# IMPROVEMENT OF THE PROCESS OF PRODUCTION OF POLYSACCHARIDE POLYRIBOSYL RIBITOL PHOSPHATE USED IN THE *HAEMOPHILUS INFLUENZAE* VACCINES

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The production of vaccines requires constant improvement of methods and tools, revision and modernization of the current technology with the aim to improve quality of the product made for the benefit of public health. The purposяe of this work was to improve the process of production of polysaccharide polyribosyl ribitol phosphate (PRP), which is the active agent of *Haemophilus influenzae* type b (Hib) vaccines. We investigated how PRP yield depends on the following factors: concentration of dissolved oxygen in the culture liquid, glucose concentration control method applied in cultivation, source of protein for the producer microorganism, stability of the polysaccharide at the culture liquid inactivation stage. As a result, we managed to increase the PRP yield in the culture liquid by 10%, ensured a 25% boost of the biomass accumulation rate during cultivation in the fermenter and reduced the cultivation time by 6.5 hours. The PRP loss rate at the culture liquid inactivation stage was reduced by 80%. Relying on the patented composition, we invented a new composition of the nutrient medium that meets the current regulatory requirements.

**Keywords:** *Haemophilus influenzae* type b, polyribosyl ribitol phosphate, nutrient medium, vaccine, cultivation, peptone, hemin, protoporphyrin

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# УСОВЕРШЕНСТВОВАНИЕ ПРОЦЕССА ПОЛУЧЕНИЯ ПОЛИСАХАРИДА ПОЛИРИБОЗИЛРИБИТОЛФОСФАТА, ИСПОЛЬЗУЕМОГО В ПРОИЗВОДСТВЕ ВАКЦИН ПРОТИВ ГЕМОФИЛЬНОЙ ИНФЕКЦИИ

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Производство вакцин требует постоянного усовершенствования методов и инструментов, пересмотра и модернизации существующих технологий, позволяющих получать качественный продукт для обеспечения здоровья населения. Целью работы было усовершенствование стадии получения полисахарида полирибозилрибитолфосфата (PRP) — активного компонента вакцин для профилактики гемофильной инфекции. Изучено влияние на выход PRP следующих факторов: концентрации растворенного кислорода в культуральной жидкости, способа регулирования концентрации глюкозы во время культивирования, источников белкового питания микроорганизма-продуцента, стабильности полисахарида на стадии инактивации культуральной жидкости в процессе получения полисахаридной вакцины для профилактики гемофильной инфекции. Выход PRP в культуральной жидкости увеличен на 10%, скорость накопления биомассы во время культивирования в ферментере — на 25%, время культивирования сокращено на 6,5 ч. Потери PRP на стадии инактивации культуральной жидкости сокращены на 80%. Предложен новый состав питательной среды на основе запатентованного состава, соответствующий актуальным требованиям нормативной документации.

**Ключевые слова:** *Haemophilus influenzae* тип b, полирибозилрибитолфосфат, питательная среда, вакцина, культивирование, пептон, гемин, протопорфирин

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*Haemophilus influenzae* type b causes severe meningitis and pneumonia in children. Before the widespread vaccination, *Haemophilus influenzae* type b (Hib) was behind 8.13 million cases of invasive diseases among children under 5 years of age and 371 thousand deaths. Vaccination campaigns in 136 brought down the associated mortality by 45.28% [1]. The risk of complications (severe deafness in children, 10% of cases) makes Hib especially dangerous [2]. Haemophilus influenzae is highly resistant to antibiotics, which makes anti-infective chemotherapy less effective against the disease associated therewith [3, 4].

The vaccines used for prevention of the *Haemophilus influenzae* disease are of the combined type. There are three such vaccines registered in Russia: Infanrix Hexa, aAPDT-HEP B+Hib, Pentaxim. However, there are no monovalent Hib vaccines registered in the country, which can be used for patients intolerant to any of the components of the combined vaccines. The current scale of Hib vaccination in Russia is insufficient [5], therefore, to secure reliable availability of the Hib prevention preparations, it is necessary to set up domestic production of such vaccines relying on the current advancements in biotechnology.

Optimization of biotechnological production means increasing the amount of the product yielded from one batch without compromising its quality, as well as making the production time shorter. Polysaccharide polyribosyl ribitol phosphate (PRP) is the active component of the vaccine used to prevent *Haemophilus influenzae* disease in children [6–8]. It induces an effective immune response upon conjugation with the carrier protein. PRP is a microbial synthesis product made at biotechnological facilities. The production technology used by a facility directly determines the amount of polysaccharide produced, with the key production stages affecting the yield being fermentation, which is expected to deliver the maximum

amount of PRP, and isolation and purification, which minimize losses.

Designing solutions, a drug developer should rely on regulations: WHO requirements and recommendations [9], GMP system, and pharmacopoeial monographs [10, 11]. The XIV$^{th}$ edition of the State Pharmacopoeia of the Russian Federation contains the first-ever monograph regulating production and control of quality of the Hib vaccine [11]. The quality of production of the *Haemophilus influenzae* disease vaccine depends on the biotechnological stage thereof: cultivation of the producer culture in a fermenter in order to synthesize PRP. The main task at this stage is to produce the maximum amount of polysaccharide that meets the requirements of the specification.

There are many factors that influence quantity and quality of the produced PRP: concentration of oxygen dissolved in the culture liquid during cultivation; concentration of glucose and method of introduction of additional nutrients to the culture fluid during cultivation; source of nitrogen nutrition and growth factors in the nutrient medium; conditions of cultivation and inactivation of the culture liquid to stabilize the end product.

The purpose of this work was to improve the PRP production technology by optimizing the stage of cultivation of the producer culture in a fermenter.

## METHODS

### Estimation of the amount of polysaccharide

The quantitative content of PRP was determined with the help of the orcinol method (ribose identification) [12]. The samples were appropriately prepared before polysaccharide quantity assessment: it was precipitated in the culture liquid with a cetyltrimethylammonium bromide (CTAB) solution, the samples were centrifuged, upper layer removed and the PRP-containing precipitate dissolved subsequently [13, 14].

### Producer microorganism

*Haemophilus influenzae* type b, a naturally occurring agent, is used in polysaccharide production. It is a gram-negative coccobacillus, auxotroph (requires growth factors in the medium, hemin and nicotinamide adenine dinucleotide (NAD)), facultative anaerobe. We used the *Haemophilus influenzae* SPB type b strain deposited in the State Collection of Pathogenic Microorganisms and Cell Cultures of the GNCPMB under the number B-7884 [15].

### Cultivation

To prepare the inoculum for cultivation in a laboratory fermenter, we added 1 ml of thawed culture from a cryovial (working inoculum) to 150 ml of a synthetic liquid nutrient medium poured into flasks. The seeded nutrient medium was kept on a Unimax 1010 incubator shaker (Heidolph; Germany) for 6 h with temperature and rpm controlled. Optical density and microbiological purity were the controlled properties of the inoculum. The entire 150 ml of inoculum were subcultured into a Biostat A laboratory fermenter (Sartorius Stedim Biotech; Germany). Cultivation lasted 18 hours and was carried out in a 2.0 L laboratory fermenter, with ceaseless stirring and oxygen supply and controlled pH at 7.2±0.2. With the aim to investigate how oxygen concentration in the culture liquid affects the yield, we maintained its level at 10, 30 and 60% during cultivation. The fermenter consisted of a borosilicate glass flask (UniVessel), a stand and a lid made of AISI 304 stainless steel, a six-blade two-tier stirrer agitating the culture liquid, a bubbler supplying sterile compressed air to the culture liquid. During cultivation, the temperature of the culture liquid was controlled with the help of a heat exchanger made of AISI 304 stainless steel complete with a heating plate mounted on the outer surface of the fermenter flask. To maintain pH at the required level, we delivered 2.0 M NaOH and 1.0 M HCl (titrating solutions) in the automatic mode. Glucose concentration was controlled manually, by introduction of a feed solution of dissolved glucose and yeast extract. A sampler mounted into the lid of the fermenter allowed taking samples during cultivation.

### Nutrient medium

For the study part, we used a semi-synthetic liquid nutrient medium [16] containing saline solutions, sources of nitrogen and carbon nutrition, and growth factors. For the experiments designed to investigate the impact of nitrogen nutrition sources and growth factors, we changed the patent composition of the nutrient medium: animal peptone was replaced by vegetable (soy) peptone (Sigma-Aldrich, P6463; Germany); pork hemin — by protoporphyrin IX.

## RESULTS

*Haemophilus influenzae* type b is a facultative anaerobe, therefore, it is possible to influence its growth and biosynthesis of the target product by controlling aeration during cultivation. In this context, at the first stage we studied the impact of aeration on the accumulation of biomass and synthesis of the target product, PRP. It was assumed that by changing the oxygen concentration in the culture liquid, it would be possible to regulate the biochemical processes of the producer microorganism and stimulate it to accumulate biomass (the target product is a polysaccharide, which is the outer protective shell of the bacterium) or accelerate synthesis of the target product when the producer is under stressful conditions.

Culture liquid was saturated with oxygen by bubbling it with sterile compressed air and stirring with a two-tier six-blade stirrer (Biostat A fermenter design). Table 1 presents the results of the experiment designed to investigate the impact of the culture liquid oxygen concentration in cultivation.

With aeration increased by early intensive stirring and bubbled supply of compressed sterile air (to maintain the oxygen concentration at 60%), biomass accumulation grows up 1.3 times, which is significant, but synthesis of the target

**Table 1.** Impact of aeration on *Haemophilus influenzae* type b biomass growth and synthesis of the target product during the preparation of inoculum

| Concentration of oxygen dissolved oxygen in the culture liquid, % | Culture liquid optical density* | Concentration of PRP, μg/ml* |
|---|---|---|
| 10 | 3,232 ± 0,109 | 350 ± 23 |
| 30 | 4,101 ± 0,095 | 423 ± 27 |
| 60 | 5,634 ± 0,145 | 450 ± 21 |

**Note:** PRP — polyribosylribitolphosphate; * — the table shows standard deviation.

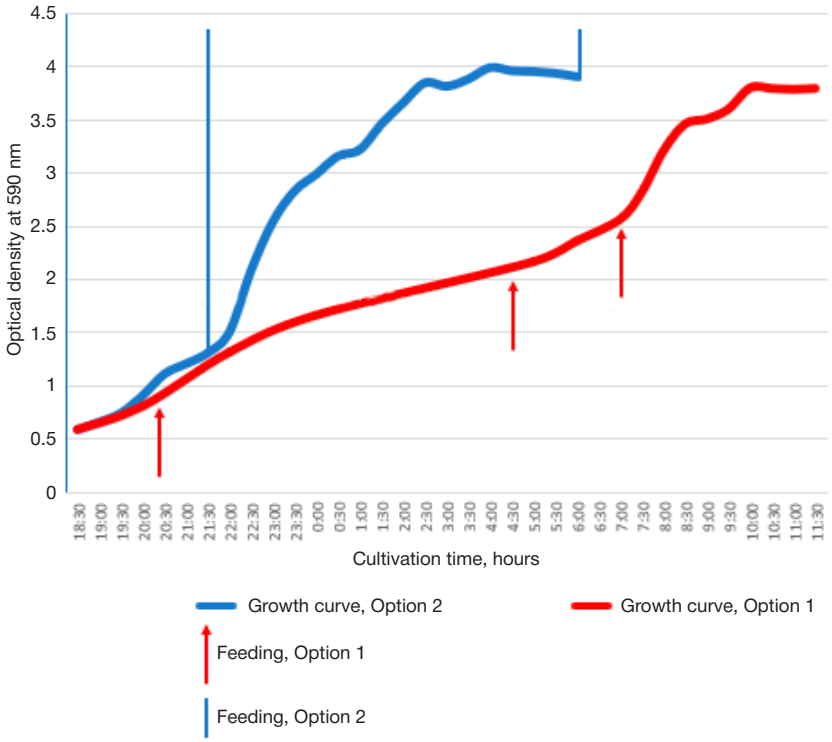


**Fig. 1.** Growth curves describing cultivation of *Haemophilus influenzae* type b

product increased insignificantly (maximum by 10%) (see Table 1).

The next step was to study the impact of the culture liquid glucose concentration on PRP yield during cultivation of *Haemophilus influenzae* type b.

We used a solution of glucose and yeast extract as a feed in cultivation of *Haemophilus influenzae* SPB type b B-7884. Glucose acts as a carbon source, and yeast extract is the source of nitrogen, vitamins and microelements. It is assumed that introduction of a large volume of feed, for example, at the beginning of the exponential phase, can significantly accelerate the formation of biomass due to the high concentration of nutrients in the culture liquid. Controlled feeding throughout the cultivation process allows responding to the microorganism's need for glucose at a given moment and adding the nutrients to the culture liquid gradually.

To study the impact of the feeding method, we compared two options: option 1, introduction of large volumes at certain stages of cultivation (upon reaching a certain optical density value), and option 2, introduction of small volumes throughout the entire cultivation, seeking to maintain glucose concentration within the range from 12 to 34 mmol/L. Figures 1 and 2 show the results of this com comparison.

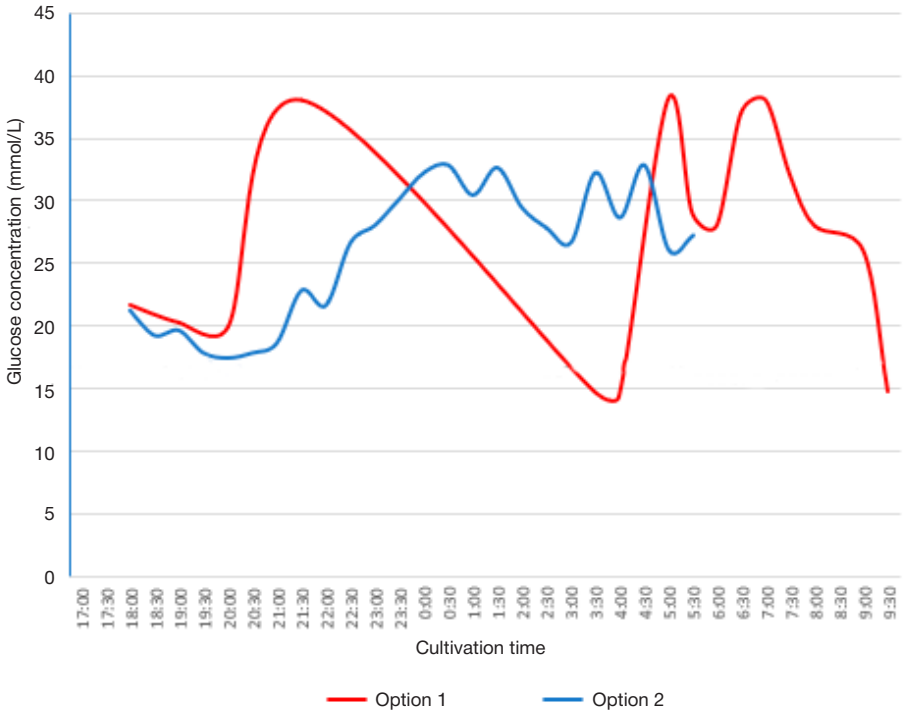


**Fig. 2.** Glucose concentration in cultivation of *Haemophilus influenzae* type b

**Table 2.** The results of cultivation of *Haemophilus influenzae* type b in nutrient media containing different sources of protein nitrogen

| Peptone | OD* before cultivation | OD* after cultivation | OD* difference | Concentration of PRP, μg/ml | Percentage of PRP |
|---|---|---|---|---|---|
| From animal raw materials | 0,245 ± 0,034 | 1,150 ± 0,097 | 0,905 ± 0,063 | 307,5 ± 5,3 | 100 |
| Soy | 1,213 ± 0,085 | 2,092 ± 0,102 | 0,879 ± 0,017 | 288,2 ± 7,4 | 93 |

**Note:** * — optical density

With the first feeding method, it took 16 hours to achieve the culture liquid optical density of 3.8 ± 0.2, which was maintained for 1 hour of cultivation. The reached optical density of 3.8 ± 0.2 and its persistence at this level indicate onset of the stationary phase. The second feeding method allowed reaching the optical density value marking transition to the stationary phase faster, in 12.5 hours.

The next step was to investigate how various sources of nitrogen and growth factors affect PRP biosynthesis.

The pharmacopoeia monograph regulating production and control of quality of the Hib vaccine sets out the requirements for PRP quality indicators and PRP production recommendations, including composition of the nutrient media used for cultivation. One of them is to use the media free from animal products with the aim to eliminate the risk of prion infection. The nutrient media used for cultivation of *Haemophilus influenzae* type b B-7884 contains some animal products. One of them is peptone, made from meat, another is hemin, the X-growth factor produced, in most cases, from pork or beef material. To ensure conformity to the regulatory requirements, it is necessary to study how the origin of nitrogen sources and growth factors in the nutrient medium influences growth of biomass and biosynthesis of the target product. Peptones from animal raw materials can be replaced with peptones derived from plants: pea, wheat, soy and proteose peptones. These peptones can differ significantly in chemical composition, and since biomass growth and synthesis of PRP depend on the amino acid composition of the medium [17–19], the chemical composition of peptones can have a significant effect on the production of the target product.

It is also important to evaluate how the origin of source of the X factor in the nutrient medium influences growth of the *Haemophilus influenzae* type b biomass and PRP biosynthesis. The X factor is involved in the synthesis of cytochrome C and other iron-containing respiratory enzymes. *Haemophilus influenzae* type b has an enzyme called ferrochelatase, which converts protoporphyrin IX to hemin [20]. Thus, hemin can be replaced with protoporphyrin IX in the nutrient media used to cultivate *Haemophilus influenzae* type b [20]. Traditionally, the X factor used for the purpose is hemin derived from the blood of cattle. This is a potentially dangerous raw material that presents the risk of prion contamination. Using pig blood as the source of hemin is one approach to mitigation of the said prion infection risk [21], but some countries outlaw pig products, which hinders imports of the vaccine and makes replacement of hemin with protoporphyrin promising.

We conducted a number of experiments to assess the possibility of replacement of animal peptone with plant peptone. The control medium was a nutrient medium containing peptone from animal raw materials and yeast extract as prescribed by the patent [16] in a ratio of 15 : 2. The peptone-to-yeast ratio in the experimental culture media with plant peptone was the same. The cultivation was done in shaking flasks on an incubator shaker; it lasted for 6 hours at a temperature of (35 ± 2) C°, with the shaking flasks constantly stirred at 150 rpm. Table 2 shows the results of the experiment designed to evaluate the possibility of replacing animal peptone with plant peptone.

The results of cultivation in the nutrient medium based on soy peptone were similar to the results peculiar to cultivation in the animal peptone nutrient medium.

Next, we experimented with replacing hemin (the X factor substance) with protoporphyrin IX. The cultivation was done in shaking flasks on an incubator shaker; it lasted for 6 hours at a temperature of (35 ± 2) C°, with the shaking flasks constantly stirred at 150 rpm.

Table 3 shows how the source of the X factor affects the biomass growth.

Culture medium with protoporphyrin IX as the X factor gave a yield resembling that produced by the medium with hemin (patent medium).

The experiments allowed suggesting a new composition of the nutrient medium that accords with recommendations set out in the regulations. This new composition has different sources of nitrogen nutrition and the X factor. Table 4 shows the results of comparison of the two media.

Experimentally, we discovered that a nutrient medium without components of animal origin insignificantly slows productivity of *Haemophilus influenzae* type b (by 8%).

The next step was to investigate the possibility to reduce PRP losses at the culture liquid inactivation stage.

Fermentation is followed by purification allowing to isolate the target product. Purification may be chemical (precipitation, extraction) and mechanical (filtration); regardless, this process implies unrecoverable losses of the target product since the stage of biological transformations (biosynthesis) is over by that time. According to the requirements for products made with the involvement of pathogenic microorganisms, the end product should contain no living pathogenic microorganisms. To eliminate the risk of such contamination, vaccine producers typically resort to inactivation, which completely kills all living microorganisms in the culture medium after cultivation. Inactivation, regardless of the method of implementation (chemically or thermally), is inevitably associated with the loss of the target product. During inactivation, exposed to high temperatures, PRP can depolymerize and degrade. It is known that the PRP polysaccharide is more stable in an acidic medium

**Table 3.** The results of cultivation with various substances acting as X factors

| Nutrient medium | OD[1] before cultivation | OD[1] after cultivation | OD[1] difference | PRP content, μg/ml | Percentage of PRP |
|---|---|---|---|---|---|
| Hemin environment[2] | 0,343 ± 0,024 | 1,649 ± 0,101 | 1,306 ± 0,077 | 307,5 ± 10,5 | 100 |
| Protoporphyrin medium[3] | 1,015 ± 0,092 | 1,757 ± 0,145 | 0,742 ± 0,053 | 300,1 ± 11,3 | 98 |

**Note:** [1] — optical density; [2] — patent composition medium; [3] — medium of patent composition, that contains same concentration of protoporphyrin instead of hemin

**Table 4.** Comparison of the two *Haemophilus influenzae* type b cultivation nutrient media by productivity and biomass accumulation

| Nutrient medium | Optical density before cultivation | Optical density after cultivation | Optical density gain | PRP content, μg/ml | Percentage of PRP |
|---|---|---|---|---|---|
| Patent composition | 0,352 ± 0,023 | 1,767 ± 0,125 | 1,41 ± 0,102 | 448,5 | 100% |
| No components of animal origin[1] | 3,064 ± 0,164 | 4,456 ± 0,312 | 1,39 ± 0,148 | 427,2 | 92,25% |

**Note:** [1] — patent composition medium with meat peptone replaced with soy and pork hemin with protoporphyrin IX.

(pH 6.5 and below), where, according to the mathematical model, it virtually does not depolymerize [22].

When the cultivation process in the fermenter was complete, we lowered the pH of the culture liquid to 6.5 by introducing titrating agents. Then the culture liquid was transferred to the inactivation stage. For the purpose of control, we relied on the cultivation data that had the pH unchanged until its completion (pH 7.2 ± 0.2). The results are shown in Table 5.

Lowering the pH during inactivation allowed to reduce the losses at this stage by 80%.

## DISCUSSION

### Investigation of the impact of the culture liquid oxygen concentration in cultivation

As shown by the experiments (see Table 1), the dependence of biomass growth and PRP synthesis may be associated with the fact that the culture of Haemophilus influenzae SPB type b B-7884 actively consumes oxygen to oxidize nutrients used in the anabolic processes of formation of the new cells, which slows down the synthesis of the target product. In addition, the increased amount of biomass can affect the parameters of the subsequent stages of isolation and purification, significantly complicating them due to the increased load on equipment and materials, which boosts the loss of the target product at these stages.

With culture liquid oxygen concentration decreased to 30%, biomass growth went down by 27% and the synthesis of the target product slowed down insignificantly (by up to 6%), allowed producing a sufficient amount of PRP, isolating and purifying it. An experiment with the culture liquid oxygen concentration at 10% ended in a significant decrease in the level of both biomass and PRP.

These results indicate that oxygen concentration in the culture liquid affects cultivation, which can be used to optimize the process of production to ensure the maximum possible yield of the PRP while keeping the subsequent isolation and purification stages as simple as possible in the view of the increased amount of biomass.

### Study of the impact of the culture liquid glucose concentration on PRP yield during cultivation of *Haemophilus influenzae* type b

The second feed introduction method tested enabled rapid transition to the stationary phase (Fig. 1), which is associated with maintaining a certain concentration of glucose in the culture fluid (Fig. 2). The first feeding method option implied introduction of large amounts of glucose and subsequent spike in its concentration, which can inhibit growth of the culture. With the glucose level kept constant, as the feeding option 2 allows, there are no sharp increases in glucose concentration, and, consequently, no inhibition of culture growth. The PRP yield in the experiment that tested option 1 was 403.2 μg/ml, and that for option 2 was 443.5 μg/ml. These results enable optimization of the glucose delivery strategy during cultivation.

### Investigation of how various sources of nitrogen and growth factors affect PRP biosynthesis

As shown experimentally (Table 2, 3, 4), replacement of animal components with plant components in the *Haemophilus influenzae* type b cultivation medium led to an insignificant (less than 9%) decrease in the amount of the produced PRP. Despite this, it was shown that it is possible to replace some animal components of the nutrient medium with components of non-animal origin, which will ensure conformity of the Hib vaccine production facilities to the latest regulatory requirements.

### Investigation of the possibility to reduce PRP losses at the culture liquid inactivation stage

Based on the results of the experiment (Table 5), it is possible to offer a fundamental possibility of reducing the loss of PRP at the inactivation stage by lowering the pH of the culture liquid to 6.5 ± 0.1 once cultivation is complete. But, regardless, it should be noted that direct cultivation at such pH values is impractical, since accumulation of the polysaccharide under such conditions is slower [23].

## CONCLUSIONS

In the course of this work, we suggested several methods of optimization of the process of producing PRP through cultivation of *Haemophilus influenzae* SPB type b B-7884. The results of this study allow optimizing a PRP production facility to ensure conformity to the regulatory requirements. The yield of PRP from the culture liquid was increased by 10%. The rate of biomass growth during cultivation in a fermenter was increased by 25%, the cultivation time is reduced by 6.5 hours. The PRP loss rate at the culture liquid inactivation stage was reduced by 80%. We also suggested a composition of a new culture medium is proposed that meets the latest requirements of regulatory documents. The results of this study allow improving the production process of PRP, which is the active component of Hib vaccines.

**Table 5.** Study of the influence of culture liquid pH of inactivation

| pH at inactivation | PRP before inactivation, μg/ml | PRP after inactivation, μg/ml | Loss of PRP during inactivation |
|---|---|---|---|
| 7,2 ± 0,2 | 405,6 ± 12,6 | 305,2 ± 10,3 | 100,4 ± 11,3 μg/ml |
| 6,5 ± 0,1 | 407,4 ± 14,1 | 388,6 ± 9,4 | 18,8 ± 12,7 μg/ml |

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