures higher than -8°C, the recovery appears to depend on the initial cell concentration. Below -8°C, where viable recovery tended to level off at its maximal value, the survival became less dependent upon initial cell concentration.

Table 1 gives a summary of viable-cell recoveries obtained, after vacuum lyophilization at bath temperatures between -8 and -12°C, from cell suspensions of different initial concentrations. It should again be emphasized that these results were obtained from triply washed organisms in the absence of added stabilizers. In the temperature range from -8 to -12°C, recoveries were essentially independent of the...
TABLE 2. Effect of pressure during lyophilization upon the survival of washed and stabilized Serratia marcescens

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>Viable cells per ml before drying ($\times 10^6$)</th>
<th>Approx pressure during drying (torr)</th>
<th>Per cent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naylor-Smith/10</td>
<td>3.9 ± 1.3*</td>
<td>2.5 $\times 10^{-2}$</td>
<td>55 ± 9*</td>
</tr>
<tr>
<td>NaCl, 0.05%</td>
<td>3.6 ± 0.9</td>
<td>2.5 $\times 10^{-2}$</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>14.6 ± 0.4</td>
<td>2.5 $\times 10^{-2}$</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.8 ± 0.9</td>
<td>2.5 $\times 10^{-2}$</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Naylor-Smith/10</td>
<td>2.3 ± 1.4</td>
<td>5 $\times 10^{-6}$</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>NaCl, 0.05%</td>
<td>2.3 ± 0.4</td>
<td>5 $\times 10^{-6}$</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Distilled water</td>
<td>—</td>
<td>5 $\times 10^{-6}$</td>
<td>56 ± 8*</td>
</tr>
</tbody>
</table>

* Indicates 95% confidence interval.
* Mean of values from Table 1.

FIG. 3. Survival of dried unstabilized Serratia marcescens after 1-day storage at room temperature in sealed ampoules at various dry air pressures. Per cent survival = $N/N_0$ (100) where $N_0$ and $N$ are the number of viable organisms after lyophilization and after storage, respectively.

Initial cell concentration of the suspensions. During this study, some samples of dried organisms were rehydrated with degassed water. Within the range of experimental error, no difference in recovery could be detected with the use of degassed water for reconstitution. Also, organism suspensions prepared by the two methods described above yielded identical results.

The results obtained by lyophilization at higher pressures (with the use of the same system without the diffusion pump) and at a lower pressure are given in Table 2. Also in Table 2, per cent survival of organisms dried from fortified and water suspensions is compared for two different pressures during lyophilization. The Naylor-Smith stabilizer and NaCl used in these experiments were adjusted to yield the cell-stabilizer ratios recommended by Benedict et al. (2). The results show that the protective additives had a very beneficial effect upon the level of survival after the high-pressure lyophilization, but had little effect compared with water suspensions after the lyophilization at a lower pressure.

Moisture determinations by the dry-weight method consistently yielded moisture contents of less than 2%. No vapor pressure could be detected by an oil manometer when dried samples (approximately 0.5 g) were held at 25 C for 1 hr in a closed system. Vapor pressures greater than $10^{-5}$ torr are easily detected by this method.
It was concluded, after comparison with reported moisture-vapor pressure data (1), that the samples had little, if any, residual moisture. Therefore, data reported in Tables 1 and 2 are considered representative of samples having a residual moisture content of less than 2%.

The influence of sealing pressure on survival after 1 day of storage at room temperature is shown in Fig. 3. The survival increased markedly with decreasing pressure. This again indicates that degree of vacuum is very important for obtaining high levels of survival after lyophilization of washed organisms.

Figure 4 shows the survival levels after 1-hr storage as a function of temperature for dried organisms held at pressures of less than $10^{-5}$ torr. No losses in viability were detected after 1 hr at temperatures between 3 and 32 C. When the dried bacteria were held at 0 C or below for 1 hr, 40 to 50% losses in viability occurred. Similar losses occurred when the bacteria were held at subzero temperatures for only 20 min. The dried samples originally at 25 C, cooled below 0 C, and warmed to 25 C (by immersing the ampoule in a water bath at 25 C) before rehydration, were, therefore, subjected to a cooling and warming cycle. When the bacteria were subjected to three temperature cycles, the resulting viability losses were the same as those observed after the first cycle. The viable population of the dried bacteria was reduced about 80% when held at 40 C for 1 hr. When the bacteria were subjected to temperatures between 40 and 75 C for 1 hr, the viability losses did not increase markedly with temperature, but at 100 C the losses in viability were so large that the extent was difficult to define experimentally.

Survival after storage for as long as 1 month of dried organisms sealed under pressures of less than $10^{-5}$ torr is shown in Fig. 5. At Dry Ice temperatures, the only viability loss appears to correlate with that found for the cooling and warming cycle previously mentioned. At 4 C, there was a gradual decrease in viability with time; the losses became more extensive at room temperature.

Figure 6 is a representation of the typical viability losses that occur when the dried organisms at 25 C are exposed to dry air at atmospheric pressure for short periods of time. In this case, approximately 85% of the dried organisms were inactivated in 15 min. The dried bacteria could not be reactivated by continual evacuation at pressures of less than $10^{-5}$ torr for periods up to 5 hr, but evacuation stopped the inactivation process immediately.

**DISCUSSION**

This study has demonstrated that high levels of survival can be obtained upon lyophilization of washed *S. marcescens* even in the absence of added protective substances. The pressures during lyophilization and storage were found to be important factors affecting the survival of the dried bacteria. The results obtained by the high-vacuum technique can best be explained in terms of molecular oxygen which is lethal to the dried bacteria (3, 10, 11). Also, the high vacuum...
appeared to have no adverse effects on the survival of the dried *S. marcescens*. This is in agreement with the results reported by Portner et al. (16), for the exposure of dried bacteria to ultra-high vacuums. The survival levels for dried *S. marcescens* after 1-day storage under various sealing pressures show that the dried bacteria are sensitive even to traces of dry air.

The results obtained from lyophilization at higher pressures (approximately $2.5 \times 10^{-2}$ torr) emphasize the importance of the degree of vacuum. Under identical conditions, i.e., time, temperature, and history of organisms, lyophilization at higher pressures gave substantially lower survival levels: 10 to 20% compared with 50 to 65% survivals for the high-vacuum technique. The addition of stabilizers (NaCl and Naylor-Smith) to cell suspensions prior to lyophilization did have a beneficial effect on the survival at the higher lyophilization pressure. However, both stabilizers failed to increase the levels of survival compared with water suspension when lyophilized at pressures less than $10^{-4}$ torr. Lion (9) suggested that perhaps the main function of stabilizers is to afford protection against the adverse effect of oxygen. The effect of pressure during lyophilization found in this study supports Lion's suggestion.

The freezing-thawing recoveries reported in Table 1 were obtained by taking samples of a cell suspension through the same temperature cycle as during lyophilization, except that the suspensions were not dried. The freezing, thawing, and warming of the liquid suspension consistently resulted in viability losses of 15% ($\pm 8$). The rate of freezing and thawing was found not to affect these viability losses. This is consistent with results reported by Heckly (7) for the same organism. The dependence of recovery after lyophilization upon bath temperatures is also compatible with the results reported by other investigators, as reviewed by Heckly (7).

The 1-hr storage studies under high vacuum gave an unexpected result below 0 C. No explanation is given for the 40 to 60% losses in viability that occurred when the dried organisms were subjected to a cooling and warming cycle. These viability losses were quite reproducible. No attempt was made to control the rates of cooling or warming in these experiments. Also, the 50 to 60% survival resulting after the cooling-warming cycle corresponds to the 50 to 65% survival obtained for unstabilized *S. marcescens* after lyophilization, in which the dried organisms were subjected to the warming phase of the cycle.

Considerable viability losses occurred during storage after approximately 1 month at 25 C, even under initial sealing pressures of less than $10^{-5}$ torr. No explanation can be given at this time concerning the nature of these losses. Naylor and Smith (15) reported high levels of storage survival for the same organism when lyophilized in the presence of effective stabilizers. It appears reasonable that stabilizers might be beneficial for long-term storage stability even when organisms are stored under pressures of less than $10^{-5}$ torr. The trend of temperature dependence of survival during storage reported in this study is in agreement with results reported by other investigators (7). Studies of survival after storage for longer times are now in progress in this laboratory.

The recoveries after lyophilization reported here are in marked disagreement with those of Wagman and Weneck (19). They reported low recoveries for washed *S. marcescens* when cells from water suspensions were freeze-dried under vacuum and higher recoveries when the organisms were freeze-dried in an atmosphere of circulating gases. They also stated that the residual moisture level was a major factor in determining this recovery of organisms, and suggested that over-drying should be avoided. In the present work, high levels of recovery (greater than 50%) were obtained even at residual moisture contents of less than 2%. These results indicate that the removal of water is not the major factor causing death of organisms during lyophilization.

**Acknowledgments**

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