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

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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.

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

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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* subsp. *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 CM70 M 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.