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EXPERIMENTAL ARTICLES

Long-Term Storage of Industrial Microbial Strains

A. A. Tsutsaeva[a†], A. E. Anan'ina[a,1], L. M. Balyberdina[a], L. V. Stepanyuk[a], and N. V. Pavlenko[b]

[a] *Institute for Problems of Cryobiology an Cryomedicine, National Academy of Sciences, Pereyaslavskaya, 23, Kharkov, 61015 Ukraine*

[b] *Kharkov State Medical University*

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Abstract—Morphofunctional characteristics of industrial microorganisms belonging to different genera, species, and strains were investigated after 15 to 20 years of storage in liquid nitrogen. The taxonomic position of microorganisms, the cell physiological state prior to storage, and the cryoconservation regime were found to affect microbial cryoresistance. Protective media, density of cell suspensions, freezing rate, and heating temperature are the parameters important for development of efficient technologies for cryoconservation of industrial microorganisms at –196°C.

Key words: industrial microbial strains, cryoconservation, storage, morphofunctional characteristics, productive activity.

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Storage of collection and industrial strains, apart from loss of viability, results in the processes of population variability. The dominant phenotype is replaced by another one, different in its characteristics and productive activity; thus, the priority features of the strains are lost. Reliable technologies for long-term storage of collection, industrial, and reference cultures for various microbial genera, species, and strains are therefore required.

Cryoconservation, transfer of biological objects to deep cold anabiosis (–196°C) with subsequent restoration of their metabolic activity under optimal culture conditions, is an efficient method for long-term storage of microorganisms [1–5]. The efficiency of maintenance of viability and productive characteristics depends on the methods of its transfer to and removal from the state of deep cold anabiosis [5]. Individual efficient cryoconservation technologies are therefore developed for various genera, species, and strains of microorganisms in order to ensure the maximal amount of viable microbial cells retaining their original characteristics [6, 7]. Low-temperature banks for storage of various taxonomic groups of microorganisms are widely used [8–10].

The goal of the present work was to investigate the characteristics of industrial microbial strains after long-term (15–20 years) storage in liquid nitrogen (–196°C).

MATERIALS AND METHODS

Industrial strains used in this work were obtained from the institutes of the USSR Ministry of Medical and Microbiological Industry. They belonged to the genera *Streptomyces* (species: *S. aureoverticillus, S. aureofaciens, S. griseus*, and *S. fradiae*), *Bacillus* (species *B. thuringiensis* subsp. *galleriae*), *Saccharomyces* (species *S. cerevisiae*), *Streptococcus* (species *Str. lactis, Str. diacetilactis*, and *Str. cremoris*), *Bifidobacterium* (species *B. adolescentis, B. longum, B. bifidum* LBA-3, and *B. bifidum* 1), *Anabaena* (species *A. variabilis),* genera *Synechocystis, Nostoc*, *Spirulina* (*S. platensis*) and bacteriophages T3, T4, and φX174.

The microorganisms have been stored in liquid nitrogen at –196°C for 15 to 20 years. Cultural, morphological, and biochemical techniques were used to assess the preservation of characteristics of the cultures. Productive activity was determined by comparisons of the values obtained after storage with those for the cryoconserved cultures defrosted without storage. The number of viable cells was determined by plating serial dilutions on agarized media with subsequent microcolony counting (CFU) [11].

Acid production by lactic acid streptococci and bifidobacteria were determined according to [12]; insecticidal activity of *B. thuringiensis* subsp. *galleriae* was determined as in [13]; antibiotic activity of streptomycetes was determined as in [14]; enzymatic activity in yeasts, according to [15]; proliferative activity and biomass accumulation by cyanobacteria, according to [16]; intracellular life cycles of bacteriophages and their adsorption rate to bacteria were determined as in [17].

† Deceased.
[1] Corresponding author; e-mail: cryo@online.kharkov.ua

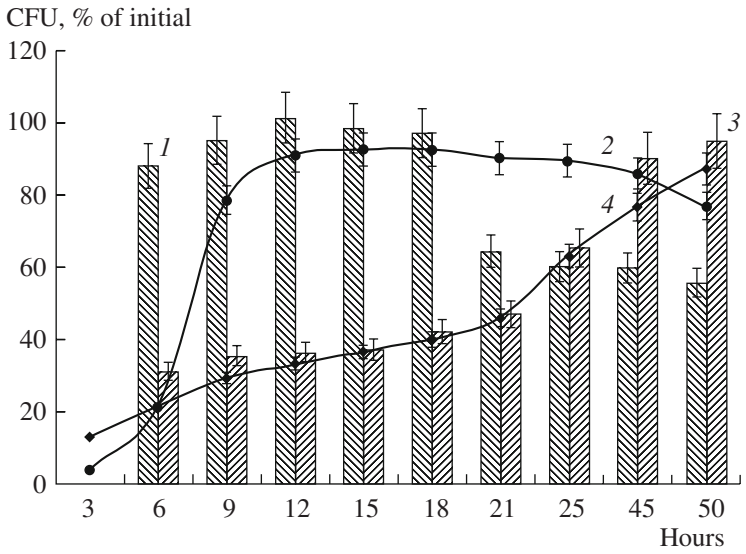

Fig. 1. Numbers of viable cells of *Bifidobacterium bifidum* 1 (*1*) and *Bacillus thuringiensis* (*3*) after cryoconservation of material collected at different growth phases of *Bifidobacterium bifidum* 1 (*2*) and *Bacillus thuringiensis* (*4*).

Cell suspensions were frozen in metal containers (1 and 10 ml) hermetically sealed with Teflon screw caps (manufactured in the Institute for Problems of Cryobiology an Cryomedicine, National Academy of Sciences of Ukraine, Kharkov).

The samples were frozen in the freezing chamber of a UOP-6 programmed freezer (manufactured in the Institute for Problems of Cryobiology an Cryomedicine, National Academy of Sciences of Ukraine, Kharkov) within the range from 18–20 to –196°C at different cooling rates.

Heating and reactivation (deconservation) were carried out in a defroster designed as a water bath with a shaker. The optimal defrosting temperatures have been previously experimentally determined for each microbial strain by heating frozen but not stored cultures.

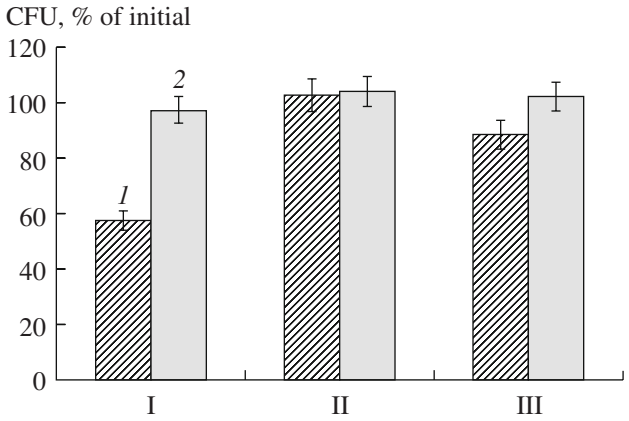

Fig. 2. Number of *Streptococcus cremoris* viable cells after cryoconservation of cell suspensions of 10^8 cell/ml (*1*) and 10^{10} cell/ml (*2*) in protective media: physiological saline (I), growth medium (II), and recovered milk (III).

Storage of the samples frozen under optimal modes was carried out in XB-05 low-temperature repositories at –196°C [4, 5, 9] with monitored liquid nitrogen levels.

Statistical treatment of the data was carried out according to accepted procedures [18].

RESULTS AND DISCUSSION

In development of the technological process for cryoconservation, the following factors were considered: original characteristics of microorganisms, growth phase of the culture, cell concentration in suspensions, composition of conservation media, cooling rates, and heating temperatures.

Efficient cryoconservation technologies have been developed for each genus, species, and strain of industrial microorganisms; these technologies enabled cryoconservation and prolonged storage of microorganisms [5, 9].

Microbial cryoresistance was found to depend on the growth phase. Most of the organisms studied had high cryoresistance in the stationary growth phase (Fig. 1) [5].

Cryoresistance of most microorganisms increased with their concentration in the conservation medium; lactic acid streptococci may serve as an example (Fig. 2). Increased concentration of cyanobacteria in suspensions did not result in increased number of viable cells after cryoconservation.

For most of the studied microorganisms, fresh growth medium was the optimal environment for cryoconservation (Fig. 2).

High cooling rates (300–400°C/min) were found to be optimal for entomopathogenic bacteria, lactic acid streptococci, spore cultures of streptomycetes. After freezing these organisms, the ratio of viable cells was 53–90%. Low and medium cooling rates (0.1–0.4°C/min; 1–4°C/min; 10–40°C/min) were optimal for bifidobacteria, vegetative streptomycete cells, bactreiophages (T4 and φX174), and bakery yeast strains. After cooling, the ratio of viable cells was 50.4–91.3% of the control values.

Spore-forming microorganisms exhibited the highest cryoresistance; the number of colony-forming units was maximal when spore suspensions were frozen within a broad range of cooling rates. Unlike spores, vegetative microbial cells were less cryoresistant. Low cooling rates (0.2–4.0°C/min) were optimal for vegetative cells of *B. thuringiensis*, streptomycetes, bakery yeast, two bifidobacteria species, and asporogenic microalgae. Heating temperature also affected survival of cryoconserved microorganisms. For example, while low heating temperatures (1.5–4.1°C) were optimal for bacteriophages, the optimal heating temperature for bacteria and yeasts was 30–41°C.

Differences in cryoresistence were revealed not only between microbial species, but between strains as well. Cryoconservation in the optimal mode resulted in a reliable decrease of viable cell numbers of some yeast strains *S. cerevisiae* (no. 14 and Tomskaya-7); only 22% of viable cells survived after heating. Cryoconservation of the yeast strain no. 608 resulted in 90% viable cells. Streptomycetes also exhibited strain differences. Although high cooling rates provided for the best survival of streptomycete spores, a medium cooling rate of 10°C/min was optimal for one of the six studied *Streptomyces fradiae* industrial strains.

Bacteriophage strains also differed in cryoresistance. Phage T3 was the most resistant to cryoconservation; phage T4 was the most cryolabile; phage φX174 occupied an intermediate position.

Cryoconservation under optimal conditions caused nonlethal damage to microbial cells; this damage was not affected by storage duration and was repaired after heating. Nonlethal damage is expressed as increased duration of the exponential growth phase of reactivated microorganisms, elongation of the intracellular assembly cycle of bacteriophages (Fig. 3), increased number of growth centers resulting from fragmentation of microalgal filaments and streptomycete hyphae, fragmentation of streptococci chains, etc. In some streptomycete strains, antibiotics synthesis was reversibly stimulated, in some yeast strains, enzyme formation, and in cyanobacteria, chlorophyll synthesis. Reversible inhibition of nucleic acid and protein synthesis immediately after heating, as well as inhibition of bioenergetic processes and synthesis of cryostress proteins have been shown to be the cause of nonlethal damage [5].

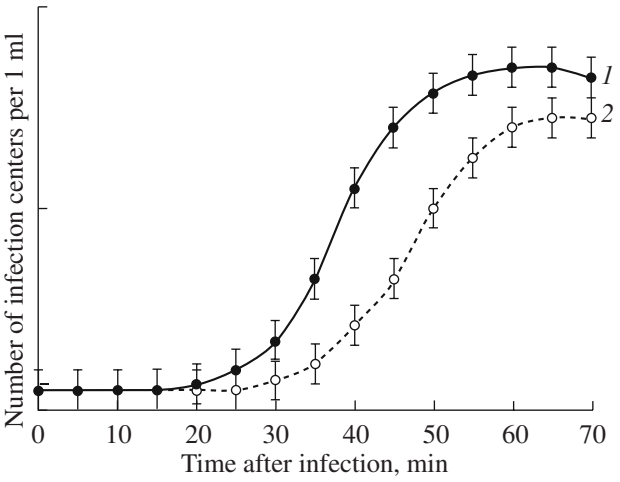

Fig. 3. Graphs of a single growth cycle of bacteriophage T4 before (*1*) and after cryoconservation (*2*).

No additional deterioration of the characteristics of microbial cultures was observed after storage for 15 to 20 years in liquid nitrogen.

Thus, the optimized procedures for cryoconservation with subsequent storage in liquid nitrogen at −196°C ensured the preservation of microbial characteristics for 15–20 years.

Our results demonstrate that not only taxonomically different microorganisms, but even strains of one species can differ significantly in cryoresistance, although the morphofunctional and cultural characteristics, as well as metabolic activity, are similar within a species [5, 19, 20]. The characteristics determining strain specificity usually have no effect on cryoresistance. Microbial cryoresistance understood from the viewpoint of traditional cryobiology is therefore a feature that can be used as an additional characteristic of a strain. Elucidation of the reasons for different cryoresistance of the strains will improve our understanding of the factors and mechanisms determining cryoresistance in biological objects [19]. Cryoconservation of microorganisms results in certain changes of their cells; their consequences are revealed during the first transfer after deconservation as changes in the number of viable units and inhibition or stimulation of antibiotic or enzyme production. High preservation of the viable cell titer (CFU) and their productive activity are the main criterion for the choice of the technological regulations for long-term cryoconservation of microbial producers of biologically active compounds [20].

Prior to long-term storage, the cryoconservation technological processes efficient for every individual microorganism should be developed in order to preserve the highest possible number of viable cells with their initial characteristics unaffected. Efficient technologies for cryoconservation of microorganisms of different taxonomic groups have been developed; they

are used for biotechnological processes. After storage for 15–20 years, no reliable decrease was found in the number of viable cells; their morphofunctional, cultural, and biochemical characteristics and productive activity did not differ from those observed in the same cultures heated immediately after freezing.

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