



# Registration of BOD using *Paracoccus yeei* bacteria isolated from activated sludge

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Received: 20 December 2019 / Accepted: 8 April 2020  
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## Abstract

This work investigated the properties of *Paracoccus yeei* VKM B-3302 bacteria isolated from activated sludge and immobilized in an N-vinylpyrrolidone-modified poly(vinyl alcohol) matrix. The developed hydrogel formed a network structure to enable the entrapment of microbial cells with their viability and biocatalytic properties preserved, which ensured the technological possibility of replicating expendable biosensor receptor elements. A new ratio of the components for the synthesis selected in this work enabled producing a copolymer of an earlier undescribed chemical structure, which can be efficiently used for immobilization of highly sensitive *P. yeei* bacteria. A biological oxygen demand (BOD) biosensor with these bacteria and matrix was shown to possess a long-time stability exceeding that described earlier, to have a broad substrate specificity and to exceed approximately tenfold the nearest analogues by its sensitivity and the lower boundary value of 0.05 mg/dm<sup>3</sup>. The biosensor enabled assays of water samples initially attributed to pure samples (the BOD range, 0.05–5.0 mg/dm<sup>3</sup>). BOD assays of water samples from various sources showed the use of the receptor element of this composition to enable the data that closely correlated with the standard method ( $R^2 = 0.9990$ ).

**Keywords** Biosensor · Biochemical oxygen demand · BOD<sub>5</sub> · Poly(vinyl alcohol) · N-vinylpyrrolidone · *Paracoccus yeei* bacteria

## Introduction

Of immediate interest at present is the development of methods and instrumentation for rapid assays of natural-water and wastewater purity. One of the parameters that determine the integral pollution of water is the biochemical oxygen demand (BOD) index. The standard BOD technique requires incubation of an oxygen-saturated sample for 5 or 20 days (BOD<sub>5</sub> or BOD<sub>20</sub>). In accordance with world practice, an efficient approach for the rapid assessment of BOD is to develop biosensors based on microorganisms oxidizing a broad range of organic substances (Jouanneau et al. 2014).

Bioreceptor elements of BOD sensors are developed using microbial associations (activated sludge, artificial consortia) as well as individual microbial cultures with certain consumer properties. An advantage of using microbial associations is their ability to oxidize a broad range of organic substrates; however, BOD biosensors on their basis are insufficiently stable because consortium's composition changes in time (Ponamoreva et al. 2011). Individual cultures are more stable in the measurement mode, are capable of oxidizing various classes of organic substances, are stable under immobilization and resistant to negative factors of the environment (Abrevaya et al. 2014).

A key point in forming the stable biosensitive element of a BOD biosensor is the immobilization of microorganisms. Efficient methods of producing immobilized cells are associated with processes of their entrapment into natural and synthetic hydrogels; as a rule, these processes provide for mild conditions of cell entrapment into the supporting matrix. Produced matrices should ensure a high diffusion of substrates and products, as well as high cell retention (Burlage and Tillmann 2017). Application of various hydrogels

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has been shown to satisfy most of these requirements for BOD biosensors (Tag et al. 2000; Martínez et al. 2007; Fine et al. 2006; Commault and Weld 2016; Buenger et al. 2012; Fang et al. 2016). For instance, poly(carbamoyl)sulfonate was used to immobilize the yeasts *Arxula adeninivorans* LS3 and *Saccharomyces cerevisiae* (Tag et al. 2000; Martínez et al. 2007); calcium alginate and poly(vinyl alcohol) (PVA) were chosen for immobilization of luminescent yeasts (Fine et al. 2006); a polyurethane-based matrix was created for immobilization of aerobic microorganisms isolated from seawater (Commault and Weld 2016). Besides these hydrogels, widely applied are matrices based on photocrosslinkable resins, polyacrylonitrile, poly(ethylene glycol), carrageenan, agarose, chitosan and various protein structures (Buenger et al. 2012; Fang et al. 2016). The major problems of using these matrices in BOD biosensors are the complexity of production, decrease of microbial activity due to temperature treatment or use of toxic substances during the synthesis of the matrix, and low mechanical strength and stability of produced gel (Guisan and editor. 2006).

PVA is often used as a synthetic gel former as it is chemically and microbiologically stable, nontoxic and biocompatible (Szczena-Antczak et al. 2004). However, its use as the base of the receptor element in a biosensor analyzer is made difficult as it forms water-soluble films of low mechanical strength. A way of creating network PVA structures is to introduce crosslinking agents (Aleshina et al. 2001). A new reagent used for modification of PVA with the view of immobilizing microorganisms to develop biosensor recognition elements is N-vinylpyrrolidone (NVP). It is known that a polymer on its basis, polyvinylpyrrolidone (PVP), readily forms complexes with many compounds (toxins, drug substances, dyes), is biocompatible, nontoxic, is the basis of blood substitutes (Zhanybekova 2013), forms films when mixed with PVA (Bazilyuk and Melnik 2003). Also, it increases the activity of some microorganisms: for instance, the presence of PVP contributes to a significantly accelerated biodegradation of drug substances by *Rhodococcus erythropolis* (Demakov 2008).

An earlier work has shown a possibility of developing a stable biosensor receptor element by entrapping microorganisms into N-vinylpyrrolidone-modified PVA gel (Arlyapov et al. 2013). Investigation of the physical and chemical structure of this hydrogel and optimization of its composition enabled improving the metrological characteristics of the developed BOD biosensor. Formation of mechanically strong hydrogel films with immobilized microorganisms makes it possible to ensure the technological possibility of replicating biosensitive elements for modification of oxygen electrodes.

The aim of this work was to develop a matrix based on N-vinylpyrrolidone-modified poly(vinyl alcohol) for immobilization of *Paracoccus yeei* bacteria isolated from activated

sludge and to study the characteristics of an amperometric BOD biosensor based on the produced biocatalyst.

## Experimental

### Production of N-vinylpyrrolidone-modified poly(vinyl alcohol)

N-Vinylpyrrolidone-modified poly(vinyl alcohol) was produced using a 5% aqueous solution of PVA, grade 16/1 (Russia); an aqueous solution of cerium (IV) ammonium nitrate  $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$  (analytical grade, technical specs TU 6-09-4762-84, Russia) as an initiator; and N-vinylpyrrolidone (99%, Acros Organics, USA) as a crosslinking agent. The modification was performed at constant stirring in a nitrogen atmosphere at a temperature of 40 °C.

### Recording of IR spectra

Dried films of NVP-modified poly(vinyl alcohol) were used. The IR spectra were recorded on an FMS 1201 infrared Fourier spectrometer (OOO Monitoring, Russia).

### Recording of NMR spectra of modified poly(vinyl alcohol) and initial substances

The NMR spectra were recorded at the Centre of Magnetic Spectroscopy, N.M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences. The measurements were carried out at a Bruker Avance III 500 spectrometer with the working frequencies of 500.2 MHz and 125.8 MHz for proton and carbon, respectively. The structure of the produced copolymer with the component ratio of 160: 7: 1 (PVA: initiator: NVP) was established by a joint analysis of 1D  $^1\text{H}$ ,  $^{13}\text{C}$ -DEPT and 2D COSY, HSQC, HMBC NMR spectra. The paper shows the data for only 1D NMR spectra.

We recorded the spectra of an aqueous solution of the reaction mixture at room temperature with the addition of 10%  $\text{D}_2\text{O}$  (to stabilize the resonance conditions) and with water signal suppression. The chemical shifts of  $^1\text{H}$  and  $^{13}\text{C}$  were calibrated relative to the tetramethylsilane signal. All two-dimensional experiments were performed according to the standard Bruker techniques. The spectra were processed using Bruker's TopSpin 3.0 software.

### Scanning electron microscopy of gel structure

NVP-modified poly(vinyl alcohol) samples containing and not containing microbial cells were analyzed using a JSM-6510 LV scanning electron microscope (Jeol, Japan).

### Determination of the fraction of crosslinked N-vinylpyrrolidone-modified poly(vinyl alcohol)

The fraction of the crosslinked polymer was determined by the extraction method. A dried sample of 0.01–0.02 g was weighed, poured with water and mixed for 2 h at 60 °C. After the extraction, water was separated by centrifugation, and the samples were dried to a constant weight at 60 °C. Extraction was repeated several times to a constant weight of the sample after drying. The experiment was conducted for 15 specimens of the same film with the statistical treatment of the obtained data. The percentage of the weight loss was determined by the formula:

$$P = m_2/m_1 \times 100\%,$$

where  $m_1$  is the weight before extraction and  $m_2$  is the weight of dried hydrogel after extraction.

### Cultivation of microbial cells

The yeast strains *Ogataea angusta* VKM Y-1397, *Debaryomyces hansenii* VKM Y-2482, *Debaryomyces hansenii* VKM Y-111 and *Arxula adeninivorans* VGI 78-6 were received from the All-Russian Collection of Microorganisms, Institute of Biochemistry and Physiology, Russian Academy of Sciences. Cells of the bacteria *Paracoccus yeei* BAA-599<sup>T</sup> and *Pseudomonas veronii* DSM 11331<sup>T</sup> were isolated from activated sludge of wastewater treatment facilities, the city of Tula (Kharkova et al. 2019). The microorganisms *Paracoccus yeei* BAA-599<sup>T</sup> were deposited in the All-Russian Collection of Microorganisms under number B-3302.

The yeasts *D. hansenii* VKM Y-2482, *D. hansenii* VKM Y-111, *A. adeninivorans* VGI 78-6 and the bacteria *P. yeei* VKM B-3302 and *Ps. veronii* DSM 11331<sup>T</sup> were cultivated on a rich mineral medium (liquid glucose–peptone nutrient medium). The composition of the liquid medium: glucose, 10 g/dm<sup>3</sup> (OOO Diaem); peptone, 5 g/dm<sup>3</sup> (OOO Diaem); yeast extract, 0.5 g/dm<sup>3</sup> (OOO Diaem). The yeast *O. angusta* VKM Y-1397 was cultivated on a rich mineral medium (liquid yeast nutrient medium). The composition of the liquid medium: yeast extract, 0.1 g/dm<sup>3</sup>; leucine, 0.034 g/dm<sup>3</sup>; glycerol, 1.66 cm<sup>3</sup>; trace elements (H<sub>3</sub>BO<sub>3</sub>, ZnSO<sub>4</sub>, Na<sub>2</sub>MoO<sub>4</sub>, MnCl<sub>2</sub>, FeCl<sub>3</sub>) 0.2 cm<sup>3</sup> (Sigma, USA).

The cultivation medium was sterilized by autoclaving at a temperature of 120 °C and pressure of 1.1 atm for 40 min. Cells were cultivated aerobically for 18–20 h in 750-cm<sup>3</sup> shaken flasks at a temperature of 29 °C. Produced biomass was centrifuged at room temperature for 10 min at 10,000 rpm (a TG16WS centrifuge, OOO Polycom, Russia).

The centrifugate was washed with a 20-mM phosphate buffer solution, pH 6.8 (or pH 7.6 for the yeast *O. angusta* VKM Y-1397). Sedimented cells were resuspended by the buffer solution, were distributed in Eppendorf microtubes and centrifuged on an Eppendorf centrifuge for 5 min at 10,000 rpm. Washed biomass was stored at a temperature of –25 °C in microtubes without using a protective agent. Biosensors can be formed using both freshly grown and frozen bacterial cultures. The loss of activity when using thawed bacterial cells was no more than 10–15% as compared with freshly grown cells.

### Growth curves

Microbial growth curves were obtained by overlapping the time intervals within which the optical density was measured. Optical densities of the suspension were measured spectrophotometrically on an SF-103 spectrophotometer (Akvilon, Russia) every two hours for 48 h at a wavelength of 540 nm and cuvette thickness of 1 cm relative to a cuvette with distilled water. The obtained dependences of optical density on time were used to plot the growth curves for the bacterial cells. The oxidative activity of cells was measured by the biosensor method; a solution of glucose and glutamic acid (GGA) at a concentration of 500 mg/dm<sup>3</sup> was used as substrate. The experiment to study the activity of bacteria with respect to growth time was conducted on a freshly grown culture.

### Immobilization of microorganisms using a dialysis membrane

For immobilization using a dialysis membrane, cell biomass was prediluted with a phosphate buffer solution at a ratio of 1:1. An amount of 5 µl of the produced suspension was applied onto a D977 dialysis membrane (Sigma, USA) and fixed on the surface of an oxygen electrode by a rubber ring.

### Immobilization of microorganisms in modified PVA hydrogel

To produce a biocatalyst immobilized in NVP-modified PVA hydrogel, 20 mg of microbial cells was added to 100 µl of hydrogel. An even distribution of cells in hydrogel was achieved by shaking on a CM70M Sky Line Elmi Centrifuge & Vortex (ELMI, Latvia) for 5 min. The produced suspension was transferred to a plate ( $d=5$  mm) and left to dry at a temperature of 18–22 °C. After drying for 24 h, the bioreceptor was fixed on the surface of a Clark electrode by means of a nylon net.

## Biosensor measurements

Electrochemical measurements were carried out using an Ekspert-009 analyzer (OOO Ekoniks-Ekspert, Russia) coupled with a computer operated by specialized software EXP2PR (OOO Ekoniks-Ekspert, Russia). Clark oxygen electrodes containing immobilized microbial cells were transducers. A biosensor response was a change of oxygen concentration ( $\text{mg}/\text{dm}^3 \cdot \text{s}$ ) in the near-electrode space owing to the biochemical reaction. As namely bacterial cells transform substrate when it is added into the cuvette, no biosensor response to the addition of an analyte was registered in their absence. Measurements were carried out in a 5-ml cuvette. For measurements, we used a sodium–potassium phosphate buffer solution (pH 6.8 or 7.6) the total concentration of the salts in which was 20 mM. The solution was mixed by a magnetic mixer (200 rpm). A mixture of glucose and glutamic acid at a ratio of 1:1 (w/w) used as the BOD<sub>5</sub> detection standard in the Russian Federation and in international practice (Federal Environmental Regulatory Documents (PND F) 2003; Szczesna-Antczak 2004) was used as a model mixture. In accordance with regulatory documentation, BOD<sub>5</sub> equal to 205  $\text{mg}/\text{dm}^3$  was taken to correspond to a solution containing 150  $\text{mg}/\text{dm}^3$  of glucose and 150  $\text{mg}/\text{dm}^3$  of glutamic acid ( $\text{BOD}_5 = 0.68 \times C_{\text{GGA}}$ ). Between assays, the biosensor was stored at room temperature.

## Determination of BOD<sub>5</sub> by the standard dilution method

The dilution method was used as the reference method of BOD<sub>5</sub> determination. Analysis was done in accordance with the technique indicated in Federal Environmental Regulatory Documents (PND F) 1997. The content of dissolved oxygen in analyzed samples was determined amperometrically in accordance with the standard technique.

## Discussion

### Development of the BOD-biosensor receptor element

Four yeast strains capable of oxidizing a broad range of organic substances and efficiently used earlier to develop BOD biosensors (Yudina et al. 2015) were used to form the biosensor receptor element. Besides, use was made of two bacterial strains isolated from activated sludge of wastewater facilities. These microorganisms were adapted to investigated samples, which ensured a high convergence of biosensor-assay results with those of the standard method (Kharkova et al. 2019).

Biological material, which made the basis of the BOD-biosensor receptor element, was chosen based on the ability of microorganisms to oxidize a broad range of organic substrates, by the stability during the immobilization on the Clark electrode and the stability during measurements. Encapsulation of the microbial suspension using a dialysis membrane was used as the simplest immobilization technique to perform.

From the data of Table 1, it is seen that the microorganisms *D. hansenii* VKM Y-2482, *D. hansenii* VKM Y-111 and *P. yeii* VKM B-3302 have the broadest spectrum of oxidized substrates. Herewith, the receptor element based on *P. yeii* has high long-time stability and the highest sensitivity. For this reason, the microorganisms *P. yeii* isolated from activated sludge were used in further work.

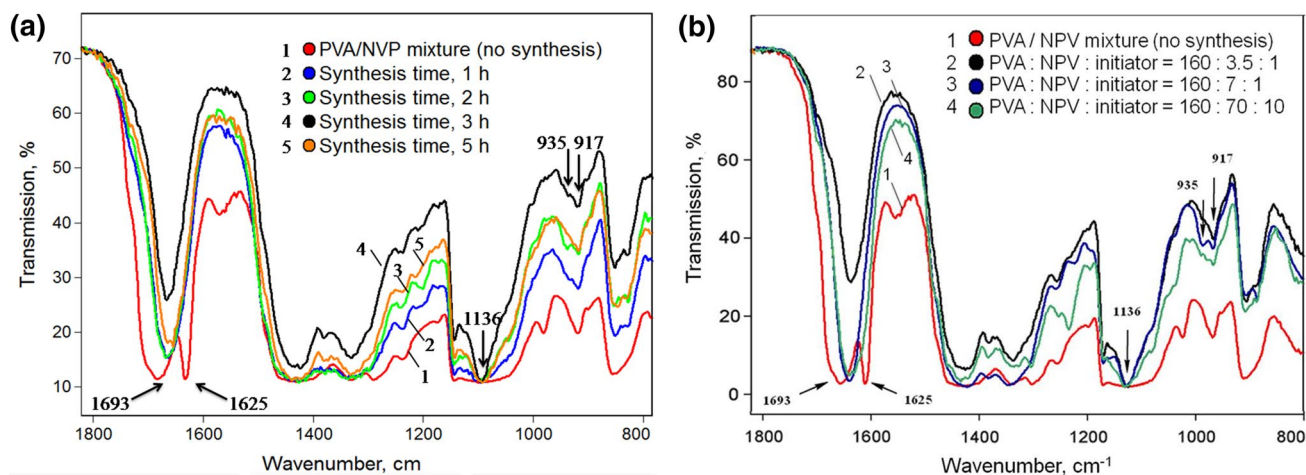
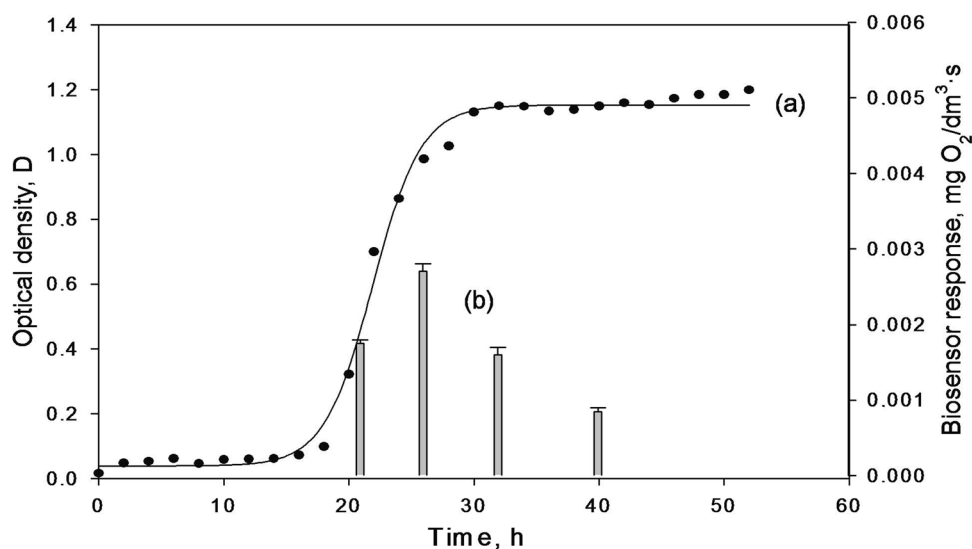
To develop a biosensor receptor element possessing a high sensitivity, it is necessary to use microorganisms with the maximum oxidative activity. An important factor affecting microbial activity is cultivation time. To choose an optimal cultivation time for *P. yeii*, we analyzed the dependence of the oxidative activity of cells, that comprise the receptor element, on growth time (Fig. 1).

From the data presented in Fig. 1, it is seen that the maximal activity of microorganisms is observed during the

**Table 1** Major characteristics of biosensitive elements based on immobilized microorganisms

| Microorganisms                            | Number of oxidized substrates (out of 12 attributed: methanol, ethanol, propanol, glycerol, glucose, fructose, saccharose, glutamic acid, glycine, SDS, potassium benzoate, 4-nitrophenol) | Long-time stability, days | Sensitivity coefficient, $10^{-5} \text{ s}^{-1}$ |
|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|---------------------------------------------------|
| <i>O. angusta</i> VKM Y-1397              | 8                                                                                                                                                                                          | 26                        | $16 \pm 2$                                        |
| <i>A. adenivorans</i> VGI 78-6            | 8                                                                                                                                                                                          | 27                        | $23 \pm 2$                                        |
| <i>D. hansenii</i> VKM Y-2482             | 12                                                                                                                                                                                         | 35                        | $30 \pm 3$                                        |
| <i>D. hansenii</i> VKM Y-111              | 12                                                                                                                                                                                         | 15                        | $12 \pm 1$                                        |
| <i>P. yeii</i> VKM B-3302                 | 12                                                                                                                                                                                         | 45                        | $110 \pm 5$                                       |
| <i>Ps. veronii</i> DSM 11331 <sup>T</sup> | 10                                                                                                                                                                                         | 47                        | $90 \pm 2$                                        |

**Fig. 1** **a** *P. yeii* growth curve obtained photometrically; **b**, a dependence of bacterial oxidative activity on growth time (a solution of GGA at a concentration of 500 mg/dm<sup>3</sup> was used as substrate)



**Fig. 2** IR spectra of NPV-modified PVA ( $\nu$ , cm<sup>-1</sup>; ATR accessory): 1693 cm<sup>-1</sup>, valence vibrations of the C=O pyrrolidone cycle; 1625 cm<sup>-1</sup>, valence vibrations of the C=C bond; 1136, 935,

917 cm<sup>-1</sup>, vibrations of the CH–O–CH bond. **a** synthesis time variation; **b** variation of initial components' ratios

cultivation for 26 h, which corresponds to the onset of the growth declining phase.

An important stage in the formation of a stable receptor element for a BOD biosensor is the immobilization of microorganisms. The main requirements imposed on microbial immobilization matrices are a high diffusion of substrates and products, insolubility in aqueous media (availability of a network structure), high capacity, mechanical strength.

To produce a PVA-based network polymer, the linear polymer was modified by N-vinylpyrrolidone in the presence of cerium (IV) ammonium nitrate as an initiator of radical crosslinking. In the course of the investigation, the synthesis time and the reagents/initiator ratio were varied. The quality of the produced materials was assessed by their IR spectra and the crosslinked polymer fraction, which was determined

**Table 2** Effects of synthesis time and components, molar ratios on polymer crosslinking

| Experiment no. | PVA: NPV: initiator molar ratio | Synthesis time, h | Fraction of crosslinked polymer, % |
|----------------|---------------------------------|-------------------|------------------------------------|
| 1              | 160: 1.75: 1                    | 3                 | Soluble                            |
| 2              | 160: 3.5: 1                     | 3                 | Soluble                            |
| 3              | 160: 7: 0.5                     | 3                 | 42 ± 2%                            |
| 4              | 160: 7: 1                       | 1                 | 50 ± 4%                            |
| 5              | 160: 7: 1                       | 2                 | 52 ± 5%                            |
| 6              | 160: 7: 1                       | 3                 | 54 ± 3%                            |
| 7              | 160: 7: 1                       | 5                 | 55 ± 5%                            |
| 8              | 160: 35: 5                      | 3                 | 60 ± 3%                            |
| 9              | 160: 70: 10                     | 3                 | 70 ± 10%                           |

by the swelling of films in water at a temperature of 60 °C. The results are given in Fig. 2 and Table 2.

The IR spectra of initial components' mixtures (Fig. 2) are observed to have an absorption band at  $\nu = 1625 \text{ cm}^{-1}$  corresponding to the C=C double bonds of N-vinylpyrrolidone. The band vanishes in an hour in the process of the synthesis, and, as the result of the running reaction, a new bond >CH–O–CH< forms. This bond yields three characteristic absorption bands at 1136, 935 and  $917 \text{ cm}^{-1}$  in its IR spectrum (Fig. 2). The fraction of the crosslinked polymer also increases with synthesis time from 50 up to 55% (Table 2).

The properties of modified polymer are also affected by the ratio of NPV. With the NPV lacking, the sample of produced copolymer dissolves at a temperature of 60 °C (Table 2). The maximal fraction of cross-linked polymer (70%) was obtained in the polymer at a ratio of PVA: NPV: initiator = 160: 70: 10 and synthesis time of 3 h. However, this polymer, as the polymer with a component ratio of 160: 35: 5, significantly hampered the diffusion of oxygen as well as the products of reaction, which could lead to a decrease in the biosensor response. Further in our research we used a copolymer with the ratio of components being 160: 7: 1 and synthesis time lasting for 3 h.

To establish the structure of the produced cross-linked polymer, we studied the reaction products by one- and two-dimensional NMR spectroscopy. The proton spectra are shown in Fig. 3a.

In the proton NMR spectrum of a copolymer solution, there is a signal of the  $\text{NH}_4^+$  group of cerium (IV) ammonium nitrate (6.90–7.22 ppm) and there are no proton signals of the N-vinylpyrrolidone group  $\text{CH}_2=\text{CH}-$ , which is indicative of the complete running of the reaction. In the proton spectrum (Fig. 3), well seen are the broadened signals of  $\text{CH}_2$  (8) (1.50–1.70 ppm) and CH (6,7) (3.86–4.06 ppm) groups of the poly(vinyl alcohol) chain. Apart from proton signals of three  $\text{CH}_2$  groups—1.96–2.14  $\text{CH}_2$  (2), 2.29–2.45  $\text{CH}_2$  (3); 3.35–3.59  $\text{CH}_2$  (1)—there is one more signal of the  $\text{CH}_2$  (4) group, 1.27 ppm. Its upfield shift indicates that this  $\text{CH}_2$  group belongs to the crosslinking fragment. Figure 3b presents a  $^{13}\text{C}$  (DEPT) spectrum of NVP-modified poly(vinyl alcohol). Three groups of signals—42.0–42.7 ppm ( $\text{CH}_2$ ), as well as 63.2–66.6 ppm and 72.3–74.7 ppm (CH)—correspond to the  $\text{CH}_2$  and CH groups in the carbon spectrum. The occurrence of two groups of signals for the CH groups is explained by the fact that one of them (at 63.2–66.6 ppm) pertains to the unsubstituted hydroxyl (CH–OH), and the other (at 72.3–74.7 ppm) belongs to the hydroxyl group, to which N-vinylpyrrolidone is attached. Besides the proton signals of three  $\text{CH}_2$  groups – 1.96–2.14  $\text{CH}_2$  (2), 2.29–2.45  $\text{CH}_2$  (3), 3.35–3.59  $\text{CH}_2$  (1) there is also one more signal of a  $\text{CH}_2$  group (4): 1.27 ppm (19.7 ppm in the carbon spectrum). Its upfield shift implies unequivocally that this  $\text{CH}_2$

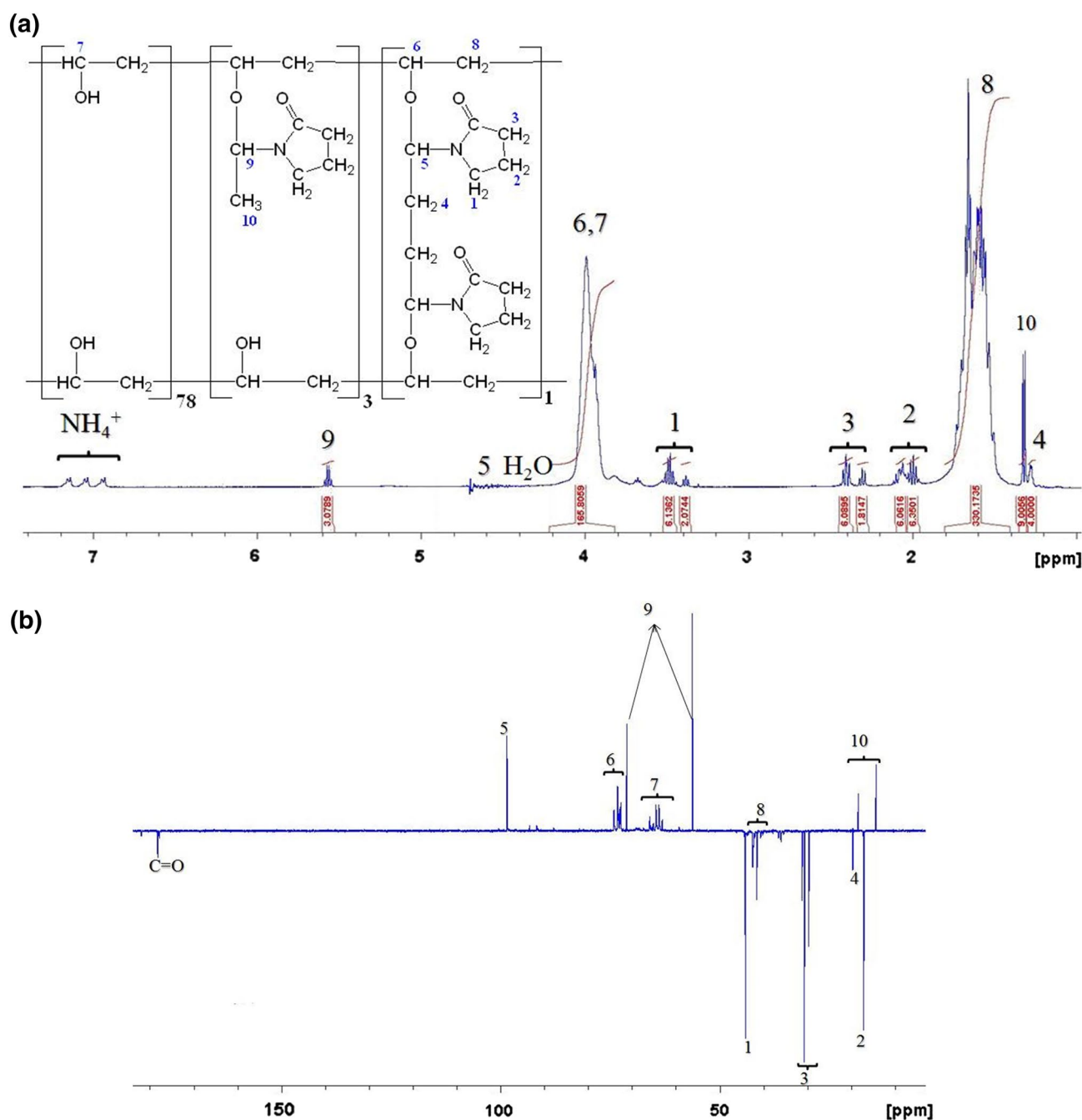
group pertains to the crosslinking fragment. The CH proton of group (5) is not seen in the  $^1\text{H}$  spectrum, because it gets under a suppressed water signal. Apart from the crosslinking signals, signals of two methyl  $\text{CH}_3$  (10) groups, 1.32 and 1.40 ppm (14.3 and 18.4 ppm in the carbon spectrum), and two CH (9) groups, 5.57 and 5.85 ppm (56.2 and 71.3 ppm in the carbon spectrum) are also seen in the proton and carbon spectra, which suggests the occurrence of an ethylpyrrolidone fragment in the polymer. Thus, proceeding from the analysis of two NMR spectra, the structure of the produced polymer corresponds to that presented in the insert of Fig. 3. It is important to emphasize that this particular structure is not similar to the one synthesized earlier for a different ratio of components in the same copolymer (Arlyapov et al. 2013).

The structure of a copolymer specimen with a molar component ratio of PVA: NPV: initiator = 160: 7: 1 was studied by scanning electron microscopy (Fig. 4).

As a result of the modification of poly(vinyl alcohol) by N-vinylpyrrolidone, a hydrogel of a network structure is produced (Fig. 4). For a chosen ratio of PVA: NPV: initiator = 160: 7: 1, a copolymer forms, which possesses a homogeneous structure and whose pores vary from 1 up to 10  $\mu\text{m}$  in size (Fig. 4a). At the entrapment of *P. yeii* cells into the matrix (the diameter of bacteria, 0.5–0.9  $\mu\text{m}$ ) they are evenly distributed along the bulk of the carrier. In Fig. 4b, it is seen that after the immobilization the bacteria are retained in the matrix, and the matrix itself has a sufficiently porous structure, which contributes to the passage of oxygen and organic substances to microorganisms and the release of enzyme reaction products from them. Thus, the produced matrix in combination with *P. yeii* microorganisms can be efficiently used for the development of a biosensor.

### 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 of detecting each component of the assayed object irrespective of others. In the case of a biosensor assay, its 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 detection it is preferable to use microbial cells that possess a broad substrate specificity (low selectivity). The broad substrate specificity is, in this case, an advantage, because it leads to an increase in the accuracy of BOD assay results. This work assessed the substrate specificity of *P. yeii* bacteria, immobilized in the N-vinylpyrrolidone-modified PVA matrix, with respect to 33 substrates of various classes of organic compounds. As substrates, we chose mainly readily oxidized organic substances, whose occurrence in water reservoirs leads to a significant decrease of the level

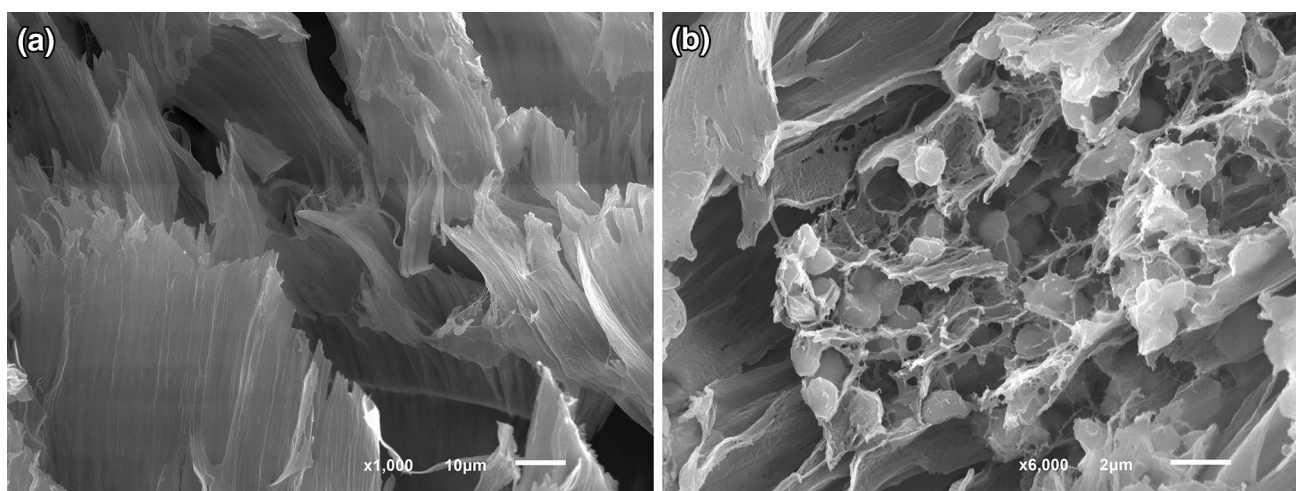


**Fig. 3** A NMR spectrum of N-vinylpyrrolidone-modified PVA at a component ratio of PVA: NPV: initiator=160: 7: 1; **a** a proton NMR spectrum; **b** a carbon NMR spectrum. The signals of the CH and  $\text{CH}_3$  groups are directed upwards; of the  $\text{CH}_2$  and quaternary carbons, downwards

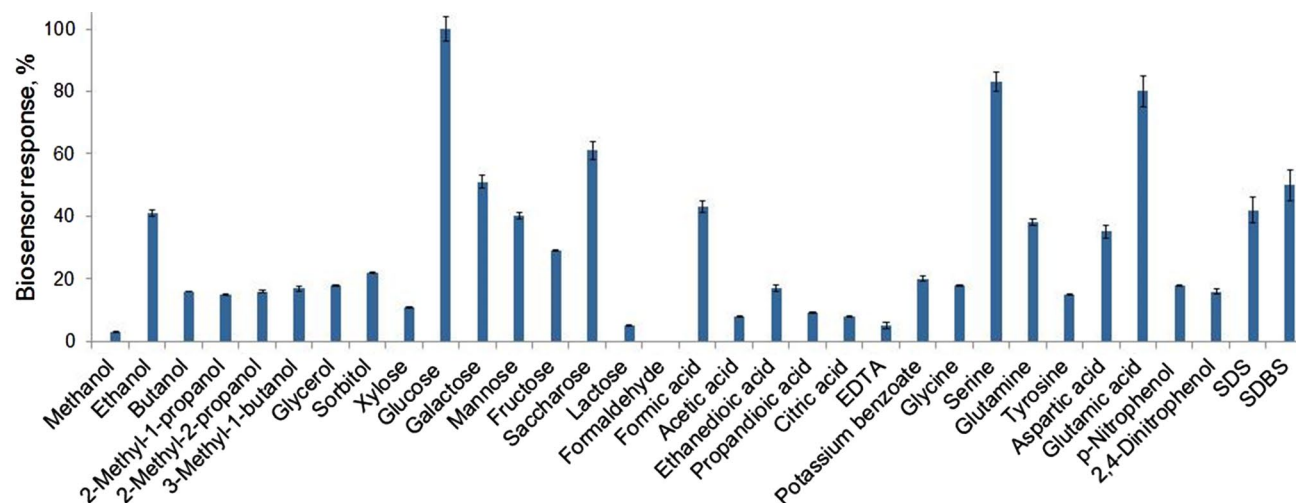
of dissolved oxygen and the further eutrophication of aquatic ecosystems. Figure 5 presents data on the substrate specificity of the biosensor based on *P. yeii* bacteria.

Bacteria *P. yeii* immobilized in PVA-modified gel possess the highest sensitivity to glucose; the response to it was taken for 100%. Bacteria oxidize substances of all presented classes of organic compounds: alcohols, carbohydrates, carboxylic acids, amino acids, nitrophenols

and surfactants that can be found in wastewaters. Valuable from the point of view of practice are responses to sodium dodecylsulfate, sodium dodecyl benzenesulfonate (detergents' components) and nitrophenols (widespread industrial toxicants), as well as the absence of a toxic effect of these substrates at their brief impact on immobilized *P. yeii* bacteria. The obtained results suggest that, when determining  $\text{BOD}_5$  values in water samples of various



**Fig. 4** Scanning electron micrographs of N-vinylpyrrolidone-modified PVA. **a** a modified PVA matrix after swelling in a buffer solution; **b** a modified PVA matrix with immobilized *P. yeii* bacteria after swelling in a buffer solution



**Fig. 5** Substrate specificity of *P. yeii* bacteria immobilized in the N-vinylpyrrolidone-modified PVA matrix (substrate concentration, 0.04 mol/dm<sup>3</sup>)

origins (industrial, household, synthetic wastewaters), a high correlation can be obtained between the readings of the developed biosensor and the standard method.

A calibration dependence of biosensor response on BOD<sub>5</sub> in a measuring cuvette for the developed biosensor is shown in Fig. 6.

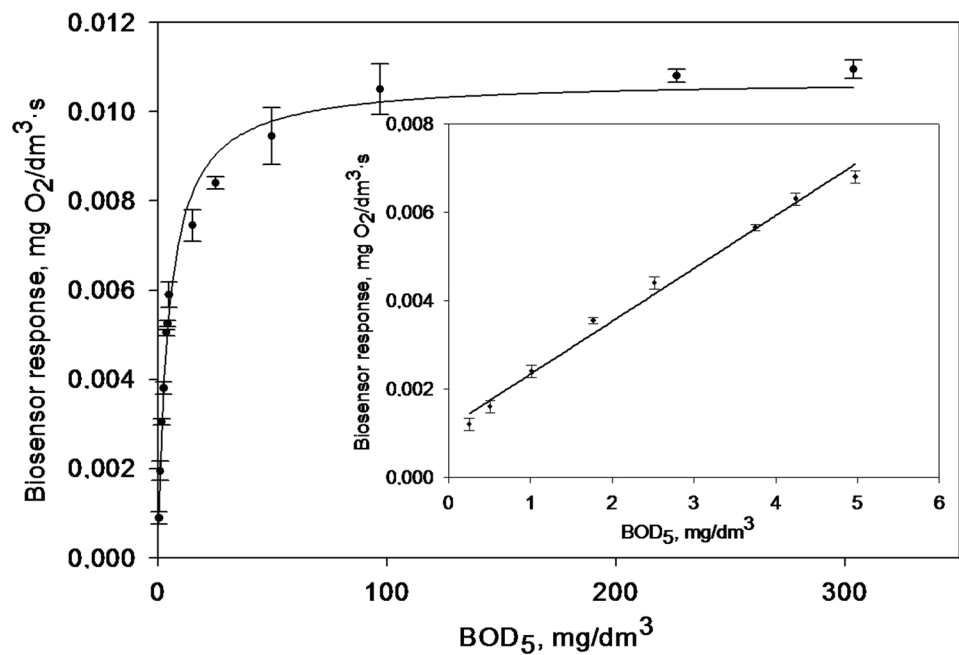
Bioreceptors based on microbial whole cells are of catalytic type, i.e., the biological response in these systems is provided for by microbial enzyme reactions. The dependences shown in Fig. 6 are satisfactorily approximated by a Michaelis–Menten-type equation

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

where  $R_{\max}$  is the maximal rate of oxygen uptake by immobilized microorganisms achieved at  $[S] \rightarrow \infty$ ;  $K_M$  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 calibration curve bounded from above by the Michaelis constant  $K_M$  (5.0 mg/dm<sup>3</sup>).

**Fig. 6** A dependence of the response of the developed biosensor on BOD<sub>5</sub>. The insert shows the linear segment of the calibration dependence



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<sub>5</sub> values was 0.05 mg/dm<sup>3</sup>. Thus, the linear range of the dependence of biosensor response on BOD<sub>5</sub> is within the limits of 0.05–5.0 mg/dm<sup>3</sup>.

Table 3 presents the major analytical and metrological characteristics of a biosensor based on *P. yeii* bacteria. The characteristics of the developed biosensor were determined using a model GGA mixture.

Thus, it can be noted that the use of N-vinylpyrrolidone-modified PVA (the component ratio, PVA: NPV: initiator = 160: 7: 1) for immobilization of *P. yeii* bacteria made it possible to improve the main characteristics of the biosensor as compared with its analog based on the yeast *D. hansenii* described in (Arlyapov et al. 2013). A new ratio of the components for the synthesis selected in this work enabled producing a copolymer of an earlier undescribed chemical structure, which can be efficiently used for immobilization of highly sensitive *P. yeii* bacteria. The lower boundary of the assayed BOD<sub>5</sub> values decreased more than tenfold, and the sensitivity of the biosensor increased ~ 50 times; herewith, the operational life of the BOD biosensor

**Table 3** Characteristics of a biosensor based on *P. yeii* bacteria

| Analogs characteristics         | Developed biosensor                                                                                                              | Analog 1 (Arlyapov et al. 2013) | Analog 2 (Jouanneau et al. 2014) | Analog 3 (Li et al. 2016) |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------|---------------------------------|----------------------------------|---------------------------|
| Microorganisms                  | <i>P. yeii</i>                                                                                                                   | <i>D. hansenii</i>              | Activated sludge                 | <i>P. fluorescens</i>     |
| Immobilization technique        | Entrapment in PVA                                                                                                                | Entrapment in PVA               | —                                | Entrapment in agarose     |
| Operational stability           | Relative standard deviation in 15 successive measurements, %                                                                     |                                 |                                  |                           |
|                                 | 7                                                                                                                                | 4.20                            | —                                | 7.7                       |
| Long-time stability             | Time within which the biosensor response to the same concentration of GGA was no less than 25% of the initial value, days        |                                 |                                  |                           |
|                                 | 45                                                                                                                               | 42                              | 20                               | —                         |
| Sensitivity                     | Slope of the linear segment in the dependence of the biosensor response on BOD <sub>5</sub> , $\times 10^{-4}$ , s <sup>-1</sup> |                                 |                                  |                           |
|                                 | $35 \pm 2$                                                                                                                       | $0.75 \pm 0.05$                 | $4.8 \text{ mV dm}^3/\text{mg}$  | —                         |
| Single-measurement time         | Response development time and activity recovery time, min                                                                        |                                 |                                  |                           |
|                                 | 4–6                                                                                                                              | 5–7                             | 15                               | 10–15                     |
| Detected BOD <sub>5</sub> range | mg/dm <sup>3</sup>                                                                                                               |                                 |                                  |                           |
|                                 | 0.05–5.0                                                                                                                         | 0.7–206.7                       | 1–10                             | 5–40                      |

without replacement of the bioreceptor element remained on the previous level (about 45 days).

The developed BOD biosensor based on this bioreceptor element exceeds the known analogs by orders of magnitude with respect to its sensitivity and is not inferior by its rapidity. In particular, it has a smaller lower limit of the determined BOD<sub>5</sub> values as compared with the predominant majority of the described prototypes (Jouanneau et al. 2014; Li et al. 2016; Wang et al. 2015). Besides, a single BOD assay using this analyzer is less time-consuming (Ponamoreva et al. 2011; Li et al. 2016; Wang et al. 2015). Herewith, the developed biosensor is distinguished with a narrower range of determined BOD values, which is easily eliminated by diluting the assayed sample.

### Analysis of water samples

We analyzed samples of water using the developed biosensor and by the standard dilution method. Samples were wastewaters of the municipal sewage treatment works taken at various stages of purification, effluents of a food processing plant, natural waters including pond water polluted with fuel and lubricants. Sample taking and BOD<sub>5</sub> determination by the standard dilution method were carried out according to the regulatory documents acting in the Russian Federation Federal Environmental Regulatory Documents (PND F) 1997. When determining the BOD<sub>5</sub> of wastewater samples using the developed biosensor, the sample was prediluted. The value of dilution was adjusted such that the sensor response is within the linear segment of the calibration dependence (Fig. 6). Figure 7 shows a correlation between

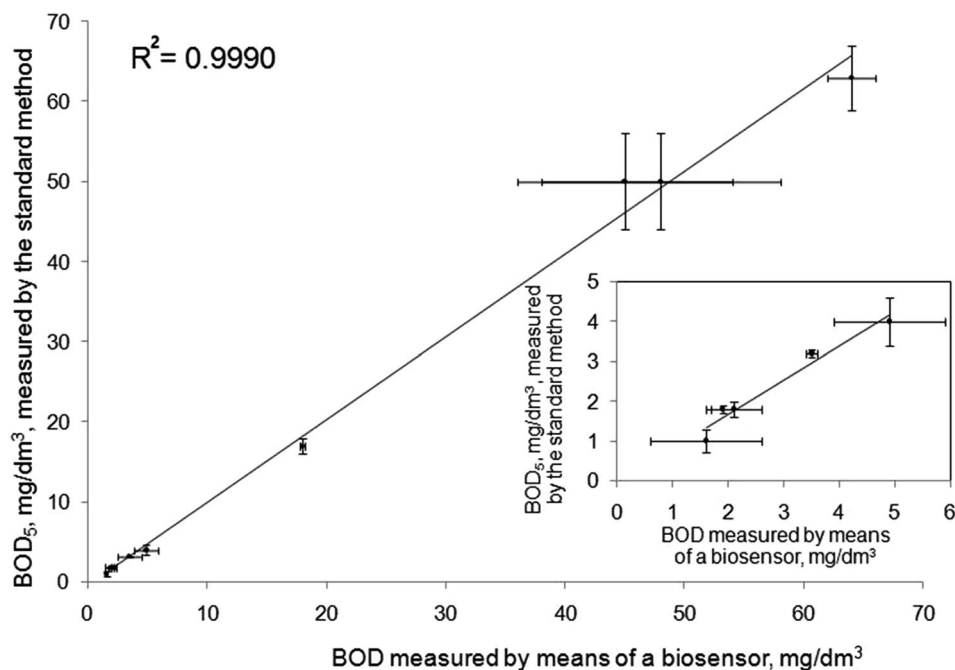
the BOD values determined using the biosensor and those by the standard dilution method.

Thus, the values of BOD<sub>5</sub> determined using a *P. yeii*-based biosensor coincide with those obtained by the standard method with account for the confidence interval. Correlation with the standard method for a biosensor based on *P. yeii* microorganisms immobilized in modified PVA hydrogel was  $R^2 = 0.9990$ , which is higher than for earlier described analogs (Jouanneau et al. 2014; Arlyapov et al. 2013; Raud et al. 2012).

### Conclusions

We developed a BOD biosensor based on *P. yeii* VKM B-3302 bacteria immobilized in N-vinylpyrrolidone-modified poly(vinyl alcohol) hydrogel. *P. yeii* bacteria were shown to possess a broad substrate specificity and to be capable of oxidizing substrates from various classes of organic compounds. The developed hydrogel formed a network structure to enable the entrapment of microbial cells with their viability and biocatalytic properties preserved, which ensured the technological possibility of replicating expendable biosensor receptor elements. A new ratio of the components for the synthesis selected in this work enabled producing a copolymer of an earlier undescribed chemical structure, which can be efficiently used for immobilization of highly sensitive *P. yeii* bacteria. The lower boundary of BOD concentrations detected by the developed biosensor was 0.05 mg/dm<sup>3</sup>, which enables assays of high-purity water samples.

**Fig. 7** Correlation of data obtained by the standard method and using a biosensor based on *P. yeii* bacteria immobilized in modified PVA hydrogel



We determined the BOD indices of water samples from various sources. The use of activated sludge cells immobilized in N-vinylpyrrolidone-modified PVA was shown to enable registration of data that highly correlated with those of the standard method. The obtained results indicate the possibility of using the developed biosensor analyzer as a prototype of pilot devices for serial application.

**Acknowledgements** The reported study was funded by the RFBR, project number 20–33-70078, and a grant of the President of the Russian Federation for the State Support of Young Russian PhD Scientists, agreement No. MK-1349.2020.3. The authors acknowledge Victor Selivanov for providing linguistic help.

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