Effect of Drying Medium on Residual Moisture Content and Viability of Freeze-Dried Lactic Acid Bacteria

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The effect of various substances on the relationship between residual moisture content and the viability of freeze-dried lactic acid bacteria has been studied. Compounds such as polymers, which display considerable ability in displacing water, showed no protective action during freeze-drying. Adonitol, on the other hand, produced the smallest change in water content at various times during drying and allowed the highest rate of survival.

The amount of water remaining after drying affects not only the viability of bacteria, as determined immediately after the process, but also the rate of loss of viability during subsequent storage. According to Webb (15), 80% of the physiological reactions of organisms depend on the movement of bound water, not of free water. Fry and Greaves (4) obtained evidence indicating that the drier the product, the lower the rate of survival. The optimum residual moisture content varies with the composition of the fluid in which organisms are dried, with the storage atmosphere, and probably with the species and physiological state of the organisms (3, 13). In this paper, we investigated the effect of various cryoprotectants, especially adonitol, in preserving the viability of freeze-dried lactic acid bacteria in relation to the residual moisture content.

MATERIALS AND METHODS

Cultures. Lactobacillus murinus ATCC 35020, Lactobacillus fermentum ATCC 9338, and Streptococcus thermophilus ATCC 19258 were obtained from the American Type Culture Collection, Rockville, Md. Lactobacillus plantarum CRL 83, Lactobacillus casei CRL 200, Streptococcus lactis CRL 215, and Streptococcus faecium CRL 175 were obtained from the stock collection of the Centro de Referencia para Lactobacilos (CERELA) and were originally isolated from Taff cheese (5).

Culture media. MRS broth (1) was used for the propagation of lactobacilli, and LAPTg30 broth (12) was used for the growth of streptococci. Cultures were incubated at 35 and 45°C for mesophilic and thermophilic bacteria, respectively, and at 30°C for S. lactis.

Rehydration medium. The following medium was used for rehydration: peptone, 15.0 g; tryptone, 10.0 g; meat extract, 5.0 g; and distilled water, 1 liter (pH 7.0; sterilized at 121°C for 15 min). This medium was also used as the diluent for viability counting. Culture preparation, cell suspension, and freeze-drying procedure are described elsewhere (2, 14). A sample of 10% nonfat skim milk was used as the control. The amount of the samples was 0.3 ml in all cases.

The number of viable cells was determined by the agar plate method. Immediately before plating, each sample of freeze-dried bacteria was brought to its original volume with the rehydration medium. Serial dilutions of each sample were plated in triplicate, and plates were incubated at the appropriate temperature. After 48 h, the resulting colonies from samples taken before and after freeze-drying were scored, and the percent survival was calculated.

Additives tested. The following additives were tested: polyethylene glycol (PEG) 1000 (0.05 M; BDH, Poole, England), PEG 6000 (8.5 mM; E. Merck AG, Darmstadt, Federal Republic of Germany), dextran B (0.33 mM; BDH), β-glycerophosphate (0.25 M; Merck), glutamate (1.0 M; Merck), and adonitol (0.75 M; Fluka AG). β-Glycerophosphate, glutamate, and adonitol were sterilized by filtration (filter type 1121; pore size, 0.2 μm; Schleicher & Schuell Inc., Keene, N.H.); other additives were sterilized at 121°C for 15 min. The pH was adjusted to 7.0 with NaOH.

Measurement of residual moisture content. The residual moisture was calculated by gravimetric methods from the relation between the initial wet weight and the dry weight at the end of the freeze-drying procedure as described by Nei et al. (10). Residual moisture content = ([A - B]/B) × 100; A is the weight of the specimen immediately after freeze-drying, and B is the weight of the dry matter, obtained by drying for 3 h at 60°C in a vacuum of 10⁻³ mmHg (1 mmHg = 133.3 Pa). Samples of 0.3 ml were employed. Residual moisture and viability determinations were effected at various times of drying, from 15 min to an overdrying of 300 min.

RESULTS AND DISCUSSION

The additives tested in this study may be divided into three groups: (i) polymers (PEG 1000, PEG 6000, and dextran); (ii) polyols (β-glycerophosphate and adonitol); and (iii) amino acids (glutamate). These protectors were selected on the basis of the results obtained in previous papers (2, 14).

Table 1 shows the effect of the suspending medium on the residual moisture content at various drying times. It was noted that polymers produced the largest reduction in water content during the interval between 15 and 45 min of drying. From then on, the decrease in residual moisture was less marked. A similar degree of desiccation was observed with 10% nonfat skim milk. By contrast, β-glycerophosphate, glutamate, and especially adonitol made possible the retention of a high water content from 15 to 60 min of drying with respect to the other cryoprotectants used. When samples were freeze-dried from 180 to 300 min, the residual moisture was less than 0.1% for all of the additives tested.

The different rate of sublimation observed might be due to the movement of free water and bound water in the cell.

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material during freeze-drying. Mellor (8) considers that the free water is eliminated by sublimation during the primary drying, and since the hydrogen bonds are relatively weak, some of the bound water is also eliminated by evaporation at the beginning of secondary drying. By contrast, most of the water bound by electrostatic forces remains in the dry product because of the difficulty in removing it.

The influence of the drying media on the degree of desiccation of lactic acid bacteria and its relation to the survival rate are shown in Fig. 1 and 2. From the results obtained in the first trial (Table 1), adonitol, glutamate, and PEG 1000, each belonging to one of the three groups of additives tested, were chosen for viability assays. The cultures were freeze-dried in 10% nonfat skim milk and in nonfat skim milk containing each of the cryoprotectants mentioned above.

No changes in viability were detected from 90 to 120 min of freeze-drying when either adonitol or glutamate was present in the suspending medium. However, the former additive exerted the strongest protective effect on the viability of lactic acid bacteria, and a higher residual moisture content was obtained.

After 120 min of drying, a remarkable decrease in viable counts was found; this reduction was less marked in L. plantarum CRL 83, L. casei CRL 200, and L. murinus ATCC 35020. On the other hand, PEG 1000 showed no protective effect, and a large loss of water was observed in the cultures at the various times used. When comparing the results obtained with the various additives tested with those obtained with adonitol (Table 1), a higher percentage of residual moisture was observed from 15 to 120 min of freeze-drying in the latter case, which partly accounts for the protective capacity of this additive (14). This effect appears to be related to the ability of various substances to displace or retain water (11).

In some cases, there was a slight increase in viability when cultures were freeze-dried for brief drying times (30 to 45 min) with respect to the results obtained at 90 or 120 min of freeze-drying. This effect was more evident in L. casei and L. plantarum when they were freeze-dried in milk as well as in L. murinus in the presence of PEG 1000 (Fig. 1). The percent survival was found when samples were processed immediately after the lyophilization procedure. However, an important loss of viability was observed in the samples within 48 to 72 h after freeze-drying (data not shown). This

### Table 1. Effect of cryoprotectants on residual moisture content at various drying times

<table>
<thead>
<tr>
<th>Drying time (min)</th>
<th>PEG 1000</th>
<th>PEG 6000</th>
<th>Dextran</th>
<th>β-Glycerophosphate</th>
<th>Glutamate</th>
<th>Adonitol</th>
<th>NFSM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3.80</td>
<td>3.70</td>
<td>3.45</td>
<td>4.35</td>
<td>5.86</td>
<td>7.20</td>
<td>5.60</td>
</tr>
<tr>
<td>30</td>
<td>1.70</td>
<td>1.70</td>
<td>1.12</td>
<td>4.00</td>
<td>5.60</td>
<td>6.60</td>
<td>3.60</td>
</tr>
<tr>
<td>45</td>
<td>0.10</td>
<td>0.09</td>
<td>0.08</td>
<td>2.70</td>
<td>3.40</td>
<td>4.30</td>
<td>1.95</td>
</tr>
<tr>
<td>60</td>
<td>0.09</td>
<td>0.05</td>
<td>0.04</td>
<td>1.40</td>
<td>1.60</td>
<td>1.60</td>
<td>0.99</td>
</tr>
<tr>
<td>90</td>
<td>0.09</td>
<td>0.05</td>
<td>0.03</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>120</td>
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<td>0.035</td>
<td>0.027</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>150</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
<td>0.10</td>
<td>0.20</td>
<td>0.20</td>
<td>—</td>
</tr>
<tr>
<td>180</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.09</td>
<td>0.03</td>
<td>0.16</td>
<td>—</td>
</tr>
<tr>
<td>300</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.06</td>
<td>0.03</td>
<td>0.04</td>
<td>—</td>
</tr>
</tbody>
</table>

*Additives suspended in 10% nonfat skim milk (NFSM).

*10% NFSM (control).

![FIG. 1. Effect of the suspending medium on the survival rate of lactic acid bacteria at various drying times. Symbols: (●) adonitol, (○) glutamate, (▲) PEG 1000, and (△) nonfat skim milk (control).](http://aem.asm.org/)

![FIG. 2. Effect of the suspending medium on the survival rate of streptococci at various drying times. Symbols: (●) adonitol, (○) glutamate, (▲) PEG 1000, and (△) nonfat skim milk (control).](http://aem.asm.org/)
fact was attributed to insufficient drying, since some areas remained moist and gradually dissolved when evacuation ceased. Under conditions of high residual moisture, free water may interact with cellular proteins, which could result in a loss of the typical structure of the native protein (8). Overdrying is also harmful to survival. The high death rate registered during excessive drying (180 to 300 min) (Fig. 1) might be related to the elimination of the three fractions of cellular water (free water, intermediate water, and structured water), with the consequent exposure of hydrophilic sites of the protein due to the O₂ in contact with the dry preparations (8).

The results obtained agree with the observations of Nei et al. (10), who determined that the survival of cells decreases as dehydration increases. Nei (9) suggested that the removal of free water does not damage the cell, but as the moisture content approaches zero, the viability of the organisms decreases as a consequence of the elimination of the un-freezable fraction of cell water.

From the results shown in Fig. 1 and 2, we are convinced that a certain minimal amount of water must be left for a satisfactory survival rate. This residual moisture in freeze-dried materials is directly related to the type of freeze-drying medium and also to the dehydration capacity of the apparatus (6).

The higher survival rate obtained at 90 and 120 min of drying could be accounted for by the optimal moisture content in the freeze-dried samples with adonitol as a cryoprotective agent.

The various degrees of affinity of drying media for water are also important factors in lyophilization. Estimations of the dehydration rate of bacterial suspensions with various types of drying media allow for their classification into two groups: (i) high-molecular-weight additives, such as PEG, which facilitated the dehydration of the cultures and had no protective effect; and (ii) those such as adonitol or glutamate, which made possible the retention of greater amounts of residual moisture. When comparing the effectiveness of the latter two, adonitol obviously provided the highest survival rate in relation to the residual water content in the freeze-dried samples; these results agree with those presented in a previous work (14). However, even though adonitol in milk is an effective protector of the viability of freeze-dried lactic acid bacteria, it is not necessarily the most adequate protective agent for all microorganisms.

In conclusion, it can be said that cellular water plays an important role in freeze-drying and that the removal of un-freezable bound water affects cell survival in microorganisms. On the other hand, residual moisture content is closely related to the drying medium, and an appropriate selection of both the suspending medium and the protector seems to be essential, since variations in the drying medium may affect the survival of bacteria during the freeze-drying process as well as their viability during the period of storage and reconstitution.

ACKNOWLEDGMENTS

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LITERATURE CITED