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EXPERIMENTAL ARTICLES

Construction of Flavocytochrome b_2-Overproducing Strains of the Thermotolerant Methylotrophic Yeast *Hansenula polymorpha* (*Pichia angusta*)

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Abstract—L-Lactate cytochrome *c* oxidoreductase (flavocytochrome b_2, FC b_2) from the thermotolerant methylotrophic yeast *Hansenula polymorpha* (*Pichia angusta*) is, unlike the enzyme form baker's yeast, a thermostable enzyme potentially important for bioanalytical technologies for highly selective assays of L-lactate in biological fluids and foods. This paper describes the construction of flavocytochrome b_2 producers with overexpression of the *H. polymorpha CYB2* gene, encoding FC b_2. The *HpCYB2* gene under the control of the strong *H. polymorpha* alcohol oxidase promoter in a plasmid for multicopy integration was transformed into the recipient strain *H. polymorpha* C-105 (*gcr1 catX*), impaired in glucose repression and devoid of catalase activity. A method was developed for preliminary screening of the transformants with increased FC b_2 activity in permeabilized yeast cells. The optimal cultivation conditions providing for the maximal yield of the target enzyme were found. The constructed strain is a promising FC b_2 producer characterized by a sixfold increased (to 3 μmol min^{-1} mg^{-1} protein in cell-free extract) activity of the enzyme.

Key words: gene *CYB2*, flavocytochrome b_2, methylotrophic yeast, *Hansenula polymorpha* (*Pichia angusta*), producer.

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Highly selective, sensitive, and reliable assay methods are required for the certification of certain food and pharmaceutical products, for environmental control, and for identification of metabolites that indicate a change in the physiological state of humans. The lactic acid (lactate) assay is of great importance for the food industry and medicine [1, 2].

Lactate assays are usually carried out with enzymatic methods using the NAD^+-dependent lactate dehydrogenase (LDH) from animal muscle (EC 1.1.1.27) [3–5] or bacterial lactate oxidase (LO) (EC 1.13.12.4) [6, 7]. When the NAD^+-dependent LDH is used, the balance is shifted towards pyruvate reduction to lactate, which requires the use of additional reagents for the balance to be shifted towards lactate oxidation, a toxic hydrazine, or an additional enzyme, glutamate pyruvate transaminase [5].

L-lactate cytochrome *c* oxidoreductase (EC 1.1.2.3; flavocytochrome b_2, FC b_2), a mitochondrial enzyme involved in lactate metabolism in the yeasts, is a promising alternative biological component for determining lactic acid [8, 9]. FC b_2 is a homotetramer with each subunit consisting of the flavin mononucleotide and protoheme IX-containing domains [10, 11]. Owing to its unique catalytic properties (absence of the necessity of the exogenous cofactor, absolute selectivity in relation to L-lactate, and nonspecificity to the electron acceptor), FC b_2 is of utmost bioanalytical importance and can replace both the NAD^+-dependent LDH and LO when lactic acid is determined by enzymatic and biosensor methods [12].

The use of *Saccharomyces cerevisiae* FC b_2 in bioanalytical practice is limited by the high lability of this enzyme and the complexity of its isolation and stabilization [13]. In our previous work, the screening of yeasts producing stable FC b_2 forms resulted in the selection of several *Hansenula polymorpha* strains as promising sources of a stable enzyme [14].

In this article, the results of construction of the producer of the thermostable FC b_2 based on the preliminarily selected strain of the thermotolerant methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) [14] are presented, as well as the optimized conditions for strain cultivation to ensure maximal synthesis of the target enzyme.

MATERIALS AND METHODS

The work used the following yeast strains from the collection of the Institute of Cell Biology, National

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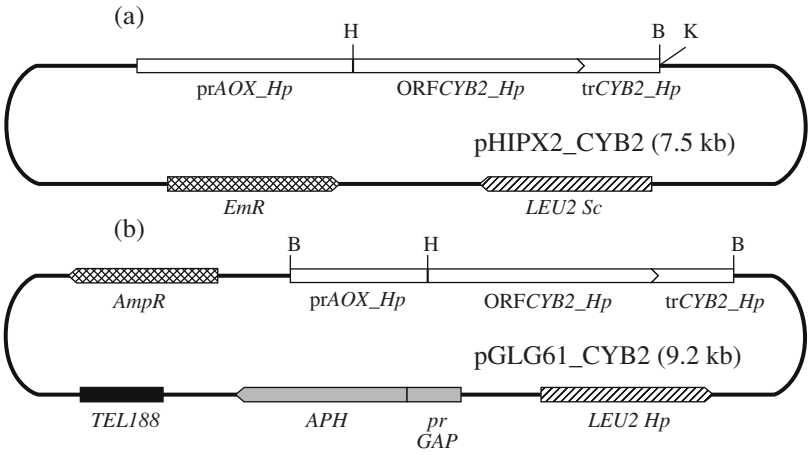

Fig. 1. Circular schemes of (a) the plasmid pHIPX2_CYB2 (7.5 kb) and (b) the plasmid pGLG61_CYB2 (9.2 kb). The expression cassette, consisting of the *AOX* gene promoter, the *CYB2* ORF, and the *H. polymorpha CYB2* terminator, is designated with the white line. *S. cerevisiae* or *H. polymorpha LEU2* genes are designated with hatched lines. Genes *EmR* and *AmpR* providing resistance to erythromycin and ampicillin are designated with cross-hatched lines. Gene *APH* providing resistance to geneticin, linked to an impaired constitutive gene promoter, encoding glyceraldehydephosphate dehydrogenase (*GAP*) is designated with the gray line. The *HARS36* ARS-element (*TEL188*) is designated with the black line. Restriction sites: H-*Hin*dIII, B-*Bam*HI, K-*Kpn*I.

Academy of Sciences of the Ukraine: *Hansenula polymorpha* 356 line DL1; *H. polymorpha* CBS 4732; and *H. polymorpha* C-105 (*gcr1 catX*) [15, 16]. The initial strain C-105 was impaired in glucose catabolic repression due to a genetic defect of the glucose sensor [17], which provides for the constitutive functioning of the alcohol oxidase promoter, and in the catalase gene, which facilitates the isolation of the target enzyme [18]. For plasmid construction and amplification, the strain *Escherichia coli* DH5α was used [19]. The plasmid DNA isolation, restriction, ligation, electrophoresis in agarose gel, electrotransformation, and PCR were carried out by the standard methods [19].

The nucleotide sequence of the *H. polymorpha CYB2* gene was obtained from the Rhein Biotech GmbH (Düsseldorf, Germany) database. The open reading frame (ORF) of the *H. polymorpha CYB2* gene along with the terminator sequence was isolated by PCR using the primers Sm1 (5'-CCC AAG CTT ATG TGG AGA ACC TCC TAT AG-3')/Sm2 (5'-CCC GGT ACC GGA TCC CAA AAT AGA GCG CAA GAT TGC-3') and the chromosomal DNA of *H. polymorpha* CBS 4732 as a template. The obtained fragment flanked by the *Hin*dIII and *Kpn*I restriction sites was cloned before the *H. polymorpha AOX* gene promoter into the pHIPX2 plasmid [20] by substituting the *AOX* gene terminator. As a result, the plasmid was obtained, designated pHIPX2_CYB2 (Fig. 1a). The initial plasmid pHIPX2 was kindly provided by Dr. M. Veenhuis. The expression cassette consisting of the *AOX* gene promoter and the *CYB2* ORF with the terminator sequence was isolated by PCR using the primers Sm3 (5'-TGT GGA TCC TCG TTT AGA ACG TCC TG-3')/Sm2 and the vector pHIPX2_CYB2 as a template. The PCR product preliminarily treated with *Bam*HI restriction endonuclease was cloned to the vector pGLG61 [21] kindly provided by Dr. H.A. Kang. The constructed plasmid was named pGLG61_CYB2 (Fig. 1b).

The yeast was grown to the mid-exponential phase in Erlenmeyer flasks (0.5 l) using an orbital shaker (240 rpm) at 37°C in Burkholder's medium supplemented with 0.2% yeast extract. Depending on the aim of the experiment, different carbon sources were used: sucrose (Suc), glucose (Glc), sodium L-lactate (Lact), or mixtures of them in different proportions. The biomass was determined turbidimetrically in a FEK-56M photoelectric colorimeter (cuvette, 3 mm, light filter no. 6) with gravimetric calibration.

The cells washed off the medium were destroyed using the planetary homogenizer with 0.5-mm glass beads (1000 rpm; r_{av}, 10 cm; 6 min; 4°C). Cell-free extracts were obtained by centrifugation of the homogenate (15000 rpm; r_{av} 8 cm; 15 min; 4°C). The FC b_2 activity in fresh cell-free extracts was determined spectrophotometrically at 20°C [13] by recording the overall ferricyanide reductase and nonspecific activity with Lact and without additional Lact, respectively. The specific activity (SA) of FC b_2 (μmol min^{-1} mg^{-1} protein) was calculated by the difference: ($SA_{FC\ b_2} = SA_{+Lact} - SA_{-Lact}$.

The preliminary screening of the FC b_2-producing transformants was carried out using the method of visualization of the enzymatic activity of permeabilized cells in petri dishes developed by us and similar to the method of formation of the Prussian blue complex in the PAG plates after subjecting the cell-free extracts to native electrophoresis [22].

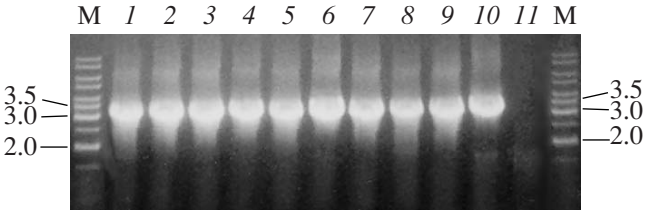

Fig. 2. Electrophoresis of the PCR assay of *H. polymorpha* C-105 stable transformants with the plasmid pGLG61_CYB2. Lanes *1* to *9*, stable transformants; *10* and *11* are a positive (the plasmid pGLG61_CYB2) and negative (strain C-105 chromosomal DNA) control, respectively. M is a marker of the molecular mass of the fragments (the fragment values are expressed in kb).

RESULTS AND DISCUSSION

The expression of the target genes in yeasts may be significantly increased by repeated amplification of the required nucleotide sequence [23, 24]. The plasmid pGLG61 contains the dominant marker, a bacterial *APH* gene (aminoglucoside-3-phosphotransferase) whose expression is impaired, and the sequence of the *HARS36* (*TEL188*) autonomic replicating sequence. Such constituents ensure multiple tandem integration of pGLG61 into *H. polymorpha* telomere regions in transformant selection on medium with the antibiotic geneticin [21].

The plasmid pGLG61_CYB2, constructed on the basis of the vector pGLG61 (Fig. 1b), was transformed into the recipient strain *H. polymorpha* C-105 (*gcr1*, *catX*). A rich medium with increasing geneticin concentrations was inoculated with the transformants. The highest geneticin concentration at which the transformants grew was 1 mg/ml. The transformants obtained were stabilized by cultivation under nonselective conditions over the course of 10–12 generations with subsequent transfer onto selective medium with genecitin. The presence of the expression cassette in the stable transformants was confirmed by PCR. Using the primers Sm3/Sm2 and the chromosomal DNA of stable transformants as a template, fragments of the predetermined magnitude (~3.3 kb) were obtained (Fig. 2).

Preliminary screening of the geneticin-resistant transformants with an increased FC b_2 activity was carried out using the method of visualization of the enzymatic activity of permeabilized cells in Petri dishes. For this purpose, the suspension of the cells preliminarily grown in 1% Suc was applied dropwise on 2% agarized medium with 1% Glc. After incubating the cells for 14 h at 37°C, the dishes were overlaid with a solution of the permeabilizing agent (cetyltrimethylammonium bromide, 3 mg/ml) in 50 mM phosphate buffer, pH 8.0, with the addition of 1mM phenylmethane sulfonyl fluoride (PMSF) and 1 mM EDTA. The permeabilization was carried out for 10 min at 30°C. The cells washed off the permeabilized solution were incubated for 15 min in a reaction mixture containing the following components: phosphate buffer, 50 mM, pH 8.0; PMSF, 1mM; EDTA, 1 mM; $K_3Fe(CN)_6$, 0.83 mM; and L-lactate, 100 mM. The FC b_2 activity of permeabilized cells was visualized with a 20 mM solution of $FeCl_3$ in 100 mM HCl for 3–5 min. In the process, the permeabilized cells acquired blue coloration of varying intensity (Fig. 3b). The most intensively colored colonies (the transformants 1, 6, and 9) were selected from the matrix dish for a more detailed study.

The optimization of the nutrient medium composition for the maximal production of FC b_2 by the cells of the selected transformants (1, 6, and 9) was carried out taking into account the optimal conditions of induction of the target gene in the *AOX* initial recipient strain *H. polymorpha* C-105 (*gcr1*, *catX*). This strain is impaired in catabolic repression; therefore, constitutive FC b_2 synthesis regulated by the *AOX* promoter occurs in the medium with glucose. In addition, it was previously shown that the maximal *AOX* expression for this strain occurred on the synthetic medium containing an increased (0.2%) concentration of yeast extract (YE) [25]. Considering this fact, the FC b_2 activities were determined in the transformants and the initial strain when the yeast grew in medium with 1% Glc or a mix-

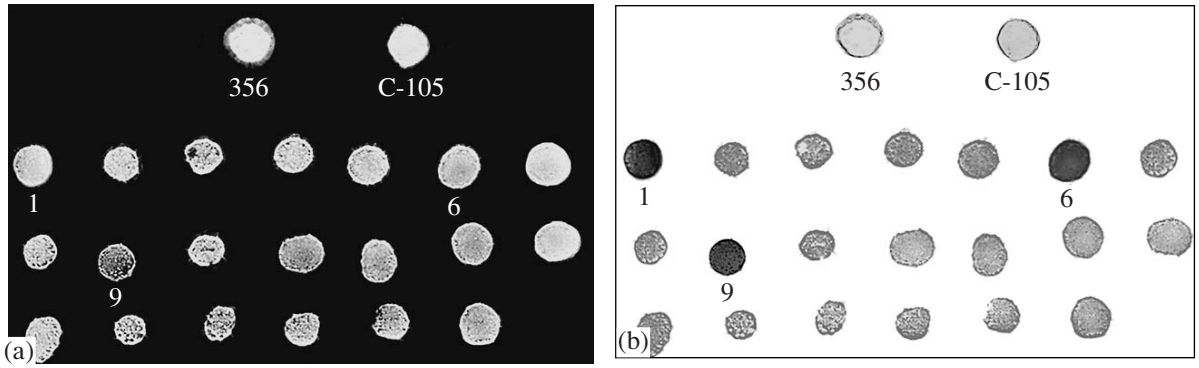

Fig. 3. Screening of FC b_2-producing transformants. (a) Permeabilized transformant colonies. (b) Transformant colonies after visualizing the enzymatic activity. 356 is the wild type strain; 1, 6, and 9 are the transformants with an increased FC b_2 activity.

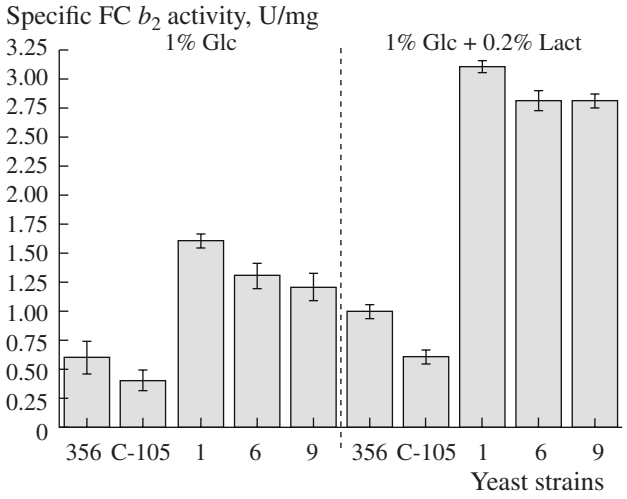

Fig. 4. Specific FC b_2 activity in cell-free extracts of the initial strains and the selected transformants grown in nutrient media with 1% Glc or a mixture of 1% Glc and 0.2% Lact.

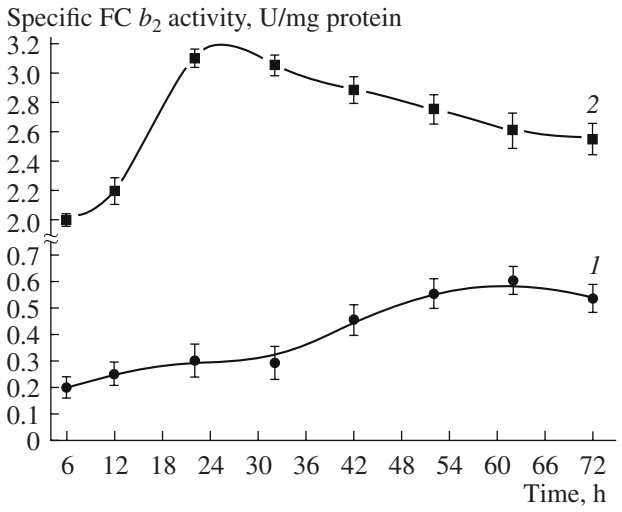

Fig. 5. Time profile of FC b_2 activity in cell-free extracts of (*1*) the initial strain C-105 and (*2*) the transformant 1 in the dynamics of the yeast growth.

ture of 1% Glc and 0.2% Lac supplemented with 0.2% of the YE.

Figure 4 shows the data on the FC b_2 activity in the cell-free extracts of the transformants and the initial strains in media of different carbon composition. The FC b_2 activities in the transformants grown in medium with 1% Glc were 1.2–1.6 μmol min^{-1} mg^{-1} protein, thus exceeding three- to fourfold the enzyme activity of the initial strain C-105 (0.4 μmol min^{-1} mg^{-1} protein). When the yeast cells grew in a mixture of 1% Glc and 0.2% Lact, the enzyme activity in the transformants constituted 2.8–3.1 μmol min^{-1} mg^{-1} protein, whereas the activity of the initial strain C-105 was 0.6 μmol min^{-1} mg^{-1} protein. The difference in the enzyme activity was 4.7 to 5.2 times. An increase in the FC b_2 activity in all the strains, when grown in medium with lactate, results from the inducing influence of the latter on the expression of the initial *CYB2* gene [26]. Thus, the medium containing 1% Glc and 0.2% Lact is the optimal one for the maximal production of an active form of flavocytochrome b_2 by the transformants.

The time profile of the FC b_2 activities of the transformant 1 and the initial strain C-105 is shown on Fig. 5.

The maximal enzyme activity value in strain C105 cell-free extracts occurs at 62 h of cell growth, which coincides with the period of intense assimilation of L-lactate by the yeast cells [14]. In the transformant 1, the peak activity of FC b_2 occurs at 24 h of cell growth. The results obtained agree well with the data on the dynamics of the *AOX* activity in *H. polymorpha* C-105 cells grown in a similar medium: the peak activity of *AOX* was observed during the same period of cell growth (24–40 h) [25]. By this time, glucose is completely consumed by *H. polymorpha*. It is possible that a decrease in the glucose concentration may lead to a deficiency and the resultant energy impairment of FC b_2 synthesis (Fig. 5).

Thus, a strain, a promising FC b_2 producer characterized by a sixfold increase in the enzyme activity, was constructed by incorporating, under the control of a strong *AOX* promoter, additional copies of the *H. polymorpha CYB2* gene into the genome of strain C-105 (*gcr1 catX*) with a special regulation of this promoter by glucose. A method for preliminary screening of the transformants with an increased FC b_2 activity in permeabilized cells was developed. The conditions for strain cultivation were optimized to ensure the maximal synthesis of the target enzyme. At present, we are actively performing the investigations aimed at using the cells of the transformants obtained, as well as the target enzyme FC b_2, for the development of the microbial and enzymatic biosensors highly selective to L-lactate.

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