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An Inducible Whole-Cell Biosensor for Detection of Formate Ions

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Abstract—Ten strains of the yeast *Yarrowia lipolytica* were constructed, the genomes of which contain the *hrGFP* gene under regulation of the formate dehydrogenase promoters. The resulting strains can act as whole-cell biosensors for the detection of formate ions in various media. By visual assessment of the biomass fluorescence, we selected the three most promising yeast strains. The main biosensor characteristics (threshold sensitivity, amplitude, and response time) of the selected strains were measured. As a result, in terms of characteristics, the B26 strain was recognized as the most suitable for the detection of formate ions. A carbon source for the nutrient medium that does not reduce the activation of the biosensor was selected. Furthermore, we showed that unlike formate and formaldehyde, methanol practically does not induce the biosensor fluorescence response.

Keywords: inducible promoter, fluorescence, formate ion, whole-cell biosensor, *Yarrowia lipolytica*

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Formic acid (formate anion, $HCOO^-$) is the simplest carboxylic compound, a metabolite of plants, insects, and microorganisms. It is used as a preservative in feeds and food products, an acidifying agent in the textile and leather industries, and a reagent in chemical production and in modern fuel elements (https://www.acs.org/molecule-of-the-week/archive.html?archive=All) [1, 2]. Formate is a side product formed in some technological processes; e.g., in enzymatic pretreatment of lignocellulosic materials, it inhibits the subsequent hydrolysis or fermentation [3, 4]. Sodium formate is added to de-icing agents to decrease their corrosive activity, which can lead to the accumulation of chemical reagents in the deep layers of roadside soil [5]. In addition, formic acid can be an indicator of intoxication from formaldehyde, methanol, or acetone [6]. All the above emphasizes the need for formate anion detection in different media and substances.

The formate anion is detected by high performance liquid and gas chromatography. However, these methods have low selectivity and are sometimes hard to use because of the complex process of sample preparation and the need for low-mobility expensive equipment and highly qualified personnel [7–10]. One of the alternatives to chromatography techniques is using biosensors. Inducible whole-cell biosensors are the cells of microorganisms with two major elements embedded in the genome: a regulatory system (the promoter–operator region) and reporter genes transcriptionally fused with the latter. The most frequently used reporter genes are *lacZ* (encoding b-galactosidase), *gfp* (encoding green fluorescent protein), and *lux*CDABE (encoding luciferase and reductase) [11, 12]. Under normal conditions (in the absence of an inducer in the medium), the promoter upstream of the reporter genes is inactive and biosensor cells do not differ from the control strain. When an inducer appears in the medium, the promoter is activated with the subsequent synthesis of the reporter protein. This results in fluorescence, luminescence, etc., of biosensor cells.

The genes of formate dehydrogenases (EC: 1.2.2.1, 1.17.1.9, 1.17.1.10, 1.17.2.3, 1.17.5.3, 1.17.98.3, 1.17.98.4) have been found in many methylotrophic and non-methylotrophic microorganisms: *Methylorubrum extorquens* [13], *Pseudomonas aeruginosa* [14], *Escherichia coli* [15], *Candida boidinii* [16], *Hansenula polymorpha* [17], *Saccharomyces cerevisiae* [18], etc. [19]. In the nonmethylotrophic yeast *Yarrowia lipolytica*, ten formate dehydrogenase genes and the respective ten promoter sequences have been identified [20]. Their regulatory regions can be a basis for constructing specific biosensors with different degrees of sensitivity.

The aim of the present work was to develop specific inducible biosensors (with the *hrGFP* reporter gene) for the detection of formate anions.

Table 1. Description of the genotypes of the biosensor strains constructed

Promoter (the *FDH* gene locus)	Plasmid for integration	*Y. lipolytica* strain	Strain designation
YALI1_A12502g	pProUA-pFDH_A1	W29 Δ*ku70* IntC2::P_{FDH_A1}-*hrGFP*-T_{LIP2}	A1
YALI1_B26033g	pProUA-pFDH_B26	W29 Δ*ku70* IntC2::P_{FDH_B26}-*hrGFP*-T_{LIP2}	B26
YALI1_B29419g	pProUA-pFDH_B29	W29 Δ*ku70* IntC2::P_{FDH_B29}-*hrGFP*-T_{LIP2}	B29
YALI1_C10762g	pProUA-pFDH_C1	W29 Δ*ku70* IntC2::P_{FDH_C1}-*hrGFP*-T_{LIP2}	C1
YALI1_C20054g	pProUA-pFDH_C2	W29 Δ*ku70* IntC2::P_{FDH_C2}-*hrGFP*-T_{LIP2}	C2
YALI1_F18740g	pProUA-pFDH_F1	W29 Δ*ku70* IntC2::P_{FDH_F1}-*hrGFP*-T_{LIP2}	F1
YALI1_F21426g	pProUA-pFDH_F2	W29 Δ*ku70* IntC2::P_{FDH_F2}-*hrGFP*-T_{LIP2}	F2
YALI1_F36552g	pProUA-pFDH_F3	W29 Δ*ku70* IntC2::P_{FDH_F3}-*hrGFP*-T_{LIP2}	F3
YALI1_E17326g	pProUA-pFDH_E17	W29 Δ*ku70* IntC2::P_{FDH_E17}-*hrGFP*-T_{LIP2}	E17
YALI1_E19014g	pProUA-pFDH_E19	W29 Δ*ku70* IntC2::P_{FDH_E19}-*hrGFP*-T_{LIP2}	E19

MATERIALS AND METHODS

Microbial strains and plasmids. The strain *Escherichia coli* TOP10 F$^-$*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(*StrR*) *endA1* λ$^-$ was used to obtain plasmid DNA [21].

The yeast strains used in this work were *Yarrowia lipolytica* Y-3178 W29 MatA (wild type) and Y-4973 W29 Δ*ku70* Δ*URA3*, both provided by the National Resource Center of the All-Russia Collection of Industrial Microorganisms.

The plasmid vector pProUA-mScarlet contains the ampicillin resistance gene (Ap^r), the green fluorescent protein gene (*hrGFP*) is optimized at the codons for the expression in *Y. lipolytica*, and the selective yeast marker *URA3* is the gene for uracil synthesis. The helper plasmid pCasNA-IntC2 contains the gene for the Cas9 protein, the target DNA (sgRNA) sequence, and the gene of resistance to nourseothricin (Nat^r) and ampicillin (Ap^r) [22].

Construction of *Y. lipolytica* biosensor strains. The formate dehydrogenase genes in the genome of *Y. lipolytica* were identified by the amino acid sequence homology with the formate dehydrogenase FDH1 of *S. cerevisiae* (EC: 1.17.1.9) using BLAST (Basic Local Alignment Search Tool, NCBI, United States). In accordance with the GenBank database (NCBI, United States), the formate dehydrogenase genes in the *Y. lipolytica* genome are located in the following loci: YALI1_A12502g, YALI1_B26033g, YALI1_B29419g, YALI1_C10762g, YALI1_C20054g, YALI1_F18740g, YALI1_F21426g, YALI1_F36552g, YALI1_E17326g, YALI1_E19014g.

The *Y. lipolytica* strains for promoter tests were constructed according to the *YaliCraft* manual with the involvement of Pro Module [22]. To this effect, the nucleotide sequences of 800 b.p. in length located upstream of the formate dehydrogenase (*FDH*) genes in the yeast *Y. lipolytica* were chemically synthesized by Twist Bioscience (United States) and inserted into the pProUA-mScarlet vector provided by the same company, immediately upstream of the *hrGFP* reporter gene. Plasmids pProUA-pFDH_A1, pProUA-pFDH_B26, pProUA-pFDH_B29, pProUA-pFDH_C1, pProUA-pFDH_C2, pProUA-pFDH_F1, pProUA-pFDH_F2, pProUA-pFDH_F3, pProUA-pFDH_E17, and pProUA-pFDH_E19 provided by Twist Bioscience were transformed into *E. coli* TOP10 cells for plasmid DNA production. Cell transformation and plasmid DNA isolation were performed according to [21].

The produced plasmids were linearized at the NotI restriction sites and inserted into chromosome C of the strain Y-4973 of *Y. lipolytica*. Yeast transformation was performed in conjunction with helper plasmid pCasNA-IntC2 according to the previously described protocol [22]. Transformants were verified by PCR. The constructed biosensor strains are presented in Table 1.

Nutrient media and growth conditions. *E. coli* TOP10 cells were grown in a Luria–Bertani (LB) broth at 37°C for 2 h to the mid-exponential growth phase. The transformed TOP10 strains were selected on the L-agar with ampicillin (100 μg/mL).

For transformation, *Y. lipolytica* Y-4973 cells were grown at 30°C for 16–20 h to the exponential growth phase on an agarized YP medium (peptone, 10 g/L; yeast extract, 5 g/L) with glucose (1% vol) as a carbon source. The transformed yeast strains were selected on an agarized YNB medium (Yeast Nitrogen Base; M139, HIMEDIA, India, 6.75 g/L) with glucose (1% vol) and nourseothricin (50 μg/mL). For further experiments, *Y. lipolytica* strains were grown on agarized or in liquid YNB media with the addition of a carbon source to the required concentration.

Enzymes and chemicals. All chemical reagents were of analytically pure grade. The enzymes for the work with DNA were obtained from Fermentas (Lithuania).

The inducers were sodium formate, formaldehyde, and methanol produced by Sigma-Aldrich (United States). All test solutions were prepared immediately before use.

Measurement and visualization of biosensor fluorescence. For visual assessment of fluorescence of the constructed biosensors, an overnight culture of yeast strains was diluted in a sterile physiological solution to an optical density of 0.8 and cell suspensions were inoculated by 5 μL onto Petri dishes with agarized YNB containing 1% glucose and different concentrations of sodium formate. The dishes were incubated at 30°C for 24 h. Biosensor activity was assessed by the intensity of green fluorescence of the grown biomass using a Clare Chemical Research DR46BLED blue light transilluminator (United States) with an orange filter.

The time dependence of the intensity of biosensor fluorescence on different inducer concentrations was measured in yeast strains inoculated on an agarized YNB with 1% vol/vol glucose and grown at 30°C for 20 h. The grown cell biomass was diluted with liquid YNB containing the required concentrations of the carbon source and the inducer to an optical density of 0.3. Control samples were prepared analogously but without the inducer. Ready samples were transferred by 100 μL into the wells of a 655096 Greiner Bio-One microplate (Germany), which was then put into a CLARIOstar Plus multimodal reader (BMG Labtech, Germany) and incubated at 30°C and 500 rpm for 6 h with measurement of the optical density and fluorescence every 15 min according to [22]. The relative fluorescence was calculated for each well, the measured fluorescence values being divided by the values of the optical density (FLU/OD_{600}). The number of replicates was five wells for each concentration of sodium formate. Data processing was performed with MARS (BMG Labtech, Germany) and Excel (Microsoft, United States).

The findings were used to determine the major three parameters characterizing the biosensor quality: response amplitude (RA), minimum response time t_m, and threshold sensitivity. RA is determined by the formula $RA = I_t - I_o/I_k - I_o$, where I_o is the relative fluorescence of the preparation at the moment of adding the inducer ($t = 0$), I_k is the relative fluorescence of the control preparation at moment t, and I_t is the relative fluorescence of the experimental preparation at moment t. The minimum response time t_m is determined by the time interval between the moment of addition of the inducer and the moment of measuring the reliable increase in the signal compared to $(I_k - I_o)$ with a fluorimeter. The threshold sensitivity is determined by the minimum concentration of the inducer, when RA is approximately equal to two.

RESULTS AND DISCUSSION

Qualitative assessment of fluorescence of the constructed strains. At the first stage, the activity of the constructed biosensors (Table 1) was assessed qualitatively: by the intensity of green fluorescence of the biomass of strains grown on an agarized medium contain-

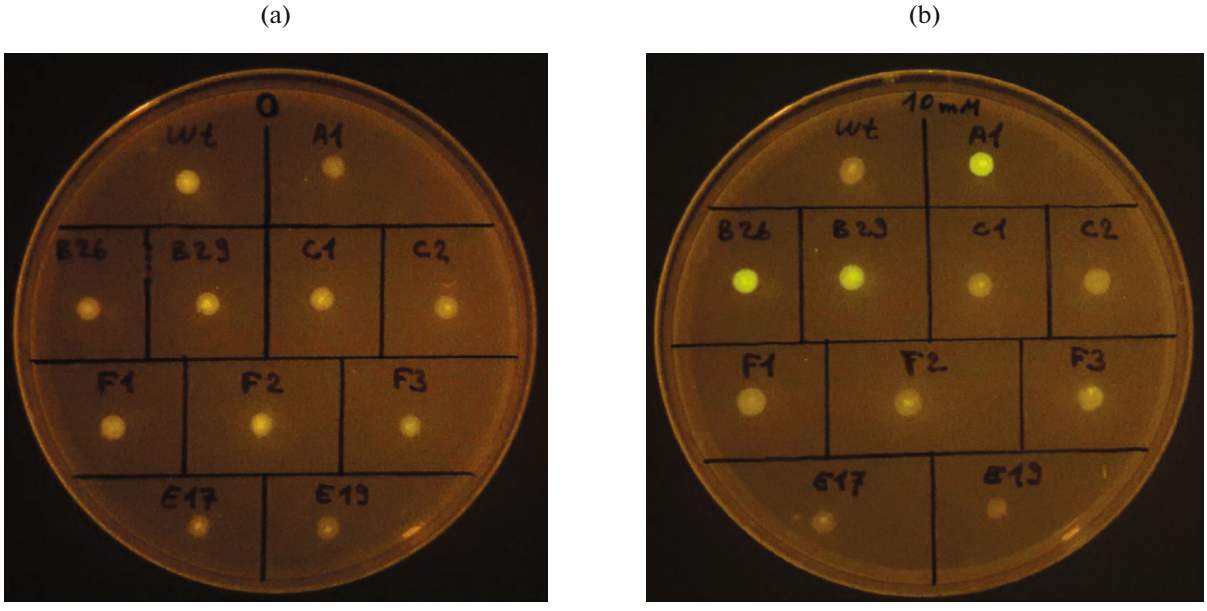

Fig. 1. The fluorescence (hrGFP) of the yeast strain biomass after cultivation for 20 h on an agarized YNB medium with 1% glucose (a) without formate and (b) with the addition of 10 mM sodium formate. The control strain Y-3178 W29 MatA (wild type) is marked as wt.

ing 0 (control) and 10 mM of sodium formate (Fig. 1). It can be seen that most of the tested strains do not fluoresce, similarly to wild type Y-3178 (wt), even in the presence of formate (Fig. 1b). The marked green fluorescence is demonstrated by three variants of the strain: A1, B26, and B29.

Based on the qualitative assessment, the A1, B26, and B29 strains were chosen as potential biosensors for fluorescence quantification and for the comparison of their characteristics.

Quantification of fluorescence of the A1, B26, and B29 strains. Figure 2 shows the curves of the fluorescence normalized by the optical density (OD) for the A1, B26, and B29 strains depending on the time of incubation in the medium with different concentrations of sodium formate. Yeast strains were inoculated on agarized YNB with 1% glucose and grown at 30°C for 20 h. The cell biomass was diluted in liquid YNB with 1% glucose to an optical density of 0.3. Then cell suspension was transferred by 100 μL into wells of the 655 096 Greiner Bio-One microplate (Germany), and sodium formate was added at given concentrations as an inducer. Then the microplate was placed into the CLARIOstar Plus multimodal reader (BMG Labtech,

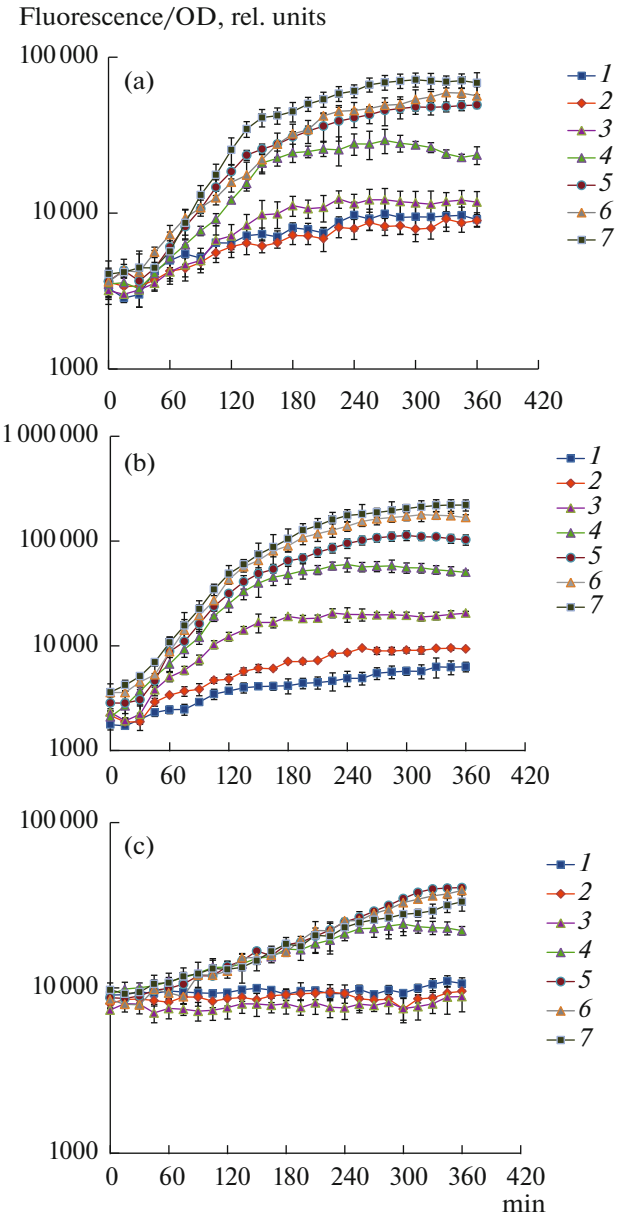

Fig. 2. The dependence of the (a) A1, (b) B26, and (c) B29 fluorescence normalized by the optical density (OD) on the time of incubation in the medium with sodium formate: *1*, without sodium formate; *2*, 10 μM; *3*, 100 μM; *4*, 1 mM; *5*, 10 mM; *6*, 90 mM; *7*, 440 mM.

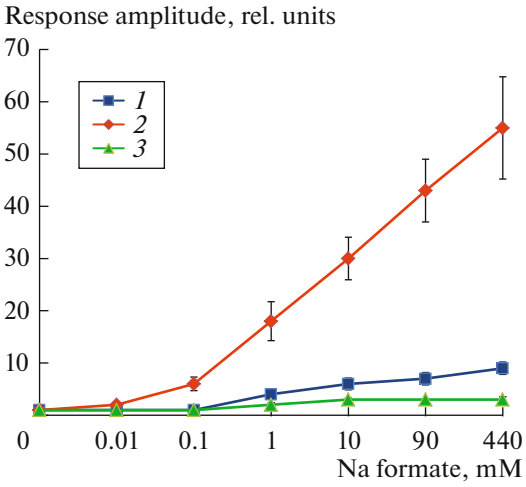

Fig. 3. The dependence of the maximum responses (RA) of the A1, B26, and B29 strains on different concentrations of sodium formate. *1*, A1; *2*, B26; *3*, B29.

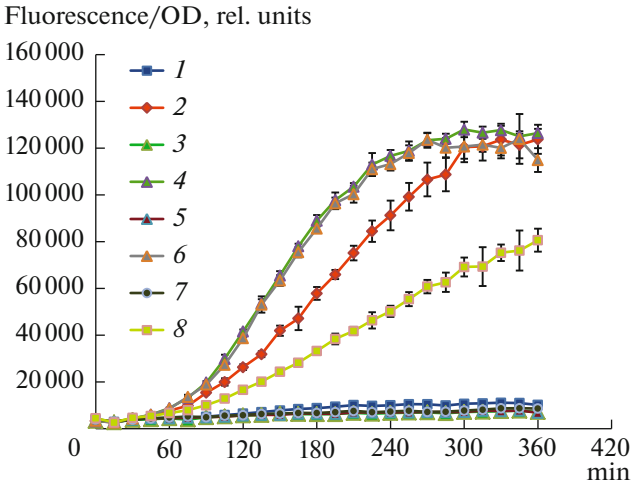

Fig. 4. The effects of carbon sources in the nutrient medium on activation of the B26 biosensor upon the addition of 10 mM formate: *1*, glucose (1%); *2*, glucose and formate; *3*, sorbitol (1.5%); *4*, sorbitol and formate; *5*, mannitol (1%); *6*, mannitol and formate; *7*, citrate (1%); *8*, citrate and formate.

Germany) and incubated at 30°C and 500 rpm for 6 h, with optical density and fluorescence measured every 15 min according to [22].

It can be seen that all three strains (A1, B26, and B29) responded to the addition of sodium formate by enhanced fluorescence. The response time was the same for all three strains at 60–70 min. However, among the tested strains, B26 demonstrated the lowest threshold concentration of 10 μM (Fig. 2b), while the threshold concentrations for A1 and B29 were 100 μM (Fig. 2a) and 1 mM (Fig. 2c), respectively. It should be noted that the B29 strain had a higher background fluorescence compared to A1 and B26, which resulted in a lower response amplitude. Thus, B26 was the optimal strain for determining the content of formate anions in the medium.

Figure 3 shows the dependencies of the maximum responses (RA) of the A1, B26, and B29 strains on the sodium formate concentration. According to the diagram, the B26 strain demonstrated the maximum RA at all of the tested concentrations of sodium formate; therefore, B26 was chosen as a whole-cell biosensor for the detection of formate anions.

Selection of the optimal carbon source. Previous works have shown that the addition of glucose as a carbon source can reduce the induction of formate dehydrogenase gene promoters [23, 24]. Glucose (1%), sorbitol (1.5%), mannitol (1%), or citrate (1%) was added to the medium as a carbon source, and the levels of the promoter activation in the B26 strain after the addition of 10 mM formate were compared. The experimental results are shown in Fig. 4.

The addition of citrate to the medium had not proper effect on biosensor activation. The addition of sorbitol or mannitol demonstrated similar levels of promoter activity; at the same time, the RA of the biosensor in the medium with mannitol or sorbitol was actually higher than in the medium with glucose. As a result, mannitol was chosen as a carbon source for the nutrient medium, because *Y. lipolytica* utilized it better than sorbitol.

Measurement of the main characteristics and verification of the specificity of the B26 biosensor. It is known that formate dehydrogenases of yeasts (both methylotrophic and nonmethylotrophic) participate in the oxidation of formaldehyde to carbon dioxide [18]. Formaldehyde produced during methanol oxidation or in other cellular processes is cytotoxic; therefore, all organisms have formaldehyde detoxification pathways [25]. Although *Y. lipolytica* is not a methylotrophic yeast, the possibility of activating the B26 biosensor by not only formaldehyde but also methanol has been verified.

Figure 5 shows the curves of dependence of the B26 biosensor fluorescence normalized by the optical density (OD) on the time of incubation in the YNB medium with 1% mannitol at different concentrations of sodium formate, formaldehyde, or methanol. The range of normalized fluorescence of biosensor cells upon the addition of sodium formate varied from 10000 to 200000 depending on the inducer concentration in the medium (Fig. 5a). The threshold concentration was 10 μM; the minimum response time was 70 min and depended on the inducer concentration. It should be noted that biosensor cells tolerated well the addition of high concentrations of sodium formate (up to 6%).

With formaldehyde used as an inducer (Fig. 5b), the range of working concentrations of the biosensor narrowed dramatically due to the high cytotoxicity of

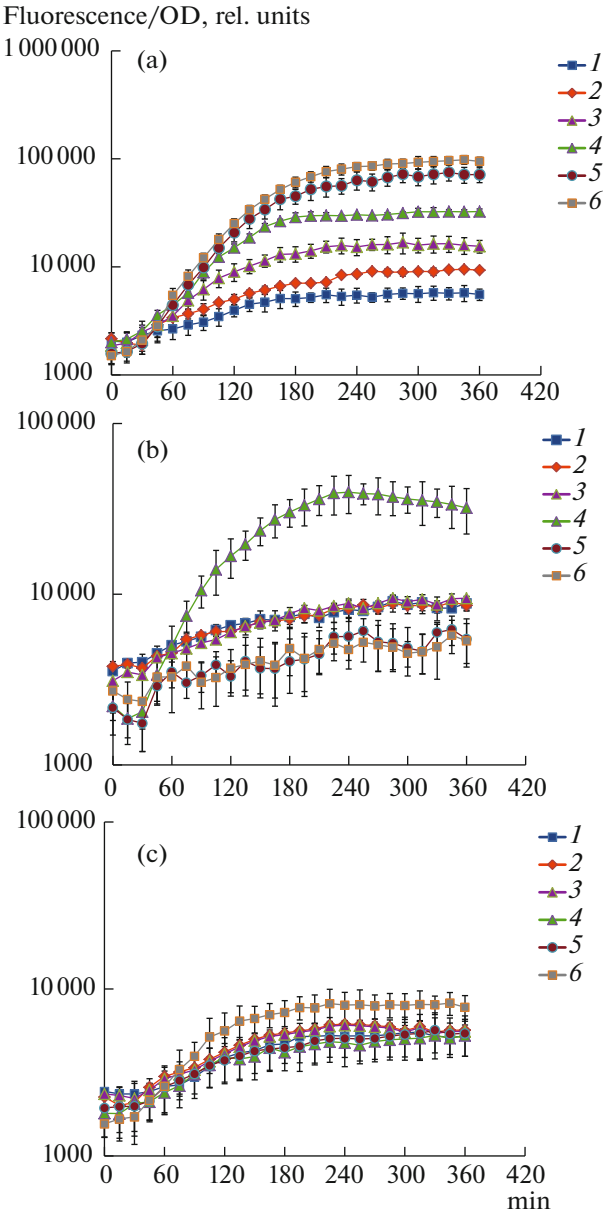

Fig. 5. The dependence of the B26 biosensor fluorescence normalized by the optical density (OD) on the time of incubation in the nutrient medium containing mannitol (1%) and (a) sodium formate, (b) formaldehyde, or (c) methanol at the following concentrations: *1*, without the addition; *2*, 10 μM; *3*, 100 μM; *4*, 1 mM; *5*, 10 mM; *6*, 100 mM.

the inducer. At the same time, the sensitivity of the biosensor to formaldehyde was rather low: the threshold concentration was 400 μM. The minimum response time was longer than the time of the biosensor response to formate anions: 1.5 h.

There was almost no biosensor activation on the addition of methanol. An insignificant increase in fluorescence was observed at a 100-mM methanol concentration in the medium (Fig. 5c). Thus, this biosensor is not recommended for methanol detection.

The calculated values of the main parameters of the B26 biosensor are given in Table 2.

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The whole-cell biosensor constructed in the present work for formate anion ($HCCO^-$) detection in different media is based on the yeast *Y. lipolytica* and has a threshold sensitivity of 10 μM, which is comparable with the sensitivity of chromatography techniques [26]. The sensitivity of the biosensor proposed in this work is lower compared to other types of biosensors based on the enzymatic activity of formate dehydrogenases functioning in a free state [6, 27, 28] or localized on the surface of microbial cells [26]. The threshold concentration of such biosensors is 2–5 μM. However,

Table 2. The main characteristics of the B26 biosensor

Inducer	[C]*, M	AO_{max}	t_m, min	Threshold sensitivity
Sodium formate	10^{-4}	6 ± 1.8	70	10^{-5} M
	10^{-3}	18 ± 4.0		
	10^{-2}	30 ± 6.0		
	0.1	43 ± 9.8		
Formaldehyde	10^{-3}	6 ± 1.3	90	0.4×10^{-3} M
Methanol	0.1	2.3 ± 0.9	120	0.1 M

*[C] is the inducer concentration when RA is maximal.

the difficulty of isolating the pure enzyme, checking its localization, stability, and activity, as well as the difficulty of detecting enzymatic reaction products, may hinder the widespread use of such systems. The developed biosensor is devoid of these disadvantages and can be used in any laboratory equipped with a fluorimeter. At formate anion concentrations above 5 mM, it is possible to assess visually the activation of the biosensor during inoculation on an agarized nutrient medium.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human or animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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