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EXPERIMENTAL ARTICLES

# The Effect of Long-Term Preservation of Bacterial Cells Immobilized in Poly(Vinyl Alcohol) Cryogel on Their Viability and Biosynthesis of Target Metabolites

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**Abstract**—The effect of cell storage at –18°C for 18–24 months on reproductive capacity was investigated for various microorganisms (gram-positive and gram-negative bacteria, yeasts, and filamentous fungi) immobilized in poly(vinyl alcohol) cryogel. To examine the viability of immobilized cells after defrosting, the bioluminescent method of intracellular ATP determination was used. A high level of metabolic activity of immobilized cells after various periods of storage was recorded for *Streptomyces anulatus, Rhizopus oryzae*, and *Escherichia coli*, which are producers of the antibiotic aurantin, L(+)-lactic acid, and the recombinant enzyme organophosphate hydrolase, respectively. It was shown that the initial concentration of immobilized cells in cryogel granules plays an important role in the survival of *Str. anulatus* and *Pseudomonas putida* after 1.5 years of storage. It was found that, after slow defrosting in the storage medium at 5°C for 18 h of immobilized cells of the yeast *Saccharomyces cerevisiae* that had been stored for nine months, the number of reproductive cells increased due to the formation of ascospores.

*Key words*: storage, cell immobilization, poly(vinyl alcohol) cryogel, lactic acid, organophosphate hydrolase, aurantin.

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The choice of the method of preservation of biological material is often crucial in microbiology, as well as in molecular biology and bioengineering, where microorganisms often act as models for investigations. One of the commonly used methods is cryopreservation, which includes freezing and preservation of cells in the presence of various substances with cryoprotector properties [1–3]. During natural cryopreservation, cells of microorganisms are often immobilized (adhered to diverse surfaces, included into biofilms, etc.) [4], which allows them to survive and preserve a high level of metabolic activity.

Poly(vinyl alcohol) (PVA) cryogel has proved itself to be a polymer carrier highly suitable for the immobilization of various microorganisms and the creation of biocatalysts possessing a chemically stable matrix with high porosity and mechanical durability [5–7]. The inclusion of cells into this matrix guarantees their survival during the process of freezing–defrosting, which is necessary for gel formation, and the preservation of the productive state of the cells during their subsequent use in various biotechnological processes. It is well-known that immobilization increases the tolerance of cells used as biocatalysts to environmental factors (pH, high concentrations of metabolites, presence of toxic substances, etc.) [8]. However, the influence of long-term storage of cryoimmobilized cells on their survival and metabolism has not been studied yet, although such studies are undoubtedly of great interest and urgency from the standpoint of the development of new methods of cell preservation and the possibility of practical application of the results obtained.

Microorganisms of various systematic groups can differ fundamentally in their sensitivity to the conditions and duration of preservation [9]. In addition to the maintenance of the viability of cells, it is also important to preserve their physiological and biochemical properties, which may alter after storage due to the heterogeneity of the microbial population and replacement of the dominant phenotype, resulting in a decrease in the antibiotic and enzymatic activity of the preserved cells [10].

The goal of the present work was to study the effect of the duration of preservation of microbial cells (gram-positive and gram-negative bacteria, yeasts, and filamentous fungi), immobilized into PVA cryogel and stored in a frozen state, on their survival and biosynthetic activity with respect to target metabolites.

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## MATERIALS AND METHODS

In the present work, PVA cryogel (trademark 16/1, Azot, Ukraine) was used. Microorganisms were used as follows: *Streptomyces anulatus* var. *chrysomallus* (Collection of Microorganisms of Moscow State University), *Saccharomyces cerevisiae* 231 (VKM), *Rhodococcus erythropolis* Ac-1514 (VKM), *Pseudomonas putida* B-1091 (VKM), *Rhizopus oryzae* F-814 (VKPM), and *Escherichia coli* DH5α/pTrcTE-OPH [11].

To incorporate microorganisms into PVA cryogel, cells of *E. coli*, *S. cerevisiae*, *R. erythropolis* and *P. putida* were grown to the end of the exponential phase in media recommended for these cultures [11–14]. The biomass of vegetative cells was separated from the culture liquid by centrifugation on a Bechman J2-21 centrifuge (United States, 6000 *g*, 15 min) and mixed thoroughly with an 11% solution of PVA on the basis of distilled water so that the cell concentration in mixture obtained was 10.4 or 30 wt %.

In the case of the cultures of the fungus *R. oryzae* and the streptomycete *Str. anulatus*, spore material was immobilized. In this case, a spore suspension ($4 \times 10^6$ spores/ml) prepared by washing spores off of solid medium was mixed with an 11% PVA solution.

Suspensions of cells or spores in a PVA solution were aseptically dispensed into wells of 96-well immunologic plates (0.2 ml per well), the plates were hermetically closed, and the samples were stored at –18°C.

To examine the reproductive capacity of microorganisms after their preservation by immobilization into the PVA cryogel and freezing, they were used as inocula for free cell growth. For this purpose, granules with immobilized cells of *Str. anulatus*, *S. cerevisiae*, *R. erythropolis*, and *P. putida* were slowly defrosted (18 h, 5°C). Then, 1 g of granules was washed with a sterile 0.9% NaCl solution (500 ml) for 10 min, and the granules were transferred to tubes with nutrient broth supplemented with 1% glucose (pH 7) so that the granule concentration in the medium was 20 g/l. Then the tubes with granules of examined microorganisms were put into an Adolf Kuhner AG temperature-controlled shaker (Switzerland; 28–30°C, 180 rpm). To evaluate the quantity of the biomass accumulated, the optical density of free cell suspensions was measured at fixed time intervals with an Agilent-UV-853 spectrophotometer (Germany) at $\lambda = 540$ nm.

Granules with immobilized recombinant cells of *E. coli* transformed with plasmid pTrcTE-OPH and producing recombinant organophosphate hydrolase (OPH, 3.1.8.1 [11, 15]) were defrosted under the same conditions, transferred to a medium with 5 g/l of yeast extract, 10 g/l of tryptone, 5 g/l of NaCl, 100 μg/l of ampicillin, and $5 \times 10^{-5}$ M of $CoCl_2$ (pH 6.5), and cultivated for 24 h. The concentration of granules with immobilized cells was 20 g/l medium. An inducer of OPH synthesis, 0.2 mM isopropyl-β-D-thiogalactopyranoside, was added to the medium 3 h after the beginning of cultivation. The activity of OPH was determined as described in [16]. The quantity of enzyme necessary for the hydrolysis of 1 μmol of substrate in 1 min at pH 9.0 and 25°C was taken as the unit of enzymatic activity.

In the case of the culture of the fungus *R. oryzae*, a L(+) lactic acid (LA) producer [17], granules with immobilized spores were transferred to medium containing 100 g/l of glucose, 3 g/l of $(NH_4)_2SO_4$, 0.3 g/l of $MgSO_4 \cdot 7H_2O$, 0.05 g/l $ZnSO_4 \cdot 7H_2O$, 0.2 g/l of $KH_2PO_4$, and 5 g/l of $CaCO_3$ (pH 6.0). The specific growth rate of immobilized mycelium was calculated from the change in the weight of dry granules, which was routinely determined by drying the granules to a constant weight. Data obtained earlier on the constancy of the polymeric constituent of granules with immobilized mycelium were taken into account [17]. Concentration of L(+)-LA accumulated in medium was assayed by an enzymatic method [18] with the use of Sentinel lactate oxidase–peroxidase reagent (Italy).

To determine the quantity of the antibiotic aurantin synthesized by the culture of *Str. anulatus* reactivated cells were cultivated in 5 ml of nutrient broth with 1% glucose for 72 h and then used for inoculation. Experimental variants infected with 5 ml of inoculum were cultivated with the use of a temperature-controlled shaker (96 h, 200 rpm) in flasks (250 ml) containing 50 ml of medium of the following composition (g/l): glucose, 30.0; $KNO_3$, 4.0; $K_2HPO_4$, 0.2; $MgSO_4$, 0.5; NaCl, 1.0; $ZnSO_4$, 0.02; $FeSO_4$, 0.02 (pH 7.0–7.2). The quantity of aurantin synthesized was assayed by a colorimetric method at $\lambda = 400$ nm using a calibration curve plotted for standard antibiotic solutions [19, 20].

The concentration of intracellular ATP in immobilized cells was determined by the bioluminescent luciferin–luciferase method using granules with cells and spores immediately after their defrosting. For this purpose, granules were weighed (90–150 μg) and transferred to dimethyl sulfoxide (1 ml) and kept in it for 5 min at 25°C to extract intracellular ATP. Then, 50 μl of the obtained extract was added to the cuvette of a Microluminometr 3560 bioluminometer (New Horizons Diagnostics, United States) with 50 μl of the luciferin–luciferase reagent (Lumtek, Russia), and the intensity of luminescence was measured. The ATP concentration was determined using the calibration curve plotted for standard ATP solutions ($10^{-11}$–$10^{-9}$ M).

To control the composition of granules, their moisture content, which was 85 ± 2%, was determined [21], and the concentration of intracellular ATP was assayed. The latter parameter reflected the number of live cells in the granules.

The specific growth rates of the cultures ($\mu$, $h^{-1}$) were assayed according to Pirt's recommendations [22]. During the treatment of all experimental data, the mean values of the examined parameters and the values of standard deviation were calculated.

Dependence of the specific growth rate of free cells ($h^{-1}$) on the time of preservation of immobilized microbial cells used as inoculum

| Initial cell concentration in granules of PVA cryogel (% of mass) | Time of preservation, months | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 3 | 5 | 9 | 12 | 12* | 17 | 24 |
| *Str. anulatus* | | | | | | | | |
| 10.4 | 0.0076 | 0.0067 | 0.0071 | 0.0075 | 0.0062 | 0.0063 | 0.0020 | 0.0017 |
| 30 | 0.091 | 0.0056 | 0.0063 | 0.0067 | 0.0063 | 0.0063 | 0.0031 | 0.0030 |
| *R. erythropolis* | | | | | | | | |
| 10.4 | 0.081 | 0.075 | 0.058 | 0.052 | 0.061 | 0.060 | 0.053 | 0.050 |
| 30 | 0.055 | 0.052 | 0.058 | 0.043 | 0.062 | 0.067 | 0.041 | 0.055 |
| *P. putida* | | | | | | | | |
| 10.4 | 0.19 | 0.14 | 0.16 | 0.22 | 0.17 | 0.11 | 0.051 | 0.042 |
| 30 | 0.18 | 0.14 | 0.19 | 0.21 | 0.23 | 0.22 | 0.18 | 0.17 |
| *S. cerevisiae* | | | | | | | | |
| 10.4 | 0.028 | 0.033 | 0.030 | 0.026 | 0.064 | 0.051 | 0.041 | 0.036 |
| 30 | 0.031 | 0.031 | 0.022 | 0.060 | 0.057 | 0.053 | 0.046 | 0.062 |

* Specific growth rate of free microbial cells after repeated freezing and defrosting of immobilized cells stored for one year and then used as inoculum.

Microscopy of samples of yeast cells (stained fixed preparation) was performed with a Laboval 4 light microscope (Carl Zeiss, Jena, Germany) according to [19].

## RESULTS AND DISCUSSION

To investigate the effect of storage of microorganisms immobilized into PVA cryogel on their growth and metabolic characteristics, representatives of various taxonomic groups were examined: the yeast *S. cerevisiae*, the filamentous fungus *R. oryzae*, the gram-negative bacteria *P. putida* and *E. coli*, and the gram-positive bacteria *R. erythropolis* and *Str. anulatus.* All these microorganisms are of biotechnological importance or serve as models in molecular and genetic research.

It should be noted that after the cell immobilization, the matrix of the polymer carrier based on PVA cryogel can be destroyed only by melting at a temperature above 80°C [6]. Therefore, reliable evaluation of the number of living cells after their preservation in granules by separation of cells from the carrier and their direct count by the corresponding microbiological methods cannot be fulfilled. In this connection, evaluation of the state of cells after their preservation by immobilization and freezing was fulfilled by analysis of their reproductive capacity and of the level of intracellular ATP of immobilized cells immediately after their defrosting. It must be emphasized that the bioluminescent method of intracellular ATP determination is widely used for the evaluation of the number and state of living cells, including immobilized cells, because of the rapidity of this method and good correlation of the results with the data of microbiological analysis [23, 24].

To analyze the influence of the conditions of preservation of immobilized cells on their survival, PVA cryogel granules with different concentrations of cells and polymer were prepared. It is known that varying of polymer concentration results in changes in the porosity and, correspondingly, mass-transfer characteristics of the polymeric matrix [5, 6], and that an increase in cell concentration may contribute to better cell survival under unfavorable conditions [25].

To evaluate the reproductive capacity of immobilized microorganisms after their preservation, the kinetics of accumulation of biomass of free cells was recorded in medium inoculated with immobilized cells, and the specific rate of free cell growth was determined.

The data in the table show that, over a year, the reproductive capacity of cells of *R. erythropolis*, *P. putida*, and *S. cerevisiae* immobilized in PVA cryogel decreased slightly, as evidenced by values of the specific growth rate of free cells accumulating in the medium.

Interesting results were obtained during the microscopic investigation of a yeast reactivated after nine-month preservation in PVA cryogel. Formation of ascospores was detected; their germination was accompanied by budding and resulted in an increase in the

number of reproductive cells in the inoculum. This phenomenon could be the reason for the twofold increase in the specific rate of cell growth in comparison with the growth of a culture from cells that had been immobilized but not stored. Probably, ascospore formation was due to the fact that the immobilized cells were diploid yeast cells grown on rich medium [12], as well as to the response of cells to the slow 18-h defrosting, when they recovered metabolic activity after the stress. As far as we know, we are the first to report on this phenomenon. Further investigation of the development of microbial cultures defrosted by this method seems promising.

It is necessary to note that preservation of immobilized yeasts in granules with an initial cell concentration of 10.4% for two years increased the lag phase of growth after their defrosting.

Preservation of the bacteria *Str. anulatus* and *P. putida* for more than 1.5 years in PVA cryogel with low cell concentration resulted in a decrease in the specific growth rate of free cells by 3.5–4 times. It was found that the growth of free cells of *Str. anulatus* from immobilized inoculum after a year of its preservation was characterized by the prolongation of the lag phase from 24 to 96 h.

No visible influence of the initial concentration of immobilized cells in PVA cryogel granules on cell survival was detected for cells of *R. erythropolis* (table).

It is of interest that repeated freezing and defrosting of samples of immobilized cells after a year of culture preservation did not influence visibly their growth characteristics, except for cells of *P. putida* preserved in granules with a low cell concentration (table).

Monitoring of intracellular ATP concentration in cells of various microorganisms preserved by immobilization and freezing (Fig. 1) revealed a lack of visible changes in comparison with the initial ATP level. Visible (fourfold) decrease in the intracellular ATP concentration was detected only for yeast cells after 1.5 years of preservation, and this was, probably, a result of the formation of ascospores, characterized by an ametabolic state.

The data obtained on ATP are, on the whole, evidence of the preservation of the viability of cells immobilized and stored in a frozen state over a long period. Inasmuch as ATP concentration is known to be a characteristic of the metabolic activity of living cells [14], the value of this parameter may be thought of as the most adequate reflection of the state of cells in the granules of PVA cryogel.

In the present work, we have evaluated not only the reproductive capacity of immobilized cells, but also analyzed the influence of the duration of cell preservation on the antibiotic activity of growing free cells of *Str. anulatus* (Fig. 2), the OPH activity of cells of a recombinant strain *E. coli* (Fig. 3), and the production of L(+)-LA by the immobilized mycelium of the fungus *R. oryzae* (Fig. 4).

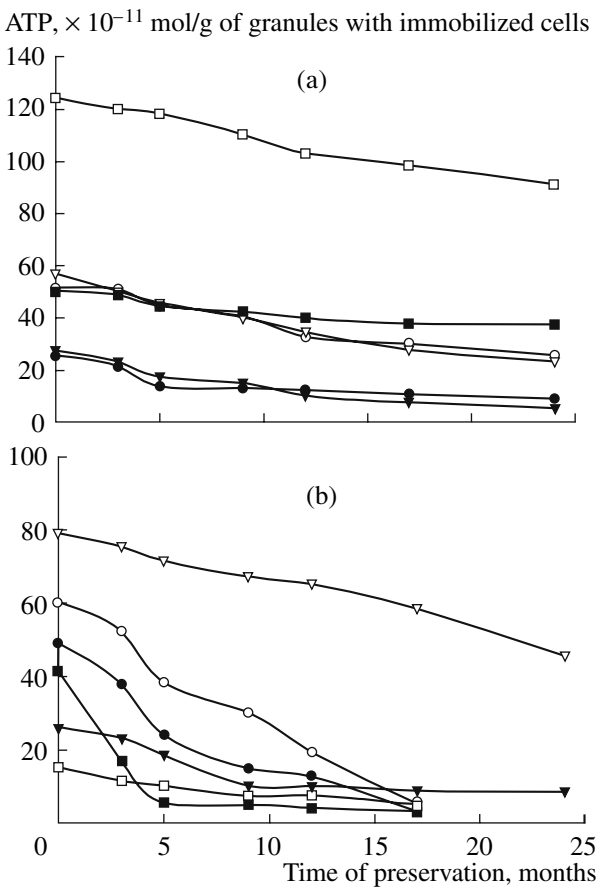


**Fig. 1.** Dependence of intracellular ATP concentration on the time of preservation in a frozen state of PVA cryogel granules with immobilized cells of various microorganisms, which initially accounted for 10.4% (unfilled symbols) and 30% (filled symbols) of the granule mass: (a) *R. erythropolis* (□, ■), *Str. anulatus* (○, ●)), and *P. putida* (▽, ▼); (b) *E. coli* (□, ■), *S. cerevisiae* (○, ●), and *R. oryzae* (▽, ▼).

The decrease in the concentration of aurantin accumulating in the medium with free cells of *Str. anulatus* correlated with worsening of culture growth caused by a decrease of the spore germination capacity and changes in the composition of the cell population due to different survival of dissociants during the preservation of immobilized inoculum for longer than 12 months.

Analysis of the OPH activity of immobilized *E. coli* cells preserved in granules of PVA cryogel (Fig. 3) revealed that they maintained a high level of productivity of a target recombinant protein over a long period (1.5 years). The specific growth rate (Fig. 3) and specific OPH activity of free cells grown for 24 h (132 ± 7 units per gram of wet cells; moisture content, 85 ± 1%) practically did not depend on the duration of inoculum preservation in the immobilized state.

The data obtained on immobilized recombinant cells are a practically important supplementation of the well-known information about the high stability exhibited by recombinant strains used in various biotechno-

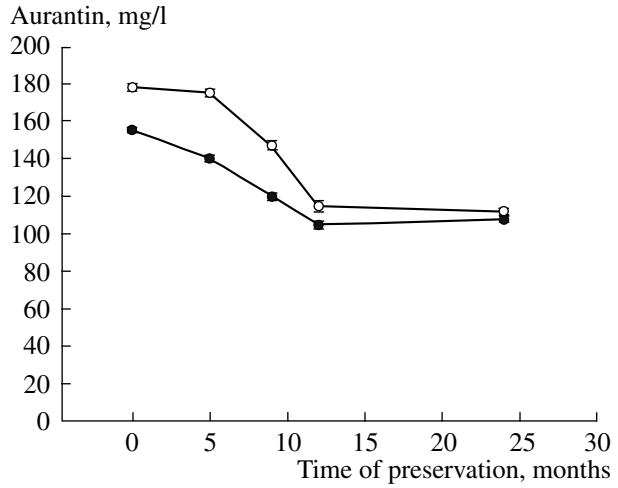


**Fig. 2.** Synthesis of the antibiotic aurantin by free cells of *Str. anulatus* grown from the inoculum consisting of immobilized cells of different time of preservation. The initial cell content in the granules amounted to (●) 10.4% and (○) 30% of the granule mass.

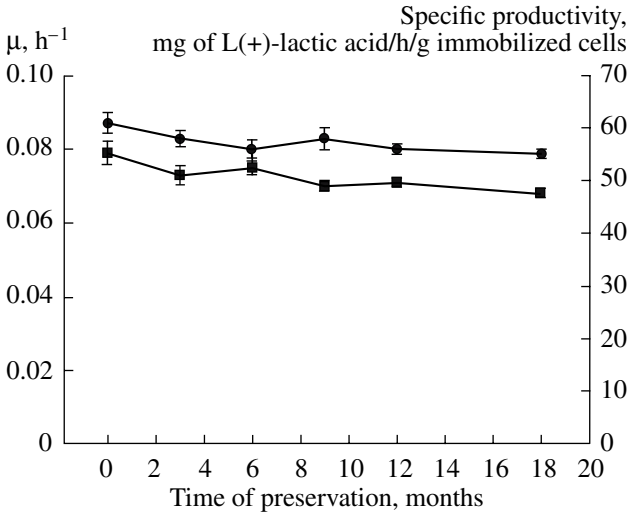


**Fig. 4.** Influence of time of preservation of *R. oryzae* spores immobilized into PVA cryogel on the (■) specific growth rate of the fungus and (●) specific production rate of L(+)-lactic acid.

logical processes in the form of immobilized catalysts [25].

The potential capacity of mycelium to grow both inside and on the surface of granules of cryogel containing immobilized *R. oryzae* spores was retained over 1.5 years of preservation (table). Periodically reactivated samples were characterized by a practically constant specific growth rate and level of accumulating biomass. The fungus productivity with respect to lactic acid was also practically at a constant level during the whole period of investigation (Fig. 4). This fact may be of great practical importance for the application of biocatalysts on the basis of filamentous fungi for industrial production of target metabolites. In this case, preservation of immobilized and frozen spores of this producer should be successful.

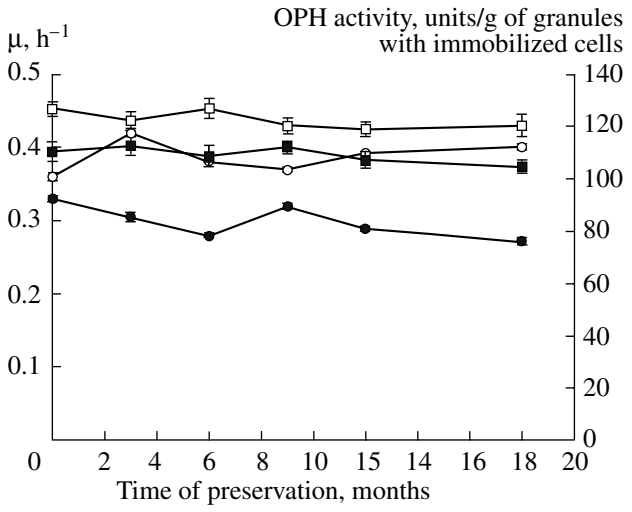


**Fig. 3.** Specific growth rate of free *E. coli* cells accumulating in the medium upon inoculation with immobilized cells ((●) 10.4% and (○) 30% of the granule mass) stored for different time periods, and the OPH activity recorded after 24 h of cultivation in medium with an inducer in the polymer granules with different initial concentrations of immobilized cells (■ and □).

Based on the data obtained, the method of preservation of prokaryotic and eukaryotic cells by their immobilization into PVA cryogel and storage at a temperature of –18°C may be recommended for wide testing. This method provides for long preservation of viability and productive capacities of microorganisms.

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