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Cryopreservation: The secret of modern preservation of brewer's yeasts – minireview

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Abstract

The aim of the long-term preservation of cells, tissues and organs is to maintain their cellular structures and biological functions for as long as possible. Cryopreservation is a process where biological material is stored and preserved at very low temperatures. However, freezing and thawing processes can cause irreversible cell damage, which is related to formation of ice crystals, osmotic stress, accumulation of reactive forms of oxygen, etc. Therefore the cell viability depends mainly on the freezing rate, the composition of the cryoprotective medium as well as on the thawing rate. Using a suitable cryoprotective medium can increase the viability rate of the yeasts after "revitalization". Appropriate pre-cultivation before freezing also plays an important role. These facts show that cell freezing and thawing processes must be controlled to avoid cell damage.

Keywords: long-term storage of yeasts, cryopreservation, cryoprotective substances, brewer's yeasts, *Saccharomyces*, cellular stress

1 Introduction

It is essential that even after a long-term storage, brewer's yeasts maintain their original properties which constitute one of the main requirements for their use in brewing.

The preservation process should not only ensure high viability of the cells, but also the preservation of their physiological as well as technological properties. Various techniques can be used for preservation of yeasts, for example regular re-inoculation on a suitable medium (a liquid or solidified soil), drying methods, freeze-drying (lyophilization), freezing in liquid nitrogen (cryopreservation), vitrification, etc. (Kumar et al., 2013; Day and Stacey, 2007; Kirsop and Doyle, 1991).

This paper is focused mainly on the long-term storage of brewing yeast using the cryopreservation technique. I addition, it also offers a brief overview of other preservation techniques to enable a comparison of their particular advantages and disadvantages.

2 Traditional techniques for non-freezing yeast preservation

The yeast preservation techniques can be generally divided according to the length of the preservation period into short term and long-term, and according to the applied process into cultivation, drying and freezing. A short-term period is considered to be the preservation of the sample of up to 1 year, while long term is considered as permanent (Nakasone et al., 2004).

Cultivation methods – periodic transfer

The repeated cultivation is a basic short-term preservation technique which maintains yeast growth on agar soils (Nakasone et al., 2004). In the brewing environment, it constitutes a widely employed technique mostly using a slant agar (e.g. malt agar) and a storage temperatures of up to 4 °C (Matoulková and Sigler, 2011). This method is simple and cheap, because specialised equipment is not

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required. On the other hand, it is rather time consuming and labour intensive since it involves the steps of re-inoculation, regular checking for contamination and desiccation (Nakasone et al., 2004). That is why this method is recommended primarily for collections with a small number of cultures which are used constantly and for short periods of time i.e. less than 1 year (Matoulková and Sigler, 2011).

It is possible to prolong the intervals between re-inoculations by using sterile mineral oil. The medium used to overlay yeast cultures is mineral oil, and thus the yeast metabolism is slowed down (Suga et al., 2000). The oil level in preservation vessels (e.g. tubes, vials, etc.) must be periodically checked, and oil should be added, if necessary. To revive an active oil-free culture, it is suggested to re-cultivate the harvested colony several times (Nakasone et al., 2004).

The use of the re-inoculation method has its limits because the yeast viability cannot be maintained for more than 5–6 months. Moreover, the multiple re-inoculation can lead to significant changes in viability, morphology, physiology and genetic stability. Respiratory-deficient mutants, changes in cell morphology as well as varieties with different flocculation properties can appear (Kirsop and Doyle, 1991; Kirsop, 1974). Furthermore, frequent handling of cultures increases the risk of contamination (Boulton and Quain, 2001).

Drying methods

Likewise, drying methods have established their strong position in the brewing industry due to the fact that they are simple, fast and cost-effective. The literature describes a number of techniques ranging from preparation of active dried yeasts (ADY) to protocols incorporating yeasts into a gel structure (Gelinas, 2019; Nyanga et al., 2012; Jekins et al., 2011; Cyr et al., 2007; Nakasone et al., 2004). However, drying methods can influence the yeast viability undesirably. Several studies point out that especially lager strains of *Saccharomyces pastorianus* are extremely sensitive to drying process; subsequently abnormal flocculation, haze formation and a less stable foam structure can arise (Jekins et al., 2011; Cyr et al., 2007; Russell and Stewart, 1981).

Fluidised bed drying has become a widespread and gentle technique for preparation of ADY for brewing. Moreover, spray drying should be mentioned as well, although it is not commonly used in brewing because it produces low viability cultures.

Desiccation using anhydrous silica gel is considered to be a medium-term and cheap drying method. Silica gel is a porous form of silica (SiO_2) with a pore diameter of 0.1 to 10 µm. This technique is based on mixing a yeast suspension with a precursor of gel, thus the process of gelatination starts. During gelatination water evaporates and gel dries. This results in incorporation of yeasts into gel structure (Uo et al., 2004). Revival of cultures involves scattering a few silica gel crystals on an agar plate. The viability of cultures stored in this way depends on the yeast strain and the medium on which it was cultivated before the preservation process began. The advantage of silica gel is that it prevents all fungal growth and metabolism. (Nakasone et al., 2004).

It should be added that also lyophilisation (freeze-drying method) can be included in this chapters, nevertheless we preferred to assign it to the next chapter dealing with freezing methods.

3 Freezing methods

Freezing methods are versatile and widely applicable in a long-term preservation of brewer's yeasts, however, these methods require specialized and expensive equipment. The easiest variant of freezing methods is to place suitably prepared cultures in mechanical freezers in a temperature range between -20 and -80 °C, although the viability decreases with the time (Bond, 2007). Electric freezers enable the yeast preservation even at lower temperatures between -100 and -150 °C. Nevertheless, a frequently utilized, rather inexpensive and simply accessible protocol is a combination of 25% (v/v) glycerol as a cryoprotectant and freezing temperatures from -60 to -80 °C (Bond, 2007; Nakasone et al., 2004).

It is relevant to add that the above-mentioned freezing technique is sometimes referred to as cryopreservation in the scientific literature (Cabrera et al., 2020; Grimalt-Alemany et al., 2020; Wing et al., 2020; Homolka, 2013). In this paper we will define the freezing techniques of deep freezing and the cryopreservation as a cell storage in liquid or vapour nitrogen.

Lyophilization/freeze-drying technique

Lyophilization is based on the sublimation of water from a sample at a low temperature and pressure. The process involves three basic steps: freezing and two high vacuum drying phases (Alonso, 2016; Morgan et al., 2006). Although the loss of the cell viability is quite high during the lyophilisation process, it is minimal during the subsequent storage of the lyophilised yeasts, which remains viable for more than 30 years (Day and Stacey, 2007). The freeze-drying technique is thus generally regarded as a gentle and suitable process of yeast preservation even from the point of view of further manipulation and transport. As about 90% of water is removed, the lyophilised cultures are very light. Although they should be kept at -18 °C to maintain

stability, the cold chain can be interrupted for a short time, e.g. during transport (Foerst and Santivarangkna, 2015). Despite its numerous advantages, it is also necessary to point out that lyophilization is energy and cost demanding (Foerst and Santivarangkna, 2015).

4 Cryopreservation – storage using nitrogen

Cryopreservation is a method of a long-term storage of viable organisms in a frozen state. To be exact, we should understand this method as a storage of (micro)organisms at the temperature below -139 °C, when the cells occur in the state of anabiosis, that means a reversible suspension of life processes (Kirsop and Doyle, 1991). In other words, the rates of biophysical processes are too slow to affect cell vital properties at -139 °C, i.e. the temperature in which not even ice crystals grow (Nakasone et al., 2004). Various liquified gases are used to reach this extremely low temperature, for example nitrogen, argon or xenon. Nevertheless, liquid or vapour phase nitrogen, with its working temperature range between -160 °C to -196 °C, is used almost exclusively due to safety and financial reasons (Matoulková and Sigler, 2011; Bond, 2007).

We may assume that the rates of mutation in cultured yeasts approximately correspond to those of cell division and metabolic activity. Therefore the cryopreservation prevents increased genetic variability of stored cultures owing to the complete halt of cell division as well as the total arrest of metabolism. In addition, the method is timesaving, reducing labour needs compared to the handling of living cells. It prevents culture contamination and increases assurance of the long-term availability of cultures. Of course, there are several drawbacks such as the high cost of the equipment and the fact that liquid nitrogen must be checked every 2 days and refilled if necessary; further a sufficient space for refrigeration units is required (Bond, 2007; Nakasone et al., 2004).

Let's briefly examine the effects of freezing of living cells. The cells are exposed to 2 basic stressors, namely osmotic and thermal stress during both processes of freezing and thawing. The cell suspension can be considered as an aqueous solution of yeast cells and various solutes coming from the cultivation medium. When such an aqueous solution begins to cool below the freezing point, the water in the external environment will freeze the first, because it is much less concentrated than the cytoplasm inside the yeasts. The frozen water starts to be released from the cell suspension in the form of ice crystals, whereby the substances dissolved in the remaining non-frozen fraction of the solution are concentrated. The osmotic pressure of the environment increases and the cells respond by expelling water from the intracellular environment. Thus the yeasts are dehydrated and wrinkle their shape (Hubálek, 1996; Morris et al., 1998).

Since yeasts are relatively sensitive to the above described osmotic and thermal stresses, the addition of cryoprotective substances to the freezing medium is often used in order to prevent damage of the cells as much as possible. In general, these substances should be readily soluble in water and non-toxic or very slightly toxic. Cryoprotectants differ in their ability to penetrate through the cell membrane. On the basis of this key ability, they show different protective effects:

i) penetrating cryoprotectants – such as glycerol or dimethyl sulfoxide (DMSO), which easily pass through the cell membrane and ensure intracellular as well as extracellular protection;

ii) non-penetrating cryoprotectants – such as various sugars (e.g. sucrose, lactose, glucose), alcohols (e.g. mannitol, sorbitol), amino acids, proteins or a number of other substances with different structure and molecular weight (e.g. dextran, polyvinyl-pyrrolidone, and hydroxyethyl starch), which protect the cells extracellularly (Nakasone et al., 2004; Hubálek, 2003; Hubálek, 1996).

The principle of the cryoprotectant effect is to increase the total concentration of all solutes in the system and thus reduce the ice formation (Bond, 2007).

The most commonly used cryoprotectants in cryopreservation of the yeasts are glycerol in concentrations 5–20%, alternatively ethylene glycol or propylene glycol in concentrations of 10% or 5% respectively (Hubálek, 2003; Hubálek, 1996).

The cryoprotective principle of glycerol as an intracellular cryoprotective agent lies in the forming of strong hydrogen bonds with water molecules through hydroxyl groups. These bonds prevent excessive cell dehydration, intracellular ice crystal formation as well as reduction of salt toxicity by decreasing the amount of frozen water. They also increase the plasticity of the cell wall and bind intracellular water, which increases the cell's resistance to hyperosmotic stress (Hubálek, 2003).

As we mentioned above, the cells are submerged in liquid nitrogen (Figure 1) usually at the temperature range from -160 to -196 °C, at which all biochemical and crystallization processes cease. Freezing, or more precisely the transition of cells from the optimal ("room") temperature to the very low temperature of e.g. -196 °C (= boiling point of nitrogen at sea level), is a controlled process, depending on the technical equipment, by means of which the efficiency of cryopreservation can be influ-

enced. The alternative to storing cultures directly submerged in liquid nitrogen is to place them in special refrigeration boxes filled with nitrogen vapours (Figure 1) with the temperature of -140 °C (Kirsop and Doyle, 1991).

The rate of freezing largely determines the degree of cell dehydration and the formation of ice crystals together with their size and location. During slow freezing (< 10 °C/min), the cells have enough time to equalize the osmotic pressure of the medium and therefore only extracellular ice crystals are formed. On the contrary, during rapid freezing (cca 102 °C/min), the cells do not have enough time to expel water to the external environment, thus intracellular as well as extracellular ice crystals are formed. In case of very fast freezing (> 103 °C/min), the cells do not lose water at all and therefore contain a high amount of very small ice crystals (Dumont et al. 2004; Dumont et al., 2003; Hubálek, 1996). In the two latter cases, there is a risk of cell damage during revitalization because the growing ice crystals inside the cells can damage cytoplasmic membrane, the cell organelles and the cell wall of the yeasts (Momose et al., 2010; Seki et al., 2009). An improperly chosen freezing method can also lead to cell damage at the level of nuclear or mitochondrial DNA (Stamenova et al., 2008; Stoycheva et al., 2007).

The most effective approach is a combination of slow freezing with fast recovery. Figure 2 illustrates how cryosamples of brewer's yeasts are pulled up from the cryogenic Dewar vessel filled with liquid nitrogen. The optimum revitalization rate is 200 °C/min. This roughly corresponds to immersion of a frozen culture, which has been placed in a polypropylene straw, into a water bath at 37 °C (Hubálek, 1996). The literature reports and practice often implements a non-programmable conventional method, which is based on pre-freezing of cultures in ultra-low temperature freezers at -80 °C and their subsequent immersion into liquid nitrogen (Yang et al., 2010). The disadvantage of this procedure is that the rate of freezing cannot be controlled, and thus there is a risk that intracellular crystals will form in the cells.

The resistance of yeast cells to thermal and osmotic stress can be increased by a suitably selected cultivation method used immediately before an immersion into liquid nitrogen. Aeration, i.e. shaking, during cultivation as well as the presence and the concentration of a cryoprotective component added into the incubation medium usually affect yeast resistance (Polezhaeva et al., 2014; Suga et al., 2000). The choice of the cryoprotective component together with a corresponding methodological procedure represent a significant aspect. For example, glycerol as the most common cryoprotective substance enters into yeast cells relatively slowly. When glycerol is used, it is recommended to leave the cells in a medium supplemented

Figure 1 *Cryopreservation of the brewer's yeasts in the cryogenic Dewar vessel filled with liquid nitrogen in RIBM laboratory*

Figure 2 *Pulling up of test tubes with brewer's yeasts from the cryogenic Dewar vessel filled with liquid nitrogen*

with this cryoprotectant at a room temperature for about 2 hours prior to freezing. This phase is called the "equilibration phase" and it gives glycerol the time necessary to enter into the cells (Hubálek, 2003). The storage of the cell suspension in a cryoprotective medium is provided in suitable vessels (cryotubes, cryovials) or in thin cryotubes, called straws, which are then placed in cryotubes. The volume of one straw is between 50–80 microlitres of the cell suspension. It means that a large enough number of straws is necessary to store each strain. Freezing is performed in a chamber with a programmable and controlled rate of freezing. An open straw with the cell suspension is placed aside for each frozen batch so that the cooling process can be monitored and the program adjusted. A record of progress is kept for each frozen batch. The freezing itself occurs when the cryotubes with straws are placed in a freezing chamber into which nitrogen vapours are fed in a controlled manner. As soon as a required temperature is reached (e.g. -160 °C), the cooling process is terminated. Finally, the frozen cryotubes are placed in racks and containers in a Dewar vessel and submersed in liquid nitrogen (Matoulková and Sigler, 2011).

5 Conclusion

The general purpose of the long-term deposition of cells is to keep them viable for as long as possible. Moreover, in the case of technologically important microorganims, it is necessary to ensure the stability of their physiological and technological properties. The cryopreservation, as a storage in liquid nitrogen, is an effective way to preserve many (micro)organisms including brewer's yeasts. However, the process requires correctly set conditions that enable the long-term storage.

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