Immobilization of bacterial cells on nanowires

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Abstract. The paper investigated the effect of immobilization of bacterial cells Lactobacillus brevis 2k.GV. on polyvinylpyrrolidone nanowires on viability and enzymatic activity of cells. The optical density of the culture solution and the growth rate of lactobacilli were measured using a Reverse-Spinner RTS-1 bioreactor to assess physiological activity of the immobilized cells. It is shown that immobilization is ‘soft’, the carrier dissolves in the culture solution after biofilm formation.

1. Introduction
Normal intestinal microflora of humans and animals includes lactobacilli, which perform a number of functions. They prevent the growth of pathogenic microorganisms and increase immunity. The enzymes they produce are involved in membrane digestion, and promote cholesterol hydrolysis and toxin decomposition [1, 2]. A decreased amount of normal microflora causes indigestion, development of infectious diseases [3], weakening of immunity [4–6], and gastrointestinal disorders [7].

Recently, the prevalence of microflora disorders has increased, which is associated with a widespread use of antibiotics, environmental disturbances, and various stresses. A disturbed normal microflora of the gastrointestinal tract of humans and animals is corrected by probiotic preparations containing live cultures of microorganisms, widely used lactobacilli in particular.

The production of probiotic preparations is faced with a number of problems, which can be solved through creation of immobilized microbial cultures.

Immobilization refers to the process of attachment of cells to carriers, after which their bioactivity decreases. As a result, immobilized cells exhibit excellent viability, stability and increased enzymatic activity.

For example, a decreased bioactivity of lactobacilli increases the rate of accumulation of metabolites, such as lactase, which control the intensity of homofermentation [8]. Thus, the speed of manufacturing various fermented milk products, and, hence, their shelf life can be controlled.

Common methods of microbial cell immobilization imply incorporation of objects into gels and capsules, adsorption on carriers or covalent binding to a carrier, cross-linking with bifunctional reagents with no carrier used, and cell self-aggregation followed by formation of flakes and granules.

However, these methods have a number of disadvantages: impossibility of a rapid outflow of metabolic products, inadequate diffusion barriers created by the carrier, insufficient supply of immobilized cells with nutrients, and potential unavailability and high cost of some types of carriers [9].
In this study, immobilization of lactobacilli on polyvinylpyrrolidone (PVP) nanowires was performed. PVP is a biocompatible material that dissolves slowly in water and other solvents. The efficiency of immobilization and enzymatic activity of cells attached to nanowires were evaluated, and the immobilization mechanism was determined.

2. Experimental technique
PVP nanowires for bacterial cell immobilization were synthesized by electrospinning – pulling of polymer wires from a capillary due to electrostatic field [10]. The solution was prepared using high molecular weight polyvinylpyrrolidone (Mr=1.3×10⁶ g/mol) dissolved in ethanol. Electrospinning was performed by means of a NewEra SyringePumpNE-300 syringe pump. The solution feed rate was about 0.3 ml/h. A potential difference of 10–13 kV was created between the capillary and the collector at a distance of 8–12 cm.

The morphology of the produced structures was studied using Hitachi SU1510 and TESCAN VEGA scanning electron microscopes, and a Keyence VK 9710 laser confocal microscope.

The study used a lactobacillus strain isolated from environmental objects. The strain was identified in the Laboratory of Molecular Diagnostics of the Center for Collective Use ‘Bioengineering’ (Moscow) using the 16S rRNA gene. For identification, universal primers 11f -1492r were employed [11]. The primary analysis of the similarity of the nucleotide sequences of the 16S rRNA genes of the studied strains was performed using the BLAST software [12] and the RDP Classifier [5]. According to the results of identification, the strain was assigned to the species *Lactobacillus brevis* [13] and referred to as *Lactobacillus brevis* 2k.Gv. General ecological and physiological characteristics of the strain are presented in Table 1.

| Species      | Ecological group | Respiration type   | Energy cycle                         |
|--------------|------------------|--------------------|--------------------------------------|
| *L. bravis*  | Symbiont         | facultative anaerobe | anaerobic pathway, presence of O₂ is permissible |

The biochemical characteristics of this strain corresponded to chemoorganotrophs, the metabolism was strictly fermentative by means of various substrates.

For the experiment on immobilization, *Lactobacillus brevis* 2k.Gv. strain was pre-grown in lactose broth at 37 °C for 48 hours. The cultivation was carried out in a sterile 250 ml Erlenmeyer flask filled with 50 ml of the nutrient base, 0.5 g of the carrier and 1 ml of the microbial mass at a concentration of 10⁹ CFU/ml. The ingredients were mixed with a a S:S-3L.A20 series shaker at a rotational speed of 110 rpm.

During the exponential phase of growth, 1 ml of lactobacillus cells was collected, centrifuged and washed with phosphate buffered saline (PBS, pH=7.2) followed by suspension in PBS (sodium phosphate buffered saline, non-toxic to cells) and incubation at 37 °C. To assess the viability of the isolated strains in the presence of PVP, 0.1 ml of the inoculation suspension was added to agar lactose medium. The number of cells was counted, and the data obtained were used to assess lactobacilli viability.

The effect of immobilization was considered positive in case of decreased concentration of cells in solution, stabilized enzymatic activity, increased volumetric productivity, expanded pH optimum, optimal conversion of the substrate into a product instead of biomass, and preserved viability of lactobacillus cells. The immobilization effect was assessed with regard to characteristics of cells: the ability to reach the maximum value of the cell titer, the logarithmic phase of growth, availability of a nutrient substrate, the ease of removal of toxic metabolic products, the possibility of reusing the by-products of the metabolism of immobilized cells.
To evaluate physiological activity of immobilized cells of *Lactobacillus brevis 2k.Gv.*, the optical density and growth rate were measured at different time points using the Reverse-Spinner RTS-1 bioreactor supplied with software and the function of real time monitoring the growth of microorganisms. Sterile TPP TubeSpin 50 vessels for aerobic culture with a membrane filter were used to incubate the immobilized cells. The volume of the culture medium was 29 ml, and the volume of the introduced reaction mixture (carrier with immobilized cells) was 1 ml. The optical density of the reaction mixture and the kinetics of the studied microbial culture growth were measured at $\lambda=850$ nm in a given range of 0–8 OD (OD is a decimal logarithm of the ratio of the incident radiation flux to the transmitted one) at a constant incubation temperature of 37 °C (temperature stability ±0.1°C).

In parallel, the parameters of the physiological state of microorganisms were assessed: the degree of the culture medium acidification, viability and biotiter. The acidity of the experimental and control culture solutions was measured using a pH meter-ionometer. Physiological activity of immobilized cells of *Lactobacillus brevis 2k.Gv.* was evaluated using a culture medium containing fish flour pancreatic hydrolyzate; baker’s yeast extract; meat extract; glucose; potassium phosphate 1-substituted; sodium acetate; ‘Twin-80’; ammonium citrate 1-substituted; magnesium sulfate; manganese chloride; agar; pH 5.7±0.3. The cultures were incubated in a thermostat at 37 °C for 24 hours. Working lactobacillus cultures were maintained on media using sterile skim milk.

3. Results and discussion

Figure 1 shows photographs of nanowires. The diameter of the wires is 310±53 nm, and spacing between them is 900±100 nm. 

*Lactobacilli* belong to the widespread nature of the physiological or acidophilus group lactic acid bacteria (LAB). Species of the genus *Lactobacillus* have a morphotype of rod-shaped cells reaching a length of 4-15 micrometers and a width of 0.5-0.6 micrometers [14]. The features of the ultrastructure of lactobacilli include a gram-positive type of cell wall, lack of mobility and the ability to form spores. By type of respiration, most members of the genus are facultative anaerobes: they do not contain the enzyme catalase, but can grow in the presence of oxygen. In the process of metabolism, homofermentation reactions is controlled by splitting glucose along the hexosodiphosphate pathway to form lactic acid [15].

The average pore size between wires is comparable to the diameter of lactobacilli; therefore, cells can easily be adsorbed in pores between wires.

As can be seen from the photographs (Figure 1), nanowires exhibit a large number of morphological defects: constrictions, inhomogeneities, roughness. There are numerous ‘droplets’ formed during synthesis. In addition, nanowires are arranged in the form of a matrix, which increases the total contact area of bacilli when they are incorporated in the pores between nanofibers. EDX analysis of pure nanowires did not reveal any foreign impurities.

![Figure 1](image-url)
Thus, the morphology of PVP nanowires indicates that they can be used to adsorb lactobacillus cells.

To immobilize cells, PVP filaments were added to the culture medium. In the control, the culture medium without PVP was used for lactobacillus cultivation. The immobilization period lasted 30 days.

In a series of experiments with immobilization of cells on nanofibers, two types of cells were used: dormant cells *Lactobacillus brevis* 2k. Gv. in lyophilization (L) and physiologically active cells *Lactobacillus brevis* 2k. Gv. (A).

The results of immobilization were assessed with respect to the changed growth rate of lactobacilli, acidity (pH), and oxidation reduction potential (ORP) of the culture medium.

The initial cell concentration was $10^9$ CFU/ml; the concentration after 30 days of the experiment is shown in Table 2.

Table 2. The number of viable cells (CFU/ml$^{-1}$) *Lactobacillus brevis* 2k. Gv. in the experiment and in the control.

| Cell variants | Medium with PVP  | Control  |
|---------------|------------------|----------|
| Dormant (L)   | $(7.4\pm0.6) \times 10^8$ | $(2.5\pm0.7) \times 10^5$ |
| Active (A)    | $(6.9\pm0.7) \times 10^9$ | $(1.9\pm0.5) \times 10^6$ |

According to the results obtained, the viability of both dormant and active cells in the medium with PVP is significantly higher than that in the control.

The acidity of the culture medium at the beginning of the experiment was pH $5.7\pm0.3$, ORP=72 mV. The values of these parameters after 30 days of the experiment are presented in Table 3.

Table 3. Acidity (pH) and ORP (Eh) of the culture medium in the experiment and in the control.

| Cell variants | Medium with PVP | Control |
|---------------|-----------------|---------|
| Dormant (L)   | Acidity (pH)    | 3.10    | 4.50    |
|               | ORP (Eh)        | 360.5   | 169.7   |
| Active (A)    | Acidity (pH)    | 2.70    | 4.02    |
|               | ORP (Eh)        | 374.0   | 204.6   |

Within 30 days of the experiment, the pH values of the culture medium decreased to 3.10 due to lactate accumulation. In the control, the pH did not exceed 4.50. Apparently, it was threshold acidity for native lactobacilli, which, with the depleted nutrient substrate and accumulated extracellular toxic metabolites, suppressed the growth of microorganisms and decreased their viability. In the experiment, Eh values increased to $+360$ mV. In the control, Eh was observed to increase only during 20 days of the experiment (from $+72$ mV to $+203$ mV), and after 30 days, Eh decreased to $+169$ mV. In all experimental versions, physiologically active cells (A) were found enzymatically stable.

Thus, within 30 experimental days, the population of immobilized cells retained a greater number of viable cells than lactobacilli that grow in liquid suspension cultures. In contrast to free cells, immobilized cells exhibit decreased activity, which ensures rapid accumulation of metabolites.

To study the immobilization mechanism, physiological activity and growth rate of cells were assessed using the Reverse-Spinner RTS-1 bioreactor.

The data on the optical density of the medium (Figure 2) show that in the first 50 hours of analysis, the cells actively expand and their number remains stable for a long period. Thus, it can be hypothesized that in the first 50 hours of life cells form biofilms, where cells actively expand. The biofilms attain a certain critical size, and the number of cells stops increasing.
A spontaneous growth of biofilms is known to be a multi-stage process: adsorption of the cell on a solid surface is followed by irreversible adhesion due to polymeric substances produced by the cell, and then the cell expands to form a biofilm [16]. After that, cells detach from the growing biofilm (Figure 4).

According to [17], in contrast to suspended cells, biofilms exhibit increased resistance to antimicrobial agents and other harmful factors.

The hypothesis of biofilm formation from lactobacilli is confirmed by the study of the growth rate of bacilli (Figure 3). The cells inside the biofilm are not capable of rapid growth, since the space for expansion is limited. Thus, in the first 20 hours of immobilization, the biofilms were not completely formed; therefore, the cells were not packed tightly and therefore showed a high growth rate. Then the growth rate began to decrease rapidly, which corresponds to the stage of biofilm formation (Figure 4).

Analysis of the graphs (Figures 2 and 3) allows assumption that in this case self-aggregation, i.e. independent biofilm formation is the main mode of lactobacilli immobilization.
For biofilm formation, cells initially need to be adsorbed. The initial culture medium does not contain carriers for lactobacillus cells to attach. In the culture medium with PVP, bacterial cells are adsorbed on nanowires to induce the initial phase of biofilm formation. Then nanowires dissolve, and the biofilm continues to form.

**Figure 5.** Photographs of lactobacillus biofilms taken using a Hitachi SU1510 scanning electron microscope: (a) x100 magnification; (b) x3000 magnification.

The photographs of the culture medium with immobilized lactobacilli confirm this hypothesis (Figure 5).

The photographs show an accumulation of locally distributed biofilms. The shape of biofilms is comparable to the theoretical one: cells of similar size growing on top of each other form clusters of random shapes.

Thus, bacilli are immobilized by self-aggregation, i.e. through biofilms formation. In this case, nanowires are material for cell adsorption only at the initial moment of immobilization. Biofilms are formed around the adsorbed cells, and nanowires gradually dissolve.

4. **Conclusions**

The data on immobilization of bacterial cells on PVP nanowires synthesized by electrospinning were obtained in the study. In contrast to free cells, immobilized cells exhibit increased viability and enzymatic activity.

The mechanism of lactobacilli immobilization on PVP nanowires is atypical: at the initial moment, cells are adsorbed on the surface of nanowires and begin to form a biofilm; in the state of biofilm, the metabolic activity of bacilli is stabilized. This can be due to the fact that after immobilization microbial enzymes acquire, in addition to stability, new properties that are not characteristic of their free state. For example, these are wider zones of optimum relative to temperature and acidity of the medium [18, 19]. In addition, cells are permanently retained in a certain place in the biofilm throughout the entire reaction, after which they can be easily separated from the reaction products and reused. The use of lactobacillus cells immobilized on PVP nanowires and products of cellular metabolism will expand the biotechnological potential of microorganisms through the increased stability, and the possibility of reuse and targeted control of enzymatic cycles. In addition, nanowires can be modified with various functional molecules with specified physicochemical properties, which can be used as adequate sources of growth factors for probiotic cultures. This will significantly reduce the cost of ingredients for their cultivation.
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