Optimization of nutrient medium composition to increase biomass of propionic acid bacteria and acidophilic bacteria

I V Boiarineva, I S Khamagaeva and I E Muruyev

1 East Siberia State University of Technology and Management, 40V, Klyuchevskaya str., Ulan-Ude, the Republic Buryatiya, 670013, Russian Federation
2 Khabarovsk State University of Economics and Law, 134, Tikhookeanskaya str., Khabarovsk, 680042, Russian Federation

E-mail: hutorokdv@gmail.com

Abstract. Numerous studies have shown that it is very important that the microorganisms included in the composition be in a strong symbiotic relationship when selecting vents for fermented dairy products. Therefore, it is the primary task to identify the relationship between microorganisms in the preparation of the starter. Strains that retain biochemical activity for a long time, depending not only on external factors (composition of nutrient medium, temperature, etc.), but also on the ratio between biochemical active and inactive cells in populations of microorganisms, which determines the viability of the culture, its practical value, are considered valuable. Microorganisms of the genus Lactobacillus are widespread in nature and are members of human microflora. They have high biological and functional activity, which determines their practical use as probiotics and in food production. Propionic acid bacteria are considered as promising probiotics, the positive impact of which on human health is generally recognized. The scientific results show the expediency of using strains of propionic acid bacteria in the production of functional dairy bio-products for the purpose of enrichment with probiotics cells and their useful exometabolites. As a result of the co-cultivation of propionic acid bacteria and acidophilic bacterium, a stable microbial association with high probiotic and biotechnological properties (inoculum) has been developed. A scheme for preparing an inoculum for producing a bacterial concentrate using pure cultures of propionic acid bacteria and an acidophilic bacterium has been developed. This article presents the results of studies on the selection of nutrient medium in order to increase the yield of biomass and the accumulation of certain metabolic products by microorganisms.

1. Introduction

One of the most important tasks of the production of bacterial preparations is to improve methods of culturing cells and bacteria taking into account the qualitative characteristics of biopreparation. The use of modern approaches to detect the presence of technological and probiotic properties in commercially significant species of bacteria allows more competent use of bacteria cultures in various biotechnological processes and selection of starting cultures of lactic acid bacteria for further production of various fermented dairy products, which is the basis for guaranteeing the quality of the finished product.

Among the different groups of microorganisms, lactic acid bacteria are among the most complex organisms in terms of the need for a variety of nutrients. In order to demonstrate their vital activity,
they require the presence of substrates, which are a source of energy and the substances they need to build a bacterial cell. Nutrient media must meet the following minimum requirements: they must contain all the elements from which the cell is built, in a form in which microorganisms are able to absorb them [1, 2, 3, 4, 5, 6, 7].

Of the numerous nutrient media used for the cultivation of lactic acid bacteria, those containing all the necessary nutrients and stimuli balanced in easy-to-assimilate form are suitable for the preparation of bacterial concentrates, providing a high rate of bacteria reproduction along with a minimum growth cycle to obtain a high concentration of viable microbial cells per unit volume of nutrient medium (not less than $10^9$ CFU/cm$^3$). Raw materials for the preparation of such media should be available, technologically feasible and inexpensive in the manufacture of bacterial concentrates in large quantities. At present, nutrient media of different composition are used to accumulate biomass of lactic acid bacteria. The base is usually defatted milk, hydrolyzed milk, whey and others. Additional sources of nutrients and growth factors: meat and vegetable peptones, acid and pancreatic casein hydrolysate, yeast extracts and autolysates, corn extracts, molasses, mineral salt solutions and other components [1-3, 5, 6, 8-10].

In the media for suspending the cultures to be frozen and freeze-dried, it is common to introduce substances that reduce the die-off of microbes during freezing, drying and dry storage. The various substances used for this purpose may have an advantageous protective effect on only certain adverse factors. In view of this fact, it is preferable to use complex protective media of two or more components. We will identify the main requirements for protective media used in freezing and drying microorganisms:

1. The medium shall provide a protective effect at all stages of freezing and freeze-drying processes.
2. Microbes frozen and lyophilized in a protective medium should be stable when stored at wide temperature ranges.
3. Once free water is removed, the medium must provide a fine, sufficiently dense structure. If this condition is not met, the dry material has a loose or powdery structure and part of the microbes will be carried away from the ampoules during drying in a water vapor stream. In addition, the material can be entrained during the opening of the ampoule, which is particularly dangerous when working with pathogens.
4. The protective medium should be sufficiently hydrophilic to ensure optimum residual moisture of the preparation.
5. The media should dissolve well, provide a standard form of tablet, have no antigenicity.

Thus, the most effective protective media are complex mixtures containing colloid and soluble substance [11].

2. The purpose of the study
The aim of the study is to select the composition of the nutrient medium by full-factor mathematical planning to obtain biomass of the combined starter based on propionic acid bacteria and acidophilic bacterium.

3. Materials and methods
The objects of research were used for production of combined starter propionic acid bacteria (PAB) strains *Propionibacterium freudenreichii* subsp. *shermanii* AC-2503 (*Pr. shermanii*), *Lactobacillus acidophilus* (*L. acidophilus*) strain of viscous race. The relative viscosity of the vents and the finished product was determined by the liquid flow rate. The optical density of the solution was determined on a spectrophotometer.
4. Discussion of the results

The basis of the nutrient medium was clarified curd serum. The use of whey for culturing microorganisms is due to carbohydrates (mono-, oligo- and aminosachars), lipids, mineral salts, vitamins, organic acids, enzymes and trace elements contained therein.

Serum lactose is an energetic substrate for the development of microorganisms that are part of the inoculum.

As a source of nitric nutrition, peptone was added to the serum. To maintain the buffer capacity, tri-substituted sodium citric acid and single-substituted potassium phosphate were added to the medium. Since propionic acid bacteria are optional anaerobes, agar-agar was used to thicken the medium.

It is known that microorganisms exhibit a demanding presence of vitamins in the nutrient medium. The acidophilic bacterium needs riboflavin, folic acid and cyanocobalamine. However, all of these vitamins acidophilic microorganisms can be produced when they are co-cultured with propionic acid bacteria, as the latter synthesize them [12]. And propionic bacteria are characterized by the need for thiamine, which causes the inclusion of this vitamin in the nutrient medium. The presence of cobalt salts in the medium is known to increase the vitamin synthetics of propionic acid bacteria [13]. Taking into account the peculiarities of vitamin $\text{B}_{12}$ synthesis, cobalt chloride was included in the medium.

Natural nutrients have a significant disadvantage – variability of chemical composition. Therefore, considering that the set of vital trace elements and vitamins for microorganisms in serum is unstable, sulfur ions are introduced into it in the form of $\text{SO}_{4}^{2-}$ stimulating proteolytic enzymes and $\text{Mg}^{2+}$ ions activating a whole group of enzymes responsible for fermentation processes. Magnesium plays a leading role in the glycolytic cycle, namely the transfer of phosphates. Magnesium ions play an important role in the phosphorylation process. Quite often $\text{Mg}^{2+}$ acts as a binder by enzyme and substrate. He takes part in the stabilization of the double helix of DNA. The optimal effect of magnesium depends on the concentration of carbon sources, the formation of hydroxy acids, the concentration of other ions for which magnesium is an antagonist [14].

The development of microorganisms is possible only within certain limits of each factor. Therefore, the mathematical planning method was used in the experiment. The purpose of this study is to select culture conditions and determine the significance of the components of the nutrient medium for the production of biomass of microorganisms by a full factor experiment.

The inoculum used was a combined starter of propionic acid bacteria and acidophilic bacterium. According to technological scheme for preparation of inoculum at the first stage activation of pure cultures is carried out $L$. acidophilus and $Pr.$ shermanii. The ratio is then 2:1. By preparation of milk for fermentation carry out sterilization at a temperature of 121 °C within 15 minutes, cool up to the temperature of souring (30±2) °C. 3% of combined starter is added to prepared milk for laboratory preparation (inoculum).

The inoculum is characterized by gentle, homogeneous consistency, good taste characteristics, has moderate acidity, high content of live cells $10^9$ CFU/cm$^3$ and sanitary reliability, which makes it possible to recommend it for biomass production in bacterial concentrate production.

In the first stage of optimization, a plan was drawn up for a two-level full factor experiment of FFE 2. The variation interval of factors is represented in the equations 1,2,3.

$$x_1 = (0 - 5) mg/l; \quad (1)$$
$$x_2 = (0,5 - 2,5) mg/l; \quad (2)$$
$$x_3 = (0,2 - 0,6) g/l; \quad (3)$$

Accordingly, 8 media were prepared in which all possible combinations of the studied factors at two levels were exhausted. Biomass growth was carried out until the end of the exponential phase for 12 hours, the dose of inoculum was 5%.

The process was controlled by optical density. The results of the studies are shown in table 1 and figure 1.
Table 1. Plan of a factorial experiment

| №  | X₁ₓ | X₂ₓ | X₃ₓ | Yₓ   |
|----|-----|-----|-----|------|
| 1  | 0   | 2   | 0.2 | 1.7  |
| 2  | 0   | 8   | 0.2 | 1.59 |
| 3  | 0.1 | 2   | 0.2 | 1.7  |
| 4  | 0.1 | 8   | 0.2 | 1.6  |
| 5  | 0   | 2   | 0.8 | 1.65 |
| 6  | 0   | 8   | 0.8 | 1.68 |
| 7  | 0.1 | 2   | 0.8 | 1.4  |
| 8  | 0.1 | 8   | 0.8 | 1.45 |

As a result of optimization by preliminary calculation of coefficients, equation 4 was obtained, describing the process of biomass accumulation.

\[
y = 1.59 - 0.058x_1 - 0.016x_2 - 0.015x_3 + 0.00375x_{12} + 0.036x_{23} - 0.06x_{13} + 0.00125x_{123}
\]  

(4)

Statistical analysis of the significance of the coefficients showed that the coefficient \( b_{123} \) is not significant. Thus, equation 5 is presented describing the biomass accumulation process.

\[
y = 1.59 - 0.058x_1 - 0.016x_2 - 0.015x_3 + 0.00375x_{12} + 0.036x_{23} - 0.06x_{13}
\]  

(5)

Testing the adequacy of the resulting equation after eliminating a minor coefficient revealed that the equation adequately describes the biomass accumulation process and can serve as a good basis for finding optimal solutions.

![Figure 1. Process exit](image)

The resulting linear model describes an inclined hyperplane in \((n + 1)\) - dimensional space. However, this linear equation will reflect with sufficient accuracy the response surface only in some local area corresponding to the studied range of factor change.
If you find the direction of the steep rise (gradient) of this plane and move in this direction (by conducting experiments under appropriate conditions) outside the studied area, you can find a combination of factors that will correspond to the beginning of the reduction of the response surface. Finding this top point when describing the process with a linear equation can only be accomplished experimentally by scheduling a series of experiments in the direction of the gradient. In this regard at the following stage of researches optimization of composition of nutrient medium comes down to finding of an optimum ratio of the most essential (significant) factors against the background of the constant level of the others (the procedure of Boxing Wilson) [15]. The optimization program can be obtained using an algorithm proposed by Box and Wilson.

At the next stage found works b, λ. At most the work was taken as basic. The change in factors is carried out as shown in the equations 6, 7.

\[ K_i = \frac{b_x \lambda_i}{b_0} \]  
\[ S_i = K_i \cdot S_i \]  

where \( S_i \) – is the base factor step, (0.3-0.7) \( \lambda_i \).

An adequate equation derived from the implementation of the full-factor planning plan is in the form of equation 8.

\[ y = 1.59 - 0.058x_1 - 0.016x_2 - 0.015x_3 + 0.00375x_1x_2 + 0.036x_2x_3 - 0.06x_1x_3 + 0.00125x_1x_2x_3 \]  

The variation interval of factors is shown in equations 9, 10, 11.

\[ x_1 - (0 - 5) mg/l; \]  
\[ x_2 - (2 - 8) mg/l; \]  
\[ x_3 - (0.2 - 0.6) g/l \]  

The center of the experiment is described by equations 12, 13, 14.

\[ C_{10} = \frac{c_1 + c_1^-}{2} = \frac{0+5}{2} = 2.5 \]  
\[ C_{20} = \frac{c_2 + c_2^-}{2} = \frac{2+8}{2} = 5 \]  
\[ C_{30} = \frac{c_3 + c_3^-}{2} = \frac{0.2+0.6}{2} = 0.4. \]  

The range of variation is represented by equations 15, 16, 17.

\[ \lambda_1 = \frac{c_1^+ + c_1^-}{2} = \frac{5-0}{2} = 2.5 \]  
\[ \lambda_2 = \frac{c_2^+ + c_2^-}{2} = \frac{8-2}{2} = 3 \]  
\[ \lambda_3 = \frac{c_3^+ + c_3^-}{2} = \frac{0.6-0.2}{2} = 0.2. \]  

To calculate the optimization program according to the Box-Wilson scheme, linearization of the adequate equation was carried out, the result is presented in the form of equation 18.

\[ \Delta y = (0.058 + 0.00375 - 0.06x_3 + 0.00125x_1x_3)\Delta x_1 + (-0.016 + 0.00375x_1 + 0.036x_2 + 0.00125x_1x_2)\Delta x_2 + (-0.05 + 0.036x_2 - 0.06x_1 + 0.00125x_1x_2)\Delta x_3. \]  

Design data of the first stage of the optimization program are presented in table 2.
Table 2. First stage of the optimization program

|          | $b_i$ | $\lambda_i$ | $b_{\lambda_i}$ | $K_i$ | $S_i$ | $c_{i0}/x_{i0}$ | $c_{il}/x_{il}$ | $c_{ill}/x_{ill}$ |
|----------|-------|-------------|-----------------|-------|-------|-----------------|-----------------|-------------------|
| Thiamine | 1     | -0.06       | 2.5             | -0.15 | -1    | -1.5            | 2.5/0.0         | 1/-0.6            |
| CoCl$_3$ | 2     | 0.036       | 3               | 0.108 | 0.72  | 1.08            | 5/0.0           | 6/0.33            |
| Mg$_2$SO$_4$·7H$_2$O | 3   | -0.06       | 0.2             | -0.012 | 0.08  | -0.12           | 0.4/0.0         | 0.28/-0.6         |

Equation 19 describes the process between the initial experience ($x_i = 0$) and the first experience of the optimization program.

$$\Delta y_{0-1} = 0.058\Delta x_1(0-1) - 0.016\Delta x_2(0-1) - 0.05\Delta x_3(0-1).$$  \hspace{1cm} (19)

Calculated data are summarized in Table 3.

Table 3. The second stage of optimization

|          | $b_i$ | $\lambda_i$ | $b_{\lambda_i}$ | $K_i$ | $S_i$ | $c_{i0}/x_{i0}$ | $c_{il}/x_{il}$ | $c_{ill}/x_{ill}$ |
|----------|-------|-------------|-----------------|-------|-------|-----------------|-----------------|-------------------|
| Thiamine | 1     | -0.06       | 2.5             | -0.15 | -1    | -1.5            | 2.5/0.0         | 1/0.6             |
| CoCl$_3$ | 2     | 0.036       | 3               | 0.108 | 0.72  | 1.08            | 5/0.0           | 6/0.33            |
| Mg$_2$SO$_4$·7H$_2$O | 3   | -0.06       | 0.2             | -0.012 | 0.08  | -0.12           | 0.4/0.0         | 0.28/-0.6         |

Between the I and II experiments of the optimization program, the process is described by equation 20.

$$\Delta y_{1-2} = 0.09\Delta x_1(1-2)0.039\Delta x_2(1-2) - 0.07\Delta x_3(1-2).$$ (20)

Table 4. Third phase of the optimization program

|          | $b_i$ | $\lambda_i$ | $b_{\lambda_i}$ | $K_i$ | $S_i$ | $c_{i0}/x_{i0}$ | $c_{il}/x_{il}$ | $c_{ill}/x_{ill}$ |
|----------|-------|-------------|-----------------|-------|-------|-----------------|-----------------|-------------------|
| Thiamine | 1     | -0.06       | 2.5             | -0.15 | -1    | -1.5            | 2.5/0.0         | 1/0.6             |
| CoCl$_3$ | 2     | 0.036       | 3               | 0.108 | 0.72  | 1.08            | 5/0.0           | 6/0.33            |
| Mg$_2$SO$_4$·7H$_2$O | 3   | -0.06       | 0.2             | -0.012 | 0.08  | -0.12           | 0.4/0.0         | 0.28/-0.6         |

Between the II and III experiments of the optimization program, the process is described by equation 21.

$$\Delta y_{2-3} = 0.11\Delta x_1 - 0.049\Delta x_2 - 0.03\Delta x_3.$$ \hspace{1cm} (21)

Calculations for process optimization are presented in the form of Table 5.

Table 5. Fourth stage of the optimization program

|          | $b_i$ | $\lambda_i$ | $b_{\lambda_i}$ | $K_i$ | $S_i$ | $c_{i0}/x_{i0}$ | $c_{il}/x_{il}$ | $c_{ill}/x_{ill}$ |
|----------|-------|-------------|-----------------|-------|-------|-----------------|-----------------|-------------------|
| Thiamine | 1     | 0.11        | 2.5             | 0.275 | 1     | 1               | 2               | 3/0.2             |
| CoCl$_3$ | 2     | -0.05       | 3               | -0.15 | -0.545| -0.545          | 5.48            | 4.9/0.16          |
| Mg$_2$SO$_4$·7H$_2$O | 3 | -0.03         | 0.2             | -0.006 | 0.02  | -0.02           | 0.22            | 0.198/-0.9      |

Equation 22 describes the process between the III and IV experiments.

$$\Delta y_{3-4} = 0.12\Delta x_1 - 0.05\Delta x_2 - 0.06\Delta x_3.$$ \hspace{1cm} (22)

According to the calculations made, the optimization program will have the form shown in Table 6.
### Table 6. Program of optimization

| Factors     | Options of environments |
|-------------|--------------------------|
|             | 1  | 2  | 3  | 4  |
| Thiamine    | 0,025 | 0,001 | 0,002 | 0,003 |
| CoCl₂       | 0,005 | 0,066 | 0,058 | 0,049 |
| Mg₂SO₄·7H₂O | 0,4   | 0,16  | 0,04  | 0 |

Biomass storage efficiency was monitored by optical density. The results of the pilot studies carried out according to this plan are presented in figure 2.

Analysis of the results showed that when microorganisms were cultured in nutrient media 1, 2, 3, an increase in cell biomass was observed. As can be seen from figure 2, when the nutrient medium 3 is used, a combination of factors is observed in which the efficiency of the biomass storage process is maximized. Next, the yield of the process is reduced, which is also illustrated in figure 2. This suggests that it is impossible to increase the efficiency of the process without limits.

![Figure 2. Results of nutrient medium composition optimization](image)

5. Conclusion.
Thus, the best variant of a combined starter culture medium consisting of propionic acid bacteria and an acidophilic bacterium is the one shown in table 7.
Table 7. Composition of nutrient medium

| Name of components                      | Contents, g/l |
|-----------------------------------------|---------------|
| Cottage cheese serum                    | 987.77        |
| Peptone                                 | 5             |
| Agar-agar                               | 0.75          |
| Potassium fosfornokisly monosubstituted | 5             |
| Sodium limonnokisly trisubstituted     | 1             |
| Magnesium sulfate                       | 0.4           |
| Thiamine                                | 0.02          |
| Cobalt chloride                         | 0.058         |

As a result of the studies carried out, the component and mass composition of the nutrient medium was determined for the production of biomass of propionic acid bacteria and acidophilic bacteria.

6. Acknowledgments
I express gratitude to my scientific head Hamagayeva I. S. for the opportunity to work out technological parameters on the basis of an innovative production center, valuable advice when planning research and recommendations on the design of the article.

References
[1] Ganina B I, Koroleva N S and Filchakova S A 2008 Technical microbiology of animal products (Moscow: DeLi print)
[2] Gottschalk G 1982 Metabolism of bacteria (Moscow: World)
[3] Kvasnikov E I and Nesterenko O A 1975 Lactic acid bacteria and ways to use them (Moscow: The science)
[4] Munch G D, Zaupe X and Steiner M 1985 Microbiology of products of animal origin (Moscow: Agropromizdat)
[5] Tamim A and Robinson R K 2003 Yoghurts and other fermented milk products (Saint-Petersburg: Profession)
[6] Schlegel G. 1987 General Microbiology (Moscow: Mir)
[7] De Man J C, Rogosa M and Sharpe M E 1960 A medium for the cultivation of lactobacillus J. Appl. Bact. 1 130-135
[8] Efstathiou J D, Mckay L L, Morris H A and Zottola E A 1975 Growth and preservation parameters for preparation of a mixed species culture concentrate for cheese manufacture J. Milk Food Technol 38 (8) 444-448
[9] Petterson H E 1975 Preservation of mixed species lactic starter concentrates by freezing and lyophilisation methods Milchwissenshaft 30 (9) 539-548
[10] Petterson H E 1975 Studies on batch production of bacterial concentrates from mixed species of lactic starters Applied microbiology 29 133-144
[11] Ohapkina V Y 2009 Methods for maintaining microbial cultures. Part 2. Lyophilization Theoretical and applied ecology 4 21-32
[12] Hettinga D H and Reinbold G W 1972 The propionic acid bacteria – a Review. I Growth J. Milk Food Technol. 35(5) 295-301
[13] Jeter R and Escalante-Semerena J C 1987 Synthesis and use of vitamin B12 Escherichia coli and Salmonella tephimurium 1 551-556
[14] Vorobyova L I and Charakhchyan I A 1983 Consumption of various sources of sulfur propionic acid bacteria Microbiology 52(26) 875-879
[15] Grachev Y P 1979 Mathematical methods for planning experiments (Moscow: Food industry)