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RAPID SANITARY AND TECHNOLOGICAL CONTROL OF CULTURING THE PRODUCERS OF AMYLOLYTIC ENZYMES

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Abstract. It has been established that the biotechnological process of culturing bacillary microbial producers of amylolytic enzymes can be express-controlled by determining their ATP bioluminescence. The advantages of the method have been shown. The analytical review of producers of hydrolytic enzymes has made it clear how practical it is to use bacillary microorganisms for targeted secretion of amylolytic enzymes in biotechnological production. After monitoring bacillary microorganisms, it has been found advisable to choose *Bacillus subtilis* ATCC 6633 as the working culture due to its time of production of amylolytic enzymes and its biosynthetic activity. Reasons have been given for using the rapid ATP control method, which is based on the principle of bioluminescence. Different growth media have been compared and evaluated in order to intensify the quantitative biosynthetic activity of the microbial culture in the technological process of culturing bacillary microorganisms. The experiments have proved that growth media can be modified by introducing a number of carbohydrate–protein substrates as inducers of amylolytic complex gene expression. The latter manifests itself in the amylolytic activity accelerated by 12–24 hours, and causes an increase in the number of microorganisms (1.87–3.99 times as many as in the reference culture). Two methods of control (rapid bioluminescent and classic microbiological) have been used for correlative determination of the quantitative growth of *Bacillus subtilis* cells. Mathematical straight-line correlations have been obtained in a semilogarithmic system for the number of cells of the bacillary producer of the amylolytic enzyme complex. These correlations allow carrying out rapid control in a production environment. Along with the traditional rapid sanitary control in biotechnological production, which includes controlling the contamination of the equipment, personnel's hands, and water, it has been suggested to perform proprietary technological express control of amylolytic enzyme biosynthesis using the culture *Bacillus subtilis* ATCC 6633.

Keywords: bacillary microorganisms, *Bacillus subtilis*, ATP (adenosine triphosphate), bioluminescence, amylolytic enzyme complex, sanitary and technological control.

Introduction. Formulation of the problem

Amylolytic enzymes are required in various branches of food manufacture and the light industry. They are used in brewing and bread-baking, in juice, fabric, and paper production, are components of detergents, etc., because they can catalyse the breakdown of starch into oligo-, di-, and monosaccharides. That is why amylolytic enzymes belong to the most important and widely produced protein products. Amylolytic enzymes include biocatalysts with hydrolytic or transferase activity capable of cleaving 1,4- or 1,6-glycosidic bonds in glucose polymers. Representatives of this family are α-amylase (Enzyme Commission number EC 3.2.1.1), β-amylase (EC 3.2.1.2), glucoamylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41), isoamylase (EC 3.2.1.68), amylosucrase (EC 2.4.1.4), cyclodextrin glucanotransferase (EC 2.4.1.19), and some other enzymes [1,2].

Bacillary microorganisms have a high and diverse spectrum of biological activity. They are pronouncedly

antagonistic towards pathogensand can produce a number of enzymes that break down starch, cellulose, proteins, fats [3]. As the genus *Bacillus* is one of the numerous producers of bioactive substances (enzymes, antibiotics, insecticides), it is advisable to monitor representatives of this particular genus of microorganisms as producers of amylolytic enzymes.

Analysis of recent research and publications

Development and practical application of new rapid methods of detecting microorganisms and their metabolites is a topical issue due to the technological problems of quality control in biotechnology, industrial microbiology, food and pharmaceutical industries, in obtaining sterile materials, etc [4]. In many cases, the classic microbiological methods prove inadequate for rapid control of bacterial microbiota and automation of production, which tells on the quality of products [5,6].

Classic microbiological methods of controlling bacterial contamination consist in the following. The samples to be tested (water, air, soil, food, wipe samples from technological equipment, etc.) are inoculated on growth media and, after incubation, the number of the colonies grown is counted. The effectiveness of determining the degree of bacterial contamination largely depends on the particular characteristics of the object under research, the inoculation technique, the composition of the growth media, the time and temperature of incubation, etc. This method makes it possible to confirm the presence of viable microorganisms in a sample with the highest possible probability [6]. The classic methods of bacterial analysis are rather laborious, take a lot of time to obtain results, and often require very expensive media to culture microorganisms [6,7]. This does not allow using them for constant monitoring and rapid analysis.

To determine the metabolic products of microorganisms, the food industry practises the impedance method using the microbiological analyser BacTrac 4300 [6]. In total, examining a sample using this analyser takes no more than 24 hours. However, such a rapid analyser is quite expensive, and many food enterprises cannot afford it.

For better hygiene and sanitation, biotechnological manufactures widely use methods based on determining any physicochemical parameter of a sample, the absolute value of which (or its change) is proportional to the number of microbial contaminants or colony-forming units (CFU). One of them is the bioluminescent method. It consists in using the luciferin–luciferase system to measure the concentration of microbial and biogenic ATP in the sample analysed. The measurement of ATP by bioluminescence using ATP reagents (a mixture of luciferase, substrates, and buffer components) takes 1–2 min, complete analysis of the sample is fast, as compared with other methods, and this is an advantage of bioluminescence ATP-metry [8]. Luminescent bacterial test methods are widely practised in all developed countries, and can be used as a primary rapid quantitative laboratory test to check whether water samples meet the sanitary requirements [9].

Rapid testing of microbial contaminants using ATP-bioluminescence is a widely accepted method to monitor the hygienic condition of food manufacturing lines and to check the effectiveness of cleaning procedures. ATP bioluminescence detects microbial cells and food residues that can remain after unsatisfactory cleaning and be a source of nutrients for microorganisms [10]. Analytical literature describes the use of the bioluminescent method to determine the microbial contamination of raw materials. Due to the high residual content of non-microbial ATP (the total of free ATP and ATP of somatic cells) in the samples analysed, the detection limit for microbial contamination was higher than 5×10^4 CFU/g. To determine microbial contamination properly using the bioluminescent method, samples should be pretreated. This pretreatment significantly disrupts the non-microbial ATP or eliminated it from the sample [11].

According to other sources, the detection limit of cells by the parameter of intracellular ATP is less than 1000 cells in 1 ml/g of the sample [12]. Unlike the traditional microbiological methods, which take 24 to 72 hours or more, bioluminescence analysis not only allows reducing considerably the time of analysis, but also makes it cheaper and less laborious. That is why bioluminescent ATP-metry is the basis for so-called "rapid microbiology". The ATP content in tissues and in cells of plants and animals indicates the energy condition of the cells.

In recent years, wider possibilities have appeared for using microorganisms as biotechnological sources of industrially important enzymes, particularly of amylosubtilin obtained from *Bacillus subtilis*. Monitoring of the range of amylolytic enzyme preparations has shown that the market demand for amylases is about 30% of the global production of enzymes [13]. That is why metabarcoding is actively developing, such as amplicon target sequencing of 16S rRNA for bacterial producers [14]. No less important is the development and application of rapid methods for quantitative control of microbial contamination of objects in all technological processes of enzyme production to simplify monitoring the critical points of the production process (according to the HACCP system).

Purpose and objectives of the research. The **aim** of the study was to develop a method of proprietary rapid control of the biotechnological process of culturing bacillary microorganisms, producers of amylolytic complex enzymes, on various growth media.

To achieve this purpose, the following **objectives** were set:

1. carrying out rapid sanitary and technological control over the process of culturing bacillary microbial producers of amylolytic enzymes;

2. substantiating the choice of the microorganism – producer of amylolytic enzymes;

3. comparing and evaluating the effect of different growth media on a microbial culture's quantitative

biosynthetic activity in the expression of amylolytic enzyme genes.

Research materials and methods

For the experiments, we used cultures of microorganisms from the collection of the Department of Biochemistry, Microbiology, and Nutrition Physiology of ONAFT: *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *B. cereus* ATCC 10702, *B. cereus* UKM V 5650, *B. cereus* UKM V 5671, *Paenibacillus polymyxa* B 5760^T, *P. macerans* $B5803^T$, and *G. stearothermophilus* VKM-V-718. Also, we used strains of the bacilli P90-4, P90-9, P90-1 isolated from raw materials obtained from plants zoned in Ukraine and grown in the Odessa region [15]. According to the chromatography data on their fatty acid composition, these microorganisms were identified as *B.thuringiensis* and *B.subtilis*.

The amylolytic activity was determined by incubating the cultures of bacteria on MPA (meat peptone agar) with 0.2% of starch added. The presence of discoloured areas around and inside the growth zones after treatment with Lugol's solution indicated a positive test result [6].

To register bioluminescence, we used a special portable device – a luminometer *Lumitester PD-30* from Kikkoman (Japan). Its principle of operation is based on registration of light emission, the intensity of which is proportional to the amount of ATP (Fig. 1). This improved method, which allows determining both ATP and AMP, was used to analyse water and the microbial culture growth, and to control objects of the technological process. Sampling was performed using the *LuciPac Pen Aqua* and *LuciPac Pen* test systems supplied with the instrument. The *LuciPac Pen Aqua* test kit was used to analyse water and other liquid samples, and the *LuciPac Pen* was used to monitor the sanitary condition of equipment and surfaces at biotechnological manufactures [16].

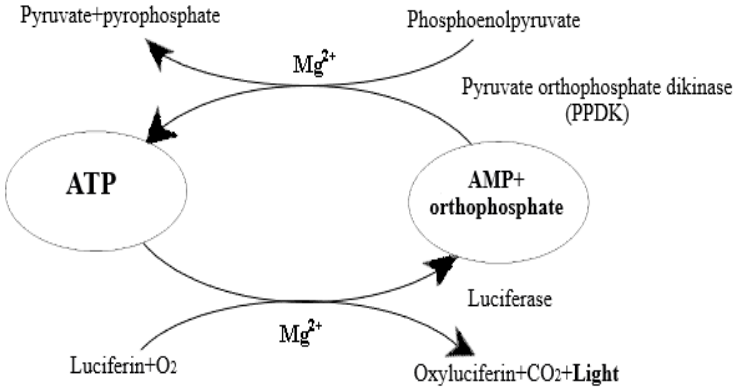


Fig. 1. Scheme of the bioluminescent ATP reaction

These kits contain reagents that ensure a stable bioluminescence reaction: luciferase, luciferin, magnesium acetate, buffer solution, and stabilisers. These kits are single-use and stored in a refrigerator at 2–8°C [16,17]. Bacillary microorganisms were cultured on a classic NB medium (nutrient broth) and on modified nutrient media with carbohydrate–protein components added: flours from wheat, soya bean, rye, and maize, and yeast autolysate. The growth media had the following composition:

– nutrient broth (PB): pancreatic hydrolysate of fishmeal – 0.8%, peptone – 0.8%, sodium chloride – 0.4%;

– medium MM-1: 1% of wheat flour, 2% of rye flour, 0.5% of yeast autolysate, 0.6% of minerals ($MgSO_4$ and $CaCO_3$);

– medium MM-2: 5% of NB, 2% of soya flour, 1% of oat flour, 0.5% of yeast autolysate, 0.6% of minerals;

– medium MM-3: 5% of NB, 1.5% of maize flour, 2% of soya flour, 0.5% of yeast autolysate, 0.6% of minerals; pH – 6.8–7.0.

The incubation was carried out at (30±1)°C during 12, 24, 36, and 48 hours. At each time interval, samples were selected, and microbial cells were quantified by the classic control method [6] and by the express bioluminescence method [17].

Results of the research and their discussion

It is known from the analytical review that for industrial use, amylolytic enzymes can be produced using bacillary microorganisms. So, we tested various species of bacilli for their ability to produce amylolytic enzymes (Table 1).

According to the results shown in Table 1, the culture of microorganisms *Bacillus subtilis* ATCC 6633 was chosen as the working culture. It has been found that this culture has the amylolytic activity accelerated by 12–24 hours and is not inferior to the microbial cultures described in the literature [18-20]. Besides, *B. subtilis* received the US Food and Drug Administration's designation GRAS (generally regarded as safe) as a safe microorganism [21,22].

To activate the gene expression system of hydrolytic enzymes of the amylolytic complex, the composition of the growth medium was modified. On the basis of the above analytical review, we used a classic NB medium and modified it by introducing various flours and a yeast autolysate. Fig. 2 allows evaluating the results of culturing *B.subtilis* ATCC 6633 on various growth media.

Table 1 – Amylolytic activity of microorganisms of the genus *Bacillus*

Species of microorganisms	Amylolytic activity during culturing (+) – present; (–) – absent; (±) – weak		
	24 h	36 h	48 h
Bacillus thuringiensis	–	–	–
Bacillus subtilis ATCC 6633	±	+	+
Bacillus subtilis P90-1	±	±	+
Bacillus subtilis P90-9	±	±	+
Bacillus thuringiensis P90-4	–	–	+
Paenibacillus polymyxa B 5760T	–	+	+
P. macerans B 5803T	–	±	+
Bacillus cereus ATCC 11778	–	±	±
B. cereus ATCC 10702	–	–	±
B. cereus UKM V 5650	–	–	–
B. cereus UKM V 5671	–	–	±
G.stearothermophilis VKM-V-718	–	–	±

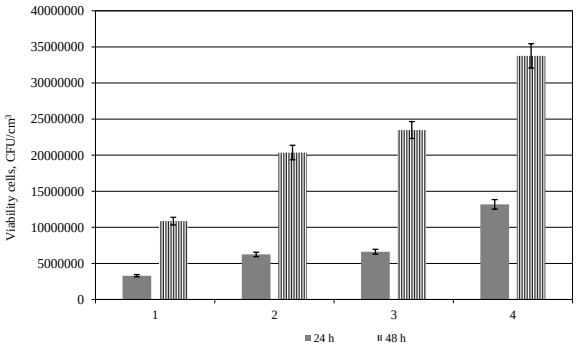


Fig. 2. Comparative bioproductive characteristics of the media by the growth of cultures *B. subtilis* ATCC 6633: *1* – nutrient broth (NB); *2* – medium MM-1; *3* – medium MM-2; *4* – medium MM-3

The results shown in Fig. 2 allow concluding that the preferred modified medium for culturing gram-positive bacilli *B.subtilis* ATCC 6633 is MM-3. Given the results obtained, it can be assumed that the biosynthetic productivity of the culture *B. subtilis* and hydrolase gene expression are induced by the presence of certain substrates. The components of the modified media contain factors that increase the number of microbial cells and regulate this expression.

Approaches to activating microbial synthesis by modifying the composition of growth media are basically known [19]. If the growth of microorganisms is suppressed or stimulated by the action of various substances, the concentration of ATP increases or decreases accordingly [23]. It should be noted that the introduction of additional components into the modified media increases the bioproductivity of the culture by 1.87 to 3.99 times in comparison with the classic medium taken as the reference. That is why for the technological process, it can be recommended to culture bacillary microorganisms using a modified growth medium, in particular, MM-3. The sanitary and hygienic level of the production has been controlled, and it has been checked how much the growth media used contributed to the RLU characteristic. The total ATP value consists of somatic ATP (animal and plant cells), microbial ATP (microbial cells) and free ATP. So, we have determined how much the components of the technological process (growth media, water, and cleanliness of the equipment) contributed to the bioluminescence index. The results of our research (n=3, $p \geq 0.95$) and the previously published data are given in Table 2.

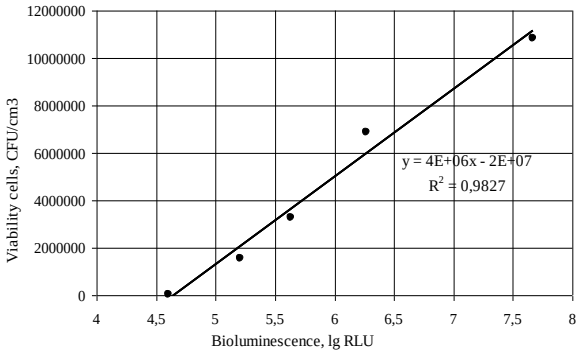
According to the results of the control, the objects of biotechnological manufacture (water and equipment) meet the requirements [17]. Biotechnological manufacture involves using growth media, but according to the RLU measurements, they contribute but insignificantly to the bioluminescence index when controlling the synthesis of microorganisms, as shown by our research results presented in Table 2.

Table 2 – Results of rapid ATP control of biotechnological and food manufactures

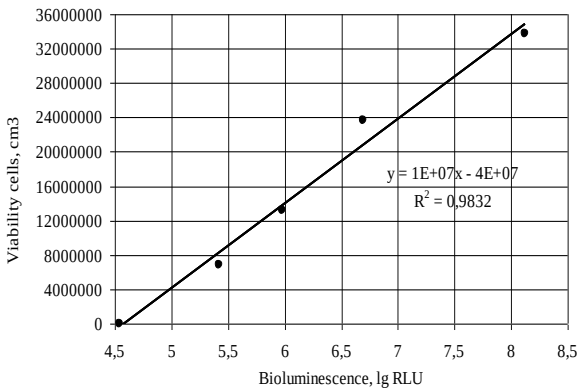
Object under control	RLU value		Source
	Satisfactory	Unsatisfactory	
Vegetable raw materials (vegetables/fruit)	500	1500	[12, 24]
Production of pasteurised milk/dairy products	70	200	[12, 24]
Ready-to-eat culinary products	500	1500	[16, 24]
Water for biotechnological manufacture	31	100	Own results, [25]
Sterile NB	2432	–	Own results
Sterile MM-1	1609	–	Own results
Sterile MM-2	2370	–	Own results
Sterile MM-3	2140	–	Own results
Technological equipment (metal surface)	175	500	Own results,[26]
Technological equipment (glass surface)	154	500	Own results, [26]

To control the technological process of biosynthesis in the microbial culture *B. subtilis*, it has been experimentally assessed how the number of microbial cells correlates with the readings of the ATP luminometer. For this, a calibration curve was built according to the results of the alternative method and the classic control method. Fig. 3 shows the correlation dependences between the results of the rapid and the classic control of the bioproductivity of the culture *B. subtilis* ATCC 6633.

As can be seen from Fig. 3, with an increasing number of cells of microorganisms, the intensity of bioluminescence increases proportionally, that is, there is linear dependence between the CFU and the logarithm of RLU. This dependence is described by equations with high values of the coefficient of determination R^2. The obtained results of mathematical description of culturing microorganisms (which description is based on the dependence of the number of amylolytic enzyme producers and on their bioluminescence index values) make it possible to recommend using rapid control in the biotechnology of manufacturing these amylolytic enzyme producers. This information was obtained by express-controlling the process of ATP accumulation while culturing the microorganisms. Unlike other classic and rapid methods of monitoring the process of culturing microorganisms, this allows rapid control and correction of the technological process.



a



b

Fig. 3. Graphical results of proprietary control of culturing *B. subtilis* ATCC 6633: *a* – on the NB medium; *b* – on the medium MM-3

Conclusion

It has been established that the biotechnological process of culturing bacillary microbial producers of amylolytic enzymes can be express-controlled by determining their ATP bioluminescence, and the advantages of the method have been shown. Based on the results of monitoring bacillary microorganisms, *B. subtilis* ATCC 6633 was selected as a potential producer of amylolytic enzymes. The compositions of growth media for culturing *B. subtilis* ATCC 6633 were tested and modified. It has been found that introducing carbohydrate–protein and mineral components into the growth media MM-1, MM-2, and MM-3 developed by us accelerates detection of the amylolytic activity by 12–24 h and increases the biosynthetic activity of the microbial culture by 1.87–3.99 times in comparison with culturing on the classic medium (NB).

Mathematical relations between the quantitative characteristics of the microbial culture growth and the bioluminescence values have been obtained. These relations are described by trends with high coefficients of determination. This proves the practical importance of using rapid bioluminescent control of sanitary conditions and rapid technological control of production of amylolytic enzymes by *B. subtilis* ATCC 6633 at biotechnological manufactures.

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САНІТАРНО-ТЕХНОЛОГІЧНИЙ ЕКСПРЕС-КОНТРОЛЬ КУЛЬТИВУВАННЯ ПРОДУЦЕНТІВ АМІЛОЛІТИЧНИХ ФЕРМЕНТІВ

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Анотація. Встановлено можливість і доцільність проведення експресного контролю біотехнологічного процесу культивування бацилярних мікробних продуцентів амілолітичних ферментів за визначенням показника їх АТФ-біолюмінесценції. Аналітичний огляд джерел продуцентів гідролітичних ферментів показав доцільність використання бацилярних мікроорганізмів для цілеспрямованого секретування ферментів амілолітичного комплексу в біотехнологічному виробництві. Проведений моніторинг бацилярних мікроорганізмів встановив, що за терміном продукування амілолітичних ферментів і показником біосинтетичної активності як робочу культуру доцільно обрати *Bacillus subtilis* АТСС 6633. Обгрунтовано використання експресного АТФ-контролю, який заснований на принципі біолюмінесценції. Для підвищення квантитативної біосинтетичної активності мікробної культури в технологічному процесі культивування бацилярних мікроорганізмів проведено порівняльну оцінку різних поживних середовищ. Експериментально доведено можливість модифікації поживних середовищ з введенням ряду вуглеводно-білкових субстратів як індукторів експресії генів амілолітичного комплексу, що виявляється прискореною на 12-24 години амілолітичною активністю та призводить до зростання кількості мікроорганізмів в 1,87-3,99 раза у порівнянні з контролем. Здійснено корелятивне визначення кількісного зростання клітин *B. subtilis* експресним біолюмінесцентним і класичним мікробіологічним методами контролю. Отримано математичні прямолінійні залежності кількості клітин бацилярного продуцента амілолітичного комплексу ферментів в напівлогарифмічній системі, які дозволяють проводити оперативний експресний контроль на виробництві. Поряд з традиційним санітарним експресним контролем

на біотехнологічному виробництві, який включає контролювання контамінації обладнання, рук робітників та води, запропоновано проводити пропрієтарний експресний технологічний контроль біосинтезу амілолітичних ферментів культурою мікроорганізмів *Bacillus subtilis* ATCC 6633.

Ключові слова: бацилярні мікроорганізми, *Bacillus subtilis*, АТФ (аденозинтрифосфат), біолюмінесценція, амілолітичний комплекс ферментів, санітарно-технологічний контроль.

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