



A kinetic approach to the formation of two-mediator systems for developing microbial biosensors as exemplified by a rapid biochemical oxygen demand assay

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Abstract

This work proposes a method of forming a microorganism–mediator(s) receptor system, in which the rates of separate stages of mediator bioelectrocatalysis are used as the basis for the development of biosensors for the biochemical oxygen demand (BOD) rapid assay. In the presence of a ferrocene mediator, the yeast *Blastobotrys adeninivorans* was shown to enable oxidation of a larger range of substrates as compared with other investigated microorganisms—bacteria *Escherichia coli* and yeast *Ogataea polymorpha*. The rate constants of the interaction of the yeast *B. adeninivorans* with nine compounds, electron transfer mediators, were determined; the best mediator for these microorganisms was found to be neutral red ($k_{\text{int}} = 0.681 \pm 0.009 \text{ dm}^3/\text{g s}$). Neutral red possesses a high rate of interaction with the ferrocene mediator ($14,200 \pm 100 \text{ dm}^3/\text{mol s}$) shown earlier to be the most promising acceptor of electrons at a carbon paste electrode ($0.4 \pm 0.1 \text{ cm/s}$). These features enabled the formation of a two-mediator ferrocene–neutral red system to be used in a biosensor. A two-mediator-based biosensor had a higher sensitivity (the lower limit of detected BOD concentrations, 0.16 mg/dm^3) than that of a one-mediator system based on neutral red and ferrocene. Analysis of ten samples from surface water reservoirs showed the combination of ferrocene, neutral red and the yeast *B. adeninivorans* to enable the data that highly correlated ($R = 0.9693$) with those of the standard method.

Keywords Biochemical oxygen demand · Biosensor · Electron transfer mediator · Ferrocene · Two-mediator system · Yeast *Blastobotrys adeninivorans*

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Introduction

Biosensor technologies are frequently used in ecological monitoring, as they enable rapid and accurate analyses of the environmental quality (Bilal and Iqbal 2019; Ejeian et al. 2018). The standard methods are not attributed to rapid techniques, as they require long-time incubation, e.g., in the assessment of biochemical oxygen demand (BOD) or general toxicity (ISO 2003, 2008), thereby providing for a high demand for biosensor research and development. At the assessment of integral parameters, such as BOD, much attention is paid to microbial biosensors. The value of BOD_5 (the subscript indicates the sample incubation time and is usually 5 days) reflects the total amount of organic pollutants in wastewaters (mg/dm^3) and shows the potential ability of wastewaters to deplete oxygen in a natural water reservoir. This parameter does not take account of organic pollutant components in the sample but makes it possible to rapidly

react to the pollution of natural waters, as well as to assess and control the wastewater cleanup.

Proceeding from features of BOD determination, whole microbial cells are used to form bioreceptors. As compared with enzymatic biosensors, microbial biosensors are less sensitive to inhibiting substances, more stable to pH/temperature measurements, have longer operating lifetimes (Nakamura 2018). A widespread approach is to use associations of microbial cells for BOD assays (Jordan et al. 2014; Liu et al. 2015; Niyomdech et al. 2017). However, the problem with associations is the reproducibility of the developed system due to the changing microbial makeup of the association. When forming portable devices, preferable microbial strains to be used are those that oxidize a broad range of organic substances (Hu et al. 2015, 2016, 2017) isolated from natural environments (Hooi et al. 2015; Kharkova et al. 2019).

Organic pollutants in wastewaters can be detected using electrochemical biosensors. Coupling of a biomaterial with an electrode can be done the most successfully in amperometric systems (Bilal and Iqbal 2019; Ejeian et al. 2018). Three generations in the development of microbial BOD biosensors are singled out (Nakamura 2018). In first-generation devices, the analytical signal is registered using sensors of the oxygen-concentration decrease caused by the response of biomaterial to organic components of the sample. First-generation biosensors have become the most widespread for the BOD assay due to their simplicity and low cost; however, they have some drawbacks. First, the amperometric measurement of oxygen requires the use of relatively high potentials, at which the effect of interference caused by substances present in assayed samples becomes significant. Second, the concentration of oxygen may vary, causing additional errors of the assay (Nakamura 2018).

The second generation of microbial amperometric mediator biosensors are analytical devices comprising microbial cells, whose electric contact with the biosensor transducer is via electron transfer mediators. A mediator possesses an electrochemical activity and can be reduced under the action of microbial cells, transferring electrons to the electrode. This approach eliminated the drawbacks of first-generation amperometric biosensors and enabled developing portable rapid BOD assay systems (Niyomdech et al. 2017; Hu et al. 2015, 2016, 2017; Hooi et al. 2015; Kharkova et al. 2019). A significant limitation of this technology, however, was that it could be used predominantly with bacterial cells. Yeast cells have a thick (100–200 nm) cell wall containing polysaccharides and proteins (Christwardana and Kwon 2017; Gal et al. 2016). Signalling systems—yeast cytochromes—are in mitochondria, and transmembrane proteins are in the cell membrane enclosed by the cell wall. Therefore, to receive an electrochemical signal from yeast cells, the mediator should pass through the cell wall and interact with the membrane and/or intracellular components, such as NAD⁺/NADH

(Rawson et al. 2012; Kasem et al. 2013). Proceeding from the above, it could be said that the use of one-mediator systems with yeast cells is low-efficient. Herewith, it should be taken into account that, from the point of view of BOD rapid monitoring, yeasts are a promising biomaterial: they are capable of metabolizing a broad range of substrates, are characterized by a stability to negative factors of the environment (high salinity, temperature/pH variations) (Bilal and Iqbal 2019; Ejeian et al. 2018; Nakamura 2018).

The third generation of BOD biosensors is based on two-mediator systems (Nakamura 2018). A system based on the yeast *Saccharomyces cerevisiae* and a potassium hexacyanoferrate(III)–vitamin K3 (menadione, 2-methyl-1,4-naphthoquinone) two-mediator system was proposed for BOD assay in Nakamura et al. (2007a, b). Menadione is a lipophilic mediator, which interacts with the microorganism, and potassium hexacyanoferrate(III) is a hydrophobic mediator, which possesses a high rate of electron transfer to the electrode. A biosensor of this setup enables assaying the organic insecticide diazinon within the range of 10^{−6} g/ml up to 10^{−2} g/ml (Mazloun-Ardakani et al. 2019). Using this biosensor, the BOD can be assayed within 7–220 mg/dm³. The use of the described system of mediators for assessing the general toxicity of wastewaters should be noted to be a success (Gao et al. 2017). Besides the above described, there are also other systems of mediators for BOD₅ assay (Ino et al. 2019; Pasco et al. 2005; Nakamura et al. 2007a, b). Combinations of some lipophilic mediators with potassium hexacyanoferrate(III) (PHC) were investigated in Pasco et al. (2005). *p*-Benzoquinone, 2,6-dichlorophenolindophenol, menadione, neutral red, *N,N'*-tetramethyl-1,4-phenylenediamine, phenazine ethosulfate, 2,3,5,6-tetramethylphenylenediamine were used as lipophilic mediators. The use of the two latter mediators jointly with PHC led to a rise in the efficiency of oxidation of a glucose–glutamate mixture (GGA); the other mediators except neutral red decreased the GGA oxidation efficiency. The decrease was explained by the toxic action of the lipophilic mediator on *Escherichia coli* cells. The use of neutral red did not lead to a significant increase of GGA oxidation efficiency, which can be explained by the low redox potential of the mediator. A vitamin K3–hydrogen peroxide two-mediator system was used to develop an optical BOD biosensor based on the yeast *S. cerevisiae* (Nakamura et al. 2007a, b). In the presence of biodegradable compounds, vitamin K3 is reduced to vitamin K4. Reverse oxidation of vitamin K4 occurs under the action of hydrogen peroxide, which reacts with luminol and hydroxide anions, the result of which is a registered chemiluminescence. The biosensor enabled the BOD assessment within the range of 11–220 mg O₂/dm³ (Nakamura et al. 2007a, b).

The use of a two-mediator system makes it possible to increase the sensitivity of the assay. In Zaitseva et al. (2017),

the yeast *Debaryomyces hansenii* was shown to be used with the ferrocene and methylene blue mediators. The lower limit of the determined BOD concentrations for the biosensor was 2.5 mg/dm^3 ; herewith, in a one-mediator system, the assay could be carried out only for samples with the BOD of no less than 5.1 mg/dm^3 . This system was the best from a series of biosensors comprising yeasts and the neutral red mediator. It should be noted that the use of two-mediator systems does not always lead to improve the biosensor characteristics (Gao et al. 2017; Zaitseva et al. 2017). The low efficiency of a potassium hexacyanoferrate(III)–vitamin K3 two-mediator system used with *Escherichia coli* cells was shown in Gao et al. (2017) and Pasco et al. (2005): introduction of the second mediator into the system failed to increase the biosensor response. Zaitseva et al. (2017) also discussed a decrease of sensitivity when passing from a one-mediator system based on neutral red, which provides for the BOD lower detection limit of 5.1 mg/dm^3 , to a ferrocene–neutral red two-mediator system (the BOD lower detection limit of 11.3 mg/dm^3). For the transfer of electrons in a two-mediator system to proceed successively, without the mediators competing, it is necessary to provide for a difference in the rates of interaction both with the biomaterial and the electrode; an ideal case is the implementation of a scheme in which one mediator interacts with the electrode at a high rate and possesses a low rate in the interaction with the biomaterial. Herewith, the second mediator should have a high rate in the interaction with the biomaterial and a low rate with the electrode (Nakamura et al. 2007a, b; Mazloum-Ardakani et al. 2019; Gao et al. 2017; Ino et al. 2019; Pasco et al. 2005; Nakamura et al. 2007a, b; Zaitseva et al. 2017). This work investigates the justification of choosing two-mediator systems from the point of view of the bioelectrochemical reaction kinetics and proposes the basis for the development of a BOD biosensor.

Experimental

Reagents and materials

Glucose, tryptone, peptone (Panreac, Spain), yeast extract (Helicon, Russia), sodium chloride, glycerol, NaH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , K_2HPO_4 , leucine (Diaem, Russia), agar-agar (Panreac, Spain) were used to grow microorganisms. To form a working carbon paste electrode, use was made of graphite powder of particle size 75 microns and high purity 99.997% (Fluka, Germany), paraffin oil (Fluka, Germany) and dialysis membrane with an MWCO of 14 kDa (Roth, Germany).

Ferrocene (Aldrich, Germany), 1'-dimethylferrocene (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 hexacyanoferrate(III) (Diaem, Russia) were used as electron transfer mediators.

A sodium–potassium phosphate buffer solution with various values of pH ($33 \text{ mM KH}_2\text{PO}_4 + 33 \text{ mM Na}_2\text{HPO}_4$, Diaem, Russia) was used for work with microorganisms.

Microorganisms

Yeasts *Blastobotrys adeninivorans* VKM Y-2677 (*B. adeninivorans*) and *Ogateae polymorpha* VKM Y-2559 (*O. polymorpha*) were provided by the All-Russian Collection of Microorganisms, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, FRC PCBR RAS (Pushchino). *Escherichia coli* K-802 (*E. coli*) bacteria were from the Laboratory of Plasmids, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, FRC PCBR RAS.

Cultivation of microbial cells

Microorganisms were grown using an ES-20/60 orbital shaker-incubator (BioSan, Latvia); TG16WS (Polikom Ltd, Russia) and MiniSpin plus (Eppendorf, Russia) centrifuges. Biomass was stored in microtubes at a temperature of -25°C .

The yeast *B. adeninivorans* was cultivated on a medium of the following composition: glucose, 1%; peptone, 0.5%; yeast extract, 0.05%. Cultivation time was 18–20 h at a temperature of 28°C . Centrifugation was carried out at $7000\times g$ for 10 min. A phosphate buffer solution, pH 6.8, was used to prepare a cell suspension.

The yeast *B. adeninivorans* was cultivated on a rich mineral medium (liquid glucose–peptone nutrient medium). The composition of the liquid medium was as follows: glucose, 1%; peptone, 0.5%; yeast extract, 0.05% (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 28°C . The produced biomass was centrifuged at room temperature for 10 min ($7000\times g$). 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 10 min at $7000\times g$. The washed biomass was weighed and stored in microtubes at a temperature of -25°C .

The yeast *O. polymorpha* was cultivated in the following medium: glycerol, 1%; NaH_2PO_4 , 0.39%; $(\text{NH}_4)_2\text{SO}_4$, 0.25%; trace elements, 0.1%; yeast extract, 0.05%; MgSO_4 , 0.04%; K_2HPO_4 , 0.09%; leucine, 0.017%. 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 36 h in 750 cm³ shake flasks at a temperature of 28 °C. The produced biomass was centrifuged at room temperature for 15 min (4500×g). The centrifugate was washed with a 20 mM phosphate buffer, pH 7.2. Sedimented cells were transferred to fresh portions of buffer, distributed by portions and sedimented at an Eppendorf centrifuge for 10 min at 4500×g. The washed biomass was weighed and stored in microtubes at a temperature of −25 °C.

Escherichia coli bacteria were grown in a Luria–Bertani medium: tryptone, 1%; NaCl, 1%; yeast extract, 0.5%. 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 24 h in 750 cm³ shake flasks at a temperature of 28 °C. The produced biomass was centrifuged at room temperature for 15 min (9000×g). The centrifugate was washed with a 20 mM phosphate buffer, pH 7.5. Sedimented cells were transferred to fresh portions of buffer, distributed by portions and sedimented at an Eppendorf centrifuge for 10 min at 9000×g. The washed biomass was weighed and stored in microtubes at a temperature of −25 °C.

The purity of the grown culture was controlled by optical microscopy.

Formation of a working electrode

A working electrode was formed by filling a plastic tube of the working surface area 6.3 mm² with a paste made from a mixture of graphite powder and mineral oil. Electrodes based on low-soluble mediators (ferrocene, 1,1'-dimethylferrocene, ferrocenecarboxaldehyde, ferroceneacetone, 2,5-dibromobenzoquinone) were formed as follows: 100 mg of graphite powder was mixed with a 1% solution of mediator in acetone in the amount sufficient for a required mediator concentration in graphite paste. Upon evaporation of acetone, 40 µl of paraffin oil was added and the paste was mixed. This modified paste filled the plastic tube of the measuring electrode. To form electrodes based on soluble mediators (thionine, neutral red, methylene blue, 2,6-dichlorophenolindophenol, potassium ferricyanide), non-modified paste was prepared (100 mg of graphite powder was mixed with 40 µl of paraffin oil) and was also used to fill the plastic tube of the measuring electrode.

Bacterial and yeast cells were immobilized on the electrode surface as follows. A microbial suspension (200 mg wet weight/ml) in the amount of 3 µl was applied onto the working surface of the electrode and dried at room temperature for 15 min. To retain cells on the electrode surface, a securing dialysis membrane was used, which was fixed by a plastic ring.

Registration of current–voltage characteristics

Cyclic voltammograms were registered by an IPC-microgalvanopotentiostat (Volta Ltd, Russia) using a three-electrode circuit. A graphite paste electrode with immobilized microbial cells served as a working electrode; a platinum electrode, as an auxiliary electrode. As a reference electrode, a saturated Ag/AgCl electrode was used, relative to which all values of potentials are presented. Cyclic voltammograms were registered at a potential sweep rate of 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.

Biosensor creation

A working graphite paste electrode with immobilized microorganisms and a reference electrode [saturated silver chloride electrode (Ag/AgCl)] were connected to an IPC-microgalvanopotentiostat. A potassium–sodium–phosphate buffer solution at a temperature of 22 °C was used as an electrolyte; the cell volume was 5.0 cm³.

Soluble mediators [2,6-dichlorophenolindophenol, thionine, neutral red, potassium hexacyanoferrate(III), methylene blue] were added to the potassium–sodium–phosphate buffer solution in which measurements were conducted. The mediator redox potential found from the voltammogram was used for the measurements. The measurement temperature was 20 °C; the cell volume, 5.0 cm³. After a stable level of current was settled, a quantity of the solution of the analyzed substance required for the specified concentration was pipetted into the cell. The cell was washed with the potassium–sodium–phosphate buffer solution after each measurement.

Determination of BOD by the standard dilution method

The dilution method was used as a reference method for BOD₅ determination. The analysis was conducted in accordance with the method in ISO 5815-1:2003. The dissolved oxygen content was determined using an Ekspert-001-4.0.1 BOD thermo-oximeter (OOO Ekoniks-ekspert, Russia).

Results and discussion

Selection of biomaterial

To correlate the results of BOD detection by the biosensor and standard assays, it is necessary that the developed biosensor register oxidations of as broad range of organic substrates as possible. As biomaterial, use was made of bacteria *E. coli* K-802, yeasts *B. adeninivorans* VKM Y-2677

and *O. polymorpha* VKM Y-2559. As biological material for BOD₅ rapid assay biosensors, yeast cells *O. polymorpha* and *B. adeninivorans* possessing a broad range of oxidized organic compounds were chosen (Ponamoreva et al. 2015; Yudina et al. 2015). Strain *B. adeninivorans* VKM Y-2677 belonging to the All-Russian Collection of Microorganisms is used as a production culture at hydrolysis plants and is characterized by a high salt tolerance (Chan et al. 1999). The yeast *O. polymorpha* is actively used in biotechnology to produce certain proteins; in biosensor technology, it is used for developing biosensing elements for methanol and ethanol assays. Depending on the growth conditions, they can change their specificity and, for this reason, can be used in the development of specialized receptor elements for certain effluents (Kamanina et al. 2016). Bacteria *E. coli* occur in natural and waste waters, they are also one of the components of the inoculate in the standard method of analysis, which increases the sensitivity of BOD₅ detection at their incorporation into the receptor element (Galler et al. 2018).

As a model electron transfer mediator, use was made of ferrocene, because the electrochemical reaction with its participation does not depend on pH of the medium, and the low solubility of ferrocene in water makes it possible to modify graphite paste, thus immobilizing the mediator on the surface of the working electrode (Zaitseva et al. 2017). All investigated microorganisms are capable of interacting with an artificial electron acceptor, ferrocene, which is confirmed by the presence of biosensor responses

at a working potential of 250 mV, used the most often in operation with modified carbon paste electrodes with immobilized microbial cells (Zaitseva et al. 2017; Babkina et al. 2006). It should be noted that the analytical signal—an increase in current strength—is recorded only in the presence of microorganisms.

An efficient biocatalyst was selected by the substrate specificity of the receptor element based on microorganism–mediator systems. Substrate specificity was assessed for 32 substrates from various classes of organic compounds (Fig. 1). Organic substances occurring the most often in wastewaters of various production facilities were chosen as substrates. The presence of these substances in water reservoirs leads to a significant increase of the biochemical oxygen demand index.

Thus, a larger number of oxidized substrates could be registered using the ferrocene mediator and the yeast *B. adeninivorans* (23 substrates). The substrate specificity of a similar biosensor based on the earlier investigated halo-/osmotolerant yeast *D. hansenii* was as low as 14 substrates (Zaitseva et al. 2017). The substrate specificity of biosensors based on *E. coli* bacteria (8 substrates) and the yeast *O. polymorpha* (17 substrates) was also narrow, so these biosensors have low prospects for further research. Thus, based on the substrate specificity as well as adaptability to negative environmental factors, namely, owing to its halo- and osmotolerance, the yeast *B. adeninivorans* was chosen for further work.

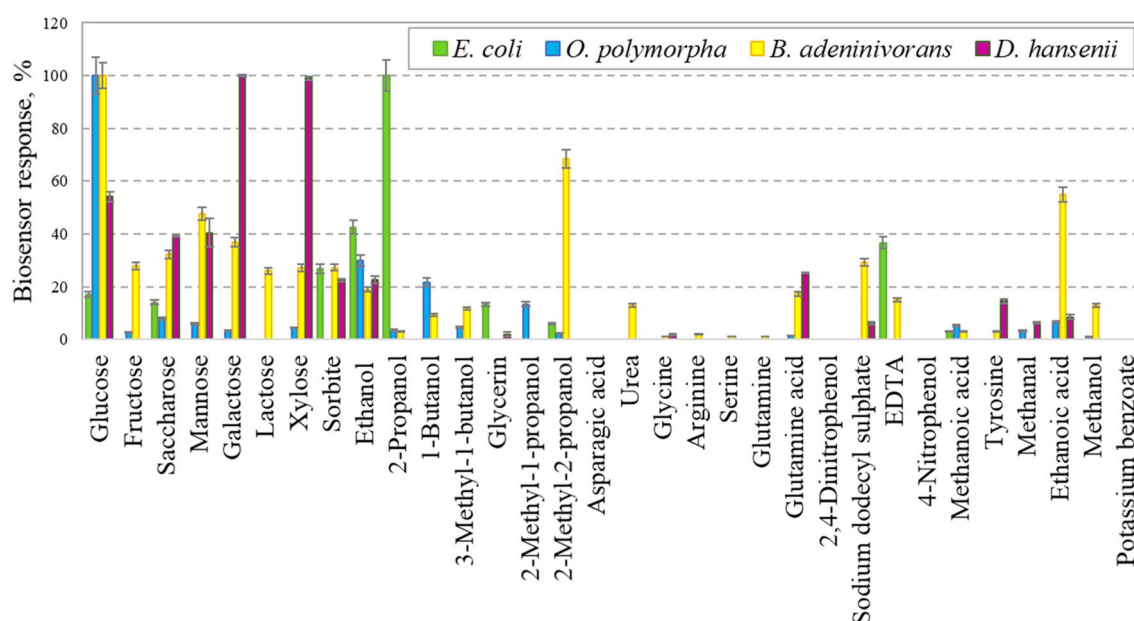


Fig. 1 Substrate specificity of *E. coli* bacteria, *O. polymorpha* and *B. adeninivorans* yeasts in the presence of the ferrocene mediator. Data on the substrate specificity of the yeast *D. hansenii* were added from the results of Zaitseva et al. (2017)

Selection of electron transfer mediator for the formation of a bioreceptor system

The electron transfer mediator was selected from nine compounds from various classes of substances by comparing the efficiency of the biocatalytic oxidation of glucose by the yeast *B. adeninivorans*. At the registration of a cyclic voltammogram, in the presence of glucose there occurs an increase of anodic current (Fig. 2a) due to the electrocatalytic oxidation of substrate by the yeast, which proceeds according to the following formula:

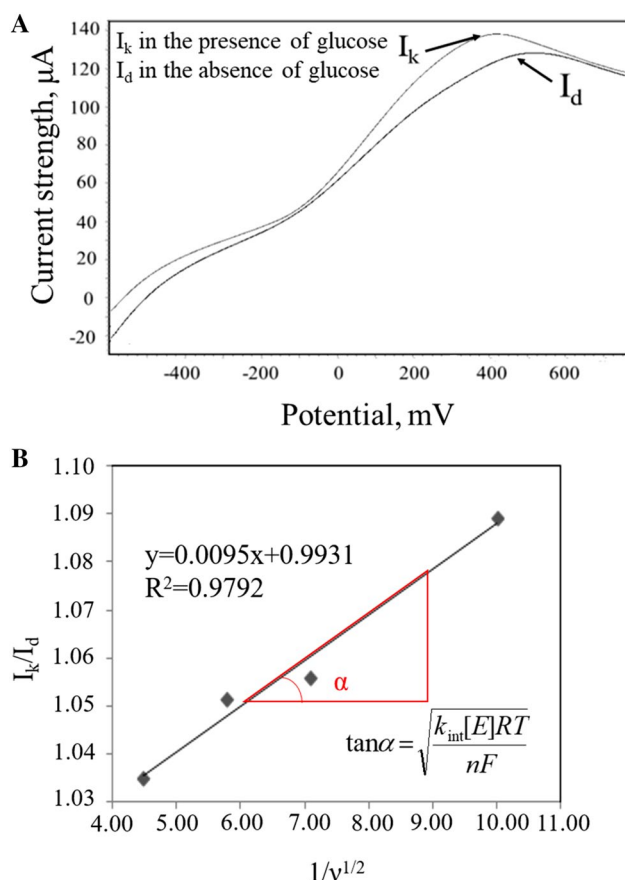
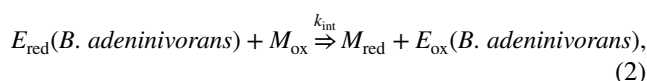
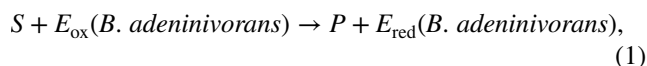


Fig. 2 Determination of the constant of interaction of the mediator with the yeast cells of *B. adeninivorans* by cyclic voltammetry. **a** Typical voltammograms exemplified by 1,1-dimethylferrocene mediator at a scan rate of 20 mV/s. **b** Dependence of the ratio of limiting currents in the presence and absence of substrate on the inverse root of the scan rate $1/v^{1/2}$ in the yeast *B. adeninivorans*–1,1-dimethylferrocene mediator system



where *S* is substrate (glucose); *P*, product; *E*_{ox} (*B. adeninivorans*) and *E*_{red} (*B. adeninivorans*), oxidized and reduced forms of the enzymes of the yeast *B. adeninivorans*; *M*_{red} and *M*_{ox}, oxidized and reduced forms of the electron transfer mediator; *n* $\bar{e}$, the number of electrons transferred to the electrode; *k*_{int}, constant of the rate of interaction between the mediator and biomaterial.

For the mediator to be an efficient electron acceptor for investigated biomaterial, it is necessary that the rate constant *k*_{int} in Eq. (2) have the greatest value; in this case, the mediator will successfully compete with the natural electron acceptor, dissolved oxygen, and no deaeration of the cell will be required during the biosensor operation. The complete description of the cyclic voltammetry method for determination of the interaction constant is given in Nicholson and Shain (1964). Certain conditions are required to be created for reaction (2) to become rate determining. First, for reaction (1) not to be limiting, it is necessary to use substrate at a concentration exceeding the Michaelis constant. In this connection, all experiments were carried out under substrate concentration excess conditions; herewith, the final concentration of glucose was 50 mM. Second, for reaction (3) not to be rate-determining, the transfer of electrons should be sufficiently fast, and the fixed current be diffusion-limited. The diffusion character of the limiting stage in the carbon paste electrode–mediator system when using non-ferrocene mediators had been established in Kharkova et al. (2020), which found the heterogeneous electron transfer rate constants for the investigated mediators. When passing from the carbon paste electrode–mediator system to the carbon paste electrode–mediator–yeast *B. adeninivorans* system provided that oxidized substrate is in excess and the concentration of mediator is lower than the Michaelis constant, the limiting current is inversely proportional to the root of the sweep rate. This is indicative of the diffusion character of the limiting process and the possibility of using the Nicholson–Shain model for all investigated mediators.

Assuming that the enzyme systems of the yeast *B. adeninivorans* are in a reduced state due to a high concentration of substrate, the constant of the interaction of the yeast with the investigated mediators can be found using Eq. (4).

To determine the rate constants, the dependences of the ratio of the limiting anodic currents in the presence and absence of substrate, (*I*_k/*I*_d), on the value of $1/v^{1/2}$ were found; by the slope of the linear regression, *k*_{int} was found. A typical form of the calculation plot is given in Fig. 2b.

$$\frac{I_k}{I_d} = \sqrt{\frac{k_{\text{int}}[E]RT}{nFv}}, \quad (4)$$

where I_k is the limiting current in the presence of substrate; I_d , the limiting current in the absence of substrate (A); k_{int} , the rate constant of the interaction of the mediator and biomaterial ($\text{dm}^3/\text{mg s}$); R , the universal gas constant (J/mol K); T , temperature, degrees Kelvin (K); $[E]$, the titre of cells (mg/dm^3); ν , sweep rate (V/s); n , the number of transferred electrons; F , Faraday constant (C/mol).

The obtained constants of interaction of the mediators with the yeast *B. adeninivorans* are given in Table 1. On the whole, it can be noted that soluble mediators interact with cells much faster than with ferrocene derivatives, which is, probably, due to the ability of phenazine and phenathiozine mediators to penetrate into the cell, thus providing for high interaction rates (Ikeda et al. 1996).

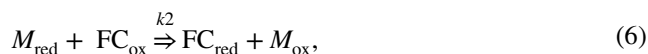
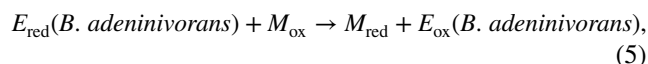
It should be noted that the yeast *B. adeninivorans* is a sufficiently promising biomaterial for its further use in mediator bioelectrocatalysis, because in a number of cases, the constants of its interaction with mediators exceed similar constants for a system based on *P. yeii* bacteria isolated from activated sludge (Kharkova et al. 2020). To form the receptor system of a BOD biosensor, the neutral red mediator was chosen, because it possesses the largest constant of the interaction with the investigated yeasts (the highest rate), and the ferrocene mediator, which exceeds the other used mediators by the rate of electron transfer to the carbon paste electrode; the constant of the heterogeneous transfer of electrons to the carbon paste electrode is $0.4 \pm 0.1 \text{ cm/s}$ (Kharkova et al. 2020).

Formation of a two-mediator bioreceptor system

As already noted, one of the approaches enabling an increase of electron transfer efficiency provides for the use of two-mediator systems. For this, it is necessary that one of the mediators rapidly interact with the microorganism, and the other mediator transfer electrons to the electrode (Nakamura et al. 2007a,

b; Mazloun-Ardakani et al. 2019; Gao et al. 2017; Ino et al. 2019; Pasco et al. 2005; Nakamura et al. 2007a, b; Zaitseva et al. 2017). Proceeding from the works described in Kharkova et al. (2020), ferrocene exceeds the other used mediators by the rate of electron transfer to the electrode; for this reason, it was chosen as one of the components of the system. To choose the other mediator for the receptor system, four mediators were tested: methylene blue, potassium hexacyanoferrate(III), thionine, neutral red; the constant of interaction with biomaterial for them exceeds more than ninefold the ferrocene interaction constant and is inferior by an order of magnitude with respect to the heterogeneous electron transfer constant (Kharkova et al. 2020, Table 2). This difference of the rates assumes the following electron transfer mechanism, which is presented in Fig. 3.

The efficiency of electron transfer in a two-mediator system will depend on the rate of interaction of two mediators (k_2)—a high value of this constant will not limit the electron transfer process in the operation of biosensors under these conditions. Besides, a high value of k_2 and a low value of the heterogeneous electron transfer constant k_3 for the acceptor of electrons from biomaterial will provide for the succession of electron transfer but not the competition of two mediators. To assess this constant, the Nicholson–Shain modelling (Nicholson and Shain 1964) under the same limitations as for the interaction constant in a one-mediator system was used:



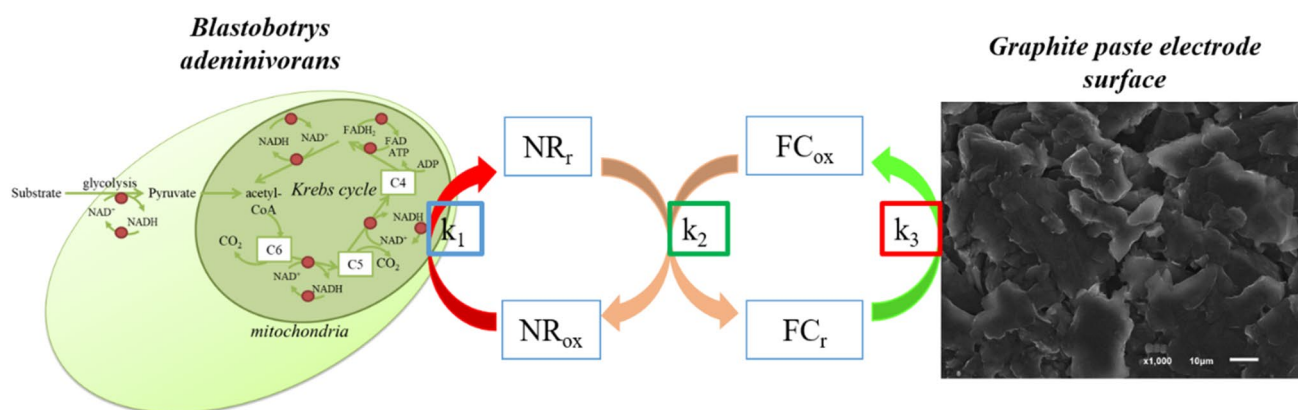
To determine the rate constants, the dependences of the ratio of limiting anodic currents (I_k/I_d) in the presence and absence of microbial cells on $1/\nu^{1/2}$ were obtained; from the

Table 1 Constants of the rate of interaction of electron transport mediators with bacteria *P. yeii* (Kharkova et al. 2020), and constants of the interaction of mediators with yeast *B. adeninivorans*, as determined in this work

Mediator	Constant of the interaction of mediators with microorganisms, $\text{dm}^3/(\text{g s})$	
	Yeast <i>B. adeninivorans</i>	Bacteria <i>P. yeii</i> (Kharkova et al. 2020)
Methylene blue	0.092 ± 0.004	0.021 ± 0.001
Potassium hexacyanoferrate(III)	0.620 ± 0.008	0.019 ± 0.003
Thionine	0.123 ± 0.007	0.013 ± 0.004
2,6-Dichlorophenolindophenol	0.040 ± 0.006	0.013 ± 0.002
Neutral red	0.681 ± 0.009	0.013 ± 0.003
Ferrocene	0.011 ± 0.005	0.023 ± 0.001
1,1'-Dimethylferrocene	0.013 ± 0.003	0.0038 ± 0.0009
Ferrocenecarboxaldehyde	0.077 ± 0.004	0.007 ± 0.001
Ferroceneacetonitrile	0.098 ± 0.005	0.0014 ± 0.0001

Table 2 Kinetic characteristics of the formed two-mediator systems

Mediator	Constant (k_1) of the interaction of the yeast <i>B. adeninivorans</i> with mediators, $\text{dm}^3/\text{g s}$	Constant (k_2) of the interaction of the mediator with ferrocene, $\text{dm}^3/\text{mol s}$	Constant (k_3) of the heterogeneous transfer of electrons to the electrode, cm s^{-1} (Kharkova et al. (2020))
Methylene blue	0.092 ± 0.004	1950 ± 90	0.025 ± 0.009
Thionine	0.123 ± 0.007	7792 ± 80	0.022 ± 0.005
Neutral red	0.681 ± 0.009	$14,200 \pm 100$	0.017 ± 0.005
Potassium hexacyanoferrate (III)	0.620 ± 0.008	$13,170 \pm 70$	0.0067 ± 0.0009
Ferrocene	0.011 ± 0.005	—	0.4 ± 0.1

**Fig. 3** Assumed mechanism for the operation of the working electrode of a two-mediator biosensor exemplified by the ferrocene–neutral red–*B. adeninivorans* cells system. NR_{ox} : oxidized form of neu-tral red; NR_r : reduced form of neutral red; FC_{ox} : oxidized form of ferrocene; FC_r : reduced form of ferrocene

slope of the linear regression (see Eq. (4)), the value of k_2 was found. Table 2 presents the kinetic characteristics of the formed two-mediator systems.

The presented results indicate that neutral red possesses the greatest constants of the interaction both with biomaterial (k_1) and with the ferrocene mediator (k_2), which enables a successive electron transfer in the two-mediator system (Fig. 3). Thus, three receptor systems based on the yeast *B. adeninivorans* were formed: two one-mediator systems based on neutral red and ferrocene, as well as one two-mediator system that included the joint use of these mediators.

Selection of the working parameters of the receptor systems

To develop mediator biosensors based on the yeast *B. adeninivorans*, it is necessary to select conditions under which the current generated by the biosensor is maximal. The current generated by the biosensor in the presence of a detected substance can change depending on conditions—the working parameters of the analytical system, at which the measurements are carried out; the concentration of the mediators;

the specific density of the biomass at the electrode; pH of the working electrolyte. To select the working parameters of one- and two-mediator receptor systems, we investigated the dependences of biosensor responses on the specific density of biomaterial at the electrode, the concentrations of the investigated mediators and pH of the working electrolyte and chose the values at which the maximal analytical signal was achieved. All experiments used the glucose solution concentration equal to 50 mmol/dm^3 .

The specific density of the biomass at the electrode is directly related to the value of the signal; however, an increase of the cell content in the biocatalytic layer can lead to diffusion limitations both for the used substrate and for the mediator. For this reason, it is important to select an optimal amount of biomaterial applied onto the electrode (Fig. 4).

At an increase of the specific density of the yeast on the electrode surface from 0.005 up to 0.01 mg/mm^2 , responses of a neutral red-based biosensor increase (Fig. 4a). This is due to the fact that the current of the electrocatalytic oxidation of glucose by the enzyme systems of the yeast is directly proportional to the concentration of the enzymes per electrode surface area unit (Ikeda et al. 1996). However,

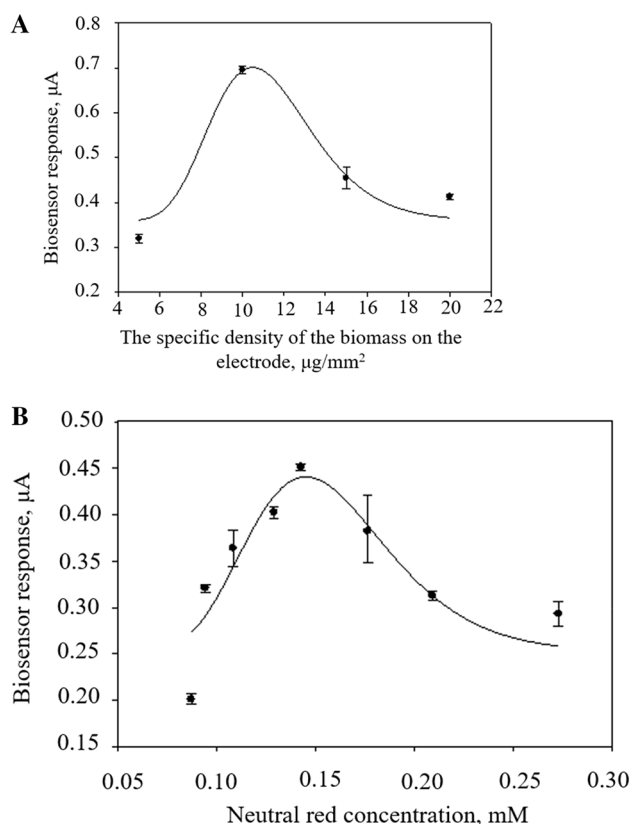


Fig. 4 Dependence of the response of a biosensor based on the mediator neutral red. On **a** the specific density of the biomass on the electrode; **b** the concentration of neutral red

at a further increase of the biocatalyst mass on the electrode surface, the biosensor responses decrease due to a diffusion limitation for the transport of substrate to the enzyme active centres owing to an increase of the thickness of the biocatalytic layer. Similar dependences were obtained in a ferrocene-based one-mediator system as well as in a ferrocene–neutral red two-mediator system. Proceeding from the maximum of the obtained dependence, the specific densities of the biomass at the electrode were chosen.

A high concentration of the mediator leads to an increase of the current generated by the biosensor; at the same time, a high content of the mediator in the graphite paste of the working electrode can change the current-conducting properties of the paste (Trosok et al. 2001) and have a toxic effect on biomaterial (Babkina et al. 2006). These conditions were determining in the choice of the working concentrations of the mediator in the investigated systems (Fig. 4b).

An important working parameter is pH of the buffer solution in which the measurements are carried out. This parameter affects the activity of the yeast enzymatic systems. In Babkina et al. (2006), it has been noted that the optimum pH of the mediator biosensor is determined not only by the properties of biomaterial but also depends on the chosen

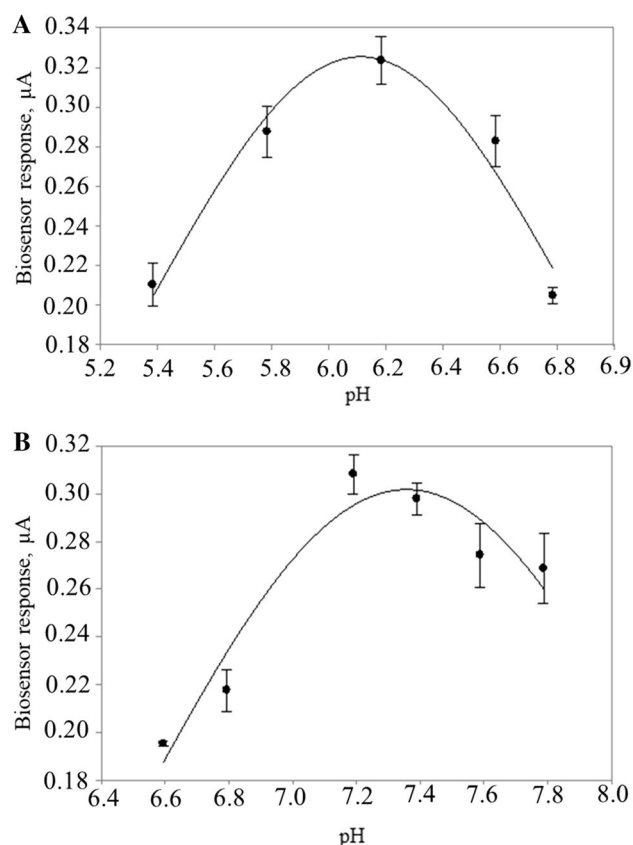


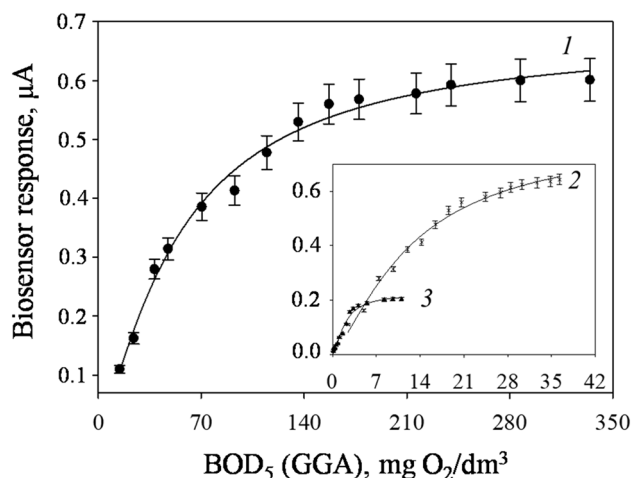
Fig. 5 Determination of the pH of the working electrolyte at the functioning of a ferrocene-based one-mediator system (**a**); of a two-mediator system (**b**)

mediator. The dependence of the value of biosensor response on pH of the medium was studied within the pH range from 5.6 to 7.8. Potassium–sodium–phosphate buffer solutions with various pH values, produced by varying the buffer system ratios, were used. Figure 5 presents dependences of mediator biosensors' responses on pH of the medium.

The redox transformation of neutral red involves two protons; this affects the sensitivity of the biosensor to changes of pH in the working electrolyte. The unprotonated form of neutral red is an efficient acceptor of electrons from biomaterial (Pasco et al. 2005); with account for acid–base interactions, an optimal value of pH using neutral red is 6.7–11.5 (Pauliukaite and Brett 2008), and the optimum pH for the oxidative activity of the yeasts is 6.0 (Bischoff et al. 2017). Thus, the close pH optimum values for neutral red and the used eukaryotes precondition the biosensor's generated response maximum at pH equal to 7.2, which was chosen as a working parameter for further research. As the electrode reaction of ferrocene in a two-mediator system occurs without the participation of protons, its effect on the shift of pH optimum is insignificant: an optimum is reached at pH equal to 6.2. Thus,

Table 3 Working parameters of BOD biosensors

Working parameter	Mediator system		
	Neutral red	Ferrocene	Ferrocene–neutral red
Specific density of biomass on the electrode, mg/mm ²	0.10	0.16	0.10
Concentration of mediator	0.14 mmol/dm ³	10%	Ferrocene 10% Neutral red 0.20 mmol/dm ³
Working pH of the buffer solution	7.2	6.2	7.2

**Fig. 6** Dependence of the biosensor response on the BOD₅ of the assayed sample: 1, one-mediator system based on the neutral red mediator; 2, one-mediator system based on the ferrocene mediator; 3, two-mediator system based on a ferrocene–neutral red combination of mediators

the working value of pH of mediator biosensors based on one- and two-mediator systems predominantly depends on the used biocatalyst—the yeast *B. adenivorans*—and the properties of neutral red.

The analytical potential of the investigated systems was further studied at the working parameters shown in Table 3.

Analytical and metrological characteristics of the developed mediator BOD biosensors

Calibration dependences of the analytical signal on the BOD₅ index for the investigated biosensors were obtained (Fig. 6).

The electrochemical response of the bioreceptor element based on whole cells is provided for by enzymatic reactions of microbial cells. Modelling within the framework of the Michaelis–Menten kinetics enables the use of Eq. (8) for approximating the produced curves:

$$R = \frac{R_{\max}[S]}{K_M + [S]}, \quad (8)$$

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

It follows from Eq. (8) that at low concentrations of substrate the analytical signal will be proportional to BOD₅, which makes it possible to single out a linear segment of the calibration curve limited from above by the value of K_M . The lower boundary of the linear segment was calculated by a statistical method, proceeding from the criterion of a relative standard deviation of the measurement results, $(\text{Sr}(\text{C})) < 0.33$. Table 4 presents the main analytical and metrological characteristics of one- and two-mediator biosensors based on the yeast *B. adenivorans*.

Based on the analysis of the metrological and analytical characteristics presented in Table 4, it can be concluded that the biosensor response is more stable when using the

Table 4 Characteristics of a biosensor based on the yeast *B. adenivorans* for one- and two-mediator systems

Characteristic	Neutral red	Ferrocene	Ferrocene–neutral red
Operational stability, %	7.6	6.6	1.5
Long-time stability, days	31	5	26
Single measurement time, min	4–5	4–5	4–5
Sensitivity coefficient, nA dm ³ /mg O ₂	5 ± 1	25 ± 6	52 ± 5
Linear range of determined BOD ₅ values, mg O ₂ /dm ³	14.5–71	2.5–21	0.16–2.7
Substrate specificity, number of oxidized substrates, of 32	27	23	30

two-mediator system—the relative standard deviation is 1.5%. With respect to the long-time stability, both biosensors operate for 30 days on average, which suggests a sufficiently long operational life of the biosensors without replacing the receptor element. The single assay time does not exceed 5 min, which enables increasing the assaying performance.

For the analysis of natural waters, where the BOD₅ value can be rather low to make ~2 mg/dm³ (clear natural waters), a one-mediator system based on neutral red is little suitable, because the lower boundary of the biosensor is rather high, 14.5 mg/dm³; the sensitivity of the one-mediator system based on ferrocene also restricts the analytical potential of the biosensor. It is most expedient to use a two-mediator system for the analysis of natural waters, because the lower boundary of the biosensor enables assessing lower BOD₅ values. Thus, the bioreceptor element based on the ferrocene–neutral red two-mediator system and the yeast *B. adeninivorans* is the most suitable for the development of a BOD biosensor with respect to such parameters as sensitivity and selectivity. For comparison, Table 5 presents some data concerning the known mediator BOD biosensors.

It is seen from the data of Table 5 that the developed mediator BOD biosensor based on a two-mediator ferrocene–neutral red system is not inferior than the known analogs: 0.16 mg O₂/dm³ is the lower limit of the developed two-mediator biosensor and 1 mg O₂/dm³ of the analogue presented in Galler et al. (2018). Thus, the proposed kinetic approach to the formation of a receptor system based on the yeast *B. adeninivorans* and the two-mediator ferrocene–neutral red system made it possible to form a more sensitive system exceeding by its characteristics one-mediator analogs, as well as that developed earlier for the yeast *D. hansenii* (Zaitseva et al. 2017), where the lower limit of 2.7 mg O₂/dm³ allows to carry out analysis of natural water samples for which the BOD value should not exceed 4 mg O₂/dm³. With the obtained value of the lower limit of the determined concentrations, the developed system can be used for the analysis of purer samples of natural water for which the BOD value should not exceed 2 mg O₂/dm³.

Analysis of water samples by the developed biosensor and by the standard BOD₅ detection method

Ten samples of surface waters were taken to test the two-mediator biosensor. Figure 7 shows the correlation between the BOD₅ values for the samples assayed by the biosensor and those determined by the standard dilution method.

The statistical treatment of the obtained data shows that the results of the standard dilution method and those using the biosensor differ insignificantly. The correlation coefficient was $R = 0.9693$ for ten water samples assayed. Thus, the developed two-mediator biosensor based on the yeast cells *B. adeninivorans* and the ferrocene–neutral red mediator system can be efficiently used for the analysis of various water samples.

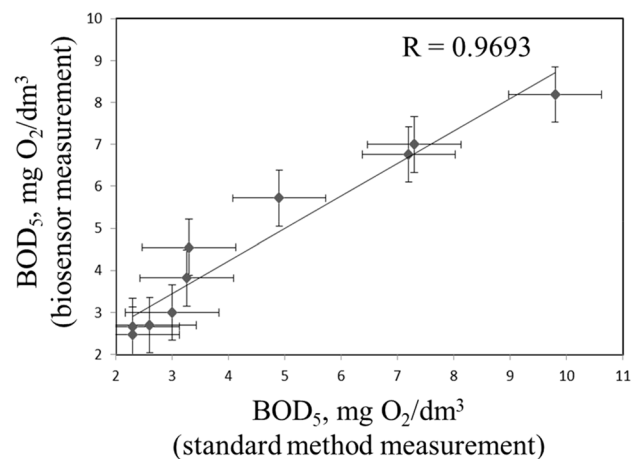


Fig. 7 Correlation of BOD₅ values, determined using a developed two-mediator biosensor based on ferrocene–neutral red and the yeast *B. adeninivorans*, and the values of BOD₅ determined by the standard method

Table 5 Developed microbial mediator biosensors and literature analogs

Microorganisms	Mediator	BOD ₅ linear range, mg O ₂ /dm ³	References
<i>B. adeninivorans</i>	Neutral red	14.5–71	This work
<i>B. adeninivorans</i>	Ferrocene	2.5–21	This work
<i>B. adeninivorans</i>	Ferrocene–neutral red	0.16–2.7	This work
<i>B. subtilis</i>	Potassium hexacyanoferrate(III)	4–60	Hu et al. (2017)
<i>P. aeruginosa</i>	Polyneutral red	3–100	Hu et al. (2015)
Activated sludge	Methylene blue	1–100	Niyomdechcha et al. (2017)
<i>C. violaceum</i>	Potassium hexacyanoferrate(III)	20–225	Hooi et al. (2015)
<i>P. yeii</i>	Ferrocene	1.3–360	Kharkova et al. (2019)
<i>D. hansenii</i>	Ferrocene–methylene blue	2.7–7.2	Zaitseva et al. (2017)

Conclusions

Thus, the constants of the interaction of graphite paste-immobilized ferrocene with phenazine mediators were obtained for the first time in the presence of *B. adeninivorans*. Based on the analysis of the obtained rate constants of the interaction of ferrocene and a number of water-soluble mediators (rate constants of the yeast *B. adeninivorans* the interaction with electron transport mediators and heterogeneous rate constants of electron transfer to the electrode), an approach to the development of two-mediator biosensor systems is proposed, which makes it possible to increase the efficiency of electron transfer from microorganisms to the electrode. The efficiency of the developed system was confirmed by the characteristics of the BOD biosensor, primarily its high sensitivity (lower limit of assayed concentrations, 0.16 mg/dm³ in the two-mediator system and 2.5 mg/dm³ in the single mediator system). High sensitivity of developed two-mediator biosensor allow carrying out analysis of natural water, for which the BOD value should not exceed 2 mg O₂/dm³. Analysis of natural water samples showed that the use of a two-mediator ferrocene-neutral red system and the yeast *B. adeninivorans* enabled the data that highly correlated with the results of the standard method ($R=0.9693$). The proposed combination of microorganisms and the mediator can be used to develop a BOD biosensor prototype with a sensitivity not inferior to that of the known analogs.

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Authors' contributions VAArl, ONP, VAAIf, ANR, conceptualization; ASK, ASI, VAArl, conducted the experiments; ASK, ASI, VAArl, ONP, data analysis; VAArl, ONP, VAAIf, ANR, wrote the manuscript with contribution from the other authors.

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Availability of data and materials The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflicts of interest The authors declare no conflicts of interest.

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