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MATHEMATICAL PLANNING OF PROPIONIC ACID BACTERIA CULTIVATION

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Abstract: *Based on our own research results and analysis of literature data we have proposed conditions and composition of nutrient media for the cultivation of Propionibacterium freudenreichii. As an inoculum we used propionic acid bacteria concentrate, which contains cells of selective strains of Propionibacterium freudenreichii. In the nutrient medium we kept the following concentrations constant: inoculum - 5% vol, cobalt chloride $CoCl_2$ at a dose of 20 mg/l, and hydrolysed milk concentration - 5% vol. The contents of yeast autolysate, ascorbic acid, ammonium sulphate and lactose were varied in the composition of the nutrient medium. We cultivated the resulting mixture in a thermostat for 7 days at 30 °C and a pH of 7.0. The cultivation process was optimized by the Box-Wilson method. Using the method of mathematical planning we designed a full factor experiment FFE 2^n, which allowed us to optimize the nutrient medium for propionic acid bacteria with the highest increase in bacterial biomass and accumulation of vitamin B_{12}. We found optimal cultivation conditions for the Propionibacterium freudenreichii biomass accumulation, where the maximum biomass value at day 5 of cultivation was 60.5 g/l with high viable probiotic bacteria cell content of 12×10^{12} CFU/cm^3 and maximum vitamin B_{12} accumulation at day 5 of cultivation equal to 108.1 μg/ml.*

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Introduction

The human body is a biological system that is closely related to its environment. As a result of anthropogenic factors - environmental conditions, stress, inadequate nutrition, use of antibiotics, medications, excessive consumption of carbohydrates - the composition of the gastrointestinal tract microflora changes qualitatively and quantitatively. In this case, normal metabolic function is disturbed - food is partially digested, pathogenic and opportunistic bacteria and *Candida* yeasts begin to develop [1-3]. To prevent the consequences of digestive disorders and the development of related diseases, the development and expansion of dietary

supplements, including those based on the principles of biotechnology, are required. In particular, such products include products containing highly active live bacteria or products produced by them [4-6]. Technologies have been developed for their production in liquid or dry forms from microbial cultures (bifidobacteria, lactobacilli, propionic acid bacteria, etc.) [7, 8], which have high activity [9, 10].

Propionic acid bacteria are particularly interesting. They are inherently antimutogenic and immunogenic, as well as the ability to accumulate vitamin B_{12} during their life activity [11]. Therefore, preparations based on propionic acid bacteria are always in demand. A prospective approach is to increase the uniqueness of the production of these preparations and to use innovations in propionic acid bacteria production in order to improve the properties of the products. Particularly relevant to the application of preparations is the challenge of ensuring conditions for increased preservation, which are primarily created by the economic environment of the production facility. Particular attention is paid to the technology of obtaining preparations as dry products.

The aim of the study is to find the optimum ratio of nutrient media used for the development of propionic acid bacteria to create conditions for maximum growth of biomass of cultured bacteria and the production of the largest amount of vitamin B_{12} during their life activity. The determining factors for the solution of this problem are: the choice of nutrient medium and the determination of the ratio of the components, as well as the cultivation conditions (temperature, pH, concentration).

Experimental part

A factor for increasing stability when removing moisture is primarily the problem of optimizing the composition of the nutrient medium for the cultivation of propionic acid bacteria. Milk serum was chosen as the main component of the nutrient medium for the cultivation of propionic acid bacteria [12]. The use of serum for the cultivation of propionate bacteria is explained by the containing of carbohydrates (lactose and glucose), lipids and milk fat, digestible protein (casein, albumin and globulin), B vitamins, ascorbic and pantothenic acids, tocopherol, organic acids (lactic and acetic, citric and formic), mineral components (phosphorus and magnesium, calcium and chlorine, zinc and sodium, potassium and iron, iodine, cobalt and molybdenum), and amino acids.

We selected a nutrient medium for the development of propionic acid bacteria, on the basis of which we tested the ratio of components to increase the accumulation of propionic acid bacteria biomass. The components included in the medium were whey, yeast autolysate, hydrolysed milk, ascorbic acid, ammonium sulphate, buffer, lactose and cobalt chloride. We kept the concentrations of: inoculum - 5% vol., $CoCl_2$ cobalt chloride at a dose of 20 mg/l, and hydrolysed milk concentration - 5% vol. The inoculum is a propionic acid bacteria concentrate containing cells of *Propionibacterium freudenreichii* strains characterized by an increased second heating temperature. By other issues the *Propionibacterium freudenreichii* inoculum for cultivation and biomass accumulation is optimal and is contained in an amount of 5% of the volume in the nutrient medium. This amount of inoculum allows optimal biomass growth and vitamin

B_{12} synthesis and leads to a cheaper final product. It is also known that the optimum concentration of hydrolysed milk is 5% in the volume of the nutrient medium [13]. We took the temperature of 30 °C and the process duration of 7 days in accordance with the studies of other authors on the cultivation of *Propionibacterium freudenreichii*. According to preliminary tests, cobalt chloride $CoCl_2$ at a dose of 20 mg/l gives the highest value of vitamin B_{12} and produces the least suppression of bacteria during growth [14]. We took the contents of: yeast autolysate, ascorbic acid, ammonium sulphate and lactose as variable components in the nutrient medium. We cultured the obtained mixture in a thermostat for 7 days at 30 °C with a pH of 7.0. We optimized the cultivation conditions of propionic acid bacteria by the method of Box-Wilson, using mathematical planning in the construction of a complete factor experiment FFE 2^n, where n - the number of varying factors [15].

The result of the two-factor experiment is a linear model:

$$y = b_0 + b_1x_1 + b_2x_2 + \ldots + b_nx_n, \quad (1)$$

where $b_0, b_1, b_2, \ldots, b_n$ - are the regression coefficients; $x_1, x_2, \ldots, x_n$ - are the values of the varying factors in coded form.

We calculated the equation regression coefficients by the average results of N experiments, using the corresponding formulas [16]:

- free coefficient of the equation:

$$b_0 = \frac{\sum_{u=1}^{N} \bar{y}_u}{N}, \quad (2)$$

- regression coefficients of the i-th factor:

$$b_1 = \frac{\sum_{u=1}^{N} x_{iu}\bar{y}_u}{N}, \quad (3)$$

where x_{iu} - is the value of the variable value in the column of the planning table; $\bar{y}_u$ - is the result of the u-th experiment, the arithmetic mean value; N - is the total number of experiments; u - is the numbering of the experiment variant; i - is the number of the factor.

The values of the coefficients affect the process if the inequality is met

$$|b_i| > t\sqrt{S_b^2}, \quad (4)$$

where $\sqrt{S_b^2}$ - is the error of finding the coefficient; t - is the Student's test, which is determined by the level of significance and the number of degrees of freedom; S_b^2 - is the variance of reproducibility.

To find the amount of bacterial biomass, we used the weighing method. According to this method, the biomass obtained after bacterial cultivation was separated from the liquid phase in a centrifuge and sent for weighing [17].

We define the vitamin B_{12} content by the spectrophotometric method. According to the method, the bacterial cells are first separated and then washed to transfer the cobalamin using hydrolysis into an aqueous solution. The hydrolysate is then exposed to light, which converts the cobalamin to oxycobalamin, and then the optical density is determined using a light wavelength of 530 nm. The value obtained determines the amount of cobalamin.

Results and Discussion

We selected the main level of varying factors based on the results of experimental researches by a number of authors [13, 18]. To identify the optimal conditions for propionic acid bacteria development, we planned and implemented a two-level full-factor FFE experiment 2^4. We considered the following as variable factors: X_1 - yeast autolysate concentration, %; X_2 - ascorbic acid concentration, %; X_3 - ammonium sulphate content, g/l; X_4 - lactose content, g/l. Optimisation criterion Y is the biomass of propionate bacteria. Table 1 shows the values of the factor levels.

Table 1. Factors and values of levels

Levels	Factors			
	X_1	X_2	X_3	X_4
Main	5.0	0.1	3.0	5.0
Variation interval	0.5	0.05	0.3	0.5
Upper	5.5	0.15	3.3	5.5
Lower	4.5	0.095	2.7	4.5

Table 2 shows the planning matrix. According to the plan shown, we prepared 16 media where we presented all possible combinations of the factors studied at two levels. We conducted biomass build-up until the end of the exponential phase for 5 days, the dose of bacterial inoculum introduced being 5%. We included cobalt chloride in the medium to enhance the vitamin-synthesising capacity of propionic acid bacteria at a dose of 20 mg/l. We conducted the experiments in three iterations.

Table 2. FFE planning matrix 2^4

№ item n/a	By the natural state				By the coded state			
	Yeast autolysate,	Ascorbic acid, %	Ammonium sulphate, g/l	Lactose, g/l	X_1	X_2	X_3	X_4
1	4.5	0.95	3.3	4.5	-1	1	-1	-1
2	4.5	0.15	2.7	5.5	-1	-1	-1	1
3	4.5	0.95	2.7	5.5	-1	-1	1	1
4	4.5	0.15	3.3	4.5	-1	1	1	-1
5	5.5	0.95	2.7	5.5	1	-1	-1	1
6	5.5	0.15	3.3	4.5	1	1	-1	-1
7	5.5	0.95	3.3	4.5	1	1	1	-1
8	5.5	0.15	2.7	5.5	1	-1	1	1
9	4.5	0.95	2.7	4.5	-1	-1	-1	-1
10	4.5	0.15	3.3	5.5	-1	1	-1	1
11	4.5	0.95	3.3	5.5	-1	1	1	1
12	4.5	0.15	2.7	4.5	-1	-1	1	-1
13	5.5	0.95	3.3	5.5	1	1	-1	1
14	5.5	0.15	2.7	4.5	1	-1	-1	-1
15	5.5	0.95	2.7	4.5	1	-1	1	-1
16	5.5	0.15	3.3	5.5	1	1	1	1

We processed the experimental results to obtain a linear model equation (1). Determination of values of coefficients included in the equation was done according to average results of N

experiments according to equations (2) and (3). From the average results of the 16 experiments, we obtained the coefficients:

$$b_0 = 43{,}4,\ b_1 = 1{,}2;\ b_2 = 1{,}36;\ b_3 = -0{,}10;\ b_4 = 1{,}11.$$

The symbol $(+)$ of the coefficient shows that the value of the factor will increase, the sign of $(-)$, on the contrary, that there is a decrease in the optimization parameter, i.e. the increase in biomass.

Significance of the regression coefficients was conducted using Student's test coefficient according to equation (4). Student's $t = 2{,}04,$ test determined at a significance level of 0.05 and degrees of freedom:

$$f = (n-1)N = (3-1)16 = 32,$$

where n - is the number of iterations of each experiment; N - is the number of experiments conducted.

The processing of experimental and calculated data allows us to find the value of reproducibility variance S_b^2 = 0.102. The value of the regression coefficient is an influence on the process according to equation (4) if the inequality is fulfilled

$$|b_i| > 2{,}04 \cdot \sqrt{0{,}102} = 0{,}652. \quad (5)$$

As a result, we determined that the inequality is not fulfilled for the third value of the regression coefficient, so that the third factor is insignificant in the composition of the nutrient media for propionic acid bacteria.

The regression equation therefore takes the form of

$$y = 43{,}4 + 1{,}2x_1 + 1{,}4x_2 + 1{,}1x_4.$$

Next, we did the calculation according to the steep ascent programme. For the cultivation process, we calculated six new nutrient media. We used the baseline as the planning centre. From the baseline we calculated steps where the value of the step is added to or subtracted from the value of the previous level, depending on the resulting sign of the regression criterion. For the factor that has the greatest influence, the last step is equal to its minimum or maximum level. Thus, we defined six compositions of the ascending media. Since the third factor according to the obtained inequality (5) has no influence on the process, the level in all compositions remained equal to the initial one. Table 3 shows the results of the calculations.

Table 3. Media for realising a steep ascent

Medium No.	X_1	X_2	X_3	X_4
1	5.28	0.17	3.0	5.28
2	5.56	0.23	3.0	5.56
3	5.84	0.30	3.0	5.84
4	6.12	0.36	3.0	6.12
5	6.40	0.43	3.0	6.40
6	6.67	0.49	3.0	6.67
Components	Yeast autolysate, %	Ascorbic acid, %	Ammonium sulphate, g/l	Lactose, g/l
Main level	5.0	0.1	3.0	5.0

In each of the experiments we conducted calculations of the amount of propionic acid bacteria biomass and the amount of accumulated vitamin B_{12} within 7 days. Fig. 1 presents the results of biomass accumulation from the steep ascent programme presented as a graphical dependence.

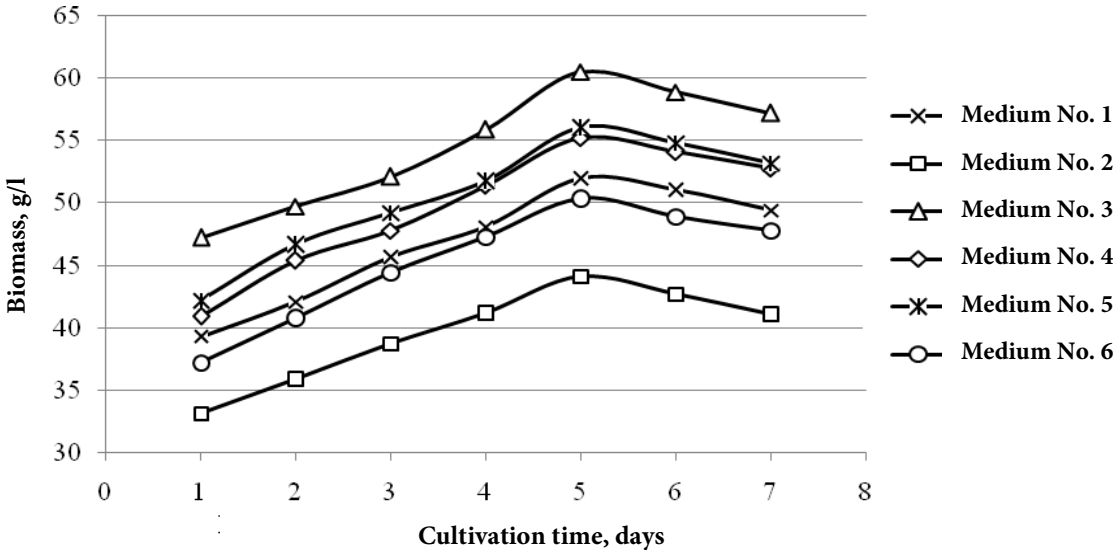

Fig. 1. Dynamics of biomass accumulation by *Pr. freudenreichii* bacteria as a function of nutrient medium composition in a steep ascent programme

Experimental results show that the greatest accumulation of bacterial biomass occurs in cultivation on medium number 3 (see Table 3), reaching a maximum value of 60.5 g/l on the 5th day of cultivation. The graph of changes in biomass accumulation shows that there is an active accumulation of biomass for the first five days, after which a drop in the bacterial content in the nutrient medium is observed. After five days of cultivation the nutrient medium is depleted of components and their combined effect has no stimulus for further bacterial synthesis.

Fig. 2 presents the results for vitamin B_{12} levels from the steep ascent programme in the form of a graphical dependence.

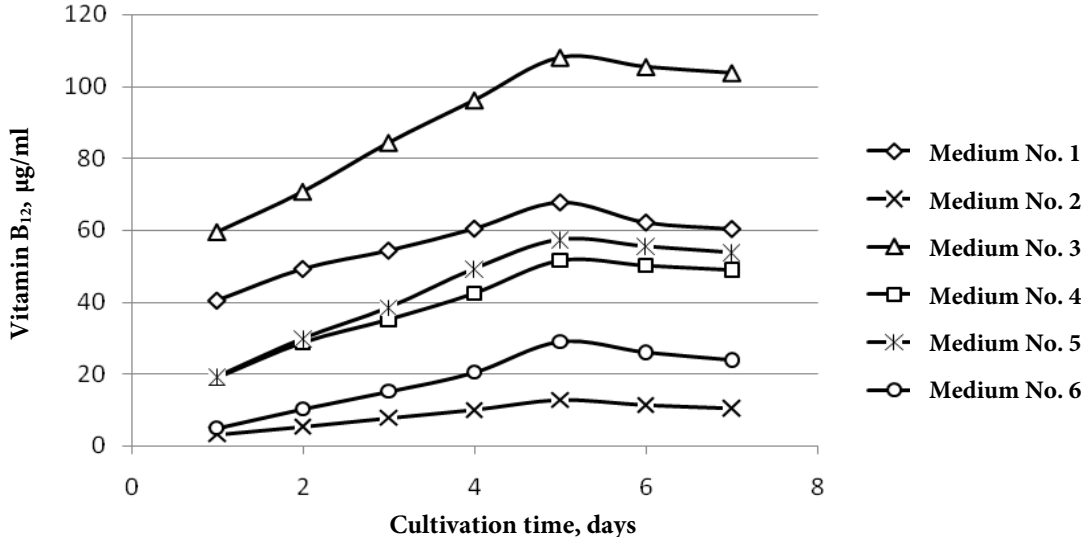

Fig. 2. Vitamin B_{12} accumulation as a function of nutrient medium composition on a steep ascent programme

The optimum medium for vitamin B_{12} accumulation is also medium number 3 (see Table 3). The maximum value of vitamin on the 5th day of cultivation reaches 108.1 μg/ml. From the graph of changes in vitamin B_{12} accumulation, it can be seen that the first five days there is an active accumulation. The maximum level of vitamin is reached on the 5th day, after which there is a drop in the level of vitamin accumulation in the nutrient medium. Table 4 shows a number of the most important characteristics of liquid starter.

Table 4. Qualitative data for liquid starter based on the results of optimisation

Indicators	Qualitative data
Taste and smell	A pleasant sour-milk flavour, specific to the product, with no extraneous odours
Consistency	Homogeneous with moderate viscosity
Bacteria biomass, g/l	60.5
Vitamin B_{12}, μg/ml	108.1
Number of propionic acid bacteria, CFU/cm^3	12×10^{12}

Conclusions

Using the method of mathematical planning we constructed a plan of full factor experiment FFE 2^n. Based on the results of the optimization problem, we determined the optimum composition of the medium and conditions for the development of *Propionibacterium freudenreichii* to achieve the highest growth of bacterial biomass with a high titer of viable cells and the highest content of vitamin B_{12}. Optimal medium composition is: inoculum - 5% vol.; whey - 5% vol.; hydrolysed milk - 5% vol.; cobalt chloride - 20 mg/l; yeast autolysate - 5.84% vol.; ascorbic acid - 0.30% vol.; ammonium sulphate - 3.0 g/l; lactose - 5.84 g/l. We selected optimum conditions under which the maximum growth of propionic acid bacteria is reached on the 5th day of cultivation and is 60.5 g/l with a high value of viable probiotic bacteria cells of 12×10^{12} CFU/cm^3. The accumulation of vitamin B_{12} is also reached at day 5 of cultivation and amounts to 108.1 μg/ml.

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