

INFLUENCE OF THE GROWTH CONDITIONS ON THE RESISTANCE OF *SACCHAROMYCES CEREVISIAE*, STRAIN NBIMCC 181, BY FREEZE-DRYING

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Summary

Experiments were carried out for determination the influence of the growth conditions on the resistance of brewery yeast Saccharomyces cerevisiae, strain 181, to freeze-drying. Growth curves were studied in parallel with the viability of the strain by three variants of cultivation – in a liquid broth, in a solid agar medium and in combination of liquid and solid agar nutrition medium. Better preservation of the cell suspensions obtained in agar medium was determined, where the maximum value was about 8.3-8.7 % after freeze-drying preservation at the 64th and 104th hour of cultivation, whereas in a liquid broth it was 0.28 – 0.25 % between the 18th and 24th hours. We propose a combined two-step method for consecutive growing in liquid and agar medium, by which viability of 13.2 % from the yeast cells after lyophilization was reached.

Introduction

The variety of results in the world literature, regarding the influence of the conditions of regeneration and cultivation on the resistance to cryoconservation of different microorganisms could be accepted more as a challenge than as an opportunity for applying the foreign experience. In the collection activity in the guidelines and instructions for work it is recommended that the microorganism must be inoculated on a solid nutrition medium [2, 3, 6, 8]. The well-grown culture is then washed with cryo-protecting medium and then freeze-dried in

ampoules [5-7]. If the culture was received in the collection grown on a solid medium then the same procedure was followed – directly got to conservation. No comparative studies have been met, regarding the preservation of the microorganisms by this method to the method by which the culture was grown in a liquid broth. Besides, since the obtained cell suspension was extremely heterogeneous in relation with its age, the appropriate period for preservation has not been defined.

In the present study an attempt was

made for finding out the conditions by which high cell concentration was reached before freeze-drying, which correlates with a high resistance of the cells after cryopreservation, in question with freeze drying. The expe-

riments were carried out with a strain 181, belonging to *Saccharomyces cerevisiae*, a species, which according to a number of studies was de-scribed as a low lyo-resistant [1, 4, 7, 9, 10].

Materials and Methods

Microorganism. An industrial strain from the collection of National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), belonging to the species of *S. cerevisiae* with NBIMCC number 181, was studied, which is generally used in the brewery production [2].

Media and packaging.

- Nutrition medium GPY(A) with pH 6: glucose 10.0 g, peptone 5.0 g, yeast extract 5.0 g, (agar-agar 20.0 g), distilled water 1.0 l.

- Protecting medium: sucrose 10 % (20 %) and gelatin 1.5 % (3 %).

- Dehydration medium and dilutions: water solution of NaCl 0.9 %.

- Package for freeze-drying: vials of 10 cm³, supplied with vacuum stopper, in which 1 ml from the cell suspension is distributed.

Methods.

- Cultivation.** The investigated strain was grown statically in aerobic conditions at 27±0.5°C in liquid and agar nutrition medium in glass tubes. Inoculum was 1 ml, with concentration of 10^{6÷7} cells/ml. The culture was tested at different age: by the received in liquid broth ones in every 6 hours during 40 hours; for the agar medium - in every 8 hours for a period of 160 hours. The concentration of the cells was controlled, before and after freeze-drying.

- Freeze-drying.** The obtained in a liquid medium cell suspension was protected by

mixing it with an equal volume of the protective medium in a double concentration, and that from the agar medium – by pouring it off with sucrose 10 % and gelatin 1.5 %. The samples, distributed in vials, were lyophilized in a freeze drying machine “SMH 15” (“Usifroid”- France) by the following conditions: at average cooling rate 0.6°C x min⁻¹ to minus 32°C, sublimation at pressure 15 Pa and secondary drying at pressure of 1 Pa and temperature 22°C for 5 hours.

- Assay of the concentration of cell suspension and the survival of the strain after preservation.** The cell concentration was determined by the indirect method of the limited logarithmic dilutions. 0.1 ml was plated on agar nutrition medium in petri dishes (ø 100 mm). The assessment was made at the 72nd hour of cultivation at 27°C. By the counting petri dishes with above 5 well-formed colonies, but not more than 500, were used. The obtained results were the average sums from the number of the colony forming units (CFU) from three dilutions in three repetitions. The determination of the strain survival after preservation was made according to the equation $b/a \times 100 = c \%$, where *a* is the average number of the colonies before conservation, *b* is the average number of the colonies after conservation and *c* is the survival of the culture in percentage.

Results and Discussion

The preservation of the strain 181 of *S. cerevisiae* was studied. Investigations were

carried out, by which the cultures were grown in three variants.

Cultivation in a liquid broth

The resistance to freeze-drying of the culture at different age was checked. The growth curve and the survival of the yeasts after freeze-drying were studied in dynamics. The results were presented graphically in Fig. 1.

It could be seen that the highest percentage

of the viable cells (0.28 %) was achieved by preservation of the strain after the second third of the logarithmic phase (at about the 18th hour of the cultivation). We estimate the result from the conservation as very low and unsatisfactory.

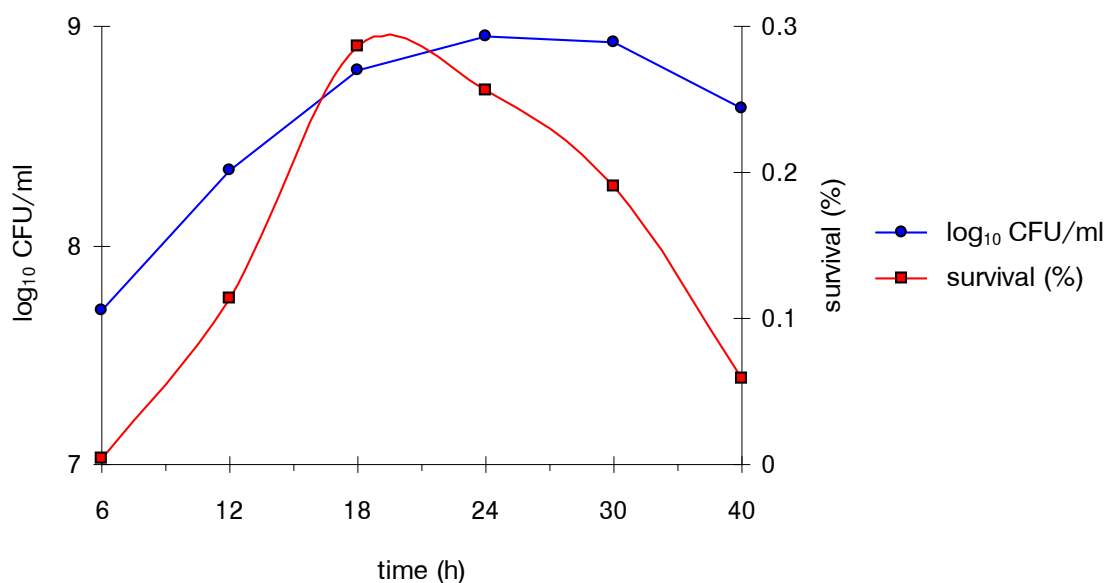


Fig. 1. Growth curve by submerge cultivation and survival of the strain 181 after freeze-drying, in relation with the cultural age.

Cultivation on agar nutrition medium

For assessment the optimal period, during which the investigated strain *S. cerevisiae* showed maximal resistance to freeze-drying preservation, we carried out experiments by which the growth curve and the survival were also studied in dynamics (Fig. 2).

It was established that the logarithmic growth of the strain was to the 64th hour (Fig. 2), as by the logarithmic phase of the submerge cultivation. The highest number of the CFUs was achieved at the 72nd hour. In the following hours the cells were grown on the free agar nutrition medium, by which it was observed that the number of the viable ones was kept relatively constant till the 120th hour. As the nutrition

medium got poorer the balance between the dividing and dieing cells was disrupted.

The results from these investigations for preserved viability of the strain 181 after freeze-drying preservation correlated with the growth curve. The survived cells, conserved at age between the 64th and 104th hour were $4.7 \times 10^6 \pm 0.7 \times 10^6$, by which the loss was about $1.07 \log_{10} \pm 0.01$. The establishment of broad range in the course of time, with near values of the viable cell after lyophilic conservation, makes the work easier for preparation of the strains for lyophilization. The maximal survival of the strain (8.7 %) was achieved by its preservation at age about the 72nd hour.

Comparing the best results, established after the freeze-drying of the culture, obtained by cultivation in non-agar nutrition medium, by this method 30 fold increasing of the survival of the cells was achieved.

In the recent study it was experimental established, that for the aim of the collection

work, when preparing the strain *S. cerevisiae* 181 for lyophilic conservation, the agar nutrition medium was more appropriate in comparison with the liquid one. We suppose that the higher survival after the freeze-drying preservation correlates with the age heterogeneity of the cells in the suspension.

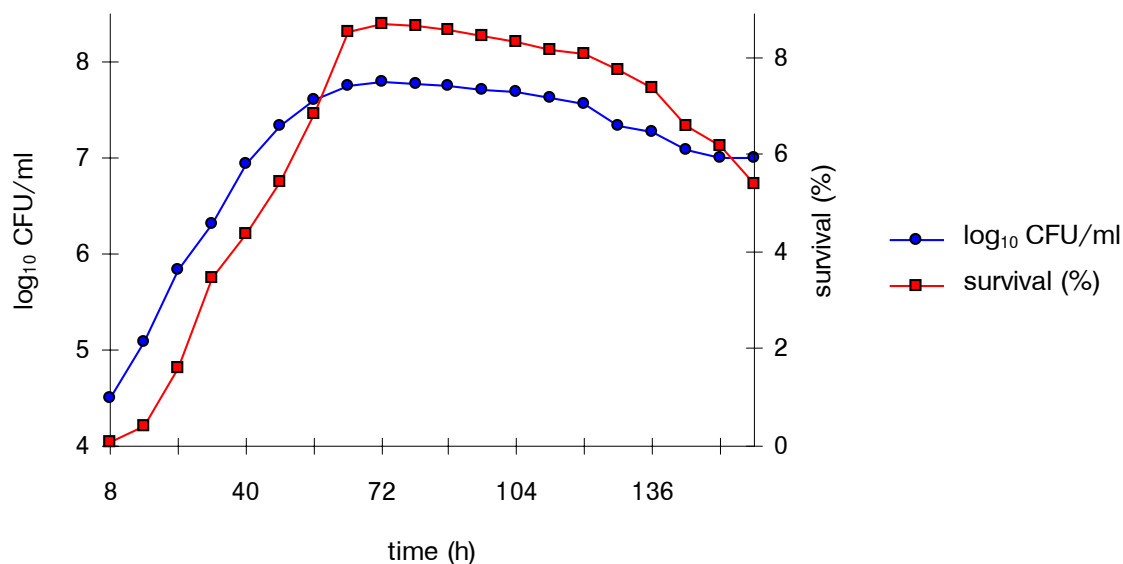


Fig. 2. Growth curve by cultivation on agar nutrition medium and survival of the strain 181 after freeze-drying, in relation with cultural age.

A combined method for cultivation

The method that we developed combines the good growth of the cultures by cultivation in liquid medium and following high-density growth on agar nutrition medium.

At the first stage, the inoculum, 1 ml cell suspension with concentration $3 \div 8 \times 10^6$ cells/ml, was homogenized with 9 ml of the liquid nutrition medium in a glass tube and then grown statically for 18÷20 hours at temperature $27 \pm 0.5^\circ\text{C}$. At the second stage, 2 ml well grown cultural suspension, with concentration of 10^8 cells/ml were poured out in glass tubes with slopping agar. The glass tubes were then left in a slopping state at angle 18° , in order the cells to slayer equally on the agar medium, after which the remaining liquid was discarded. The strain was

grown statically at temperature $27 \pm 0.5^\circ\text{C}$. For the aim of the study samples from the culture were taken in every 8 hours for freeze-drying. The results were described in Fig. 3. The growth curve and the survival of the culture after freeze-drying were depicted.

By the so proposed two-stepped method for propagation, the culture from the second stage before washing differed visually in a significant extend from the culture grown by the classical surface cultivation. It represented a white pellicle, equally covered the agar nutrition medium. The obtained results showed increasing in concentration of the cells, both before and after freeze-drying of the strain, with one order, the maximum survival had been increased from 8.71 % to 13.54 %, i.e. above 55 %.

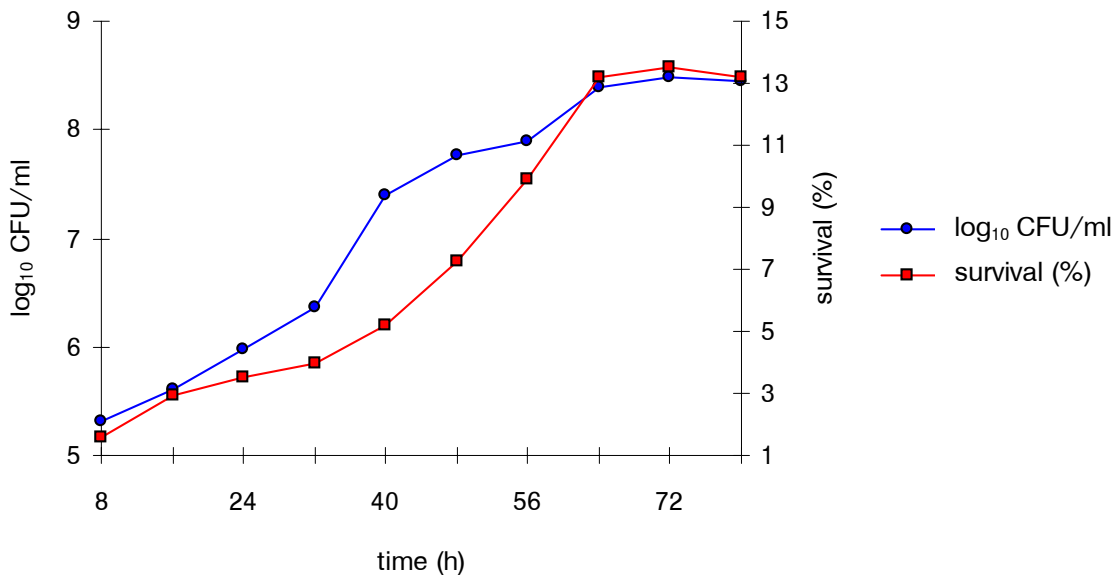


Fig. 3. Growth curve at the second stage from the combined cultivation on agar nutrition medium and survival of the strain 181 after freeze-drying, in correlation with the cultural age.

On the basis of the recent study we recommend when preparing the cell suspension of *S. cerevisiae* for freeze-drying a combined two-stepped method for propa-

gation of the strains to be applied. Similar experiments were carried out with other yeast strains belonging to this species and analogical results had been obtained.

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ВЛИЯНИЕ НА УСЛОВИЯТА НА КУЛТИВИРАНЕ ВЪРХУ УСТОЙЧИВОСТТА НА *SACCHAROMYCES CEREVISIAE* ЩАМ 181 ПРИ ЛИОФИЛНО КОНСЕРВИРАНЕ

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Резюме

*Проведени са опити за определяне на влиянието на условията на култивиране върху устойчивостта на пивни дрожди *Saccharomyces cerevisiae*, щам NBIMCC 181, към вакуумно-сублимационно сушене. Изследвани са растежните криви в паралел с преживяемостта на щама при три варианта на култивиране – в течна, на агаризирана и комбинация от течна и агаризирана хранителна среда. Установено е по-добро запазване при клетъчните суспензии, получени върху агаризирана среда, като максималната стойност е около $8.3 \div 8.7\%$ при лиофилно консервиране на $64^{\text{УЯ}}$ до $104^{\text{УЯ}}$ час от култивирането, докато при течната тя е $0.28 \div 0.25\%$ между $18^{\text{УЯ}}$ и $24^{\text{УЯ}}$ часове. Предлага се комбиниран двустъпален метод на последователно култивиране върху течна и агаризирана среда, при който е достигнато преживяване на 13.2% от дрождевите клетки след лиофилизацията им.*