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DEVELOPMENT OF THE TECHNOLOGY OF DEEP CULTIVATION OF A TRANSFORMED *B. SUBTILIS* STRAIN

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Abstract

Deep cultivation of recombinant cultures in bioreactors makes it possible to accumulate a large number of specific antigens used in the production of immunobiological preparations and diagnostic test systems in a short period of time, taking into account stimulating and limiting growth factors. The main purpose of the research was to determine the influence of the factors of seed concentration, pH level and the level of dissolved oxygen on deep cultivation, with the control of accumulation kinetics by turbidimetry. During the research, it was found that the most optimal inoculation concentration is 1 million cells/cm^3 with a maximum final concentration of *B. subtilis* 4x10^6 cells/cm^3, the most optimal pH level is a dynamic mode from 8.0 to 6.0 with a maximum final concentration of 5x10^6 cells/cm^3, the most optimal level of dissolved oxygen is stationary mode with a minimum oxygen level of 25%, with a maximum final concentration of 4x10^6 cells/cm^3.

Bacteriological, technological and biochemical methods were used in the work to develop the modes of deep cultivation of the transformed *B. subtilis* strain with control of pH parameters and the level of dissolved oxygen.

The scientific significance of the research is based on the development of deep cultivation, with an assessment of external growth factors and their influence on the kinetics of accumulation of the transformed *B. subtilis* strain, which will provide effective conditions for the growth and production of microorganisms. The practical significance of the research is based on the possibility of optimizing the biotechnological stages of the cultivation of the transformed B. subtilis strain, which will positively affect the efficiency of submerged cultivation as the main method for the accumulation of specific antigens.

Key words: bioreactor; biotechnology; *B. subtilis*; cultivation; growth factors; microbiology; nutrient medium.

Introduction

Chlamydia abortus is a Gram-negative obligate intracellular bacterium responsible for abortion and reproductive problems. The disease has a high zoonotic potential and causes great economic damage to ruminant farmers [1]. The main method of combating this disease is the timely diagnosis and quarantine of animals. *Chlamydia psittaci (C. psittaci)* is distributed throughout the country and can be transmitted from animals to humans through close contact [2, 3]. Avian chlamydia (AC), mainly caused by *C. psittaci*, is an acute, severe or chronic asymptomatic disease of poultry, poultry and mammalian hosts. In the poultry industry, *C. psittaci* has been identified as the main causative agent of eye, respiratory, intestinal, and arthritis diseases, as well as abortions [4]. Moreover, a retrospective study has shown that *C. psittaci* may be a risk factor for atherosclerosis in poultry [5]. Thus, *C. psittaci* not only threatens the poultry industry and domestic animals, but also causes serious economic damage to the poultry industry. However, *C. psittaci* infections are often underestimated due to the lack of rapid and reliable testing kits. In addition, there is a multi-infection of *Chlamydia pecorum* (*C.pecorum*), *Chlamydia gallinacean* (*C.gallinacean*) and *Chlamydia avium* (*C.avium*) in poultry [6, 7].

According to the OIE recommendation, an enzyme immunoassay based on recombinant proteins can be used as one of the main methods for diagnosing chlamydia in farm animals [8].

As the main carrier of recombinant genes, we chose the *Bacilus subtilis* strain, which is a widely used commercial strain with wide application in the field of bioengineering and biotechnology, since they are considered safe for use [9]. The main methods of cultivation of *B. subtilis* are stationary on solid nutrient media and submerged cultivation in bioreactors, each method has its advantages and disadvantages, primarily associated with high requirements for the availability of equipment and qualified personnel, only very recently, advances in the study of submerged cultivation penetrate into the field of hybrid modeling. [10]. The submerged culture method was chosen by us first of all with the possibility of combining modern recombinant technologies together with the technological stages of production in bioreactors, with low risks of fungal and bacterial contamination, and greater control over external parameters of cultivation. Over the past few decades, there has been significant progress in the production of pharmaceutical compounds using both microbial and mammalian cellular systems in large-scale bioreactors. For each new process, this includes scaling production steps from laboratory bioreactors (eg 250 ml to 2 L) to larger pilot and production bioreactors (eg 500 L to 15,000 L). However, scaling up is not a trivial task, since bioreactors are often considered one of the most difficult pieces of equipment to scale up [11]. Several factors exacerbate the complexity of scaling. These include the complexity of biological systems and significant costs, including media and reagents, associated with the transfer and testing of processes in large-scale bioreactors [12]. The environment in a large-scale bioreactor is also heterogeneous, which consequently leads to increased intercellular variability

and greater process variability at scale. Similar process differences are observed between large bioreactors and downscale models and are one of the main limitations of downscale models, which may not be accurate indicators of production scale performance [13].

To obtain a transformed culture of *B. subtilis* for research, we chose the chlamydia outer membrane complex (COMC), which is a protein-rich insoluble shell of the outer membrane, which includes outer membrane proteins (OMPs), in particular, the main outer membrane protein (MOMP) and polymorphic membrane proteins (Pmps), which are used as the main candidates for vaccines against CD4 T cells and enzyme immunoassay diagnostic test systems [14].

Materials and Methods

2.1 Strain

B. subtilis strain transformed with specific fragments of Pmps (Polymorphic Membrane Proteins - polymorphic membrane proteins). The strain has the typical properties of bacilli, causing sporulation upon contact with oxygen.

2.2 Deep cultivation in bioreactor

Deep cultivation was carried out in a Bailun BlBio 30 bioreactor (Produced by Bailun Biotechnology Co Ltd (Bailun), China), with a volume of 30 liters, with automatic control systems for cultivation parameters, including temperature, pH, dissolved oxygen (DO), stirrer speed, etc. (Figures 1 and 2).

Figure 1 - Bioreactor for suspension cultivation.

Figure 2 - Automatic control systems of the bioreactor.

2.3 Growth phases of bacteria

Bacterial growth dynamics was examined based on the traditional 4 phases, (I) lag phase (II) logarithmic phase (III) stationary phase (IV) deceleration phase. The growth phases were determined by the concentration of viable bacteria incapable of spore formation per 1 cm^3. The growth phases were determined by microscopy, which will determine the degree of sporulation of strain *B. subtilis*, and the method of determining the number of cells using a densitometer, which will determine the total concentration in the samples.

2.4 Culture medium

In the studies, a modified Hottinger's digest was used, enriched with glucose, pH (7.4±0.1), containing NaCl - 0.5%, Na_2HPO_4 - 0.06%, peptone up to 1%. The prepared nutrient medium was tested for microbial and fungal contamination. This nutrient medium was developed and standardized at the Scientific and Production Enterprise "Antigen" and controlled by a system of standard operating procedures.

2.5 Concentration control

The concentration of streptococci was monitored using a DEN-1B MF-Units densitometer (Produced by GRANT USA INC.), measuring the turbidity of cell suspensions within the range of 0.0 - 6.0 McFarland units (McF) (0 - 180 x 10^7 cells/cm^3), along with McFarland turbidity standards of 0.5; 1.0 and 2.0 ($BaSO_4$), which corresponds to a *B. subtilis* cell size of 0.25-1 micrometers in width and 2-4 micrometers in length, and varies slightly during the sporulation stage. The turbidity of the samples taken from the bioreactor was determined, followed by taking into account the size of the cells.

Results

To develop the technology of deep cultivation of the transformed strain *B. subtilis*, 3 main areas of research were selected, (I) Culture inoculum regimes (II) Culture pH regimes (III) Dissolved oxygen culture regimes. The modes presented will make it possible to determine the main factors and aspects of deep cultivation, which will serve as the basis for the development of modern tools and methods of mathematical modeling. The timing of the growth phases was examined using a densitometer (Figure 3) and Gram staining (Figures 4 and 5).

Figure 3 - Densitometer McFarland DEN-1B.

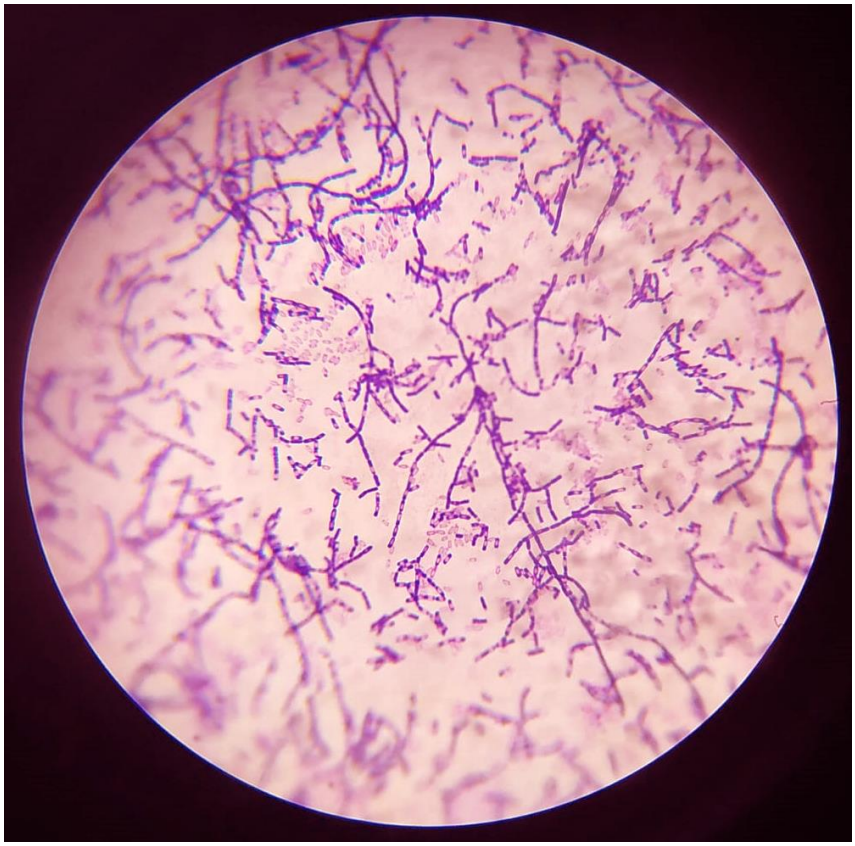

Figure 4 - Transient phase of sporulation.

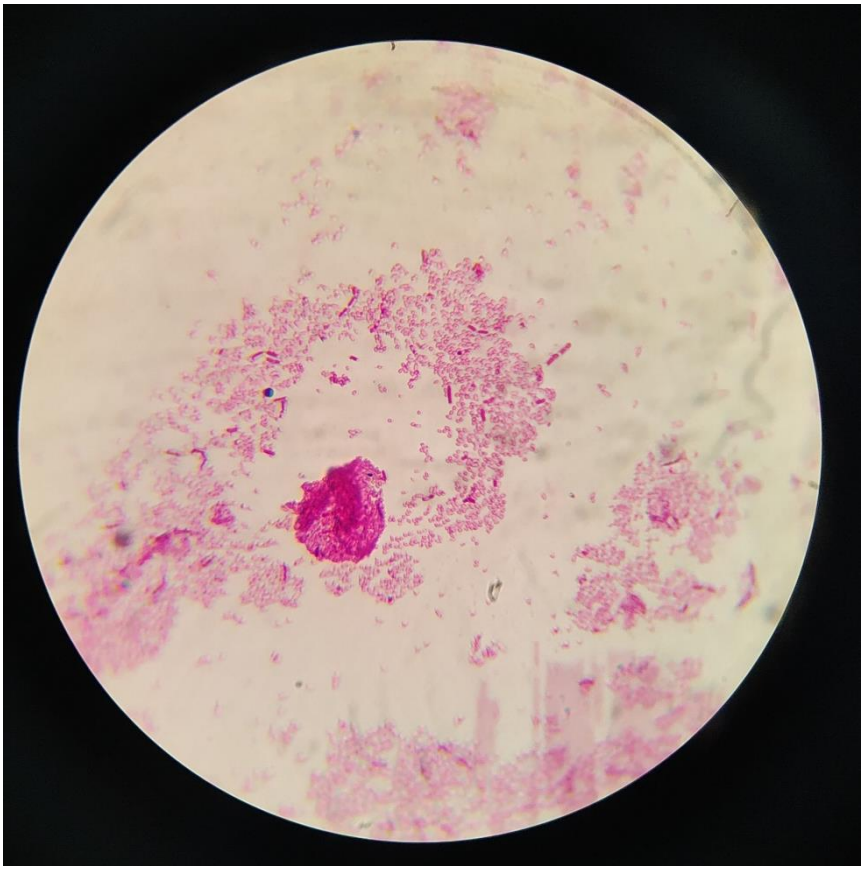

Figure 5 - the final phase of sporulation.

Modes of seed concentration during cultivation

The development of modes of seed concentration during cultivation will allow the cost-effective use of seed material, taking into account 4 phases of bacterial growth. To work out the seed concentration, 5 modes were selected with an increasing concentration of bacterial cells per 1 cm^3. These modes take into account the maximum and minimum seed concentration for the study. The results of the conducted studies are presented in table 1.

Table 1 - Results of testing modes of sowing concentration

№	Sowing concentration, cells/cm3	Growth phase time, h				Maximum concentration cells/cm^3
		I	II	III	IV	
1.	100 thousand	20	72	96	108	$2x10^6$
2.	250 thousand	20	60	84	108	$3x10^6$
3.	500 thousand	20	56	72	108	$4x10^6$
4.	1 million	20	44	92	120	$4x10^6$
5.	2 million	20	44	92	120	$4x10^6$

According to the presented table, the most effective inoculation concentration is 1 million, where the maximum concentration reached $4x10^6$ cells/cm^3, and the phase of logarithmic growth and stationary growth is the longest, which will allow efficient cultivation of strain *B. subtilis* in a bioreactor. Whereas low seed concentration cultivation regimes are characterized by slow reaching of the logarithmic and stationary growth phases, and fast reaching of the slow growth phase. Cultivation mode 5 with an inoculation concentration of 2 million cells/cm^3 does not differ from mode 4, so it is not advisable to spend extra money during cultivation.

pH levels during cultivation

The development of the pH level regimes will make it possible to take this factor into account during submerged cultivation in a bioreactor. The pH level during deep cultivation is one of the main factors that stimulate or limit the growth of bacteria. For the study, 5 modes were selected, where 3 modes are stationary, and 2 are dynamic with an increase / decrease in the pH level depending on the growth phases (Table 2).

Table 2 - results of testing the pH level modes

№	pH level	Growth phase time, h				Maximum concentration cells/cm^3
		I	II	III	IV	
1.	6,0	20	56	72	108	$3x10^6$
2.	7,0	20	56	72	108	$3x10^6$
3.	8,0	20	56	72	108	$3x10^6$
4.	6,0 → 8,0	20	72	96	108	$2x10^6$
5.	8,0 → 6,0	20	44	92	120	$5x10^6$

According to the results obtained, the most effective pH regimes for deep cultivation is dynamic regime 5, where the maximum concentration reached $5x10^6$ cells/cm^3. In modes 1 to 3, the maximum concentration practically does not differ and is $3x10^6$ cells/cm^3, while mode 4 turned out to be limiting and the maximum concentration was $2x10^6$ cells/cm^3, this is due to the sensitivity of strain *B. subtilis* in the first phases to acidic environmental conditions, and the need for a pH level of 6.0 during growth phases 3 and 4.

Dissolved oxygen regimes during cultivation

The level of dissolved oxygen during deep cultivation is one of the most important factors, since the cultivated strain *B. subtilis* being a facultative aerobe is particularly susceptible to dissolved oxygen levels. For the study, there were 5 modes of the level of dissolved oxygen, 3 stationary and 2 dynamic. These modes take into account the effects of both low, high and varying dissolved oxygen conditions.

Table 3 - results of working out the modes of dissolved oxygen

№	Dissolved oxygen level	Growth phase time, h				Maximum concentration cells/cm^3
		I	II	III	IV	
1.	25%	20	44	92	120	$4x10^6$
2.	50%	20	44	78	92	$2x10^6$
3.	75%	20	44	60	78	$2x10^6$
4.	25% → 75%	20	44	60	78	$2x10^6$
5.	75% → 25%	20	44	60	78	$2x10^6$

According to the results obtained, the most effective regime is the lowest level of dissolved oxygen, where the maximum concentration was. It is worth noting that modes 1 and 4 reached growth phase 2 in the same amount of time, while phases 3 and 4 are strikingly different. And also, at high levels of dissolved oxygen, active spore formation is observed, limiting further growth of *B. subtilis*.

Discussion

The use of recombinant technologies for the transformation of producer organisms makes it possible to obtain a high concentration of specific antigens, without the risk of fungal or extraneous bacterial contamination. The combined use of recombinant technologies, together with industrial methods of deep cultivation in a bioreactor, allows the most efficient accumulation of specific antigens. The use of modern recombinant technologies makes it possible to develop highly effective vaccines and diagnostic test systems. O'Neill and colleagues have developed a new vaccine based on recombinant major intrinsic protein and chlamydial protease activity factor proteins against *Chlamydia abortus* enzootic abortion in sheep. According to the results of their studies, these recombinant proteins are effective in inducing immune responses important for the treatment of chlamydial infections [15]. As a rule, diagnosing chlamydial infection by isolating the pathogen is difficult, since detection requires more than 2 weeks [16], which is why it is necessary to use modern diagnostic methods, such as ELISA. Specific detection of *Chlamydophila psittaci* can be analyzed

using ELISA based on synthetic peptides. Recombinant major outer membrane protein [17] or outer membrane polymorphic protein [18, 19.]. A recently developed indirect ELISA based on recombinant proteins has shown its sensitivity and specificity for *Chlamydophila psittaci* [20]. The conducted studies will allow to determine the specific conditions of deep cultivation in bioreactors, as the main method for obtaining a large number of specific antigens, taking into account external and internal factors of growth, productivity, with the possibility of manual and automatic regulation of biotechnological stages. Deep cultivation in bioreactors is a modern high-performance solution that helps create unique conditions for each type of bacteria, including those in the presented studies, which will allow in the future to create libraries of cultivation models in accordance with the international Good Manufacturing Practice standard.

Conclusion

The most optimal parameters for submerged cultivation of the transformed *B. subtilis* strain were chosen: seed concentration of 1 million cells/cm^3, at a dynamic pH level of 8.0→6.0 and at a level of dissolved oxygen of 25%, these cultivation parameters make it possible to obtain the concentration of the transformed strain *B. subtilis* within 4x10^6 and 5x10^6 cells/cm^3.

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