JOURNAL OF CULTURE COLLECTIONS
Volume 4, 2004-2005, pp. 17-28

ANABIOSIS AND CONSERVATION OF MICROORGANISMS

Tsonka Uzunova-Doneva* and Todor Donev

National Bank for Industrial Microorganisms and Cell Cultures, 1113 Sofia,
P.O.Box 239, Bulgaria; e-mail: donevi@cablebg.net

Summary

A literature review is made about the applied methods for microorganisms preservation in the collection activity. Two groups of conservation methods are described – in hypobiotic and anabiotic states. The first group aims reducing to minimum the cell vital activity by storage under mineral oils, in water and water solutions, inactivation in dryers etc. The second group methods lead the microorganisms to an anabiotic state by means of freezing and low temperature storage and by freeze-drying (lyophilization). The main factors in the culture preparation before the conservation are analyzed. They affect the cell resistance and survival and include cultivation method, nutrient medium composition and pH, temperature regime, aeration, culture physiological state, cell concentration, protection, suspension equilibration etc. The influence of the factors and methods on the survival of different microorganism groups – actinomycetes, bacteria, yeasts and fungi, is considered.

Introduction

The support of culture collections is an essential element of the microbiological science, practice and their development. The man has accomplished to choose and select thousands of useful microorganisms, which now are the base of the biotechnological processes. Some of them are strains involved in the production of dairy, bakery, spirits, alcohol, vaccines, antibiotics, enzymes, silage, vinegar etc. Parallel with the process of isolation, selection and genetic engineering a need arises for the preservation of strains, their vitality, specificity, activity, immunogenicity and other properties in laboratory conditions. The production standard and quality depend on the right choice of preservation methods of the industrial strains. A variety of methods are available for strain preservation, which keep their vitality and authenticity. The main difference between them is the time they provide for the hopeful strain using. Depending on the aims, the methods could be divided into: laboratory with short time between cultivations – from several months to years (subcultivation, storage under mineral oil, in water and water buffers, by cooling to 4 – 8 °C or drying etc.); long-term storage methods (freezing at different temperature, freeze-drying),

by which the specimens could be preserved unchanged for decades. The conservation results could be affected by lots of factors during the preliminary culture preparation, by the choice of protectants, preservation and regeneration methods with minimum consequences for the strains. With the development of microbiology the requirements for the culture preservation increase. It is not enough to perform a successful conservation; it is also necessary to keep the strain for a long–term period.

Anabiosis of microorganisms

Anabiosis is a state and ability of the organisms to reduce or stop reversibly their vital activity. A connection between the evolutionary processes of the organic compounds and Earth's life is found, during study the anabiosis. It is a form of adaptation, way of survival and preservation of organized matter [2, 8, 36, 37, 38, 55, 108].

The term "anabiosis" was formulated for the first time in 1900 in the journal "Scientific review" ("Nauchno obozrenie") by the emigrated in Bulgaria Russian scientist P. Bahmetiev and it expressed the absence of organism vitality, not meaning its death [7].

The microbiologists show interest in the anabiosis phenomenon. The microorganisms are constantly disposed on atmospheric influences in their natural environment – warmth, cold, freezing and thawing, drying and moisturizing. Yeasts are the first investigated microbiological object. Nowadays for the definition of anabiosis M. Becker suggests the following conclusion – the organism ability to stop convertibly and utmost reduce the vitality [9].

Characters of this condition are:

- absence or utmost reduction of metabolism;
- structure preservation for a long time;
- absence of noticeable amounts of free water as permanent medium in the liquid phase;
- increased resistance to extreme conditions;
- ability to recover the vital activity.

The study of anabiosis continues to engage the scientists. Expeditions have isolated microorganisms from the permanent frost world regions – Arctic, Antarctic and Siberian tundra. Samples of ice and turf with supposed age between 300 and 3 million years are studied. Rich and various microflora – spore-forming and non-sporeforming bacteria, actinomycetes, fungi and yeasts are found in anabiotic state. The representatives of the following genera are isolated and defined: *Basidiomycota, Ascomycota, Zygomycota, Pseudomonas, Bacillus, Nocardiopsis, Streptomyces, Aspergillus, Penicillium, Cladosporium, Mucor, Cryptococcus, Rhodotorulla etc* [2, 45]. Data for the anabiotic state continuance in Antarctic ice exist, which show that the longevity limit is many times over compared to laboratory results. Many of the isolated strains are defined as psychrotolerant and even psychrophilic with optimal growth temperature from 2 – 5 °C to 18 – 20 °C. The natural conditions determine adaptive structural and functional changes in the cell that lead to modifications in the regulatory mechanisms, slow down the culture development and metabolism. This structural and biochemical organization allows keeping the viability of the yeast cell for a long period of time [9].

In the beginning of the twentieth century conditions to develop the methods for microorganisms conservation are available. Mainly they could be divided in two groups.

The first group aims detention of the cell vitality to minimum – hypobiosis, a state known as "cells at rest".

The second group leading the microorganisms to an anabiotic state is: drying, freezing at low temperatures and lyophilization.

Methods for maintenance and preservation

Subcultivation. The subcultivation is a method of periodical cultivation on agar nutrient medium and it is the oldest method used for microorganisms maintenance and preservation in laboratory and industrial conditions. A main principle in the cultivation is taking cell material from great amount of colonies. Using a single colony is not recommended because this increases the unwanted selection probability. Thus the control of the innate strain characteristics, activity change and vitality could be impossible [19, 27, 30, 82].

The choice of nutrient medium for strain cultivation is essential for the method application. Choosing correct nutrient compounds is the base of further preservation of taxonomical, morphological and biochemical culture properties. The regularity of the cultivation is different for the separate microorganisms groups and varies from 30 days to several years, at preservation temperature 3 – 5 °C. According to some scientists the temperature increasing over 5 °C leads to quick lost of cell viability [82]. The average conservation longevity for yeasts is 1 to 3 months. Data exist that some bacteria are conserved for 5 to 12 months and microscopic fungi over 5 years. Announcements are found that representatives of genus *Streptomyces* keep their vitality by this method for 26 years. Fungi are the longest preserved by subcultivation strains and have been kept since 1895 [4, 15, 27, 65, 91, 103].

Mineral oils. Other cultures preservation method is under mineral oil [17, 41, 48]. It was applied for the first time in 1914 by A. Limier to keep the gonorrhea agent *(Neisseria gonorrhoeae).* In 1921 by this way M. Michelle preserved in broth gonococci, meningococci and pneumococci [46]. The method essence is covering the well grown culture on liquid or agar nutrient medium with sterile non-toxic mineral oil. The most common used oil is paraffin or vaseline with layer thickness 1 to 2 cm. The aim is to limit the oxygen access that reduces the microorganisms' metabolism and growth, as well as to restrict the cell drying during preservation in freezing conditions.

According to some investigations the microorganisms conservation period under Vaseline oil without subcultivation is 1 to 12 years depending on their properties. Optimal and utmost time limits are established for cultivation of different taxonomical groups. The preservation period for bacteria from the genera *Azotobacter* and *Mycobacterium* is from 7 to 10 years, for *Bacillus* – 8 - 12 years. In twelve month preservation of genus *Lactobacillus* under Vaseline oil the titer decreases 2 – 3 orders but there are some data for conservation of the genus to 6 years. Other bacteria need re-cultivation at intervals of 6 months to 1 year. *Acetobacter* and *Gluconobacter* have to be undergone to five passages for biochemical properties recovering. Different genera yeasts are studied and it is determined that the conservation period varied from 1 to 7 years (*Candida, Endomycetes, Hansenula, Saccharomyces, Schizosaccharomyces, Pichia)* [5, 46, 50, 63, 69].

Water or water–salt solutions. There are data for microbiological objects preserving in water or water–salt solutions. The cells are placed in indifferent liquid medium and they approach a hypobiotic state. The suspension density, the presence of Ca^{2+} ions in the medium, the solution composition and pH, the preservation temperature influence the quantity and protection of the cells at rest. For example, it is determined that direct cause for the accelerated death of *Escherichia coli* with population number over 10^9 cells/ml is the accumulation of lethal metabolites in the intercellular medium. Their concentration grows with the cell density increasing. The optimal pH for 1 month preservation of *E. coli* is pH 8 and for *S. cerevisiae* – pH 5.5. This method is recommended for short term storage at 4 – 8 °C for 1 week to 12 months [5, 17, 59, 92, 102].

Drying. The cultures conservation method that imitates the natural conditions is drying preservation. It is based on the natural microorganism properties to fall into anabiosis. Sand, soil, mud, active carbon, saw-dust, synthetic balls and tablets, polymer matrixes, high disperse materials, filter paper etc are used as microbic material carrier. The large carrier surface adsorbs part of the moisture. The drying is performed at room temperature or by heating up at 36 – 40 °C.

In 1966 Coe and Clark applied the method for strains *Staphylococcus aureus*, announcing preservation date 6 months [63]. There are references for stability investigations after conservation on different carriers and following drying of representatives of the genera *Shigella, Salmonella, Proteus, Bacillus, Streptococcus, Pseudomonas, Corynebacterium, Rhodococcus, Serratia, Mycobacterium,* with advisable conservation time to 12 month at temperature 4 °C [11, 12, 16, 25, 47]. M. Litvinov carried out extensive research in the 40ties on the strain preservation by drying species *S. cerevisiae* [54]. He developed drying procedures on a filter paper or glass and determined the conditions – in exicator using vacuum 18 mmHg, at 25 – 32 °C in the presence of dryer – calcium chloride. This preservation method is widely used for brewery and bakery yeasts. M. Becker and A. Rapoport carry out numerous investigations connected to the structural and functional characteristics of the cytoplasmic membrane after the cell anabiotic state [66-69]. A convective drying of starter yeast cultures of species *S. cerevisiae* is applied at 37 °C with ultimate humidity 8 – 10 % [8, 9, 77, 104, 110].

In 1954 Anner for the first time used vacuum to speed up the process of microbial suspension drying. The method is named "L-drying". It is used for the conservation of spyrochetas, leptospyras, salmonellas and some yeast and virus strains [57, 63].

Cryogenic conservation. In the last decades of twentieth century the cryogenic conservation was characterized with quick accumulation of significant results from fundamental investigations in microbiological science. It is well known that the laboratory microorganisms could not always be lead to anabiosis by cooling and freezing. Often the cells die or remain alive but not viable. The temperature decrease affects the biological systems and series of mechanical, physiological and biochemical changes occur. Depending on the cooling and overcooling level, the cells suffer different damage consequences by the "temperature shock" [9, 18, 22, 26, 52, 75, 83, 94].

During the cryogenic treatment of the cells a great number of damage factors are known [73, 86, 99, 100] and the basic are:

- in and out water crystallization, accomplished by biomacromolecule dehydration and damaging barrier membrane properties, mechanical disturbances of the cell structures, caused by the mixed forms of ice – hexagonal and cubic with destructive action [32, 43, 106].
- increasing concentration of the cell electrolytes – a result of overcooling that leads to hydrogen ions concentration changes in the cell and pH disbalance. The salts reach hypertonic concentrations and critically affect the cell protein structures [34, 96, 106];
- conglomeration – spatial connection between cells and macromolecules that usually do not interact.

The literature gives theoretical and experimental data referred to the influence of the freezing and thawing rate upon the form of the crystals and their destructive action to the cell [6, 19, 64, 72, 74, 100]. The scientists have different opinions about the choice of the freezing rate for the biological suspensions preservation. According to N. Pushckar and A. Bellows the slow freezing (0.3 °C.min^{-1} – 1°C.min^{-1}) causes a formation of big crystal structures in the samples independently on the primary structure. With speed of 10 °C.min^{-1} dendrite recrystallization do not occur and with 60 °C.min^{-1} small crystal structures appear in the specimen and do not grow. A high

speed freezing from 100 °C.min^{-1} to 400 °C.min^{-1} is used in laboratory conditions and the liquid phase freezes almost immediately. The structure is defined as "non-crystal". The phenomenon term is vitrification, because of the vitreous amorphous condition of the system. There are data for freezing rates to 6000 °C.min^{-1} [18, 73-75, 84].

The thawing of different microorganisms is usually in water bath at temperature 25 °C to 41 °C. The slower static thawing of the cells (stored at low temperatures of minus 135 °C – 196 °C) at room temperature could lead to lethal recrystallization in the temperature range between minus 130 °C – minus 110 °C [31, 40, 94].

Freezing in liquid nitrogen. The method of freezing to minus 196 °C and preservation in liquid nitrogen or its vapor is basic for most of the culture collections. Actinomycetes, bacteria, yeasts, fungi, plant and animal viruses and cell cultures are conserved that way. According to some scientists the optimal cooling rate for fungi is 1°C.min^{-1}, for yeasts 7-10 °C.min^{-1}, for bacteria and actinomycetes 2-45°C.min^{-1} [88]. In the 60ties of the twentieth century Maussori carried out experiments of yeasts freezing with different rates including 6000°C.min^{-1} [19]. Some authors recommend this freezing speed for the species *Lactobacillus delbrueckii ssp. bulgaricus*, independently on the protective media [41]. Tsutsaeva et al. pay attention to the aeration of the culture grown in liquid medium as well as to the higher resistance of the cells cultivated on rich nutrient medium. The influence of some physicochemical factors on the yeasts sensitivity to freezing is studied and the slow freezing is recommended – 0.4 °C.min^{-1}. The experiments with *E. coli* bacteriophages do not show longevity change at different freezing rates from 1 °C.min^{-1} to 400 °C.min^{-1} [94, 95, 97, 98]. B. Kirsop analyzes the conservation of about 500 strains from 75 species of genus *Saccharomyces* in liquid nitrogen and reveals that the average survival is 66.1 % with deviation 34.8 % [39].

Lyophilization. Lyophilization, vacuum – sublimation drying or freeze-drying are the commonly accepted names for the process of taking away the entire quantity freeze moisture from the solid matrix of the wet containing materials by vacuum sublimation. The lyophilization consists of the following stages:

- material freezing to low temperatures – below the eutectic temperature;
- primary drying when the ice crystals sublimate influenced by the passed in the system heat energy in vacuum conditions;
- secondary drying when after the ice separation the remained material moisture is desorbed in maximum deep vacuum conditions [75].

There are a great number of literature data for the microbiological cultures lyophilization [3, 13, 44, 49, 65-68, 71, 101, 106, 107]. The method has a wide application for the vaccine production, veterinary and humane purposes [99, 100]. Most of the viruses survive very well the vacuum-sublimation drying. Only small part of them is sensitive to freezing and drying [86, 101].

From all microorganisms groups the bacteria sustain lyophilization the best [56]. According to their resistance to drying some authors relate the bacteria to three groups:

- Strongly resistant, such as the genera *Streptococcus, Staphylococcus, Brevibacterium, Corynebacterium, Lactobacillus, Salmonella, Bacillus* etc. Their viability reaches 100 % after drying;
- Medium resistant, such as the genera *Brucella, Salmonella, Seratia, Pseudomonas.* Their survival reaches 70 %.
- Sensible to drying are some representatives of the genera *Spirochete, Methylobacter, Methylococcus* [58, 67, 85].

With long storage terms 33 – 36 years are the genera *Aspergillus, Fusarium, Citromyces, Acetobacter, Alcaligenes, Bacillus, Mycobacterium* etc [50].

The yeasts resistance to freeze-drying preservation is considerably smaller [6, 58]. Yeasts of species *S. uvarum* with concentration 10^8 cells/ml in water suspension, frozen at different rates of 0.1 °C.min^{-1} to 1 °C.min^{-1}, die over 99 % after the vacuum – sublimation drying [19, 54].

Protectants

Protective compounds – cryoprotectants, are found to eliminate most of the multiple destructive factors during freezing of biological structures. This cryobiology branch is based on studying the natural protection models of the cell, being in hypo- and anabiotic state. The basic indicators of the protective media are determined by the experimental data of scientists such as Louie, Lovelock, Shickama:

- to keep the microorganism viability, morphological, biochemical, taxonomical and genetic properties during conservation and preservation;
- to be non-toxic;
- to have good water solubility;
- to connect easily with water, performing coligative properties;
- to have low eutectic temperature;
- to prevent salt hyperconcentration in the suspension;
- to stabilize hydrogen connections in the crystal lattice and to prevent large crystal forming;
- to penetrate good the cells (only endocellular mechanism of cryoprotecting);

According to the location of their action the cryoprotectants are divided in two groups:

- endocellular cryoprotectants – media, penetrating cells;
- extracellular cryoprotectants – media, connecting with the extracellular water.

Endocellular protectants. The application of protective media with endocellular mechanism presents a cell penetration. The media overcooling before the freezing contribute to small crystal formation, which restricts the mechanical disturbing action during the cryogenic treatment. The main endocellular protective media are glycerol and dimethylsulphoxid.

Glycerol **(**glycerin, 1, 2, 3 propantriol, $C_3H_8O_3$). Some microorganisms in natural environment or after adding extra amount of glycerol to the nutrient media increase their synthetic processes before the anabiotic transition. Such examples are *S. rouxii, E. coli, Ps. putida.* The increasing glycerol concentration assumes to play an important part in the microorganisms' stability toward dehydration, increases the resistance at lowering the active water (a_w) and secures the vitality during freezing [62, 87, 105]. A lot of cryogenic conservation experiments are carried out with varying the glycerol concentrations at different temperatures and freezing rates. Glycerol is successfully applied to freezing *Serratia marcescens, Erwinia aroideae, T4 phage, Claviceps sp., Acremorium chrysogenum, S. cerevisiae, C. utilis* and others with high percentage survival and preserved properties [62, 91].

Dimethylsulphoxid (DMSO, C_2H_6SO). The cryoprotective properties of this compound depend on the concentration. For example, the study of DMSO concentrations from 10 % to 20 % at freezing myocardial tissue cultures in liquid nitrogen shows the best results at 17.5 % [76]. The major part of the world culture collections recommends 10 % protecting concentration [87].

There is lots of information in the literature for the survival of the microorganisms stored in liquid nitrogen when glycerol and DMSO are used as protective media. The optimal concentrations are different depending on the specificity of the treated cells and vary between 5 – 20 % [28, 29, 42, 70].

Extracellular protectants. Some of the extracellular protectants applied to preservation of the biological objects in frozen state are polyvinylpyrrolidone, hydroxyethyl starch and dextran.

Polyvinylpyrrolidone (PVP, $(-C_6H_9NO-)_n$). There are several hypotheses about the cell cryoprotection mechanism of PVP. According to one of them the protective effect is connected with its ability of cell penetration by pinocytosis. Other hypothesis refers the protecting mechanism to the connection ability of

the polymer molecules with the cell membrane and to the envelope formation around.

Hydroxyethyl starch (HES). It is non-toxic, biologically inert, indifferent protectant. These properties make HES useful as a plasma substitute. It is an appropriate protectant for some blood cells.

Dextran. This compound is successfully applied as cryoprotectant for viruses' and microorganisms' conservation [20]. It is possible to use this substance as a protective medium combined with other cryoprotectants because it is chemically inert [7, 20, 21]. Dextran is also used in medicine for blood transfusion as a plasma substitute.

Combined media for cryo- and lyo-conservation and preservation of micro-organisms are applied, containing different sugar concentrations (sucrose, glucose, trehalose), colloids (gelatin, agar, peptone, milk and sera), salts (sodium glutamate) etc. In the experiments and practice, using combined media higher percentage cell viability is established compared to the single component protective media, during and after conservation [1, 14, 51, 61, 64, 93].

When the influence of different factors upon bacteria and actinomycetes resistance during lyophilization is studied the most commonly used protective medium is sucrose 10 % + gelatin 1 % [3, 43, 44, 67, 90]. Other media such as 0.1 – 10 % peptone, 10 % sucrose, 10 % lactose, 10 % trehalose, 10 – 20 % skimmed milk, 5 % Na glutamate, casein hydrolysate and many others are applied successfully to the genera *Bacillus, Pantoea, Seratia, Erwinia, Lactobacillus, Acetobacter, Streptococcus* etc [16, 24, 49, 85].

Preparation of the cultures for conservation

The basic factors in microbiological cultures preparation before conservation by which the strain preservation could be purposively influenced are: cultivation method (deep and surface), temperature regime, composition and pH of the nutrient medium, aeration. The age, physiological condition and culture concentration at the moment of conservation are of great importance too [101].

Nutrient medium. The nutrient medium composition affects the cell resistance. Two basic opposite opinions exist about the choice of the propagation media – poor and rich one. Cultivation on rich nutrient media is recommended in some conservation prescriptions because the percentage of viable cells is higher compared to those cultivated on poor media. According to other authors the poor and becoming poor by the time of propagation nutrient media is a signal for cell metabolism reorganization that leads to energy storage. Probably the cells prepare themselves for a hypobiosis from which the transition to anabiotic state is easier [92]. The right choice of nutrient medium leads to protein, carbohydrate and lipid accumulation that could increase the cell resistance to lyophilic treatment. For example, extra addition of tween 80 and oleic acid to meat peptone broth (MPB) during cultivation of *Ps. denitrificans, S. marcescens, E. aroideae, Acinetobacter calcoaceticus* etc raises cell viability after conservation [3, 24].

The acidity (pH). Cell resistance during conservation depends on the cultivation medium acidity. It is well-known that the cultivation medium pH influences the cell propagation: there is optimal pH range for every culture and the cell growth is slow or absent out of it. Studying *S. cerevisiae* brewery and bakery yeasts a correlation between the cryoresistance and the medium acidity exists. Although at pH 4.2 cells grow faster their vitality after lyophilization is minimal. The survival increasing on an average with 10 – 25 % at pH 5.4 is observed, compared to the lowest studied pH 3 [34, 35, 109].

Cultivation method. In the literature and in the practice of different culture collections liquid broth or agar nutrient media

are used, and deep or surface cultivation respectively, for the preparation of the cell culture before conservation. There are no data comparing both methods in relation to the culture resistance to conservation by freezing or freeze-drying.

Temperature regime. The optimal incubation temperature for a given microorganism is enlisted in its databases (catalogue information) and varies up to some limits. A lot of strains have equal temperature values in the catalogues. For example, more than 90 % of the strains *E. coli* in NBIMCC have optimal cultivation temperature 37 °C. The temperature regime of lactic acid bacteria is different – from 37 °C to 52 °C according to their specificity. Species *S. cerevisiae* are conventionally propagated at temperature 25 - 28 °C [14].

In *S. cerevisiae* studies, when the cultivation temperature is gone down from 30 °C to 15 °C the cell resistance increases and a linear correlation is established. It is necessary a separate temperature determination for every strain considering the cell structure and biochemical reactions. Studying the growth of *E. coli* in the range of 13 °C to 46 °C the lowest percentage of the cell biochemical changes is established and the optimal strain viability is at 23 - 37 °C [10, 106].

Aeration. It is proven that the cells have different conservation resistance depending on the aeration conditions during the cultivation. For example in yeasts, the medium aeration performs the following functions: providing the necessary oxygen for yeast metabolism; supplying the intensive mixing of the nutrient medium and equalizing the cultivation parameters; removing the carbon dioxide released as a vital activity product of the yeast cells, that leads to resistance increasing.

The representatives of species *S. cerevisiae* cultivated in aerobic conditions are significantly more resistant to hypo- and hypertonic shock. The anaerobic propagation reliably reduces the cryoresistance that is explained with the change in the structural organization of the cytoplasmic membrane and the cell bioorganization [33, 89, 96, 97]. According to other authors, one of the basic reasons for cell disturbance by cryoconservation and lyophilization is the action of the dissolved in the medium oxygen that activates during processes and joins one or two electrons and oxidizes important cell building compounds [66].

Culture age (physiological condition). When planning the experiment the choice of culture age is of great importance for receiving high percentage vitality after microorganism lyophilization. Biriuzova and Rapoport recommend 48 hours culture of *S. cerevisiae* as the most appropriate for conservation. Other scientists propose preserving *S. cerevisiae* at the beginning of the stationary growth phase not specifying the age [35, 106].

There are data confirming the fact that the microorganisms are more resistant to freezing and dehydration at the end of the logarithmic growth phase or at the beginning of the stable one [3, 34, 43, 60, 68].

Cell concentration. There are different points of view about the influence of the cell suspension concentration on the strain viability preservation. When studying the correlation between the population density and the survival after lyophilization of *Streptococcus cremoris* it is established that with the concentration increasing from 10^6 to 10^9 cells/ml the vitality percentage also increases [19]. For *Ps. denitrificans* freeze-drying, Arkadieva et al. recommend starting concentration of 10^9–10^{10} cells/ml [3]. Experiments are also carried out with species *S. cerevisiae* about the cell suspension density. The results show that the injured cell percentage in extreme conditions raises with decreasing the cell concentration but on the other side the increasing concentration leads to intercellular contacts, toxic metabolite production etc that reduces the vitality [109]. Most of the culture collections recommend using initial concentration 10^8–10^{10} cells/ml [50].

Raising the initial concentration of the viable cells on one hand increases the chance of cell preservation even at low survival level after conservation but on the other the lysed cells and the cell compounds could act as cryoprotecting medium for the cells [60].

Equilibration. The equilibration is the first stage connected with the microorganisms' preparation, necessary for the cell structure stabilizing before conservation. It is possible a series of cell biochemical changes to occur during the equilibration causing either positive effect or negative influence on the metabolic processes before microorganisms preservation. During that period the cells pass through a transfer stage that prepares them to stand the extreme conditions of conservation (osmotic and temperature shock).

The inner cell properties and characteristics could be lost or the microorganisms could die under the stress influence attending the conservation processes. The different microorganism groups have different resistance. The sporeforming cultures keep very well their viability at almost all conservation methods. This has connection with the fact that they are natural preservation form and also with the smaller water amounts in the spore. Non-sporeforming microorganisms stand less the cryogenic and lyophilic treatment. It is also known that the prokaryotes are more resistant than the eukaryotes as well as Gram positive bacteria than Gram negative ones [59, 60, 87].

A different cryo- and xero-resistance exists for separate strains of one spesies. For example, when *S. uvarum* and *S. cerevisiae* are cultivated under same conditions in concentration 2-5.10^8 cells/ml and protected by 10 % glycerol and 10 % DMSO, their viability varies within wide limits from 34 to 100 % after preservation in liquid nitrogen (minus 196 °C). After three years of storage in liquid nitrogen some of the strains preserve their vitality unchanged however, the survival of the others decreases significantly.

Consequently, for the preservation of the initial properties of every microorganism group in long term storage an individual approach is needed either during the preliminary culture preparation or when choosing preservation and recovering methods.

References

1. Abadias, M., N. Teixido, J. Usall, A. Benabarre, I. Vinas, 2001. *J. Food Prot.*, **64** (6), 856-861.
2. Abysov, S. S., V. I. Biryazova, N. A. Kostrikina, 1990. *Microbiology (Moskva),* **59** (6), 1094-1101 (in Russian).
3. Arkadieva, Z. A., E. I. Kozlova, T. V. Kovalenko, 1975. *Appl. Biochem. Microbiol. (Moskva)*, **9** (4), 519-522 (in Russian).
4. Arkadieva, Z. A., N. L. Olsinskaya, M. A. Alekseeva, 1988. *Appl. Biochem. Microbiol. (Moskva)*, **24** (6), 839-845 (in Russian).
5. Arkadieva, Z. A., M. N. Pimenova, 1985. *Appl. Biochem. Microbiol. (Moskva)*, **21** (5), 645-648 (in Russian).
6. Bakalivanov, St., Sl. Ivanova, Ts. Tsvetkov, 1998. *Agricult. Sci. (Sofia)*, **36** (2), 6-10 (in Bulgarian).
7. Bahmetiev, P. I., 1900. *Scientific Rev. (Moskva),* **1**, 3-9 (in Russian).
8. Becker, M. E., B. E. Danberg, A. I. Rapoport, 1981. *Anabiosis of microorganisms,* Riga: Zinatne, 253 (in Russian).
9. Becker, M. E., A. I. Rapoport, L. B. Kalakutsi, 1987. *Detention of the cell vitality,* Riga: Zinatne, 240 (in Russian).
10. Belukov, S. V., V. J. Ivanova, N. A. Kustova, 1997. *Biotechnology (Moskva)*, **3**, 39–44 (in Russian).
11. Bilko, I. P., 1988. *J. Microbiol. (Kiev)*, **50** (1), 96-97 (in Russian).
12. Bilko, I. P., V. V. Gashitskii, A. A. Furman, 1979. *J. Microbiol. (Kiev)*, **5**, 557-559 (in Russian).
13. Blankov, B. I., D. L. Klebanov, 1961. *Application of the lyophilization in microbiology,* Moskva: Medgiz, 263 (in Russian).
14. Catalogue of National Bank for Industrial Microorganisms and Cell Cultures, 1995. Sofia: NBIMCC, 128.
15. CBS – 75 years culture collection, 1979. Delft: CBS, 61.

16. Champagne, Cl. P., N. J. Gardner, 2001. *Elect. J. Biotechnol.,* **4** (3), 146-152.
17. Claudia, C., L. Lastra, A. E. Hajek, R. A. Humber, 2002. *Can. J. Bot.*, **80** (10), 1126-1130.
18. De Valdez, G. F., G. S. Giori, 1993. *Cryobiology*, **30**, 329-334.
19. Donev, T. N., 2001. *Methods for conservation of industrial microorganisms*, Sofia: NBIMCC, 93.
20. Donev, T. N., L. Mehandjiska, E. Builieva, 1987. *Third national school "Cryobiology and freeze-drying"*, 2-9 Aug., Smolyan, Bulgaria, 279–292 (in Russian).
21. Donev, T. N., I. Savova, M. Ralchevska, 1987. *Third national school "Cryobiology and freeze-drying"*, 2-9 Aug., Smolyan, Bulgaria, 277–279 (in Russian).
22. Donev, T. N., I. Tepavicharova, 1991. *Inform. NBIMCC (*Sofia), **15** (1), 4–8 (in Bulgarian).
23. Donev, T. N., Ts. Tsvetkov, 1984. *Meat Industry (Sofia)*, **4**, 90-91 (in Bulgarian).
24. Emtzeva, T. V., L. N. Lavrova, N. D. Konstantinova, 1991. *Microbiology (Moskva)*, **60** (5), 879-888 (in Russian).
25. Fedorov, A. J., E. V. Volchenko, I. N. Singircev, V. I. Korgenevich, G. M. Shub, 2000. *Appl. Biochem. Microbiol. (Moskva)*, **36** (1), 59-67 (in Russian).
26. Feofilova, E. P., 2003. *Appl. Biochem. Microbiol. (Moskva)*, **39** (1), 5-24 (in Russian).
27. Gern, R., 1983. *Methods of general bacteriology,* Moskva: Mir, 521–534 (in Russian).
28. Henry, J., B. Kirsop, 1989. *Cryopreservation of yeasts in polypropylene straws*, UNESCO/WFCC, Technical information sheet 3.
29. Hoffmann, P., 1989. *Cryopreservation of fungi,* UNESCO/WFCC, Technical information sheet 5.
30. Idelchik, M. S., G. I. Pavlovskaia, E. A. Neginetz, 1981. *Appl. Biochem. Microbiol. (Moskva)*, **17** (6), 850-853 (in Russian).
31. Ivanov, S. A., E. O. Puchkov, 1989. *Microbiology (Moskva)*, **58**, 699-701 (in Russian).
32. Ivanov, V. N., A. I. Rapoport, A. A. Pindrus, L. A. Saulite, T. A. Shifruk, 1987. *Microbiology (Moskva)*, **56** (2), 341-349 (in Russian).
33. Ivanova, V. J., S. V. Belukov, 1996. *Biotechnology (Moskva)*, **8**, 50-52 (in Russian).
34. Ivanova, V. J., S. V. Belukov, A. Z. Korshunov, 1996. *Biotechnology* (Moskva), **11**, 38–41 (in Russian).
35. Ivanova, V. J., S. V. Belukov, A. Z. Korshunov, 1996. *Biotechnology (Moskva)*, **11**, 42–45 (in Russian).
36. Jackobson, J. O. (Ed.), 1973. *Anabiosis and preanabiosis of microorganisms*, Riga: Zinatne, 136 (in Russian).
37. Kalakutskij, L. V., T. M. Sidiakina, 1988. *Cryobiology* (Moskva), **4**, 3 – 9 (in Russian).
38. Kashner, D. (Ed.), 1981. *Life of the microorganisms in extreme conditions,* Moskva: Mir, 519 (in Russian).
39. Khursheed, M. A., 1990, *Freeze-drying of microorganisms using a simple apparatus*, UNESCO/ WFCC, Education Committee.
40. Kirsop, B. E., C. P. Kurtzman, 1988. *Yeasts*, Cambridge: University Press, 233.
41. Kish, J. C., 1998. *Resurrecting a better method for long-term storage of mushroom cultures*. http://www.mycoculture.org/ftpdir/jjkish - culture-storage-technique.
42. Kitamoto, Y., A. Suzuki, S. Shimada, K. Yamanaka, 2002. *Mycoscience*, **43** (2), 143-149.
43. Klickov, S. P., J. P. Paderin, M. M. Sadikov, V. P. Chuprunov, V. V. Debrishev, V. V. Gusev, 1966. *Biotechnology (Moskva)*, **1**, 3-39 (in Russian).
44. Kolesov, S. G., 1952. *Drying of microorganisms and biopreparations,* Moskva: Selhozgiz, 192 (in Russian).
45. Kochkina, G. A., N. E. Ivanushkina, S. G. Karasev, E. J. Gavrish, L. V. Gurina, L. I. Evtushenko, E. V. Spirina, E. A. Vorobeva, D. A. Gilichinskii, S. M. Ozerskaia, 2001. *Microbiology (Moskva)*, **70** (3), 412-420 (in Russian).
46. Krasilnikov, N. A., 1967. *Preservation methods for culture collection microorganisms,* Moskva: Nauka, 151 (in Russian).
47. Kudrish, I. K., A. A. Roy, A. D. Gragulya, E. A. Kiprianova, 1999. *Microbiology (Moskva),* **68** (3), 387-391 (in Russian).
48. Kudryavcev, V. I., M. V. Fateeva, T. N. Nikitina, 1972. *Microbiology (Moskva)*, **41** (5), 903–908 (in Russian).
49. Kupletskaya, M. B., 1987. *Microbiology (Moskva)*, **56** (3), 488–491 (in Russian).
50. Kupletskaya, M. B., Z. A. Arkadieva, 1997. *Microbiology (Moskva),* **66** (2), 283–288 (in Russian).
51. Kuznetsov, V. D., S. N. Filipova, E. K. Ruge, 1978. *Microbiology (Moskva)*, **47** (4), 706-710 (in Russian).
52. Lewis, J. G., R. P. Learmonth, K. Watson, 1994. *Cryobiology*, **31**, 193-198.
53. Likov, A. V., 1974. *Drying*, Sofia: Technika, 539 (in Bulgarian).

54. Litvinov, M. A., 1939. *Modern Botany (Moskva)*, **6-7**, 169 – 198 (in Russian).
55. Lozina – Lozinski, L. K., 1972. *Cryobiology articles,* Leningrad: Nauka, 288 (in Russian).
56. Mehandjiska, L., I. Tepavicharova, T. Donev, E. Buylieva, 1989. *Fourth international school "Cryobiology and freeze-drying"*, 29 July – 06 August 1989, Borovets, Bulgaria, P 34.
57. *Microbial diversity in safe hands*, 1999. ECCO, 3th edn.
58. Miyamoto-Shinohara, Y., T. Imaizumi, J. Sukenobe, Y. Murakami, S. Kawamura, Y. Komatsu, 2000. *Cryobiology*, **41**, 251-255.
59. Mulyukin, A. L., A. K. Lusta, N. M. Gryaznova, E. S. Babusenko, N. A. Kozlova, V. M. Duga, A. L. Mityushina, V. I. Duda, G. I. El-Registan, 1997. *Microbiology (Moskva)*, **66** (1), 42-49 (in Russian).
60. Mulyukin, A. L., V. V. Sorokin, N. G. Loyko, N. E. Suzina, V. I. Duda, E. A. Vorobeva, G. I. El-Registan, 2002. *Microbiology (Moskva)*, **71** (1), 37-48 (in Russian).
61. Nezhyuta, A. A., E. S. Serbis, 2001. *Biotechnology (Moskva)*, **6**, 59-67 (in Russian).
62. Nikolaev, J. A., 1997. *Microbiology (Moskva),* **66** (6), 790-795 (in Russian).
63. Norris, J. R., D. W. Ribbons, 1970. *Methods in microbiology*, London, New York: Acad. Press, 319.
64. Novik, G. I., N. I. Astapovich, N. G. Kadrinkova, N. E. Ryabaya, 1998. *Microbiology (Moskva)*, **67** (5), 637-642 (in Russian).
65. Novik, G. I., N. I. Astapovich, N. E. Ryabaya, 1998. *Microbiology (Moskva)*, **67** (5), 631-636 (in Russian).
66. Oparin, J. G., 1996. *Biotechnology (Moskva)*, **7**, 3–13 (in Russian).
67. Oparin, J. G., Z. F. Bogautdinov, I. K. Pivovarova, B. J. Shuster, V. I. Zaerko, 1996. *Biotechnology (Moskva)*, **9**, 29-31 (in Russian).
68. Osadchaya, A. I., V. A. Kudryavtsev, A. L. Safronova, 2002. *Biotechnology (Moskva)*, **3**, 45-54 (in Russian).
69. Overchenko, M. B., G. M. Dobrolinskaya, D. R. Djigure, 1982. *J. Microbiol. (Kiev)*, **44**, 636-639 (in Russian).
70. Panoff, J. M., B. Thammavongs, M. Gueguen, 2000. *Cryobiology*, **40**, 264-269.
71. Pitombo, R. N. M., Cl. Spring, R. F. Passos, M. Tonato, M. Vitolo, 1994. *Cryobiology*, **31**, 383-392.
72. Platov, A. V., S. V. Belukov, A. Z. Korshunov, 1996. *Biotechnology (Moskva)*, **11**, 60-62 (in Russian).
73. Pushkar, N. S., A. M. Bellows, 1975. *Introduction in cryobiology,* Kiev: Naukova Dumka, 344 (in Russian).
74. Pushkar, N. S., A. M. Bellows, Ju. A. Utkin, V. I. Vishnevskij, 1977. *Low temperature crystallization in the biological systems*, Kiev: Naukova Dumka, 242 (in Russian).
75. Pushkar, N. S., A. M. Bellows, Ts. D. Tsvetkov, 1984, *Theory and practice of the cryogenic sublimation preservation.* Kiev; Naukova Dumka, 259 (in Russian).
76. Pushkar, N. S., M. I. Shrago, A. M. Belows, J. V. Kalugin, 1978. *Cryoprotectants,* Kiev: Naukova Dumka, 204 (in Russian).
77. Rapoport, A. I., M. E. Becker, 1983. *Microbiology (Moskva)*, **52** (5), 719-722 (in Russian).
78. Rapoport, A. I., M. E. Becker, 1985. *Microbiology (Moskva)*, **54** (3), 450-453 (in Russian).
79. Rapoport, A. I., V. I. Birozova, M. E. Becker, 1983. *Microbiology (Moskva)*, **52** (2), 259-262 (in Russian).
80. Rapoport, A. I., G. Y. Chrustaleva, G. Y. Chamanis, M. E. Becker, 1995. *Microbiology (Moskva)*, **64** (2), 275-278 (in Russian).
81. Rapoport, A. I., A. B. Markovskii, M. E. Becker, 1982. *Microbiology (Moskva)*, **51** (6), 901-904 (in Russian).
82. Ruban, E. L., 1989. *Appl. Biochem. Microbiol. (Moskva)*, **25** (3), 291-301 (in Russian).
83. Rudolph, A. S., J. H. Crowe, 1985. *Cryobiology*, **22**, 367-377.
84. Rumian, N., M. Angelov, Ts. Tsvetkov, 1993. *Cryobiology*, **30**, 438-442.
85. Rumian, N., Ts. D. Tsvetkov, M. Angelov, 1993. *Cryobiology*, **30**, 536-540.
86. Safonova, V. I., N. I. Novikova, T. M. Sidyakina, L. T. Bozjeva, 1991. *Microbiology (Moskva)*, **60** (2), 368 – 376 (in Russian).
87. Sidyakina, T. M., 1985. *Conservation of microorganisms. Ser. Conservation of genetic resources,* Pushchino: AS USSR, 63 (in Russian).
88. Sidyakina, T. M., 1988. *Methods for conservation of microorganisms. Ser. Conservation of genetic resources,* Pushchino: AS USSR, 59 (in Russian).
89. Sidyakina, T. M., S. V. Ustyuzhanina, N. D. Novikova, S. E. Gorin, V. V. Esipova, L. V. Kalakutskii, 1990. *Antibiotics and chemotherapy,* Pushchino: AS USSR, 11-14 (in Russian).
90. Stoyanova, L. G., Z. A. Arkadieva, 2000. *Microbiology (Moskva)*, **69** (1), 98-104 (in Russian).
91. Sudenko, V. I., E. I. Kvasnikov, 1986. *J. Microbiol. (Kiev)*, **48** (2), 82-84 (in Russian).

92. Tepavicharova, I., T. Donev, 1987. *Methods for preservation of microorganisms,* Sofia: DKNTP, 80 (in Bulgarian).
93. Terashita, T., K. Yoshida, A. Suzuki, T. Sakai, K. Yoshikawa, M. Nagai, 2003. *Mycoscience*, **44** (1), 71-74.
94. Tsutsayeva, A. A. (Ed.), 1987. *Cryobiology and biotechnology*, Kiev: Naukova Dumka, 216 (in Russian).
95. Tsutsaeva, A. A., L. N. Kazanskaya, L. M. Balaiberdina, V. M. Markova, O. V. Kudokotseva, N. G. Kadnikova, 1987. *Microbiology (Moskva)*, **56** (2), 338-340 (in Russian).
96. Tsutsaeva, A. A., O. V. Kudoktseva, 1989. *Microbiology (Moskva)*, **58** (2), 339–345 (in Russian).
97. Tsutsaeva, A. A., O. V. Kudoktseva, 1989. *Microbiology (Moskva)*, **58** (5), 785–790 (in Russian).
98. Tsutsaeva, A. A., I. P. Visekantsev, 1982. *Microbiology (Moskva)*, **51** (4), 519-520 (in Russian).
99. Tsvetkov, Ts. D., 1979. *Cryobiology and lyophilization*, Sofia: Zemizdat, 159 (in Bulgarian).
100. Tsvetkov, Ts. D., 1982. *Manual of cryobiology*, Sofia: Zemizdat, 317 (in Bulgarian).
101. Urakov, N. N., V. Y. Volkov, R. V. Borovik, 1988. *Biotechnology (Moskva)*, **4,** 420-432 (in Russian).
102. Vachitov, T. Y., L. N. Petrov, 1992. *Microbiology (Moskva)*, **61** (6), 1087-1095 (in Russian).
103. Valagurova, E. V., V. E. Kozairitskaya, A. A. Pindrus, O. A. Azimtseva, 2000. *J. Microbiol. (Kiev)*, **62** (4), 3-8 (in Ukrain).
104. Ventinya, E. J., L. A. Saulite, A. I. Rapoport, M. E. Becker, 1984. *Microbiology (Moskva)*, **53** (4), 658-664 (in Russian).
105. Visekantsev, I. P., T. K. Krasheninnikova, E. V. Olechnovich, L. V. Stepanyuk, 1992. *Microbiology (Moskva)*, **61** (6), 1098-1099 (in Russian).
106. Volkov, V. Y., 1994. *Microbiology (Moskva)*, **63** (1), 5-16 (in Russian).
107. Volkov, V. Y., B. V. Saharov, V. D. Shtepkin, G. N. Fedyukina, A. A. Kashuba, 1992. *Microbiology (Moskva)*, **61** (1), 214-222 (in Russian).
108. Zvyagintsev, D. G., D. A. Gilichinskii, S. A. Blagodatskii, 1985. *Microbiology (Moskva)*, **54** (1), 155 – 161 (in Russian).
109. Zickmanis, P. B., L. P. Auzinya, R. V. Kruche, M. V. Margevicha, S. I. Auzan, M. E. Becker, 1989. *Microbiology (Moskva)*, **58** (3), 471–474 (in Russian).
110. Zickmanis, P. B., R. V. Kruche, L. P. Auzinya, M. V. Margevicha, M. E. Becker, 1988. *Microbiology (Moskva)*, **57** (3), 491-493 (in Russian).

АНАБИОЗА И КОНСЕРВИРАНЕ НА МИКРООРГАНИЗМИ

Цонка Узунова – Донева*, Тодор Донев

Резюме

Направен е литературен преглед на прилаганите методи за съхранение на микроорганизми при колекционирането им. Описани са две групи методи за запазване – в хипобиотично и в анабиотично състояние. Първата група цели забавяне до минимум на жизнената дейност на клетките – съхранение под минерални масла, във вода и водни разтвори, инактивиране в сушители и други. Втората група методи цели довеждането на микроорганизмите до състояние на анабиоза, чрез замразяване и съхранение при ниски температури и чрез вакуумно сублимационно сушене (лиофилизация). Описани са основите фактори при подготовката на микробиологичните култури преди консервирането, влияещи върху устойчивостта и преживяемостта на клетките - метод на култивиране, състав на хранителна среда, pH, температурен режим, аерация, физиологично състояние на културата, клетъчна концентрация, протектиране, еквилибрация на суспензията и т.н. Проследено е влиянието на факторите и методите върху преживяемостта на различни групи микроорганизми – актиномицети, бактерии, дрожди и плесени.