Resistance of Probiotic Bacteria Immobilized on PVP Nanofilaments in Gastrointestinal Juice

P.O. Lyashkov, *O.Ya.* Berezina, *N.A* Sidorova, *A.D.* Rikkinen and *M.A.* Germanova

Petrozavodsk State University, Petrozavodsk, Russian Federation

Abstract. The article discusses the stability of various types of immobilized forms of probiotic bacteria in the analogue of gastric juice. Immobilization was carried out in an innovative way: bacterial cells are first fixed to the carrier in the form of PVP nanofilaments, after which the nanofilaments dissolve in the culture medium, and the bacteria, fixing on each other, form biofilms.

1 Introduction

In the production of agricultural products, in most cases, animals, birds and fish are kept very crowded, which requires the use of antibiotics for the treatment and prevention of various diseases. Intestinal dysbiosis appears in animals, the environment is harmed, the remains of antibiotics in meat enter the human body [1].

Antibiotics can be replaced in whole or in part with probiotic feed additives. In this case, both producers and consumers of agricultural products benefit. The costs associated with the treatment and death of animals from infectious diseases will decrease, the productivity of animals will increase, the products produced will not harm human health.

In the production of probiotic drugs, the method of immobilization of bacterial cells on solid carriers is widely used. We also propose a method of "soft" immobilization on nanofilaments of biocompatible soluble polymers [2]. This method makes it possible to simplify the technological process of production of probiotics and obtain highly effective drugs.

At the synthesis stage, various metals can be added to the nanofilament in the right concentrations, which, as part of the probiotic, will make up for the lack of a number of trace elements in the organisms of farm animals and fish.

The therapeutic effect of a probiotic drug begins in the intestines of an animal or a person. Therefore, it is important that, after passing through the esophagus and stomach with its aggressive acidic environment, the drug does not collapse. To assess the possibility of using a probiotic made by the proposed method, it is necessary to test the drug for resistance in gastric juice.

2 Materials and methods

2.1 Materials

2.1.1 Polyvinylpyrollidone

Polyvinylpyrrolidone (PVP) is an aminobutyric acid polymer, chemical formula $(C_6H_{12}NO)_n$. Vinyl radical easily enters into polymerization reactions, which allows, by changing the concentration of the catalyst, to obtain polymers both low-molecular (6000-10000 AMU) and high-molecular (50000-60000 AMU).

Vinylpyrrolidone itself is obtained from acetylene, which makes polyvinylpyrrolidone a relatively cheap polymer. The solubility of PVP is due to the presence of a lactam group.

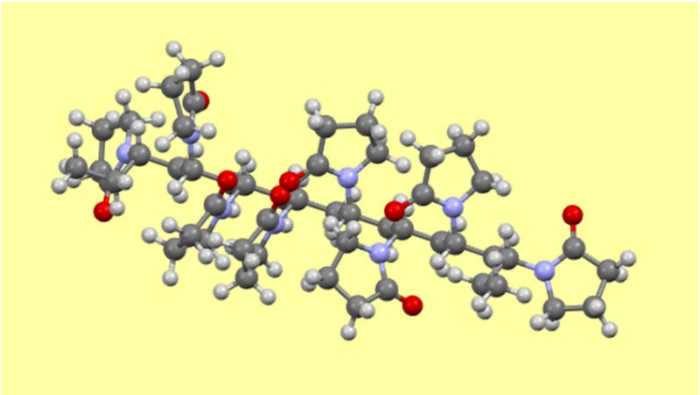

Fig. 1. 3D image of a part of the PVP molecule.

The PVP molecule (Figure 1) contains the N-C=O group, which makes polyvinylpyrrolidone a good adsorbent.

2.1.2 Zinc

In the experiment, PVP nanofilaments were alloyed with zinc. The choice of zinc is due to its importance for the vital activity of the body, along with iron, iodine, magnesium.

The growth rate of young animals depends on the amount of zinc, because it participates in metabolic processes – it promotes the absorption of nitrogenous substances and vitamins [3]. In the animal body, the highest concentration of zinc is in the bones and skin.

The ability of zinc to have an antibacterial effect makes it possible to control the enzymatic activity of bacteria immobilized on PVP nanofilaments doped with zinc.

Zinc doping of nanofilaments was carried out at the stage of preparation of the working solution – zinc acetate $Zn(O_2CCH_3)_2$was added to it. It exists in two types: dehydrated (molar mass 219.5 g/mol) and anhydrated (molar mass 183.48 g/mol). Under normal conditions, salt is colorless crystals, perfectly soluble in water and various organic compounds.

During the manufacture of the working solution, it was dissolved in ethanol, which made it possible to obtain a PVP solution of lower viscosity at the same concentration than when dissolved in water [4].

2.2 Methods

Incubation of immobilized cells was carried out in sterile TPP TubeSpin 50 vessels with a volume of 50 ml for aerobic cultivation with a membrane filter. The volume of the nutrient medium was 29 ml, and the volume of the introduced reaction mixture (carrier with cells immobilized on it) was 1 ml.

To assess the growth rate of microbial cultures and the effectiveness of immobilization, the dependence of the optical density of the nutrient medium with bacteria on time was measured. The optical density (OD - optical density) of the reaction mixture and the growth kinetics of the studied microbial cultures were measured at a wavelength of 850 nm in the range 0 - 8 OD, where OD is the decimal logarithm of the ratio of incident and transmitted radiation fluxes. At OD = 0, the light passes completely, at OD = 8, the light is weakened by 10^8 times. The measurement accuracy was ± 0.3 OD, incubation temperature (37 ± 0.1) °C.

At the same time, the parameters of the physiological state of microorganisms were evaluated: the degree of acidification of the culture medium, viability and biotitre for experimental and control culture solutions. The cultivation of bacteria was carried out in the Reverse-Spinner RTC-1C bioreactor. The culture medium, together with nanofilaments and lactobacilli in 50 ml test tubes, was stirred by rotation when the direction of rotation changed.

Artificial gastric juice of the following composition (g/l) was used to simulate gastric stress: NaCl (SigmaS9625) – 2.2; L-lactic acid (SigmaL1750) – 9.9 (0.11 M); pepsin (pork) (SigmaP7125) – 3.5 units/mg. pH: 2.7 ±0.1. pH after dilution 3.1± 0.1. In the experiment, 0.1 ml of the studied culture in the stationary phase was added to 1 ml of artificial gastric juice, and in the control to 1 ml of the medium. Both variants are incubated for 30 min at 37 °C, then samples are taken. The experiment is performed three times. The selected samples after incubation are diluted from 10^2 to 10^{10} in a nutrient medium (MRS broth is a medium for cultivating and counting lactobacilli or LBB — lacto and bifidobacteria), dilution is plated by a deep method on cups with agar medium (MRS agar or LAB — lactic acid bacteria) and incubated for 24 - 48 hours at 37 °C in the thermostat. Determine the number of surviving bacterial cells by counting the number of colonies that have grown.

The viability of immobilized forms in gastric juice was studied with three types of bacteria: *Lactobacilus brevis, Bacillus subtilis* and *Bifidobacterium bifidum.* These bacteria synthesize enzymes that restore the intestinal microflora and can be used as probiotics. *Bacillus subtilis* bacteria are resistant to antibiotics, so the resistance of their immobilized form to zinc supplementation is of interest.

3 Discussion of results

Tests for resistance to gastric juice were carried out for bacteria immobilized on pure PVP nanofilaments and PVP filaments doped with zinc at concentrations of 6, 9, 15%. Lactobacillus cultures with the addition of powdered polyvinylpyrrolidone instead of nanofilaments served as a control. The initial concentration of bacteria was $1.5 \cdot 10^7$ CFU/ml. In imitation of gastric juice, the bacteria were kept for 24 hours.

The test results are shown in Table 1.

Table 1. The concentration of bacteria immobilized on PVP nanofilaments with zinc addition after exposure in imitation of gastric juice.

Sample	PVP + Zinc 6% at., CFU/ml	PVP+zinc 9% at., CFU/ml	PVP+zinc 15% at., CFU/ml	PVP powder, CFU/ml
L.brevis	$0.21 \cdot 10^5$	$0.36 \cdot 10^7$	$0.11 \cdot 10^2$	$0.26 \cdot 10^3$
B.subtilis	$0.43 \cdot 10^4$	$0.41 \cdot 10^6$	$0.14 \cdot 10^2$	$0.11 \cdot 10^2$
Bifidum	$0.11 \cdot 10^2$	$0.17 \cdot 10^6$	$0.36 \cdot 10^2$	$0.22 \cdot 10^3$

From the data in the table, it can be seen that in the control forms of bacteria, resistance to gastric juice is low. This is due to the fact that the immobilization of bacteria to PVP powder practically does not occur, and the viability of non-immobilized forms is low.

The most resistant to gastric juice are all types of bacteria immobilized on nanofilaments with a zinc concentration of 9% at. After exposure to simulated gastric juice, the concentration of bacteria decreased slightly compared to the initial one. Thus, an additive of zink 9% at. to PVP nanofilaments makes it possible to enhance the viability of lactobacilli immobilized on such filaments.

The viability of bacteria immobilized on filaments with a zinc concentration of 15% at. was lower than in the control or approximately the same. It can be assumed that a high concentration of zinc has an antibacterial effect.

The viability of bacteria immobilized on filaments with a zinc concentration of 6% at. is high for *L.brevis* and *B.subtilis* and below control for *Bifidum.*

The survival rate of *Bifidum* is high only at a zinc concentration of 9% at. At other zinc concentrations, the viability is low. It can be assumed that low survival is associated with anaerobicity (oxygen is not required for vital activity). The presence of a large amount of free oxygen in the applied imitation of gastric juice has a bad effect on the *Bifidum* [6]. The bacteria *L.brevis and B.subtilis* are aerobic, so their survival in the immobilized form is high and with a small concentration of zinc.

The most resistant to gastric juice were the immobilized forms of *L.brevis*.

4 Conclusions

The results of the experiments showed that the probiotic feed additive, made by the method of "soft" immobilization of lactobacilli on a biocompatible soluble polymer, withstands the destructive effects of the acidic environment of the stomach. The optimal concentration of zinc in PVP filaments used for immobilization is about 9% at. To use the resulting drug as an antibiotic, it is necessary to use nanofilament with a zinc concentration close to 15% at.

The most resistant to the aggressive environment of the stomach was a drug based on *Lactobacilus brevis* bacteria.

Acknowledgments

The work was carried out as part of the implementation R&D Support Programs for students and postgraduates of PetrSU, funded by the Government of the Republic of Karelia, Agreement No. KGRK-21/ NZ-16.

References

1. O. I. Kalnitskaya, Veterinary and sanitary assessment of animal and poultry meat containing antibiotics, AVU, **7** (2008).
2. O. Ya. Berezina, N. P. Markova, A. V. Semenov, N. A. Sidorova, Possibilities of using nanofilaments based on polyvinylpyrrolidone for immobilization of Lactobacillusacidophilus cells, Izvestiya vuzov. Applied chemistry and biotechnology, **2(25)** (2018).
3. M. A. Wattio, Digestion and feeding: A technical guide to milk production, 22 (Babcock Institute, 1994).
4. A. V. Capilla, R. A. Aranda, Anhydrous Zinc(II) Acetate (CH3-COO)2Zn (рум.), Crystal Structure Communications, **8**, 795-797 (1979).

5. M. Jungersen, A. Wind, E. Johansen et al., The science behind the probiotic strain Bifidobacterium animalis subsp. lactis BB-12®, Microorganisms, **2**, 92-110 (2014).