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EXPERIMENTAL ARTICLES

Activities of the Enzymes of Formaldehyde Catabolism in Recombinant Strains of *Hansenula polymorpha*

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Abstract—Activities of the enzymes of formaldehyde (FA) catabolism in recombinant strains of the methylotrophic yeast *Hansenula polymorpha* overproducing NAD^+- and glutathione-dependent formaldehyde dehydrogenase (FADH) were studied under different cultivation conditions and at elevated FA content. Southern dot-blot analysis confirmed the presence of six to eight copies of the target *FLD1* gene in stable recombinant clones of *H. polymorpha*. Under certain cultivation conditions, the transformants resistant to elevated FA concentrations were shown to produce FADH and other bioanalytically important enzymes: formate dehydrogenase, alcohol dehydrogenase, alcohol oxidase, and formaldehyde reductase. The optimal cultivation conditions for recombinants were determined, resulting in maximum synthesis of FADH: methanol as a carbon source, methylamine as a nitrogen source, FA as an inducer, temperature of 37°C, and cells in the early exponential phase of growth.

Keywords: formaldehyde, NAD^+- and glutathione-dependent formaldehyde dehydrogenase, *Hansenula polymorpha*, recombinant overproducers.

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Understanding the mechanisms of formaldehyde (FA) formation and detoxification in living systems is important, because FA is a central natural metabolite, as well as a product of chemical synthesis extensively used in industry, pharmacology, medicine, and agriculture [1, 2]. FA has cytotoxic and mutagenic, as well as immuno- and oncogenic effects [2–5]. Interest in the probable role of FA in the pathogenesis of diabetic angiopathies, atherosclerosis, and cardiovascular and other diseases has recently developed [6–8]. The high toxicity of FA is due to its capacity to react spontaneously with many bioactive compounds, including the formation of DNA–peptide cross links involving the NH_2-groups of histone lysine and exocyclic amino groups of the purine bases of DNA [9, 10].

Its place at the branching point of the dissimilation and assimilation pathways determines the key role of FA in methanol metabolism in yeasts. Initial oxidation of methanol to FA and hydrogen peroxide is catalyzed in peroxisomes by alcohol oxidase (AO) [11, 12]. Then FA spontaneously reacts with reduced glutathione (GSH) forming S-hydroxymethyl glutathione ($GSCH_2OH$). The latter is transported into the cytosol, where it is oxidized to CO_2 with participation of NAD^+ and GSH-dependent FADH, S-formylglutathione hydrolase, and formate dehydrogenase (FDH). The GSH-dependent pathway of FA oxidation has two important functions: FA detoxification and energy generation through NADH [1].

FADH plays the main role in FA dissimilation and detoxification in yeasts [13, 14]. Certain isoenzymes of alcohol dehydrogenase (ADH) also participate in FA detoxification. At least five structural genes of this enzyme, *ADH1–ADH5* are known in the baker's yeast *Saccharomyces cerevisiae* [15–17]. The product Adh1p, a cytosolic isoenzyme of ADH, catalyzes NADH-dependent reduction of acetaldehyde to ethanol and of FA to methanol without participation of GSH [15]. In *S. cerevisiae*, Adh1p is homologous to formaldehyde reductase (FR) of methylotrophic yeasts [18, 19]. A significant role in FA detoxification in yeasts belongs to methylformate synthase (MFS), a NAD^+ dependent mitochondrial enzyme catalyzing oxidation of long-chain aliphatic alcohols (similar to ADH) and formation of methylformate from the hemiacetal adduct of methanol and FA [20].

In view of the above, we searched for and genetically constructed the producers of the enzymes involved in FA metabolism in the methylotrophic yeast *Hansenula polymorpha*. As a result, we obtained a highly purified preparation of thermostable FADH (EC 1.1.1.284) from the recombinant yeast producer and developed the enzymatic and biosensor methods of FA analysis using FADH and the producer strain [22–26].

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Conditions of the assay of enzyme activities

Reaction mixture components	FADH	FDH	FDH	FR	MFS
50 mM phosphate buffer	pH 8.0	pH 7.0	pH 8.0	pH 7.0	pH 7.0
NAD^+	1 mM	1 mM	1 mM	–	10 mM
NADH	–	–	–	0.2 mM	–
GSH	2 mM	–	–	–	–
FA	1 mM	–	–	60 mM	–
Ethanol	–	500 mM	–	–	–
Decanol	–	–	–	–	500 mM
Na formate	–	–	100 mM	–	–

The goal of this work was molecular genetic and metabolic characterization of the FADH-overproducing recombinant strains of *H. polymorpha* under different cultivation conditions.

MATERIALS AND METHODS

The strains used were NCYC 495 (*leu1-1*) and CBS 4732 (*leu2-2*) of the thermotolerant methylotrophic yeast *H. polymorpha* from the collection of the Institute of Cell Biology, National Academy of Sciences of Ukraine, as well as the FADH overproducers: stable transformants Tf 11-6 and Tf-142 obtained from these strains [21, 26].

The yeast cells were grown in flasks at 28, 37, or 42°C in a synthetic Burkholder medium containing the following (g/l): KH_2PO_4, 1; $(NH_4)_2SO_4$, NH_4Cl, or $CH_3NH_2 \cdot HCl$, 3.5; $MgSO_4 \cdot 7H_2O$, 0.5; and $CaCl_2$, 0.1, with the standard content of trace elements and 0.05% (wt/vol) yeast extract. The influence of carbon sources on yeast growth and enzyme synthesis was studied using 2% (wt/vol) glucose, 2% (wt/vol) glycerol, and 1% methanol and 1% ethanol (vol/vol). In other experiments, the yeast was cultivated in the presence of 1% CH_3OH and 3.5 g/l $(NH_4)_2SO_4$. The media were inoculated up to the concentration of 0.005 mg/ml. For obtaining the inoculum, liquid medium with the respective carbon source and $(NH_4)_2SO_4$ was inoculated with the cells from fresh plates and cultivated for 24 h at 28°C.

Cell-free extracts were obtained as described earlier [21]; protein was assayed according to Lowry [27]. Enzyme activities in the cell-free extracts were determined by spectrophotometry (Shimadzu-UV-1650) at 340 nm by the rates of NADH formation for formaldehyde dehydrogenase (FADH), formate dehydrogenase (FDH), methylformate synthase (MFS), and alcohol dehydrogenase (ADH), or NADH uptake for formaldehyde reductase (FR) in the respective reaction mixtures (table) at room temperature (20–25°C). The activity (A) for each enzyme (in μmol min^{-1} mg^{-1} protein) was calculated by the formula: $A = A_{+substrate} - A_{-substrate}$, taking into account the nonspecific background reactions.

The activities of NADH-generating enzymes in 8% native polyacrylamide gel (PAG) were visualized in the respective reaction mixtures (see table) by the formazan method [22]. For detection of the zones of alcohol oxidase (AO), PAG was incubated in a mixture of 10 mM methanol, 0.6 mM benzidine, and 0.05 mg/ml peroxidase in 50 mM phosphate buffer, pH 7.0. The FA solution was prepared by hydrolysis of paraformaldehyde [22]; FA concentration was measured with 3-methyl-2-benzothiazolinone hydrazone [28].

RESULTS AND DISCUSSION

Previously, we reported that the most effective producers showed four- to fivefold higher FADH activity than the parent strains *leu1-1* and *leu2-2*, respectively [21, 26]. This work presents the molecular genetic characterization of the most active recombinant strains, particularly determination of the copy number of the plasmid pHpFLD1 integrated into the genome of the transformants (Fig. 1). Using Southern hybridization (Fig. 1a) and Southern dot-blotting (Fig. 1b), it was found that the elevated FADH activity and FA resistance in the transformants correlated with the copy number of a vector integrated into the transformant genome.

Figure 1a shows that the FADH-producing strains contain at least eight copies of the plasmid pHpFLD1 compared to the signal of one copy of the *FLD1* gene in the initial strain. It is an indication of tandem integration of the pHpFLD1 vector into the genome of the *H. polymorpha* recipient strain. Quantitative assessment of the copy number of the *FLD1* gene in the genome of the integrants confirmed the data obtained by Southern hybridization: the maximum number of the *FLD1* gene copies (no less than eight) was present in the integrant Tf 11-6 (Fig. 1b, well 7). Such a high copy number during transformation was achieved due to application of the plasmid $pHp(FLD1)_2$ containing two inserts of the target gene.

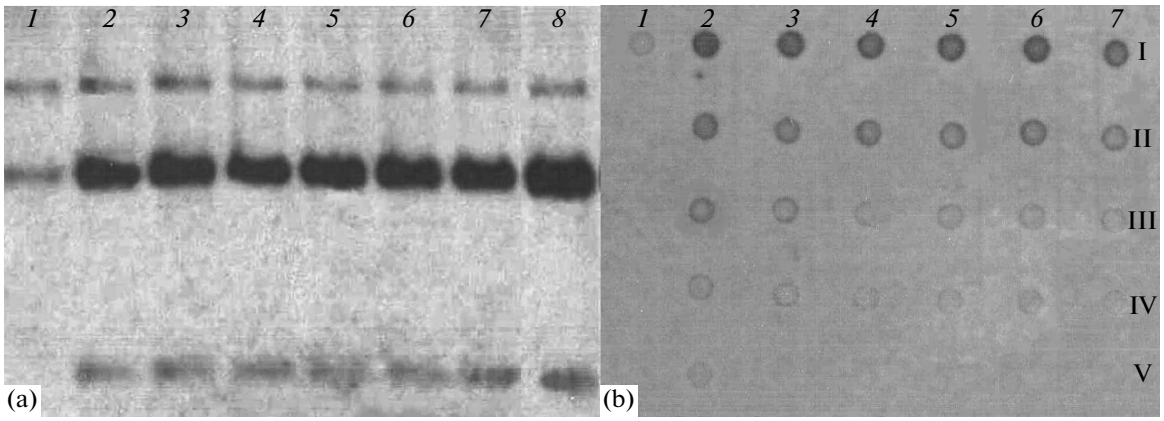

Fig. 1. Assessment of the copy number of the integrated vectors by Southern hybridization (a) and Southern dot-blot hybridization (b) of *H. polymorpha* recipient strains (*1*) and transformants (*2–8*). a: *leu2-2* (*1*), Tf-42 (*2*), Tf-74 (*3*), Tf-79 (*4*), Tf-126 (*5*), Tf-142 (*6*), Tf-166 (*7*), and Tf 11-6 (*8*). b: *leu2-2* (*1*), Tf 11-6 (*2*), Tf-126 (*3*), Tf-142 (*4*), Tf-148 (*5*), Tf-166 (*6*), and Tf-79 (*7*). DNA quantity (μg) in each dot: 0.2 (I), 0.1 (II), 0.05 (III), 0.025 (IV), and 0.0125 (V).

Further studies of recombinant FADH producers were carried out with the transformants Tf 11-6 and Tf-142. When studying the dynamics of yeast growth in the medium with methanol, we registered an increased biomass yield of the FADH producers compared to the parent strains (Fig. 2). Such growth characteristics may result from different rates of FA detoxification, especially at the initial stages of cell cultivation.

Previously, based on the physiological and biochemical analysis of the specially constructed *H. polymorpha* mutant strains with certain metabolic defects, we suggested participation of additional mechanisms of FA and formate detoxification under extreme conditions [29]. Neutralization of the toxic effects of FA and formate was shown to involve the system of energy-dependent extrusion of formic acid into the medium. Under conditions of formaldehyde stress, wild type cells with a lower FADH activity degrade FA, probably not only via the oxidative pathway, but also using FR. FA is converted into less toxic methanol, which is energetically equivalent to consumption of two ATP molecules during utilization of 1 mole of the cytosol NADH. On the contrary, more favorable conditions for FA detoxification in the reactions involving FADH and FDH develop in recombinant strains with a high FADH activity; accordingly, the role of energy-demanding reductase reactions in this process decreases and the recombinant strains are characterized by the higher yield of biomass in FA-containing medium.

During the growth of strain *leu1-1* and its transformant Tf 11-6 under standard conditions on methanol, the pH values for both strains were practically the same: 5.1–5.3 in the beginning of the experiment and 3.2–3.3 after 75 h of cultivation. Consequently, the difference in the biomass yields of strains *leu1-1* and Tf 11-6 is not associated with the theoretically possible differences in formic acid excretion into the medium, which could have a negative effect on the energetic efficiency of methanol utilization [14, 29].

Investigation of the effect of temperature on the growth and FADH activity of the recombinant strains cultivated in the medium with methanol showed that these parameters were maximal at 37°C but minimal at 28°C. While the maximum FADH activity was observed during the early logarithmic phase of growth at all temperatures, the highest enzyme activity (6.7 ± 0.1 μmol min^{-1} mg^{-1} protein) was observed at 37°C after 17 h of growth. This value was 1.3-fold higher than at 42°C and 1.6-fold higher than at 28°C. Consequently, as was expected, 37°C was the optimal temperature for the growth of recombinants and FADH biosynthesis.

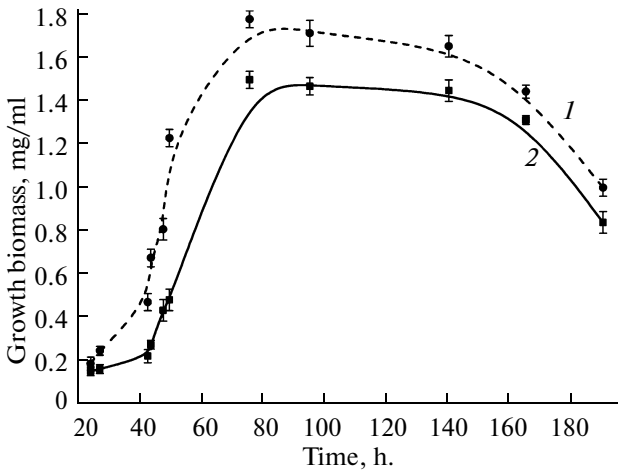

Fig. 2. Growth dynamics of the parent strain *leu1-1* (*1*) and the transformant Tf 11-6 (*2*) under standard cultivation conditions (1% CH_3OH, 3.5 g/l $(NH_4)_2SO_4$).

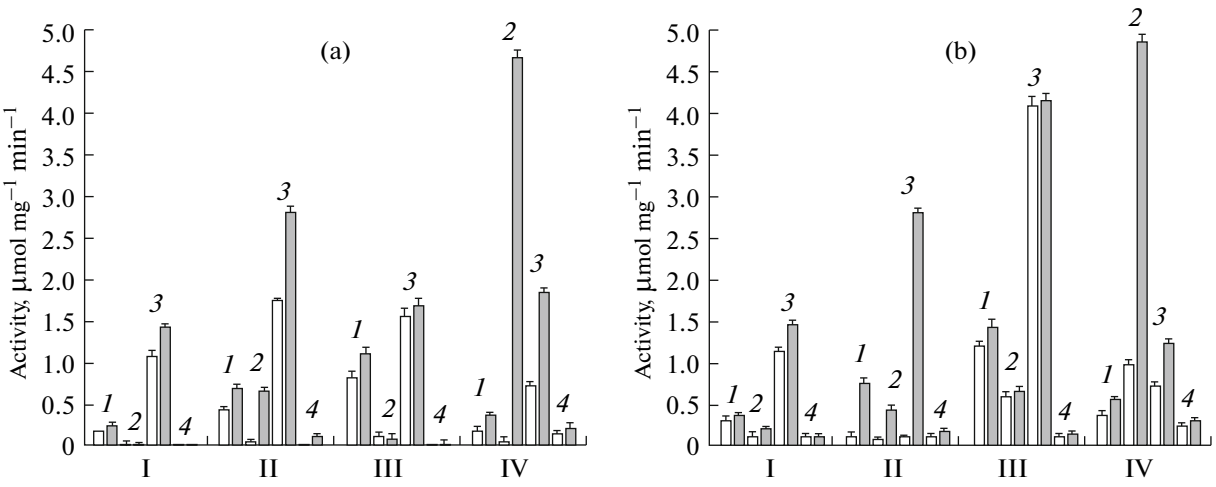

Fig. 3. Effect of the carbon sources on the activities of ADH (*1*); FADH (*2*); FR (*3*); and FDH (*4*) in the cell-free extracts of strains *leu1-1*, Tf 11-6 (a) and *leu2-2*, Tf-142 (b). White columns signify *leu1-1* and *leu2-2*; gray columns, Tf 11-6 and Tf-142. Carbon sources: glucose (I), glycerol (II), ethanol (III), and methanol (IV). Cell concentrations are 0.7-1.0 mg/ml.

Comparison of activities of the FA-metabolizing enzymes in the cell-free extracts of recombinant and parent strains showed that the nature of the carbon source in the growth medium determined preferable synthesis of some or other enzyme. It is known that the synthesis of AO, FADH, and FDH in methylotrophic yeast is induced by methanol but repressed by ethanol and glucose [1, 30, 31]. The results presented in Figs. 3 and 4 make it possible to assess the effect of the carbon source on the activities of different enzymes in cell-free extracts. The maximum AO, FADH, and FDH activities were found in the cells grown on methanol, while the maximum ADH and FR activities were found in the cells grown in the media with ethanol and glycerol, and these latter activities were different in strains *leu1-1* and *leu2-2* and in the recombinants.

The highest FR activity was revealed in the strains *leu2-2* and TF-142 grown in the medium with ethanol (Fig. 3b) and in the strains *leu1-1* and Tf 11-6 grown in the medium with glycerol (Fig. 3a).

Visualization of the activities of some yeast FA-utilizing enzymes in PAG (Fig. 4) illustrates the quantitative data presented on Fig. 3. As is shown on Fig. 4, methanol was the most effective carbon source for accumulation of FADH and AO by the cells. The maximum ADH and MFS activities were observed during cultivation of the transformants on ethanol and glycerol. Practically in all cases, the activities of FA-utilizing enzymes were higher in the recombinants than in the parent strains.

Thus, depending on the cultivation conditions, particularly on the carbon source, the recombinant strains of *H. polymorpha* may be producers of not only FADH, but also of other enzymes with prospects for bioanalytical application: FDH, ADH, and FR.

The FADH and FDH activities in the Tf 11-6 cells grown under different conditions are presented on Fig. 5. With methanol as a carbon source, methylamine as a nitrogen source, and FA as an additional inducer, the specific activities of FADH and FDH in the cell-free extracts reached 6.2 and 0.3 U/mg, respectively (Fig. 5). It is 1.4-fold higher than in the cells grown in the medium with methanol and ammonium sulfate without FA (Fig. 3). Replacement of ammonium sulfate by ammonium chloride as a nitrogen source had no effect on the activities of the enzymes under study. Consequently, the higher FADH activity resulted in appropriate "adjustment" of FDH activity in the transformants. Parallel increase in the FDH and FADH activities in methylotrophic yeast was probably the response to the more intensive production of formic acid as a result of FA oxidation in the presence of FADH. The FADH and FDH activities increased upon addition of FA to the media with various carbon sources, underlining the important role of these enzymes in FA detoxification.

For verification of the hypothesis about the role of FA as an additional inducer of FADH biosynthesis, FADH activity was measured during the growth of strain Tf 11-6 in the standard medium with 1% methanol at different FA concentrations: 5 and 10 mM. When using the medium with 10 mM FA, the maximum FADH activity (8.3 μmol min^{-1} mg^{-1} protein) was twice as high as in the same strain cultivated without FA (Fig. 6). In the presence of 5 mM FA in the growth medium, the maximum FADH activity (5.9 μmol min^{-1} mg^{-1} protein) was 1.4-fold higher than that without FA induction. Monitoring of the FA concentration in the medium showed that elevated FADH activity in the cells resulted in rapid utilization of FA: the level of FA (at the initial concentration of 5 mM) decreased 2.5 times after 20 h and 10 times after 50 h. At the initial concentration of 10 mM, the level of FA decreased 2.5 times after 50 h and 20 times

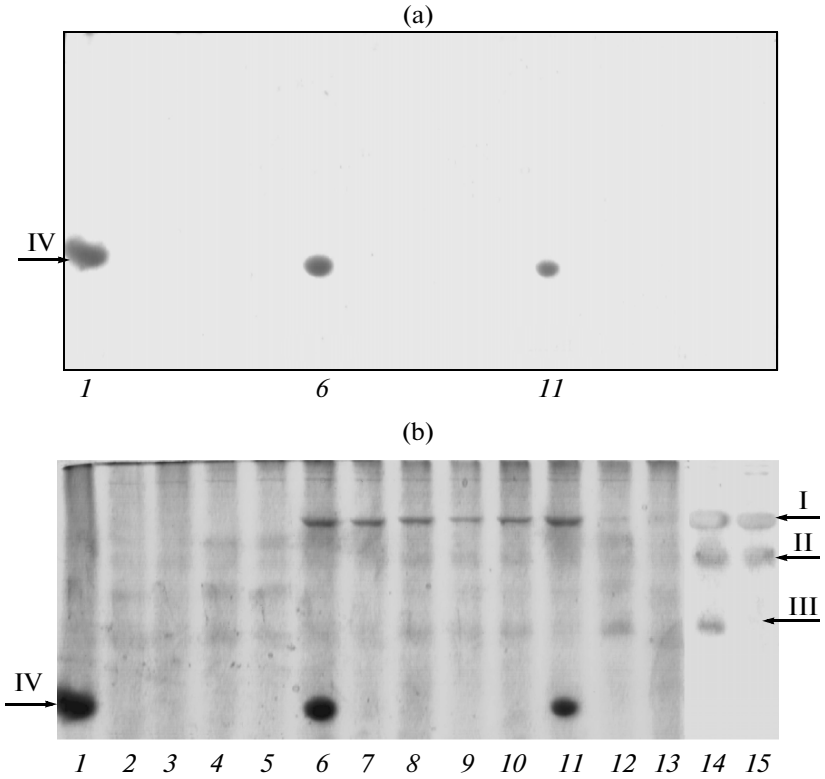

Fig. 4. Visualization of enzyme activities of cell-free extracts of the cells grown under different conditions within PAG: AO (I); ADH (II); MFS (III); FADH (IV). Protein was added by 20 μg into holes 2–15; 10 μg of purified FADH (17 U/mg) was added into hole 1. A part of PAG (*1–13*) after FADH detection (a) was stained with Coomassie G-250 (b). Another part of the gel (*14–15*) was gradually developed on ADH (b), then on MFS, and finally on AO. Carbon sources: glucose (*2*, *3*, *13*), glycerol (*8*, *9*, *14*), ethanol (*4*, *5*, *12*), and methanol (*6*, *7*, *11*, *15*). Strains: Tf 11-6 (*2*, *4*, *6*, *8*, *14*, *15*), Tf-142 (*11–13*), and *leu1-1* (*3*, *5*, *7*, *9*).

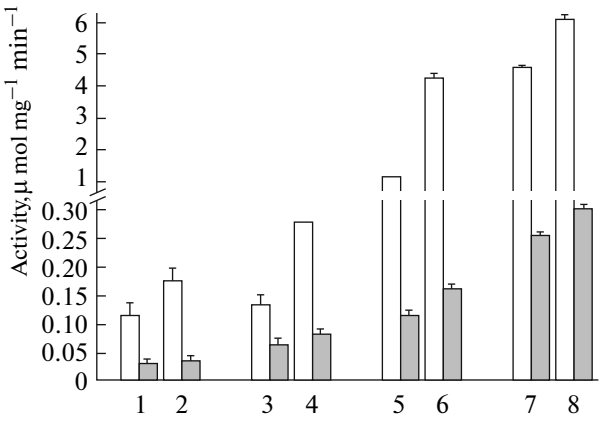

Fig. 5. Dependence of the FADH (white columns) and FDH (shaded columns) activities in the cell-free extract of strain Tf 11-6 on the medium composition and the presence of FA (5 mM). Carbon sources: glucose (1), glucose and FA (2), ethanol (3), ethanol and FA (4), glycerol (5), glycerol and FA (6), methanol (7), and methanol and FA (8). Methylamine was used as the nitrogen source.

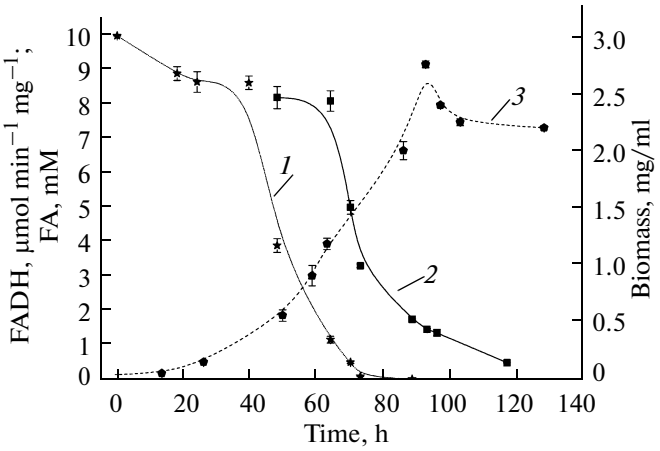

Fig. 6. FA utilization (*1*) in the culture liquid and the level of FADH activity (*2*) during accumulation of the biomass (*3*) of transformant Tf 11-6 in the medium with 1% methanol and 10 mM FA.

after 70 h of growth (Fig. 6). The evident correlation between the levels of FADH activity and residual FA concentrations in the medium additionally confirms the key role of FA as an inducer of FADH synthesis.

ACKNOWLEDGMENTS

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