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BOD biosensor based on the yeast *Debaryomyces hansenii* immobilized in poly(vinyl alcohol) modified by N-vinylpyrrolidone

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ABSTRACT

An amperometric biosensor for assessing the biochemical oxygen demand (BOD) was formed by immobilizing *Debaryomyces hansenii* VKM Y-2482 yeast cells in poly(vinyl alcohol) modified by N-vinylpyrrolidone. Modification provided for a high sensitivity and stability of the bioreceptor. A high oxidative activity of the receptor element and the absence of any toxic effect of assayed compounds were shown for 34 substrates (alcohols, carbohydrates, carboxylic acids, amino acids, nitrophenols and surfactants) that may occur in wastewaters. Estimates of the measurement range and region of the linear dependence of signals on the BOD level, pH and temperature sensitivities, dependences of signals on concentrations of salts, stability, Michaelis kinetic constants and assay rates were obtained. The BOD values determined by the biosensor in assayed wastewater samples were shown to have a high correlation with those obtained by the standard dilution method.

1. Introduction

To date, a large number of laboratory models and commercially manufactured BOD biosensor analyzers have been described [1]. Biosensors enable BOD assays within the average range of 2–300 mg/l over a time of several minutes. However, numerous regularly published papers on this topic are an indication that characteristics, which would put a stop on further search, have not yet been obtained. Topical problems in the development of BOD sensors are assay sensitivity increase, larger lifetimes of biomaterial in receptor elements, complex requirements of maintenance and insufficient stability of applied microbial cultures with respect to heavy metals and various toxic substances. The issues described can be sorted out, on the one hand, by screening for microbial strains with broad substrate specificities and high resistance to toxic substances contained in wastewaters. On the other hand, search for new ways can be focused on the development of novel biomaterial-immobilization methods that would provide for long-time biosensor operation with high biocatalyst activity preserved.

Yeasts are the most preferable biomaterials for the development of BOD biosensors as they are resistant to negative environmental factors and can provide for long-time operation in biosensor's recognition element [2]. The most frequently used yeasts in BOD biosensor developments are of the genera *Trichosporon* [3], *Saccharomyces* and *Issatchenkia* [4]. Promising applications for BOD biosensors are yeasts of the genus *Debaryomyces*. According to available data, the yeast *Debaryomyces hansenii* has a broad substrate specificity and is capable of oxidizing many alcohols, carbohydrates, amino acids and other organic substances [5]. This yeast is also known to be resistant to high concentrations of salts. These features suggest that it can be efficiently used in the development of a BOD biosensor with a high measurement accuracy and resistance to negative environmental factors.

A promising immobilization technique for whole cells is the entrapment in gel based on poly(vinyl alcohol) (PVA). PVA is chemically and biologically stable, non-toxic and biocompatible [6], which determines its efficient use as a cell immobilization matrix. However, it is little suitable as the base of biosensor receptor elements, because thin films of gel have a low mechanical strength. There are methods of producing mechanically stable PVA gels by UV irradiation, by the action of boric acid solution [7] and co-polymerization with N-vinyl pyridine. However, those methods are associated with the use of reagents or reaction conditions negatively affecting living microorganisms and leading to a decrease of biosensor sensitivity. A novel approach in immobilization of microorganisms for fabricating biosensors' recognition elements is the use of N-vinylpyrrolidone for modification of PVA. N-vinylpyrrolidone is not only practically non-toxic but enhances the activity of some microorganisms. The use of N-vinylpyrrolidone

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for modification of PVA makes it possible to significantly increase the long-time stability of fabricated receptor elements with high sensitivity and broad substrate specificity preserved [8].

The aim of this work was to form and study the characteristics of an amperometric BOD biosensor based on the yeast strain *D. hansenii* VKM Y-2482 immobilized in poly(vinyl alcohol) modified by N-vinylpyrrolidone.

2. Materials and methods

2.1. Biosensor measurements

Electrochemical measurements were done using an EKSPERT-001-4.0.1 pH meter/ion meter/BOD thermo-oximeter (Ekoniks-ekspert, Russia) coupled to a personal computer operated by specialized software EXP2PR (Ekoniks-ekspert, Russia), which enabled recording and processing of sensor signals. The measured parameter (biosensor response) was the maximum rate of oxygen concentration change at the addition of substrates (mg/dm^3 min). The sensors were Clark oxygen electrodes (Kronas, Russia) with immobilized microbial cells. Measurements were carried out in a 5-ml cuvette. A sodium–potassium phosphate buffer solution (pH = 6.8), the salt concentration in which was 20 mM, was used for the measurements. The solution was mixed by a magnetic mixer (200 rpm). Samples were added by automatic variable-volume micropipettes (200–1000 μl, 20–200 μl, 0.5–10 μl; Biotech, USA). A mixture of glucose and glutamic acid (GGA) at a ratio of 1:1 (w/w) was taken as a model mixture; it is used as the standard in BOD_5 detection in the Russian Federation and in international practice. In accordance with the norms, BOD_5 equal to 205 mg/dm^3 was taken to correspond to a solution containing 150 mg/dm^3 of glucose and 150 mg/dm^3 of glutamic acid ($BOD_5 = 0.68 \times C_{GGA}$). All measurements were done in triplicate.

2.2. Cultivation of yeast biomass

Cells of the strain *D. hansenii* VKM Y-2482 were obtained from the All-Russian Collection of Microorganisms, Institute of Biochemistry and Microbiology of Microorganisms, Russian Academy of Sciences (Pushchino). The yeast was grown on a rich medium of the following composition: glucose, 10 g/dm^3; peptone, 5 g/dm^3; yeast extract, 0.5 g/dm^3; at a temperature of 28 °C as described in [5]. After cultivation, cells were harvested by centrifugation at 4500 rpm for 15 min and washed twice with a 20-mM sodium–potassium phosphate buffer solution, pH 6.8. Washed biomass was weighed and kept in micro test tubes at +4 °C.

2.3. Acquisition of yeast growth curves

The yeast growth curves were registered spectrophotometrically by measuring the dependence of the optical density of the culture liquid on time. Optical density measurements of the suspension were done on an SF-103 spectrophotometer (Akvilon, Russia) at a wavelength of 450 nm and cuvette thickness of 1 cm with respect to a cuvette with distilled water. The measurements were repeated each 2 h for 48 h.

2.4. Immobilization of D. hansenii cells in gel based on PVA modified by N-vinylpyrrolidone

To prepare PVA modified by N-vinylpyrrolidone, an aqueous solution of ceric ammonium nitrate $(NH_4)_2Ce(NO_3)_6$ and N-vinylpyrrolidone was added to an aqueous solution of PVA (molecular weight, 1×10^5–1.1×10^5 AWU) at constant stirring. The mixture was stirred at 40 °C for 3 h.

The immobilized catalyst was obtained by adding 36 mg of *D. hansenii* cells to 200 μl of PVA gel modified by N-vinylpyrrolidone. The uniform distribution of cells in gel was achieved by shaking on a CM70M centrifuge (ELMI, Latvia) for 5 min. The obtained suspension was transferred onto an object plate and dried for 24 h at room temperature. The film obtained was 15 μm thick; the specific density of immobilized cells was 5.8×10^{-5} g/dm^2. The immobilized biocatalyst was stored at a temperature of +4 °C. To form the biosensor, a fragment of obtained gel 4 mm in diameter was applied to the surface of an oxygen electrode and fixed by a capron (nylon-6) mesh.

2.5. Determination of BOD_5 by the standard dilution method

The dilution method was used as a reference method of BOD_5 determination. Analysis was done in accordance with the technique indicated in Federative Environmental Normative Documents. The content of dissolved oxygen in analyzed samples was determined by the Winkler's iodometric method in accordance with the standard technique.

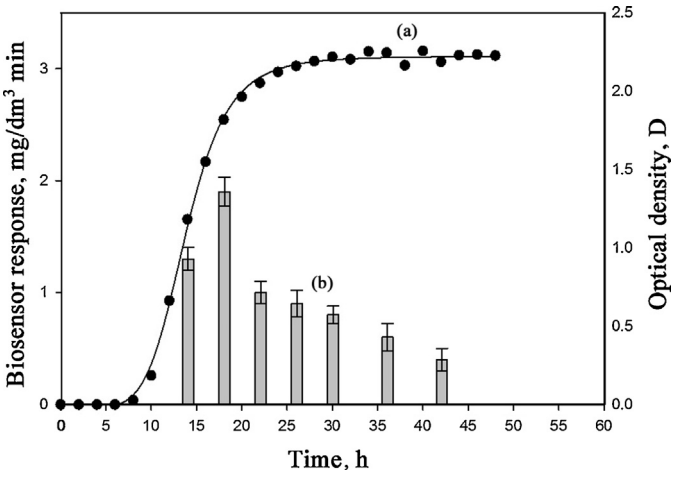

Fig. 1. (a) A growth curve obtained spectrophotometrically for the yeast *D. hansenii*; (b) a dependence of the oxidative activity of the yeast *D. hansenii* on growth time (a GGA solution at a concentration of 500 mg/dm^3 was used as substrate).

3. Results and discussion

3.1. Development of the BOD biosensor receptor element

The receptor element of a high-sensitivity biosensor should be developed using microorganisms with a high oxidative activity. Cultivation time is the main factor affecting the activity of microorganisms. To choose an optimal cultivation time for the strain *D. hansenii*, we analyzed the dependence of the activity of cells constituting the receptor element on growth time (Fig. 1).

From the presented data the maximal activity of the yeast microorganisms is seen to occur at cultivation for 18 h, which corresponds to the end of the linear growth phase and the start of the growth slowdown phase.

Gel of poly(vinyl alcohol) modified by N-vinylpyrrolidone was used to immobilize microorganisms. Modification was in the presence of ceric ammonium nitrate as initiator; it was chosen based on the literature data in analogy with the described PVA–NVP interaction reaction [9]. The assumed radical copolymerization mechanism with Ce^{4+} ions as initiator comprises several stages (Fig. 2). At the first stage, cerium ions are reduced to form oxyl radicals (Fig. 2(a)). Further on, oxyl radicals formed at the first stage interact with N-vinylpyrrolidone to form polymer radicals (Fig. 2(b)). The cross-linking occurs due to the recombination reaction of polymer radicals formed (Fig. 2(c)).

The synthesis conditions do not rule out the formation of branched polymer in the interaction of polymer radicals with N-vinylpyrrolidone, either. The mechanism of PVA–NVP copolymerization is described in detail in [8].

To determine the optimal amount of cells in gel mass, receptor elements with cell weight of 10–300 mg per 1 ml gel were formed (specific density of cells in gel, 0.5–12 mg/cm^2). It was found that, to form a receptor element, it is sufficient to incorporate 180 mg of *D. hansenii* cells into 1 ml of chemically modified PVA; the specific density of cells is in this case 5.8 mg/cm^2. At an increase of the microbial content in gel the activity of the receptor element does not practically change.

3.2. Determination of the characteristics of a BOD biosensor based on the developed receptor element

An important characteristic of the assay is its selectivity, i.e., the possibility to determine each component of an analyzed item independently of the others. In the case of the biosensor assay, the

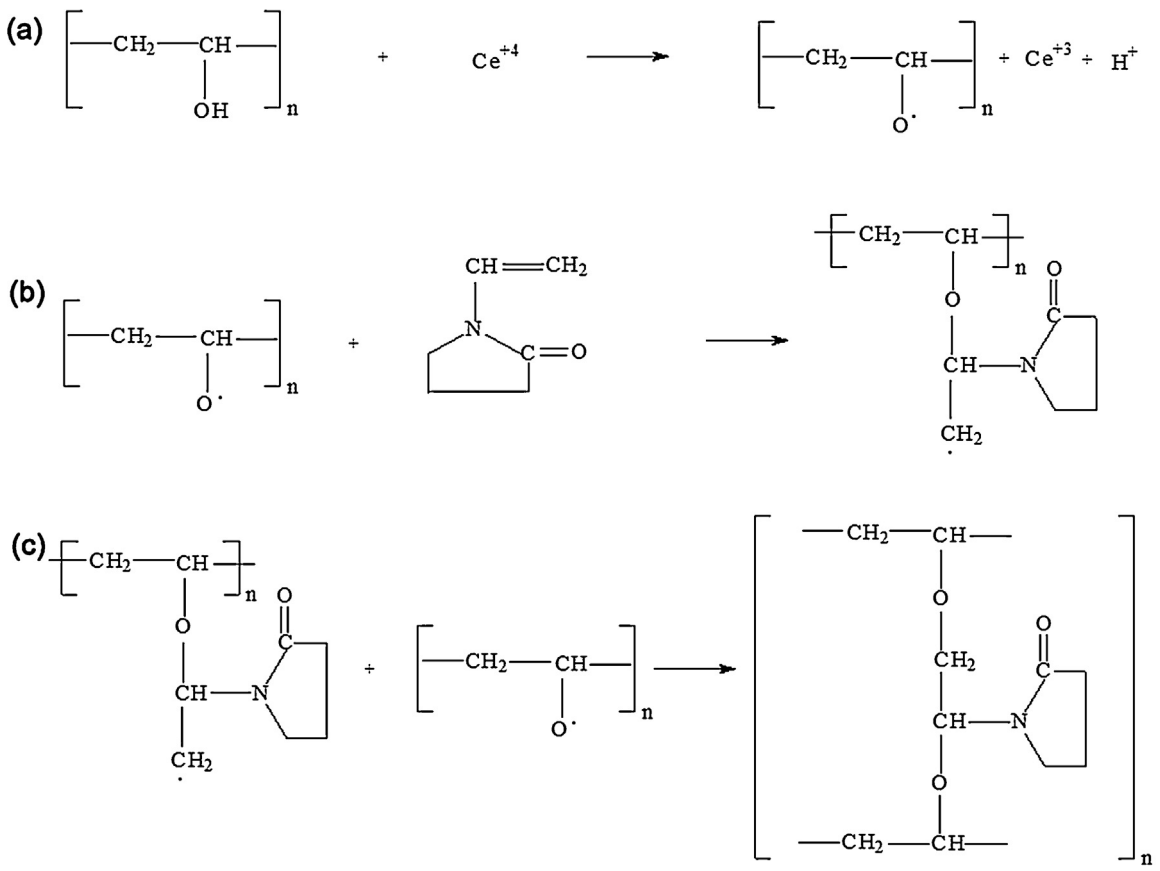

Fig. 2. The assumed mechanism of radical co-polymerization of PVA and N-vinylpyrrolidone.

selectivity is determined by the substrate specificity of biomaterial used to form the sensor's receptor element. When developing the receptor element of a biosensor for BOD assays, it is preferable to use whole microbial cells possessing a broad substrate specificity (low selectivity). Herewith, the broad substrate specificity is an advantage, as it leads to increase the correctness of BOD assay results. We assessed the substrate specificity of *D. hansenii* immobilized in a N-vinylpyrrolidone-modified PVA film with respect to 34 substrates from various classes of organic compounds. Mainly readily oxidizable organic compounds, whose occurrence in water bodies leads to a significant decrease of dissolved oxygen and further eutrophication, were chosen as substrates [10]. Fig. 3 presents

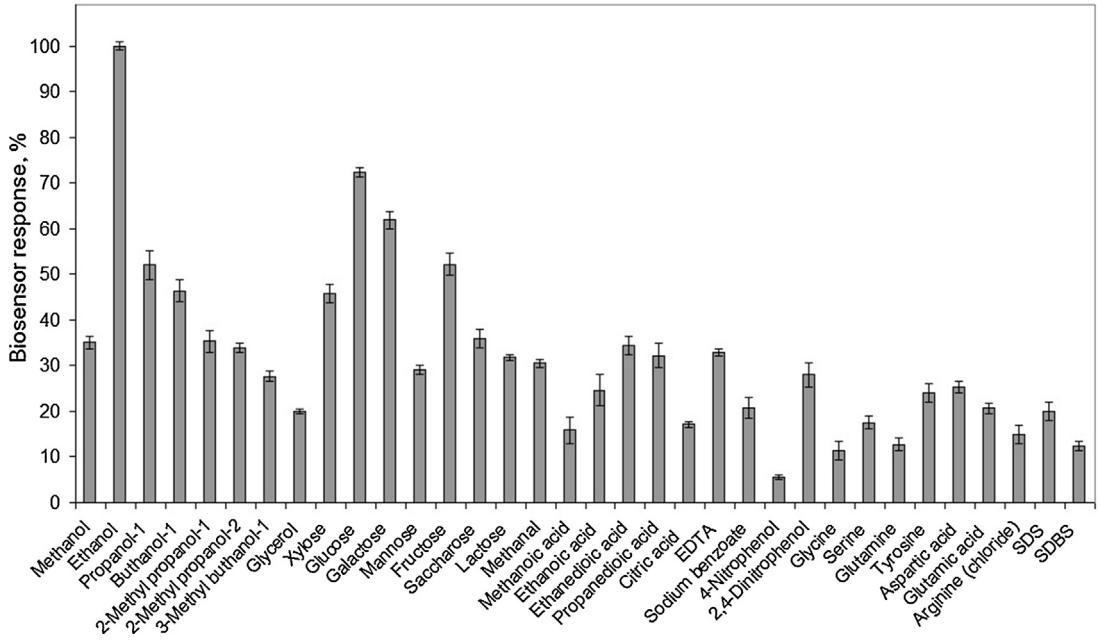

Fig. 3. Substrate specificity of a biosensor based on the yeast *D. hansenii* immobilized in a PVA film modified by N-vinylpyrrolidone (substrate concentration, 0.04 mol/dm^3).

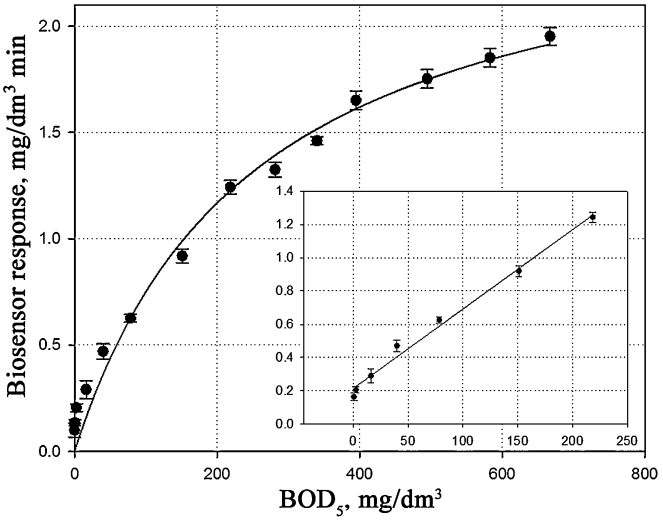

Fig. 4. A dependence of the response of the developed biosensor on BOD_5 in a measuring cuvette.

data on substrate specificity of the biosensor based on the yeast *D. hansenii*.

The maximal signal was obtained for ethanol, the response to it was taken to be 100%. The yeast cells oxidize substances of all represented classes of organic compounds: alcohols, carbohydrates, carboxylic acids, amino acids, nitrophenols and surfactants that can be found in wastewaters. Valuable from the practical point of view are responses to sodium dodecyl sulfate, sodium dodecylbenzene sulfonate (components of detergents) and nitrophenols (widespread commercial toxicants), as well as no toxic action of these substrates at their short-time effect on the immobilized yeast *D. hansenii*. The obtained results suggest that BOD assays of real-life samples would ensure a high degree of correlation between the readings of the developed biosensor and the standard method.

A graduation dependence of biosensor response on BOD_5 in a measuring cuvette is given in Fig. 4.

Bioreceptors based on whole microbial cells are of catalytic type, i.e., the biological response in such systems is provided for by microbial enzyme reactions. Thus, the dependences shown in Fig. 4 are well approximated by a Michaelis–Menten-type equation:

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

where $R_{\max}$ is the maximal rate of oxygen uptake by immobilized microorganisms achieved at $[S] \to \infty$; K_M is the effective Michaelis constant, i.e., the substrate concentration at which $R = R_{\max}/2$.

To reduce the error of the assay, use is made, as a rule, of the linear segment of the graduation dependence bounded from above by K_M (207 ± 8 mg/dm^3). The lower boundary of the linear segment corresponds to the lower boundary of the determined contents and was calculated by the statistical method, proceeding from the criterion of the value of the relative standard deviation of the measurement results ($S_r(C)$) < 0.33. The lower boundary of the determined BOD_5 values was 0.7 ± 0.1 mg/dm^3. Thus, the linear segment of the dependence of biosensor response on BOD_5 is within the limits of 0.7–207 mg/dm^3.

Table 1 presents the major analytical and metrological characteristics of a biosensor based on the yeast *D. hansenii*. The characteristics of the developed biosensor were determined using a model GGA mixture.

In this way, the use of a novel carrier for immobilization of the yeast *D. hansenii* made it possible to improve the earlier described [5] major characteristics of the biosensor based on this yeast. Thus, the lower limit of the determined BOD_5 contents became more than 3 times lower, and the duration of a single measurement more than 2 times smaller as compared with a similar biosensor based on *D. hansenii* immobilized by adsorption.

It is important to note that by its analytical and metrological characteristics the developed biosensor is not only competitive with world analogs [11,12] but partially surpasses them. Thus, it has a broader range of assayed BOD_5 values as compared with described prototypes [13,14]. Besides, a single BOD assay by means of the developed analyzer requires less time compared with BOD analysis by other biosensor models [2,15,16].

3.3. Effect of the composition of assayed samples and assay conditions on BOD biosensor operation

Wastewaters of industrial enterprises can contain not only organic compounds oxidized by microorganisms contained in the bioreceptor, but also inorganic substances that affect negatively the oxidative microbial activities. For this reason, when developing a BOD biosensor, it is essential to study the effects of negative environmental factors on its operation.

pH of the medium is one of the factors that affect the activity of cell enzymes and sensitivity of bioreceptor to various substrates. Responses of biosensors to the standard GGA solution were obtained within the range of pH 5.4–7.8 (Fig. 5). The maximal response of the biosensor based on the yeast *D. hansenii* was observed within the range of pH 6.8–7.2.

The dependence of biosensor response on ionic strength of solution reflects the ability of cells to oxidize substrates in media with high osmotic pressure. It is known that the yeast *D. hansenii* is a typical moderate halophile and is capable of growth at salt concentrations of up to 2 mol/dm^3 [17]. To establish an ionic strength, we used a phosphate buffer system with pH 6.8 and a total salt concentration range of 16.5–132 mM (Fig. 6). At an eightfold increase of the concentration of buffer solution the decrease of responses was 50% of the initial value, which indicates the availability of a

Table 1
Characteristics of a biosensor based on *D. hansenii* microorganisms.

Characteristic	Description	Value	Units
Operational stability	Relative standard deviation by 15 consecutive measurements	4.2	%
Long-time stability	Time within which the value of biosensor response to the same concentration of GGA was no less than 25% of the initial value, temperature 20 °C	30	Days
Sensitivity	Slope of the linear segment of sensor response from BOD5	0.0045 ± 0.0003	min^{-1}
Duration of single measurement	Response development time	1	min
	Receptor element washing time	4–6	min
	Duration of single measurement	5–7	min
Range of determined BOD5 contents	–	0.7–206.7	mg/dm^3

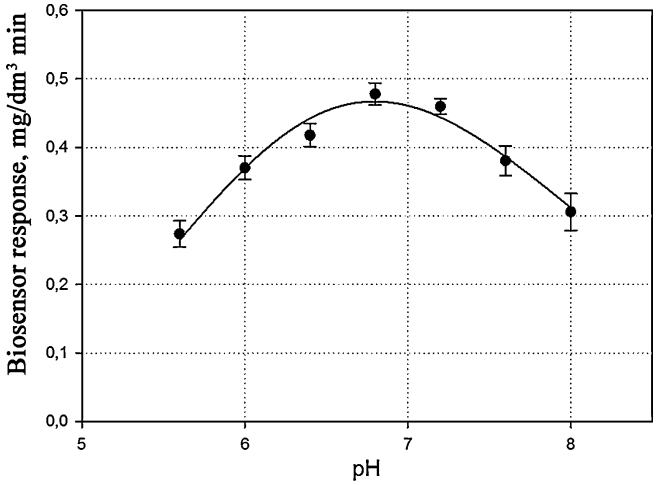

Fig. 5. Dependence of the response of a *D. hansenii*-based biosensor on pH.

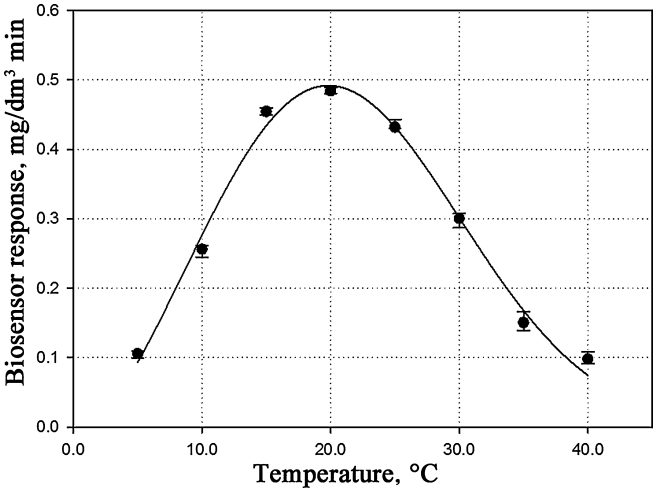

Fig. 7. Dependence of the response of a biosensor based on the yeast *D. hansenii* on temperature.

sufficiently efficient system for regulation of intracellular osmotic pressure in the yeast *D. hansenii*.

Temperature is an important factor of vital activities for all yeast cells. For each of the diverse functions of yeast cells – respiration, fermentation, growth – there are optimal, minimal and maximal temperature conditions. This work investigated the character of biosensor-response change at a temperature change from 5 °C to 40 °C (Fig. 7).

The maximal response of a biosensor based on the yeast *D. hansenii* is observed within the temperature range of 15–25 °C. Thus, the developed biosensor can be efficiently operated at room temperature without any thermostatting equipment, which is an evident advantage as compared with a number of described BOD biosensors [2,18].

The main factor that can reduce the biosensor response and even lead to the death of bioreceptor is the occurrence of heavy metal ions in wastewaters. Heavy metal ions have both bacteriostatic and bactericidal action. The mechanisms of their interaction with enzymes are diverse: substitution of physiologically significant cations and nonmetal-containing oxy anions in enzymes' active sites, binding of functional sulfhydryl groups etc. To study the inhibitory action of heavy metal compounds, we investigated the dependence of the oxidative ability of the yeast *D. hansenii* in the presence of Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Bi^{3+}, Sn^{2+}, Hg^{3+}, Cr^{3+} and $Cr_2O_7^{2-}$ ions in solution within the range of concentrations exceeding the maximally permissible concentrations (MPC) in fishery water bodies 10–100 times [19].

The greatest effect on the respiratory activity of the yeast *D. hansenii* is rendered by the presence of Fe^{3+}, Sn^{2+} and Pb^{2+} in solution; the decrease of responses at a 100-fold excess of the MPC was, respectively, 85, 77 and 72%. The presence of Fe^{2+}, Zn^{2+}, Cd^{2+}, Bi^{3+} and Cr^{3+} salts in the system evokes a twofold decrease of sensor responses. Ni^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} ions occurring in the system at a 100-fold excess with respect to the MPC reduce the response of immobilized yeast by 30–40%. At a 10-fold excess of the MPC for Pb^{2+} ions the decrease of responses was 53%; for all the other heavy metal ions investigated the decrease of responses is no more than 20%, which can indicate a stress resistance of the yeast *D. hansenii*. The stability of particular enzymes and activation of enzyme systems responsible for stress metabolism can be one of the possible causes of low susceptibility of the yeast to the excess of heavy metals.

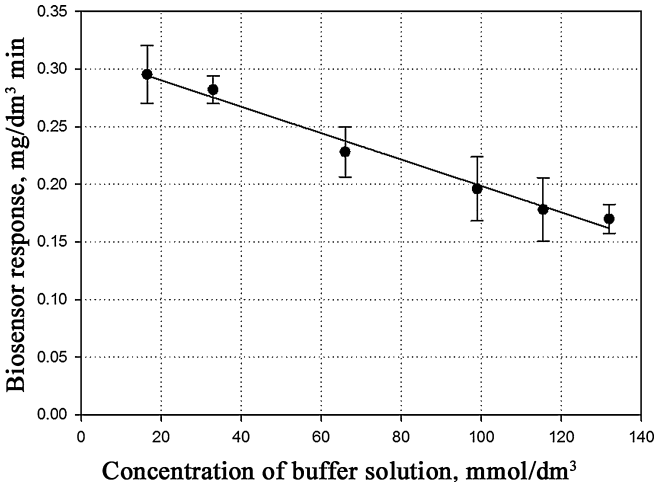

Fig. 6. Dependence of the response of a *D. hansenii*-based biosensor on the ionic strength of solution.

3.4. Analysis of wastewater samples

Samples for BOD_5 analysis were taken at the Federal State Healthcare Institution "Center of Hygiene and Epidemiology in Tula Region". The samples represented wastewaters of municipal water treatment facilities at various stages of purification, wastewaters of the food factory and snowmelt from the territory of the metal recycling plant. Sampling was done in accordance with the standard protocol [20]. Determination of BOD_5 in wastewaters by the standard dilution method was carried out according to the norms acting in the Russian Federation. The measurement error in the standard method is 10%. When determining BOD_5 in wastewater samples using the developed biosensor, a sample was preliminarily diluted. The dilution value was chosen such that the sensor response should be inside the linear segment of the calibration dependence (Fig. 4). Fig. 8 shows a correlation between the BOD values determined by means of the biosensor and those assayed by the standard dilution method ($R^2 = 0.9911$).

Thus, the values of BOD_5 determined using the biosensor based on *D. hansenii* cells coincide with the BOD_5 values obtained by the standard method with account for the confidence interval.

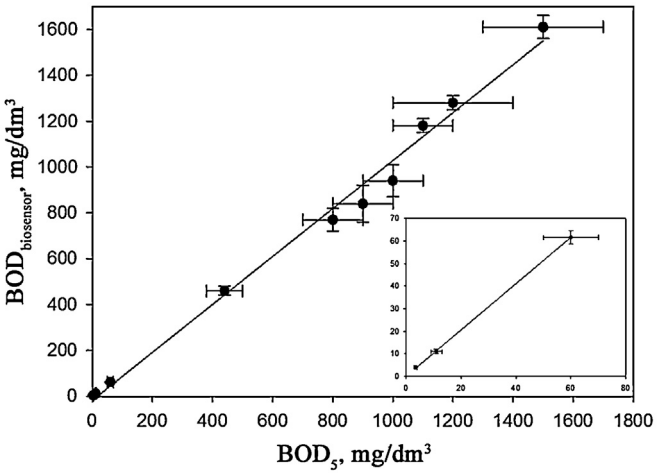

Fig. 8. Correlation between the BOD values determined using the developed biosensor and those determined by the standard method.

4. Conclusion

We developed a BOD biosensor based on the yeast *D. hansenii* immobilized in PVA modified by N-vinylpyrrolidone. The yeast was shown to possess a broad substrate specificity, was capable of oxidizing substrates in various classes of organic compounds. The range of BOD concentrations assayed by the developed biosensor was 0.7–207 mg/dm^3.

Effects of the compositions of samples and assay conditions studied (pH, ionic strength, temperature, heavy metal compounds) on biosensor signals were investigated. The biosensor response was observed to be maximal within the range of pH 6.8–7.2 and temperature 15–25 °C. At a maximum permissible concentrations exceeded 10-fold for all investigated heavy metal ions, except Pb^{2+}, responses decreased by no more than 20%, which can be indicative of the stress resistance of the yeast *D. hansenii* to the given type of toxicants.

The BOD index for wastewaters of water-treatment facilities was determined. The use of *D. hansenii* yeast cells immobilized in N-vinylpyrrolidine-modified PVA as the base of biosensor's receptor element for assaying the wastewater BOD in the treatment facilities, was found to yield data highly correlating with the data of the standard method.

Thus, the use of *D. hansenii* yeast immobilized in PVA modified by N-vinylpyrrolidone made it possible to develop a BOD biosensor with high performance characteristics. The results indicate a possibility of using the developed biosensor analyzer as a prototype pilot model of devices for commercial production.

Acknowledgements

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