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Expression of glucose dehydrogenase–synuclein hybrid in *Saccharomyces cerevisiae*

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Yeast expression vectors bearing glucose dehydrogenase gene fused with α-Synuclein (GDHsyn) were constructed and successfully introduced into *Saccharomyces cerevisiae* cells. Optimization of growing conditions of yeast transformants and a partial purification on the CM column of hybrid proteins were performed. The effect of culturing conditions on glucose dehydrogenase activity of hybrid construct-bearing yeasts was determined. Evaluation of expression level and localization of GDHsynEco and GDHsynPvu proteins identified major GDH activity in the cytoplasmic fractions of both producents. The genetic construction of the hybrid and the growing conditions of producents was found to influence not only the level of protein production, but also its localization in the cell. GDHsynPvu and GDHsynEco hybrids are cleaved into individual components, and only traces of the hybrid protein are detectable on SDS-PAGE.

Key words: *S. cerevisiae*, synuclein, glucose dehydrogenase, hybrid protein

INTRODUCTION

Heterologous protein production is an important biotechnological topic. Unicellular microorganisms, both prokaryotic and eukaryotic, are the preferred heterologous protein producers for obvious reasons: they can be cultured in big amounts at low costs, their manipulation is simple and genetic background is well known. Some proteins can be secreted into the medium and easily recovered and purified [1]. As producents, a large and ever-increasing number of unicellular organisms are being used: prokaryotic – *Escherichia coli*, *Bacillus subtilis*, etc; eukaryotic – *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia pastoris*. The advantage of bacterial producers is the high yield of production and the ease of genetic manipulation. Still, they lack the ability of a correct protein processing, and in many cases heterologous proteins build up inclusion bodies which often cause the proteins to lose their three-dimensional structure or / and enzymatic activity [1, 2].

Among the microbial eukaryotic host systems, yeasts combine the advantages of unicellular organisms with the capability of protein processing, assembly and folding. *S. cerevisiae* has been widely employed as a host organism for the expression of heterologous proteins; the use of regulated systems enables to provide an accurate control of gene expression in the functional analysis and the timely recombinant protein synthesis during fermentative production [3, 4].

In the present work, cloning of fused glucose dehydrogenase-synuclein genes into a *S. cerevisiae* expression system was performed and the peculiarities of hybrid constructs expression were examined. α-Synuclein is a 140-aa cytoplasmic and membrane-attached protein found in presynaptic terminals of neuronal cells. Although the precise physiological role of this protein is not fully understood, it has been suggested that α-syn is involved in the modulation of neurotransmitter release, ER / Golgi trafficking [5]. There are observations that accumulation of α-synuclein, resulting in the formation of oligomers, has been linked to Parkinson disease affecting about 2% of the population over the age of 65 [6]. A fusion component – pyrroloquinoline quinone (PQQ)-dependent dimeric glucose dehydrogenase (EC 1.1.5.2) from *Acinetobacter calcoaceticus* – was chosen as a marker that allows monitoring synuclein

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during culturing optimization, protein extraction and purification. A yeast model was generated for future studies of α-synuclein toxicity against eukaryotic organisms. The aim of this work was to analyse glucose dehydrogenase-synuclein expression possibilities in yeast developed into an attractive host for the expression and processing of heterologous proteins.

MATERIALS AND METHODS

The multicopy expression plasmid pBK [7] replicating in *E. coli* as well as in *S. cerevisiae* was used for constructing recombinant plasmids pBK-GDHsynEco and pBK-GDHsynPvu. The vector was prepared by restricting pBK by *SacI* and *PstI* endonucleases and removing 5'-phosphate by alkaline phosphatase. DNA was purified from the agarose gel and prepared for ligation. GDHsyn hybrid constructs were amplified by PCR (primers used: direct ($GDHsyn_{Fw}$) – GCATGAGCTCAAATGAATAAACATTTATTG; reverse ($GDHsyn_{Rw}$) – GCATCTGCAGCACTTCACAGGTCAAGC) from pTsynEco and pTsynPvu bacterial vectors (kindly provided by Dr. V. Časaitė). The target products were isolated from agarose gel, purified using a glass particle suspension and the following digestion with *SacI* and *PstI* restriction enzymes combined with vector DNA using T4 DNA ligase.

The *E. coli* strain DH5α (*F*$^-$ (φ*80d*Δ(*lacZ*)*M15*) *recA1 endA1 gyrA96 thi1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*) *U169*) was used for cloning experiments, plasmid isolation and maintenance [8]. *E. coli* transformation was carried out by the calcium chloride method or electroporation [9].

For the expression of hybrid proteins, *S. cerevisiae* strain 21PMR (*MATα leu2 ura3-52*) was used [10]. The transformation was performed using the LiAc / PEG method [11] and transformants selected by *URA3* auxotrophy complementation. The media growth for *S. cerevisiae* (SC-gal, synthetic complete medium with 2% galactose; YEPD, rich medium with 2% glucose; YEPG, rich medium with 3% galactose) as well as standard genetic techniques were as described in Ausubel [9].

Hybrid protein production and GDH activity were analyzed after the cultivation of clones under appropriate conditions. One option was to grow yeast for 4 days in a minimal synthetic medium at a temperature of 18 °C until reaching the cell density maximum. Another option was cultivation of yeast transformants in YEPG medium for 2 days or 1 day in YEPD and for the next 24 h under inducing conditions in the YEPG medium. Yeast cells were harvested by centrifugation (3000 g) at 4 °C for 10 min, the biomass (1 g) was resuspended in 2 ml of A buffer (50 mM Tris-HCl, pH 8.5) and ground using liquid nitrogen. The cell extract (cytoplasmic fraction) was cleared by centrifugation (11000 g) at 4 °C for 15 min. Sediments (membrane fraction) were dissolved by suspending and incubation in A buffer with 1% Triton X-100 at 4 °C for 1 h. GDHsyn purification was performed by ion-exchange chromatography on a CM-cellulose column according to the manufacturer's instruction (GE Healthcare). The specific activity of GDH (U/ml) was determined in a crude yeast extract and in fractions by measuring the decrease in DCIP absorbance at 600 nm [12]. Yeast growth, GDH-syn extraction and enzyme activity measuring were triplicated, and the results were averaged providing a standard deviation. Presence of hybrid protein or individual components was identified on SDS-PAGE data after visualization using Coomassie Brilliant Blue [9].

RESULTS AND DISCUSSION

On the basis of *S. cerevisiae* vector pBK, the recombinant plasmids pBK-GDHsynEco and pBK-GDHsynPvu were constructed by inserting an PCR-amplified GDHsyn sequence (Fig. 1). It is of importance that the successful constructs were obtained only by introducing the ligation mixture into DH5α cells by electroporation (resulting in just several colonies out of 0.25 mg of the vector / fragment mixture), while multiple chemical transformation experiments weren't successful. It is possible that the lower transformation efficiency was caused by the toxic effect of synuclein. The synuclein gene in pBK-GDHsynEco plasmid was sequentially connected to the 3'end of GDH, while in case of pBK-GDHsynPvu it was inserted into the glucose dehydrogenase gene. Two different hybrid arrangements were chosen to obtain a higher GDH activity and the optimal yield of hybrid protein, still avoiding synuclein toxicity.

The obtained constructs were analyzed by restriction mapping and electrophoretic analysis. The expression systems pBK-GDHsynPvu and pBK-GDHsynEco bearing hybrid derivatives aligned to the GAL-CYC1 promoter were transformed into the yeast strain 21PMR, and the transformants were selected by *URA3* complementation. Stable 21PMR-[pBK-GDHsynPvu] and 21PMR-[pBK-GDHsynEco] strains were grown in different liquid media (YEPG, YEPD–YEPG, SC-gal) for optimizing the time and temperature of cultivation. In all cases, the cultivation temperature was reduced to avoid the toxicity of a synthesized synuclein protein.

It is known from previous data that α-synuclein tends to accumulate in the membrane [13]. Thus, we decided to verify the hydrophobic (transmembrane) areas of GDH-syn derivative. Examination of hydrophilic–hydrophobic profiles showed three potential transmembrane motives: a strong hydrophobic N-terminal region and two smaller areas in the middle and C-terminus of the hybrid protein. Hence, the hybrid construct could attach to the membrane and also may be found in the cytoplasm. Therefore, the sub-

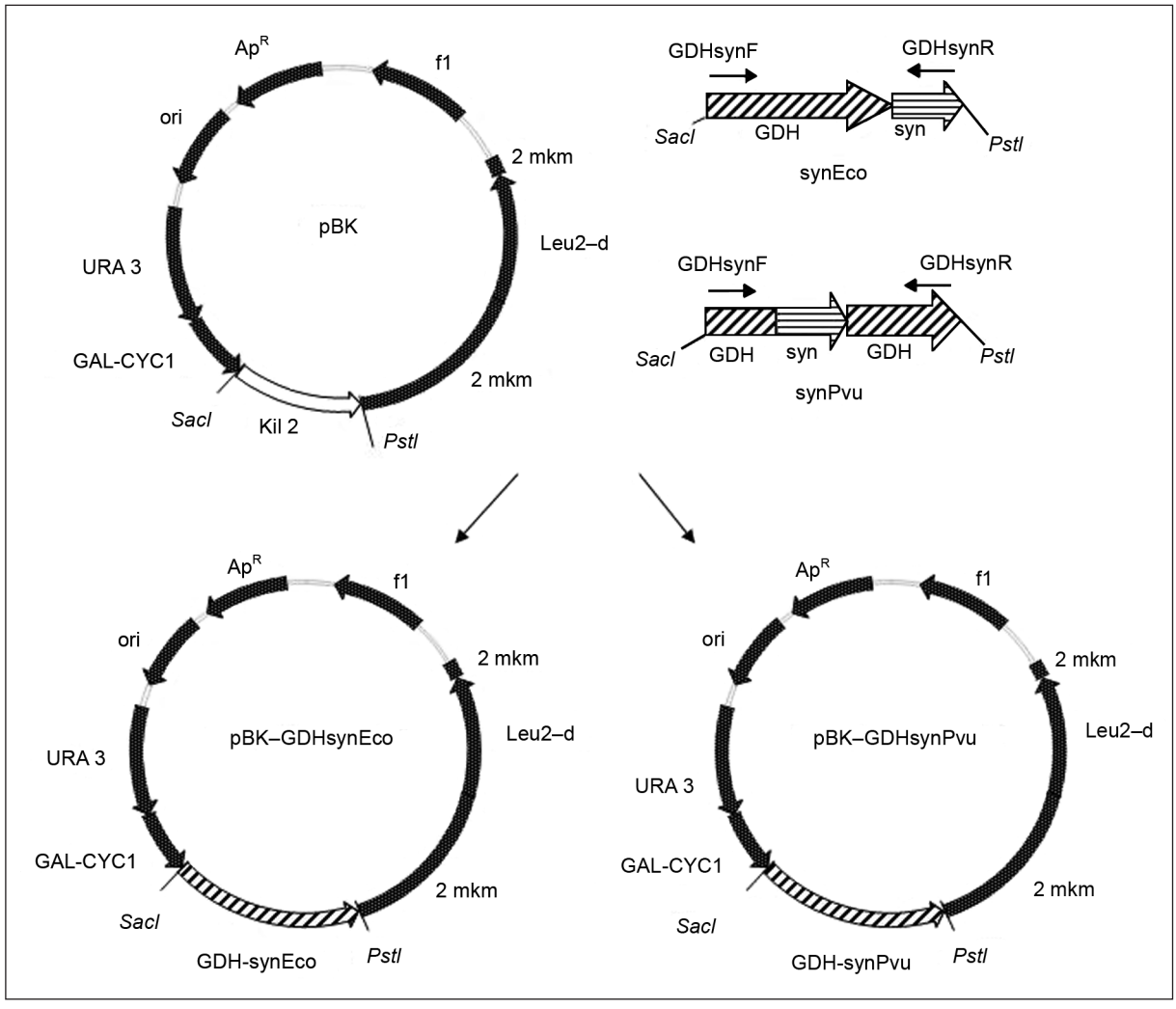

Fig. 1. Principal scheme of constructing pBK-GDHsynEco and pBK-GDHsynPvu plasmids

URA3, *Leu2*-d – genetic markers; 2 mkm – sequence originated from 2 μ plasmid of *S. cerevisiae*; ApR – gene for β-lactamase; ori – pMB1 replication origin; GAL-CYC1 – galactose inducible promoter; GDH – glucose dehydrogenase gene; syn – synuclein sequence; GDHsyn – glucose dehydrogenase gene fused with synuclein; *SacI, PstI* – restriction endonuclease sites; GDHsynF and GDHsynR – direct and forward primers used for PCR amplification

sequent fractions (cytoplasmic and membrane) of yeast cultures were grown as described in Materials and Methods and studied in parallel. The collected yeast biomass (~1 g) was disrupted by mechanical grinding with liquid nitrogen, the intracellular and membrane fractions were separated by centrifugation and then purified on the carboxymethyl column. In both cases, we observed small amounts of eluted proteins (15–30 mAU). Five fractions covering the peak were selected, the GDH activity was measured and compared to enzyme activity in a crude extract. The cytoplamic or membrane fractions of GDHsynEco (sequential GDHsyn connection) show a higher enzyme activity compared to the GDH–synPvu version (see Fig. 2). Interruption of the integrity of the GDH gene and insertion of the synuclein gene (GDHsynPvu) decrease the enzymatic activity two–three (crude cytoplasmic extract and partially purified fractions) or even two–ten times (purified fractions and crude membrane extract) (Fig. 2 *A, B*). Yeast cultures grown in different media accumulate various amounts of active protein. Transformants with pBK–GDHsynPvu cultivated in rich media show a higher enzymatic activity compared to ones grown in a minimal medium (Fig. 2, column YEPG). Meanwhile, yeast cells containing the pBK–GDHsynEco plasmid accumulate higher levels of active protein when grown in a minimal medium (Fig. 2, SC-gal column).

The assessment of GDH-synuclein transmembrane motive location allowed to expect that hybrid GDHsyn could prefer membrane-bound localization. Therefore, GDH activity was measured in all fractions of chromatographic purification (Fig. 2, *B*) and crude membrane extract (obtained by treatment with Triton X100) (Fig. 2, *A*). It was determined that GDH activity in crude membrane extracts was lower as compared with cytoplasmic variants: in case of GDHsynEco – 1.188 U/ml (in cytoplasm) and 0.629 U/ml

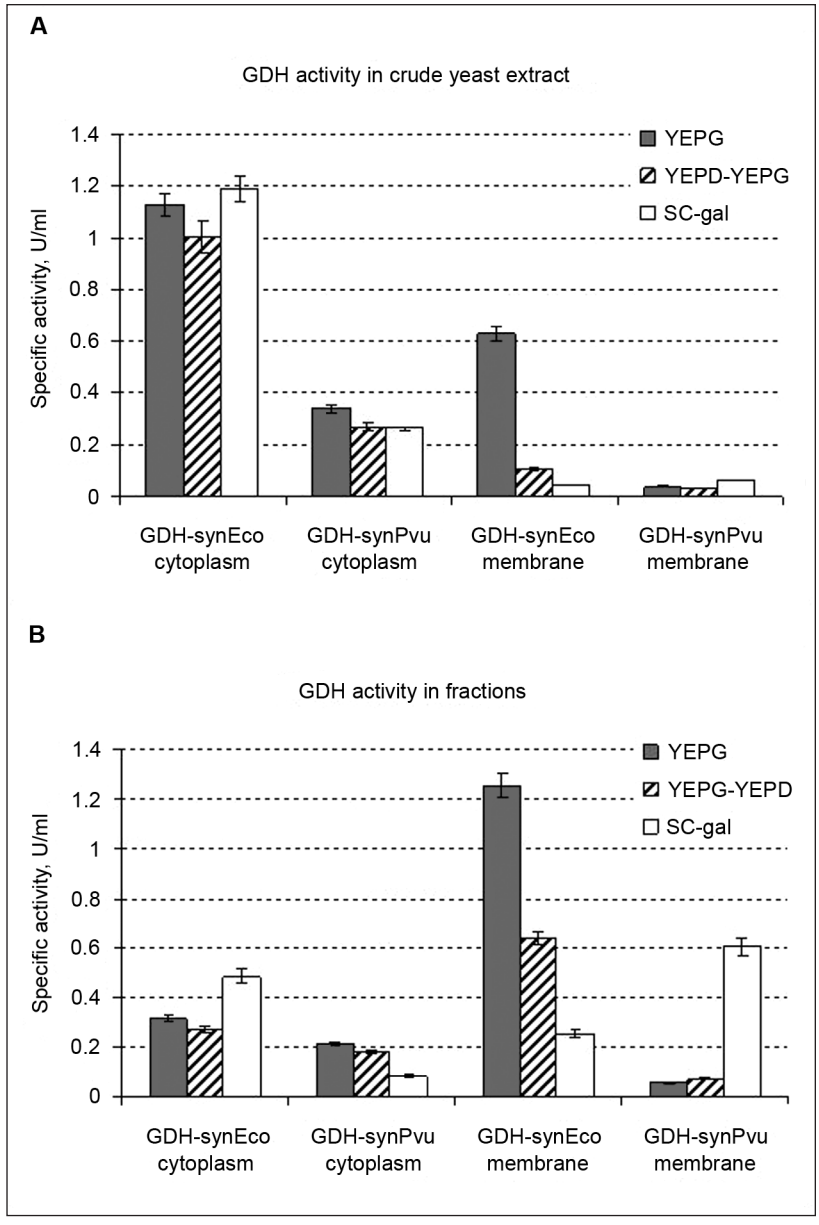

Fig. 2. Determination of GDH activity in crude yeast extract (A) and CM – column samples (B)

Yeasts were cultivated in YEPG, YEPD-YEPG or SC-gal media and GDH activity was measured in cytoplasmic or membrane extracts as well as partially purified samples

(in membrane); GDHsynPvu – 0.341 U/ml (cytoplasm) and 0.065 U/ml (membrane). However, after purification the GDH activity increased and exceeded the cytoplasmically located versions up to 3-fold (Fig. 2, *B*). The distribution of hybrid protein in the membrane (detected from glucose dehydrogenase activity) was also found to depend on yeast cultivation. The maximum of enzyme activity was observed in membraneous GDHsynPvu expressing yeasts cultivated in a minimal medium (Fig. 2, SC-gal column), while the largest amount of active GDHsynEco was found in the membrane of yeasts grown in YEPG medium (Fig. 2, column YEPG). These results suggest that growing conditions affect not only the target protein production level but also localization. It is likely that a different genetic structure of a hybrid arrangement affects not only a particular protein activity but also a target protein production in definite conditions.

Fractions demonstrating the highest enzyme activity were analyzed by SDS–PAGE: intracellular GDHsynEco grown in SC-gal medium, followed by chromatographic purification on a CM column (GDH activity in fraction 0.486 U/ml); intracellular GDHsynPvu cultivated in YEPG medium (0.213 U/ml); membrane fraction of GDHsynEco grown in YEPG medium (1.127 U/ml); membraneous GDHsynPvu grown in SC-gal medium (activity 0.607 U/ml). Analysis of cytoplasmic fractions has shown that the GDHsyn hybrid tends to split into separate components. On SDS-PAGE we observed a small amount of a protein corresponding to the hybrid derivative, as well as about 45 and 13 kDa bands as possible GDH and synuclein proteins (Fig. 3). The protein of interest wasn't detected by electrophoretic analysis in membrane fractions. Existence of a hybrid protein can be predicted taking into account GDH

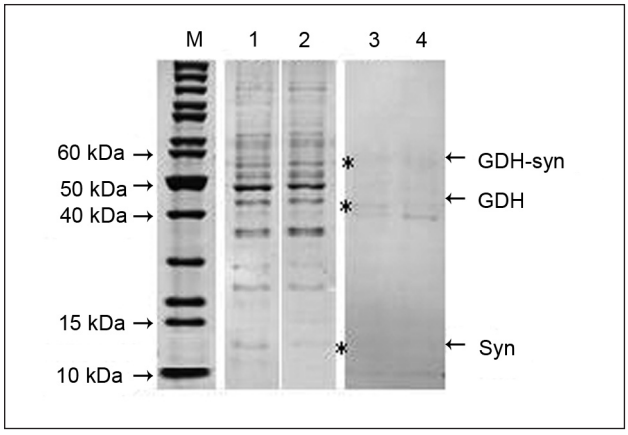

Fig. 3. SDS-PAGE electrophoresis of hybrid protein

Coomassie blue-stained gel of cytoplasmic and membrane proteins extracted from yeast transformants and purified on CM column. Lane M – Page Ruler™ unstained protein molecular weight marker (Fermentas); Lane *1* – cytoplasmic GDHsynEco; Lane *2* – cytoplasmic GDHsynPvu; Lane *3* – membraneous GDHsynEco; Lane *4* – membraneous GDHsynPvu

activity observed in membranes (Fig. 2), and the target protein could be proven by the Western analysis only. Our data are in line with the GFPsyn fusion protein analysis [14], demonstrating that hybrid protein was detected by immunodetection only. Despite such a low level of α-synuclein, Zabrocki and co-authors were able to the examine aggregation and toxicity of the target protein [14].

In summary, our study demonstrated the value of hybrid genetic constructions and yeast cultivation conditions in the synthesis and localization of active proteins of interest. The current *S. cerevisiae* yeast expression system is barely the best option for a high-level hybrid GDHsyn synthesis. However, our investigation has demonstrated the possibility of using the GDHsyn model system for testing synuclein toxicity by the analysis of yeast cell viability and growth dynamics.

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References

1. Petrescu-Danila E, Voicu M, Stanescu R et al. Rom Biotech Let 2009; 14: 4201–10.
2. Porro D, Sauer M, Branduardi P et al. Mol Biotechnol 2005; 31: 245–59.
3. Quintero MJ, Maya D, Arevalo-Rodriguez M et al. Microbial Cell Fact 2007; 6: 1–9.
4. Ton V, Rao R. Am J Physiol Cell Physiol 2004; 287: C580–8.
5. Vilar M, Chou H, Luhrs T et al. PNAS 2008; 105: 8637–42.
6. Crews L, Tsigelny I, Hashimoto M et al. Neurotox Res 2009; 16: 306–17.
7. Serviene E, Melvydas V. Biologija 1999; 2: 14–17.
8. Woodcock DM, Crowther PJ, Doherty J et al. Nucleic Acids Res 1989; 17: 3469–78.
9. Ausubel F. Current Protocols in Molecular Biology. NY: John Wiley and Sons, Inc., 1999.
10. Jokantaite T, Melvydas V. Proceeding of the Institute of Botany, Lithuania, 1995: 82–91.
11. Gietz R, Woods R. Methods Enzymol 2002; 350: 87–96.
12. Tanaka S, Igarashi S, Ferri S et al. BMC Biochemistry 2005; 6: 1–6.
13. Liang J, Clarck-Dixon C, Wang S et al. Hum Mol Genet 2008; 17: 3784–95.
14. Zabrocki P, Pellens K, Vanhelmont T et al. FEBS J 2005; 272: 1386–400.

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GDH-SINUKLEINO SINTEZĖS *SACCHAROMYCES CEREVISIAE* MIELĖSE TYRIMAS

Santrauka

Šiame darbe buvo tiriamos heterologinio baltymo – gliukozės dehidrogenazės-sinukleino – raiškos *Saccharomyces cerevisiae* mielėse galimybės. GDHsyn hibridai buvo klonuoti iš bakterinių vektorių į mielių raiškos sistemas, parinktos optimalios kultūrų auginimo sąlygos bei atliktas dalinis baltymų gryninimas ant karboksimetilinės kolonėlės. Nustatyta, kad genetinė struktūra ir augimo sąlygos turi įtakos ne tik produkuojamo baltymo kiekiui, bet ir jo lokalizacijai ląstelėje. Įvertinus hibridinių darinių GDHsynEco ir GDHsynPvu raišką ir lokalizaciją, didžiausias GDH aktyvumas pastebėtas abiejų producentų citoplazminėse frakcijose. Nustatyta, kad *S. cerevisiae* mielėse hibridinis darinys yra linkęs skilti į atskirus komponentus.

Raktažodžiai: *Saccharomyces cerevisiae*, gliukozės dehidrogenazė, sinukleinas, hibridinis darinys