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EXPERIMENTAL ARTICLES

Screening of Yeasts Producing Stable L-Lactate Cytochrome *c* Oxidoreductase and Study of the Regulation of Enzyme Synthesis

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Abstract—Screening of strains producing a stable form of L-lactate cytochrome *c* oxidoreductase (flavocytochrome b_2, FC b_2) was carried out among 14 yeast species. Enzyme activity was detected in polyacrylamide gel after the electrophoresis of cell-free extracts. The FC b_2 of *Hansenula polymorpha, Rhodotorula pilimanae*, and *Kluyveromyces lactis* are characterized by high thermostability; in particular, the FC b_2 of *H. polymorpha* retains its activity and tetrameric structure even after heating at 60°C for 10 min. Constitutive synthesis of FC b_2 was observed in *H. polymorpha* grown on either glucose, ethanol, or glycerol. L-Lactate induces de novo synthesis of FC b_2, as proved by the use of cycloheximide, an inhibitor of protein synthesis.

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Key words: yeasts, screening, synthesis regulation, flavocytochrome b_2, thermostability.

Lactic acid is a common metabolite of almost all living organisms and a natural component of various food products. Lactate monitoring is essential for quality control in brewing, wine-making [1], and dairy industries [2]. A quantitative analysis of lactic acid in blood is widely used in the diagnostics of hypoxia [3], acidosis [4], and cardio-vascular diseases, as well as for the development of optimal regimes of sportsman training [5]. Since chemical methods of lactate analysis are nonspecific, the development of enzymatic and biosensory methods for testing this metabolite is of great importance.

Conventional enzymatic methods of lactate analysis are based on the use of muscular NAD-dependent lactate dehydrogenase (LDH) (EC 1.1.1.27) [6, 7] or bacterial lactate oxidase (LO) (EC 1.13.12.4) [8, 9]. The disadvantages of these methods are their insufficient specificity and high cost, due to the additional cofactors and enzymes required, LDH and LO, respectively.

L-lactate cytochrome *c* oxidoreductase (EC 1.1.2.3, flavocytochrome b_2) (FC b_2), which is encoded by the *CYB2* gene, is an enzyme involved in the lactate metabolism of yeasts [10, 11]. The FC b_2 from *Saccharomyces cerevisiae* and *Hansenula anomala* are described as homotetramers, each subunit of which includes flavin mononucleotide- and protoheme IX-containing domains [12, 13]. FC b_2 is characterized by unique catalytic properties. Namely, it does not require an exogenic cofactor for its activity, exhibits absolutely specificity to L-lactate, and a nonspecificity for electron acceptors. Due to these characteristics, FC b_2 can be used for the analysis of lactic acid by enzymatic and biosensory methods instead of the NAD-dependent LDH and bacterial LO [14].

The wide application of FC b_2 in analytical studies is hindered by a relatively high liability of this enzyme and difficulties in its isolation and stabilization [15]. For this reason, the screening of yeasts that produce stable forms of FC b_2 is of great importance.

This paper describes the screening of yeasts producing stable forms of FC b_2 among 14 strains. Since the methylotrophic yeast *H. polymorpha* was found to be a promising producer of FC b_2, the regulation in this yeast of its enzyme synthesis by some carbon sources and L-lactate as an inducer was studied.

MATERIALS AND METHODS

The study was conducted with the following yeast strains obtained from the collection at the Institute of Cell Biology, National Academy of Sciences of Ukraine: *Candida boidinii* T2A, *Hansenula ano-mala* D-84, *H. polymorpha* 356 (line DL1), *H. polymorpha* K-105 (*gcr1 cat*), *Pichia fermentas* D-38, *Rhodotorula pilimanae* D-76, and *Saccharomyces cerevisiae* S-288C. The thermotolerant strains *S. cerevisiae* IZR-42 and *S. cerevisiae* IZR-106, which have been isolated from Evolutionary Canyon, were kindly provided by Nevo (Israel). The strains *Kluyveromyces lactis* Y-762, *K. thermotolerance* Y-894, and *Yarrowia lipolytica*

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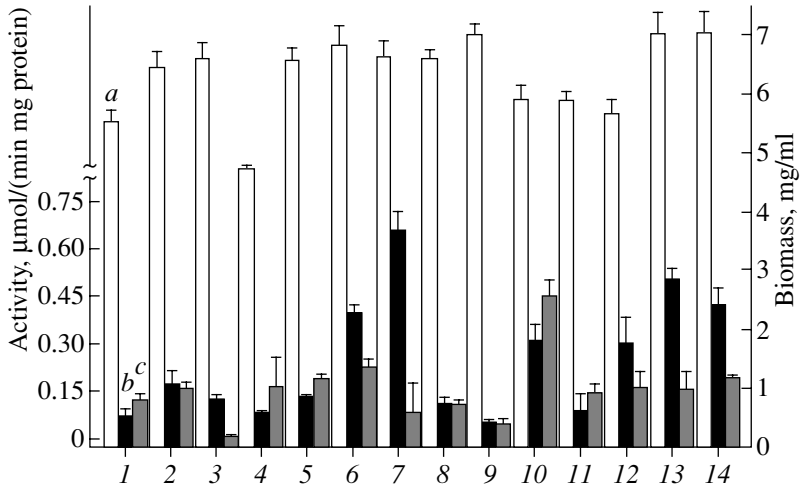

Fig. 1. (*a*) Biomass, (*b*) activity of L-lactate-dependent FC b_2, and (*c*) nonspecific ferricyanide reductase activity after 60-hour cultivation in a medium containing 1% glucose and 0.2% L-lactate: (*1*) *P. guilliermondii* ATCC 9058; (*2*) *P. fermentas* D-38; (*3*) *Rh. pilimanae* D-76; (*4*) *C. boidinii* T2A; (*5*) *S. cerevisiae* S-288C; (*6*) *S. cerevisiae* IZR-42; (*7*) *S. cerevisiae* IZR-106; (*8*) *Sch. pombe* D-9-S; (*9*) *Y. lipolytica* Y-917; (*10*) *K. lactis* Y-762; (*11*) *K. thermotolerans* Y-894; (*12*) *H. anomala* D-84; (*13*) *H. polymorpha* 356; and (*14*) *H. polymorpha* K-105.

Y-917 were obtained from the All-Russia Collection of Microorganisms (VKM). The strains *Pichia guilliermondii* ATCC 9058 and *Schizosaccharomyces pombe* D-9-S were obtained from the Microbiology Department of the Franco Lviv National University (Ukraine).

The yeasts were grown to the mid-logarithmic growth phase at 30°C on a shaker (240 rpm) in Erlenmeyer flasks in the Burkholder medium supplemented with yeast extract (up to 0.075%) and one of the following sources of carbon and energy: glucose, sodium L-lactate, ethanol, glycerol, and their combinations in different ratios. The cell concentration was determined nephelometrically using an FEK-56M photoelectrocolorimeter (3-mm cuvette; optical filter 6) with gravimetric calibration.

Cells were washed to remove the components of the growth medium and disrupted in a homogenizer (0.5-mm glass beads, 1000 rpm, $r = 10$ cm, 4°C, 6 min). To prepare a cell-free extract, the homogenate was centrifuged at 15000 *g* for 15 min (4°C). The activity of FC b_2 in fresh cell-free extracts was determined spectrophotometrically at 20°C [15]. The activities of the total and nonspecific ferricyanide reductases were recorded with and without lactate, respectively. The specific activity (SA) of FC b_2 (μmol/(min mg protein)) was calculated as the difference between the two values: $SA_{FC_{b_2}} = SA_{(\text{with lactate})} - SA_{(\text{without lactate})}$. The electrophoresis of proteins in 7.5% PAAG under nondenaturing conditions was according to the Orstein–Davis method [16] on a VE-2M device for vertical electrophoresis (Khelikon, Moscow).

To select strains that produce stable forms of FC b_2, we used the method developed earlier of the visualization of enzyme activity in protein bands based on the formation of a complex with Prussian blue on PAAG slabs, after the nondenaturing electrophoresis of cell-free extracts [17]. The thermostability of FC b_2 in cell-free extracts was determined by comparing the enzyme activity of protein bands in PAAG [17] before and after heating the samples at 20, 40, 60, and 80°C for 10 min. To investigate the induction of FC b_2 synthesis by L-lactate, yeast strains were grown in a medium with 1% glucose until the biomass reached 3 mg/ml, then cells were washed aseptically and transferred into a medium with 1% L-lactate. The glucose concentration in the culture broth was measured using a Diaglyuk-2 analyzer [18]. L-lactate was assayed with bovine heart NAD-dependent LDH [7].

RESULTS AND DISCUSSION

The design of screening experiments was based on the specific features of expression of the *CYB2* gene encoding FC b_2 in *S. cerevisiae*, such as its repression by glucose under fermentative conditions, its induction by substrate (lactic acid), and its poor expression under anaerobic conditions [10, 11, 19]. The preliminary screening of the strains showed that most of them were unable to grow in the medium with 1% L-lactate as the sole source of carbon and energy. For this reason, the yeasts we cultivated in the medium containing both 1% glucose and 0.2% sodium L-lactate.

The growth of the yeast strains and the activity of FC b_2 in their cell-free extracts are illustrated in Fig. 1. In the medium containing both glucose and L-lactate, the best growth was observed in the case of *H. polymorpha* (strains 356 and K-105), *Y. lipolytica*, and *S. cerevisiae* IZR-42 (the biomass reached 6.90 ± 0.84, 6.85 ± 0.35, and 6.40 ±0.78 mg/ml, respectively). The

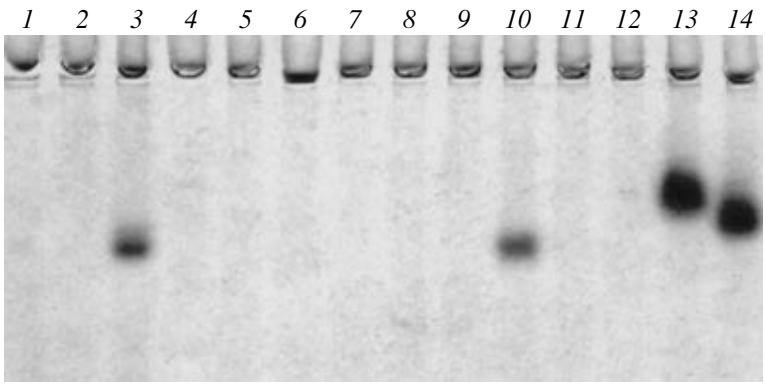

Fig. 2. Screening of the stable form producers of FC b_2 by the method of visualization of the enzyme activity of protein bands in PAAG after the electrophoresis (under nondenaturing conditions) of cell-free extracts prepared from yeast cells grown in a medium containing 1% glucose and 0.2% L-lactate. Conditions of nondenaturing electrophoresis: 7.5% PAAG; pH 8.3; 6.5 h; 180 V; 25 mA; without chamber cooling. Strain designations as in Fig. 1.

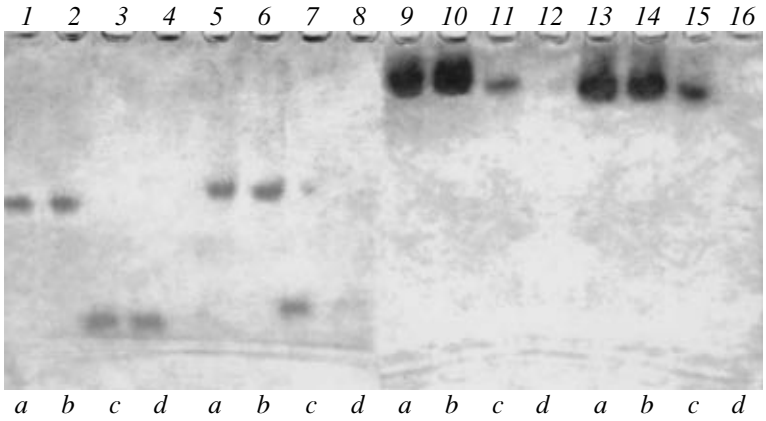

Fig. 3. Determination of the thermostability of FC b_2 in protein bands after the electrophoresis of the cell-free extracts of yeasts: (*1–4*) *Rh. pilimanae* D-76; (*5–8*) *K. lactis* Y-762; (*9–12*) *H. polymorpha* 356; (*13–16*) *H. polymorpha* K-105. Conditions of electrophoresis: 7.5% PAAG; pH 8.3; 3 h; 60 V; 12 mA; 4°C. Prior to electrophoresis, the cell-free extracts were heated for 10 min at (*a*) 20; (*b*) 40; (*c*) 60, and (*d*) 80°C.

highest activity of FC b_2 was found in the cell-free extracts of the thermotolerant yeast strains *S. cerevisiae* IZR-106, *H. polymorpha* 356, *H. polymorpha* K-105, and *S. cerevisiae* IZR-42 (0.66 ± 0.06, 0.51 ± 0.03, 0.43 ± 0.05, and 0.40 ± 0.02 μmol/(min mg protein), respectively).

To select the producers of stable forms of FC b_2, we measured enzyme activity in fresh cell-free extracts, carried out electrophoresis under nondenaturing but still rather harsh conditions (without chamber cooling for more than 6 h, 180 V, 25 mA,), and visualized zones of enzyme activity on PAAG plates (Fig. 2). Using these approaches, we found that most of the strains under study are unable to produce stable forms of FC b_2. However, four yeast strains (*Rh. pilimanae, K. lactis, H. polymorpha* 356, and *H. polymorpha* K-105) retained L-lactate oxidoreductase activity under the above conditions. These strains were chosen for detailed investigation of enzyme thermostability.

As can be seen from Fig. 3, the activity of FC b_2 remained in the cell-free extracts of the selected yeasts after their heating at 60°C for 10 min. Of interest is the fact that the electrophoretic mobility of enzymes from *Rh. pilimanae* and *K. lactis* increased after thermal treatment. A plausible explanation of this phenomenon is that some thermal decomposition products of the tetrameric structure of native proteins, such as monomers or even FMN-containing domains, retain enzymatic activity. At the same time, in the case of the thermostable strains *H. polymorpha* 356 and *H. polymorpha* K-105, protein bands with enzyme activity in PAAG did not change their electrophoretic mobility after thermal treatment, which suggests that the native structure of the holoenzyme is retained after heating at 60°C. One of the thermotolerant strains, *H. polymorpha* 356, was chosen to study the regulation of FC b_2 synthesis.

The effect of different carbon sources on the growth of *H. polymorpha* and FC b_2 synthesis is illustrated in Figs. 4–6. This strain grew well (the biomass reached

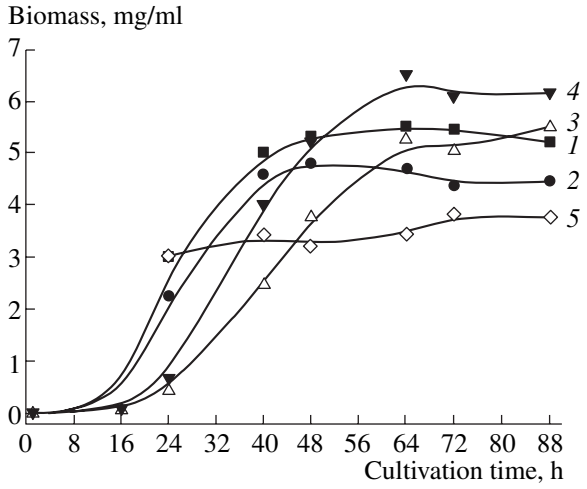

Fig. 4. Growth of *H. polymorpha* 356 in a medium with different sources of carbon and energy: (*1*) 1% glucose, (*2*) 1% glucose + 0.2% L-lactate, (*3*) 1% ethanol, and (*4*) 1% glycerol. In variant (*5*), the yeast was grown in a medium with 1% glucose until the biomass reached 3 mg/ml, and then cultivation was continued in a medium with 1% L-lactate.

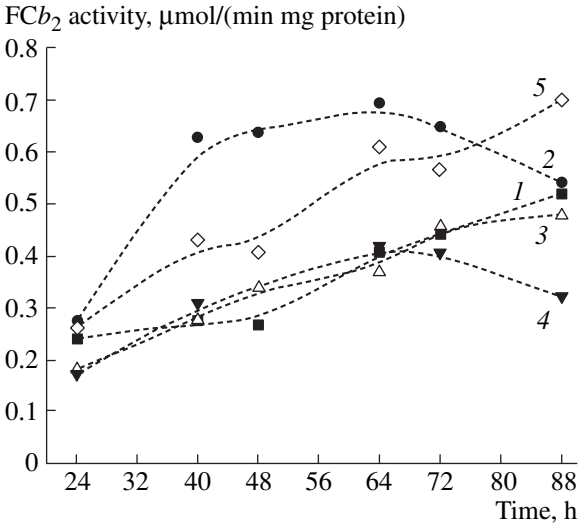

Fig. 5. FC b_2 activity in the cell-free extracts of the yeasts grown in media with different sources of carbon and energy. Designations as in Fig. 4.

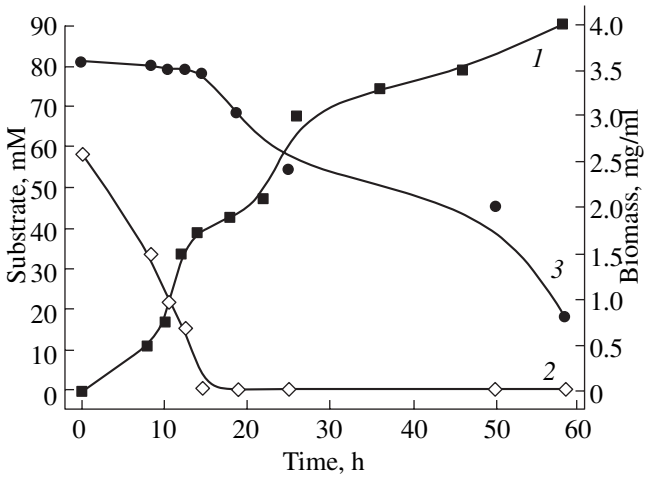

Fig. 6. (*1*) Biomass accumulation and the utilization of (*2*) glucose and (*3*) L-lactate during the growth of *H. polymorpha* 356 in the medium containing glucose and L-lactate (1% each). The utilization of substrates was determined by the measuring residual concentrations in the medium.

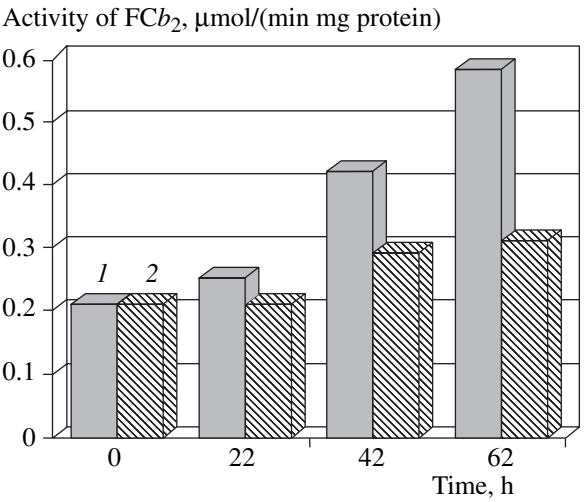

Fig. 7. The effect of cycloheximide (0.1 mg/ml) on the synthesis of FC b_2 during the growth of *H. polymorpha* 356 cells in a medium with 1% L-lactate. The activity of FC b_2 was determined in cell-free extracts (*1*) in the absence and (*2*) in the presence of the inhibitor.

5.5–6.2 mg/ml) in the medium containing glucose, ethanol, or glycerol at a concentration of 1% (Fig. 4). The FC b_2 activity amounted to 0.40–0.46 μmol/(min mg protein) by the 64th day of cultivation (Fig. 5). Growth was not observed in the medium containing 1% lactate as the sole source of carbon and energy. When the yeast cells grown in the medium with 1% glucose were transferred to the medium with 1% L-lactate cell growth was also absent (Fig. 4), however, FC b_2 activity increased to 0.7 μmol/(min mg protein). This circumstance is indicative of the positive regulation of enzyme synthesis by L-lactate. The medium containing 1% glucose and 0.2% L-lactate was favorable for both cell growth and enzyme synthesis. In this case, the FC b_2 activity reached 0.7 μmol/(min mg protein) by the 64th day of cultivation (Fig. 5).The synthesis of FC b_2 in *H. polymorpha* 356 was not markedly repressed by glucose; in particular, the increase in glucose concentration by 10% resulted in the decline in the FC b_2 activity by 30%. It can be inferred that the regulation of FC b_2 synthesis in the methylotrophic yeast *H. polymorpha* 356 considerably differs from that in the yeast of bak-

ers, in which the effect of glucose repression on enzyme synthesis is relatively strong, especially under fermentative conditions [11]. The observed differences in the regulation of FC b_2 synthesis by glucose in these yeast species can be explained by the fact that *H. polymorpha*, unlike *S. cerevisiae*, is a strict aerobe incapable of fermentative metabolism. The repression of FC b_2 synthesis in *H. polymorpha* by other carbon substrates, such as ethanol and glycerol, was insignificant, which is indicative of the constitutive synthesis of FC b_2 in this methylotrophic yeast.

As can be seen from Fig. 5, the synthesis of FC b_2 in the thermotolerant species *H. polymorpha* 356 reached a maximum in the middle of the stationary growth phase (60–65 h). The optimum temperature for enzyme synthesis (approximately 36°C) coincided with that growth.

To study the utilization kinetics of glucose and lactate, the strain *H. polymorpha* 356 was grown in the medium containing glucose and L-lactate (1% each). In this case, the time course of cell growth was typical diauxic with a biphasic utilization of substrates: the utilization of L-lactate began only after glucose exhaustion (after 16–18 h of growth) (Fig. 6).

To elucidate the induction mechanism of FC b_2 synthesis by L-lactate, we used cycloheximide, an inhibitor of protein synthesis [20]. Preliminary experiments showed that cycloheximide at a concentration of 0.1 mg/ml caused a twofold retardation of the growth of *H. polymorpha* 356. The addition of cycloheximide at this concentration into the medium with 1% L-lactate considerably inhibited FC b_2 synthesis (Fig. 7). Therefore, it can be assumed that the induction of FC b_2 synthesis by L-lactate occurs at the level of de novo synthesis (most probably, at the level of transcription).

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