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SURVIVAL OF *BACILLUS* SP., *PSEUDOMONAS* SP. AND LACTIC ACID BACTERIA SUBJECTED TO FREEZE-DRYING AND LONG-TERM STORAGE

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ABSTRACT

National Collection of Nonpathogenic Microorganisms (NCNM) contains bacterial diversity of micromycetes, yeasts, actinomycetes, bacteria, cyanobacteria, microalgae with antimicrobial activity, stimulator effect on plant productivity, producers of vitamins, porphyrins, siderophores, exopolysaccharides, phytohormones, polypeptide antibiotic substances, lactic and acetic acids, etc. Freeze-drying provides high cell viability, is used for the long-term preservation, also it plays a fundamental role in scientific and practical fields.

Key words: freeze-drying, bacteria, viability.

Introduction

Lyophilization is a widespread and safe method of preserving the stability of the biological properties of microorganisms.

The National Collection of Nonpathogenic Microorganisms (NCNM) contains bacterial species like *Pseudomonas* sp., *Bacillus* sp., which are known like biological control agent, source of vitamins, phytohormones, bioinsecticide, exometabolites for plant protection with antimicrobial activity and stimulator effect on plant productivity. For these reasons it can be applied for advantageous bacterial preparations insect and plant disease control.

Authors Bogdan, Petatán-Sagahón, Barreto demonstrated that due to high antifungal activity against phytopathogens *Alternaria alternata*, *Botrytis cinerea*, *Fusarium solani*, *Fusarium oxysporum* and *Aspergillus niger, Bacillus* sp. and *Pseudomonas* sp. cultures are characterized are very promising for biotechnology field (2, 3, 12). NCNM also contains lactic acid bacteria isolated from naturally fermented homemade dairy foods. Lactic acid bacteria and are destined for sour cream, fresh cheese, yoghurt, soy milk, brined cheese production. These bacteria have the ability to produce better taste, flavour and texture of the fermented food, ensures the product manufacture with beneficial microorganisms in abundance, extending the shelf-life and enhancing the safety of food products due to produce of lactic acid as antimicrobial substances.

Freeze-drying provides higher cell viability needs for the long-term preservation, also it plays a fundamental role in scientific and practical fields. Depending on the resistance against freeze-drying process bacterial strains can be divided in three groups: resistant bacteria, medium resistant and sensitive bacteria. According to this classification, bacteria belonging *Pseudomonas* and *Bacillus* genus are medium and resistant to lyophilization process.

Conservation of scientific and industrially valuable strains of microorganisms (fungi, yeasts, actinomycetes, bacteria, cyanobacteria, microalgae) for long-term storage, without transfers, which can change the properties of microorganisms present the main goal for every collection inclusive for NCNM.

Lyophilization of bacteria is carried out with the use of protective media containing sugars and protein compounds, due to what milk is better than water or saline where suspended cells do not survive (1, 5, 14). Authors Kupletskaya and Netrusov mentioned the fact that sugars from protective media increase the osmotic pressure into the cells and preventing formation of ice crystals and destruction of the cells in the course of freezing (7).

But not all bacteria can be successfully freeze-dried. According to Miyamoto-Shinohara certain strains might not survive after freeze-dried. The best way to determine if a strain is amenable to freeze-drying is to evaluate its stability post–freeze-drying (8, 9, 10).

The aim of the research was to check the viability and stability of some pure *Bacillus* sp., *Pseudomonas* sp. and lactic acid bacteria strains after 15 years storage in NCNM. Lactic acid bacteria pure cultures used in this study included *Lactococcus* sp. strains and *Streptococcus thermophilus* strains.

Cell viability during storage is important for cultures used for the direct application to matrices for food and/or agriculture industries.

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Materials and methods

For cultivation of mentioned strains nutrient agar medium and sterile skimmed milk were used as a nutrient medium at a temperature of $+30^0$C, for up to 72 hours. Skimmed milk served as a protective medium for lyoflization. Samples were subjected to freezing at temperature -80^0C, then lyophilized at the Free Zone Plus installation. Research was carried out according to the standard method (17, 18, 19).
Bacterial strains were activated according to the conditions. The number of viable cells was determined as colony forming units per ml (CFU/ml). The survival rate was calculated as CFU/ml after freeze-drying divided by CFU/ml before freeze-drying (15). For lactic acid bacteria coagulation properties, acidity and lactic acid production, organoleptic properties and microscopy at OPTIKA B-510PH also were investigated. All the experiments were carried out in triplicate, and results were statistically processed.

Results and discussions

Freeze-drying is a useful, effective and gentle technology for preserve microorganisms. Freeze-dried cultures of *Bacillus* sp. and *Pseudomonas* sp. are used in agriculture and microbiological industry. Strains, culture conditions and cryoprotectants choosing should be considered. Protective agents such as skimmed milk, whey, glycerol, glucose are commonly employed to protect bacterial cultures (5).

Accordingly, *Bacillus* sp. and *Pseudomonas* sp. strains are viable and their titer was ranged from 6,8 to 7,6 $\log_{10}$UFCml^{-1} for *Bacillus* sp. (fig.1) and from 7,9 to 8,1 $\log_{10}$UFCml^{-1} for *Pseudomonas* sp. (fig.2).

It is known that *Pseudomonas* and *Bacillus* bacteria can be stored for over 30 years in freeze-dried form with no changes of high level cell viability at 6-7 $\log_{10}$UFCml^{-1}.

Sharma et al. reported skim milk as cryoprotectant for lactic acid bacteria lyophilization (13) at the same time Yeo et al. recommended a mix of 10% skim milk and 10% sucrose with 2.5% sodium glutamate (16). Author Mostafa studied two media for protecting *Bifidobacterium longum* and *Lactobacillus helveticus* skim milk and gelatin/glycerol medium were the most suitable. Viability loss of *B. longum* and *L. helveticus* was 20-92% at gelatin/glycerol medium and 36-44% at skimmed milk (11).

Lactic acid bacteria strains after 15 years of storage in freeze-dried form demonstrated a survival percent of more than 70% with titer ranged from 6,2 to 8,3 $\log_{10}$UFCml^{-1}. According to other studies viability of species *Streptococcus, Staphylococcus, Brevibacterium, Pseudomonas, Corynebacterium, Lactobacillus, Salmonella, Bacillus* after freeze-drying amount to min. 70%. Thus, the numbers of viable cells remaining in the ampoules are sufficient to maintain the culture. In tab. 1 summarizes the viability data.

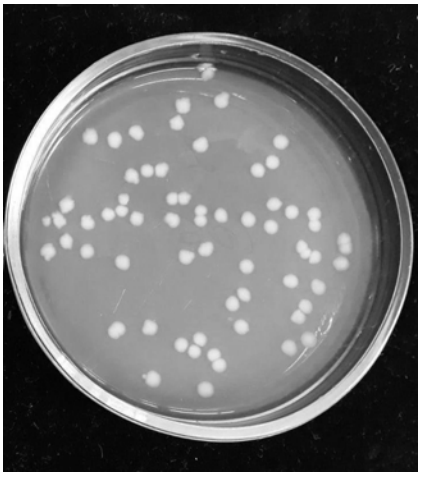

Fig. 1 - *Bacillus* sp. colonies morphology on nutrient agar medium

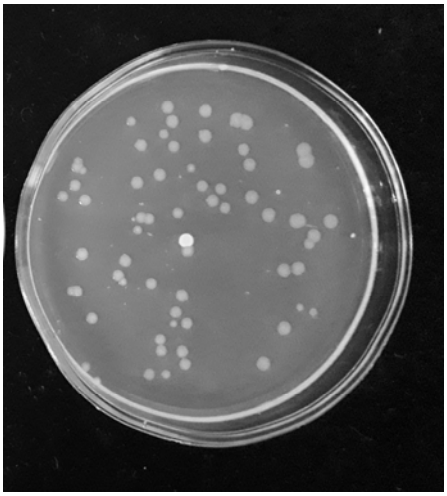

Fig. 2 - *Pseudomonas* sp. colonies morphology on nutrient agar medium

Table 1 - Viability data of freeze dried bacteria

Freeze dried bacteria of NCNM	Survival rate (%)
Bacillus sp.	82,07
Pseudomonas sp.	87,2
Lactococcus lactis ssp.	91,57
Streptococcus thermophilus	88,62

Microscopic examination confirmed the purity of cultures, *Bacillus* sp. represented rod-shaped Gram-positive cells and *Pseudomonas* sp. Gram-negative. Lactic acid bacteria present cocci in short or long chains (fig.3) and were able to active milk coagulation, dense consistency, without gas eruption.

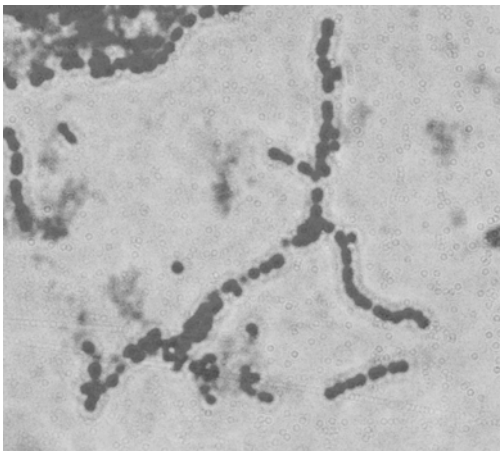

Fig. 3 – *Lactococcus lactis ssp.* microscopic characterization

Researchers Harrison and Pelczar say that the reason why lyophilized material appears to maintain its characters so well may be because the usual holding a period a few years is relatively short as compared with the potential longevity of the suspension. They also refer to the viability of *Lactobacillus bifidus*, *Lactobacillus fermenti, Micrococcus albus, Pseudomonas* sp. of level cell viability at 6-8 $\log_{10}$UFCml^{-1} (6).

The authors Chiselitsa et al. highlight the effectiveness of freeze-drying as a method of conservation and sustainable retention of bacteria due to what that *Bacillus* sp. had 5,7 to 7,5 log10 UFC ml^{-1} and *Pseudomonas* sp. bacteria had titer of 6,6-6,8 log10 UFC ml^{-1} (4).

Further, all strains of lactic acid bacteria showed active coagulation, forming homogeneous dense consistency curd and with acidity limit of 99 ^{O}T to *Streptococcus thermophilus* and 128^{O}T to *Lactococcus lactis ssp.* The obtained results confirm that NCNM stored biotechnological potential of *Bacillus* sp., *Pseudomonas* sp., *Lactococcus lactis ssp.* and *Streptococcus thermophilus* strains.

Conclusions

Our experiments have shown that after 15 years of storage the viability of lyophilized bacterial strains decreased, but the titer of viable cells is maintained at 10^7 to preserve valuable properties.

The results of the morphological, cultural and technological properties of lactic acid bacteria correspond to the species of the studied strains and to the requirements of the normative documents.

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Rezumat

Colecția Națională de Microorganisme Nonpatogene (CNMN) conține o diversitate de micromicete, drojdii, actinomicete, bacterii, cianobacterii, microalge cu activitate antimicrobiană, efecte stimulatoare asupra productivității plantelor și capacitatea de a produce vitamine, porfirine, siderofori, exopolizaharide, fitohormoni, substanțe antibiotice polipeptidice, acizi lactic și acetic, etc.

Liofilizarea asigură o viabilitate celulară ridicată, fiind utilizată pentru conservarea pe termen lung și având un rol fundamental în domeniile științifice și practice.

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