Enzyme and Microbial Technology 98 (2017) 43–51

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Use of one- and two-mediator systems for developing a BOD biosensor based on the yeast *Debaryomyces hansenii*

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ARTICLE INFO

Article history:
Received 8 July 2016
Received in revised form
23 December 2016
Accepted 23 December 2016
Available online 26 December 2016

Keywords:
Biosensor
BOD (biochemical oxygen demand)
BOD_5
Mediator
Yeast *Debaryomyces hansenii*

ABSTRACT

We investigated the use of one- and two-mediator systems in amperometric BOD biosensors (BOD, biochemical oxygen demand) based on the yeast *Debaryomyces hansenii*. Screening of nine mediators potentially capable of electron transfer – ferrocene, 1,1′-dimethylferrocene, ferrocenecarboxaldehyde, ferroceneacetonitrile, neutral red, 2,6-dichlorophenolindophenol, thionine, methylene blue and potassium ferricyanide – showed only ferrocene and neutral red to be efficient electron carriers for the eukaryotes studied. Two-mediator systems based on combinations of the investigated compounds were used to increase the efficiency of electron transfer. The developed two-mediator biosensors exceeded their one-mediator analogs by their characteristics. The most preferable two-mediator system for developing a BOD biosensor was a ferrocene–methylene blue combination that ensured a satisfactory long-time stability (43 days), selectivity, sensitivity (the lower limit of the determined BOD_5 concentrations, 2.5 mg O_2/dm^3) and speed (assay time for one sample, not greater than 10 min) of BOD determination. Analysis of water samples showed that the use of a ferrocene–methylene blue two-mediator system and the yeast *D. hansenii* enabled registration of data that highly correlated with the results of the standard method (R = 0.9913).

1. Introduction

Biochemical oxygen demand (BOD) is a main index characterizing the pollution of water objects by organic substances. By definition, BOD is the amount of oxygen consumed for aerobic biochemical oxidation within a registered period of time (usually 5 days, BOD_5) under the action of microorganisms occurring in the investigated sample. The standard BOD determination method specified in the international standards ISO5815-1:2003 [1] and ISO5815-2:2003 [2] is based on measuring the difference in the content of dissolved oxygen before and after the incubation under conditions set by the regulatory documents. The main drawback of the method is assay duration (5 days), which does not make it possible to assess the quality of wastewater purification quickly and efficiently. Besides, the produced results of interlaboratory studies may differ by more than 20% due to the different composition of activated sludge used in the BOD assay [3]. Demand for the BOD assay contributed to the emergence of alternative rapid techniques, in particular, to the development of biosensor analyzers based on the use of microorganisms metabolizing a broad range of organic compounds [4]. A distinction of this technique from the standard method is the assay time reduced from 5 days to 10 min.

There are two major approaches to the development of amperometric BOD biosensors. The first is based on the use of Clark's oxygen electrode registering the speed of oxygen consumption by microorganisms, which is equivalent to the amount of analyzed substrate [5,6]. This approach has a number of drawbacks, one of which is that the results of the biochemical oxygen demand measurement by Clark's electrode are subjected the most to the effect of diffusive exchange flows of oxygen between the assayed sample and the atmosphere. The approach assumes the use of low molecular weight compounds, electron transport mediators, that transfer electrons between microbial cells and the electrode. The mediator facilitates the thermodynamically advantageous but spatially and/or kinetically hindered electron transfer. Herewith, the redox potential value of the mediator used should provide for the required gradient to transfer the electron between the enzymes' and cells' redox centre and the electrode [7]. Thus, for oxidative bioelectrocatalysis the mediator's standard redox potential should be higher than the standard redox potential of the enzyme's active centre.

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http://dx.doi.org/10.1016/j.enzmictec.2016.12.005
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For this reason, amperometric measurements are conducted at a potential equal to mediator's redox potential [8]. The possibility of operation at lower potentials enables reducing the effect of fluctuations of dissolved oxygen and electroactive impurities on the assay.

An important stage in the development of a BOD biosensor is the choice of biomaterial, which should oxidize a broad range of organic compounds so that the produced data correlate with the results of the standard method. Bioreceptor elements of mediator BOD biosensors are often fabricated from prokaryotic cells [9–14]. This is due to the membrane localization of enzyme systems, which facilitates their interaction with electron transport mediators [15]. Yeast cells are used comparatively rarely [13,16]. One of the reasons for the rare use of yeasts in biosensors is, apparently, due to the fact that the mediator transfer of electrons from eukaryotic cells to the electrode is made difficult [16]. However, in contrast to bacteria, yeasts are capable of oxidizing a broader spectrum of organic compounds and are stable to the action of various toxicants and negative environmental factors [17]. In [16], the authors considered the possibility of developing a mediator BOD biosensor based on ten mediators and *Candida* yeasts. Of ten mediators, electron transfer was registered only in the presence of potassium ferricyanide (potassium hexacyanoferrate (III)) and hydroxyferrocene. The complexity of developing yeast mediator biosensors is associated with the structure of the eukaryotes – the enzyme systems in yeast cells are often localized inside the microorganism (in the cytoplasm or in organelles), which makes difficult their interaction with electron transfer mediators. Still, the use of yeast cells for developing biosensors for the determination of integral indices, such as BOD, is sufficiently promising.

A two-mediator system can be used for efficient transfer of electrons from eukaryotic cells to the electrode. Thus, for instance, Nakamura et al. [18] developed an analyzer based on the yeast *Saccharomyces cerevisiae* and the two-mediator system of potassium hexacyanoferrate (III) and vitamin K3 (menadione, 2-methyl-1,4-naphthoquinone). Menadione was chosen because it is capable of interacting with NADH, as well as with elements of the electron transfer respiratory chain [19], and ferricyanide was taken based on the data of [16] as the most efficient hydrophilic mediator. The biosensor enabled BOD assessments within the limits of 6.6–220 mg O_2/dm^3. The two-mediator system of vitamin K3–hydrogen peroxide was used for developing an optical BOD biosensor based on the yeast *S. cerevisiae* [20]. In the presence of biodegradable compounds vitamin K3 is restored to vitamin K4. Inverse oxidation of vitamin K4 occurs under the action of hydrogen peroxide, which reacts with luminol and hydroxide anions, as the result of which a chemiluminescence is registered. The biosensor made it possible to assess BOD within the limits of 11–220 mg O_2/dm^3 [20]. However, the described biosensors possess a low sensitivity and do not enable BOD determination in pure surface waters, where BOD is a value of about 2 mg O_2/dm^3.

Our earlier studies have shown a BOD biosensor based on the oxygen electrode and the yeast *D. hansenii* to feature a high long-time stability and sensitivity [5,6]. Besides, the yeast *D. hansenii* is a promising biocatalyst in the BOD biosensor due to its halo-, osmo- and cryotolerance [21,22], as well as stability with respect to heavy metals [6]. The aim of this work was to investigate the possibility of using *D. hansenii* yeast cells in combination with the amperometric graphite paste electrode for developing highly stable and sensitive one- and two-mediator BOD biosensors.

2. Materials and methods

2.1. Reagents and materials

D-glucose (Panreac, Spain), peptone (Condra, Spain), yeast extract (Helicon, Russia) were used to grow the yeast *D. hansenii*. Graphite powder (Fluka, Germany), paraffin oil (Fluka, Germany), dialysis membrane (Roth, Germany; molecular weight cutoff, 14 kDa) were used to fabricate the working graphite paste electrode.

As electron transfer mediators, we used ferrocene (Aldrich, Germany), 1,1′-dimethylferrocene (Aldrich, Germany), ferrocenecarboxaldehyde (Aldrich, Germany), ferroceneacetonitrile (Aldrich, Germany), neutral red (Diaem, Russia), 2.6-dichlorophenolindophenol (Diaem, Russia), thionine (Diaem, Russia), methylene blue (Diaem, Russia), potassium ferricyanide (Diaem, Russia).

2.2. Cultivation of microbial cells

Cells of strain *D. hansenii* VKM Y-2482 were obtained from the All-Russian Collection of Microorganisms, IBPhM RAS (Pushchino, Moscow Region). The yeast was grown on a rich mineral medium (liquid glucose–peptone nutrient medium). The composition of the liquid medium was as follows: glucose, 10 g/dm^3; peptone, 5 g/dm^3; yeast extract, 0.5 g/dm^3 (Sigma, USA). The medium for cell growth was sterilized by autoclaving at a pressure of 1 atm and temperature of 121 °C for 45 min. Cells were grown aerobically for 18–20 h in 750-cm^3 shake flasks at a temperature of 29 °C. The produced biomass was centrifuged at room temperature for 10 min (10,000 rpm). The centrifugate was washed with a 20-mM phosphate buffer, pH 6.8. Sedimented cells were transferred to fresh portions of buffer, distributed by portions and sedimented at an Eppendorf centrifuge for 5 min at 10,000 rpm. The washed biomass was weighed and stored in microtubes at a temperature of 25 °C.

2.3. Formation of the working electrode

The working electrode was formed according to the technique described in [23] by filling a plastic tube (working surface area, 6.3 mm^2) with prepared graphite powder–mineral oil paste. Electrodes based on slightly soluble mediators (ferrocene, 1,1′-dimethylferrocene, ferrocenecarboxaldehyde, ferroceneacetonitrile, 2,5-dibromobenzoquinone) were formed as follows: 100 mg graphite powder was mixed with 500 μl of 1% solution of a mediator in acetone necessary to produce the required concentration of mediator. After the evaporation of acetone, 40 μl of paraffin oil was added and the paste was mixed. The plastic tube of the measuring electrode was filled with this modified paste. To form electrodes based on soluble mediators (thionine, neutral red, methylene blue, 2.6-dichlorophenolindophenol, potassium ferricyanide), we prepared a non-modified paste (100 mg graphite powder mixed with 40 μl paraffin oil) and also filled with it the plastic tube of the measuring electrode.

D. hansenii cells were immobilized on the electrode surface as follows: 3 μl of *D. hansenii* suspension (cell content, 200 mg wet weight/ml) was applied to the working surface of the electrode and dried at room temperature for 15 min. To retain cells on the electrode surface, we used a dialysis membrane fixed by a plastic ring.

2.4. Registration of current–voltage dependences

Cyclic voltammograms were registered by an IPC-micro galvanopotentiostat (Volta Co. Ltd, Russia) using the three-electrode circuit. A graphite paste electrode with immobilized *D. hansenii* cells served as the working electrode; a platinum electrode, as the auxiliary electrode. A silver/silver chloride (Ag/AgCl) electrode with saturated potassium chloride electrolyte was used as the reference electrode with respect to which all values of potentials are given. The cyclic voltammograms were registered at a scan rate of

Table 1
Working potentials in mediator biosensors.

Mediator	Working potential, mV	
	Experiment	Literature data
Ferrocene	250	315 [26], 280 [23]
1,1′-Dimethylferrocene	409	200 [26], 220 [23]
Ferrocenecarboxaldehyde	601	450 [26], 400 [23]
Ferroceneacetonitrile	338	210 [27]
Neutral red	335	−500 [28], −350 [29]
2,6-Dichlorophenolindophenol	416	100 [30], 217 [31]
Thionine	829	64 [31], −200 [32]
Methylene blue	401	−300 [32], 11 [30]
Potassium ferricyanide	288	600 [9], 400 [10], 450 [33]

10 mV/s in a 0.15-M potassium sodium phosphate buffer, pH 6.8, at a temperature of 22 °C. The cell volume was 15 ml.

2.5. Biosensor measurements

The measurements were carried out using the IPC-micro galvanopotentiostat mentioned in the previous section. The two-electrode circuit was used to register biosensor responses. A graphite paste insoluble mediator-modified electrode with immobilized *D. hansenii* cells served as the working electrode; a silver/silver chloride electrode with saturated potassium chloride electrolyte, as the reference electrode.

The soluble mediators (2.6-dichlorophenolindophenol, thionine, neutral red, potassium hexacyanoferrate, methylene blue) were added to the potassium sodium phosphate buffer in which the measurements were made. The potential found from the voltammogram was used for the measurements. The measurement temperature was 20 °C; the volume of the cell, 5.0 cm^3. After a stable level of current was set, the amount of the solution of an analyzed substance required to obtain the given concentration was introduced into the cell. After each measurement, the cell was washed with potassium sodium phosphate buffer.

2.6. BOD determination by the standard dilution method

The dilution method was used as the reference method for BOD_5 determination. Analysis was carried out in accordance with the technique given in [1,2]. The concentration of dissolved oxygen was determined using an EKSPERT-001-4.01 BOD thermooximeter (Ekoniks-ekspert, Russia).

3. Results and discussion

3.1. Selection of electron transport mediators

One of the main tasks in the development of BOD biosensors is to choose an efficient mediator of electron transfer from microbial enzyme systems to the electrode. The ability of microorganisms to interact with electron transport mediators is determined by the accessibility of the enzyme systems of these compounds and depends both on the nature of cells and the structure and properties of the mediator. To develop a BOD biosensor, it is necessary to choose mediators capable of transferring electrons from *D. hansenii* cells to the electrode. The possibility of using compounds as mediators is determined by standard redox potentials (Table 1) and peculiarities of the *D. hansenii* respiratory chain. The respiratory chain of *D. hansenii* has been well investigated [24]: it is sufficiently branched and has several alternative redox complexes; for this reason, the yeast is sufficiently tolerant to negative factors of the environment, such as high saline and heavy-metal concentrations. Despite the complex process of substrate oxidation by the yeast *D. hansenii*, all reactions occur within the range of −0.48 to +0.55 V [25]; thus, the flow of electrons can be directed to the selected mediators, because a potential gradient between the redox centres of cells' enzymes and the electrode is created (oxidative bioelectrocatalysis). Herewith, the working potential of the electrode, at which measurements are conducted, can differ significantly from the standard potential of the mediator. Depending on conditions, various potentials can be chosen for the same mediators. The working redox potential was determined proceeding from the results of the cyclic voltammograms (Fig. 1).

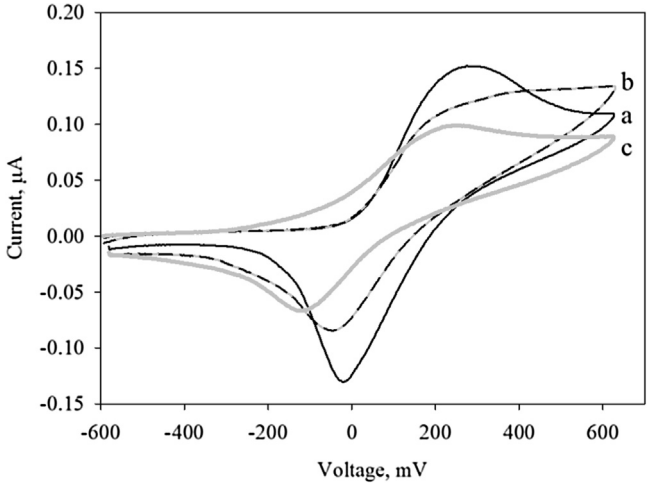

Fig. 1. Current–voltage curves of a ferrocene-modified graphite paste electrode (a), after immobilization of *D. hansenii* yeast cells on the surface of the electrode (b) and addition of 50-mM glucose to the measuring cuvette (c).

There are two peaks on the cyclic voltammogram of the ferrocene-modified graphite paste electrode (Fig. 1, curve *a*). The anodic peak reflects the oxidation of the reduced form; the cathodic peak, the reduction of the oxidized form of ferrocene. The current values of the anodic and cathodic peaks in the absence of a biocatalyst are approximately equal, which is indicative of the reversibility of ferrocene as a mediator. Similar voltammograms were obtained in the case of using 1,1′-dimethylferrocene and ferrocenecarboxaldehyde. However, the voltammogram for ferroceneacetonitrile differs significantly (data not shown). The absence of coupling of the CN group with the pentadiene ring decreases the reversibility of this system, because when going from the reduced to oxidized form the coordination spheres and the geometry of the complex may change. Using soluble mediators, the values of the anodic and cathodic peaks on the voltammograms are weakly pronounced. Possibly, this is due to the fact that the electrochemical oxidation reaction of soluble mediators on the electrode leads to a less intensive generation of receptor's oxidized and reduced forms, which is observed in the case of ferrocene and its derivatives.

From the current–voltage dependences for the graphite paste electrode, we found the redox potentials of mediators in systems with immobilized yeast cells. The anodic peak potential on the voltammogram was taken to be the working potential. The experimentally found potentials are given in Table 1.

Comparison of the obtained results with the literature data enables a conclusion that the working potential can depend on the analysis conditions and biomaterial used. Systems for BOD biosensors formed based on the yeast *D. hansenii* and water-soluble/slightly soluble mediators were tested for the analytical signal at the found redox potentials of mediators used. Biosensor responses to the addition of substrate were produced only using the slightly soluble mediator neutral red and the slightly soluble mediator ferrocene. Ferrocene and neutral red were used in further work to form a BOD biosensor.

3.2. Selection of the working parameters of one-mediator BOD biosensors

An important parameter in the development of biosensors is the determination of the working parameters at which the current generated by the biosensor will be maximal. The value of current generated by the biosensor in the presence of substrate can change depending on measurement conditions (concentrations of mediators, mass of biomaterial on the electrode). The amount of biomaterial on the electrode directly affects the parameters of the developed biosensor; however, an increased layer of biomaterial can lead to diffusion restrictions both for the substrate used and the mediator [16]. A high concentration of mediator can change the current-conducting properties of the electrode and have a toxic effect on biomaterial [16,26].

To select the working parameters of mediator biosensors based on the yeast *D. hansenii*, we obtained the dependences of biosensor responses on biomaterial mass on the electrode and mediator concentration. The tests were conducted using a glucose glutamate mixture (GGM), which is a model solution used to calibrate BOD biosensors [5,6,18,20].

The values of biosensor responses, as shown in Fig. 2, increase linearly at an increase of the specific density of biomass (the amount of biomaterial applied on an electrode 6.3 mm^2 in area) within the range of ~0.06 up to 0.10 mg/mm^2, owing to the fact that the current of the electrocatalytic oxidation of substrate by the yeast enzyme systems is directly proportional to the concentration of enzymes contained on unit area of the electrode surface [33,34]. However, at a further increase of the amount of biocatalyst on the electrode surface the biosensor responses do not change when using the ferrocene mediator, and decrease in the case of the neutral red mediator. This is due to the emerging diffusion restrictions for the transport of substrate to the enzymes' active centres as a consequence of the increase in the thickness of the biomaterial layer on the electrode surface [35]. Thus, biosensors based on the investigated mediators possess a maximal analytical signal at a specific density of yeast cell biomass of ~0.16 mg/mm^2.

Dependences of biosensor-generated currents on mediator concentrations are given in Fig. 3. Usually, dependences of generated current on mediator concentration for mediator biosensors have the shape of a hyperbola [26,34]. However, when using neutral red this dependence is peak-shaped. At high mediator concentrations, biosensor responses decrease, which can be explained by a possible inhibition of the enzyme systems of substrate's biochemical oxidation at high concentrations of neutral red. Based on the produced dependences, the following working concentrations of mediators were chosen: the concentration of ferrocene in the paste should be 10% of the total weight, and the concentration of neutral red in the cuvette should be about 0.2 mmol/l. The further studies were conducted at these working parameters.

3.3. Development of two-mediator BOD biosensors

Efficient transfer of electrons from eukaryotic cells can be performed by various strategies of using the two-mediator system [18,36,37]. We assume that thionine, neutral red, methylene blue, 2,6-dichlorophenolindophenol are capable, as lipophilic compounds, of penetrating inside the cell through the lipid membrane, interacting with the reduced form of the enzyme, taking electrons and passing them on to the immobilized mediator, which transfers electrons to the electrode (Fig. 4). The value of the working potential, at which the measurements are to be carried out, should provide for the required gradient for the transfer of electron between the enzyme's redox centre and the electrode, i.e., the value of the redox potential of an immobilized mediator should be higher than the standard redox potential of the soluble mediator [38]. Thus, for oxidative biocatalysis the value of the working potential is chosen by the value of the redox potential of the end electron acceptor, in this case, by the value of the redox potential of the insoluble mediator.

Based on combinations of four insoluble and five soluble mediators, we formed 20 two-mediator systems for developing biosensors on the basis of the yeast *D. hansenii*. In the course of the study, the presence or absence of the biosensor response was registered. Out of 20 systems, the biosensor response was obtained only for four combinations. For each, the working parameters indicated in Table 2 were chosen.

The use of other combinations of mediators was restricted by their properties. For an efficient electron transfer, and, therefore, for producing a biosensor response, it is necessary that the soluble mediator were rapidly restored by *D. hansenii* cells, and the insoluble mediator were subjected to rapid electrochemical oxidation [36]. Apparently, when using ferrocene derivatives, the rate of heterogeneous electron transfer significantly decreases as compared with ferrocene [39]. For this reason, a more efficient electrochemical oxidation is achieved in ferrocene-based two-mediator systems. The possibility of using ferrocene jointly with soluble mediators that function only in a two-mediator system supports the assumed scheme.

With the parameters given in Table 2, the maximal biosensor response is achieved at the constant values of the other parameters. The further characteristics of the biosensors were determined at the parameters indicated in Table 2.

3.4. Analytical and metrological characteristics of the developed mediator BOD biosensors

In the course of the work, we developed biosensors for rapid BOD assays based on the investigated mediator systems and the yeast *D. hansenii*. Using a model solution of a glucose–glutamate mixture, we produced the calibration dependences of a biosensor response on BOD_5 in the measuring cuvette (Fig. 5).

The biological response in bioreceptor elements based on whole cells is provided for by the enzyme reactions of microorganisms. The dependences given on Fig. 5 were fitted by the Michaelis–Menten equation:

$$R = \frac{R_{\max}[S]}{K_M + [S]},$$

where R_{max} is the maximal enzyme reaction rate achieved at $[S] \to \infty$, K_M is the apparent effective Michaelis constant, i.e., the concentration of substrate at which $R = R_{max}/2$.

When $[S] \ll K_M$, where K_M is the upper limit of the linear range, sensor responses are linear. The linear segments of the calibration curves were used for BOD assessment. The lower limit of the determined BOD_5 concentrations was calculated by the statistical method, proceeding from the criterion of the relative standard deviation of the measurement results ($S_r(C)$) < 0.33. Table 3 presents the major analytical and metrological characteristics of one- and two-mediator biosensors based on the yeast *D. hansenii*.

Based on the analysis of the metrological and analytical characteristics given in Table 3, it can be concluded that the developed one- and two-mediator biosensors enable generation of a stable analytical signal: operational stability (relative standard deviation) does not exceed 5%. Herewith, biosensors based on one-mediator systems are characterized by a low sensitivity, a higher value of the determined BOD lower limit (25.2 mg O_2/dm^3, when using ferrocene) and lower long-time stability. The stability was 16 days using neutral red; the analytical signal decreased in this case by 50%, so these systems can be said to be little suitable for assaying real samples.

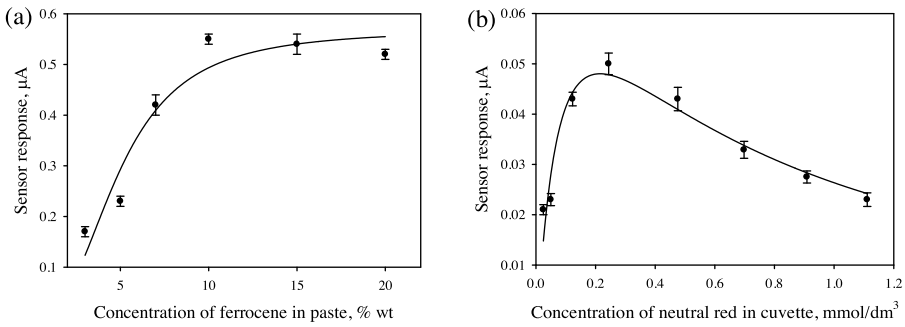

Fig. 2. Response of a biosensor based on mediators ferrocene (a) and neutral red (b) versus specific density of *D. hansenii* cell biomass.

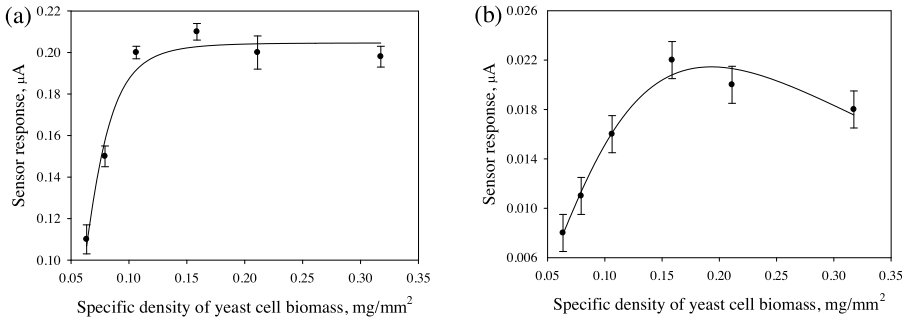

Fig. 3. Responses of mediator biosensors based on the yeast *D. hansenii* versus concentrations of ferrocene (a) and neutral red (b).

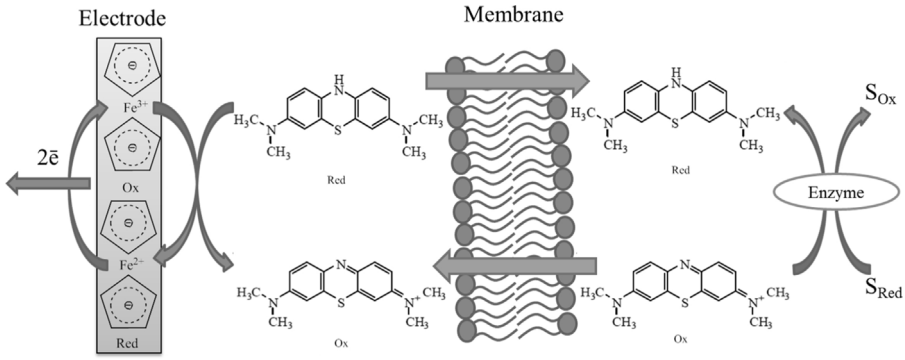

Fig. 4. Assumed mechanism for the operation of the working electrode of a two-mediator biosensor as exemplified by the ferrocene–methylene blue system and *D. hansenii* cells.

Table 2
Working parameters for a biosensor based on *D. hansenii* and two-mediator systems.

Insoluble–soluble mediator system	Concentration of soluble mediator, mmol/dm^3	Content of insoluble mediator, %	Specific density of biomass, mg/mm^2
Ferrocene–methylene blue	1.1	10	0.16
Ferrocene–potassium ferricyanide	0.4	10	0.11
Ferrocene–neutral red	1.1	10	0.16
Ferrocene–thionine	2.2	10	0.16

Table 3
Characteristics of a biosensor based on *D. hansenii* and a two-mediator system.

Investigated system	FC	NR	FC–MB	FC–PFC	FC–NR	FC–TN
Operational stability, %	2.2	2	1.2	3.1	2.0	2.2
Long-time stability, days	39	16	43	5	24	37
Single measurement time, min	8–20	5–10	3.5–6	20–26	7–10	8–20
Sensitivity coefficient, nA·dm^3/mg O_2	0.5 ± 0.2	0.4 ± 0.1	0.81 ± 0.04	1.1 ± 0.1	0.11 ± 0.01	0.22 ± 0.01
Range of determined BOD concentrations, mg O_2/dm^3	25.2–320	5.1–53	2.5–7.2	2.0–18.0	11.3–19.5	4.6–16.7

The use of two-mediator systems for developing a biosensor showed a higher efficiency of electron transfer from yeast cells to the electrode and, as a consequence, improved parameters of the BOD biosensor. An exception is a ferrocene–potassium ferricyanide system. For this system, the biosensor featured a low long-time stability (5 days) and high duration of measurement (20 min). These

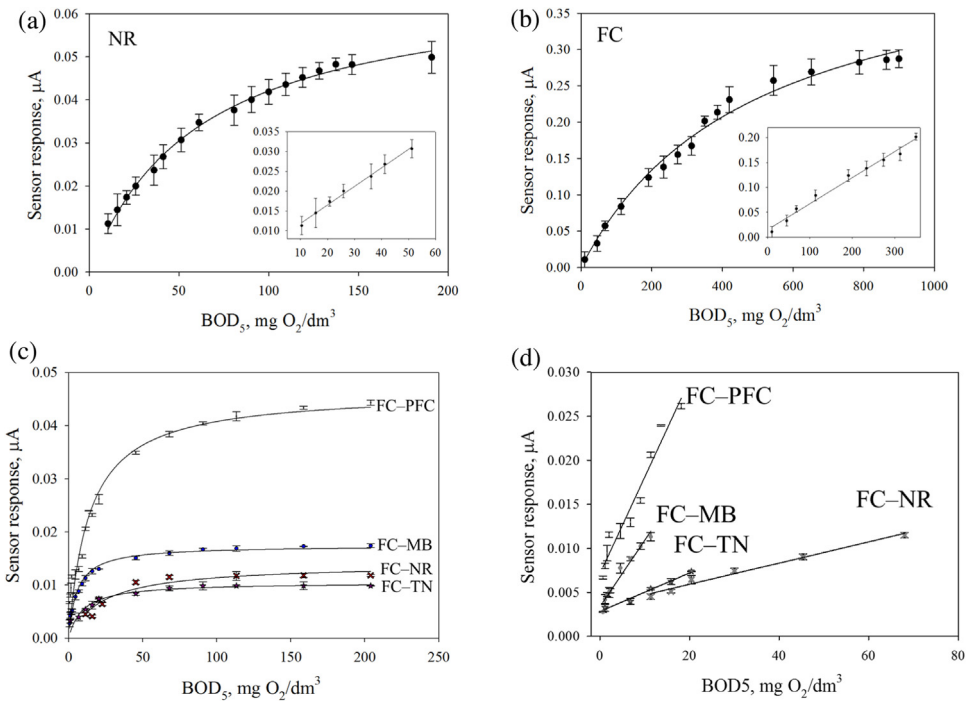

Fig. 5. Dependence of the response of one- and two-mediator biosensors on BOD_5 in the measured sample: (a) the calibration curve of single mediator (NR) neutral red and (b) (FC) ferrocene; (c) the calibration curves of two-mediator system: (FC–MB) ferrocene–methylene blue; (FC–PFC) ferrocene–potassium ferricyanide; (FC–NR) ferrocene–neutral red; (FC–TN) ferrocene–thionine and (d) there the linear parts.

values of the parameters significantly restrict the possibility of its further use, although this biosensor is characterized by the highest sensitivity in BOD determination – the detection limit when using the ferrocene–potassium ferricyanide was 2.0 mg O_2/dm^3.

The use of a two-mediator combination of ferrocene–neutral red increased the long-time stability of the receptor element by 10 days as compared with the one-mediator system based on neutral red. Probably, in the presence of ferrocene, which has a more positive potential (Table 1), the reduced form of neutral red is reoxidized and does not accumulate in the cell. Herewith, the use of this two-mediator system does not make it possible to rule out the toxic effect of neutral red: the long-time stability of the ferrocene-based biosensor is 15 days greater than that in the ferrocene–neutral red two-mediator system. Besides, the use of a two-mediator system increases the sensitivity of the assay. Thus, the lower limit of the determined BOD_5 concentrations based on ferrocene is almost 2.5 times higher than in the two-mediator system. Nevertheless, the high detection limit for the ferrocene–neutral red system, which is 11.3 mg O_2/dm^3, restricts its further use for assaying real samples.

By the long-time stability (43 days), sensitivity (the lower limit of the determined BOD_5 concentrations, 2.5 mg O_2/dm^3) and rapidity of assay the ferrocene–methylene blue pair is the most suitable for creating a mediator-type BOD analyzer based on *D. hansenii*. It is important to note that this biosensor is not inferior by its characteristics to its analogues [18,20] and even partially exceeds them; thus, a single BOD assay using the developed analyzer requires less time as compared with the BOD assay by other biosensor models [9–14,18,20]. The lower limit of the determined BOD_5 concentration values of the developed biosensors is slightly inferior to the detection limit of the biosensor based on the yeast *D. hansenii* VKM Y-2482 immobilized in chemically modified PVA gel as described in [6]. However, for the analysis of most real samples this value of the detection limit is acceptable.

For correlation of the results of the biosensor and standard method of assay, it is necessary that the used receptor element oxidize a broad range of organic compounds, i.e., possess a low selectivity. Proceeding from this, we assessed the substrate specificity of investigated yeast cells under conditions of the electrocatalytic oxidation of substrates. The results are given in Fig. 6. For the ferrocene–potassium ferricyanide system, no assessment was made due to a considerable duration of the measurement cycle.

Analysis of the substrate specificity profiles shown in Fig. 6 enables a conclusion that in the presence of a one-mediator system (ferrocene) it is virtually impossible to register the oxidation of alcohols. Probably, this is due to the fact that the interaction of the mediator with the enzyme systems of alcohols' oxidation by *D. hansenii* yeast cells is made difficult. In other two-mediator systems, the tendencies of electrocatalytic oxidation of carbohydrates by the yeast *D. hansenii* are rather close – the mediator systems generated the largest responses at the introduction of hydrocarbons into the systems, and the bioelectrocatalytic oxidation of alcohols was less efficient than that of sugars. In the presence of the ferrocene–methylene blue and ferrocene–neutral red mediator systems the responses to alcohols decreased with the length of the hydrocarbon chain increasing, as well as when going from primary to secondary alcohols. When using the ferrocene–neutral red system, there was no bioelectrocatalytic oxidation of propanol-2 and 2-methylpropanol-1 by *D. hansenii* cells. The character of oxidation of alcohols changed in the presence of the ferrocene–thionine mediator system; the response increased with the length of the hydrocarbon chain increasing. Thus, an intermediate conclusion can be made – the selectivity of biosensors can be changed using various mediator systems.

For the ferrocene–methylene blue and ferrocene–neutral red, responses to all investigated amino acids were registered. However, the sensor sensitivity to amino acids decreased in the ferrocene–thionine system compared with other two-mediator systems. At the same time, only this system enabled registering the oxidation of ethylenediaminetetraacetic acid (EDTA), which is important from the practical point of view, because EDTA is used in production of household products and synthetic detergents, as well as in cleaning of heat-power equipment, pipes, boilers, which

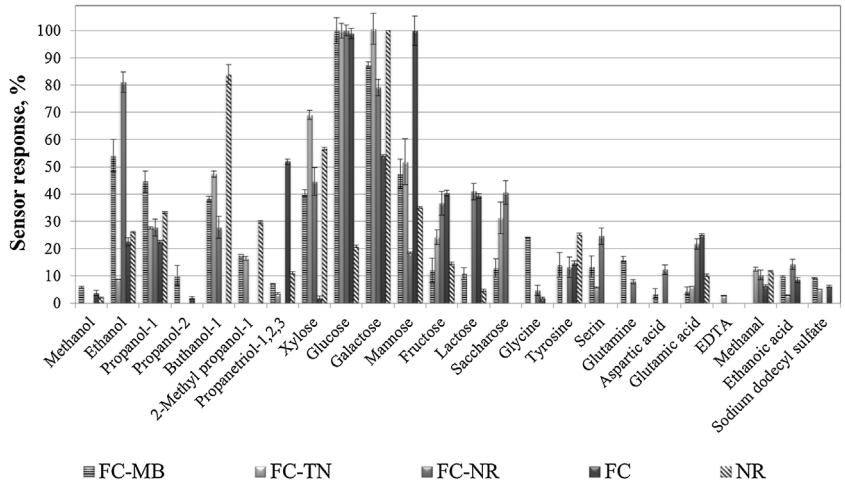

Fig. 6. Selectivity of receptor elements based on *D. hansenii* microorganisms under conditions of electrocatalytic oxidation of substrates (abbreviations are the same as in the legend to Fig. 5).

leads to this reagent getting to the environment. For this system, an important fact from the practical point of view are responses to sodium dodecyl sulfate, as well as the absence of a toxic effect of these substrates at their short-time impact on *D. hansenii* cells present in the bioreceptor.

In conclusion of the section on the assessment of substrate specificity, we can note that the broadest range of substrates can be registered by the receptor element based on the yeast *D. hansenii* in the presence of the ferrocene–methylene blue mediator system. This fact assumes that it is possible to obtain a high correlation between biosensor readings and the results of the standard method in BOD determination of real samples. By such parameters as sensitivity, long-time stability, rapidity and selectivity, the ferrocene–methylene blue mediator system is the most suitable for developing a BOD biosensor, as seen from the data of Table 3 and Fig. 6. For this reason, the biosensor based on *D. hansenii* and the ferrocene–methylene blue mediator system was chosen for analysis of real water samples.

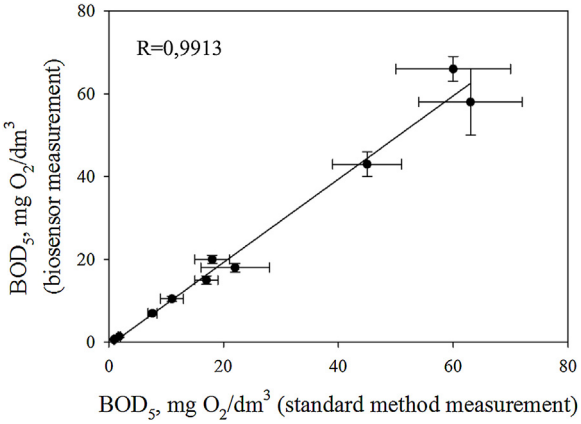

Fig. 7. Correlation between BOD values determined using the developed two-mediator biosensor (based on FC–MB and the yeast *D. hansenii*) and BOD values determined by the standard method.

3.5. Analysis of water samples using the developed two-mediator biosensor

We analyzed ten water samples using the developed biosensor and the standard dilution method. Samples of river and swamp waters, wastewaters of municipal water treatment facilities, meltwaters were taken. The samplings were conducted in accordance with the standard method. The values of wastewaters' BOD_5 by the standard dilution method were determined according to the current normative documents [1,2]. Fig. 7 shows a correlation between the BOD values of water samples determined using a biosensor and the BOD values determined by the standard dilution method.

The statistical treatment of the obtained data shows that the results of assay by the standard dilution method and by the biosensor method differ insignificantly. The correlation with the results of the standard method is sufficiently high ($R^2 = 0.99$; the sensitivity coefficient $S \approx 1$). Thus, the developed two-mediator biosensor based on *D. hansenii* yeast cells and the ferrocene–methylene blue mediator system can be efficiently used for analysis of various water samples.

The most promising approaches to the BOD rapid assessment found in the literature are given in Table 4. The known analogs were compared with the developed *D. hansenii*-based biosensor and the ferrocene–methylene blue mediator system.

The two-mediator biosensor is not inferior by its characteristics to other mediator BOD sensors that mainly use bacterial cells, but this biomaterial, as a rule, is not stable in operation. The use of stable eukaryotes that oxidize a broad range of organic substances, such as the yeast *D. hansenii*, is made difficult in a one-mediator system. To solve this problem, we used a two-mediator system with the yeast *D. hansenii*. The developed system is inferior than the biosensor based on the yeast *D. hansenii* VKM Y-2482 immobilized in chemically modified poly (vinyl alcohol) gel described in [6]. Still, this value of the lower limit is acceptable for assaying real samples.

4. Conclusion

Studies of the possibility of using soluble and slightly soluble mediators in the biosensor based on the yeast *D. hansenii* revealed that of nine mediators only ferrocene and neutral red were applicable for these studies. One-mediator BOD biosensors featured unsatisfactory parameters, which can be explained by the difficulty of achieving optimal electron transfer under these conditions. The use of two-mediator systems enables solving this problem. In a two-mediator composition, one mediator is restored by microbial cells at a high rate, while the other provides for a fast electrochemical reaction and transfers the charge to the electrode. The ferrocene–methylene blue two-mediator system is the most suitable for creating a prototype model of a BOD biosensor. This system

Table 4
Summary of BOD approaches from the literature.

Microorganisms	Electron acceptor	Response time (min)	Working range (mg O_2/dm^3)	Correlationwith BOD_5			Origins of the validation samples	Refs
				R^2	S	n		
Activated sludge	PFC	240	2.1–40	0.92	0.94	33	Wastewater treatment plant	[14]
Saccharomyces cerevisiae	M + PFC	15	6.6–220	0.71	1.00	6	River and sea samples	[18]
Debaryomyces hansenii	Oxygen	10–17	0.7–207	0.99	1.03	10	Wastewater treatment plant and food industry, snowmelt	[6]
Debaryomyces hansenii	FC + MB	10	2.7–7.2	0.99	1.01	10	River and swamp samples, wastewaters treatment plants, snowmelt	This study

provides for a satisfactory long-time stability (43 days), selectivity, sensitivity (the lower limit of the determined BOD_5 concentrations is 2.5 mg O_2/dm^3), rapidity (the time of assaying one sample does not exceed 10 min). Analysis of real samples from waters of various origins showed that the data obtained by the standard dilution method and by the BOD biosensor differ insignificantly. The produced results indicate the possibility of using the developed biosensor analyzer as a prototype of pilot devices for batch application.

Acknowledgements

The work was supported by the Federal Targeted Programme "Research and Development in Priority Areas of Development of the Russian Scientific and Technological Complex for 2014–2020", Agreement No 14.574.21.0062 (unique identifier RFMEFI57414X0062).

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