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LONG-TERM PRESERVATION OF FUNGI

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Introduction

In the fungal kingdom about 60.000 species are recognized at this moment, but the actual number of existing species is estimated between 200.000 and over a million. Many of these species can only be identified after culturing. For the conservation of these genetic resources, culture collections are playing an essential role. A prerequisite is an optimal long-term preservation method with inactive metabolism to avoid mutation of the genome and selection. Freeze-drying and cryopreservation are the most commonly used long term preservation methods for fungi. Freeze-drying has two advantages over cryopreservation: no special conditions are needed to store the product, and dispatch of the product does not require cooling facilities. However, cryopreservation is the more generally applicable and reliable method, as only spores survive freeze-drying and the drying damage surpasses that of the freezing step. Survival of freeze-dried spores of the fungus *Arthrobotrys superba* for example is one fifth of that of cryopreserved spores. Moreover, the lag phase of freeze-dried cultures is longer than that of cryopreserved cultures and it increases during storage. At the CBS, cryopreservation and lyophilization are both applied when possible, but for dispatch freeze-dried ampoules are preferred.

Freeze-drying

Before freeze-drying cells are suspended in a lyoprotectant, containing a saccharide and a macromolecule. Saccharides protect membranes during freezing and drying (1, 2, 4), against phase transition by hydrogen bonding to the phospholipid head groups (6). Disaccharides are believed to be best, particularly trehalose (1). The efficacy of various saccharides (glucose, sucrose, maltose, trehalose, lactose, raffinose) and a sugar-alcohol (*myo*-inositol) was studied for freeze-drying of fungal spores (14), with the fungi *Arthrobotrys superba* CBS 643.80 and *Dactylella gampsospora* CBS 127.83 as model organisms, the former because of its fragile, thin-walled, ovoid, two-celled spores (25 x 56 µm), that are highly susceptible to the adverse effects of freezing and drying and the latter because of its very fragile fusiform, thin-walled, five-celled spores (80 x 20 µm).

When tested directly after freeze-drying lactose provided optimal protection followed by inositol and trehalose respectively. However, the cultures with inositol deteriorated markedly during storage for two months at 30°C (Fig. 1). The physical stability of the dried formulations was evaluated by differential scanning calorimetry (DSC) (14). Glass transition temperatures (T_g) are shown in Table 1. In addition to the T_g, the onset of the transition curve (T_{on}) was estimated. With the exception of raffinose, the ranking order of survival after storage at 30°C corresponded to the ranking order of the various T_g's. T_{on} proved to be the critical parameter. The onset of the glass transition of protectants containing *myo*-inositol, glucose and sucrose was at or below 30°C. Likewise, after storage at 30°C survival decreased substantially with these compounds while the best survival was obtained with lactose followed by trehalose (Fig. 1). Despite a high T_g, survival rates with raffinose were low compared to the disaccharides, possibly because disaccharides bond hydrogen to the phospholipid headgroups better (5, 3).

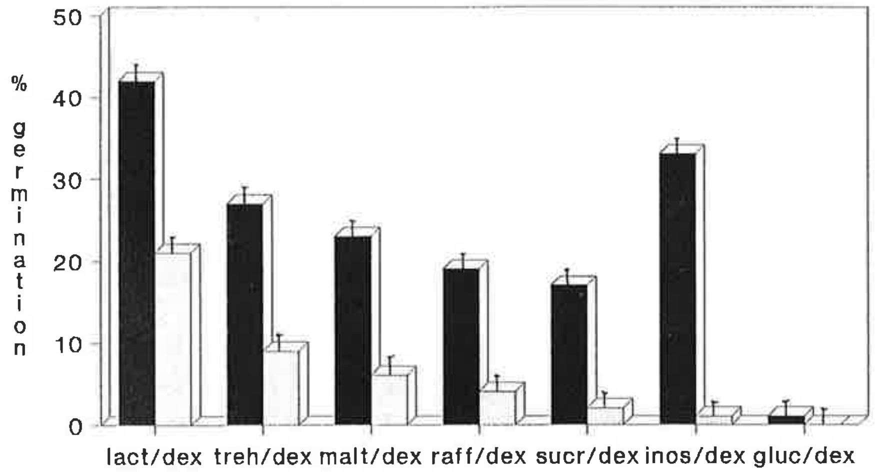

Fig. 1. Survival of spores of *Arthrobotrys superba*, freeze-dried to 2% RMC, protected by mixtures of 5% dextran plus 7% saccharide ■ 16 h; □ 2 mth 30°C.

Table 1. Glass transition temperatures (T_g) and the onset temperatures of the glass transition (T_{on}) of mixtures of 5% dextran plus 7% saccharide, freeze-dried to 2% RMC.

Protectant	T_g (°C)	T_{on} (°C)
glucose	41	31
inositol	51	24
sucrose	78	35
maltose	91	56
lactose	93	66
trehalose	94	67
raffinose	112	79

The large-molecular compound in the protectant serves as a bulking agent. In freeze-drying of biological material, proteins or polysaccharides are usually applied, because they are not toxic. The efficacy of the following compounds was studied according to the methods of Tan et al. (14): the proteins bacto-peptone, gelatin, polygelin, skimmed milk, lactalbumin, Ca-lactobionate plus lactalbumin and casein; the polysaccharides dextran

(Mw 38.000), hydroxyethyl starch (HES, Mw > 1.000.000), carboxymethylcellulose (CMC, Mw ± 500.000), ficoll (Mw 70.000) and ß-cyclodextrin and the macromolecule polyvinylpyrrolidone (PVP) (Mw 10.000). A concentration of 5% was used in combination with 7% trehalose with the exception of skimmed milk (12%), gelatin (1%), Ca-lactobionate (1%) plus lactalbumin (1%) and CMC (1%). Germination was scored for 8 x 100 spores in duplo. Data were analyzed 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 (11). Highest viability was obtained in preparations with skimmed milk and with compounds derived from milk (Fig. 2). Lowest survival was obtained with the protein peptone.

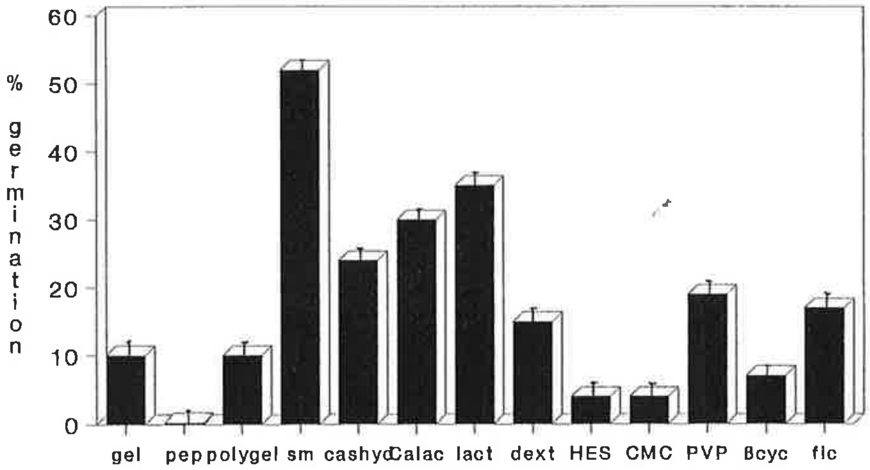

Figure 2. Percentage germination immediately after freeze-drying of spores of *Arthrobotrys superba*, freeze-dried to 2% RMC, protected by various macromolecules plus 7% trehalose.

The lag phase increased in the tests with polysaccharides and PVP; the percentage of spores still alive but not germinating was generally higher than with the proteins (Table 2). The amino-acids from the latter protectants might have helped in restoring the energy charge and repairing damaged proteins.

Table 2. Percentage of spores alive but not germinating of *Arthrobotrys superba*, freeze-dried to 2% RMC, protected by various macromolecules plus 7% trehalose n = 16.

Macromolecule	% non-germ. spores	Macromolecule	% non-germ. spores
12% Skimmed milk	11 ± 3.0	5% dextran	40 ± 2.0
1% Ca-lactobionate + 1% lactalbumin	16 ± 3.2	1% CMC	13 ± 1.0
5% casein	11 ± 2.6	5% HES	31 ± 2.7
5% peptone	20 ± 1.0	5% PVP	27 ± 2.8
1% gelatin	5 ± 2.5	5% ficoll	15 ± 2.9
5% polygelin	8 ± 2.1	5% ß-cyclodextrin	35 ± 2.3

Physical stability of the dried product was estimated by DSC (Table 3). In addition, T_{on} of the frozen product was estimated and the collapse temperature (T_{coll}) was determined with a freeze-drying microscope according to the methods of Tan et al. (13). T_{coll} was found to lower than T_{on} for all mixtures (Table 3). T_{coll} seems to be the most reliable parameter to base the freeze-drying process on. The minimum product temperature that could be attained in the freeze-drying device is approximately -40°C; results of mixtures with a T_{coll} of approximately -40°C were very inconsistent, and remained so after several repetitions. Mixtures, containing lactalbumin and peptone could not always be dried successfully, most probably as a result of their low T_{coll}, but when it was successful, stable pellets were produced with T_g values of 70 and 77 respectively. T_{coll} of mixtures with dextran, casein, gelatin, ß-cyclodextrin and polygelin were above -38°C; consequently these suspensions could be dried satisfactorily. T_g values of the dried mixtures of CMC and PVP were very variable. Moreover, T_{coll} of the frozen mixtures of HES and these compounds could hardly be estimated because ice-crystals could be detected only with difficulty in the gel-like substances produced during cooling under the microscope. The variation in T_g of their dried pellets might be explained by the fact that these 200 µl trehalose/macromolecule mixtures, cooled slowly at -1°C/min, were so amorphous that they scarcely could be freeze-dried. Fig. 3 shows SEM photographs of the dried pellets of dextran, HES, CMC or PVP, all with trehalose. The pellets with dextran (Fig. 3A) and HES (Figs 3B-C) show a regular open network structure, that of HES a bit coarser than that of dextran. In contrast the structure in pellets with CMC is irregular with mazes of various size (Figs 3D, 3F), while some parts of the pellet show a severe collapse (Fig. 3H). Pellets with PVP also show an irregular structure (Fig. 3E), and the exposed surface of the pellet is pellicular without

Table 3. Glass transition temperatures (T_g) of macromolecules plus 7% trehalose, freeze-dried to 2% RMC and collapse temperatures (T_{coll}) and onset temperatures of the glass transition (T_{on}) of the frozen mixtures.

Protectant	T_{coll} (°C) frozen	T_{on} (°C) frozen	T_g (°C) dried
12% Skimmed milk	- 39	- 32	93
1% Ca-lactobionate/ 1% lactalbumin	- 40	- 25	90
5% lactalbumin	- 43	- 33	70
5% casein	- 37	- 29	89
5% peptone	- 42	- 29	77
1% gelatin	- 38	- 22	59
5% polygelin	- 36	- 24	89
5% dextran	- 33	- 20	94
1% CMC	- 9/ - 52	- 28	54-97
5% HES	- 4/ - 38	- 25	85
5% PVP	- 43	- 27	55-104
5% ficoll	- 43	- 28	92
5% ß-cyclodextrin	- 37	- 25	80

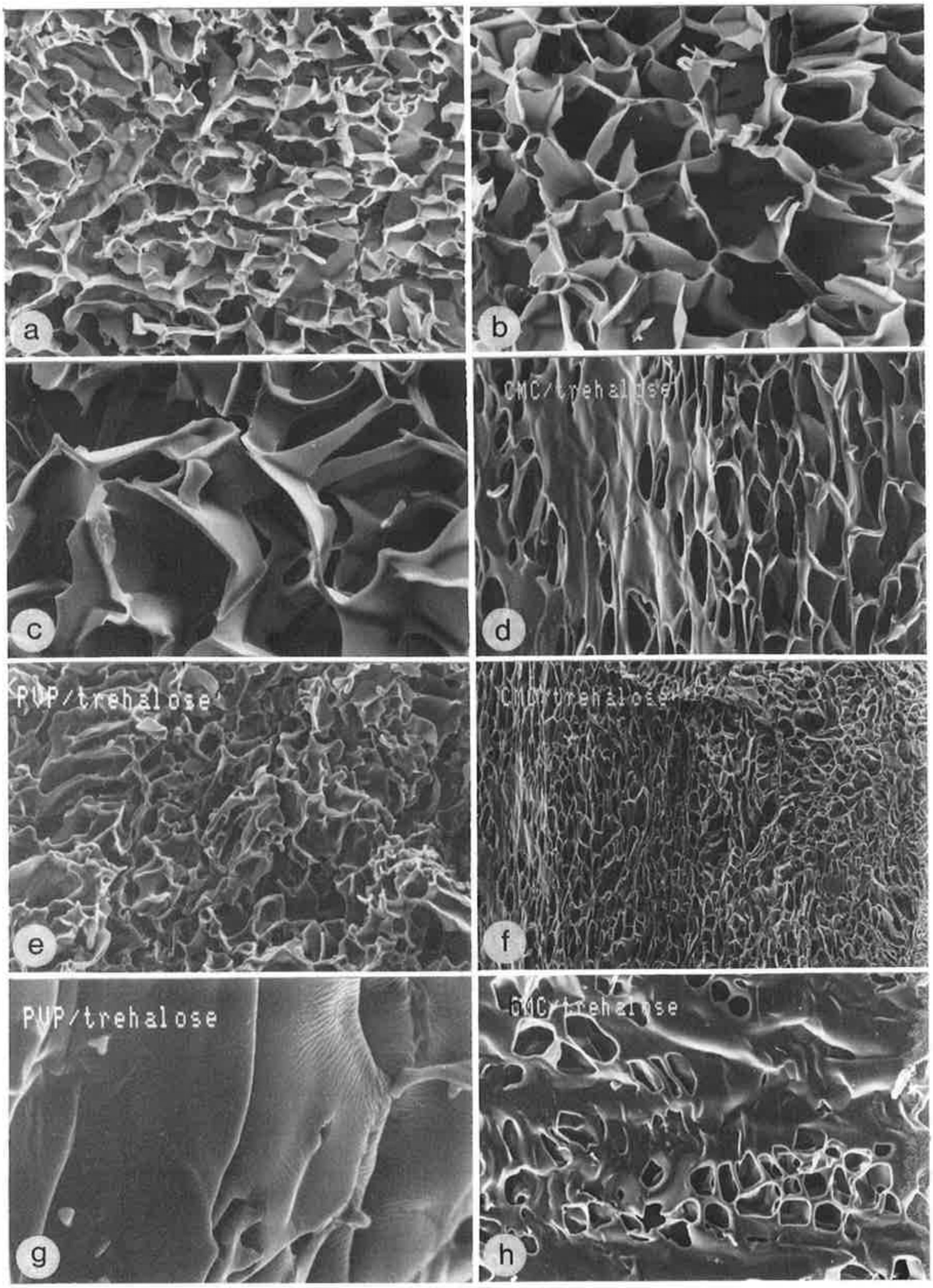

Fig. 3. SEM photographs of pellets of macromolecules plus 7% trehalose, freeze-dried to 2% RMC: 3A: dextran 300X, regular maze; 3B HES 300X, regular maze; 3C HES 700X; 3D CMC 300X, zoned area; 3E PVP 300X, somewhat irregular maze; 3F CMC 100X, zonate; 3G PVP 1000X, surface of pellet; 3H CMC 250X, collapsed area.

pores (Fig. 3G), possibly indicating a separation of phases during freezing, which hampers the freeze-drying process. Most probably, incipients (e.g. mannitol) must be added to compounds of this type. Skimmed milk, Ca-lactobionate/lactalbumin and ficoll were dried successfully despite a low T_{coll}. This might be due to a greater capacity to form a regular crystal structure, e.g. by the incipient lactose in milk.

Cryopreservation

In commonly used cryopreservation procedures, fungi are cooled slowly at -1°C/min to dehydrate the cells to such an extent as to preclude or at least reduce the formation of crystals within the cells (7). Either glycerol or dimethylsulfoxide (DMSO) is applied as cryoprotectant. However, some fungi, e.g., Oomycetes and those producing large fragile thin-walled spores do not withstand slow cooling. The possibility of vitrifying (8) these organisms was examined. To set up a vitrification procedure, toxicity and the time required for protectants (glycerol, DMSO, ethylene-glycol, polyethylene-glycol (PEG) Mw 200, PEG Mw 300, 1.2 propane-diol, 1.2 butane-diol, 1.3 butane-diol, ethanol and methanol) to permeate fungal propagules at room temperature was examined. This was done by suspending spores in a drop of water on a slide under a coverslip. Subsequently a drop of 2M cryoprotectant was placed at one edge of the coverslip and the water was subtracted and replaced by the cryoprotectant by soaking it up at the opposite edge with a piece of filter-paper. The period of time required for the protectant to permeate the cells was established by recording shrinkage and subsequent swelling of the cell. The effect of washing cells in 1.2 M sucrose after exposure to the cryoprotectant was studied by placing a drop of 1.2 M sucrose next to the coverslip and replacing the cryoprotectant by sucrose with the filterpaper. Viability was estimated by replacing the cryoprotectant or 1.2 M sucrose by the vital stain Hoechst 33285 with the aid of filterpaper and subsequently observing the slide using a 365 nm filter. The percentage living spores was scored for 6 x 50 propagules of *Arthrobotrys superba* CBS 643.80 and *Dactylella gampsospora* CBS 127.83.

The period of time required for permeation and survival were almost equal for both organisms (Table 4,5). Glycerol permeated very slowly followed by DMSO (10 to

Table 4. Period of time required for permeation of 2M cryoprotectants (time), toxicity (% spores alive) and effect of washing spores in 1.2 M sucrose for *Arthrobotrys superba* $n = 6$.

Protectant	time	% alive	1.2M sucrose
glycerol	8-16 h	67.2 ± 4.9	
PEG Mw 300	<<1 sec	8.0 ± 3	10.2 ± 3.9
PEG Mw 200	40 sec	28.4 ± 3	91.6 ± 5.8
DMSO	10 min	36.3 ± 9.3	67.3 ± 5.9
1.2 propanediol	1.5 min	57.0 ± 6.2	69.1 ± 2.8
1.2 butanediol	<<1 sec	4.7 ± 3.7	18.0 ± 8.8
1.3 butanediol	3 min	37.6 ± 3.8	78.7 ± 8
ethyleneglycol	1 min	84.3 ± 6.8	95.3 ± 3.0

Table 5. Period of time required for permeation of 2M cryoprotectants (time), toxicity (% spores alive) and effect of washing spores in 1.2 M sucrose for *Dactylella gampsospora* $n = 6$.

Protectant	time	% alive	1.2M sucrose
glycerol	8-16 h	60.8 ± 5.7	
PEG Mw 300	<<1 sec		49.4 ± 7.2
PEG Mw 200	1 min	15.6 ± 5.4	34.3 ± 5.4
DMSO	15 min	59.6 ± 7.3	87.0 ± 4.1
1.2-propanediol	2 min	78.6 ± 4.6	92.4 ± 2.1
1.2-butanediol	<<1 sec	15.4 ± 3.6	41.4 ± 3.4
1.3-butanediol	4 min	60.7 ± 5.2	89.3 ± 5.4
ethyleneglycol	1 min	61.6 ± 7.5	50.7 ± 10.6

15 minutes). The other cryoprotectants entered within 4 minutes where PEG Mw 300 and 1.2 butane-diol entered the cell almost instantaneously. 1.2 propane-diol, ethylene-glycol, glycerol, 1.3 butane-diol and DMSO were the least toxic. Ethylene-glycol gave the best results with *A. superba*; the best results with *D. gampsospora* were obtained with 1.2 propane-diol. Most toxic were both types of PEG and 1.2 butane-diol. The slower permeating 1.3-butane-diol and PEG Mw 200 were less toxic then the faster entering 1.2 butane-diol and PEG Mw 300. We have not yet been able to obtain survival data for *D. gampsospora* with PEG Mw 300 but preliminary results showed rather low survival rates. Ethanol and methanol were so toxic that they were excluded from further experiments. When cells were washed in 1.2 M sucrose viability increased substantially (Tables 4-5). With *A. superba* survival doubled for DMSO and 1.3-butane-diol while it tripled for the toxic PEG Mw 200 and 1.2-butane-diol. In *D. gampsospora*, increase in viability was observed with all cryoprotectants with the exception of ethylene-glycol.

Since ethylene-glycol and 1.2 propane-diol were the least toxic, a vitrification solution was prepared with these compounds (loading solution: 0.5 M 1.2-propane-diol, 0.5 M ethylene-glycol, 0.6 M glycerol, 0.4 M sucrose; vitrification solution: 2.5 M 1.2 propane-diol, 2.5 M ethylene-glycol, 3 M glycerol, 0.4 M sucrose (CBS-solution)). Glycerol was added to stimulate dehydration of the cells while sucrose was added to reduce toxicity. This vitrification medium was tested together with PVS2 (10) (loading solution: 0.38 M DMSO, 0.5 M ethylene-glycol, 0.65 M glycerol, 0.4 M sucrose; vitrification solution: 1.9 M DMSO, 2.5 M ethylene-glycol, 3.25 M glycerol, 0.4 M sucrose), and the vitrification solution of Steponkus (12) (loading solution: 1.75 M ethylene-glycol; vitrification solution: 7 M ethylene-glycol, 0.88 M sorbitol, 6% (w/v) lactalbumin). Vitrification versus slow cooling at -1°C/min (cryoprotectant 10% glycerol) was compared for *A. superba*. Spores suspensions were vitrified according to the method of Reinhoud (9). Viability after vitrification was estimated by scoring germination percentage for 8 x 100 spores (Table 6). Better results were obtained with vitrification than with slow cooling for all the solutions tested. Because viability after slow cooling varied, we were not able yet to establish which vitrification solution gave the best survival.

In conclusion 12% skimmed milk/7% trehalose is the best lyoprotectant for fungi. More experiments are needed to establish the optimal vitrification solution.

Table 6. Vitrification of *Arthrobotrys superba* versus cooling at -1°C/min in 10% glycerol $n = 8$.

Protectant	cooling rate	% spores germinating	% spores alive
CBS	vitr.	80.7 ± 4.7	17.1 ± 4.1
glycerol	-1°C/min	68.1 ± 3.1	6.1 ± 2.6
PVS	vitr.	97.1 ± 1.9	1.4 ± 0.9
glycerol	-1°C/min	74.7 ± 3.4	2.5 ± 1.1
Steponkus	vitr.	0.5 ± 0.8	91.2 ± 1.3
glycerol	-1°C/min	3.3 ± 1.3	43.9 ± 2.8

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