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FUNDAMENTAL BASIS OF CREATION OF PROBIOTIC WITH PROVITAMIN ACTIVITY BASED ON STRAINS BACILLUS AMYLOLIQUEFACIENS IMV B-7513 AND IMV B-7525

Dysbiotic conditions prevail in both humans and animals. Intestinal dysbiosis is accompanied, in addition to the quantitative and qualitative changes of the microflora, by immunosuppression and metabolic disorders, such as avitaminosis A. Therefore, the search and study of microorganism strains, which may be used for correction of dysbiosis and allow to normalize the homeostasis of a macroorganism, are highly relevant today. In relation to that the use of carotene synthesizing strains of bacteria of Bacillus genus is very perspective. This would allow connecting in one preparation the properties of probiotics and ability to compensate the deficiency of vitamin A in a macroorganism.

The article presents the analysis of literature data along with the results of own research concerning carotene synthesizing activity of bacteria of Bacillus genus, the nature of their carotenoid pigments and their function in a macroorganism. It was determined that carotenoid pigments of B. amyloliquefaciens IMB B-7313 and IMB B-7325 strains were presented by a complex with lipids and carbohydrates, and belonged to C₃₀-apocarotenoids, including apo-8'-phytofluene, apo-8'-ζ-carotene, apo-8'-neurosporene and apo-8'-β-carotene-3-ol. A possible way of carotene synthesis was proposed. The end product of its pathway synthesis probably is apo-8'-β-carotene-3-ol, which is able to be metabolized into vitamin A. Earlier it was shown that the investigated strains of bacteria are safe for warm-blooded animals and exhibit probiotic and provitamin activity in vivo.

The obtained results provide a fundamental basis for creation of a probiotic preparation with provitamin activity based on carotene producing strains B. amyloliquefaciens IMV B-7513 and B-7525.

K e y  w o r d s: bacteria of the genus Bacillus, carotenoid pigments, probiotic features, provitamin activity.

Dysbiotic conditions present a serious problem being widespread among people, agricultural animals and birds. The solution of this problem involves an application of probiotics based on living cultures of representatives of normal microflora of gastrointestinal tract. In recent years probiotics consisting of bacteria of Bacillus genus have become widely used.

Usually intestinal dysbiosis is accompanied by quantitative and qualitative changes of microbiota by immunosuppression and metabolic violations, in particular by avitaminosis A. Therefore, a research is necessary to identify and study microorganisms, the use of which for treatment of dysbiosis will allow to normalize the homoeostasis of macroorganism. In relation to that it is very relevant to use carotene synthesizing activity of bacteria of the Bacillus genus, that would allow to unite in one preparation the properties of probiotics and the ability to compensate for the deficiency of vitamin A in a macroorganism.
Carotenoids are a group of natural pigments of yellow, orange or red colors. They are synthesized by plants, protozoa (Dunaliella salina), fungi (Blakeslea trispora) and bacteria (Streptomyces, Staphylococcus spp., etc.). Some species of bacteria of the Bacillus genus, particularly B. indicus, B. vedderi, B. jeotgali, B. okuhidensis, B. clarkii, B. pseudofirmus, B. firmus, have yellow and orange pigmentation of vegetative cells and spores, due to the synthesis of various carotenoids. These pigments are referred to isoprenoid substances. They contain in its structure molecules of isopentenyl diphosphate, and are usually represented by apo- or diapocarotenoids with lipophilic properties [1, 2]. Unlike eukaryotes, bacterial carotenoids are associated with cytoplasmic membrane. They are mainly bound to proteins, lipids and carbohydrates.

Thus, using mass spectrometry methyl-1-(6-C_{10:0})-glycosyl-3,4-dehydro-apo-8′-lycopenoate, methyl-1-glycosyl-3,4-dehydro-apo-8′-lycopenoate, methyl-1-(6-C_{10:0})-glycosyl-3,4-dehydro-apo-8′-lycopene and 1-glycosyl-3,4-dehydro-apo-8′-lycopene were identified in vegetative cells and spores of strain B. indicus HU36 [3]. Carotenoids of the vegetative cells had yellow coloring and spores pigmentation was orange.

It is known that the vegetative cells and spores of bacteria of the genus Bacillus contain not only the C_{30}-carotenoids, but C_{40}-1-HO-demethylspheroidene and keto/hydroxy-γ-carotene derivatives. Also in the cells and spores of these bacteria neurosporene was found [1].

Despite the presence of a large number of published scientific papers on bacilli pigments, few studies are conducted about carotene synthesizing activity of bacteria of the genus Bacillus, properties of bacterial carotenes. There are no data on the patterns of carotenoid pigments biosynthesis, their metabolic transformation in a macroorganism. The methods of extraction, qualitative and quantitative analysis of these pigments are imperfect.

Several schemes of synthesis of carotenes in various representatives of Bacillus genus were suggested [1, 3] and different ways of formation of pigments in vegetative cells and spores of these bacteria were observed.

The strains B. amyloliquefaciens IMV В-7513 and IMB В-7525 from the collection of antibiotics department of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Science of Ukraine were the objects of our investigation. These strains synthesize orange pigment after cultivation on meat-peptone and tryptone soy agar.

The class determination and carotenes identification were performed by means of thin layer chromatography (TLC) and high performance liquid chromatography with mass spectrometry (LC-MS) on liquid chromatography Agilet Technologies 1200 with mass-detector Agilent Technologies G1956B. Chromatographic column Zorbax SB-C18 (2.1×150 mm, 3.5 µm) was used for pigment separation. The mobile phase included acetone and formic acid. Detection was done by diode-array detector with specter detection range191–700 nm. Ionization was conducted in ESI and APSI modes with the fixation of positive ions in SCAN mode within the range of 100–1200 m/z [4].

The presence of the gene encoding phytoene synthase which was required for the first steps of carotenoid synthesis was determined by PCR. [5]. The nucleotide sequences of primers for fragment gene amplification were picked using programs MEGA 5.0 and Primer 3.

The evaluation of yis P gene expression was performed by quantitative real
time PCR using q Tower 2.2 («Analytik Jena AG», Germany). The relative level of gene expression was calculated with $2^{-\Delta\Delta CT}$ method [6]. The expression of 16S rRNA was used as an endogenous control.

Statistical analysis of the results was carried out by determination of mean arithmetic values, their standard deviation and mean square error with significance levels 0.05 and 0.01.

An optimal system for pigments extraction from strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 was chosen for the investigation of their nature. It was established that full extraction of pigments from bacterial biomass is achieved by using chloroform and methanol solution (2:1). The standard systems for extraction of carotenoids from plants, streptomycetes and fungi were ineffective because of the differences and specificity of the structure of bacilli carotenoids. The obtained pigment extracts gave positive qualitative reaction with FeSO$_4$, H$_2$SO$_4$, SbCl$_3$ and specific for carotenes absorption maxima. However, absorption maxima comparatively to β-, γ- and ε-carotenes shifted to the short-wave region.

The extracts were separated by TLC using an adapted system – chloroform:methanol:ethyl acetate (1:1:2). The treatment of chromatograms with group reagents showed that pigments were located in complex with lipids and proteins. Pigments of the investigated strains were divided into 3 different components with Rf 0.3; 0.7 and 0.9.

Since the obtained data could be used to assign the pigments of the investigated strains to the carotenes but not to a specific class, we have determined the presence of the gene, responsible for production of the initial product of the carotene synthesis pathway, in the genomes of the investigated strains of the *Bacillus* genus. Carotenoid synthesis in all carotene synthesizing organisms begins with the formation of phytoene by phytoene synthase [7]. In species *B. subtilis*, *B. amyloliquefaciens* and *B. methylotrophicus* this enzyme is encoded by the gene *yisP*. The presence of *yisP* gene was determined in the genomes of both investigated strains after molecular genetic analysis (Fig. 1), moreover the level of *yisP* expression was higher in the strain *B. amyloliquefaciens* IMV B-7513 than in the *B. amyloliquefaciens* IMV B-7525 (Fig. 2).

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**Fig. 1. Electrophoregram of phytoene synthase gene amplification (yisP) of the Bacillus genus bacteria. M – marker, 1 – *B. amyloliquefaciens* IMV B-7513, 2 – *B. amyloliquefaciens* IMV B-7525**
Thus, the presence of \textit{yisP} indicates that the synthesis of carotenoids by the investigated strains could include two pathways with the production of $C_{30}$ or $C_{40}$ carotenes.

For further identification of pigments HPLC-MS analyzes of carotene extracts of strains \textit{B. amyloliquefaciens} IMV В-7513 and IMV В-7525 was performed. As a result three compounds were determined. Their molecular masses and maxima of absorption spectra corresponded with apo-8-ζ-carotene, apo-8-neurosporene and apo-8´-β-carotene-3-ol which was bound with glucose and a $C_{8:0}$ fatty acid (Table 1). Moreover, during the strain cultivation with diphenylamine, a well-known inhibitor of phytoenedesaturases, the apophytofluene was identified in the extract.

<table>
<thead>
<tr>
<th>No</th>
<th>Elution time, min</th>
<th>$\lambda_{max}$, nm</th>
<th>Molecular weight</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.4</td>
<td>380, 398, 422</td>
<td>405</td>
<td>apo-8-ζ-carotene</td>
</tr>
<tr>
<td>2</td>
<td>18.9</td>
<td>415, 440, 465</td>
<td>498</td>
<td>apo-8-neurosporene</td>
</tr>
<tr>
<td>3</td>
<td>22.6</td>
<td>395, 418, 440</td>
<td>723</td>
<td>($C_{8:0}$)-glycosil-apo-8´-β-carotene-3-ol</td>
</tr>
<tr>
<td>4*</td>
<td>18.4</td>
<td>330, 345, 364</td>
<td>565</td>
<td>apo-8-phytofluene</td>
</tr>
</tbody>
</table>

* - Data were obtained as a result of diphenylamine inhibition analysis.

It should be noted that in the pigment extracts of strains \textit{B. amyloliquefaciens} IMV B-7513 and IMV B-7525apolycope were not found. However, according to the literature data, the microorganisms of the \textit{Bacillus} genus often form apolycope derivatives linked with sugar and fatty acid residues during the synthesis of $C_{30}$-apocarotenoids [8, 9]. Therefore, we have assumed that in the investigated strains \textit{B. amyloliquefaciens} after the neurosporene formation.
dehydrogenation and cyclization or only cyclization of the isoprenoid chain occurred with the formation of apo-8'-β-carotene-3-ol. This phenomenon requires further investigations using molecular genetic techniques.

In addition to the antioxidant function, carotenoid pigments perform photo and radioprotective activity, are involved in photosynthetic process. In the body of warm-blooded animals some carotenes serve as the provitamin A. Nonoxidased carotenoids also have antioxidant, antitumor and immunostimulatory activity [10]. Some apo-carotenoids reveal similar properties to C_{40} carotenoids, for example provitamin activity [11, 8].

Considering the abovementioned and the fact that the carotenes in a macroorganism may exhibit prebiotic properties, a few of veterinary medications were created based on carotene synthesizing streptomycetes and yeasts. Their application can significantly increase the growth rate of animals, improve their physiological state, prevent diseases, including those associated with metabolic disorders [12]. However, none of the known drugs combines provitamin and probiotic properties simultaneously.

So, strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 synthesize C_{30}-apocarotenoids. The end product of their synthesis pathway probably is apo-8'-β-carotene-3-ol, which according to the literature [6], is able to be metabolized into vitamin A. Based on our data, we propose the following way of carotene synthesis by the investigated bacillus strains (Fig. 3).

![Fig. 3. Possible way of carotene synthesis by B. amyloliquefaciens IMV B-7513 and IMV B-7525](image_url)

Thus, we isolated carotenoid pigments of strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 for the first time. It was established that they represent a complex with lipids and carbohydrates and are related to C_{30}-apocarotenoids, particularly apo-8-phytofluene, apo-8-ζ-carotene, apo-8-neurosporene and apo-8'-β-carotene-3-ol.
Our data provide a fundamental base for using carotene producing bacillus strains as potential components of biological products with provitamin activity. The necessity of such drugs exists not only in medicine but in veterinary due to the diseases of humans, mammals and birds caused by the deficiency of vitamin A, induced by intestine dysbiosis so widespread nowadays. The probiotics based on Bacillus strains have been widely used for correction of dysbiotic states in recent years. Synthetic vitamin A or β-carotene that is biotechnologically obtained and is not totally metabolized in the body is used for the prevention and treatment of vitamin A deficiency. Attempts to combine probiotic properties of bacteria and carotene synthesizing abilities in one product have recently emerged. There is a probiotic drug based on strain Bacillus indicus HU 36, which produces C<sub>30</sub>-diapocarotenones [12], characterized only by antioxidant, anticancer and immunomodulating activities.

Information about the simultaneous display of probiotic and provitamin activity by bacilli in world literature is not available. We determined that strains B. amyloliquefaciens IMB B-7513 and B-7525 IMB in addition to the ability to synthesize carotenes are also characterized by probiotic properties. In particular, these probiotic properties include the antagonistic activity against opportunistic microorganisms, resistance to low pH levels and concentrations of bile acids, low adhesive and high enzymatic (proteo-, lipolitic, xylano-, cellulolytic, pectinesterase) activity [13]. Strains are harmless for warm-blooded animals [14]. After per os ingestion of strains (separately or in a mix) qualitative and quantitative composition of intestinal microbiocenosis in mice and birds is normalised, the microstructure of organs is restored, vitamin A is accumulated in the liver, the cellular immunity is activated [15, 16].

Thus, the study of carotene synthesizing activity of the representatives of Bacillus genus highly relevant. These bacteria are characterized by a marked antagonism against opportunistic and pathogenic microorganisms, immunostimulating effect and high biosynthetic activity, manufacturability in the production. We have not only determined the identity of carotenoid pigments of strains B. amyloliquefaciens IMV B-7513 and IMV B-7525 to C<sub>30</sub>-apocarotenones, but also we have developed the fundamentals for creation of a new Ukrainian probiotic preparation with provitamin activity.

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тивним є використання каротинсинтезувальних штамів бактерій роду *Bacillus*, що дозволить поєднати в одному препараті властивості пробіотиків і здатність компенсувати дефіцит вітаміну А в макроорганізмі.

У статті проаналізовано дані літератури і представлено результати власних досліджень щодо каротинсинтезувальної здатності бактерій роду *Bacillus*, природи їх каротиноїдних пігментів та їх функції в макроорганізмі. Встановлено, що каротиноїдні пігменти штамів *B. amyloliquefaciens* IMB В-7513 та IMB В-7525, представлені комплексом з ліпідами та углеводами, відносяться до $C_{30}$-апокаротиноїдів, зокрема, апо-8´-фітофлуену, апо-ζ-8´-каротину, апо-8´-нейроспорину та апо-8´-β-каротен-3-олу. Запропоновано можливі шляхи їх синтезу, кінцевим продуктом якого є апо-8´-β-каротен-3-ол, що здатний метаболізуватися у вітамін А. Раніше в дослідях *in vivo* показано, що штами безпечні для теплокровних, проявляють пробіотичну і провітамінну активність.

Отримані результати є фундаментальною основою створення на основі каротинсинтезувальних штамів *B. amyloliquefaciens* IMB В-7513 та IMB В-7525 пробіотичного препарату з провітамінною активністю.

**Ключові слова:** бактерії роду *Bacillus*, каротиноїдні пігменти, пробіотичні властивості, провітамінна активність.

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**ФУНДАМЕНТАЛЬНІ ОСНОВИ СОЗДАННЯ ПРОБІОТИЧЕСКОГО ПРЕПАРАТА С ПРОВІТАМИННОЮ АКТИВНОСТЬЮ НА ОСНОВЕ ШТАММОВ BACILLUS AMYLOLIQUEFACIENS IMB В-7513 ТА IMB В-7525**

**Резюме**

Дисбиотичні стани широко розпространилися як у людей, так і у животних. Дисбіоз кишечника супроводжується, крім колівочних і качественных ізменений микробіоти, імунносупресією і метаболічними нарушениями, в частині авітамінозом А. Проте актуальним є пошук і введення штамів бактерій, відіграючих важливу роль у поддержании домашніх макроорганізмів. Вноси другим перспективним є використання каротинсинтезирующего штамма виду *Bacillus*, що дозволить поєднати в одному препараті властивості пробіотиків і здатність компенсувати дефіцит вітаміну А в макроорганізмі.

В статті проаналізовано дані літератури і представлено результати власних досліджень щодо каротинсинтезуючої активності бактерій роду *Bacillus*, природи їх каротиноїдних пігментів та їх функції в макроорганізмі. Установлено, що каротиноїдні пігменти штамів *B. amyloliquefaciens* IMB В-7513 та IMB В-7525, представлені комплексом з ліпідами та углеводами, відносяться до $C_{30}$-апокаротиноїдів, зокрема, апо-8´-фітофлуену, апо-ζ-8´-каротину, апо-8´-нейроспорину та апо-8´-β-каротен-3-олу. Предложено можливий шлях їх синтезу, кінцевим продуктом якого є апо-8´-β-каротен-3-ол, що здатний метаболізуватися у вітамін А. Раніше в дослідах *in vivo* показано, що штами безпечні для теплокровних, проявляють пробіотичну і провітамінну активність.

Полученные результаты являются фундаментальной основой создания на основе
The study investigated the carotenogenic properties of strains of bacillus, specifically Bacillus amyloliquefaciens IMV В-7513 and IMV В-7525, and their probiotic characteristics. The key findings were the evaluation of these strains' potential for producing provitamin A activity. 

**Key words:** bacteria of the genus Bacillus, carotenoid pigments, probiotic properties, provitamin activity.
