Research Concerning Use of Long-Term Preservation Techniques for Microorganisms

Adriana Criste[1], Mihaela Giuburuncă[1], Octavian Negrea[1], Sorin Dan[2], Marius Zăhan[1]

[1]Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Manastur 3-5 Str., Romania
[2]Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, 400372, Cluj-Napoca, Manastur 3-5 Str., Romania

Abstract
From the large number of methods used for storage of microorganisms, the more effective methods are long-term cryopreservation and lyophilization. The temperature change rate, controls transport of water around cell membranes and indirectly likelihood of intracellular freezing. If the cooling is too fast, the membranes cannot carry water out of the cell and freezing inside. Each cell has an optimum cooling rate, while the survival of very low absolute except that cryoprotector is present to reduce freezing damage.In the present study we proposed to determine the efficiency of some techniques on long term microorganisms conservation as cryopreservation and freeze-drying and the influence of cryoprotectants used in various concentrations on survival rate of bacterial strains during cryopreservtion at -80 ° C in freezer and - 196 ° C in liquid nitrogen. These 7 bacterial strains used for this work came from our collection of cultures, and are represented by *Escherichia coli, Klebsiella pneumonie, Staphylococcus aureus, Bacillus cereus, Salmonella enterica., Pseudomonas aeruginosa, Lactobacillus casei.* The cryoprotectant used are: dimethyl sulphoxide (DMSO) at a concentration of 5% and 10%, ethylene glycol (EG) at a concentration of 5% and 10%, glycerol (Gl) at a concentration of 5% and 10%, propylene glycol (PG) at a concentration of 5% to 10%.

Keywords: bacterial strains, cryopreservation, cryoprotectant, lyophilization, microorganism.

1.**Introduction**

Different preservation methods and drying of microorganisms has been used for decades. Among the many methods used to preserve microorganisms, the most effective and lasting methods are cryopreservation and lyophilization.
Cryopreservation and freeze-drying ensure safekeeping crop viability (over 20 years), but their effectiveness is determined both by culture subject to these preservation techniques and storage conditions: temperature, used cryoprotectants and shelf life.
Cryoprotectant plays an important role in maintaining viability during storage of certain microorganisms (bacteria, viruses, fungi), it includes a wide variety of chemical compounds, but few are widely used as cryoprotectants and with satisfactory results, they are: dimethyl sulfoxide (DMSO), glycerol, propylene glycol, ethylene glycol and sorbitol.
Cryopreservation involves the use of very low temperatures to keep the structure and tissues of the cells intact. The freezing process involves complex phenomena that after decades of the research are not fully understood. Metabolism stops when all the water in the system is converted into ice. When ice forms, water is removed from the extracellular medium, thus forming an osmotic imbalance by the membrane leaves the water within the cell to escape.
Microbial cells, especially bacteria and yeasts grown in aerated conditions and show a greater

*Corresponding author: Adriana Criste, 0040264.596384/247, adriana.criste@usamvcluj.ro

resistance to adverse effects of cooling and freezing than those raised in airless conditions [1]. The permeability of the cell is higher in aerated cultures and the cells are dried more rapidly while cooling [2].

Cryoprotection usually involves treatment with solvents cryoprotectants cells and tissues, usually in higher concentrations, and this allows water circulation by osmosis and diffusion of solutes. These processes are important for cryopreservation [3].

Cryoprotectants may be added during the growth of micro-organisms and before freezing or drying. The type of used cryoprotectants depends largely of the type of microorganism. These include skim milk solids, whey, glycerol, betaine, sucrose, glucose, lactose, dextran and propylene glycol [4]. Matrix forming substances include carbohydrates such as proteins and polymers. These additives which were formed on glass have shown to have greater protection during lyophilization [5], [6]. Harmful crystal formation could damage the cell membrane, thus compromising the integrity of the cells and allowing exit, after thawing, substances within the cell. Sucrose and trehalose retain the structure and function of proteins isolated during the drying process by preventing protein denaturation via the H-bond formation [6]. Many studies suggest that the use of trehalose as a cryoprotectant allows survival of a greater number of organisms than using sucrose [5], [7], [8]. Skimmed milk and serum are used as protection in drying [4]. The most effective cryoprotectants are formed from a mixture of proteins and sugars. [9] It was demonstrated that the *Candida sake* cells recoveries ranged from 45-85%, using a mixture of skimmed milk and sugars [9]. Similarly, it was found that survival of *Lactobacillus paracasei* was significantly increased up to 1000 times, when using 10% skim milk and 10% acacia gum [10].

A traditional classification of cryoprotectants additives (CPAs) depends on the rate of penetration:

-additives which enter rapidly, in 30 minutes approximately, include methanol, ethanol, ethylene glycol (EG), propylene glycol, Me_2SO dimethylformamide;

-additives which penetrate slowly, such as glycerol, mono-, oligo-and polysaccharides, mannitol, dextran, sorbitol, hydroxyethyl starch (HES), albumin, protein, gelatin, polyethylene glycol (PEG), which induce a cryoprotectant in concentrations of 10 -40%.

Some cryoprotectants additives only penetrate the cell wall and do not penetrate the cytoplasmic membrane and thus have three categories:

-cryoprotectants additives penetrating both cell wall and cytoplasm membrane: Me_2SO and glycerol;

-cryoprotectants additives penetrating the cell wall but not the cytoplasmatic membrane: mono-and disaccharides, amino acids;

-cryoprotectants additives which not penetrates the cell wall: polysaccharides, dextran, PEG-6000 [11].

Preservation and storage of microorganisms through different preservation methods and drying has been used for decades. Among the many methods used to preserve microorganisms, the most effective and lasting methods are cryopreservation and lyophilization.

The purpose of this paper is to determine the effectiveness of microorganism's conservation techniques on long term as cryopreservation and lyophilization.

Also it is aimed to know the influence of some cryoprotectants which were used in different concentrations on the survival rate of bacterial cultures during cryopreservation at -80 ° C in freezer and -196 °C in liquid nitrogen.

2.Material and Methods

Cultures of microorganisms: the 7 bacterial strains used for this work came from our collection of cultures, and are represented by *Escherichia coli, Klebsiella pneumonie., Staphylococcus aureus, Bacillus cereus, Salmonella enterica., Pseudomonas aeruginosa, Lactobacillus casei*. These cultures were initially passed on solid nutrient agar and then in liquid nutrient-broth. After inoculation, the cultures were incubated for 24 h at 37 °C followed by a new seeding in liquid medium. Then again were incubated for 24 h at 37 °C.

The cryoprotectant used are: dimethyl sulphoxide (DMSO) at a concentration of 5% and 10%, ethylene glycol (EG) at a concentration of 5% and 10%, glycerol at a concentration of 5% and 10%, propylene glycol (PG) at a concentration of 5% to 10%.

The lyophilization of the microorganisms

We used 500 µl from each 7 bacterial strains, placed in 2 ml cryovials. They were initially plunged in liquid nitrogen for 20 seconds, and then they were kept in a freezer at -80 °C until lyophilization. The interior pressure during the freeze-drying was 39.9 $x10^{-3}$ mbar. After 7 h the tubes were removed from the lyophilizer and closed but without removing the oxygen from inside. After completion of the lyophilization process, we rehydrated the cultures with 500 ml of normal saline solution. After rehydration, 150 µl of the culture has been rolled into tubes with 5 ml of nutrient broth followed by incubation for 24 hours, at room temperature on shaker. After 24 hours we read the samples OD at the photometer at 600 nm, and the absorbance was given by the bacterial cell density in the medium.

The cryopreservation of the microorganisms

The aim of this technique was to determine the best cryoprotectant on the seven microorganism's culture. From the cultures we used 750 µl for each sample where we added 750 µl of cryoprotectant in 5 or 10% (v/v). For each culture we made two samples: one was placed in a freezer at -80˚C and one in liquid nitrogen at -196˚C. We made 160 Eppendorf tubes for the ten cultures, in two concentrations with 4 cryoprotectants.

For each of the seven cultures we made two control samples, where the cryoprotectants were not added. Again one sample was placed in the freezer and one in liquid nitrogen. After 5 days, from all samples we took 150 µl and added it to 5 ml nutritive broth. Also, for each of the culture we made a new control sample which was not subjected to freezing or to any cryoprotectant (C1). All the tubes were placed in an incubator at room temperature for 24 h. After the incubation time the absorbance was read at photometer at 600 nm. The absorbance is given by the bacterial density in growth medium.

3.Results and discussion

Preservation and storage of micro-organisms by various methods of preservation and drying has been used for decades. From the large number of methods used for storage of microorganisms, the more effective methods are long-term cryopreservation and lyophilization.

In the present study we proposed to determine the efficiency of some techniques on long term microorganism's conservation as cryopreservation and freeze-drying and the influence of cryoprotectants used in various concentrations on survival rate of bacterial strains during cryopreservation at -80 °C in freezer and - 196 °C in liquid nitrogen.

As a result of studies carried out, it was shown that the survival rate of the culture depends on the microbial strains of the microorganism used, the method of storage, cryopreservation or lyophilization, and in the case of cryopreservation, on the cryoprotectant agent used and the cryopreservation temperature -80 °C or -196 °C.

Freeze-drying is one of the most commonly used methods for the conservation of micro-organisms in the collections of microorganisms, because it allows storage in a small volume at a temperature of 1-4 °C which affords easy transportation of these.

In this respect we would like to point out that the survival rate of the lyophilized microorganisms, the results obtained are summarized in the table below:

Table 1. The viability of pathogenic and lactic bacterial cultures subjected to lyophilization process (absorbance 600nm)

	Klebsiella pneumonie	*E.coli*	*Salmonella enterica*	*Staph. aureus*	*Bacillus cereus*	*Ps. aeruginosa*	*Lactobacillus casei*
Control	2.562	1.965	2.088	2.401	2.089	1.901	2.382
Lyophilized culture	1.98	1.759	1.167	1.94	1.697	1.81	1.872

In the case of freeze-drying of the bacterial cultures, without using any protective agent, it has been observed that, in some bacterial species, the survival rates are closely to the control and other species are at half of the value observed in the control group.

At *Staphylococcus aureus*, absorbance of the control sample was 2.401 cm^{-1} and the absorbance of the freeze-dried sample was 1.94 cm^{-1}. The

species *Bacillus cereus* absorbance's of the control sample was 2.089 cm^{-1} and the freeze-dried sample was 1.697 cm^{-1}. The higher survival rate was observed in *Pseudomonas aeruginosa*, the difference between the absorbance of the control sample and the freeze-dried sample is lowest of all the samples, i.e. 1.901 cm^{-1} in the control sample and 1.81 cm^{-1} at freeze-drying sample. At *Lactobacillus casei* species, the absorbance of the blank was 2.382 cm^{-1} and the freeze-dried sample was 1.872 cm^{-1}.

Considering the survival rate of the bacterial cultures, in descending order, they are: Pseudomonas aeruginosa, *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Lactobacillus casei, Klebsiella pneumonie* and *Salmonella enterica*.

At *Klebsiella*, the best cryoprotectant at -80˚C was DMSO 10% and at -196˚C the best cryoprotectant was glycerol 10% while the most weakest from the cryoprotectants was ethylene-glycol 5 and 10%. DMSO was introduced in cryobiology because it is a very efficient, fast penetrating and universal cryoprotectant additive [12]. DMSO or Me_2SO was utilized in viruses, bacteria, mycroplasmas, cyanobacteria, yeast and fungi cryopreservation [12]. For this culture the best preservation method is cryopreservation at -196˚C, because it appears that the sudden freezing increased the survival rate for this bacterial genus.

Table 2. The viability of pathogenic and lactic bacterial cultures subjected to cryopreservation at -80 °C and -196 °C

Cryoprotectants	Storage temperature	Bacterial cultures						
		Klebsiella pneumonie	*E.coli*	*Salmonella enterica*	*Staph. aureus*	*Bacillus cereus*	*Ps. aeruginosa*	*Lactobacillus casei*
Control 1		2.562	2.265	2.088	2.701	1.897	2.181	2.372
Control 2	-80°C	1.369	1.646	1.159	2.013	0.676	1.5	1.595
	-196°C	2.184	1.524	1.099	2.323	1.439	1.915	1.577
Gl 5%	-80°C	1.832	2.129	1.226	2.381	1.869	1.767	1.752
	-196°C	2.209	1.892	1.194	2.328	1.506	1.717	1.637
Gl 10%	-80°C	1.782	2.24	1.21	2.417	1.619	1.781	1.897
	-196°C	2.259	1.892	1.217	2.666	1.184	2.084	1.384
PG 5%	-80°C	1.76	1.685	1.392	2.185	1.549	1.565	1.344
	-196°C	1.916	1.49	1.267	2.182	1.549	1.704	1.552
PG 10%	-80°C	1.746	1.613	1.523	2.246	1.256	1.538	1.362
	-196°C	2.068	1.542	1.037	2.401	1.494	1.46	1.947
EG 5%	-80°C	1.614	1.635	1.304	2.119	1.521	1.483	1.089
	-196°C	1.922	1.592	1.324	2.267	1.461	1.554	1.566
EG 10%	-80°C	1.974	1.844	1.226	2.27	1.282	1.553	1.251
	-196°C	1.851	1.65	1.238	2.218	1.628	1.271	1.423
DMSO 5%	-80°C	2.004	1.859	1.276	2.417	1.368	1.435	2.248
	-196°C	1.87	1.777	1.461	2.157	1.718	1.016	2.149
DMSO 10%	-80°C	2.141	1.838	1.152	2.002	1.374	1.617	2.246
	-196°C	1.881	1.891	1.315	2.233	1.794	1.469	1.688

At *Escherichia coli* the best cryoprotectant was 5% glycerol for -80˚C and 5% glycerol and 10% glycerol for 196˚C. The weakest cryoprotectant for the same temperature was propylene- glycol 5 and 10%. Glycerol and Me_2SO were the most utilized additives in microbiology. A plus of 5 to 42% glycerol utilized at *E. coli* suspensions allowed survival rate on a long term of these cultures. The glycerol toxicity was observed at *Chlamydia* ssp., *Staphylococcus, Micrococcus, Lactococcus, and Streptococcus.* Thereby, for this bacterial specie, the best preservation method is cryopreservation at -80˚C.

For *Bacillus cereus* the best cryoprotectant was 5 % glycerol for -80˚C and 10% DMSO for -196˚C. The weakest for the same temperatures was propylene-glycol 10 % and glycerol in 10 % concentration. For this culture the best preservation method is cryopreservation at -80˚C.

At *Stahpylococcus aureus* the best cryoprotectant was glycerol and DMSO 5 % for -80˚C and glycerol 10% for -196˚C. The weakest cryoprotectants for the same temperature were ethylene-glycol and DMSO 5%. For this culture, the best cryopreservation method is cryopreservation at -196˚C.

For *Salmonella* genus the best cryoprotectant was propylene-glycol 10% for -80℃ and DMSO 5% for -195℃. For the same temperature, the weakest cryoprotectants were DMSO and propylene-glycol 10%. For this culture the best cryopreservation method is at -80˚C because the survival rate is larger.

The best cryoprotectant for *Pseudomonas aeruginosa* was 5 and 10% glycol for -80℃ and 10% glycerol for -196˚C. For the same temperatures the weakest cryoprotectant was 5% DMSO. For this culture the best preservation method is cryopreservation at -196˚C.

For *Lactobacillus casei* culture the best cryoprotectant was DMSO in 5 and 10% concentrations for -80˚C and 5 % DMSO had the best results for -196˚C. The weakest cryoprotectant for the same temperatures was 5% ethylene-glycol and 10 % glycerol. For this culture the best preservation method is cryopreservation at -80˚C.

4. Conclusions

The temperature change rate, controls transport of water around cell membranes and indirectly likelihood of intracellular freezing. If the cooling is too fast, the membranes cannot carry water out of the cell and freezing inside. Each cell has an optimum cooling rate, while the survival of very low absolute except that cryoprotector is present to reduce freezing damage.

In the case of freeze-drying of the bacterial cultures, without using any protective agent, it has been observed that, in some bacterial species, the survival rates are closely to the control and other species are at half of the value observed in the control group.

The storage for long term of microorganisms it can be made by using the lyophilization method or by using the cryopreservation method. In cryopreservation the addition of a cryoprotectant to the samples is essential. From the four cryoprotectants that we used, the best results were given by DMSO or glycerol utilization.

References

1. Alexander, M.T., Simione, F.P. Factors affecting the recovery of freeze-dried Saccharomyces cerevisiae. Abstracts of the Annual Meeting of the American Society for Microbiology, 1980, p. 86.

2. Nei, T., T. Araki, T. Matsusaka. Freezing injury to aerated and non-aerated cultures of Escherichia coli. In T. Nei, Ed. Freezing and Drying of Microorganisms. University of Tokyo Press, 1969, Tokyo, Japan.

3. Kleinhans, F. W. Membrane permeability modelling: Kedem-Katchalsky vs a two-parameter formalism. Cryobiology, 1989, 37, 271–289.

4. Hubalek, Z. Protectants used in the cryopreservation of microorganisms. Cryobiology, 2003, 46, 205–229.

5. Israeli, E., Shaffer, B.T., Lighthart, B. Protection of freeze-dried Escherichia coli by trehalose upon exposure to environmental conditions. Cryobiology, 1993, 30, 519–523.

6. Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H., Crowe, L.M. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Applied and Environmental Microbiology, 1995, 61 (10), 3592–3597.

7. Crowe, J.H., Carpenter, J.F., Crowe, L.M. The role of vitrification in anhydrobiosis. Annual Review of Physiology, 1998, 60 (1), 73–103.

8. Streeter, J.G. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J. Appl. Microbiology, 2003, 95, 484-491.

9. Abadias, M., Teixido, N., Usall, J., Benabarre, A., Vinas, I. Viability, efficacy, and storage stability of freeze-dried biocontrol agent Candida sake using different protective and rehydration media. Journal of Food Protection, 2001, 64 (6), 856–861.

10. Desmond, C., Ross, R.P, O'Callaghan, E., Fitzgerald, G., Stanton, C. Improved survival of Lactobacillus paracasei NFBC 338 in spray-dried powders containing gum acacia. Journal of Applied Microbiology, 2002, 93, 1003–1011.

11. Tao, D., Li, P.H. Classification of plant cell cryoprotectants, J. Theor. Biol., 1986, 123, 305–310.

12. Bodas, K., Brennig, C., Diller, K.R., Brand, J.J. Cryopreservation of blue-green and eukaryotic algae in the culture collection at the University of Texas at Austin., Cryo-Letters, 1995, 16, 267-274.