

## **Mathematical Evaluation of Factors that Influence on the Survivability of Some Prokaryotes and Eukaryotes after Freeze-Drying**

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### **Abstract**

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The mathematical evaluation of the importance of the factors “regime of freezing” and “cryoprotective medium” on the survivability of probiotic microorganisms of prokaryotic and eukaryotic type after freeze-drying is conducted.

For the first time, the cryoprotective effect of some natural polymeric compounds types, such as rubbers (complex plant oligo- and polysaccharides) is investigated.

It is found that the application of investigated cryoprotectors in optimal concentrations increases the survivability of probiotic microorganisms after freeze-drying. The results obtained, are compared with analytical samples without cryoprotectors and samples including saccharose as traditional cryoprotector.

*Key words:* mathematical evaluation, freeze-drying, survivability, prokaryotes, eukaryotes

### **Introduction**

The effectiveness of cryoconservation, as a main method for preservation of microorganisms and their specific properties, depends on a complex of various factors, including cell specifications, their physiological state influenced by the temperature factor, level of cell stability, genetic potential, level and speed of freezing, ambient conditions etc. (Georgieva et al., 2006). During the time of cryogenic treat-

ment the cells are subjected to temperature and osmotic stress. Therefore the use of cryoprotective medium will protect the cells through eliminating some of the damaging factors (Belouse et al., 1985). The importance of the cryoprotectors for the development of science and practice suggests the implementation of scientific investigations into different substances and compounds of various origin that have protective influence on the cells at low temperatures (Georgieva et al., 2006).

For the objective outlined, the cryoprotective media are required to have the following characteristics:

- Dehydration activity
- Easy absorption in tissue
- Water bond effectiveness, the so-called “coligative properties”
- Relatively low toxicity
- low eutectic temperature

In the present paper for the first time we investigate the cryoprotective effect of some types natural water soluble polymeric compounds such as rubbers - complex plant oligo- and polysaccharides (Obretenov, 2002). It is well known that rubbers are used in the food industry as to improve some quality indicators such as stabilizers, jelly agents, emulsifiers etc. Under their influence the quality of free water changes, respectively the water bonds that determine the physiological properties of the products (Kratchanov, 2001). Furthermore, they influence on the technological processes: better water retain ability, lower the speed of their evaporation, change the freezing speed, influence on the modification of the formed crystals in the product, etc., i.e. rubbers have properties that are of significant importance for biomaterials lyophilization of various origin (Tsvetkov, 1982).

*Main objective of our investigations:* determination through mathematical evaluation of the importance of the factors “regime of freezing” and “cryoprotective medium” on the survivability of probiotic microorganisms of prokaryotic and eukaryotic type after freeze-drying.

## Materials and Methods

Object of experiments conducted as prokaryotes are lactic acid microorganisms provided by the collection of ICFT:

*Lactobacillus bulgaricus* strain 3556  
*Streptococcus thermophilus* strain 1374 and yeasts (eukaryotes) *Saccharomyces cerevisiae* strain 1248

Cryoprotective media applied are:

1. Saccharose
2. Microseaweeds as a source of fibre-like oligosaccharides /produced by “Medica AD”, Sandanski/
3. Fructooligosaccharide “Frutafit IQ” /produced by “Foods Consulting”/

According to common standardized methods physico-chemical investigations are conducted and following **microbiological indicators** determined:

- **Determination of the number of viable lactic acid bacteria** according to the method of limited dilutions and calculations based on the table of McCrady
- **Determination of the number of viable yeasts** (KOE/ml) according to the Koch method.
- **the survivability of the lactic acid microflora and yeasts** after freeze-drying, according to the formula:

$$\% \text{ survivable cells} = (b/a) \times 100,$$

where: b- number of viable cells before lyophilization; a- number of viable cells after lyophilization

### *Mathematical evaluation of results:*

To the data analysis of the method of one-factor and two-factor dispersion analysis and standard statistical computer programs, such as Excel are applied.

**Technological approaches:** In the process of technological investigations we mix the suspensions of lactic acid bacteria and yeasts in colloid solutions of the respective protective media in proportion 1:1 and control samples – strains without protector.

Freezing- two regimes of freezing are applied: 1) Slow freezing on air media with compulsory air convection at temperature from -30 to -35 °C and shock freezing with liquid nitrogen at temperature -196° C.

Freeze-drying is conducted in vacuum sublimate installation TG 16-50, with contact plates heating for 24 hours at final temperature of the dried products: + 30° C.

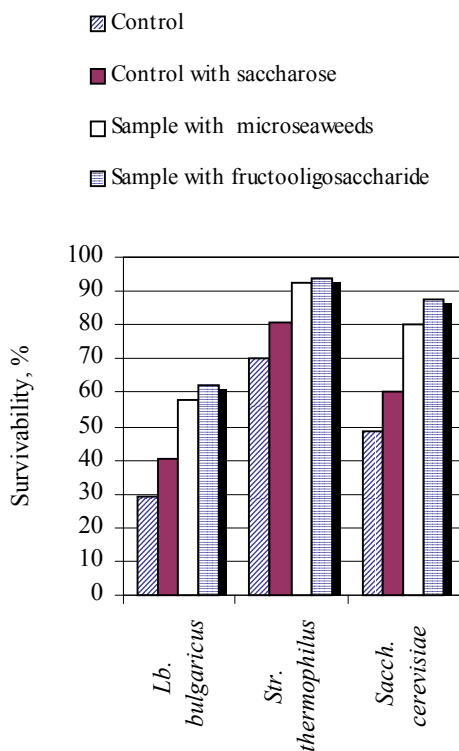
### Results and Discussion

The obtained data, related to the survivability of strains lactic acid bacteria and yeasts after lyophilization at two regimes of freezing and three cryoprotective me-

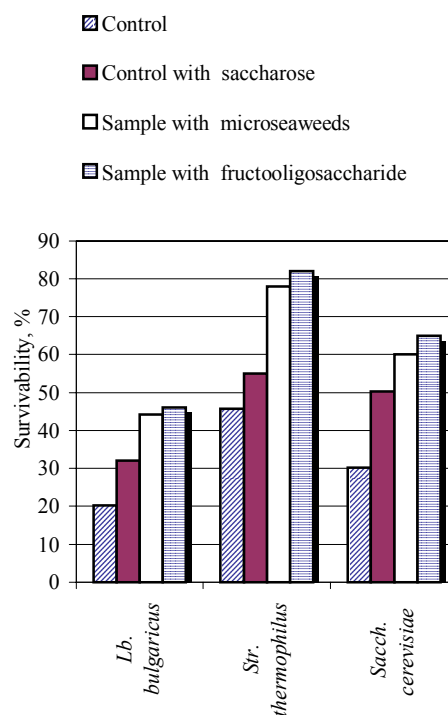
dia used, are presented in Figure 1 and Figure 2.

**In the process of the experiments conducted, the following conclusions are drawn:**

1. Figures 1 and 2 show that the survivability is lowest in the cases of the lactic acid bacteria and yeasts in the control sample without cryoprotector and the saccharose as a cryoprotective medium used. The highest survivability on the microorganisms of prokaryotic type (*Str. thermophilus*, *Lb. bulgaricus*) is achieved by means of the cryoprotector fructo-oli-



**Fig.1. Survivability of probiotic microorganisms after lyophilization, during the freezing regime (-196 °C)**



**Fig.2. Survivability of probiotic microorganisms after lyophilization, during the freezing regime (-30 -35 °C)**

gosaccharide. In the case of eukaryotic microorganisms (*Sacch. cerevisiae*), in spite of the slight difference, the seaweeds show better cryoprotective effect. In all control samples the negative influence of the low temperature can be optimal eliminated as a result of the colloid character of the samples, due to the cryoprotectors - seaweeds and oligosaccharides. The quantity of water in the samples, influenced by the osmotic pressure, depends on the molar concentration of the soluble substances and degree of their ionization.

2. The obtained data for the survivability of lactic acid bacteria and yeasts at various regimes of freezing range variously. In all control and variant samples, treated before lyophilization, according to both methods of freezing, high degree of survivability of *Str. thermophilus* to freeze-drying is reported – at quick freezing (from 70.4 to 94%), at slow drying (from 45.8 to 88.24%) in comparison with *Lb. bulgaricus* and *Saccharomyces cerevisiae* that are thermo labile. It is also observed a considerable advantage of low temperature, high speed (shock) freezing respectively from 70.4 to 94.0 % in *Str. thermophilus*; from 29.0 to 62.0 % in *Lb. bulgaricus* and from 48.6 to 77.5 % in *Saccharomyces cerevisiae*. It is likely the reason for the various resistances of the microorganisms to the drying can be found in the various sensitivity of the cell wall to the pressures, occurring at freezing and drying, or the different ability of the cell enzymes to denaturation (Wakerbauer et al., 2003).

In the mathematical evaluation of results we apply the one-factor dispersion analysis for determination of the importance of the factor –“regime of freezing” and the two-factor analysis for investiga-

tion the influence of the cryoprotective media used and the temperature factor on the survivability of lactic acid bacteria and yeasts after lyophilization.

This mathematical evaluation is necessary for comparison of the theoretical with the practical results and their scientific reasoning.

In principal the method of dispersion analysis is based on the comparison of two independent and different evaluations for the total dispersion of data and also a method for checking the hypotheses for difference between average quantities (Miteva, 2003).

In Table 1 are presented the data from the statistical evaluation, based on the one-factor dispersion analysis for the influence of the factor –“regime of freezing” (-196°C) and (-30 -35°C) on the survivability of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae* in various types of cryoprotective media.

In order to be clarified the degree and reliability on the influence of the respective factor, it is necessary to estimate and evaluate this part of the total diversity that is induced by the factor. The evaluation is accomplished with the help of two quantities – dispersion (the availability of diversity within the group and the primary value as a determination of the diversity degree) and deviation (dispersion equivalent to one element of free diversity or one degree of freedom).

In our investigations, the regime of freezing (-196 °C) of *Streptococcus thermophilus*, influences to a significant degree on the organized factors - 87.0%, whereas the casual factors are measured up to 13.0%. In the case of *Lactobacillus bulgaricus* the influence of the observed factor is more obviously expressed-

**Table 1**  
**Influence of the factor “regime of freezing” on the survivability of lactic acid bacteria and yeasts**

| Factor x                 | Microorganisms                    | $\bar{x}$           | F <sub>x</sub>         | F <sub>t</sub>   | $\eta^2_x$ (%) | $\eta^2_z$ (%) |
|--------------------------|-----------------------------------|---------------------|------------------------|------------------|----------------|----------------|
| Regime of freezing       | <i>Streptococcus thermophilus</i> | 7,5.10 <sup>8</sup> | 27,53926 <sub>3</sub>  | 4.8 <sub>3</sub> | 87             | 13             |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |
| (-196 <sup>0</sup> C)    | <i>Lactobacillus bulgaricus</i>   | 9,5.10 <sup>7</sup> | 712,1685 <sub>3</sub>  | 4.8 <sub>3</sub> | 98             | 2              |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |
|                          | <i>Saccharomyces cerevisiae</i>   | 3,4.10 <sup>8</sup> | 4790,2368 <sub>3</sub> | 4.8 <sub>3</sub> | 96             | 4              |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |
| Regime of freezing       | <i>Streptococcus thermophilus</i> | 9,5.10 <sup>8</sup> | 58,4457 <sub>3</sub>   | 4.8 <sub>3</sub> | 94             | 6              |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |
| (- 30-35 <sup>0</sup> C) | <i>Lactobacillus bulgaricus</i>   | 6,5.10 <sup>7</sup> | 371,7368 <sub>3</sub>  | 4.8 <sub>3</sub> | 91             | 9              |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |
|                          | <i>Saccharomyces cerevisiae</i>   | 3,1.10 <sup>7</sup> | 209,4244 <sub>3</sub>  | 4.8 <sub>3</sub> | 90             | 10             |
|                          |                                   |                     |                        | 3.3 <sub>2</sub> |                |                |
|                          |                                   |                     |                        | 2.3 <sub>1</sub> |                |                |

F<sub>x</sub>- indicator for reliability on the influence of the factor – “regime of freezing”

98% of the factor is due to the type of freezing and only 2% as a result of the casual factors. Similar proportion is observed in the case of yeasts - 96%: 4%, i.e. the survivability of lactobacilli and yeasts depends to a higher degree on the type of freezing. This statement is confirmed not only by our analytical analyses and other authors as well (Uzunova – Doneva et al., 2002).

At the regime of freezing (-30 °C - 35°C) of *Streptococcus thermophilus*, the

influence of the organized factors is increased in comparison with the values at slow freezing- 94%, whereas the influence of the casual factors is considerably low compared to quick freezing-6.0%.

In the case of *Lb. bulgaricus* and *Str. cerevisiae* the influence of the regime of freezing inconsiderably decreases, but also the values are also significant-respectively 91.0% and 90.0% for the organized factors and increase of the casual factors respectively 9.0% and 10%.

**Table 2**  
**Influence of the factors “regime of freezing” and “cryoprotective medium” on the survivability of lactic acid bacteria and yeasts after freeze-drying**

| Microorganisms                    | (F <sub>A</sub> ) | (F <sub>B</sub> ) | (F <sub>AB</sub> ) | (F <sub>X</sub> )         | $\eta^2_{A(\%)}$ | $\eta^2_{B(\%)}$ | $\eta^2_{AB(\%)}$ | $\eta^2_{X(\%)}$ | $\eta^2_{Z(\%)}$ |
|-----------------------------------|-------------------|-------------------|--------------------|---------------------------|------------------|------------------|-------------------|------------------|------------------|
| <i>Streptococcus thermophilus</i> | 129               | 27.97             | 3.56               | <u>92.45</u> <sub>3</sub> | 42.6             | 49               | 8.4               | 6.9              | 93.1             |
| <i>Lactobacillus bulgaricus</i>   | 462               | 508               | 28.25              | <u>74.50</u> <sub>3</sub> | 43.12            | 46.94            | 9.94              | 3.18             | 96.82            |
| <i>Saccharomyces cerevisiae</i>   | 367               | 408               | 31.01              | <u>84.63</u> <sub>3</sub> | 46.27            | 42.23            | 11.50             | 2.11             | 97.89            |

(F<sub>A</sub>) - indicator for reliability on the influence of the factor – “regime of freezing”

(F<sub>B</sub>) - indicator for reliability on the influence of the factor – “cryoprotective medium”

(F<sub>AB</sub>)- interaction between (F<sub>A</sub>)and (F<sub>B</sub>)

$\eta^2_A$  - degree of factor influence” regime of freezing” ( %)

$\eta^2_B$  - degree of factor influence ” cryoprotective medium” ( %)

$\eta^2_{AB}$  - interaction between  $\eta^2_A$  and  $\eta^2_B$  ( %)

$\eta^2_X$  - total influence of organized factors ( %)

$\eta^2_Z$  - total influence of casual factors ( %)

As to prove the obtained indicator (F<sub>X</sub>) is sufficient and the influence of the organized factors is reliable, we compare the criteria F<sub>X</sub> with values in the table at the given degrees of freedom. In our calculations the criterion F<sub>X</sub> for *Str. thermophilus*, *Lb. bulgaricus* and *Saccharomyces cerevisiae* in both regimes of freezing exceeds the three degrees of probability for reliability on the results: P<sub>1</sub> = 0.095, P<sub>2</sub> = 0.99, P<sub>3</sub> = 0.999. Consequently, we can assume the obtained results for sufficiently reliable, that is also confirmed by the electronic results processing.

In Table 2 are presented the data based on the statistical processing according to the two-factor dispersion analysis for the influence of the factors “regime of freezing” (F<sub>A</sub>) and “cryoprotective medium” (F<sub>B</sub>) on the survivability of *Str. ther-*

*mophilus*, *Lb. bulgaricus* and *Saccharomyces cerevisiae* after freeze-drying.

The mathematical processing proves that:

- In the case of *Streptococcus thermophilus*, the influence of the regime of freezing has a weaker influence on the survivability of the microorganisms – 42.6 % in comparison with the respective cryoprotective medium – 49.0 %.

The interaction between both factors is 8.4 %.

- Analogical is the situation of *Lactobacillus bulgaricus* after lyophilization. The influence of the regime of freezing is weaker- 43.12 % in comparison with the influence of cryoprotective medium applied – 46.94%.

- The regime of freezing has consider-

able influence on the survivability of *Saccharomyces cerevisiae* after freeze-drying. Compared to streptococci and lactobacilli, this value is more significant – 46.27%. Regarding the influence of the cryoprotectors applied on the survivability of *Saccharomyces cerevisiae*, it is determined that the value is lower than in the case of streptococci and lactobacilli – 42.23%. Furthermore, the interaction between both factors is more active – 11.5%.

On the basis of investigations conducted, the criteria  $F_x$  are equivalent to  $P_3=0.999$  according to the respective value in the table. This proves the influence of both factors and their interaction as well, is sufficiently reliable.

## Conclusions

The lactic acid microorganism and yeasts after freeze-drying, conducted after quick freezing with liquid nitrogen, show higher survivability, compared to those in the control samples and experimental samples, containing saccharose.

For the first time are conducted investigations for determination of the cryoprotective effect of the natural water soluble polymers such as rubbers. The results obtained prove that rubbers can be classified as effective endocellular cryoprotectors during probiotics lyophilization.

The mathematical evaluation of the factors, influencing on the survivability of lactic acid bacteria and yeasts, proves the prevailing influence of the cryoprotective media on prokaryotic microorganisms and stronger influence of the regime of freezing on the eukaryotes.

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