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## SHORT COMMUNICATIONS

# Optimization of Protective Media for Actinomycetes Storage in Liquid Nitrogen

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The problem of long-term preservation of microbial cells includes a series of issues concerning the selection of optimal conditions for cell protection from damage during storage.

The preservation of cell viability, as well as of taxonomically or practically important properties of various species of actinomycetes is associated with some problems due to their high level of genetic instability. It has been established that storage in liquid nitrogen has no destabilizing effect on the genomes of these microorganisms [1]. Cryoprotective compounds play an important role in this method of storage [2–4]. Glycerol and dimethyl sulfoxide (DMSO) are the best known and most widely used cryoprotective agents employed for cryoconservation of microorganisms [5]. Heavy water ($D_2O$) is also used for cryoconservation of various biological objects, including microorganisms [6].

The formation of resting microbial cells is one of the mechanisms of natural resistance to unfavorable conditions. The state of metabolic rest is most pronounced in bacterial endospores. The formation of resting bacterial cells is accompanied by the synthesis of a number of regulatory compounds that control the process of cell transition to anabiosis, the state of metabolic rest. These compounds include such anabiosis autoinducers as factor $d_1$ in some microbial species, represented by alkylhydroxybenzenes (AHB) [7]. These compounds, which function as intracellular protective agents, have not yet been used for the development of protective mechanisms; their application shows considerable promise.

The purpose of this work is to study the cryoprotective effect of various low-molecular weight compounds with protective features during the storage of actinomycete strains in liquid nitrogen.

Viability indices and changes in the culture population composition (dissociant percentages) were used as the main criteria of cryoprotectant efficiency during storage.

The following strains were used as objects of study: *Streptomyces ruber* K-3946, proposed as a neotype [8] (= VKM Ac-611=ATCC 43916), *Mycobacterium phlei* K-4140$^T$ (= VKM Ac-1291), and *Rhodococcus rhodochrous* K-3285 (= RIA 408 = VKM Ac-1283). The cultures were grown on an agarized oatmeal medium supplemented with 0.25% yeast extract, as well as on CP1 medium with glucose [9]. Water solutions of glycerol (REAKHIM, Russia) (15% vol/vol), DMCO (3% and 10%, vol/vol-) [5], and $D_2O$ (10% and 20% vol/vol) (kindly offered by Dr. D.A. Skladnev), as well as 4*n*-hexylresorcinol (HR), a chemical analogue of bacterial factor $d_1$ (Sigma), were used as protective media during the cryoconservation of these microorganisms. The latter compound was introduced in the form of a solution in 3% DMSO (vol/vol) to the final concentrations of 0.01%, 0.005%, 0.001%, and 0.0001% (wt/vol).

The 7–10-day cultures of *S. ruber,* as well as the 3–5-day cultures of *M. phlei* and *R. rhodochrous* grown on oatmeal agarized medium and resuspended in sterile tap water, were filtered through sterile cotton-wool filters and placed in 1.5-ml polypropylene vials with screw tops (Nalgene).

The efficiency of the protective compounds during the culture freezing and thawing was tested using a model suspension of *S. ruber* cells. The suspension was placed in vials and put into Dewar flasks with liquid nitrogen (10 min). Then the activation of microbial cells was carried out at room temperature. To study the cryoprotective effect of the media on actinomycetes in the course of long-term storage, the vials with cell suspensions were placed in special containers (Nalgene) and stored in liquid nitrogen for three months. The quantitative assessment of cell viability was performed by inoculation of the suspensions on the agarized oatmeal medium with the subsequent counting of colony-forming units (CFU). Within the population, the population heterogeneity level was determined from the ratio (%) of colonies that have the morphological properties of the dominant and minor types. In the case of *S. ruber,* all variants were classified according to the literature data on the population composition of this microorganism [8]. The dissociant forms of *M. phlei* and *R. rhodochrous* were differentiated by their morphological and cultural properties according to

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**Table 1.** Effect of the protective medium composition on the survival of the studied actinomycete cultures after three-month storage in liquid nitrogen

| Protective medium | Actinomycete culture | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. ruber* K-3946 | | | *M. phlei* K-4041 | | *R. rhodochrous* K-3285 | |
| | Survival | | | | | | |
| | before storage (CFU, cells/ml) | after freezing (%) | fter storage (%) | before storage (CFU, cells/ml) | after storage (%) | before storage (CFU, cells/ml) | after storage (%) |
| Glycerol (15%) | $(3.2 \pm 0.3) \times 10^9$ | 100 | 70.2 | $(2.3 \pm 0.2) \times 10^{10}$ | 64.8 | $(1.5 \pm 0.1) \times 10^{11}$ | 18.2 |
| $D_2O$ (20%) | $(1.0 \pm 0.2) \times 10^9$ | 76.3 | 16.1 | $(2.0 \pm 0.2) \times 10^{10}$ | 46 | $(1.0 \pm 0.1) \times 10^{10}$ | 100 |
| DMSO (10%) | $(1.2 \pm 0.1) \times 10^9$ | 92 | 25.6 | $(2.8 \pm 0.3) \times 10^{10}$ | 100 | $(1.2 \pm 0.1) \times 10^{11}$ | 33 |
| DMSO (3%) + 0.01% HR | $(3.2 \pm 0.4) \times 10^8$ | 33 | 10 | $(1.0 \pm 0.1) \times 10^{10}$ | 0.12 | $(1.3 \pm 0.1) \times 10^{11}$ | 0.6 |
| DMSO (3%) + 0.005% HR | $(4.3 \pm 0.5) \times 10^8$ | 32 | 23 | $(1.5 \pm 0.1) \times 10^{10}$ | 0.91 | $(1.5 \pm 0.1) \times 10^{11}$ | 0.18 |

[10–12]. The results were statistically examined. For each value, a confidence interval was determined. Sets of the data obtained were considered homogeneous if the standard deviation did not exceed 11%. The level of the confidence interval was assumed equal to 0.05% ($p < 0.05$).

The selection of efficient concentrations of protective compounds at the freezing and thawing stages was performed using the model *S. ruber* K-3946. As a result, we selected the following concentrations of protectants for further investigation of the medium-term storage (3 months) of actinomycetes: 15% glycerol solution, 10% DMSO, and 20% $D_2O$. These concentrations provide for a sufficiently high percentage of surviving cells and do not affect the population variability. Moreover, in our study, we used a composition based on 3% DMSO supplemented with 0.01% or 0.005% HR due to the narrowing of the spectrum of the streptomycete population variability. It should be noted that a decrease in the HR concentration in the protective medium to 0.001–0.0001% reduced the toxic effect on *Streptomyces* cells; however, the effect of stabilizing the population variability practically disappeared.

Table 1 shows the results of our experiments performed on *S. ruber* K-3946, *M. phlei* K-4041, and *R. rhodochrous* K-3285 after storage in liquid nitrogen in the media supplemented with selected protectants. The numbers of viable cells of *S. ruber* K-3946 decreased drastically during storage in all the protective media, except for 15% glycerol solution. In the case of 3% DMSO supplemented with 0.005% HR, the sharp decrease in the *S. ruber* K-3946 viability occurred mainly at the stage of mixing the cells with protective medium, whereas, during the three-month storage, the numbers of viable cell stabilized, and the number of CFU decreased by only 9% as compared to the number of CFU during the freezing–thawing experiments. The viability of *M. phlei* K-4041 after storage for three months was maximal in a 10% solution of DMSO and a 15% glycerol solution; the viability of *R. rhodochrous* K-3285 was the highest during preservation in 20% $D_2O$. The sharpest decrease in the viability of mycobacteria and rhodococci was observed when we used complex media based on a 3% solution of DMSO supplemented with 0.01% or 0.005% HR as cryoprotectants (Table 1).

Table 2 shows the effect of various protective media on the population variability of *S. ruber* K-3946 as a result of three-month storage in liquid nitrogen. The stability of the dissociant percentages of streptomycetes was highest during storage in 15% glycerol, as well as in media supplemented with 3% DMSO and HR (0.01% or 0.005%). The obtained population of streptomycetes consisted almost completely of dominant colonies. A marked increase in the population variability of *S. ruber* K-3946 after storage in liquid nitrogen was observed when using 20% $D_2O$.

The dissociant types were detected in populations of *M. phlei* K-4041 and *R. rhodochrous* K-3285 and identified on the basis of their characteristic colony structure and pigmentation parameters. The *M. phlei* K-4041 population was represented by three morphological types of colonies. The dominant colonies were convex with uneven edges, wrinkled, dry, and yellowish. The pale colony type included small colonies with elevated uncolored centers. The dwarf type included small (about 2 mm in diameter), elevated, finely wrinkled colonies. The *R. rhodochrous* K-3285 population included

**Table 2.** Effect of the protective medium composition on the population variability of *S. ruber* K-3946 after three-month storage in liquid nitrogen

| Protective medium | Population composition (variants), % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | before storage | | | | | after storage | | | | |
| | basic | pale | white | nonspore-forming red | nonspore-forming yellow | basic | pale | white | nons-pore-forming red | nons-pore-forming yellow |
| Glycerol (15%) | 99.8 | 0.1 | – | 0.1 | – | 99.0 | – | 0.45 | 0.55 | – |
| $D_2O$ (20%) | 99.0 | 0.1 | 0.1 | 0.8 | – | 83.5 | 1.5 | 8.5 | 6.45 | 0.05 |
| DMSO (10%) | 88.1 | 2.5 | 7.5 | 1.9 | – | 89.0 | 3.5 | 3.5 | 4.0 | – |
| DMSO (3%) + 0.01% HR | 98.5 | 1.5 | – | – | – | 98.0 | – | 1.5 | 0.5 | – |
| DMSO (3%) + 0.005% HR | 99.9 | – | – | 0.1 | – | 99.8 | – | 0.1 | 0.1 | – |

**Table 3.** Effect of the protective medium composition on the population variability of *M. phlei* K-4041 and *R. rhodochrous* K-3285 after three-month storage in liquid nitrogen

| Protective medium | Population composition of *M. phlei* K-4041 (%) | | | | | | Population composition of *R. rhodochrous* K-3285 (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Before storage | | | After storage | | | Before storage | | | After storage |
| | basic | light-colored | dwarf | basic | light-colored | dwarf | basic | dwarf | basic | dwarf |
| Glycerol (15%) | 100 | – | – | 93.2 | 6.8 | – | 100 | – | 100 | – |
| $D_2O$ (20%) | 100 | – | – | 96.3 | 3.4 | 0.3 | 100 | – | 95.8 | 4.2 |
| DMSO (10%) | 100 | – | – | 97.6 | 2.3 | 0.1 | 100 | – | 96.2 | 3.8 |
| DMSO (3%) + 0.01% HR | 100 | – | – | 97.4 | 2.6 | – | 100 | – | 98.6 | 1.4 |
| DMSO (3%) + 0.005% HR | 100 | – | – | 98.0 | 2.0 | – | 100 | – | 98.5 | 1.5 |

two types of colonies. The dominant colonies were smooth with even edges and bright pink coloration; the dwarf colonies were small and pale.

After storage of *M. phlei* K-4041 in various protective media, minor variants were observed; however, the numbers of dominant colonies in all experiments were high (Table 3). The application of 15% glycerol solution as a protective medium for *R. rhodochrous* K-3285 storage was found to be optimal for maintaining the phenotype homogeneity of the strain population (Table 3).

Hence, the results obtained in our experiments have shown the selectivity of the cryprotective effect of various protective media on actinobacteria belonging to various taxonomic groups during their storage in liquid nitrogen. It was established that the following solutions had a stabilizing effect that promotes cell survival and reduces the population variability during storage: 15% glycerol and 3% DMSO supplemented with 0.005% 4*n*-hexylresorcinol (for *S. ruber* K-3946), 10% DMSO (for *M. phlei* K-4041), and 20% $D_2O$ (for *R. rhodochrous* K-3285).

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