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Biosensor analyzer for BOD index express control on the basis of the yeast microorganisms *Candida maltosa*, *Candida blankii*, and *Debaryomyces hansenii*

Viacheslav Arlyapov[a], Stanislav Kamanin[a], Olga Ponamoreva[a], Anatoly Reshetilov[b,*]

[a] *Tula State University, Pr. Lenina 92, Tula, 300600 Russia*
[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pr. Nauki 5, Pushchino, Moscow Region, 142290 Russia*

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ABSTRACT

The parameters of biosensors based on the yeast strains *Candida maltosa* VKM Y-2359, *Candida blankii* VKM Y-2675, and *Debaryomyces hansenii* VKM Y-2482 for biochemical oxygen demand (BOD) detection are compared. The catalytic activity of the strains was analyzed in relation to the growth phase. The possibility of using *D. hansenii* as a basis for receptor element of a biosensor for BOD detection in municipal and biotechnological wastewaters was shown.

1. Introduction

The most important integral characteristic of water quality is biochemical oxygen demand (BOD), i.e., the amount of dissolved oxygen required for oxidation of all biodegradable organic compounds [1]. BOD assessment is an empirical test employing the standard laboratory procedure for determining oxygen uptake in analyzed water samples. The conventional method of BOD detection requires incubation of an oxygen-saturated sample for at least 5 days [2]. Due to considerable time it takes, the method is not adequate under modern conditions, because the results of analysis are made available with a significant delay. Hence, environmentally hazardous situations can occur, when an emergency inflow of polluted waters to water treatment facilities passes unnoticed or, the other way round, they are insufficiently purified in the course of regeneration.

Alternatives are express methods of BOD detection by biosensor analyzers based on microorganisms that can metabolize a wide range of organic compounds [3]. At present, commercially produced biosensor analyzers detect BOD within a few minutes in the range of 2–500 mg/dm^3 [4–6].

The bioreceptor elements of BOD sensors are based on either pure cultures with certain functional properties (a wide range of oxidized substrates, resistance to negative environmental factors, or specificity to certain wastes), or a mixture of identified microorganisms (artificial associations), or activated sludge. Each of these approaches has its own advantages and disadvantages. Yeasts are the preferable biomaterial for nearly all types of biosensors as they oxidize a broad spectrum of substances, are resistant to negative environmental factors, and can function in the recognizing biosensor element for a long time. Thus, a sensor based on the yeast *Saccharomyces cerevisiae* encapsulated in calcium alginate was described in [7]. The sensor was characterized by a satisfactory stability and was used for the analysis of wastewater samples. A good correlation between BOD values determined by the biosensor and by the standard BOD_5 approach was shown. In [8], a BOD sensor based on the yeast *Arxula adeninovorans* immobilized in a polyurethane-based polymer, polycarbomoyl sulfonate, was used for BOD detection in municipal and industrial wastewaters from different sources, including wastes with a high salt content. Application of various yeast strains for BOD sensors development is not confined to these examples [9–11].

Development of biosensor methods of analysis places demands on the quality of BOD sensors and accuracy of measurements, which primarily depend on the properties of bioreceptor elements. The promising research trend is to examine the possibility of using bioreceptor elements that would improve measurement quality. This work used the following yeast strains: *Candida maltosa* VKM Y-2359, *Candida blankii* VKM Y-2675, and *Debaryomyces hansenii* VKM Y-2482. According to the literature data, the yeasts *C. maltosa*, *C. blankii*, and *D. hansenii* have a broad substrate specificity and can oxidize many alcohols, carbohydrates, amino acids, and

* Corresponding author. Fax: +7 495 956 33 70.
E-mail addresses: gwinbleidd@rambler.ru (V. Arlyapov), gwinbleidd@rambler.ru (S. Kamanin), olga@tsu.tula.ru (O. Ponamoreva), anatol@ibpm.pushchino.ru (A. Reshetilov).

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other organic substances. The strain *C. maltosa* VKM Y-2359 is resistant to heavy metals, in particular, chromates, and is used for biological purification of petroleum products from pollutants [12,13]. It is known that the yeast *D. hansenii* is resistant to high salt concentrations [14]. The above peculiarities suggest efficient application of these microorganisms in the development of BOD sensors that would have high accuracy and resistance to negative environmental factors. Still, the parameters of biosensors with joint application of these strains have not been considered in the literature.

This work considered the parameters of biosensors based on each of the strains, which enabled their comparative assessment. Adsorption was used as a method of immobilization preserving the native state of metabolic characteristics of the strains to a high degree. Activity of the strains was considered in relation to the growth phase. Glucose–glutamate mixture was used as a calibration standard. The possibility of using *D. hansenii* as a basis of receptor element of the biosensor for BOD detection in municipal and biotechnological wastewaters was demonstrated.

2. Materials and methods

2.1. Biosensor measurements

The multi-channel flow-injection biosensor analyzer used in this work was controlled by a PC. Sensor signals were recorded and processed by special software (Kronas, Russia). The sensors were Clark oxygen electrodes with immobilized microbial cells. The mean current magnitude corresponding to an oxygen content of 9.2 mg/dm^3 in distilled water was 30 nA at a noise ±0.25 nA.

All measurements were made in the flow mode at a flow rate of 0.5 cm^3/min and sample volume of 300 μl. The primary data processing included smoothing and subsequent differentiation of the curve for $I(t)$. Sensor response was calculated as a maximum rate of current variation in time.

The measurements were made in a sodium–potassium phosphate buffer (pH 6.8); salt concentration, 20 mM. Samples were added by variable-volume automatic micropipettes (200–1000 μl, 20–200 μl, and 0.5–10 μl) (Biotech, USA). A mixture of glucose and glutamic-acid sodium salt (GGA) applied as a standard in BOD_5 detection was used for calibration [2]. In accordance with the definition, 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.

2.2. Cultivation of microbial cells

Cells of *C. maltosa* VKM Y-2359, *C. blankii* VKM Y-2675, and *D. hansenii* VKM Y-2482 were obtained from the All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino).

Yeast microorganisms were grown in a rich mineral medium (liquid glucose–peptone nutrient medium). The cultivation medium was prepared by the standard method for growing yeast strains. The procedure is based on the application of glucose. Peptone and yeast extract were used as a nitrogen source. The medium contained: glucose, 10 g/dm^3; peptone, 5 g/dm^3; yeast extract, 0.5 g/dm^3 (Sigma, USA); distilled water, 200 cm^3. Cell growth medium was sterilized by autoclaving at 1 atm for 45 min. Cells were grown aerobically for 18–20 h in 750 cm^3 shaken flasks at 29 °C. Then the biomass was centrifuged at room temperature at 8000 rpm for 10 min. The centrifugate was washed with 20 mM phosphate buffer, pH 6.8. Sedimented cells were suspended in a fresh portion of the buffer, distributed by portions, and sedimented in an Eppendorf centrifuge for 3 min at 8000 rpm. The washed biomass was weighed and stored in micro test tubes at +4 °C.

2.3. Formation of the receptor element

The grown microbial cells were washed with phosphate buffer solution (20 mM, pH 6.8) and centrifuged (2000 rpm, 10 min). The wet cell sediment was weighed and diluted with a certain quantity of buffer solution to a concentration of 150 mg/cm^3. The obtained mixture was applied to a Whatman GF/A glass-fiber filter (Sigma, USA) in the amount of 3 μl. The element (3 × 3 mm^2) was dried on air for 15 min at 20 °C. The prepared bioreceptor element was placed onto the surface of a Clark-type oxygen electrode and fixed with a nylon mesh.

2.4. Quantification of viable microbial cells

The colony-forming units (CFU) (living cell concentrations) in a suspension were quantified by the method of standard serial dilutions. Test tubes were filled with 4.5 ml each of sterile saline solution. The first dilution was done as follows: 0.5 ml of the initial cell suspension (null dilution) was transferred into the first test tube and mixed by a mixer (Heidolph, Germany). At the second dilution, 0.5 ml of the solution from the first tube was poured into the second tube, and then the third etc. dilution was done. Further on, the suspension from a test tube with required dilution was inoculated on Petri dishes with rich nutrient medium (LB agar). For this purpose, 0.1 ml of the suspension was applied to a dish and pounded with a curved glass rod (Drigalsky spatula), spreading the liquid evenly over the agar surface. Immediately before use, the spatula was dipped into 96% ethanol, passed through the flame of a Bunsen burner, and then cooled on the Petri dish cover.

The dishes were placed into a thermal room at a temperature optimal for microbial growth (24 °C) for 24–48 h; then the grown colonies were counted and the concentration of microbial cells in the initial suspension was calculated.

Table 1
The growth phases of the yeasts *Candida maltosa*, *Candida blankii*, and *Debaryomyces hansenii*.

Growth phase	*C. maltosa*	*C. blankii*	*D. hansenii*
Lag phase (h)	0–8	0–14	0–18
Exponential phase (h)	8–20	14–38	18–30
Stationary phase (h)	20–30	38–42	30–40
Death phase (h)	30–48	42–48	40–48
Maximum oxidative activity (h)	14	18	24

2.5. BOD_5 detection by the standard method of dilution

The method of dilution was used as a reference method for BOD_5 detection. The analysis was carried out strictly according to the technique given in [2]. The content of dissolved oxygen in samples under study was determined by Winkler's iodometric method according to the standard procedure.

3. Results and discussion

3.1. Dependence of the oxidative activity of the used yeast microorganisms on growth time

Microorganisms with high oxidative activity should be used for developing biosensors capable of giving reliable and reproducible results at low BOD values. The oxidative activity of microorganisms may vary depending on the growth period; for this reason, to obtain microorganisms with high oxidative activities, it was of interest to study the growth dynamics of the yeasts under study.

The dependence of the CFU number on cultivation time is of shape typical of yeast strains [15]. Several phases can be distinguished in the growth of the strains studied (Table 1).

Comparison of the growth dynamics for the three yeast strains shows that the yeasts *D. hansenii* and *C. blankii* have longer lag phases and exponential growth phases, resulting in a later transition to the stationary phase as compared with *C. maltosa*.

The oxidative activities of the yeast strains were studied by the electrochemical method of quantitative determination of substrate oxidation intensity based on the registration of oxygen content in the liquid medium by the Clark electrode. The principle of measurement by the oxygen electrode is based on the registration of current obtained in molecular oxygen reduction. Current strength is proportional to oxygen concentration in the analyzed sample. The change in oxygen concentration recorded by such an electrode characterizes the respiratory activity of microorganisms immobilized on the electrode surface.

To determine the dependence of the oxidative activity of the yeast strains on growth time, cell suspension samples were taken at equal time intervals, centrifuged, and washed three times with the buffer solution. The washed cells were immobilized and applied to the electrode. The glucose and glutamic acid mixture (GGA) was used as a standard solution. All three yeast strains showed the highest oxidative activity at the end of the exponential growth phase or at the beginning of the linear phase. All microorganisms were grown with the maximum oxidative activity during the time indicated in Table 1.

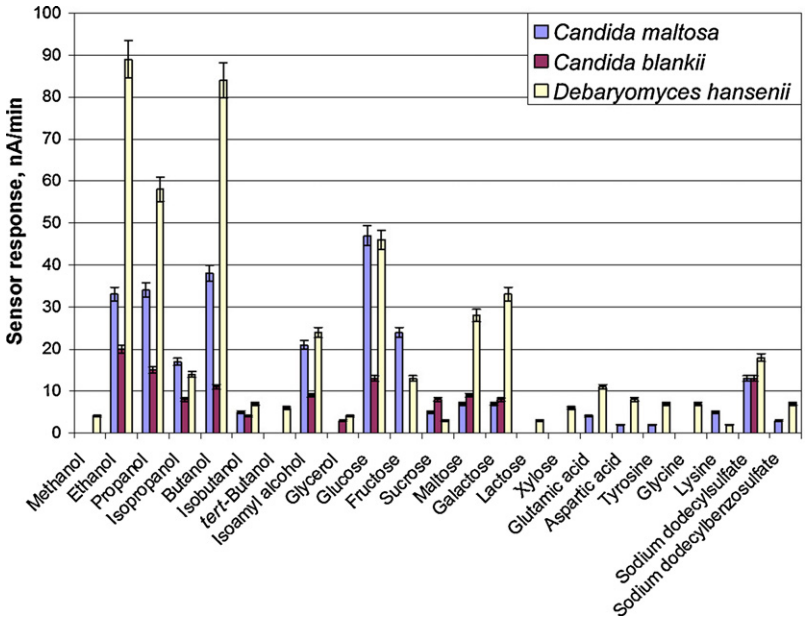

Fig. 1. Substrate specificity of receptor elements based on the strains under study (concentration of substrates, 0.09 mol/dm^3).

3.2. Characteristics of yeast-based BOD biosensors

One of the important characteristics of an assay is its selectivity, i.e., the possibility of detecting each component of an analyte irrespective of the other components. In the case of a biosensor assay, selectivity is determined by substrate specificity of biomaterial used in the receptor element. For BOD detection, it is preferable to use whole cells of microorganisms with broad substrate specificity (low selectivity) as a receptor element. Broad substrate specificity is in this case an advantage, as it increases the accuracy of the results and their identity to the standard BOD_5 value. The substrate specificity of receptor elements based on *C. maltosa*, *C. blankii*, and *D. hansenii* was estimated for 20 different substrates. The substrates were easily oxidizable organic substances considerably decreasing the level of dissolved oxygen in water reservoirs resulting in their eutrophication [1]. For estimation of substrate specificity, biosensor response was recorded during the introduction of equal amounts of substrates into the measuring cell. Fig. 1 shows the data on the substrate specificities of receptor elements based on adsorbed *C. maltosa*, *C. blankii*, and *D. hansenii*. It could be noted that a comparison with the described biosensors by the given parameter seems to be problematic, because substrate specificity is presented very rarely. The specificities given here characterize the ability of cells to oxidize a wide range of compounds. This extends the capabilities of their use in biosensors. Various biosensors can be used for different types of wastewaters. This approach provides for more reliable measurements.

The *D. hansenii* based sensor has the broadest substrate specificity and can oxidize all substances under study. The practically valuable facts are the presence of responses to sodium dodecylsulfate (SDS) and sodium dodecylbenzosulfate (SDBS) (components of detergents) and the absence of a toxic effect of these substrates on bioreceptor microorganisms during a short-time contact.

These results suggest that BOD detection in real samples can provide for a high degree of correlation between the readings of biosensors and the standard method, because all three microbial strains can oxidize a wide range of organic substances.

The calibration dependence of the response on analyte concentration is the most important metrological characteristic of a sensor. We obtained the calibration dependences of biosensor response on GGA concentration in the measuring cell for biosensors based on each receptor element (Fig. 2).

Bioreceptors based on intact microbial cells are catalytic bioreceptors, i.e., the biological response in such systems is provided for by enzymatic reactions of microorganisms. The dependences shown in Fig. 2 are well approximated by the Michaelis–Menten equation:

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

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

Analytical errors can be lowered by using only the linear region of the calibration curve. The upper limit of the linear region is limited by the K_M constant, because the response depends linearly on substrate concentration only in the vicinity of the calibration point corresponding to the K_M value. The dependence of BOD_5 on biosensor responses (R) for the linear region of the calibration

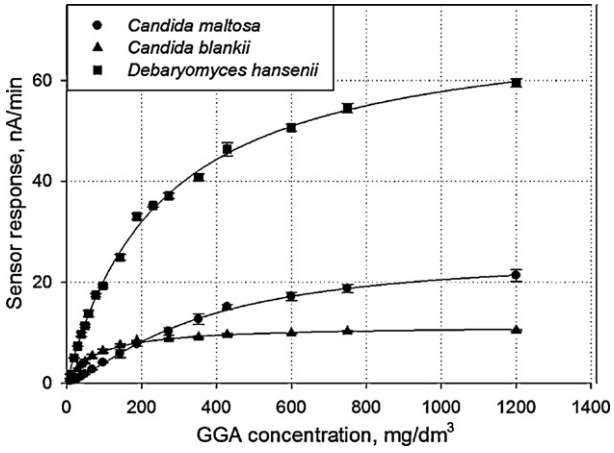

Fig. 2. Dependences of responses on GGA concentrations for biosensors based on the developed receptor elements.

Table 2
Characteristics of BOD biosensors based on various microorganisms.

Basis of receptor element	Relative standard deviation (%)	Long-term stability (days)	Sensitivity (nA dm^3/min mg)	Duration of single measurement (min)	Linear range of biosensor response dependence on BOD_5 (mg/dm^3)
Candida maltosa	3	>30	0.05	8–20	9.3–422
Candida blankii	5	8	0.11	10–22	3.0–56
Debaryomyces hansenii	4	>30	0.21	10–17	2.2–177

dependences appears as $BOD_5 = 21.7R - 13.1$ for the *C. maltosa* based biosensor; as $BOD_5 = 9.4R - 3.7$ for the *C. blankii* based biosensor; and as $BOD_5 = 4.8R - 18.1$ for the *D. hansenii* based biosensor. Thus, it can be suggested that the linear region of calibration is within 9.3–422.0 BOD_5 for the *C. maltosa* based biosensor, within 3.0–56.0 for the *C. blankii* based biosensor, and within 2.2–177.0 for the *D. hansenii* based biosensor. Measurements can be performed in situ; due to its low value, BOD of natural water bodies can be estimated without usually obligatory dilution. Dilution is required in measurements of higher BOD values.

The analytical and metrological characteristics of BOD biosensors based on the developed receptor elements were determined with glucose–glutamate mixture used as a standard solution. The results are given in Table 2.

Biosensors with the developed receptor elements are characterized by close levels of operational stability and time-dependent parameters. It should be noted that the responses of the *C. blankii* based biosensor decrease by 50% in 8 days. It is the evidence of low long-term stability of receptor elements based on this culture. Long-term biosensor stability implies the stability of sensor response values during a long period of measurement expressed in days. The receptor elements based on *D. hansenii* and *C. maltosa* have the best long-term stability. The *D. hansenii* based biosensor is characterized by the highest sensitivity and, accordingly, the minimal lower limit of linear range. Thus, the *D. hansenii* biosensor has the best characteristics of those tested. It is important to note that the *D. hansenii* biosensor is highly competitive with its analogs by its characteristics [16].

3.3. The effect of environmental conditions on the performance of BOD biosensors

The pH of the medium is one of the factors influencing the activity of cellular enzymes and, accordingly, bioreceptor sensitivity to various substrates. Biosensor responses to the standard GGA solution were obtained in the pH range of 5.4–7.8 (Fig. 3).

The maximum biosensor response is observed at pH 6.5 for *C. maltosa*, at pH 6.8 for *C. blankii*, and at pH 7.0 for *D. hansenii*. Besides, the results show that *D. hansenii* can work in a broader pH range compared to the tested yeasts of the genus *Candida*.

One more factor that influences bioreceptor sensitivity is ionic strength, which first of all affects the pressure inside the cell. The ionic strength gradient in solution was created by using a phosphate buffer system with pH 6.8 and a concentration range of 16.5–132.0 mM. Fig. 4 shows a dependence of *D. hansenii* oxidative ability on the ionic strength of solution; for two other strains, the dependence had a similar shape. The data obtained demonstrate a practically linear character of cell oxidative activity on the ionic strength of solution. Besides, the yeasts *D. hansenii* and *C. blankii* resist better the increasing ionic strength of solution, which can be due to the more efficient system of the regulation of intracellular osmotic pressure compared with *C. maltosa*.

The dominant factor that can reduce biosensor response and even lead to the death of bioreceptor is the presence of heavy metal ions in many wastewaters. Heavy metal ions have both bacteriostatic and bactericidal effects. The mechanisms of their interactions with enzymes are different: substitution of physiologically significant cations and non-metal-containing oxyanions in active enzyme centers, binding of functional sulfhydryl groups, etc. The inhibitory effect of heavy metal compounds was studied by investigating the dependence of the oxidative ability of *C. maltosa*, *C. blankii*, and *D. hansenii* on the presence of Zn, Ni, and Cr(VI) in solution in the concentration range of $0–6 \times 10^{-4}$ mol/dm^3 (for wastewaters, MPC of Zn^{2+} is 1.5×10^{-4} mg/l; MPC of Ni^{2+} is 3.4×10^{-7} mol/dm^3; and MPC of Cr(VI) is 2×10^{-7} mol/dm^3). It was shown that the receptor element based on *C. blankii* was liable to the negative effect of Ni^{2+} and Cr(VI) ions: its oxidative activity decreased. However, the presence of heavy metals in solution in the concentration range of $0–6 \times 10^{-4}$ mol/dm^3 had no significant effect on *C. maltosa* and *D. hansenii*, probably due to the resistance of the yeasts under study to these compounds and the short time of contact of a sample with the bioreceptor element owing to the flow-injection mode of

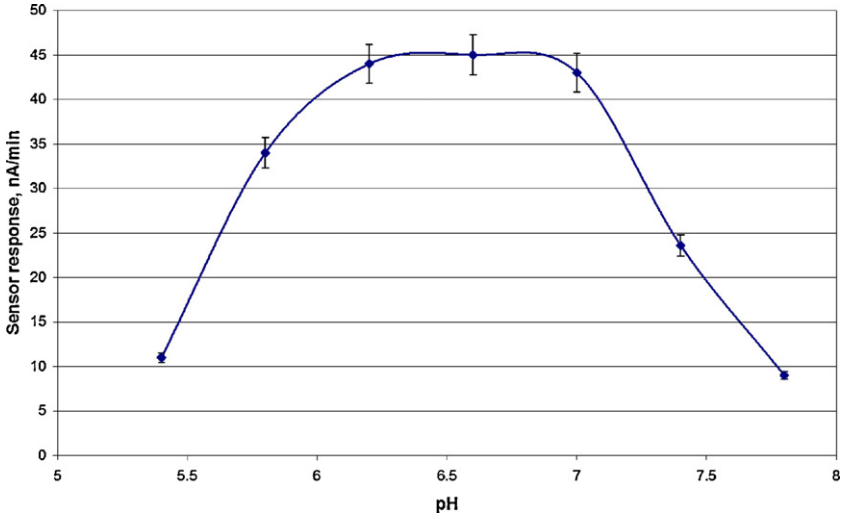

Fig. 3. The pH dependence of responses of *D. hansenii* based biosensors (concentration of GGA, 3 g/dm^3).

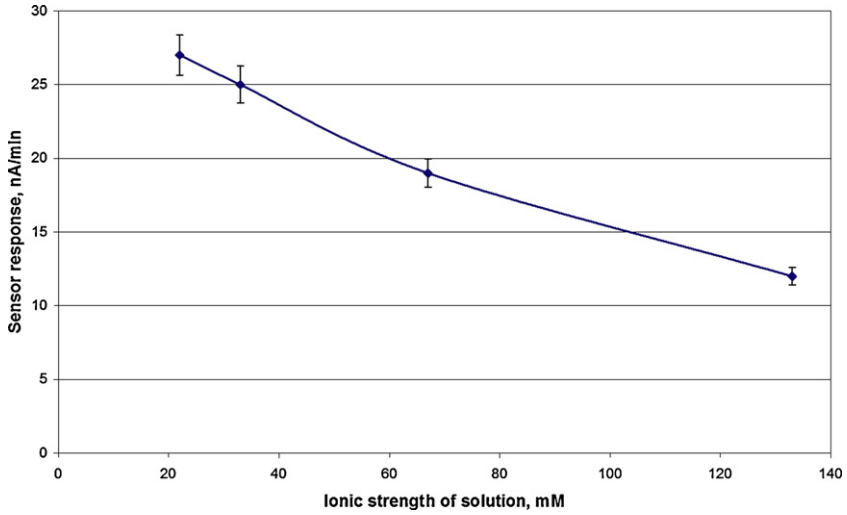

Fig. 4. Dependence of the oxidative ability of *D. hansenii* cells on the ionic strength of solution (concentration of GGA, 3 g/dm^3).

Table 3
Results of BOD_5 measurements by biosensors based on the yeast strains and by the standard method.

Sample	BOD value (mg/dm^3)			
	Biosensor based on *Candida maltosa*	Biosensor based on *Debaryomyces hansenii*	Biosensor based on *Candida blankii*	Standard dilution method
Water treatment facilities of Pushchino				
1	860 ± 10	1610 ± 50	1040 ± 30	1500 ± 200
2	700 ± 10	1280 ± 30	800 ± 20	1200 ± 200
3	570 ± 10	1180 ± 20	550 ± 10	1100 ± 100
4	220 ± 50	460 ± 20	240 ± 10	440 ± 60
Glucose-molasses plant				
1	1010 ± 70	840 ± 80	860 ± 70	900 ± 100
2	1100 ± 100	940 ± 70	910 ± 80	1000 ± 100
3	840 ± 60	770 ± 50	730 ± 40	800 ± 100

measurement. Advantages of the considered biosensors over commercial devices are a broad substrate specificity and resistance to negative environmental factors.

3.4. Wastewater sample analysis

BOD_5 values were determined in samples from wastewaters of the water treatment facilities of the town of Pushchino and of a food-manufacturing plant. The samples were taken in accordance with the standard procedure [17] (Table 3).

The biosensors based on *C. maltosa* and *C. blankii* have a large error during the analysis of wastewaters from the water treatment facilities. It is most probably associated with the presence of substances not oxidized by these microorganisms. The BOD values determined by the biosensor based on the yeast culture of *D. hansenii* coincide in all cases with the BOD values determined by the standard method (the correlation coefficient, 0.98). Thus, the *D. hansenii* based biosensor can be efficiently used for BOD assays of wastewaters, including in the presence of Zn, Ni, and Cr(VI) compounds and at a high ionic strength of solution.

4. Conclusion

The growth dynamics of the yeast strains *C. maltosa* VKM Y-2359, *C. blankii* VKM Y-2675, and *D. hansenii* VKM Y-2482 in the liquid glucose–peptone medium was described. All three yeast strains demonstrated the highest oxidative activity against GGA in the late exponential or early linear growth phase.

The developed laboratory-scale models of biosensors for BOD_5 detection are based on receptor elements with the yeast cells of *C. maltosa*, *C. blankii*, and *D. hansenii*. The *D. hansenii* based biosensor showed the best characteristics among those studied and was highly competitive with the possible analogs. The effects of pH, ionic strength, and the content of heavy metals on the above yeast strains were studied. The presence of Zn^{2+}, Ni^{2+}, and Cr(VI) ions in the concentration range of $0–6 \times 10^{-4}$ mol/dm^3 in the medium had no significant influence on the oxidative ability of any strain during the measurement.

The possibility of using *D. hansenii* microorganisms as a basis of the biosensor receptor element for BOD detection in municipal and biotechnological wastewaters was shown for the first time. The described biosensor analyzer based on this microbial culture can be a prototype model of a device for serial production and application.

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