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Резюме

У проведених дослідженнях вивчались білки дистрофін та дистроглікан, які є основними компонентами дистрофін-глікопротеїнового комплексу Drosophila melanogaster. У отриманих мутантів втрата дистрофіну та дистроглікану призводила до зниження локомоторної активності, дегенерації м'язів та порушення полярності аксонів фоторецепторів.

В проведенных исследованиях изучались белки дистрофин и дистрогликан, которые являются основными компонентами дистрофин-гликопротеинового комплекса Drosophila melanogaster. У полученных мутантов потеря дистрофина и дистрогликана вызывала снижение локомоторной активности, дегенерацию мышц и нарушение полярности аксонов фоторецепторов. Сходные фенотипы были описаны при мышечных дистрофиях человека.

We have studied dystrophin and dystroglycan – highly conserved components of Dystophin glycoprotein complex (DGC) in Drosophila melanogaster. Dystrophin and dystoglycan mutants in drosophila showed decreased climbing ability, muscle degeneration and disruption of axon guidance which resemble human case of muscular dystrophy.

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CONSTRUCTION OF URICASE-OVERPRODUCING STRAINS OF THE METHYLOTROPHIC YEAST *HANSENULA POLYMORPHA*

Uric acid is an end product from purine derivates in human metabolism. The assay of uric acid in body fluids (e.g., serum and urine) is a clinically valuable diagnostic indicator [1].The presence of elevated uric acid levels is a sign of gout, hyperuricemia, or Lesch-Nyhan

syndrome [2]. Similarly, elevated uric acid levels are related to other conditions including increased alcohol consumption, obesity, diabetes, high cholesterol, kidney disease, and heart diseases. Many epidemiological studies have suggested that serum uric acid is also a risk factor for cardiovascular disease [3].

Uricase (urate-oxygen oxidoreductase; urate oxidase, EC 1.7.3.3), is an enzyme in the purine degradation pathway and catalyzes the oxidation of uric acid in the presence of oxygen as an oxidizing agent, producing allantoin and CO_2 as oxidation products of uric acid and hydrogen peroxide as a reduction of O_2. Uricase does not require any cofactor for the enzymatic oxidation, therefore, is widely used for the enzymatic determination of uric acid to diagnose gout and mentioned above diseases and conditions.

The methylotrophic thermotolerant yeast *Hansenula polymorpha* is one of the most important industrially applied non-conventional yeasts [4]. The utility of *H. polymorpha* in the production of recombinant therapeutic proteins as well as source and producer of technical enzymes has been well established [4]. Microbial sensors for alcohols, formaldehyde and L-lactate assays based on the employment of yeast cells *H. polymorpha* were constructed [5, 6, 7].

In this report we describe the construction of strains of *H. polymorpha* overproducing own uricase on the base of previously selected strain of *H. polymorpha* C-105 (*gcr1 catX*) [5]. The optimal cultivation conditions for maximal yield of the target enzyme were found.

Materials and methods

Strains and media

Cultivation of the *H. polymorpha* CBS4732 *(leu2-2)* [8] and C-105 (*gcr1 catX*) [5] were performed in flasks on a shaker (200 rpm) at 37 °C in a medium containing (g L^{-1}): $(NH_4)_2SO_4$ - 3.5; KH_2PO_4 - 1.0; $MgSO_4$ x $7H_2O$ - 0.5; $CaCl_2$ - 0.1; yeast extract - 6. A glucose (20 g L^{-1}) was used as a carbon source. For induction of uricase the cells from the mid-exponential growth phase were washed once in the mineral medium and transferred to the shake flask cultures supplemented with 4 g L^{-1} uric acid as the carbon source. C-105 (*gcr1 catX*) was grown on YPS at 37 °C and used as a recipient for transformation experiments. For the selection of yeast transformants on YPS, 0.5–1.5 mg mL^{-1} of the antibiotic G418 was added. The *Escherichia coli* strain DH5α (Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169) was used as a host for propagation of plasmids. The strain DH5α was grown at 37 °C in LB medium as described previously [9]. Transformed *E. coli* cells were maintained on a medium containing 100 mg L^{-1} ampicillin.

Plasmid construction and molecular techniques

The *H. polymorpha* uricase gene with terminator region and alcohol oxidase gene promoter were amplified from the genomic DNA of *H. polymorpha* strain CBS4732 using the corresponding pairs of primers Ko189 5'-CAA TCT AAA GTA CAA AAA CAA AGG TAC CAT GGC TGT CCT GCA ATC GTC-3' (*Kpn*I) / Ko190 5'-CCG GGA TCC TAC TCT TTG ATT GCC TCC-3' (*Bam*HI) and Ko184 5'-CGC GGA TCC CCA TCG AAT GTA ATG AGC-3' (*Bam*HI) / Ko188 5'-GAC GAT TGC AGG ACA GCC ATG GTA CCT TTG TTT TTG TAC TTT AGA TTG-3' (*Kpn*I). Primers Ko184 and Ko190 were used for obtaining of the ~ 2.3 kb fragment containing the *H. polymorpha* uricase gene with terminator region driven by the alcohol oxidase gene promoter by overlap PCR. This fragment was treated with restriction endonuclease *Bam*HI and cloned into the *Bam*HI linearized and dephosphorylated plasmid pGLG61 [10], resulting in the recombinant constructs pGLG61_UOX.

Standard cloning techniques were applied [9].

Urate oxidase assay

Urate oxidase activity in cell extracts was determined spectrophotometrically by following the decrease of the absorbance at 293 nm in an assay mixture containing 0.1 mM

uric acid in 0.1 M Tris-HC1 buffer (pH 8.9) [11]. The reaction was started with cell extract addition.

Strains with elevated UOX enzyme activity were screened by a plate patch assay, on the basis of visualization of UOX activity by the rate of hydrogen peroxide formation in reaction with urate as monitored by the peroxidative oxidation of *o*-dianisidine in the presence of peroxidase resulted purple colour of patches. For this purpose, the transformants formed were patched onto agar mineral medium plates supplemented with glucose. After 18 h of incubation at 37 °C, the plates were overlaid with 9 ml of the urate oxidase activity assay mixture, containing 0.1 M Tris-HC1 buffer (pH 8.9), 0.7% (wt/vol) agar, digitonin (1 mg·mL$^-$ ^{1}), *o*-dianisidine (0.5 mg·mL^{-1}), peroxidase Sigma RZ 1.1 (0.13 mg·mL^{-1}), and uric acid (0.1 mM). The overlying assay mixture was allowed to set, and the plates were incubated at 37 °C for up to 1 h. Patches that stained purple faster, were selected.

All assay experiments were repeated at least twice.

Results and discussion

The recombinant plasmid pGLG61_UOX was transformed to the recipient strain *H. polymorpha* C-105 (*gcr1*, *cat*X). The transformants were grown on YPS medium in the presence of increasing concentrations of G418. The highest concentration of G418 which allow the transformants to growth was 1 mg mL^{-1}. The presence of the expression cassette in the stable transformants was examined by diagnostic PCR using primers Ko184/Ko190 and genomic DNA of stable transformants as a template. Fragments of predictable size (~2.3 kb) were obtained (data not shown).

Parental strain C-105 has impairment in glucose catabolite repression of AOX synthesis resulting induction of AOX in a glucose containing growth medium [5]. Stable transformants bearing UOX under control of AOX promoter with increased UOX enzyme activity were screened by a plate patches assay in solid glucose containing medium as described in the Material and methods section. Finally, six positive recombinant strains (20, 22, 24, 28, 32 and 36) which stained more intensive in the presence of 0.1 mM uric acid were selected.

The level of UOX activity in cell-free extracts of the selected strains was measured. Recombinant strains were cultivated in glucose containing medium. As shown in Fig. 1 A, UOX activity of the selected strains was ranged between 0.4 and 2.67 U ml−1, while the initial strain *H. polymorpha* C-105 at the same growth conditions had barely perceptible enzyme activity. For induction of uricase the cells of C-105 were cultivated in urate containing medium. UOX activity of C-105 cultivated under inducing conditions was 0.067 U ml−1. Thus the best strain 22 showed forty-fold higher UOX activity as compared to the initial strain.

The dependence of UOX activity of strain 22 from growth phase represented on Fig. 1 B. The maximal level of UOX activity in cell-free extracts occurred on 64 h of cells cultivation and reached to 3.36 U ml^{-1}. Obtained results are in good arrangement with the dynamics of AOX activity in C- 105 during cultivation on the same medium. The peak of AOX activity observed on 50-60 h of cells cultivation (unpublished data).

Although constructed strains are significantly inferior to commercial available procedures of recombinant uricase from *E. coli* or *Saccharomyces cerevisiae* in productivity [12, 13], the genetic background makes constructed strains an ideal biorecognition element for the construction of microbial urate-selective amperometric biosensor. Block in catalase activity provides the generation of hydrogen peroxide as a result of urate oxidation that could be easily detected amperometrically. The impairment in the glucose repression of constructed strains possessed overproduction of UOX in a low-priced glucose-containing growth medium. Currently, the cells of constructed UOX-overproducing strain 22 extensively investigated for further utilization as biorecognition element for the construction of microbial urate-selective amperometric biosensor.
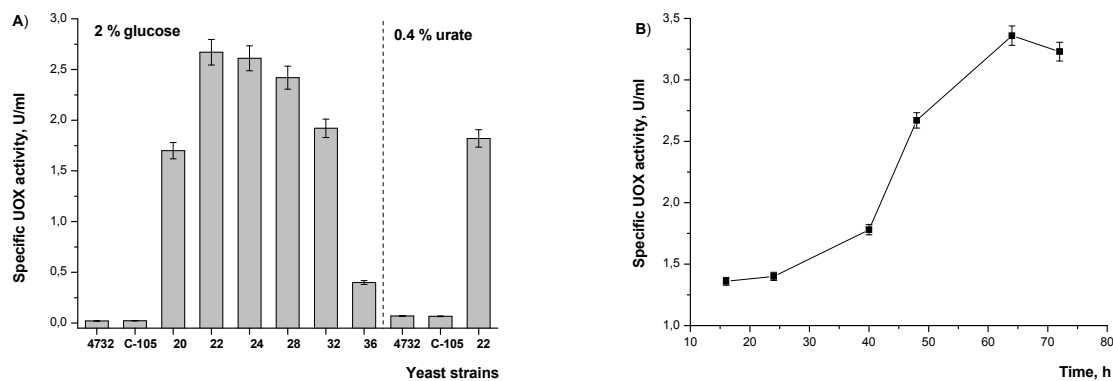
Figure 1. A) Specific UOX activity in cell-free extracts of the initial strains and the recombinant strains grown in media supplemented with 2% glucose or 0.4% urate during. B) Time profile of UOX activity in cell-free extracts of the recombinant strain 22 in the dynamics of the yeast growth.

Conclusions

Strain of *H. polymorpha* overproducing own UOX have been constructed. Genomic integration of expression cassette of uricase gene under the control of the strong *H. polymorpha* AOX promoter resulted increasing the UOX activity up to 3.36 U ml^{-1}. A method for preliminary screening of the recombinant strains with an increased UOX activity in permeabilized cells was developed. The conditions for strain cultivation were optimized to ensure the maximal synthesis of the target enzyme. The constructed strain may be used for the development of microbial urate-selective amperometric biosensor.

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Abstract

Strains of *Hansenula polymorpha* overproducing own uricase (UOX) have been constructed. Uricase gene under the control of the strong *H. polymorpha* alcohol oxidase promoter was multicopy integrated to the recipient strain *H. polymorpha* C-105 (*gcr1 catX*). The recombinant producer characterized by a forty-fold increased UOX enzyme activity (up to 3.36 U ml^{-1} in cell-free extract) compared to the initial strain.

Сконструированы штаммы *Hansenula polymorpha* с усиленной экспрессией собственной уриказы. Ген уриказы под контролем сильного промотора гена алкогольоксидазы *H. polymorpha* введён в геном реципиентного штамма *H. polymorpha* С-105 (*gcr1 catX*). Рекомбинантный штамм характеризировался сорокакратным увеличением активности уриказы (3.36 МЕ мл$^{-1}$ в бесклеточных экстрактах) в сравнении с исходным штаммом.

Сконструйовані штами *Hansenula polymorpha* з посиленою експресією власної уркази. Ген уркази під контролем сильного промотора алкогольоксидази *H. polymorpha* було введено в геном реципієнтного штаму *H. polymorpha* С-105 (*gcr1 catX*). Рекомбінантний штам характеризувався підвищенням активності уркази в 40 разів (3.36 МЕ мл$^{-1}$ в безклітинних екстрактах) у порівнянні з вихідним штамом.

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ANALYSIS OF TESTIS - EXPRESSED *STELLATE* GENES PROMOTER REGION IN *D. melanogaster*

The first described case of a natural RNA-mediated silencing found to be necessary for male fertility maintenance was discovered in *Drosophila* (Aravin et al., 2001). In wild-type testes, hyperexpression of tandemly repeated X-linked *Stellate* genes is prevented by homologous, bidirectionally transcribed Y-linked *Suppressor of Stellate* repeats (*Su(Ste)*)**,** and deletion of *Su*(*Ste*) leads to abnormalities of spermatogenesis (Palumbo et al., 1994). In the *Drosophila* germline, selfish genetic elements, such as retrotransposons and repetitive sequences, are suppressed by rasiRNAs (Vagin et al., 2006). However, a little is known about classical transcription regulation of the *Stellate* genes. The expression of the *Stellate* genes is restricted to *D. melanogaster* testes. The transcription start site was determined in (Livak,