

# Yeast Exoglycoproteins Produced Under NaCl-Stress Conditions as Efficient Cryoprotective Agents

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## ABSTRACT

Six extracellular yeast glycoproteins were prepared from three yeast species in osmotic equilibrium and unequilibrium environments and used as non-penetrating cryoadditives. Glycoproteins secreted by the strain *Dipodascus australiensis* into growth medium containing NaCl (8% w/v) were found to be the most effective cryoadditives. It was possible to use these glycoproteins alone (without DMSO as penetrating agent) for the cryoprotection of the studied yeasts.

## INTRODUCTION

Many factors that affect the successful freeze preservation of yeast cells have been documented (1). The crucial problem during preservation is to ensure genetic stability. The viability of the cells after preservation may correlate with the extent of protection against dehydration stress and with the transition of water to ice crystals (2,3). Survival and stability of all strains preserved in liquid nitrogen (-196°C) is always higher in the presence of a mixture of penetrating and non-penetrating cryoprotective agents (4) than in the presence of non-penetrating cryoagents only (5). The basic feature of non-penetrating cryoprotective agents is the ability of their hydrophilic groups to bind water, giving rise to a phenomenon often described as "bound water" (6). In higher cells, glycoproteins have various functions such as surface receptors, cell-cell mediators, components of the extracellular matrix, cryoprotectors, etc. The protein moiety of most glycoproteins is the functional part, while the carbohydrate moiety contributes to the attainment of an adequate tertiary structure and modifies the glycoprotein molecule making it more resistant to degradation, and facilitates its secretion (7).

This paper describes the cryoprotective activity of six extracellular yeast glycoproteins prepared from three yeast species cultivated under two different conditions.

## MATERIALS AND METHODS

### Yeast strains

The following psychrophilic species were used: *Candida graminis* CCY 29-133-1, *Candida capsuligena*

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CCY 29-143-1, *Leucosporidium scottii* CCY 64-1-1, *Phaffia rhodozyma* CCY 77-1-1, *Leucosporidium antarcticum* CCY 64-3-1, *Candida diffluens* CCY 29-132-1, *Sympodiomyces parvus* CCY 73-1-1, *Dioszegia hungarica* CCY 18-1-1, *Candida frigida* CCY 29-144-1, *Candida gelida* CCY 29-121-1. All yeast strains were obtained from the Culture Collection of Yeasts (Institute of Chemistry, SAS, Bratislava, Slovakia).

### **Basal cryoprotective medium**

Basal cryomedium consisted of 7g wort extract, 0.3g yeast extract (Difco), 0.5g peptone (Difco) per 100 ml of tap water. The medium was three times sterilized by heating at 100°C for 1h on three separate days.

### **Cryoadditives**

After sterilization of the basal cryomedium, DMSO (Fluka, Switzerland) was added as a penetrating cryoagent to give a final concentration of 10% (v/v) in the first series of cryomedia. The second series was prepared without DMSO. All cryomedia contained the crude ethanol precipitates of the different extracellular yeast glycoproteins of three yeast species. These non-penetrating cryoagents were added before sterilization into cryomedia to give a final concentration of 0.27%.

### **Cultivation conditions for preparation of the extracellular yeast glycoproteins**

Control condition optimal mineral cultivation medium with 2% glucose contained per l: 4g yeast extract, 10g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1g KH<sub>2</sub>PO<sub>4</sub>, 0.2g K<sub>2</sub>HPO<sub>4</sub>, 0.1g NaCl, 0.1g CaCl<sub>2</sub>, 0.5g MgSO<sub>4</sub>, and 1 ml microelements solution (1.25g H<sub>3</sub>BO<sub>3</sub>, 0.1g CuSO<sub>4</sub>, 0.25g KI, 1g MnSO<sub>4</sub>, 0.5g FeCl<sub>3</sub>, 0.5g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 1g ZnSO<sub>4</sub> contained per l). The optimal cultivation temperatures used were 28°C for *Dipodascus australiensis* and 17°C for *Waltomyces kononenkoe* and *W. lipofer* (Table 1). The cells were precultured aerobically in the test tubes to the stationary phase of growth and then cultivated on a reciprocal shaker to the stationary phase in 1 l flasks with 500 ml of optimal mineral medium. After centrifugation crude extracellular glycoproteins were isolated by precipitation from the supernatant by adding two volumes of 96% (v/v) ethanol and subsequent centrifugation. The ethanol precipitate was dissolved in distilled water, dialyzed against distilled water and freeze-dried.

To produce the stress conditions, 4% (*W. kononenkoe*, *W. lipofer*) and 8% NaCl (*D. australiensis*), was added to the control medium.

### **Preservation in liquid nitrogen**

The cell suspension (0.1 ml, 10<sup>7</sup> - 10<sup>8</sup> cells in 1 ml) and cryoprotective medium (0.5 ml) were pipetted into sterile 2-ml polyethylene ampoules (Koh-i-noor, Dalecin), and rapidly frozen in liquid nitrogen (no later than 1h after pipetting). Three ampoules were used for each strain as well as cryomedium. Cultures had been stored for one year. After 1 year of storage in liquid nitrogen, the cultures were thawed in a 37°C water bath for 30 min. opened, and recultivated.

## Survival of the storage culture

Qualitative evaluation was performed by visual screening of growth on wort agar after spreading. Growth was evaluated by 1-3 crosses for the culture grown taking into account optimal time of growth (1 cross = weak growth, 2 crosses = medium growth, 3 crosses = good growth); the cultures could maximally reach 3 crosses = 100 %.

**Table 1.** Designation of the glycoproteins described in the paper

Strains		Glycoprotein designation		
Name	No.	% NaCl in cultivation medium		
	CCY	0	4	8
<i>Dipodascus australiensis</i>	52-6-1	G <sub>1</sub>	NG	G <sub>2</sub>
<i>Waltomyces kononenkoe</i>	33-4-2	G <sub>3</sub>	G <sub>4</sub>	NG
<i>Waltomyces lipofer</i>	33-2-4	G <sub>5</sub>	G <sub>6</sub>	NG

NG, not grown.

## RESULTS AND DISCUSSION

The six crude glycoproteins were tested to determine their effect on the survival of the 10 psychrophilic yeast strains stored in liquid nitrogen.

The strains could be divided into three groups on the basis of their survival rates in the basal medium containing DMSO (8) (Figs. 1, 2). The strains survived only to a limited extent (to 4%) or did not survive in the basal medium without any cryoaddivitive.

Group I comprised strains with the highest survival rates ( $20 \pm 6\%$ ). These strains were typical psychrophilic species. Each of them produced some protective agent such as carotenoid pigment or slime. The most suitable glycoprotein cryoprotectants were G<sub>2</sub>, G<sub>4</sub> and G<sub>6</sub> produced under NaCl-induced stress conditions. Without DMSO (Figs. 1, 2) the most effective cryomedium was the one containing The average survival rate was about 75%.

Group II comprised four strains with lower survival rates (about  $15 \pm 5\%$ ). The strains of *D. hungarica* and *C. gelida* survived somewhat, while *C. graminis* and *L. scottii* survived to the lesser extent.

Group III comprised the most cryosensitive strains (survival rates 0 - 5%).

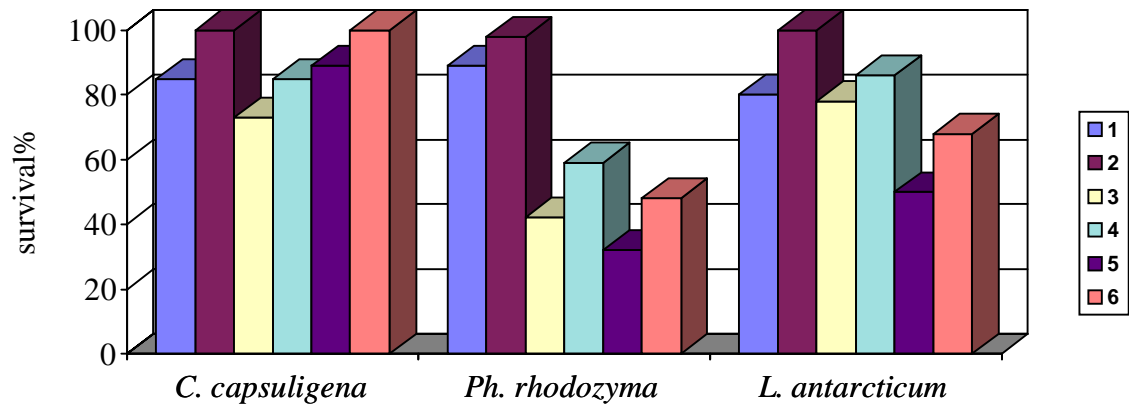
In the DMSO-free cryomedium only small differences in the percentage of survival when using various glycoproteins were observed. The glycoproteins produced by the strains of *D. australiensis* under stress conditions (8% NaCl in the cultivation medium) had the highest influence on the cryosensitive psychrophilic yeast species (Figs. 1, 2). The glycoprotein G<sub>2</sub> was the most effective for all strains. The order of the effectivity of glycoproteins was  $G_1 > G_6 > G_5 > G_3 > G_4$ .

The extracellular yeast glycoproteins produced by the genus *Waltomyces* (G<sub>3</sub>, G<sub>4</sub>, G<sub>5</sub> and G<sub>6</sub>) were not very effective as cryoprotective agents. The ability of these cultures to grow at the higher concentration of NaCl is probably dependent on the structure of the glycoproteins produced. During freezing, the

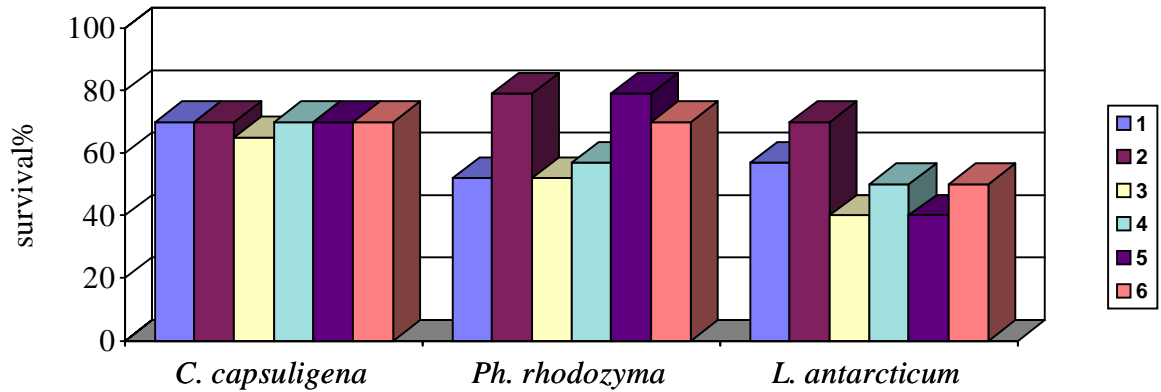
residual solution becomes hypertonic with regard to the intracellular compartment, and thus the cells are subjected to a fast decrease of osmotic pressure. The glycoproteins isolated from the 8% NaCl medium were efficient as cryoprotective agents.

The results of the study of the survival rates will be used to develop experimental protocols that minimize osmotic stress without the penetrating cryoprotectant DMSO. The application of the above results to another type of eucaryotic cells has also been described (9).

## Group I (with DMSO):



## Group I (without DMSO):



## Group II (with DMSO):

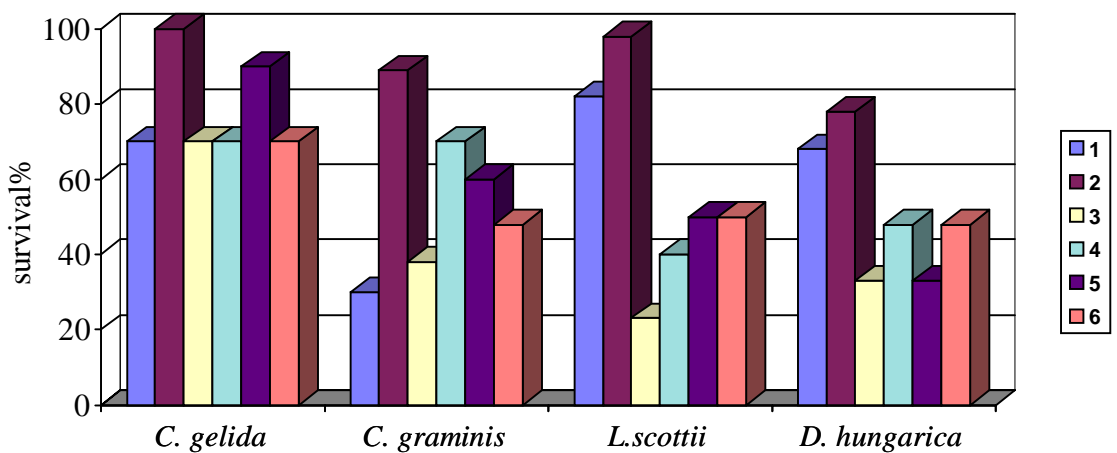
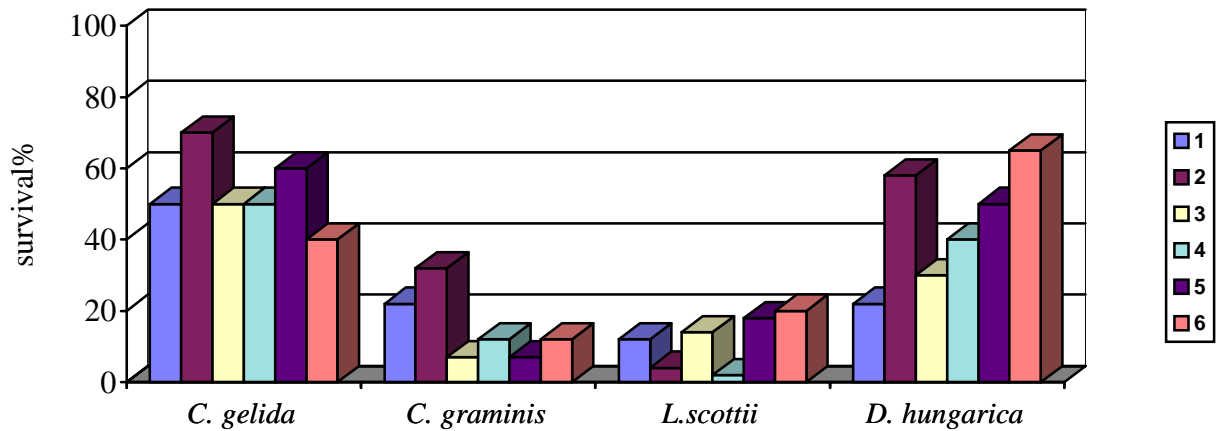
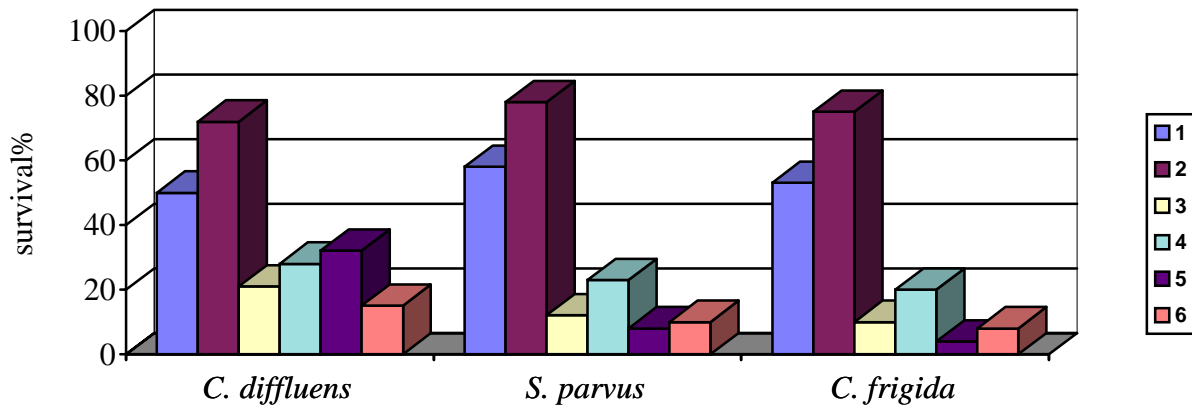


Fig. 1. Survival of the strains tested depending on the combination of basal medium (BM) and nonpenetrating cryoagents with and without DMSO. 1, BM+G<sub>1</sub>; 2, BM+G<sub>2</sub>; 3, BM+G<sub>3</sub>; 4, BM+G<sub>4</sub>; 5, BM+G<sub>5</sub>; 6, BM+G<sub>6</sub>. Data represent the mean percentage from three independent experiments

## Group II (without DMSO):



## Group III (with DMSO):



## Group III (without DMSO):

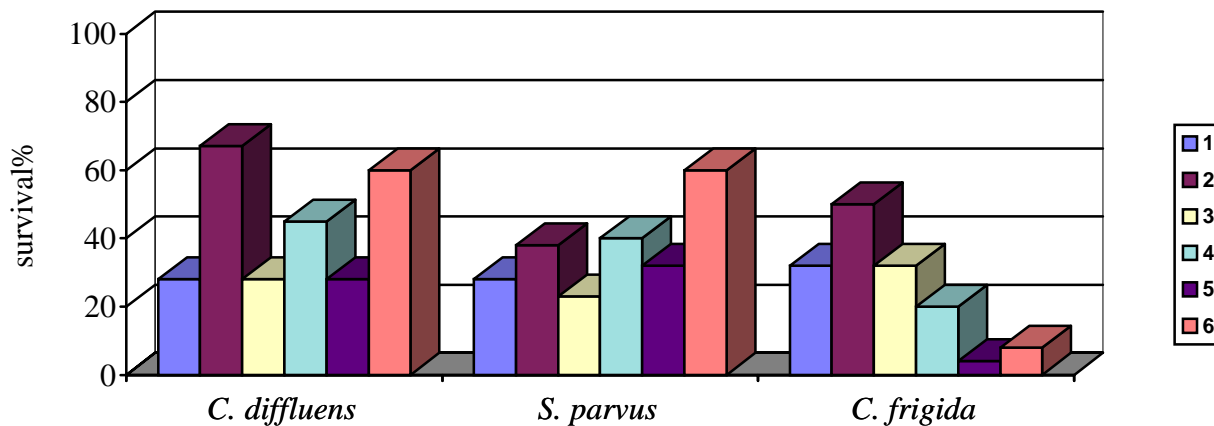


Fig. 2. Fig. 1 cont... Survival of the strains tested depending on the combination of basal medium (BM) and nonpenetrating cryoagents with and without DMSO. 1, BM+G<sub>1</sub>; 2, BM+G<sub>2</sub>; 3, BM+G<sub>3</sub>; 4, BM+G<sub>4</sub>; 5, BM+G<sub>5</sub>; 6, BM+G<sub>6</sub>. Data represent the mean percentage from three independent experiments

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