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THE INFLUENCE OF LYOPHILIZATION CONDITIONS ON YEAST VITALITY AND FERMENTATION ACTIVITY

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***Abstract**: The aim of this reserach was to consider the influence of the age of yeast culture on their survival after lyophilization and in the process of further storage. Two-, three-, seven- and thirtheen-day-old cultures were used for lyophilization, which were grown in tubes on shira-agar. Properly performed lyophilization significantly affects the survival and fermentation activity of yeasts. Three-day-old yeast cultures are most vital during lyophilization. Yeasts survive best in protective media containing 10% sucrose, 1% gelatin, 0.1% agar-agar and 2% $CaCO_3$, or 10% skim milk and 1% sodium glutamate. Prolonged storage in the dried state and rehydration lead to decreasing in reproductive energy and fermentation activity of yeasts.*

***Key words**: yeasts, lyophilization, survival, fermentation.*

INTRODUCTION

Lyophilization is a well known dehydration method widely used to preserve microorganisms. It is also commonly used in food preservation and for a variety of pharmaceutical applications, including protein-based drugs (Cleland et al., 2001). When a single microorganism or a microbial consortium is selected as a starter culture, its conservation for subsequent use in fermentations is required. Thus, storage at low temperatures (freezing or refrigeration) and lyophilization techniques are often applied to maintain the viability of starter cultures. Lyophilization is an easy way to keep a high number of viable microorganisms and the powder forms would require a low number of procedural steps (Pradelles et al., 2009). By its ability of combining freezing and drying in a unique operation, this process can create final dried products with the highest quality, but the freeze-drying step is especially

critical as it negatively affects both viability and physiological state of the yeasts. The formation of ice crystals induces mechanical damage that leads to cellular death during freezing (Tymczyszyn et al., 2005).
Although freezing itself has not a lethal effect for cells, it can induce physical stress that can injure a part of these cells, thus decreasing the proportion of viable cells (Pehkonen et al., 2008). If freezing is extremely slow, intracellular water can flow to the outer environment by osmosis and create extracellular crystals, thus causing extracellular water removing and solute concentration increase that lead to an osmotic imbalance (Miyamoto-Shinohara et al., 2008). Conversely, if freezing is too fast, cells cannot lose water fast enough to maintain the balance, so intracellular ice crystals can appear thus producing damaging or even lethal effects.

This is a very complex physical process in which cell survival is affected by many physico-chemical and biological parameters, such as cell growth conditions, type of lyoprotectant, freezing, sublimation and thawing temperatures, degree of dehydration achieved, reconstitution medium, and time and storage and rehydration conditions have been described to have great influence on the survival of microorganisms (Peiren et al., 2018). On the other hand, beside this factors, several biological factors affects microbial survival during this process including the intrinsic resistance traits of the strains, initial concentration of microorganisms, growth conditions, and the drying medium (Morgan et al. 2006). An enhanced stability of microorganisms can be achieved by adding many protective compounds such as disaccharides, polyols, monosaccharides, skim milk, and other organic molecules (Hubalek, 2003).

Lyophilization of yeast is one of the optimal ways of storing cultures and is widely used in many laboratories that have collections of microorganisms. Experience working with collections of microorganisms shows that the conditions of lyophilization, such as the age of the culture and the composition of the applied protective media, have a significant impact on the survival of microorganisms in the drying process and during their further storage (Kursop and Doybe, 2001; Abadias et al., 2001; Miyamoto-Shinohara et al., 2010).

The aim of this reserach was to consider the influence of the age of yeast culture on their survival after lyophilization and in the process of further storage.

MATERIAL AND METHODS

Two-, three-, seven- and thirtheen-day-old cultures were used for lyophilization, which were grown in tubes on shira-agar. The yeast from the oblique agar was washed with 3 mL of protective medium (0.1% sucrose-gelatin agar). 0.1 mL of yeast suspension in a protective medium was added to

2 mL glass ampoules. In order to determine the number of surviving cells in the dried material, their concentration in the initial suspension was determined before lyophilization. Ampoules with lyophilized yeast were stored at +4 to +6 ^{o}C in the refrigerator. Through 24 hours, half a year and one year of storage, the yeast was rehydrated by adding sterile distilled water to open ampoules and keeping the cells for 60 minutes at room temperature before sowing on broad agar. The titer of live yeasts was expressed as a percentage of the number of grown colonies after 24 h, half a year and one year of storage in the lyophilized state according to their number before conservation, taken as 100%.

Statistical analysis

The obtained results were statistically processed using the software package SPSS 20. To determine the statistical significant differences between the obtained values, one-way analysis of variance (ANOVA test) was performed, followed by post hoc Tukey's test ($p = 0.05$).

RESULTS AND DISCUSSION

Lyophilisation is one of the most successful methods for preserving bacteria, yeasts and sporulating fungi (Spadaro et al., 2010). This method offers convenience of storage and postage, and it keeps the microorganisms viable for long periods of time (Miyamoto-Shinohara et al., 2006). However, lyophilisation is relatively expensive as it requires sophisticated equipment and adequate power supply.

In the Table 1 are shown the results for the the influence of the age of lyophilized yeasts on their survival. In accordance, the highest titer of vital yeasts, both one day after lyophilization and during storage for one year, was observed in three-day-old cultures. After one year of storage, it decreased from 95% to 60%, while in cultures of other ages, the vitality titer was only 20 to 30%. Therefore, three-day-old yeast cultures were optimal (by age) for successful lyophilization (Fateeva et al., 2016). Neverthelses, can be seen that there is statistically significant differences ($p<0.05$) between the number of living cells at different yeast ages.

This is in line with the study from Donev et al. (1995) who pointed out that in the lyophilization of yeasts of genera *Candida* and *Saccharomyces* for collection purposes three-day-old cultures obtained through surfaced cultivation on solid medium have to be preferred. This fact has been proved many times in the routine work of the National Bank for Industrial Microorganisms and Cell Cultures.

Table 1. The influence of the age of lyophilized yeasts on their survival

Yeast age, days	Before lyophilization of yeast		A day after lyophilization		Half a year after lyophilization		One year after lyophilization	
	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)
2	$3.4^{a} \times 10^{7} \pm 0.01$	100	$2.2^{b} \times 10^{7} \pm 0.03$	65	$1.4^{c} \times 10^{7} \pm 0.03$	40	$1.0^{d} \times 10^{7} \pm 0.02$	30
3	$5.0^{a} \times 10^{7} \pm 0.02$	100	$4.8^{a} \times 10^{7} \pm 0.01$	95	$3.6^{b} \times 10^{7} \pm 0.03$	70	$3.0^{c} \times 10^{7} \pm 0.01$	60
7	$2.2^{a} \times 10^{7} \pm 0.00$	100	$1.5^{b} \times 10^{7} \pm 0.02$	70	$9.0^{c} \times 10^{6} \pm 0.02$	40	$6.0^{d} \times 10^{6} \pm 0.01$	27
13	$2.0^{a} \times 10^{7} \pm 0.01$	100	$1.0^{b} \times 10^{7} \pm 0.02$	50	$6.0^{c} \times 10^{6} \pm 0.03$	35	$4.0^{d} \times 10^{6} \pm 0.02$	20

$^{a, b, c, d}$ - values marked with different letters, have a statistically significant difference ($p<0.05$).

In order to determined the optimal protective media for lyophilization of three-day yeast cultures, 4 protecting media were prepared: 1. media - 10% sucrose, 1% gelatin, 0.1% agar-agar, 100 mL of distilled water; 2. media - first media with 2% $CaCO_3$; 3. media - 10% gelatin, 0.25% ascorbic acid, 2% $CaCO_3$, 100 mL of distilled water; 4. media - 10% skim milk, 1% sodium glutanate, 100 mL of distilled water.

The titer of vital yeasts during storage on all tread media was reduced and was minimal with using of third medium (Table 2). Maximum survival was observed in yeasts, both one day after lyophilization and after one year of storage with using of second and fourth medium. On the other hand, can be seen that there is statistically significant differences ($p<0.05$) between the number of living cells on different protective media.

Table 2. The influence of protective media on the survival of lyophilized yeasts during storage

Protective media	Before lyophilization of yeast		A day after lyophilization		Half a year after lyophilization		One year after lyophilization	
	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)	Number of living cells in mL $\bar{x}$ ± SD	Survival (%)
1	$9.0^{a} \times 10^{7} \pm 0.02$	100	$8.5^{b} \times 10^{7} \pm 0.01$	90	$7.2^{c} \times 10^{7} \pm 0.03$	80	$5.6^{d} \times 10^{7} \pm 0.01$	62
2	$7.0^{a} \times 10^{7} \pm 0.02$	100	$6.3^{b} \times 10^{7} \pm 0.03$	90	$5.3^{c} \times 10^{7} \pm 0.03$	75	$4.5^{d} \times 10^{7} \pm 0.02$	65
3	$5.0^{a} \times 10^{7} \pm 0.02$	100	$1.0^{b} \times 10^{7} \pm 0.01$	20	$8.0^{c} \times 10^{6} \pm 0.02$	16	$2.1^{d} \times 10^{6} \pm 0.03$	11
4	$3.1^{a} \times 10^{7} \pm 0.04$	100	$2.8^{b} \times 10^{7} \pm 0.02$	90	$2.1^{c} \times 10^{7} \pm 0.02$	85	$1.9^{d} \times 10^{7} \pm 0.01$	70

$^{a, b, c, d}$ - values marked with different letters, have a statistically significant difference ($p<0.05$).

The fermentation activity of yeasts, which are in a lyophilized state for one year, was checked on concentrated whey, from which proteins have been removed. Whey contains 10.5% lactose and 0.05% $(NH_4)_2SO_4$ was added, while the pH was adjusted in the range of 5.0 - 5.2. Revival of lyophilized yeasts was performed by their rehydration in distilled water for one hour (Blankov and Klebanov, 2011). After rehydration, the yeasts were sown in tubes on a broad-agar hair. After three days, the grown yeasts of the first passage were washed from the oblique agar in a 200 mL flask with 150 mL of whey. Fermentation of whey lactose was performed in flasks on a shaker with 120 rpm at a temperature of 25 ^{o}C. In the same conditions, whey was fermented with the yeasts of the second and third passages after their revival. The alcohol content in the fermentation broth was determined after distillation by the aerometric method. Moreover, stress effects on yeast cells, due to longer storage in the dried state and rehydration, lead to decreasing in the rate of fermentation of lactose milk by the yeasts of the first and second passages.

Thus, after 60 hours of fermentation, the yeasts of the first passage leaved 2.0 - 2.5% of lactose in the media, the second passage had a 0.5 -1.5% of lactose, while at the same time the yeasts of the third passage during that time practically completely fermented whey lactose. The reduction of reproductive energy and, therefore, fermentation activity in lyophilized alcoholic and wine yeasts has been observed by numerous researchers, including Burjan (2006). However, in some yeasts, lyophilized with protective second and fourth media, the fermentation activity was completely restored in the third passage.

Bearing in mind that the survival of yeasts, which were lyophilized with these protectors, is the highest, it can be considered that media 2 and 4 are optimal for lyophilization of yeasts in terms of maintaining their vitality and fermentation activity.

Thus, according to the study of Berny and Hennebert (1991), by using skim milk as a support material in combination with two compounds among honey, sodium glutamate, trehalose or raffinose, the viability of *Saccharomyces cerevisiae* cells increased from 30% to 96–98%. Abadias et al. (2001) reported a survival rate of 28.9% for *Candida sake* when 10% skim milk was used. Zhao and Zhang (2005) obtained the highest viability (53.6%) after freeze-drying of *Oenococcus oeni* H-2 by using 2.5% sodium glutamate. Generally, each single protectant has its own advantages and disadvantages which can be made up by other protectants. Therefore, several protectants are mixed together according to a certain formula for a better performance (Wang, 2000).

Fungal strains may be kept alive in freeze-drying ampoules for more than 40 years. The advantages of freeze-drying are the protection from contamination or infestation during storage, long viability, and facility for strain distribution. However, not all strains survive the process and, among those surviving, quantitative viability rates as low as 0.1% have been reported (Zhao and Zhang, 2005).

The freezing effect on cell morphology can be observed under a cryomicroscope (Smith et al., 1986). At the optimum cooling rate (3 °C/min to 10 °C/min) cells of *Saccharomyces cerevisiae* are extensively shrunken while at higher cooling rate, intracellular ice is associated with loss of viability. Smith et al. (1986) report that *Penicillium expansum* survives at all cooling rates between 0.5 °C/min and 200 °C/min, the optimum ranging between 1 and 10 °C/min. At optimum cooling rates, hyphae of *Penicillium expansum* shrink extensively. At higher rates (> 50 °C/min) intracellular ice nucleated without killing the cells.

The microorganisms viability during con-servation and storage is influenced by their nature, cultivation conditions, age, culture and protecting medium concentrations, regime of the process, etc. In order to preserve the

strain specific characteristics it is necessary to apply routine approaches, based on preliminary tested models. The main task is maximum number of initial cells to be kept and the first tool avail-able is cultivation (Donev et al., 1995).

CONCLUSION

Based on the analysis of works in this area, it can be concluded that three-day-old yeast cultures were the most vital during the lyophilization process. The best survive was obtained in protective medias with 10% sucrose, 1% gelatin, 2% $CaCO_3$ and 0.1% agar-agar, ie with 10% skim milk and 1% sodium glutamate. According to that, both of second and fourth media are recomended for future use as procective media for yeasts during the lyophilization process. Moreover, the storage of yeast in a dried state and rehydration lead to decreasing in their reproductive energy and fermentation activity.

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