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BROMOTHYMOL BLUE AS THE UNIVERSAL INDICATOR FOR DETERMINING THE STEREOMETRIC ALLOCATION OF PH AND EH IN THE MEDIUM IN HETEROPHASE MICROORGANISMS CULTIVATION

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Purpose. *The purpose of this research was the investigation of the possibility of using bromothymol blue as a RedOx indicator and the measuring of RedOx potential value of bromothymol blue decolouring.* ***Methods****. The research was run using conventional microbiological, potentiometrical, physical and chemical methods.* ***Results.*** *It is shown for the first time, that bromothymol blue is a multipurpose indicator for the determination of pH and RedOx potential (Eh) of the medium during the heterophase solid substrate fermentation by microorganisms. The bromothymol blue solution provides the visual control over the pH value dynamics in the range of 4,5 – 7,5 and Eh ≥ –200 mV (at pH 7,0).* ***Conclusions.*** *Application of bromothymol blue in the heterophase fermentation of solid substrate allows visualising of the zones with altered pH and Eh, their stereometric distribution and changing configuration in time. The ability of bromothymol blue to be a RedOx indicator as well as pH indicator expands the opportunity of its use in microbiological and biotechnological research.*

Keywords: bromothymol blue, Eh indicator, heterophase cultivation.

The effectiveness of a biotechnology substantially depends on the direction and activeness of microbial metabolism. The regularities of microbial metabolism and its regulation determine the quantitative and qualitative characteristics of the end products obtained. Values of pH and oxidation-reduction potential (Eh) of the medium are both important physical-chemical parameters that are determining the direction of microbial metabolic process.

The measurement of pH and Eh is performed using electrometric (potentiometric) or colorimetric method (application of water-soluble dyes and indicators). The electrometric method is widely used in microbiology and biotechnology researches, although it has a range of significant imperfections. These include the duration of the Eh measuring process (no less than 10 min), complicacy and low reliance of electrode sterilization. The complicated and long-termed operations frequently make the simultaneous pH and Eh measuring in several experimental variations impossible.

Let us consider the peculiarities of potentiometry. There are two variations of potentiometric method pH and Eh measuring. In the first variation all three electrodes (for measurement of pH, Eh and the reference electrode) are hermetically embedded into the cultivator. In the second method the sample of liquid culture is taken from the hermetic cultivator through the rubber tip by a syringe with needle and is translocated into the measuring cell or flask

with the electrodes. Both these methods have the imperfections, which make the measuring of pH and Eh impossible or very difficult. When using the first method the surface of the hermetically embedded electrodes become covered with a thick layer of microorganisms (biofilm) in 8 – 10 hours. This leads to alterations of the measuring results. Besides, the saturated solution of KCl is slowly diffusing into the medium from the reference electrode certainly inhibiting microbial growth. Finally, serious difficulties may occur in the process of electrode sterilization.

Using the second variation causes another problem. During the growth of aerobic, facultative anaerobic and strictly anaerobic microorganisms in the hermetic cultivator, Eh of the medium decreases from +300…+400 mV to –150…–380 mV. In the process of translocation of the low-potential culture liquid to the opened for the air measuring cylinder (or flask) the Eh indicator is inevitably altered. Culture liquid is partially oxidized by the air oxygen. Even overfilling the cylinder with argon does not provide the reliable protection of the culture liquid from oxygen.

The main imperfection of the potentiometric method, though, is the principal impossibility of the simultaneous measurement of the stereometric distribution of pH and Eh in the whole volume of the medium. The stereometric distribution of Eh and pH is of great importance in the investigations of heterophase microbial processes. The example of such a process could be the microbial destruction of solid polymeric compounds (meat proteins, cellulose, starch, etc.) in the liquid medium. Under the heterophase conditions pH and Eh values in the liquid phase (culture medium) and on the solid phase surface (insoluble source of carbon and energy, biofilm on the inert carrier, etc.) are significantly different. The same goes for the distribution in the liquid medium of different oxygen-tolerant microorganisms. In the subsurface of the medium, that is in contact with the air oxygen, mainly aerobic microorganisms will develop. In the middle part facultative anaerobes will prevail. In the lower part, under the lack of oxygen, strict anaerobes will grow. Thus, in the medium of one cultivator three zones of different metabolic activities and pH as well as Eh values are formed. Under this stereometric gradient the values of Eh and pH in all three zones will be significantly different.

The stereometric distribution of Eh and pH can be accurately determined by the colorimetric method, using indicator dyes. The pH indicators have been widely used in microbiology studies since the end of XIX century [1; 2; 3]. Starting from first half of XX century dyes as RedOx-indicators also are commonly used in biology [4]. Both types of indicators change their colour (or decolourise) under some specific for each of them values of pH or Eh. But for the possibility of their application in the microbiological research it is essential that the indicators should not be toxic in the visually detectable concentrations, allow the simultaneous measurement of pH and Eh in the particular value range. For pH this range is from 4 to 9, and for Eh from +100 to –300 mV.

The application of water-soluble indicators is the alternative method of measuring the pH and Eh values in the heterophase medium. Liquid indicator is suitable for the investigation of heterophase cultivation because it is spread in the whole medium volume and makes possible the local visual measurement of pH and Eh.

However, the simultaneous applying of two indicators (Eh and pH) is impossible. In fact, they form a "colour mixture" in the solution (culture medium), where it is impossible to detect the changes of Eh and pH using these indicators.

Thus, the determination of the stereometric gradient of pH and Eh required the multifunctional indicator, which could display the values of both these characteristics.

Therefore, the purpose of our research was the investigation of the non-toxic water-soluble indicator that was allowing simultaneous measuring of pH and Eh in the range of the values formed by heterotrophic microorganisms in the process of polymeric substrate (potatoes in this case) fermentation. We suggest bromothymol blue (BTB) as such an indicator.

In our previous research [5], when investigating the hydrogen-producing fermentation of potatoes by the *Bacillus* and *Clostridium* microbial community, we used BTB for measuring pH. In the visually detectable concentrations of BTB (0.75 ml water solution of BTB, saturated under 20°C, for 1 l of the medium) does not inhibit microbial growth. The possibility of BTB application in low concentrations is due to its high sensibility to pH changes [4; 6; 7; 8]. As the pH indicator, BTB is being used for the indication of growth of some physiological groups of microorganisms for more than hundred years. [1; 4].

The bespoke advantage of BTB, compared to other indicators, is its ability to measure pH accurately in a wide range of values. Thus, it is yellow at pH = 6,2 (and lower), green at pH = 7,0 and blue at pH = 7,1 (and higher).

That was the reason why we used BTB while investigating the regularities of potato anaerobic fermentation by the *Bacillus* and *Clostridium* community.

However, there is no data on the possibility of using BTB as RedOx indicator in literature sources. On the other hand, it is known that most pH-indicators at the specific RedOx potential values are able to form leicoform [9] that is they also can be RedOx indicators. Therefore, the purpose of our investigation was the discovery of RedOx potential value of BTB decolouring and the possibility of using the latter as a RedOx indicator.

Materials and methods. For the direct measurement of RedOx value of BTB leycoform formation we used a strong reductant – Titanium citrate, that can reduce RedOx potential of solutions (including culture medium) to –400 mV and lower [10].

BTB preparation. To obtain the visually detectable colour of the culture medium we added to it the saturated water solution of BTB in the ratio of 0,75 ml for 1 l of the medium. The saturated BTB solution was obtained by dissolving of the excessive amount of BTB in the 60°C warm distilled water with further cooling the latter to the temperature of 20°C.

Reducing agent preparation. We used the sterile solution of Ti(III) citrate after Zehnder in Tashyrev's modification for the estimation of the Eh value that causes BTB to decolorize [11; 12]. Ti(III) citrate has Eh = –600…–680 mV, and pH – 7,0 [12].

Estimating value of the RedOx potential of BTB decolourization. For the determination of the Eh of the decolourisation of BTB it was reduced in the distilled water solution by adding Ti(III) citrate. Distilled water (40 ml) was poured into the beaker (50 ml) and then the saturated BTB solution was added

until the visually detectable yellowish-green colour appeared. The solution characteristics were as follows: pH 6,49 and Eh +390 mV. The beaker was fixed in the tripod for further measuring of pH and Eh. The upper part of the beaker over the solution surface was under a flow of sterile argon.

The reducing agent was added in drops to gradually lower the RedOx potential value due to the increase of the reducing agent's concentration in the medium. Ti(III) citrate was added to the solution until the moment of full decolouration of BTB in it. The presence of electrodes allowed tracing the RedOx potential value accurately.

RedOx potential (Eh) and pH of the medium were measured by pH-meter-milivoltmeter "pH-150 MA" using three electrodes. For Eh measurement we used platinum electrode "ЭПВ-1" matching with chlorine-silver flowing comparison electrode "ЭВЛ-1М3", and for pH measurement – glass electrode "ЭСК-10603/4". Before the measuring the electrodes were checked by convention buffer solutions. For pH check conventional pH buffer solutions were used: solution of KH_2PO_4 (pH=1,68); NaH_2PO_4 (pH = 6,86); $Na_2B_4O_7 \cdot 10H_2O$ (pH = 9,18). Conventional pH buffers were prepared according to the producer's manual (OJSC "Kiev plant RIAP"). For the Eh measuring integrity check three buffer solutions were used. First – ferrycyanidic, with Eh = + 273 mV (13,5 g/l $K_3[Fe(CN)_6]$ and 3,8 g/l $K_4[Fe(CN)_6] \cdot 3H_2O$), second – Fe(II) citrate (10 g Fe(II) in 1 litre) with Eh = – 150 mV [13] and third – Ti(III) citrate 1,5% with Eh = – 440 mV [12].

Heterophase cultivation. For determining of the stereometric gradient of Eh and pH using BTB under the conditions of heterophase cultivation we used the fermentation of the chopped potatoes by the soil spore-forming microorganism community (*Bacillus* spp. and *Clostridium* spp.). In 250 ml bottles we inserted 50 g of potatoes (cubes measuring 1 cm^3) and 1 ml of inoculum. Substrate and inoculum were preheated by 100°C for 5 minutes. This measure is used to eliminate the non-desirable physiological groups of microorganisms that can compete with clostridia for sources of carbon and energy or reduce the effectiveness of hydrogen synthesis. These could be denitrificating ($H_2 + NO_3^- \rightarrow$ $\rightarrow 2H^+ + N_2$) or methanogenic ($H_2 + CO_2 \rightarrow CH_4 + H_2O$) bacteria. The ratio of the volumes of gas and liquid phases was 1: 1. The inoculum consisted of the community of spore-forming microorganisms of aerobic *Bacillus* and anaerobic *Clostridium* genera. Spore-forming bacteria were chosen because they are widely spread in the environment, and are resistant to adverse factors through the formation of endospores. They are easy to be cultivated in vitro, fermenting starch.

The expediency of using the mentioned microbial community lies in the following: aerobic bacilli begin hydrolyzing starch in potato and reduce RedOx potential in the process of their growth to the value levels of –270 …–300 mV that is optimal for the obligatory anaerobic clostridia growth. Furthermore, bacilli hydrolyze starch to hydrocarbons, which are further fermented by clostridia. So anaerobic conditions created by bacilli are optimal for hydrogen synthesis by clostridia in the process of starch hydrolysis.

Thus, this microbial community decreases the RedOx potential of the medium for hundreds of millivolts. It was shown earlier, that in the process of potatoes fermentation the medium parameters alter in a very vide range (pH from 7,8 to 3,5, and Eh from +470 to –270 mV) [5].

The substrate and inoculum were preboiled during 5 minutes. This averted the appearing of the non-spore-forming microorganisms, which could compete with hydrogen producers for the substrate. Cultivation was taking place under 21°C

The alterations in indicator colour were determined visually. Based on the obtained data the spatial distribution of pH and Eh values in the process of heterophase cultivation were determined.

Flow cultivation. The flow cultivator was presented by the system of the successively connected hermetic boxes (Fig. 1). The fermentation of potato by *Bacillus* and *Clostridium* community was taking place in every box. The emitted gas from every box was going to a gas holding system, which allowed its volume and content to be measured. The directed flow of the culture medium was going through all the boxes. The flow was supplied by the input of sterile water into the first box and the drainage of the excessive medium from the last box. To use BTB as the indicator its solution was added to the water before its inlet to the first box. Thus, the indicator was getting into all boxes and was spreading in the liquid phase.

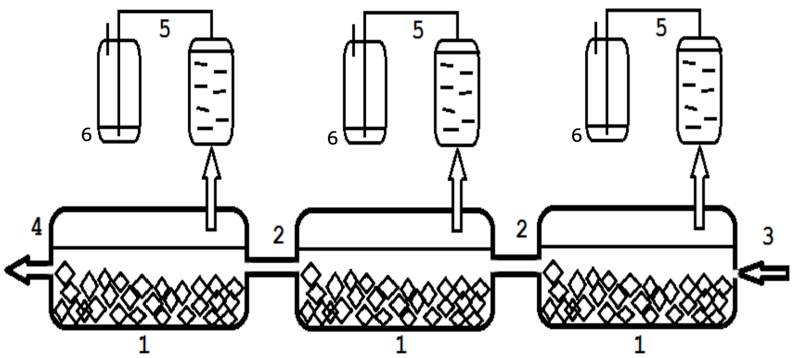

Fig. 1. Scheme of the flow system cultivator: 1- hermetic successively connected boxes (with potato and microorganisms); 2 – joining pipes; 3,4 – inlet and exhaust system pipes; 5 – gas holding system, 6 – water seal

Results. We found that the RedOx potential of the formation of BTB leicoform (discolouration) is –200 mV (pH = 7.0)

In the process of reduction by Ti(III) the decolouration of BTB began under Eh = – 190 mV (Fig. 2). Under Eh = – 200 mV the BTB solution was completely colourless. Colour remained only in the thin (3 mm) subsurface layer that can be explained by the high RedOx value in the zone due to oxidizing of the solution by trace air oxygen in the almost complete argon atmosphere. The colour of the indicator retrieved after increasing RedOx potential, thus the process was reversible.

The results of using of BTB for visual control of Eh distribution in the culture medium are displayed on Fig. 3. As it is shown during the first 9 hours of cultivation, as the result of acid hydrolysis of potato starch, the value of pH decreased, which was accompanied by BTB colour change from green (pH ≈ 7) to yellowish-green (pH ≈ 6,5).

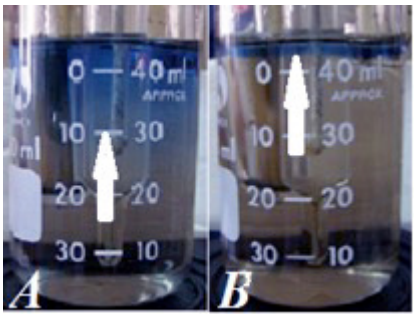

Fig. 2. Decolouration of bromothymol blue (BTB) at Eh = –200 mV.
A – decolouration starts, Eh = –190 mV,
B – complete decolouration, Eh = –200 mV

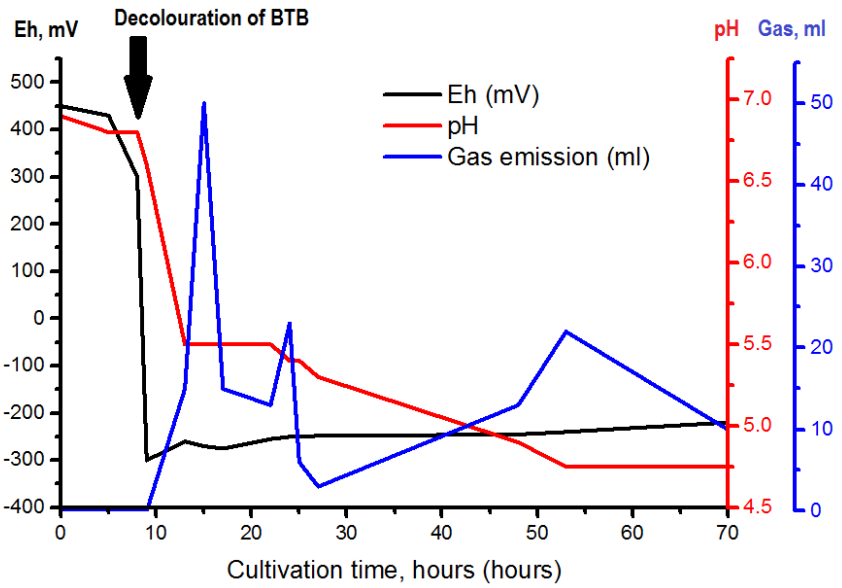

Fig. 3. The dynamics of pH, Eh and gas emission in the process of potato fermentation by *Bacillus* and *Clostridium* community. The arrow indicates the moment of BTB decolouration in the medium.

At ninth hour of cultivation the RedOx potential decreased from the initial +460 mV to –315 mV. Due to this the decolouration of BTB was observed. BTB is known as a very stable compound. In fact, it consists of aromatic rings that cannot be used as the carbon source for the microorganisms. So, the discolouration of BTB can be explained only by its leicoform formation under the RedOx potential lower than –200 mV.

The microorganisms of the *Bacillus* and *Clostridium* genera, which were in the community, are not able to use BTB as a substrate. The chemical reduction of BTB by soluble products in the medium (alcohols, organic acids, sugars) is also impossible.

To the pH change BTB is responding by colour change but not by decolouring. Thus we have discovered that coloured BTB was transformed into its colourless leucoform at the low values of RedOx potential, Eh = = –200 mV (pH = 7,0).

When BTB was added to the heterophase medium, during potato fermentation the alteration of its colour from light blue (pH = 7,3) to green (pH = 6,8) was observed. On the ninth hour of cultivation the decolouration of BTB solution was observed. This coincide in time with the start of hydrogen-producing fermentation by *Clostridia* at Eh lower than –200 mV. After some time the combustion of acid metabolites on the potato pieces had started. In these zones the indicator was oxidized to the coloured form and got bright yellow colour, that indicated the decrease of pH value (pH≤5,0) and increase of Eh (Eh > – 200 mV). As shown on Fig. 4, the application of the indicator allowed spotting the acidification and simultaneously high potential zones. On Fig. 4 the parts of cultivation boxes in the different metabolic periods are shown, that is:

1) The fermentation of potato cubes before adding the indicator;

2) BTB, added in the active fermentation phase gets dark olive colour (pH ≈ 7) and gets decoloured in five minutes (Eh < – 200 mV);

3) As the result of microbial metabolism the acidification of the medium takes place – the fermentation is slowing down – the zones of yellow coloured BTB appear;

4) pH of the medium gets lower 5,0, fermentation stops. On the surface of potato cubes the acid metabolites are combusted (bright yellow BTB colour).

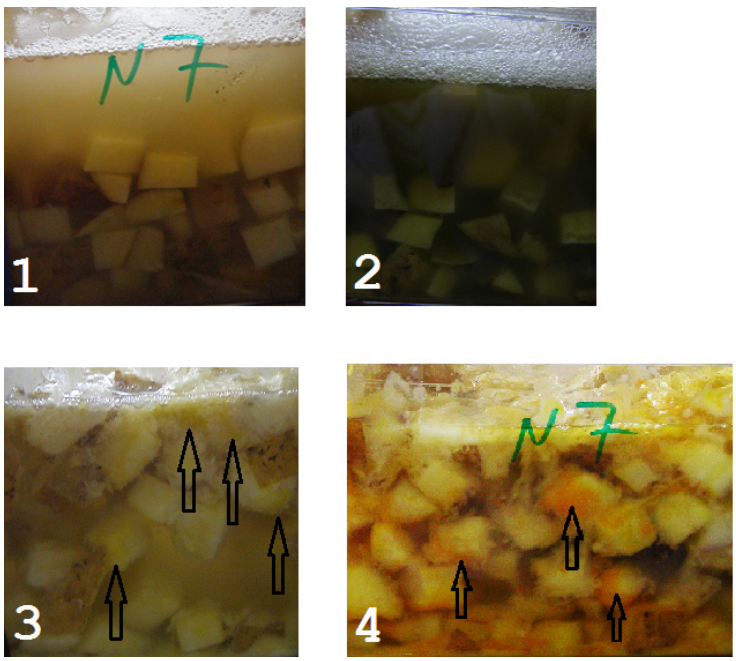

Fig. 4. Discovering of zones of the acid microbial metabolites combustion due to bright yellow BTB colour appearance (pH≤ 5,0)
Note: arrows on photos 3 and 4 show the most contrast acidification zones.

It is clear, that the acid metabolites combustion takes place on the solid substrate surface, which is indicated by the appearance of the bright yellow colour there. At the same time in the culture liquid itself Eh value remained lower than –200 mV, which was indicated by the colour of the indicator.

It is obvious, that the culture liquid pH value remained on the initial level, while it was significantly lover on the substrate surface. Application of the potentiometric method in this case would have given an invalid result, as it would register only Eh of the liquid.

Using of indicator relieves us from the necessity to perform frequent measurements of Eh and pH. The appearance of yellow colour of BTB in the cultivator was the signal of local strong decreasing of pH and accordingly the necessity of immediate compensation of this negative process. The reduction of hydrogen synthesis by microorganisms and stopping of starch destruction is the evidence to the negative effect of the local acidification on the surface of potato bits. For instance starting from the third day of cultivation the activity of hydrogen producing was sharply dropping to zero level, the pH value measured at the same time was 5.0 – 5.5. If not regulated at that level, the potato bits stopped being decomposed. Obviously for avoiding of the negative local acidification the adding of alkaline compounds in order to increase pH value is needed. For this purpose, we added saturated sodium hydrocarbonate solution and tightly mixed culture liquid. The decolouration of BTB after pH regulation indicated the renovation of optimal medium characteristics, i.e. pH close to 7,0 and Eh close to –200 mV. The decrease of Eh to –200 mV indicated on active starch fermentation by *Clostridium*.

Thus, application of BTB allows us quickly determining the necessity of applying to any particular method of metabolic characteristics (Eh and pH) regulation.

Using BTB in the direct-flow system allowed us also optimizing the position of inlet and outlet pipes and the flow speed. The alkaline solution was added through the inlet pipe into the flow system, filled with acidic solution with BTB. The flow was going in the direction from the inlet pipe. As shown on Fig. 5, when the inlet and outlet pipes were situated on the same level, the necessary mixing did not take place. The distribution of the blue coloured BTB indicator demonstrates that in this case the metabolism regulator (added the same way) is not spread through all volume of the box. Under such conditions

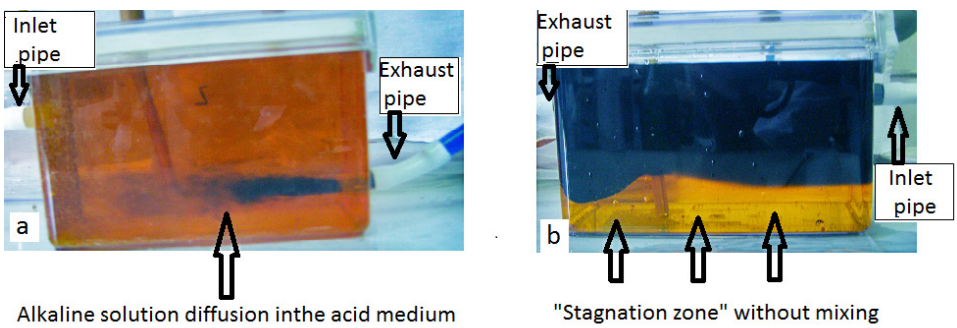

Fig. 5. Visualization of different pH zones distribution while adding alkaline solution in the flow system and positioning of the flow using BTB
a. beginning of alkaline solution diffusion in the yellow acidic solution. The spreading of the liquid (blue coloured) incoming through the pipe is visible;
b. the visualisation of 'stagnation zone' (bright yellow colour, low pH), which is inaccessible for the added alkaline solution when the inlet and outlet pipes are located at the same vertical levels.

the regulation of pH is ineffective. Application of BTB allowed determining the importance and effectiveness of mixing in the cultivation process. Thanks to mixing the complete destruction of the hydrolysed potato cubes and the relieving the excess of metabolites from microbial activity zone was performed.

Discussion. As the result of the investigation it is shown for the first time, that bromothymol blue (BTB) is a multipurpose indicator for the determination of physical and chemical parameters (pH, Eh) during the heterophase solid substrate fermentation by microorganisms.

The BTB solution provides the visual control over the pH value dynamics in the range of 4,5 – 7,5 and Eh ≥ –200 мВ (at pH 7,0). Application of BTB in the heterophase fermentation of solid substrate allows visualising of the zones with altered pH and Eh, their stereometric distribution and changing configuration in time.

Application of BTB as the Eh and pH indicator allows optimization of the process of developing new biotechnologies with use of heterophase cultivating conditions.

The ability of bromothymol blue to be a RedOx indicator as well as pH indicator expands the opportunity of its use in microbiological and biothechnological research.

БРОМТИМОЛОВИЙ СИНІЙ ЯК УНІВЕРСАЛЬНИЙ ІНДИКАТОР ДЛЯ ВИЗНАЧЕННЯ СТЕРЕОМЕТРИЧНОГО РОЗПОДІЛУ РН І ЕН В СЕРЕДОВИЩІ ПРИ ГЕТЕРОФАЗНОМУ КУЛЬТИВУВАННІ МІКРООРГАНІЗМІВ.

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Резюме

Мета. Метою дослідження було вивчення можливості використання бромтимолового синього як редокс-індикатора і визначення значення редокс-потенціалу, при якому настає його знебарвлення. **Методи**. Роботу було проведено з використанням стандартних мікробіологічних, потенціометричних та фізико-хімічних методів. **Результати.** Вперше показано, що бромтимоловий синій є універсальним індикатором для визначення pH і редокс-потенціалу (Eh) під час гетерофазного зброджування твердого субстрату мікроорганізмами. Використання розчину бромтимолового синього дозволяє візуально контролювати динаміку значення pH у діапазоні від 4,5 до 7,5 і Eh ≥ –200 мВ (при pH 7,0). **Висновки.** Використання бромтимолового синього у гетерофазному зброджуванні твердого субстрату дозволяє візуалізувати зони зі зміненим pH та Eh, їх стереометричне положення і зміну конфігурації в часі. Дана властивість індикатора дозволяє розширити сферу його використання у мікробіологічних та біотехнологічних дослідженнях.

Ключові слова: бромтимоловий синій, Eh-індикатор, гетерофазне культивування.

БРОМТИМОЛОВЫЙ СИНИЙ КАК УНИВЕРСАЛЬНЫЙ ИНДИКАТОР ДЛЯ ОПРЕДЕЛЕНИЯ СТЕРЕОМЕТРИЧЕСКОГО РАСПРЕДЕЛЕНИЯ РН И ЕН В СРЕДЕ ПРИ ГЕТЕРОФАЗНОМ КУЛЬТИВИРОВАНИИ МИКРООРГАНИЗМОВ.

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Резюме

Цель. Целью исследования было изучение возможности использования бромтимолового синего как редокс-индикатора и определения значения редокс-потенциала, при котором наступает его обесцвечивание. **Методы**. Работа проводилась с использованием стандартных микробиологических, потенциометрических и физико-химических методов. **Результаты.** Впервые показано, что бромтимоловый синий является универсальным индикатором для определения pH и редокс-потенциала (Eh) во время гетерофазного сбраживания твердого субстрата микроорганизмами. Использование раствора бромтимолового синего позволяет визуально контролировать динамику значения pH в диапазоне от 4,5 до 7,5 и Eh ≥ –200 мВ (при pH 7,0). **Выводы.** Использование бромтимолового синего в гетерофазном сбраживании твердого субстрата позволяет визуализировать зоны с измененным pH и Eh, их стереометрическое положение и изменение конфигурации во времени. Данное свойство индикатора позволяет расширить сферу его использования в микробиологических и биотехнологических исследованиях.

Ключевые слова: бромтимоловый синий, Eh-индикатор, гетерофазное культивирование.

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