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TECHNOLOGY OF PRODUCING SYMBIOTIC BIOLOGICALLY ACTIVE ADDITIVE

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Abstract. The article presents data on the development of the technology of multicomponent probiotics from two bacterial strains: *Bifidobacterium longum-Ya3* and *Propionibacterium shermanii-4*. The ability of bacteria of the genus *Propionibacterium* to have a selective stimulating effect on the growth of bacteria of the genus *Bifidobacterium* has been characterised. Based on the experimental data obtained with the MATLAB software, optimal conditions were determined for the accumulation of the maximum amount of biomass of the consortium of *Bifidobacterium longum-Ya3* and *Propionibacterium shermanii-4*. The main parameters that determined the yield of biomass in the process of cultivation under different temperature conditions (T=30°C, T=34 °C, T=37 °C) have been taken as the optimality criteria. These parameters are the number of colony-forming units and the active acidity. It has been established that the optimal time for cultivating a consortium of bifidobacteria and propionibacteria in a soy-lactose medium is 24 hours at a temperature of 34°C. On the basis of the data obtained, we have created a symbiotic BAA (biologically active additive) and developed a basic technological scheme for its production. The biologically active additive was created on the basis of the symbiotic consortium of bifido and propionibacteria containing 4×10^{10} CFU/cm^3 of *B. longum-Ya3*, and 3×10^{10} CFU/cm^3 of *P. shermanii-4*. The microbiological control of the quality of the obtained dietary supplement based on the consortium of the bacteria *Bifidobacterium longum-Ya3* and *Propionibacterium shermanii-4* has found no pathogenic and sanitary indicator microorganisms. It means that the finished product is safe and suitable for consumption. As for the organoleptic parameters, the BAA obtained is of a powder-like structure, beige-coloured, with a specific taste and smell.

Keywords: bifidobacteria, propionibacteria, consortium, biologically active additive

ТЕХНОЛОГІЯ ВИРОБНИЦТВА СИМБІОТИЧНОЇ БІОЛОГІЧНО АКТИВНОЇ ДОБАВКИ

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Анотація. У статті наведено дані стосовно розробки технології полікомпонентного пробіотику з двох штамів бактерій: *Bifidobacterium longum-Я3* та *Propionibacterium shermanii-4.* Охарактеризовано здатність бактерій роду *Propionibacterium* здійснювати селективний стимулювальний ефект щодо зростання бактерій роду *Bifidobacterium.* На основі отриманих експериментальних даних за допомогою програмного забезпечення MATLAB визначено оптимальні умови для накопичення максимальної кількості біомаси консорціуму *Bifidobacterium longum-Я3* та *Propionibacterium shermanii-4*. У якості критеріїв оптимальності було обрано основні показники, які характеризували вихід біомаси в процесі культивування за різних температурних режимів (T=30°C, T=34°C, T=37°C) – це кількість колонієутворювальних одиниць та активна кислотність. Встановлено, що оптимальним часом культивування культури консорціуму біфідобактерій та пропіоновокислих бактерій на соєво-лактозному середовищі було 24 години за температури 34°C. Ґрунтуючись на отриманих даних, створено симбіотичну біологічно активну добавку і розроблено принципову технологічну схема її виробництва. Біологічно активна добавка на основі симбіотичного консорціуму біфідо- та прорпіоновокислих бактерій із вмістом *Bifidobacterium longum-Я3* у кількості 4×10^{10} КУО/см^3, а *Propionibacterium shermanii-4* – 3×10^{10} КУО/см^3. Після проведення мікробіологічного контролю якості отриманої дієтичної добавки встановлено, що санітарно-показові та патогенні мікроорганізми відсутні, що свідчить про безпечність та придатність до споживання готового продукту. За органолептичними показниками отримана біологічна активна добавка характеризувалась порошкоподібною структурою бежевого кольору із специфічним смаком та запахом.

Ключові слова: біфідобактерії, пропіоновокислі бактерії, консорціум, біологічно активна добавка.

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Introduction. Formulation of the problem

Today, as a result of the deterioration of the environment, radiation poisoning, misuse of antibiotics, and unbalanced diet, the quantitative and qualitative composition of the gastrointestinal tract microbiota (GIT microbiota) is changing, which leads to dysbiosis. That is why, developing a new effective biocorrector for microbial rebalancing of the human GIT is an urgent problem. Nowadays, to treat dysbiosis, medicines are used containing living microorganisms that have a positive effect on the composition of the intestinal microbiocenosis. These medicines are called probiotics.

According to the forecasts of the world's leading experts in nutrition and medicine, probiotics and functional products account for 30% of the entire food market. Intensive research is under way to improve the existing probiotic drugs and functional products and the technology of their preparation. The most effective and safest for people is the use of probiotics medicines and probiotic-purpose foodstuffs [1]. Thus, the improvement of existing probiotics and the development of new compositions of probiotic bacteria in dietary supplements is still a topical issue.

Analysis of recent research and publications

The positive effect of probiotics on the body manifests itself both at the local level (through the normalisation of the microbial ecology of the GIT) and systematically. In the latter case, a probiotic preparation should consist of microorganisms that must be present in the human GIT. These are mainly bacteria of the genera *Bifidobacterium, Lactobacillus*, and *Propionibacterium* [2,3].

Bifidobacteria occupy a special place among various representatives of a person's obligate microbiota, which is composed of 85–98% of bifidobacteria. Bifidobacterium flora plays an important role in maintaining and normalising the microbiocenosis of the GIT. Bifidobacteria deficiency is one of the pathogenetic factors of prolonged intestinal dysfunctions in children and adults, which leads to mineral metabolism disorder and intestinal absorption processes, to disturbance of protein and fat metabolism, to the formation of chronic digestive disorders [4].

Today, a fairly wide range of probiotic drugs is produced on the basis of bifidobacteria, lactococci, and lactobacilli that have a positive effect on the physiological, biochemical, and immune responses of the body. In recent years, scientists have been interested in the propionibacteria of the milk group. They have a number of distinctive features: they are not digested in the human GIT, are resistant to bile acids, withstand the acidity of gastric juice, can biosynthesise B vitamins (especially B12), polysaccharides, amino acids (especially methionine), enzymes, antimicrobial compounds, or propionines (that are not only active against pathogenic bacteria, but against fungi and viruses as well), and are active bifidogenic growth factors. It is proved that they have powerful immunomodulating and antioxidant properties, are capable of destroying mutagens and carcinogens, protecting the genetic material of cells from the action of ultrasonic radiation, of free radicals and other genotoxic compounds. Propionic acid bacteria normalise lipid metabolism by regulating the level of cholesterol in the blood, preventing anaemia, cardiovascular and oncological pathology. They protect the body from infections, and optimise digestion and assimilation of food. Numerous useful properties of propionibacteria and the complete absence of toxicity make their extensive use in probiotic therapy and health-improving drugs highly prospective [5-7].

The safety of propionibacteria is defined by the European Food Safety Authorities (EFSA), with the status of QPS (Qualified Presumption of Safety). In the US, the Food and Drug Administration (FDA) has also included this bacterium in the GRAS (Generally Recognised as Safe) list with the number 21 CFR133.195. The positive role of propionibacteria as probiotics is also due to their ability of forming propionic acid, minor organic acids, bacteriocins, enzymes. Besides, when propionibacteria and bifidobacteria were cultivated simultaneously, the viable cells of both genera increased [7,8].

Stimulating the growth of bifidobacteria by these BGS from the propionibacteria is based on a mechanism quite different from that by oligosaccharides. In glucose metabolism of bifidobacteria in the presence of ACNQ and Fe(CN)63-, NAD(P)H in the cells is oxidised by ACNQ with the aid of diaphorase activity, and the oxidised ACNQ donates an electron to Fe(CN)63-. The exogenous oxidation of NADH by the ACNQ/Fe(CN)63- system results in the generation of pyruvate and a decrease in lactate production. DHNA is known as a precursor of menaquinone (vitamin K_2) and a stronger growth stimulator for bifidobacteria than ACNQ [9]. It is interesting to note that the effects of DHNA are positive not only for bifidobacteria but also for humans. In the study [10], the author reported that DHNA does not only restore colonic microbiocenosis by balancing the intestinal microflora, but also by suppressing lymphocyte infiltration through restoring the mucosal addressin cell adhesion molecule 1 (MAdCAM-1)

All of the above suggests that creating a symbiotic consortium based on bacteria of the genera *Bifidobacterium* and *Propionibacterium* is an interesting and practical task of food biotechnology, since the Ukrainian market does not have similar dietary supplements.

The aim of the work was to select optimal cultivation conditions that would make it possible to obtain the maximum yield of biomass of bifidobacteria and propionibacteria and to create a biologically active additive on their basis.

Objectives of the study:

1. To determine the number of colony-forming cells of *B. longum-Ya3* and *P. shermanii-4* genotypes and the change of the active acidity index during 72 hours of cultivation at different temperatures.

2. On the basis of the experimental data obtained using the MATLAB software, to determine the optimal conditions for the accumulation of the maximum amount of biomass of the bifidobacterium and propionibacteria consortium.

3. To develop a basic technological scheme for the production of a symbiotic BAA based on *B. longum-Ya3* and *P. shermanii-4*

4. To conduct microbiological control of the quality and safety of the product obtained.

Research Materials and Methods

The museum cultures of *B. longum-Ya3* and *P. shermanii-4* from the Department of Biochemistry, Microbiology, and Food Physiology of the Odessa National Academy of Food Technologies were used in the work. The incubation of the consortium was carried out in a soy-lactose medium at different temperatures for 72 hours. The inoculum of daily cultures was added to the flasks simultaneously in equal volumes in the amount of 5% of the total volume of the medium.

The criterion for choosing the temperature conditions for the co-cultivation of propionibacteria and bifidobacteria was comparison of the dynamics of consortium cells accumulation at different temperatures. It is known that the temperature optimum for propionibacteria is 30±1ºC, and for bifidobacteria 37±1ºC, so these points were chosen as the limiting ones. The doses of inocula were standardised to 1×10^6 CFU/cm^3. The calculation of the results was carried out by direct calculation of the propionibacteria in haemocytometers (Gorjaev's count chambers) and by spreading the bifidobacteria in a semi-liquid nutrient medium [11].

Using the MATLAB programme, a three-dimensional graph was built by means of the function *meshgrid.*

Microbiological control of the quality and safety of the product obtained was carried out by the state standards "GOST 30518-97 Bacteriological control. Bacteria of the *E. coli* group" and "GOST 10444-94 Bacteriological control. Salmonellae."

Results of the research and their discussion

At the initial stage of the research, on the basis of the experimental data by means of the MATLAB software, the optimal conditions were determined for the accumulation of biomass of a consortium of bifido and propionibacteria. As the optimality criteria, the main parameters characterising the biomass yield during the cultivation were taken. These parameters were the number of colony-forming units and the active acidity. The number of colony-forming units (CFU/cm^3) characterises in general the yield of the biomass of microorganisms in the process of cultivation. Table 1 shows the changes in the biomass accumulation rate and in the reaction of the medium during incubation for 72 h under different temperature conditions (T=30°C, T=34°C, T=37°C):

Table 1 – Change of cultivating parameters at different temperatures

Temperature T = 30 °C			Temperature T = 34 °C			Temperature T = 37 °C		
Time, (h)	Number of cells, lg (CFU/cm^3)	pH	Time, (h)	Number of cells, lg (CFU/cm^3)	pH	Time, (h)	Number of cells, lg (CFU/cm^3)	pH
0	6	7	0	6	7	0	6	7
3	6.2	6.9	3	6.2	6.9	3	6.3	6.6
6	6.5	6.7	6	6.7	6.7	6	6.5	6.5
9	7.0	6.5	9	7.9	6.5	9	7.1	6.0
12	8.0	6.2	12	8.8	5.9	12	8.0	5.7
15	8.5	6.0	15	9.7	5.5	15	8.5	5.1
18	9.0	5.7	18	10.5	5.3	18	8.9	4.8
21	9.0	5.6	21	10.6	5.2	21	9.2	4.6
24	9.0	5.5	24	10.7	5.1	24	9.3	4.5
27	9.0	5.45	27	10.7	5.1	27	9.3	4.5
30	9.0	5.2	30	10.7	5.0	30	9.3	4.45
33	8.9	5.15	33	10.6	5.0	33	9.2	4.4
36	8.8	5.1	36	10.5	4.9	36	9.0	4.37
39	8.8	5.1	39	10.5	4.9	39	9.0	4.35
42	8.8	5.05	42	10.4	4.85	42	8.9	4.3
48	8.7	5.0	48	10.4	4.8	48	8.8	4.29
60	8.6	4.9	60	10.1	4.7	60	8.6	4.25
72	8.4	4.7	72	9.8	4.5	72	8.5	4.23

The indicators of active acidity make it possible to determine the intensity and rate of development of the bacteria biomass and the accumulation of their metabolites in the culture medium. The main metabolites that create the reaction of the environment during the cultivation are lactic, propionic, succinic, formic, and acetic acids.

The largest biomass yield in the cultivation process is observed in the logarithmic phase of cultivating the consortium of *B. longum-Ya3* and *P. shermanii-4* bacteria cells ($8 \le t < 25$ hours). That is, the target function is determined by the kinetic equation:

$$B = a + \frac{bc}{d-c}\left(e^{-ct} - e^{-dt}\right) \qquad (1)$$

where $a = 0.09113 \cdot T^2 - 5.942 \cdot T + 95.17$,

$b = -0.4248 \cdot T^2 - 28.31 \cdot T + 456.3$,

$c = 0.003615 \cdot T^2 - 0.2446 \cdot T + 4.25$,

$d = 0.000642 \cdot T^2 - 0.042855 \cdot T + 0.7067.$

Consider the graph of this function B=B (t, T), which is plotted using the MATLAB mathematical modelling programme [11]. The MATLAB programme allowed creating a three-dimensional graph by means of the function *meshgrid* and has the form:

```
[X,Y]=meshgrid(7.5:0.5:24.30:0.5:37);
a1 = 0.09113.*Y.^2-5.9424.*Y+95.1682;
b1 = -0.4248.*Y.^2+28.3074.*Y-456.3301;
c1 = 0.003615.*Y.^2-0.2446.*Y+4.2498;
d1 = -0.000642*Y.^2+0.04285*Y-0.7067;
Z = a1-((b1.*c1)./(c1-d1)).*(exp(-c1.*X)-exp(-d1.*X));
surf(X,Y,Z)
```

As shown in Fig. 1, the surface B=B (t, T) has the maximum for some values of time (t) and temperature (T). To determine the maximum of the function B=B (t, T), a non-linear programming problem was used, which was also solved in the MATLAB system. The corresponding optimisation program has the form:

```
function f=myfun(x)
a1 = 0.09113*x(2)^2-5.9424*x(2)+95.1682;
b1 = -0.4248*x(2)^2+28.3074*x(2)-456.3301;
c1 = 0.003615*x(2)^2-0.2446*x(2)+4.2498;
d1 = -0.000642*x(2)^2+0.04285*x(2)-0.7067;
f = -a1+((b1*c1)/(c1-d1))*(exp(-c1*x(1))-exp(-d1*x(1)));
end
A = [-1 0;1 0;0 -1;0 1];
b = [7.5;24;30;37];
x0 = [7.5;30];
[x,fval]=fmincon('myfun',x0,A,b)
X (t) = 24.0000
X (T) = 33.7440
fval = 10.7494
```

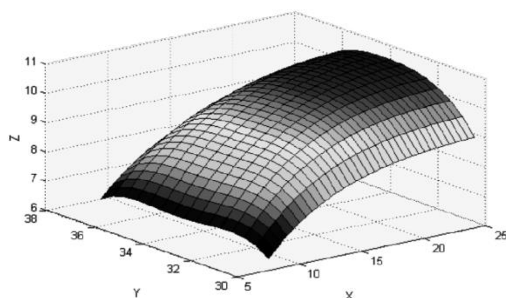

Fig.1. Graph of the function *meshgrid*. Changes in CFU/cm^3 of the consortium of bifido and propionibacteria at different temperatures: x – time of cultivation, h; y – temperature of cultivation, °C; z – number of viable cells, lg CFU/cm^3.

Thus, the greatest amount of biomass yield of the consortium for bifido and propionibacteria was B = 10.75 CFU/cm^3, which reached its maximum in 24 hours of cultivation (t=24 h) at a temperature of T = 33.74°C.

The active acidity of the medium (pH) during the logarithmic phase of biomass growth of the consortium *B. longum-Ya3* and *P. shermanii-4* is determined by the ratio:

$$pH = \exp(a(T) + b(T) \cdot B), \quad (2)$$

where $a(T)= 0.01469 \cdot T^2 - 0.9396\, T + 17.33$,

$b(T)= - 0.00272 \cdot T^2 + \cdot 0.1781\, T - 2.919.$

Under the optimisation conditions B=10.75 CFU/cm^3 and T=33.74°C, the acidity of the pH medium is pH=5.1881

Based on the results of the experimental work on optimisation of cultivation, a technology for the production of a symbiotic BAA has been developed (Fig. 2).

Thus, the technology of obtaining a biologically active additive based on a symbiotic consortium has been developed.

In previous experimental studies [11], a soy lactose nutrient medium was created to co-cultivate bifido and propionibacteria. To exclude unwanted microbiota, sterilisation was carried out at a temperature of 121°C for 20 minutes, followed by cooling to 34°C – the fermentation temperature of the symbiotic consortium *B. longum-Ya3* and *P. shermanii-4*. Cultures of the second generation *B. longum-Ya3* and *P. shermanii-4* in the proportion 1:1 in equal volumes of 5% were simultaneously inoculated into the culture medium. The cultivation process was carried out for 24 hours at a temperature of 34°C.

After obtaining, in optimal conditions, the maximum amount of the biomass of the consortium *B. longum-Ya3* and *P. shermanii-4*, the microorganisms were separated from the culture liquid by centrifugation at 10,000 rpm. for 15 minutes.

The next step was introducing a protective medium containing milk, sucrose, and gelatose [13], with further lyophilic drying. The mixture is dried after prefreezing at –20°C for 12 hours. After 8 hours from the beginning of the freezing, the temperature is lowered to -30 °C. To obtain a dry powdered product with a residual moisture of 5%, sublimation is carried out for 20–24 hours, followed by tabletting the mixture.

To obtain the tableted form, a hydraulic tableting machine was used. Tableting was done by direct packing, with a pressure of 30 MPa and heating of the matrix up to 45°C.

Microbiological analysis plays an important role in assessing the quality of probiotic food products and probiotic preparations and is carried out according to a number of requirements. To prevent microbial contamination with external microbiota from the environment, the microbiological control of the test samples was carried out according to the aseptic rules. The inoculations were carried out by the plate method on solid media.

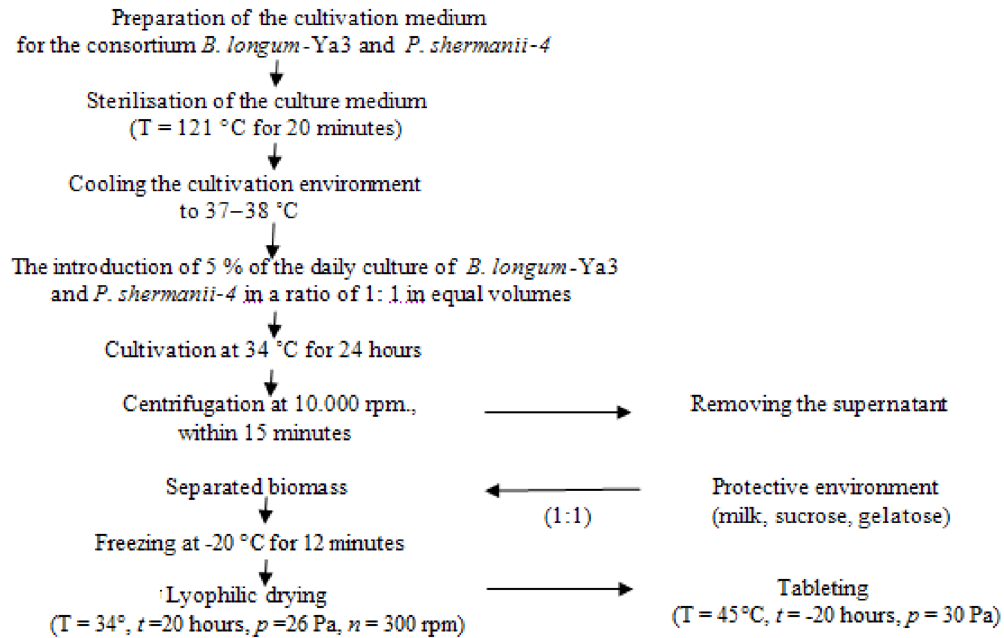

Fig. 2. The general technological scheme of obtaining symbiotic BAA

The microbiological indicators of the quality and safety of the product obtained are presented in Table 2.

Table 2 – Microbiological characteristics of the product

Indicator	Characteristics
The quantitative content of *B. longum-Ya3* *P. shermanii-4*	4×10^{10} CFU/cm^3 3×10^{10} CFU/cm^3
Mould fungi CFU/cm^3	missing
Pathogenic microorganisms, including salmonella	missing
Coliform bacteria 0.1 g	missing

The data of Table 2 show that the quantitative content of probiotic bacteria in the newly developed supplement complies with the probiotic norm, and there are no pathogenic and sanitary indicator microorganisms, which means that the additive is safe and ready-to-use.

The resulting BAA had a powdery structure, was beige in colour, with a specific taste and smell.

Conclusions

Propionibacteria have the biotechnological potential for creating both monostrain probiotics and symbiotics. In the course of the logarithmic phase of cultivation, not only the biomass of the microorganisms under study is accumulated, but their metabolic products as well, including lactic, propionic, and acetic acids. These acids form the environment (pH). Indicators of active acidity make it possible to determine the intensity and rate of bacteria biomass development and the accumulation of their metabolites in the culture medium. Based on scientifically substantiated experiments and optimisation of the key parameters of bacterial biomass accumulation, a technology for the production of a symbiotic biologically active additive has been developed.

According to the results of the experimental data, it has been established that the optimal time for cultivating a consortium of bifidobacteria and propionibacteria in a soy-lactose environment is 24 hours at a temperature of 34°C. A BAA based on the symbiotic consortium *B. longum-Ya3* and *P. shermanii-4* with a quantitative contents of microorganisms 4×10^{10} CFU/cm^3 and 3×10^{10} CFU/cm^3, respectively, has been created.

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