Title: A yeast co-culture-based biosensor for determination of waste water contamination levels

Author: N.Yu. Yudina V.A. Arlyapov M.A. Chepurnova S.V. Alferov A.N. Reshetilov

PII: S0141-0229(15)30015-6
DOI: http://dx.doi.org/doi:10.1016/j.enzmictec.2015.06.008
Reference: EMT 8770

To appear in: *Enzyme and Microbial Technology*

Received date: 3-11-2014
Revised date: 9-6-2015
Accepted date: 10-6-2015

Please cite this article as: Yudina NYu, Arlyapov VA, Chepurnova MA, Alferov SV, Reshetilov A.N.A yeast co-culture-based biosensor for determination of waste water contamination levels.*Enzyme and Microbial Technology* http://dx.doi.org/10.1016/j.enzmictec.2015.06.008

A yeast co-culture-based biosensor for determination of waste water contamination levels

N.Yu. Yudina, V.A. Arlyapov, M.A. Chepurnova, S.V. Alferov, A.N. Reshetilov[a*]

Federal State Budgetary Educational Institution of Higher Professional Education "Tula State University", 92 Lenin Prosp., Tula 300600, Russia

[a]*Federal State Budgetary Institution of Science "G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms", Russian Academy of Sciences, 5 Prosp. Nauki, Pushchino, Moscow Region 142290, Russia*

[*]Corresponding author Tel.: +7 926 573 73 62; fax: +7 495 956 33 70

Email address: anatol@ibpm.pushchino.ru

Abstract

Artificial microbial co-cultures were formed to develop the receptor element of a biosensor for assessment of biological oxygen demand (BOD). The co-cultures possessed broad substrate specificities and enabled assays of water and fermentation products within a broad BOD range (2.4–80 mg/dm^3) with a high correlation to the standard method (R = 0.9988). The use of the co-cultures of the yeasts *Pichia angusta*, *Arxula adeninivorans* and *Debaryomyces hansenii* immobilized in *N*-vinylpyrrolidone-modified poly(vinyl alcohol) enabled developing a BOD biosensor possessing the characteristics not inferior to those in the known biosensors. The results are indicative of a potential of using these co-cultures as the receptor element base in prototype models of instruments for broad application.

Key words: co-culture, bacteria, yeasts, biochemical oxygen demand (BOD), biosensor analyzer, growth curves

Highlights

- stability of two yeast co-cultures in a BOD sensor was studied
- substrate specificities of microbial co-culture-based receptor elements were assessed

- new polymer for immobilization of NVP-modified PVA improved the characteristics
- the co-culture of three strains provided for biosensor's higher analytical signals

1. Introduction

Biochemical oxygen demand (BOD) is one of the most broadly used indices for monitoring the purity of aqueous environments [1]. By definition, it represents the amount of oxygen required for biochemical oxidation of organic substances contained in the sample. For drinking and clean water the BOD value should not exceed 2 mg/dm^3; at BOD higher than 4 mg/dm^3, water is considered to be polluted. The duration of classical BOD tests is 5 days (BOD_5) and more, which is too long for the on-line assessment of an ecological situation [2]. BOD assessment methods for express assays are being developed based on biosensor analyzers [1].

As recognition elements, BOD biosensors use microorganisms capable of metabolizing a broad range of organic compounds. The biorecognition elements of BOD sensors are formed by either pure microbial cultures with certain consumer properties (a broad range of oxidized substrates, resistance to negative factors of the environment) or microbial consortia (artificial co-cultures, activated sludge) [3].

Microbial co-cultures make it possible to significantly broaden the range of oxidized substrates and, correspondingly, to increase the accuracy of BOD determination [1]. At the same time, BOD biosensors based on microbial co-cultures can be insufficiently stable due to a gradual change of their composition [4]. BOD biosensors based on a complex microbial population, as, for instance, in activated sludge, have the best capability of detecting a broad range of substrates; still, due to the instability of the consortium, in the course of time these biosensors yield the least reproducible results. Several solutions of this problem have been proposed. Thus, the biosensor can be periodically calibrated or else thermally killed activated sludge can be used [5]. Still, inconvenience of operation and technical complexity of these approaches restrict applicability.

Microbial co-cultures consisting of no more than two or three strains are used to increase the number of oxidized substrates with preserved reproducibility of biosensor responses [6]. This leads to broader substrate specificities and stabilization of the biosensor functioning for a long period of time. Thus, in [7] the authors used the co-culture of *Trichosporon cutaneum* and *Bacillus subtilis* to form a BOD biosensor. The biosensor was used to determine BOD in lake water and municipal wastewaters; its lifetime was more than 40 days. In [8–9], microorganisms *Bacillus licheniformis*, *Dietzia maris* and *Marinobacter marinus* isolated from sea water were used. The biosensor described was capable of stable operation for up to 10 months and was successfully used to assay sea water samples.

Despite the available descriptions of BOD biosensors based on microbial co-cultures, the predominant number of studies have used consortia isolated from various natural sources. A more promising approach is to use artificial microbial co-cultures formed in an empirical way based on the analysis of their physiological and metabolic properties. This approach enables developing bioreceptor elements of biosensors with required consumer qualities without time consuming experiments to isolate and identify natural microorganisms.

The aim of this work was to search for a stable artificial microbial co-culture with broad substrate specificity and to form an amperometric BOD biosensor on its basis. The work used cells of the yeasts *Pichia angusta, Arxula adeninivorans, Debaryomyces hansenii, Candida boidinii* and of the bacteria *Gluconobacter oxydans*.

2. Materials and methods

2.1. Microorganisms

Pure cultures of the yeasts *P. angusta* VKM Y-2518, *P. angusta* VKM Y-1397, *A. adeninivorans* VKM Y-2677, *D. hansenii* VKM Y-2482, *C. boidinii* VKM Y-2356, as well as of the bacteria *G. oxydans* sbsp. *industries* VKM B-1280 were used to form co-cultures. The cultures were obtained from the All-Russian Collection of Microorganisms, FSBIS G.K.

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

2.2. Cultivation of microorganisms

Bacteria were cultivated at 28°C for 18–20 h on a nutrient medium of the following composition: D-sorbitol, 200 g/dm^3; yeast extract, 20 g/dm^3; distilled water, 100 cm^3; pH of the medium, 5.2–5.5. After cultivation, cells were harvested by centrifugation (10 min at 4300 *g*) and were washed with sodium phosphate buffer, pH 6.0. Bacterial biomass was dried for 1 h in microtubes on air and was frozen for prolonged storage at –15°C.

A. adeninivorans, *D. hansenii*, *C. boidinii* yeast cells were grown on a rich medium: glucose, 10 g/dm^3; peptone, 5 g/dm^3 and yeast extract, 0.5 g/dm^3. *P. angusta* yeast cells were grown on a rich mineral medium containing yeast extract, 0.1 g/dm^3; leucine, 0.034 g/dm^3; glycerol, 1.66 cm^3; trace elements ($MnSO_4$, $CoCl_2 \cdot 6H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$); 0.2 cm^3 (Sigma, USA); distilled water, 200 cm^3. Cells were grown aerobically for 18–20 h at 29°C and mixed at 120 rpm. Biomass was centrifuged for 10 min at room temperature at 4300 *g*. The centrifugate was washed with 20 mM phosphate buffer, pH 6.8. Sedimented cells were resuspended in a fresh buffer solution and sedimented in an Eppendorf centrifuge for 3 min at 4300 *g*. Washed biomass was weighed and stored in microtubes at –15°C.

2.3. 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 yeast and bacterial cells.

2.4. Optical microscopy

Pure cultures of the yeasts and bacteria and their co-cultures were investigated by the method of light field microscopy. A Biomed-6 triocular microscope (Biomed, Russia) was used for microscopic analysis; total magnification of the object was ×1600. Before viewing, preparations were fixed and stained. Yeast cells were stained with a fucsin solution; bacterial cells were Gram stained.

2.5. Determination of the amount of viable cells in co-cultures by inoculation onto dense nutrient media (Koch method)

A number of successively diluted suspensions of microbial co-cultures (degree of dilution, 10^{-6}) were prepared to produce separate colonies. Suspensions were inoculated by the surface method from three last dilutions (4 parallel inoculations). Colonies were counted after 7 days of incubation.

2.6. Biosensor measurements

Electrochemical measurements were carried out using an Ekspert-001-4.0.1 pH meter–ionometer–BOD thermal oximeter (Ekonics-expert Ltd, Russia) coupled with a computer operated by EXP2PR specialized software (Ekonics-expert Ltd, Russia). The software enables registration and processing of biosensor signals. The maximal rate of oxygen concentration change at the addition of substrates ($mg/dm^3 \cdot s$) was the measured parameter (biosensor response). Clark-type oxygen electrodes containing immobilized microbial cells were used as transducers. Measurements were done in a 5-cm^3 cuvette. A sodium–potassium phosphate buffer solution (pH 6.8) was used; the total concentration of the salts, 20 mM. The solution was mixed by a magnetic mixer (200 rpm). A mixture of glucose and glutamic acid (GGA) at a mass ratio of 1:1, applied as the BOD_5 determination standard in the Russian Federation and in international practice [2], was used as a model mixture. In accordance with the regulatory

documents, a value of BOD_5 equal to 205 mg/dm^3 was taken to correspond to a solution containing 150 mg/dm^3 glucose and 150 mg/dm^3 glutamic acid ($BOD_5 = 0.68 \times C_{GGA}$).

2.7. Immobilization of microorganisms by adsorption

To form a receptor element, microbial biomass was diluted with phosphate buffer to a concentration of 200 mg/cm^3. Solutions containing pure microbial cells were mixed at a ratio of 1:1 (1:1:1 for a co-culture consisting of 3 microorganisms); 5 μl of the produced suspension was applied onto a 3×3 mm^2 fragment of a Whatman GF/A glass fibre filter. The receptor element was dried for 10–15 min on air and fixed by means of a nylon mesh on the surface of an oxygen electrode.

2.8. Immobilization of microorganisms in gel based on PVA modified by N-vinylpyrrolidone

To prepare PVA modified by *N*-vinylpyrrolidone, an aqueous solution of cerium ammonium nitrate $(NH_4)_2Ce(NO_3)_6$ and *N*-vinylpyrrolidone were added to an aqueous solution of PVA (molecular mass, $1.0 \cdot 10^5$–$1.1 \cdot 10^5$ amu) at constant mixing at 40°C for 3 h [10].

To produce the immobilized biocatalyst, 36 mg of microbial cells mixed at a ratio of 1:1 (1:1:1 for the association consisting of 3 microorganisms) was added to 200 μl of PVA gel modified by *N*-vinylpyrrolidone. A uniform distribution of cells in gel was achieved by shaking on a CM70M centrifuge (ELMI, Latvia) for 5 min. The suspension produced was transferred onto a slide and dried for 24 h at room temperature. The thickness of the film produced was 15 μm; the specific density of immobilized cells, 5.8 mg/cm^2. The immobilized biocatalyst was stored at 4°C. To form the biosensor, a fragment of produced gel 4 mm in diameter was fixed by a nylon mesh on the surface of an oxygen electrode.

2.9. Determination of BOD_5 by the standard dilution method

BOD_5 was determined by the dilution method as indicated in [2]. Dissolved oxygen concentrations in a sample were measured before and after the incubation period and adjusted by the sample corresponding dilution factor. To ensure that all other conditions were equal, a very small amount of microorganism seed was added to each sample tested. This seed was generated by diluting organisms with buffered dilution water. The BOD test was carried out by diluting the sample with oxygen saturated dilution water, inoculating it with a fixed aliquot of seed, measuring the dissolved oxygen (DO) and then sealing the sample to prevent further oxygen dissolving in. The sample was kept at 20°C in the dark to prevent photosynthesis (and thereby the addition of oxygen) for five days, and the dissolved oxygen was measured again. The difference between the final DO and initial DO was the required BOD value.

3. Results and discussion

3.1. Formation of artificial microbial co-cultures

The main requirements of artificial microbial co-cultures to be used in the development of BOD biosensors are a broad range of oxidized substrates and temporal stability of composition. For this reason, the main criteria in choosing microorganisms for a co-culture were substrate specificity and specific growth rate of pure cultures.

The studies showed that the substrate consumption rate of a co-culture was not always equal to the sum of consumption rates of individual strains; besides, in a number of cases the activity of a co-culture could be even lower than those of its constituent cultures [11]. This effect can be explained by a competition between co-culture's strains for the most readily utilizable substrate. For this reason, to make up a composition possessing a broad substrate specificity, without competing interactions between its constituent strains, it is required that the microorganisms possess different sets of enzyme systems and, thus, supplement each other. One of the approaches based on this principle is to include into co-cultures strains of different taxonomic groups.

Yeasts are the preferable biomaterial for the development of BOD biosensors, because they are resistant to negative factors of the environment (pH, ionic strength of solution, temperature, heavy metal ions) and can function in the recognition element of the biosensor for a long time [6, 12, 13]. Yeasts of the species *A. adeninivorans* possess a broad substrate specificity and could be potentially used in biosensors for BOD assays [14, 15]. Yeasts *P. angusta* are distinguished by a high sensitivity to alcohols [16]. Microorganisms *C. boidinii* possess a broad substrate specificity [17]. Yeasts *D. hansenii* are also distinguished by a broad substrate specificity and resistance to negative environmental factors [17]. Bacteria *G. oxydans* possess a special organization of the metabolic system, characterized by the surface localization of a number of major oxidative enzymes and a high operational efficiency of the electron transport chain, which provides for a rapid biosensor response [18]. The substrate specificities of particular microbial strains studied were assessed using substrates from various classes of organic compounds – alcohols, carbohydrates, amino acids, carboxylic acids, aldehydes. The data obtained are given in Table 1.

From the data, it could be concluded that the microorganisms *D. hansenii* and *A. adeninivorans* possess the broadest substrate specificities. These yeasts oxidize substances of a large group of the represented classes of organic compounds, which includes alcohols, carbohydrates, carboxylic acids, amino acids and surfactants that can be found in waste waters (Table 1). The ability of the yeasts *D. hansenii* and *A. adeninivorans* to actively oxidize secondary and branched alcohols enables the use of biosensors on their basis for BOD assays of waste waters in enterprises of liquor and spirits industry. Valuable from a practical point of view are responses to sodium dodecyl sulfate and sodium dodecylbenzene sulfonate (components of detergents), as well as the absence of a toxic action of these substrates at their short-time effect on immobilized yeasts.

The yeast *C. boidinii* is capable of oxidizing a sufficiently broad range of substrates except surfactants, branched alcohols, some disaccharides and lysine (Table 1). A characteristic

feature of both *P. angusta* strains is their capability of actively oxidizing secondary and branched alcohols, which enables using the biosensor on its basis for BOD assays of waste waters in liquor enterprises. The receptor based on *G. oxydans* bacteria is characterized by the most narrow range of oxidized substrates. Responses of a biosensor based on these bacterial cells were obtained for normal structure alcohols (except methanol), some carbohydrates and amino acids.

It is known that microorganisms are capable of growth and propagation in immobilized form [19]. As the functioning of a biosensor is associated with the oxidation of organic substances (nitrogen-containing substances including) by microorganisms, it can be assumed that in the course of time the composition of the co-culture in the bioreceptor may change. More rapidly growing microorganisms may drive out those that grow slower. This work obtained the growth curves of the microorganisms used. The dependences of the optical density of culture liquid on time are given in Table 2.

Having studied the obtained data on the substrate specificities of the investigated microorganisms, as well as having compared the durations of the cultures' main growth phases, we chose the microbial strains to form co-cultures.

Co-culture 1 included microorganisms *A. adeninivorans* and *P. angusta* VKM Y-1397. *A. adeninivorans* possess a broad substrate specificity and are the basic microorganisms in the co-culture. Addition of *P. angusta* Y-1397 to them enables expanding the range of oxidized substrates to include methanol, glycerol, secondary and branched alcohols. Besides, cells of *A. adeninivorans* and *P. angusta* Y-1397 have similar growth phases and arrive at the growth retardation phase in approximately 30 h. They are also characterized by the prolonged lag phase (10–12 h) and prolonged linear growth phase (12–14 h). Thus, it can be assumed that there will be no domination of one yeast species over the other.

Co-culture 2 was composed of microorganisms *A. adeninivorans, P. angusta* VKM Y-1397 and *D. hansenii.* Addition of the yeast *D. hansenii* to co-culture 1 enables an even greater broadening of the range of oxidized substrates to include some carbohydrates and amino acids

(xylose, aspartic acid). However, the durations of the cultures' main growth phases strongly differ. The yeast *D. hansenii* is the most rapidly developing species of the three microbial cultures.

Co-culture 3 was made up of *G. oxydans* and *P. angusta* Y-2518. The yeast *P. angusta* actively oxidizes alcohols, and the bacteria *G. oxydans* actively oxidize many carbohydrates, not oxidizable by *P. angusta.* This co-culture has the most narrow range of oxidized substrates. Still, both these strains are known to be actively used in the development of biosensors for assaying carbohydrates and alcohols and to possess high sensitivities [16, 20]. This suggests that a biosensor based on the co-culture of these microorganisms shall also possess high metrological characteristics. Besides, *G. oxydans* and *P. angusta* have similar growth phases, namely, the adaptation phase (up to 8 h), a short exponential growth phase (up to 2 h) and by 16 h they reach the growth retardation phase, which, as is the case with co-culture 1, suggests a high stability of the biosensor.

3.2. Stability of the developed co-cultures in time

The main obstacle on the way of introducing BOD biosensors into practice is temporal instability of microbial co-cultures' composition. A previous work [21] has shown a high long-time stability (31 days) of a biosensor based on the co-culture of *D. hansenii* VKM Y-2482, *Arxula adeninivorans* VGI (78)-6 and *P. angusta* VKM Y-2518. However, the long-term stability can be due to the high survival rate of only two or one of the yeasts used. For this reason, it appeared to be important to study the species stability of the microorganisms in the co-cultures formed. The species stability of microorganisms in the co-cultures in time (42 days) was studied by optical microscopy and the Koch method. The storage conditions corresponded to the conditions of use in a biosensor analyzer (room temperature, daily washing with GGA solution with BOD_5 = 205 mg/dm^3 and triple washing with buffer solution).

The results of the studies showed that the cell ratio of different microbial species in the co-culture of *A. adeninivorans* and *P. angusta* VKM Y-1397 did not change in the course of time (Fig. 1).

Fig. **1 is here**

The ratio of microorganisms in the co-culture of *A. adeninivorans, P. angusta* VKM Y-1397 and *D. hansenii* changed by the end of the study period. In the course of time, *D. hansenii* became the dominating species. By the 42nd day of investigation, the ratio of the yeasts *A. adeninivorans* : *P. angusta* : *D. hansenii* was 1:1:4. It should be noted that the domination of *D. hansenii* in the co-culture begins to be observed by the end of the investigation time. According to the results of the analysis by the Koch method, confirmed by the data of optical microscopy up to the 23rd day of investigation, the ratio of microorganisms in this co-culture did not change. A low stability of the co-culture of the yeasts *A. adeninivorans*, *P. angusta* and *D. hansenii* is, most likely, due to a higher growth rate of and active metabolic processes in *D. hansenii*. The specific growth rate of the *D. hansenii* cell culture is 0.259 h^{-1}, whereas for the yeasts *A. adeninivorans* and *P. angusta* the specific growth rates are 0.158 h^{-1} and 0.160 h^{-1}, respectively.

The co-culture of *G. oxydans* and *P. angusta* Y-2518 significantly changed its composition in the course of time. Thus, already by the 10th day of experiment the ratio of *G. oxydans* : *P. angusta* Y-2518 was 2:1. By the 40th day of the measurements the ratio was 8:1. Thus, the co-culture, despite the close growth rates of the microorganisms, is unstable. This is associated, most likely, with a higher resistance of the bacteria to the action of the changed conditions of the external medium (immobilization). Thus, it is known that in the case of a joint immobilization of yeast and bacterial cells the bacteria may drive out the yeasts in 20 days of operation [14]. Based on these experimental data, this co-culture was not used in further work.

3.3. Characteristics of the biosensors based on the co-cultures formed

In the course of the work, biosensors for BOD express analysis were formed based on the developed co-cultures. Gel of poly(vinyl alcohol) modified by *N*-vinylpyrrolidone was used in the development of biosensors' recognition elements to immobilize the co-cultures. The use of *N*-vinylpyrrolidone for modification of PVA makes it possible to significantly increase the long-time stability of produced receptor elements at a high sensitivity and broad substrate specificity preserved [10].

A graduation dependence of biosensor response on BOD_5 in the measuring cuvette for the developed biosensors was plotted (Fig. 2).

Fig. **2 is here**

The receptor elements based on whole microbial cells belong to catalytic type bioreceptors, i.e., a biological response in such systems is provided for by enzymic reactions of microorganisms. For this reason, the dependences presented in Fig. 2 were fit by the 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 $[S] \rightarrow \infty$, K'_M is the apparent Michaelis constant, i.e, the concentration of substrate at which $R = R_{max}/2$.

To reduce errors of analysis, it is common practice to use the linear segment of the graduation dependence bounded from above by the apparent constant K'_M. The lower boundary of the linear segment corresponds to the lower boundary of the determined contents and is calculated by the statistical method proceeding from the criterion of the value for the relative standard deviation of the measurement results, $(S_r(C)) < 0.33$. For a sensor based on the

association of the yeasts *A. adeninivorans* and *P. angusta*, the lower boundary of the determined BOD values was 2.2 mg/dm^3; for a sensor on the association of *A. adeninivorans*, *P. angusta* and D. hansenii, 2.4 mg/dm^3.

The operational stability of the biosensors was determined as a change of activity (in per cent of the initial value) after 15 successive measurements. The time within which the value of the signal was no less than 25% of the initial activity is taken as the time of the stable operation of the receptor element. Table 3 presents the main analytical and metrological characteristics of the biosensors based on the stable co-cultures formed.

Thus, the developed biosensors possess close analytical and metrological characteristics. It is important to note that by their characteristics these biosensors are not inferior to their counterparts [3, 4] and even partially surpass them. Thus, a single BOD assay using the developed analyzer requires less time (3–4 min) as compared with a BOD assay done by means of other models of biosensor analyzers (10–15 min) [13, 22, 23]. The lower boundary of the determined BOD_5 values by the developed biosensors is somewhat inferior to the biosensor based on the single strain *D. hansenii* VKM Y-2482, immobilized in gel of chemically modified PVA, described in [10]. However, for most real-life samples this value of the lower boundary is acceptable.

As the substrate specificities of a co-culture and its constituent microorganisms may differ, the substrate specificities of receptor elements based on the developed microbial co-cultures immobilized in a film of PVA modified by *N*-vinylpyrrolidone were assessed. Predominantly readily oxidizable organic substances were taken as substrates, since their ingress into water reservoirs leads to a significant reduction in the level of dissolved oxygen and subsequent eutrophication [24]. Figure 3 presents the data for the substrate specificities of the developed biosensors.

Fig. **3 is here**

Thus, the co-culture based on strains *P. angusta* VKM Y-1397, *A. adeninivorans* VKM Y-2677, *D. hansenii* VKM Y-2482 possesses the broadest substrate specificity. This co-culture oxidizes mono- and disaccharides, monoatomic and polyatomic alcohols, carboxylic acids, amino acids, some aromatic compounds. Sodium benzoate (a food additive having a suppressive action on yeasts by inhibiting the enzyme activities in the cell) and *p*-nitrophenol (a widespread commercial toxicant) are oxidized only by the co-culture of *P. angusta*, *A. adeninivorans* and *D. hansenii*.

It is important to note that, unlike the biosensor based on the single strain *D. hansenii* immobilized in gel of chemically modified PVA [10], the use of the biosensor based on the co-culture of three microorganisms makes it possible to significantly broaden the range of substrates oxidized by the bioreceptor. This will lead to increase the correctness of assaying real-life samples of waste waters and will ensure a wide application of the developed biosensor in practice.

3.4. Assays of water samples and fermentation products using biosensors based on the co-cultures formed

Water samples and fermentation products were assayed using the developed biosensors and by the standard dilution method. The samples were taken from river and bog water, waste waters of municipal sewage facilities, meltwaters, fermentation products of starchy raw materials (distillery stillage). Sampling was done in accordance with the standard technique [2]. Determination of BOD_5 in wastes by the standard dilution method was carried out according to the current regulatory documents (item 2.9) [2]. Figure 4 (a, b) shows a correlation between the BOD values of water samples determined using the biosensors (for three and two co-cultures, correspondigly) and those determined by the standard dilution method. The greatest correlation with the standard method is observed for the sensor based on the co-culture of *P. angusta*, *A. adeninivorans* and *D. hansenii* immobilized in PVA modified by *N*-vinylpyrrolidone. It is

important to note that the correlation coefficient for the data obtained by the standard method and using a biosensor based on the developed co-cultures was higher than in most counterparts described [4, 6], including the biosensor based on the single *D. hansenii* strain [10]. For all biosensors the BOD values coincide with those obtained by the standard method with account for confidential intervals.

Fig. **4 is here**

Thus, the systematic approach to the formation of artificial co-cultures of microorganisms based on the similarity of their growth parameters and substrate specificity differences enabled forming a co-culture with a broad range of oxidized substrates and stable for over 20 days. The use of co-cultures of the yeasts *P. angusta, A. adeninivorans* and *D. hansenii* immobilized in PVA modified by *N*-vinylpyrrolidone made it possible to develop a BOD biosensor of practical significance. The results indicate a possibility of using the developed biosensor analyzer as a prototype model of commercial instruments.

Acknowledgements

The work was carried out within the framework of the State Assignment of the Ministry of Education and Science of the Russian Federation; the Federal Targeted Programme for Research and Development in Priority Areas of Development of the Russian Scientific and Technological Complex for 2014–2020 (Project No.14.574.21.0062); and a grant of the President of the Russian Federation for state support of young Russian PhD scientists, agreement No. 14.Z56.14.330-MK.

References

1. Bourgeois W, Burgess JE, Stuetz RM. On-line monitoring of wastewater quality: a review. J Chem Technol Biotechnol 2001; 76:337–48.

2. APHA. Standard Methods for the Examination of Water and Wastewater, Section 5210, 18th ed. American Public Health Association Water Works Association, American Water Environment Federation, Washington: DC; 1992; 5.1–5.6.

3. D'Souza SF. Microbial biosensors. Biosens Bioelectron 2001; 16:337–53.

4. Rodriguez-Mozaz SL, López de Alda MJ, Barcelo D. Biosensors as useful tools for environmental analysis and monitoring. Anal Bioanal Chem 2006; 386(4):1025–41.

5. Tan TC, Lim EWC. Thermally killed cells of complex microbial culture for biosensor measurement of BOD of wastewater. Sens Act B 2005; 107(2):546–51.

6. Ponamoreva ON, Arlyapov VA, Alferov VA, Reshetilov AN. Microbial biosensors for detection of biological oxygen demand (review). Appl Biochem Microbiol 2011; 47(1):1–11.

7. Jianbo J, Tang M, Chen X, Qi L, Dong S. Co-immobilized microbial biosensor for BOD estimation based on sol-gel derived composite material. Biosens Bioelectron 2003; 18(8):1023–9.

8. Lin L, Xiao L-L, Huang S, Zhao L, Cui J-S, Wang X-H, Chen X Novel BOD optical fiber biosensor based on co-immobilized microorganisms in ormosils matrix. Biosens Bioelectron 2006; 21(9):1703–9.

9. Jiang Y, Xiao L-L, Zhao L, Chen X, Wang X, Wong K-Y. Optical biosensor for the determination of BOD in seawater. Talanta 2006; 70(1):97–103.

10. Arlyapov VA, Yudina NYu, Asulyan LD, Alferov SV, Alferov VA, Reshetilov AN BOD biosensor based on the yeast *Debaryomyces hansenii* immobilized in poly(vinyl alcohol) modified by N-vinylpyrrolidone. Enzyme Microb Technol 2013; 53:257–62.

11. Kobzev EN, Petrikevich SB, Shkidchenko AN. Study of the stability association of oil-degrading microorganisms in an open system. Prikl Biokhim Mikrobiol 2001; 37(4):413–7.

12. Seo KS, Choo KH, Chang HN, Park JK. A flow injection analysis system with encapsulated high-density *Saccharomyces cerevisiae* cells for rapid determination of biochemical oxygen demand. Appl Microbiol Biotechnol 2009; 83(2):217–23.

13. Dhall P, Kumar A, Joshi A, Saxsena TK, Manoharan A, Makhijani SD, Kumar R. Quick and reliable estimation of BOD load of beverage industrial wastewater by developing BOD biosensor. Sens Act B 2008; 133(2):478–83.

14. Chan C, Lehmann M, Tag K, Lung M, Kunze G, Riedeld K, Gruendige B, Renneberg R. Measurement of biodegradable substances using the salt-tolerant yeast *Arxula adeninivorans* for a microbial sensor immobilized with poly(carbamoyl) sulfonate (PCS): Part II: application of the novel biosensor to real samples from coastal and island regions. Biosens Bioelectron 1999; 14(3):295–302.

15. Arlyapov VA, Ponamoreva ON, Alferov VA, Rogova TV, Blokhin IV, Chepkova IF, Reshetilov AN. Microbial biosensors to express determine bod in effluents from food industry enterprises. Water: Chemistry and Ecology 2008; 3:23–30.

16. Voronova EA, Iliasov PV, Reshetilov AN. Development, investigation of parameters and estimation of possibility of adaptation of *Pichia angusta* based microbial sensor for ethanol detection. Anal Lett 2008; 41(3):377–91.

17. Arlyapov VA, Kamanin SS, Ponamoreva ON, Reshetilov AN. Biosensor analyzer for BOD index express control on the basis of the yeast microorganisms *Candida maltosa*, *Candida blankii* and *Debaryomyces hansenii*. Enzyme Microb Technol 2012; 50 (4–5): 215–20.

18. Tkac J, Vostiar I, Gorton L, Gemeiner P, Sturdik E. Application to the analysis of ethanol during fermentation. Biosens Bioelectron 2003; 18:1125–34.

19. Efremenko EN, Tatarinova NY. The effect of long-term preservation of bacterial cells immobilized in poly(vinyl alcohol) cryogel on their viability and biosynthesis of target metabolites. Microbiology 2007; 76(3):336–41.

20. Arlyapov VA, Ponamoreva ON, Alferov SV, Alferov VA, Reshetilov AN. Application of the low-selective microbial biosensors for the determination of components in multicomponent aqueous solutions. Sensorn Sistemy 2011; 25(4):352–60.

21. Kamanin SS, Arlyapov VA, Ponamoreva ON, Alferov VA, Reshetilov AN. BOD-biosensor based on yeast strains. Water: Chemistry and Ecology 2012; 3:74–81.

22. Chee G-J, Nomura Y, Ikebukuro K, Karube I. Optical fiber biosensor for the determination of low biochemical oxygen demand. Biosens Bioelectron 2000; 15(7–8):371–6.

23. Chang IS, Moon H, Jang JK, Kim BH. Fluorescence and bioluminescence of bacterial luciferase intermediates. Biosens Bioelectron 2005; 20(9):1856–9.

24. Henze M, Harremoës P, La Cour Jansen J, Arvin E. (eds.) Wastewater treatment. Biological and chemical processes, 2nd edn. Berlin: Springer, 1997; 383 pp.

Figure legends

Fig. **1.** Determination of the amounts of viable cells in the co-cultures in the course of time by inoculation onto dense nutrient media: (a) the co-culture of *A. adeninivorans* and *P. angusta* VKM Y-1397; (b) the co-culture of *G. oxydans* and *P. angusta* Y-2518; (c) the co-culture of *A. adeninivorans*, *P. angusta* VKM Y-1397 and *D. hansenii*.

Fig. **2.** A graduation dependence of biosensor response on BOD_5 for a biosensor based on the co-cultures used.

Fig. **3.** Substrate specificities of receptor elements based on the co-cultures used.

Fig. **4.** Correlation of the data obtained by the standard method and using biosensors based on the co-cultures of: (a) *P. angusta*, *A. adeninivorans* and *D. hansenii* immobilized in poly(vinyl alcohol) modified by *N*-vinylpyrrolidone; (b) *P. angusta* and *A. adeninivorans* immobilized in poly(vinyl alcohol) modified by *N*-vinylpyrrolidone.

Table 1. Comparison of substrate specificities for receptor elements based on the strains studied.

Substrate	*D. hansenii*	*A. adenini-vorans*	*P. angusta* Y-2518	*P. angusta* Y-1397	*C. boidinii*	*G. oxydans*
Methanol	+/–	–	+	+	+	–
Ethanol	+	+	+	+	+	+
1-Propanol	+	+	+	+	+	+
2- Propanol	+/–	–	+	+	+/–	+/–
1-Butanol	+	+	+	+	+/–	+
2-Butanol	+/-	–	+	+	+	+
2-Methyl-2-propanol	+/–	–	+/–	+/–	–	–
3-Methyl-1-butanol	+	+/–	–	–	–	–
Glycerol	+/-	–	+/–	+	+	–
Glucose	+	+	+	+	+	+
Sucrose	+/-	+	–	+/–	+/–	–
Galactose	+	+	+/–	–	+/–	+
Lactose	+/–	+	–	–	–	+/–
Xylose	+/–	–	–	–	+/–	+
Formaldehyde	+	–	+	+	+	–
Formic acid	+	–	+/–	+/–	+/–	–
Acetic acid	+	+	+/–	+/–	+/–	–
Glutamic acid	+/–	+	+/–	+	+/–	+/–
Aspartic acid	+/–	–	–	–	+	–
Tyrosine	+/–	+/-	+/–	+/–	+/–	+/–
Lysine	+/–	+/–	–	–	–	+/–
Sodium DDS	+	+/–	–	–	–	–
Sodium DDBS	+/–	+/–	–	–	–	–

\+ strong biosensor response (31–100% of the maximal response)

+/– weak biosensor response (1–30% of the maximal response)

– no biosensor response

Table 2. Microbial growth phases obtained based on optical density measurements.

Strain	Lag phase, h	Exponential phase, h	Linear growth phase, h	Growth retardation phase, h	Stationary phase, h	Specific cell culture growth rate (μ), h^{-1}
P. angusta VKM Y-1397	0–12	12–16	16–30	30–38	38–50	0.160
P. angusta VKM Y-2518	0–8	8–10	10–12	12–22	22–50	0.312
A. adeninivorans VKM Y-2677	0–10	10–14	14–28	28–34	34–50	0.158
D. hansenii VKM Y-2482	0–18	18–24	24–26	26–30	30–50	0.259
C. boidinii VKM Y-2356	0–10	10–18	18–32	32–38	38–50	0.043
G. oxydans VKM B-1280	0–8	8–10	10–16	16–24	24–50	0.333

Table 3. Characteristics of the yeast co-culture-based BOD biosensor.

Characteristics	Microorganisms, the basis of the bioreceptor	
	P. angusta, *A. adeninivorans*	*P. angusta*, *A. adeninivorans*, *D. hansenii*
Sensitivity coefficient[a], $s^{-1} \cdot 10^{-5}$	8 ± 2	10 ± 2
Range of determined BOD_5 values, mg/dm^3	2.2–42	2.4–80
Operational stability[b], %	8.5	8.9
Long-time stability[c], days	19	17
Unit assay time[d], min	4	3

[a] Slope of the linear segment in the dependence of sensor response on the value of BOD_5

[b] Relative standard deviation by 15 consecutive measurements

[c] Time within which the value of biosensor response to the same concentration of GGA was no less than 25% of the initial value, temperature 20ºC

[d] Duration of single measurement

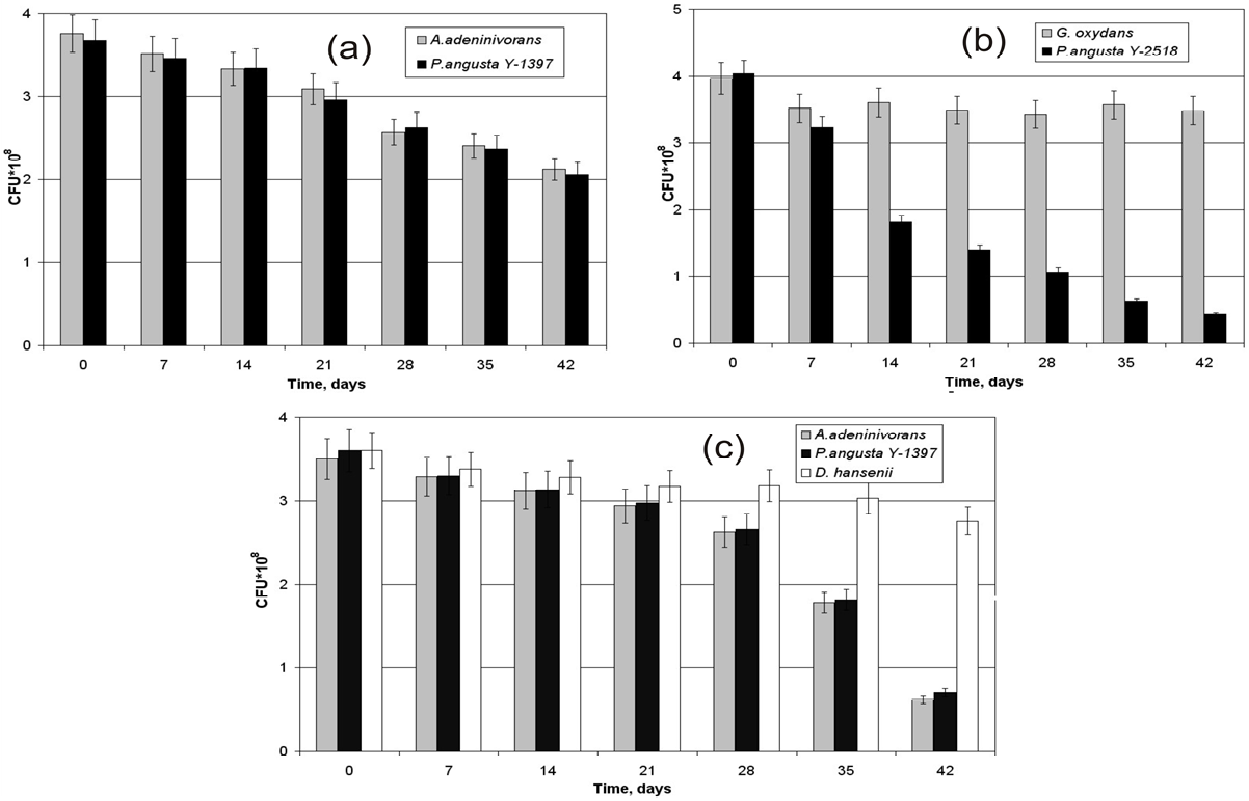

fig_1(4) .

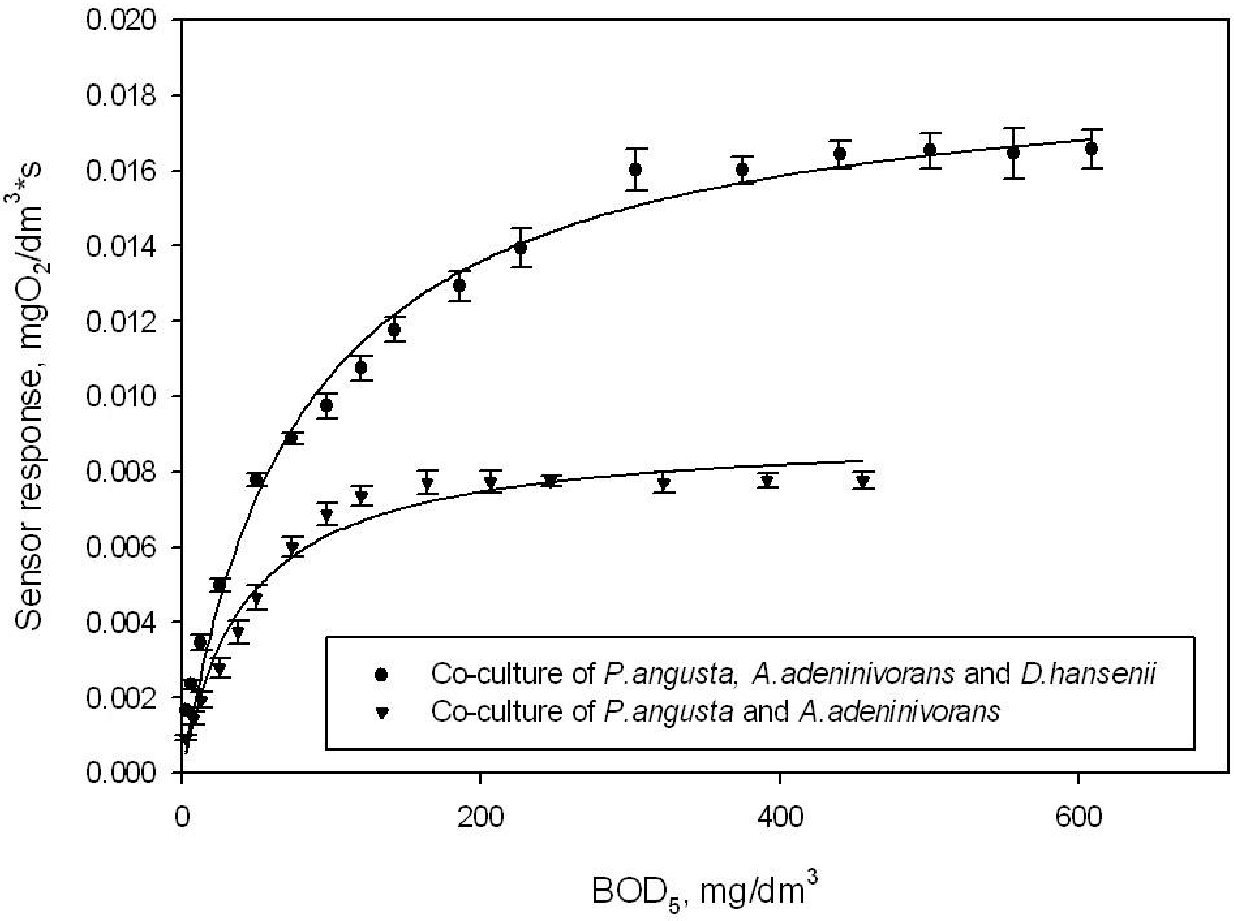

fig_2(4) .

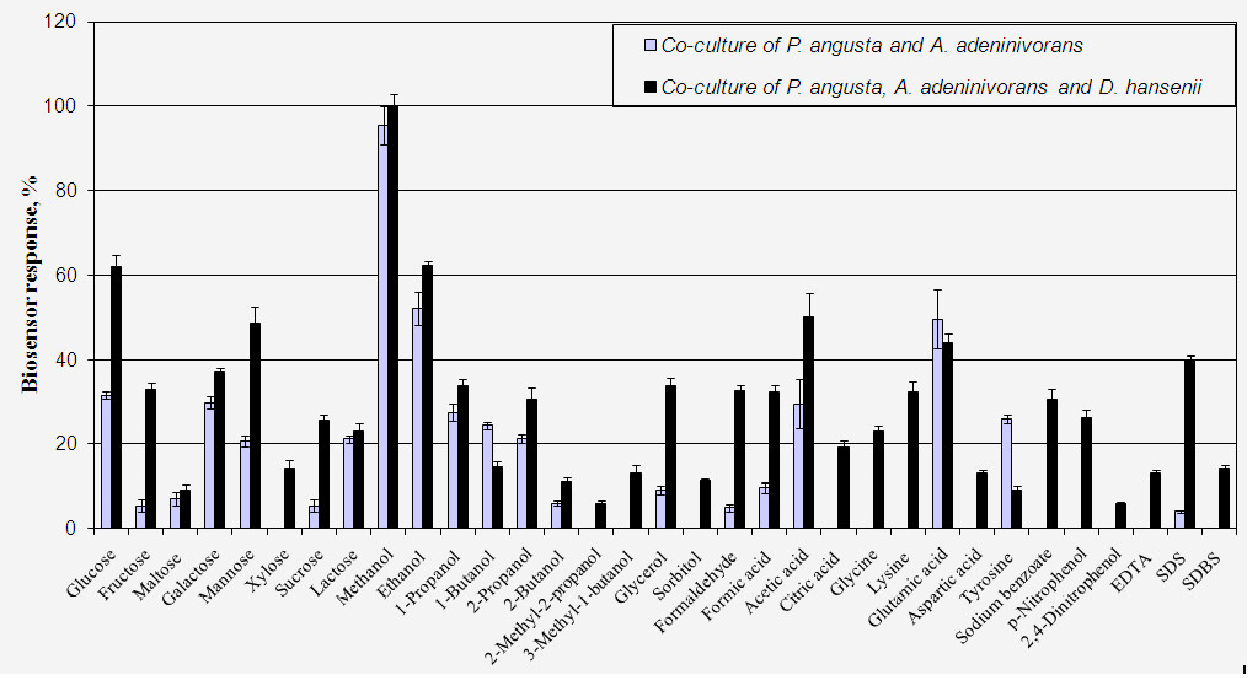

fig_3(4) .

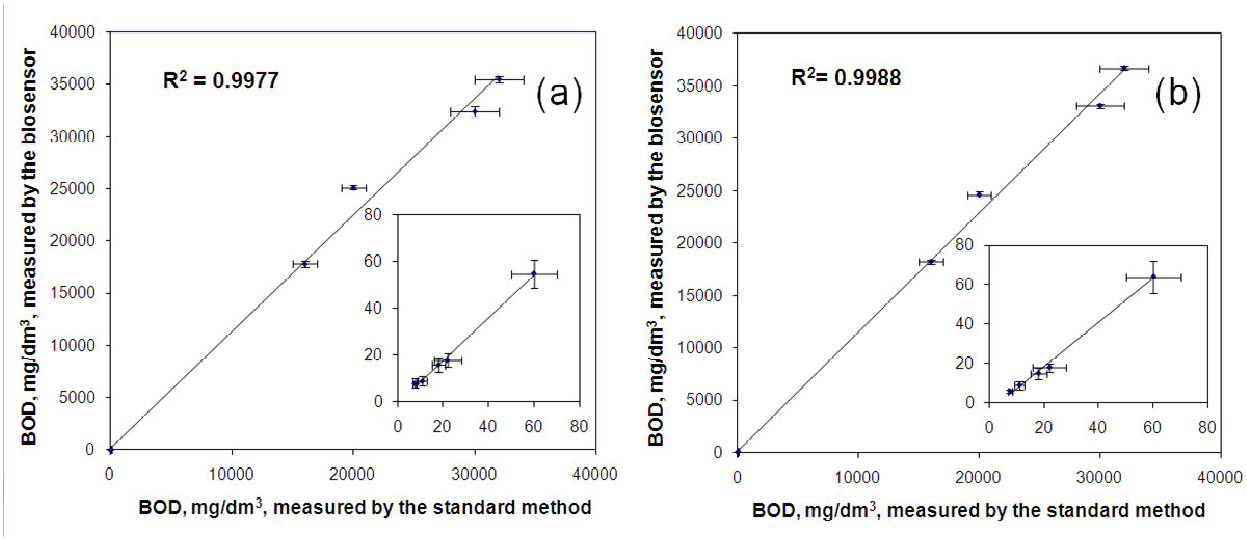

fig_4(4) .