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Protective Effect of Adonitol on Lactic Acid Bacteria Subjected to Freeze-Drying

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The protective effects of glycerol, adonitol, and four other related polyhydric alcohols on lactic acid bacteria subjected to freeze-drying were examined. The presence of adonitol in the suspending medium markedly protected the viabilities of the 12 stains tested. Dulcitol, mannitol, *m*-inositol, and sorbitol were found to provide little or no protection.

The choice of an appropriate suspending medium is of primary importance to increasing the survival rate of microorganisms during and after freeze-drying. Miller et al. (5) demonstrated that some low-molecular-weight compounds such as sodium glutamate and aspartate protect BCG during freeze-drying. The ability of a compound to preserve the viability of cells during periods of desiccation has been found to be associated with the presence of an amino group, a secondary alcohol group, or both. The aim of the present work was to compare the cryoprotective action of adonitol with those of glycerol and other polyols widely used for cryoprotection.

Cultures. *Streptococcus thermophilus* ATCC 19258, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus helveticus* ATCC 15009, *Lactobacillus leichmannii* ATCC 4797, *Lactobacillus casei* ATCC 393, and *Leuconostoc cremoris* ATCC 19254 were obtained from the American Type Culture Collection. *Lactobacillus murinus* CNRZ 313, isolated by Raibaud et al. (7) and described as a new species by Hemme et al. (2), was obtained from the Centre National de la Recherche Zootechnique. *Streptococcus lactis* T164 and T215, *Streptococcus cremoris* T162 and T55, *Lactobacillus bulgaricus* T142, and *Streptococcus faecium* T175 were obtained from the stock collection of the Centro de Referencia para Lactobacilos and were originally isolated from Tafí cheese.

MRS broth (1) incubated at 45°C for 12 h was used for the propagation of thermophilic lactobacilli; mesophilic lactobacilli and *Leuconostoc* species were grown at 30°C. Streptococci were grown in $LAPTg_{10}$ (6), which contained 15.0 g of peptone, 10.0 g of tryptone, 10.0 g of glucose, 10 g of yeast extract, 1.0 ml of Tween 80, and 1 liter of distilled water. The pH was 6.5 to 6.6. The culture were incubated at 30°C for 14 h.

Stock solutions of adonitol (2 M; Fluka AG), dulcitol (0.8 M; Fluka), *m*-inositol (0.8 M; Fluka), glycerol (1 M; Sigma Chemical Co.), mannitol (0.8 M (Sigma), and sorbitol (1 M; Sigma) were prepared with distilled water and sterilized by filtration (filter type 1121; pore size, 0.2 μm; Schleicher & Schuell Co.); the pH was adjusted to 7.0 with NaOH. Sterile distilled water and sterile (121°C, 15 min) 10% nonfat skim milk (NFSM) were used for resuspending the cells to be freeze-dried. Each polyol was added to a final concentration of 0.32 M. Cells were rehydrated in a medium containing 1.5% peptone, 1% tryptone, and 0.5% meat extract (pH 7.0). This medium was also used as the viable count diluent.

Bacterial cells were harvested at the beginning of the stationary phase and washed once with sterile distilled water. The washed cells were then resuspended in the suspending media to a final concentration of 1×10^9 to 2×10^9 CFU/ml. Aliquots (0.3 ml) of each bacterial suspension were placed in a series of sterile vials and subjected to freeze-drying.

Freeze-drying was carried out in a manifold-type freezer-dryer (Thermovac FD-ULT-10) with independent refrigeration systems for condenser and shelling bath. Samples (0.3-ml) of the bacterial suspensions were dispensed into 1-ml glass ampoules and cooled to a final temperature of −60°C in the shelling bath. The frosted samples were dessicated under vaccum (5×10^{-2} torr 90 min). The total cycle time was 2 h. Residual moisture was <1% in all samples, as determined by a gravimetric method.

The number of viable cells was determined by the agar plate method. Inmmediately before plating, each sample of freeze-dried bacteria was brought to the original volume with the rehydra-

TABLE 1. Protective effects of adonitol and five related polyols during the freeze-drying of lactic acid bacteria

Organism	Survival (%)													
	Adonitol		Dulcitol		Glycerol		*m*-Inositol		Mannitol		Sorbitol		Control	
	dH_2O[a]	NFSM	dH_2O	NFSM	dH_2O	NFSM	dH_2O	NFSM	dH_2O	HFSM	dH_2O	HFSM	dH_2O	NFSM
S. thermophilus ATCC 19258	6	44	2	5	10	21	3	5	23	4	1	3	0.6	7
L. plantarum ATCC 8014	10	72	<1	8	10	33	<1	10	<1	9.5	5	11	1	8
L. casei ATCC 393	12	60	<1	10	15	38	<1	9.3	3	11	8	15	0.9	10
L. murinus CNRZ 313	15	42	3	10	12	29	2	8.4	<1	3.2	2	10.3	1.3	6

[a] dH_2O, Distilled water.

tion medium. Serial dilutions of each sample were plated in triplicate, and the plates were incubated at 37 and 45°C for mesophilic and thermophilic bacteria, respectively, and at 30°C for lactic streptococci and *L. cremoris* ATCC 19254. After 48 to 72 h, the resulting colonies from samples taken before and after freeze-drying were scored, and the percent survival was calculated.

Table 1 shows the viabilities of the freeze-dried bacterial suspensions in the presence of glycerol, adonitol, and four related sugar alcohols. When the suspending medium was distilled water, the incorporated additive provided little or no protection to the cells. Viable counts were slightly higher in the samples to which glycerol or adonitol had been added. However, for the four organisms tested, adonitol in 10% NFSM suspending medium exerted 1.45 to 2.18 times the protective effect exerted by glycerol at equal concentration. The other polyols (dulcitol, mannitol, *m*-inositol, and sorbitol) had no protective effect: the survival rate for organisms freeze-dried with them was similar to that obtained for the NFSM control.

TABLE 2. Survival of freeze-dried microorganisms in adonitol and glycerol

Organism	% Survival		
	Adonitol[a]	Glycerol[b]	NFSM 10%[c]
S. lactis T164	100	53	10
S. lactis T215	81	26	9
S. cremoris T162	86	40	12
S. cremoris T55	96	38	10
S. faecium T175	100	5	13
S. thermophilus ATCC 19258	98	43	13
L. cremoris ATCC 19254	98.3	12	4.6
L. plantarum ATCC 8014	100	35	12
L. casei ATCC 393	98.2	32	9
L. murinus CNRZ 313	88	41	8
L. fermentum ATCC 9338	56	40	1.5
L. leichmanii ATCC 4797	87	90	<1
L. bulgaricus T142	53	2.5	3.6
L. helveticus ATCC 15009	49	20	1.4

[a] Concentration in NFSM, 0.75 M.
[b] Concentration in NFSM, 1 M.
[c] Control.

The cryoprotective action of adonitol reached its highest level at 0.75 M. On the basis of this result, the effects of 0.75 M adonitol and 1 M glycerol were compared (Table 2).

When adonitol was present in the suspending medium, all freeze-dried cultures tested exhibited a survival rate after freeze-drying that was higher than that obtained when glycerol was used. For 11 of the 13 organisms tested, the degree of protection conferred by adonitol was >80%, and for only 1 of 13 strains, the degree of protection conferred by glycerol was high. The survival rates for *L. fermentum* ATCC 9338 treated with both protective agents were similar, whereas *L. cremoris* ATCC 19254, *L. bulgaricus* T142, and *S. faecium* T175 were not adequately protected by glycerol.

The results demonstrate that adonitol has a strong protective effect on lactic acid bacteria during freeze-drying (Table 2). Among the polyols tested, mannitol and sorbitol, which are metabolized by lactobacilli, were not effective as cryoprotectors. On the other hand, adonitol, which cannot be metabolized, had a strong protective effect. This suggests that the effect is of a physicochemical nature. In agreement with the results of Lion et al. (4), no correlation was found between the protective efficiency of a sugar or sugar alcohol and its fermentability by the bacteria.

Since the polyols tested are chemically relat-

ed, the efficiency of adonitol as a cryoprotective agent might be due to the esteric structure of its hydroxyl groups. The secondary alcohol group of some polyhydric alcohols could presumably be capable of replacing water molecules in the protein structure by hydrogen bonding. Furthermore, if esteric factors are involved in the process, a certain combination between the cellular protein and the protective compound might prevent the collapse of the protein structure during desiccation. The loss of viability in the desiccated cultures and the effectiveness of some cryoprotectors might be due to interaction of the additive with the cell membrane (3, 8). The mode of action of adonitol during freeze-drying has not yet been determined. However, there is no doubt that this polyol affords strong protection for lactic acid bacteria, and its use is recommended for the production of freeze-dried cultures used as starters in various branches of the food industry.

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