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# NUTRIENT MEDIUM SELECTION AND OPTIMIZATION OF *AVIBACTERIUM PARAGALLINARUM* DEEP CULTURE METHOD

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**SUMMARY**

Different liquid nutrient media supplemented with growth factors intended for *Avibacterium paragallinarum* strain No. 5111 cultivation were compared. The highest specific growth rate ($\mu = 0.787 \pm 0.041$ $h^{-1}$) and the maximal accumulation of the agent's biomass ($X = 9.52 \pm 0.04$ lg CFU/$cm^3$) were reported when cultured in casein soybean broth. Herewith, the mean time of the live microbial cell concentration doubling was minimal (td = 0.88 h), and the exponential growth phase lasted for 6 hours. The optimal method for *Avibacterium paragallinarum* cultivation in casein soybean broth in laboratory bioreactor Biotron LiFlus GX was determined through the measurements and adjustment of basic physical and chemical parameters. The time period until the culture reached the stationary growth phase was maximal with aeration at 1.0 l/min; herewith, the $O_2$ partial pressure in the nutrient medium did not exceed 25%. The period of the intense decrease of medium's pH was accompanied with the exponential phase of the bacterial growth. The nutrient medium's pH ranging from $7.30 \pm 0.02$ to $7.90 \pm 0.06$ had no significant impact on the specific growth rate of the strain and the lag phase duration was minimal – 0.36–0.45 h. The strain cultivation in the nutrient medium with pH $7.90 \pm 0.06$ demonstrated maximal aggregation of the bacteria ($9.76 \pm 0.04$ lg CFU/$cm^3$). 40% glucose solution added at 0.6-0.8 g/l during cultivation facilitated the decrease of the suspension's pH. Minimal redox value (–75 mV) was indicative of the completion of the exponential phase of the strain growth.

Key words: avian infectious coryza (Glasser's disease), *Avibacterium paragallinarum*, strain No. 5111, cultivation.

## INTRODUCTION

Avian infectious diseases characterized by lesions in the respiratory tract presently remain one of the most relevant and economically significant problems facing veterinary medicine. Among avian respiratory diseases infectious coryza in chicken (Glasser's disease) presents a particular interest to veterinary specialists. This is due to scarce information on the disease on the one hand, and to the lack of domestic tools for its specific prophylaxis on the other hand [2, 5, 6, 11].

The technology of microbial biomass production has undergone fundamental changes over the last 30–40 years. The cultivation of microorganisms in solid nutrient media is often replaced by deep cultivation in liquid protein-hydrolyzate or synthetic media [3, 4].

Suspension cultivation compared with bacteria growth on agar has several advantages, one of which is the possibility to provide standard conditions when using automation means, especially when transferring from laboratory and semi-production conditions to automated production lines [3, 8].

Selection of nutrient (growth) medium is of great importance in terms of quality of the prepared microbial suspension. The causative agent of infectious coryza in chicken (*Avibacterium paragallinarum*) is a very fastidious microorganism that requires complex nutrient media supplemented with blood serum and nicotinamide adenine dinucleotide phosphate. In addition, *A. paragallinarum* bacteria have narrow-spectrum adaptation to cultivation conditions and are very sensitive to acidification of nutrient media, and their virulence and immunogenicity decrease after repeated passages [9, 10, 12].

One of the most important tasks in preparing a vaccine against avian infectious coryza is to develop a technology for the cultivation of its pathogen. Without studying the processes of bacterial activity in order to obtain a sufficient amount of bacterial mass with stable immunobiological

properties, it is impossible to select cultivation modes quickly [1, 7, 9].

When choosing growth conditions for each microorganism, much attention shall be paid to the selection of cultivation modes, such as temperature, mixing and aeration intensity, concentration of hydrogen ions (pH), redox potential (eH), partial pressure ($pO_2$) and glucose concentration (Sr) [4, 9].

The most suitable method for obtaining *A. paragallinarum* bacterial biomass sufficient for vaccine production is deep cultivation [3, 8]. However, information on this type of *A. paragallinarum* cultivation in order to obtain antigens is insufficient in foreign and domestic literature and many aspects are not fully understood, which significantly complicates the development of domestic tools for specific disease prophylaxis.

Therefore, the aim of this study was to select a nutrient medium and optimize the mode of deep cultivation of *A. paragallinarum* strain.

## MATERIALS AND METHODS

*Avibacterium paragallinarum* serovar B strain No. 5111 deposited in the collection of microorganism strains of FGBI "ARRIAH" in 2017 was used.

For cultivation of *A. paragallinarum* the following was used: Hottinger broth based on meat tryptic hydrolyzate (THH), broth based on pancreatic casein hydrolyzate (PCH) "Bacto Tryptone" (Difco), soy-casein broth (SCB) based on enzymatic casein and soy hydrolyzate (Sigma), Tryptone-D tryptic casein hydrolyzate (THC) broth (HiMedia), broth and agar for cultivation of pleuropneumonia-like organisms (PPLO broth and PPLO agar) (Difco). Nicotinamide adenine dinucleotide (1% prepared solution) by Roth was added as a V-growth factor. The nutrient media was supplemented with horse serum (NPP "Microgen") and hemin (1% of the prepared solution) (Serva) as growth stimulators.

Bacteria were cultivated in a liquid nutrient medium in conical flasks in an orbital shaker-incubator at 150 rpm for 12 h at 37 °C under elevated carbon dioxide conditions.

*A. paragallinarum* deep cultivation was carried out in a Biotron LiFlus GX laboratory bioreactor with pH, eH, $pO_2$, and Sr measurements.

The concentration of live microbial cells was determined by titration using solid nutrient media. Culture cultivation was conducted after adding seed material and every 2 h during cultivation [4].

The specific strain growth rate was determined by the formula:

$$\mu = 2{,}3\ (\lg X / \lg X_0)/t,$$

where $\mu$ is the specific growth rate of microorganisms ($h^{-1}$);

$X_0$ and X – the initial and final concentration of microbial cells (CFU/cm³);

t is the time of cultivation of microorganisms (h).

The doubling time of the concentration of microbial cells (td, h) was calculated by the formula:

$$td = \ln 2/\mu.$$

## RESULTS AND DISCUSSIONS

The main criterion for selecting the optimal conditions for the cultivation of the pathogen of infectious coryza in chickens is to obtain the maximum biomass amount.

Selection of nutrient medium was based on the study of *A. paragallinarum* growth dynamics in various media. The results of these studies are presented in Figure 1.

As Figure 1 shows, the highest levels of specific growth rate during the exponential phase were when SCB and a medium based on PPLO ($\mu = 0.787 \pm 0.041\ h^{-1}$ and $\mu = 0.824 \pm 0.034\ h^{-1}$, respectively) were used. The mean time of doubling the concentration of live microbial cells in such cultures was less than an hour (td = 0.88 h and td = 0.84 h, respectively). During the cultivation of *A. paragallinarum* on SCB and PPLO, the longest exponential growth phase (6 h) and the highest pathogen accumulation in these nutrient media (9.52 ± 0.04 lg CFU/cm³ and 9.48 ± 0.03 lg CFU)/cm³, respectively) were demonstrated.

The minimal specific growth rate ($m = 0.360 \pm 0.030\ h^{-1}$) and the accumulation of microbial cells ($X = 8.36 \pm 0.05$ lg CFU/cm³) were observed on THH medium. The mean time for doubling the concentration of live microbial cells in this case was td = 1.43 h.

Thus, the obtained data indicate that the most suitable media for producing the *A. paragallinarum* bacterial mass are those based on SCB and PPLO broth. Given that SCB is 1.5–2.0 times cheaper than PPLO broth, its use was more economically justified in further experiments.

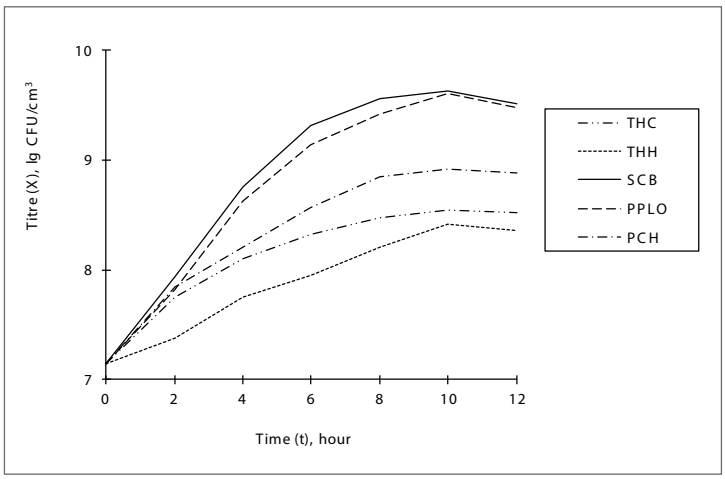
The mode of deep cultivation of the strain was optimized in a laboratory bioreactor with the measurement of the main physico-chemical parameters.

To study the effect of the degree of aeration of the nutrient medium on the *A. paragallinarum* growth, periodic deep cultivation was carried out at a stirrer rotation speed of 200 to 1,000 rpm in the established aeration modes of 1.0; 2.0 and 4.0 l/min, and also without forced air supply.

The study results presented in Table 1 show that such parameters as the specific growth rate and pathogen accumulation in the nutrient medium, depending on the selected aeration mode, have significant differences. The initial inoculum concentration of bacteria was 7.14 lg CFU/cm³. After 12 h of cultivation the maximum accumulation of live microbial cells was 9.76 ± 0.04 lg CFU/cm³, which significantly exceeds those obtained during cultivation in a flask. The time during which the cultures reached the beginning of the stationary growth phase (the time of the greatest accumulation of the pathogen in the nutrient medium) was maximal during the cultivation of bacteria without forced air supply and at aeration level not exceeding 1.0 litre/min.

In the course of the studies it was established that low accumulation of the *A. paragallinarum* bacterial

*Fig. 1. Dynamics of A. paragallinarum strain No. 5111 growth in different nutrient media*



mass under conditions of intensive air supply (4.0 l/min) resulted in a long lag phase, a low specific growth rate, and a short time for the culture to reach the stationary phase. The level of the partial pressure of oxygen in the nutrient medium during the cultivation process ranged from 50 to 75%. Higher bacterial growth rates were observed during cultivation without forced air supply or minimal aeration (1.0 l/min). Herewith, in the first case the partial pressure of oxygen in the nutrient medium was 5%, in the second case – no more than 25%. In subsequent experiments the $pO_2$ value was maintained no higher than the critical level of 25%.

A sharp decrease in bacterial survival during intensive aeration was probably due to the inhibitory effect of peroxide compounds that reduced efficiency of glycolytic processes in cells.

The optimal pH level for many types of bacteria, including *A. paragallinarum* is 7.2–7.4. And although the processes of bacterial catabolism and anabolism can occur in a fairly wide pH range (from 6.0 to 8.0), their activity at different pH levels can be different, which can affect the nature of bacterial growth during biomass production. Therefore, the next step in the experiment was to study the effect of pH on the growth of *A. paragallinarum* bacteria.

The initial nutrient medium based on SCB had a pH of 7.45 ± 0.05. The level of the hydrogen ion concentration was recorded every hour throughout the entire cultivation period.

The obtained research results indicate that after the seed material was added and the bioreactor reached the established operational mode, a decrease in pH by 0.10 ± 0.02 units was observed during the first hour of cultivation, which was associated with a short lag phase of the culture.

During the second hour of cultivation, the drop in pH remained unchanged. From the third hour of cultivation, the pH decrease gradually intensified, reaching maximum levels by hour 7–8 (by 0.20 ± 0.05 units/hour).

As a result of the studies, it was determined that the period of intensive decrease in the pH of the nutrient medium corresponded to the exponential phase of bacterial growth, which lasted up to 9 h of cultivation. By this time, the pH value had decreased up to 6.50 ± 0.05, and the concentration of live microbial cells reached a maximum of X = 9.72 ± 0.04 lg CFU/cm$^3$.

The obtained research results showed that in the process of periodic deep cultivation of *A. paragallinarum*, a constant decrease in the pH of the nutrient medium occurs, which at fairly low values (6.50 ± 0.05) can cause the culture's early reaching the stationary growth phase and, therefore, prevent further accumulation of the pathogen. To find this out studies on the effect of various pH values of the nutrient medium on *A. paragallinarum* growth and accumulation during periodic deep cultivation were conducted.

For this purpose, nutrient media with pH levels of 7.00; 7.30; 7.60; 7.90 and 8.20 were prepared and used for deep cultivation of *A. paragallinarum* strain. In this case, the specific growth rate, the time of culture reaching the stationary growth phase, and the accumulation of the pathogen in the nutrient medium were taken into account. The results of these studies are presented in Table 2.

In the course of the studies it was noted that the nutrient medium pH ranging from 7.30 ± 0.02 to 7.90 ± 0.06 did not significantly affect the strain specific rate, the duration of the lag phase was minimum – 0.36–0.45 h.

**Table 1**
**General parameters for *A. paragallinarum* growth at different aeration levels in nutrient medium**
*n* = 3

| Growth parameters ($M \pm m$) | Forced air supply (A), l/min | | | |
|---|---|---|---|---|
| | 0 | 1.0 | 2.0 | 4.0 |
| Lag-phase, h | 0.45 ± 0.09 | 0.48 ± 0.05 | 1.34 ± 0.05 | 1.54 ± 0.07 |
| m, h$^{-1}$ | 0.94 ± 0.03 | 0.97 ± 0.04 | 0.81 ± 0.03 | 0.72 ± 0.05 |
| $t_{stat}$, h | 8.65 ± 0.35 | 8.78 ± 0.25 | 7.15 ± 0.5 | 6.26 ± 0.4 |
| X, lg CFU/cm$^3$ | 9.64 ± 0.07 | 9.76 ± 0.04 | 8.20 ± 0.04 | 8.02 ± 0.09 |

m – specific growth rate during the exponential phase;
$t_{stat}$ – time of cultures' reaching the stationary phase;
X – maximum concentration of live microbial cells.

**Table 2**
**Effect of nutrient medium pH on basic parameters of *A. paragallinarum* growth**
*n* = 3

| pH value | Growth parameters during deep cultivation (M ± m) | | |
|---|---|---|---|
| | m, h$^{-1}$ | $t_{stat}$, h | X, lg CFU/cm$^3$ |
| 7.00 ± 0.04 | 0.80 ± 0.05 | 8.00 ± 0.5 | 9.50 ± 0.03 |
| 7.30 ± 0.02 | 0.91 ± 0.03 | 9.40 ± 0.4 | 9.67 ± 0.02 |
| 7.60 ± 0.04 | 0.96 ± 0.04 | 9.45 ± 0.3 | 9.74 ± 0.03 |
| 7.90 ± 0.06 | 0.97 ± 0.03 | 9.45 ± 0.5 | 9.76 ± 0.04 |
| 8.20 ± 0.05 | 0.95 ± 0.03 | 9.30 ± 0.4 | 9.72 ± 0.04 |

m – specific growth rate during the exponential phase;
$t_{stat}$ – time of the culture reaching the stationary phase;
X – maximum concentration of live microbial cells.

Analysis of *A. paragallinarum* accumulation data showed that when the strain was cultivated in nutrient media with radical pH values of 7.00 ± 0.04 and 8.20 ± 0.05, the agent's growth was characterized by the lowest value of X (9.50 ± 0.03lg CFU/cm$^3$ and 9.72 ± 0.04 lg CFU/cm$^3$, respectively) compared with the pH range 7.60 ± 0.04 – 7.90 ± 0.06 (9.74 ± 0.03 – 9.76± 0.04 lg CFU/cm$^3$, respectively).

A greater biomass accumulation was facilitated by the time the culture entered the stationary growth phase, which was higher when cultured in a nutrient medium with a pH of 7.90 ± 0.06 ($t_{stat}$ = 9.45 ± 0.5 h) and 7.60 ± 0.04 ($t_{stat}$ = 9.45 ± 0.3 h).

Based on the obtained experimental data, an additional cultivation of *A. paragallinarum* strain No. 5111 was carried out setting a "floating" range for automatic control of pH from 7.6 to 7.9. As soon as the acidification process of the bacterial suspension was over and pH level was increased by 0.2 units, 40% glucose solution was delivered by impulse-flow using a peristaltic pump.

As the microorganisms grew, the pH regulator was triggered with increasing frequency until the acidification of the medium ceased. When glucose solution was added, acidification started again, which indicates that this substrate (0.6–0.8 g/l) limited the culture growth. It should be noted that even with the limitation of the

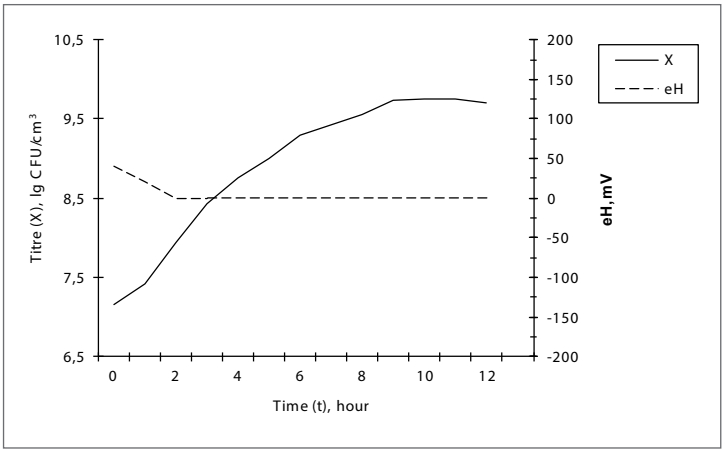



*Fig 2. A. paragallinarum growth curve and dynamics of nutrient medium eH during periodic deep cultivation*

*A. paragallinarum* growth by glucose, a gradual tendency for pH decrease was observed during the cultivation. Apparently, the organic acids formed during glucose fermentation do not undergo further degradation and, as a result, accumulate in the nutrient medium.

It is known that in the process of aerobic cultivation of microorganisms, the indicator of the redox potential is unstable, since eH is a complex function that depends on such parameters as pH, $pO_2$, temperature, as well as the properties of the medium and the characteristics of the growth of the microorganism.

The optimal eH level for most aerobic and facultative anaerobic bacteria ranges from –200 to +200 mV. Although the processes of bacterial metabolism can occur at a wider range of eH (from –350 to +350 mV), their activity can be different at different eH values, which can affect the value of the final concentration of microorganisms. Therefore, the next step in the study was to determine the dynamics of eH change during the deep cultivation of *A. paragallinarum* (Fig. 2).

The initial eH level of the nutrient medium before the introduction of the seed material was +40 mV. During cultivation, a gradual eH decline down to –105 mV, stabilization of the parameter, and a slight increase up to –75 mV were observed. The minimum eH value correlated with the completion of the exponential growth phase of microorganisms.

Thus, the process of periodic deep cultivation of *A. paragallinarum* strain No. 5111 when controlling the main physical and chemical parameters was characterized by the following indicators: duration of the adaptation phase (lag phase) – 0.46 ± 0.05 h (1/26 part of the total cultivation time); the duration of the phase of logarithmic growth (exponent) is 9.08 ± 0.10 h; maximum specific growth rate of 0.97 ± 0.03 $h^{-1}$; biomass doubling time – 0.72 ± 0.02 h; the maximum accumulation of live microbial cells is 9.76 ± 0.04 lg CFU/$cm^3$.

## CONCLUSION

In the course of the studies, the optimal nutrient medium for *A. paragallinarum* cultivation was selected. The maximum specific growth rate, doubling time and accumulation of microbial cells were observed in SCB. Taking into account the changes in the physicochemical parameters of the nutrient medium during the periodic deep cultivation of *A. paragallinarum*, it is possible to obtain a bacterial mass that would contain bacterial populations taken at the end of the exponential phase and at the beginning of the stationary growth phase (8–9 h), which makes it possible to prepare antigen with pronounced antigenic and immunogenic properties.

**Conflict of interest.** The authors claim no conflict of interest.

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