

Freeze-Drying of Fungi: Influence of Composition and Glass Transition Temperature of the Protectant

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Effects of the composition and the physical stability of the protectant on the viability of freeze-dried fungal spores of *Arthrobotrys superba* were studied. Effects of various saccharides (trehalose, lactose, maltose, sucrose, raffinose, glucose) and a sugar-alcohol (*myo*-inositol), divalent cations (CaCl_2 , MgSO_4 , ZnSO_4 , CuSO_4), and cryoprotectants (polyethylene glycol, dimethyl sulfoxide, glycerol) were investigated. Glass transition temperatures (T_g) of the dried formulations were recorded by differential scanning calorimetry for the various saccharides. Suspensions prepared in dextran plus glucose, *myo*-inositol, or sucrose showed onset temperatures of the glass transition at or below 30°C. Shelf life of these suspensions was limited when stored at 30°C. Onset temperatures were higher for the other saccharides tested. The best survival after storage at 30°C was obtained by using protectants containing mixtures of dextran with either trehalose or lactose, followed by maltose and raffinose, respectively. Increasing the concentration of trehalose to 20% resulted in better survival, while the T_g of the product did not change. Addition of cryoprotectants and divalent cations to the lyoprotectant did not improve survival. © 1995 Academic Press, Inc.

Freeze-drying is a commonly used method of preserving fungal propagules. Freeze-drying has two big advantages over cryopreservation. No special requirements are needed to store the product, and dispatch of the product does not need cooling facilities. Still, freeze-drying is not always successful. Viability is frequently low or declines during storage.

Viability of freeze-dried fungi depends on the rate of freezing (28, 31, 33), the size of the propagules, the thickness of the cell wall (33), and the composition of the protectant. This study examines the efficacy of the protectant, with special emphasis on saccharides, cryoprotectants, and divalent cations, using the fungus *Arthrobotrys superba* as a model organism. This fungus was chosen because it produces large, thin-walled spores, making it very sensitive to freeze-drying damage.

Saccharides protect membranes during freezing and drying (11, 13, 15) by hydrogen bonding to the phospholipid head groups (17). This interaction increases head group spacing, hence lowering the transition temperature of the phospholipids (18). Disaccharides, particularly trehalose, are found to be optimal.

Timasheff and his colleagues provided evidence that saccharides stabilize proteins during cooling because they are preferentially excluded from the surface of the proteins in aqueous solution. They repel the hydrophobic parts of the amino acid chains, thus preventing unfolding of the protein at the melting temperature (2, 4, 7). Moreover, hydrogen bonding between the saccharide and the protein in the final stages of desiccation is required for stabilization of the dried proteins (7, 8). Divalent cations are assumed to strengthen preferential exclusion by enhancing the hydrophobic character of the protein (5, 6).

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Finally, addition of small concentrations of the cryoprotectants glycerol, dimethyl sulfoxide, or polyethylene glycol reduces formation of lethal intracellular ice crystals (26).

During freeze-drying, the protectant is converted into a glass (20, 21). Dehydrated organisms, enclosed in this glass, can be stored successfully below the glass transition temperature (T_g) of the dried formulation. Above T_g , water mobility increases and consequently the product deteriorates. The T_g , which can be estimated by differential scanning calorimetry (DSC) (24), is determined by the composition of the protectant and the residual moisture content. In this study, physical stability of the dried formulations is evaluated by DSC with respect to the saccharides. In addition to the T_g , the onset of the transition curve (T_{on}) and the temperature at which the curve returns to a straight line (T_c) are estimated.

MATERIALS AND METHODS

Protectants

Saccharides: 5% dextran (Serva; M_r 38,000) in combination with either 7% glucose (Merck), *myo*-inositol (Serva), raffinose (Fluka), sucrose (Merck), maltose (Fluka), lactose (Brocades), or trehalose (Serva); 5% dextran in combination with either 12% or 20% trehalose.

Divalent cations: 5% dextran and 7% trehalose in combination with 0.06 mM CuSO_4 (Brocades), ZnSO_4 (Merck), MgSO_4 (Brocades), or CaCl_2 (Analar).

Cryoprotectants: 5% dextran and 7% trehalose in combination with either 1% dimethyl sulfoxide (Me_2SO ; Fluka), polyethylene glycol (PEG) (ICN; M_r 300), or glycerol (Merck).

Freeze-Drying of Suspensions

Spore suspensions of *A. superba* CBS 643.89 were prepared at a concentration of 5×10^7 spores/ml protectant. The 1.5-ml

vials (Müller & Müller, Holzminden, Germany) were filled in duplo with 300 μl of the spore suspension. Vials were closed with a rubber lyophilization stopper (diameter, 12.7 mm) (Helvoet, Alken, Belgium). The vials were cooled at a rate of $-1^\circ\text{C}/\text{min}$ to -45°C followed by cooling at a rate of $-50^\circ\text{C}/\text{min}$ to -75°C using a Sylab Icecube 1610 programmable freezer (SyLab, Purkersdorf, Austria).

The suspensions were lyophilized using a Leybold Heraeus GT 4 freeze-drying device (vacuum: 0.6 mbar; condenser temp: -60°C). Primary drying was performed 5°C below the temperature of complete solidification as estimated with an Edwards freezing analyzer (Edwards, Marburg, Deutschland). The end of the primary drying phase was indicated by a pressure rise in 1 min of less than 2.5%. The rate of heating to reach the secondary drying phase was $1^\circ\text{C}/\text{min}$. Temperature during secondary drying was 20°C . Vials were stoppered with a computer-controlled stoppering device to obtain residual moisture contents (RMC) of 1, 2, and 3.5%.

Revival of Dried Suspensions

Organisms were revived immediately after freeze-drying and after storage for 2 months at 30°C . For revival, pellets were resuscitated in 1 ml demineralized water for 30 min at 22°C . Two hundred μl of the spore suspension was plated in triplicate on 8-ml potato-carrot agar (PCA: 15 g agar, filtered extract of 20 g chopped boiled carrots and 20 g potatoes in 1 liter demineralized water). The cultures were incubated at 24°C for 16 h. Germination was scored for 8×100 spores. The data were analyzed statistically using a nested ANOVA ($P = 0.05$) after transformation of germination percentages into arcsine (square root) values. Multiple comparisons based on Newman-Keuls ($P = 0.05$) were used to calculate significance of the differences between means (32).

Estimation of Residual Moisture Content (RMC)

RMC was measured by the titrimetric procedure of Karl-Fischer (1) with a Mitsubishi CA05 Moisture Analyzer (Mitsubishi Chemical Industries, Ltd., Tokyo, Japan). The freeze-dried material was dissolved in Coulomat A (Riedel de Haën A.G., Seelze, Germany). The generator solution cell was filled with 100 ml Coulomat C (Riedel de Haën A.G.); the cathode solution cell was filled with 5 ml Coulomat A.

Differential Scanning Calorimetry (DSC)

A Perkin-Elmer DSC 2 calorimeter was recalibrated with indium, water, benzoic acid, and acetamide. Data were obtained at a scanning speed of 5°C/min. The glass transition temperature (T_g) was taken as the inflexion point of the sigmoidal curve. The point at which the curve started to deviate from a straight line was taken as the onset temperature of the glass transition (T_{on}) and the point at which the curve returned to a straight line as the end temperature (T_e).

RESULTS

The viability of fungal spores of *A. superba* was significantly influenced, immediately after freeze-drying as well as after

storage at 30°C, by the saccharide present in the protectant. Viability immediately after freeze-drying was optimal with lactose, followed by *myo*-inositol, trehalose, maltose, raffinose, and sucrose, respectively. With dextran plus glucose, survival was low immediately after freeze-drying and absent after storage at 30°C. Viability of spores protected by dextran plus *myo*-inositol decreased considerably during storage. Viability of the suspensions prepared in the tri- or disaccharides decreased approximately 20% during storage at 30°C. In these types of saccharides, survival was the lowest when the protectant contained sucrose, followed by raffinose, maltose, trehalose, and lactose, respectively (Fig. 1).

Increasing the concentration of trehalose to 20% resulted in a significantly better survival both immediately after freeze-drying and after storage at 30°C (Fig. 2).

Addition of Me_2SO to the dextran plus trehalose mixture significantly improved survival immediately after freeze-drying. However, survival rates decreased considerably during storage at 30°C, particularly when Me_2SO or PEG was added to the protectant (Fig. 3). Results could not be analyzed by ANOVA after storage at 30°C because survival rates were too low.

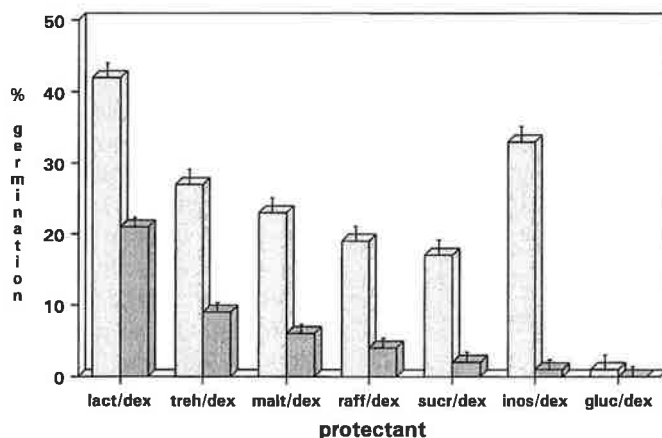


FIG. 1. Survival of spores of *Arthrotrichum superba*, freeze-dried to 2% RMC, protected by mixtures of 5% dextran and 7% of various saccharides. □ Percentage germination immediately after freeze-drying; ■ percentage germination after 2 months storage at 30°C.

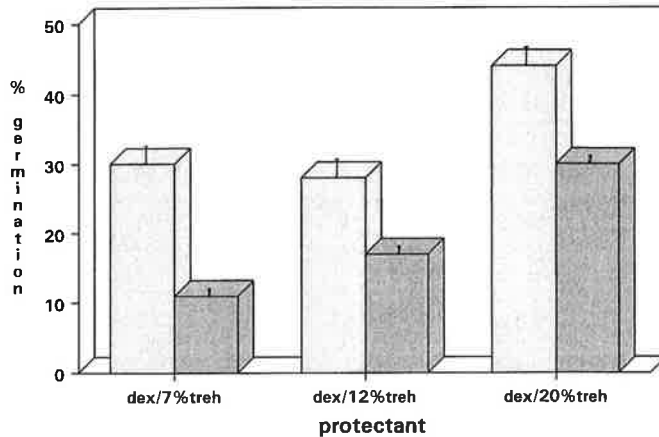


FIG. 2. Survival of spores of *A. superba*, freeze-dried to 1% RMC, protected by mixtures of 5% dextran and 7% trehalose or 12% trehalose or 20% trehalose. □ Percentage germination immediately after freeze-drying; ■ percentage germination after 2 months storage at 30°C.

Addition of 0.06 mM CuSO_4 or ZnSO_4 to the dextran plus trehalose mixture reduced survival significantly even immediately after freeze-drying. Efficacy of CaCl_2 and MgSO_4 was not significant (Fig. 4). Results could not be analyzed by ANOVA after storage at 30°C because survival rates were too low.

T_g , the onset temperature of the curve (T_{on}), and the temperature at which the curve returned to a straight line (T_e) are summarized in Table 1 with respect to the

dextran plus saccharide mixtures. T_g and T_{on} of the mixtures containing glucose and *myo*-inositol were the lowest. Within the series of the di- and trisaccharides, T_g and T_{on} of the dextran plus sucrose mixture were the lowest followed by dextran mixtures containing maltose, lactose, trehalose, or raffinose, respectively.

In Fig. 5 T_{on} of the various dextran plus disaccharide mixtures are plotted against the residual moisture content. Values were scored for disaccharides only, because di-

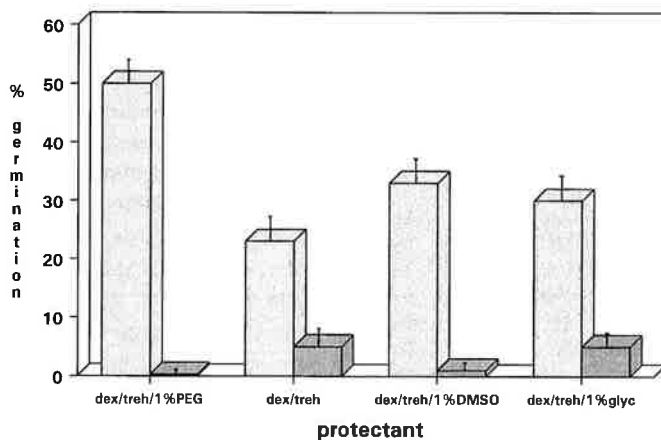


FIG. 3. Survival of spores of *A. superba*, freeze-dried to 2% RMC, protected by mixtures of 5% dextran and 7% trehalose with 1% polyethylene glycol or dimethyl sulfoxide or glycerol. □ Percentage germination immediately after freeze-drying; ■ percentage germination after 2 months storage at 30°C.

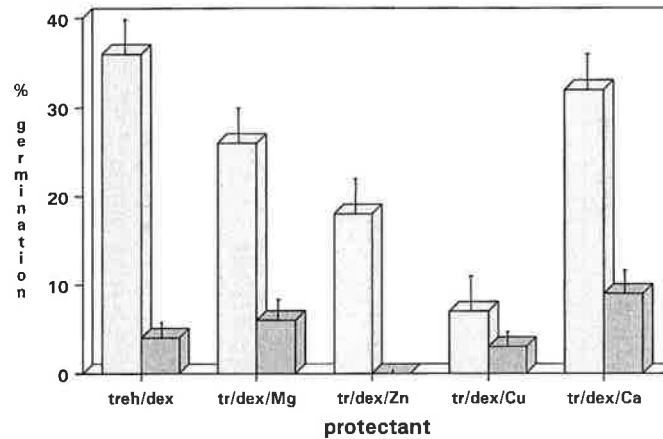


FIG. 4. Survival of spores of *A. superba*, freeze-dried to 2% RMC, protected by mixtures of 5% dextran and 7% trehalose with 0.06 mM $MgSO_4$ or $CaCl_2$ or $ZnSO_4$ or $CuSO_4$. \square Percentage germination immediately after freeze-drying; \blacksquare percentage germination after 2 months storage at 30°C.

saccharides had been found to provide optimal protection (12, 16, 17, 36). The onset temperatures of the mixtures containing lactose and trehalose were the highest, being above 60°C for RMCs lower than 3.5%. With maltose, onset temperatures ranged

between 47° and 70°C, depending on the RMC. Onset temperatures of the dextran plus sucrose mixture were the lowest, being 32°, 35°, and 53°C for 3.5, 2, and 1% RMC, respectively.

DISCUSSION

Differences in physical stability of the glasses produced with the various saccharides explained their ranking order of effectiveness, with the exception of raffinose. This was also suggested by Green and Angell (23). Excluding raffinose, survival data obtained corresponded to the data on physical stability of the dried formulations. Viability after storage at 30°C was optimal with lactose, followed by trehalose, maltose, sucrose, *myo*-inositol, and glucose, respectively. Likewise, T_g values were highest for lactose and trehalose, followed by maltose, sucrose, *myo*-inositol, and glucose, respectively. Evaluation of the results of Fig. 1 and the data in Table 1 proved the onset temperature of the glass transition curve to be a critical parameter. Protectants containing *myo*-inositol and glucose showed an onset of the glass transition curve at or below 30°C. Viability of propagules dried in these protectants decreased immediately after freeze-drying or

TABLE 1

Glass Transition Temperatures (T_g), the Onset Temperatures of the Glass Transition (T_{on}), and the End Temperatures of the Glass Transition (T_e) of Mixtures of Dextran plus Various Saccharides, Dried to Moisture Contents of 1, 2, and 3.5%

Protectant	T_g (°C)	$T_{on}-T_e$ (°C)
5% Dextran/7% glucose 2% RMC	41	31-60
5% Dextran/7% inositol 2% RMC	51	24-62
5% Dextran/7% sucrose 3.5% RMC	65	32-79
5% Dextran/7% sucrose 2% RMC	78	35-94
5% Dextran/7% sucrose 1% RMC	88	53-96
5% Dextran/7% maltose 3.5% RMC	81	47-89
5% Dextran/7% maltose 2% RMC	91	56-101
5% Dextran/7% maltose 1% RMC	103	70-114
5% Dextran/7% lactose 3.5% RMC	80	63-94
5% Dextran/7% lactose 2% RMC	93	66-109
5% Dextran/7% lactose 1% RMC	110	79-119
5% Dextran/7% trehalose 3.5% RMC	89	63-99
5% Dextran/7% trehalose 2% RMC	94	67-125
5% Dextran/7% trehalose 1% RMC	112	78-128
5% Dextran/12% trehalose 1% RMC	100	87-118
5% Dextran/20% trehalose 1% RMC	102	89-116
5% Dextran/7% raffinose 2% RMC	112	79-117

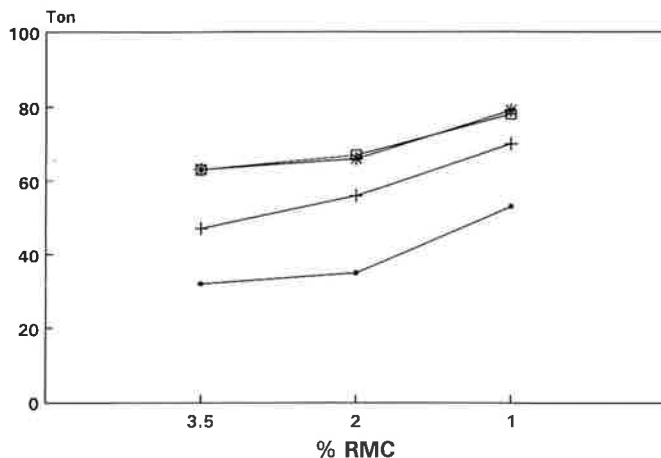


FIG. 5. Onset temperatures of glass transition (T_{on}) curves of mixtures of dextran and trehalose, lactose, maltose, or sucrose dried to 1, 2 and 3.5% RMC. (*) Lactose; (□) trehalose; (+) maltose; (■) sucrose.

after storage at 30°C. Likewise, the worst results within the series of di- and trisaccharides were obtained with sucrose, which has a T_{on} of 35°C.

Survival data did not coincide with the data on the physical stability for raffinose. The glass produced by raffinose was equally as stable as that produced by lactose or trehalose, but survival was lower. This is explained by the fact that disaccharides show a better hydrogen bonding capacity to the phospholipid headgroups (17) than the trisaccharide (14, 16).

Stability of the glass is known to be directly proportional to the size of the saccharide molecule (25, 30). This was confirmed by the results obtained. Glasses produced with the small molecules of the monosaccharide glucose and the sugar-alcohol *myo*-inositol were less stable than those produced with disaccharides or the trisaccharide raffinose (Table 1).

The ranking order of percentages of survival coincided with that of the T_g and T_{on} of the respective saccharides, even when T_{on} was above the storage temperature. This might indicate that molecular motion started at a lower temperature than the T_{on} measured by DSC. A more accurate indica-

tion of the starting temperature of this motion may be obtained by electron spin resonance (29). Differences in hydrogen bonding capacity to the phospholipid head groups (17) may also contribute to the observed ranking order. In Crowe *et al.* (16), membranes were optimally protected at low water activity by trehalose followed by lactose, maltose, sucrose, glucose, raffinose, and *myo*-inositol, respectively. Likewise, in Crowe *et al.* (14) dried liposomes were optimally protected by trehalose, maltose, sucrose, raffinose, glucose, and *myo*-inositol, respectively. These ranking orders roughly coincided with that observed in this investigation. Thus, differences in hydrogen bonding capacity and physical stability might contribute jointly to the ranking order observed with the various saccharides.

Raising the concentration of trehalose to 20% resulted in better survival both immediately after freeze-drying and after storage at 30°C while the T_g of the protectant hardly changed. Obviously, differences in survival rate could not be explained by variation in physical stability. The large amount of trehalose may either have formed a thicker glass layer surrounding the dehydrated spores or have enhanced the amount of hy-

drogen bonding to the phospholipid head groups (17) and protection of proteins against denaturation (2, 4). Alternatively, at the high concentration used, some of the trehalose may have entered the cells, thus promoting formation of a stable glass inside the cell and a better protection of intracellular proteins and membranes. Finally, dehydration of the fungal spores before freeze-drying might be increased by the higher initial concentration. Dehydration is essential, because it prevents intracellular freezing, which is lethal (33).

Protectants containing lactose provided the best protection during freeze-drying. Moreover, formulations containing lactose dried faster than formulations with other saccharides and T_g values of dextran plus trehalose and dextran plus lactose mixtures were almost the same. In contrast to this, trehalose is produced by organisms tolerant to desiccation (9, 10) including yeasts and fungi (27, 34, 35). Use of lactose during freeze-drying may have some advantages over trehalose. It has a strong tendency to crystallize. Consequently, amorphous glass is produced in addition to crystals, providing both a good structure for freeze-drying and a glass to protect the organisms. However, the stronger tendency of lactose to crystallize combined with a lower capacity to hydrogen bond to the phospholipid head-groups may make it a less suitable vehicle for preservation.

Efficacy of the divalent cations was insignificant or absent. Most probably copper and zinc were too toxic to be suitable as protectants. Both compounds stimulate production of hydroxyl radicals, resulting in peroxidation of the membranes (22).

Addition of PEG improved initial survival but the dried suspensions could not be stored. DSC analysis revealed that glass transitions occurred below 4°C (data not shown). Survival decreased more with PEG and Me_2SO_4 than with glycerol, most probably because the latter compound is less toxic (3, 19).

We conclude that freeze-dried suspensions of *A. superba* for long-term preservation at temperatures between 20° and 30°C can best be prepared with trehalose or lactose. Experiments have been extended to other fungi. Preliminary results show that protectants prepared with trehalose and lactose provide optimal protection for these organisms as well.

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