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EFFECT OF TREHALOSE AND GLYCEROL ON THE RESISTANCE OF RECOMBINANT *SACCHAROMYCES CEREVISIAE* STRAINS TO DESICCATION, FREEZE-THAW AND OSMOTIC STRESSES

Introduction. *Baker's yeast Saccharomyces cerevisiae has been used for manufacturing bakery products, food and feed supplements, alcoholic fermentation etc. In biotechnological processes, yeast cells are exposed to stress factors (high concentration of sugars and ethanol, high temperature, desiccation or freezing etc.), which negatively affects their viability. Yeasts possess certain stress protection systems, including increased accumulation of disaccharide trehalose and glycerol synthesis.*

Problem Statement. *The strengthening of yeast protective systems by increasing glycerol or trehalose concentrations can help to get increased stress robustness of the S. cerevisiae strains.*

Purpose. *To construct the recombinant strains of S. cerevisiae with increased trehalose accumulation or glycerol production and to estimate the obtained recombinant strains resistance to a range of stress factors.*

Materials and Methods. *S. cerevisiae transformation has been performed using Li-Ac-PEG method. Alcoholic fermentation has been carried out at a temperature of 30 °C with stirring at a rate of 120 rpm.*

Results. *The recombinant strains of S. cerevisiae with enhanced glycerol production (up to 19 g/L) have been constructed based on BY4742. The industrial ethanol-producing strain Y-563 has been used as parental one for construction of recombinant strains with up to 3.3-fold increase in the intracellular trehalose level. The resistance of obtained recombinant strains to different stress factors has been evaluated. BY/TPI25/gpd1gpp2f/fps1 strain with the highest glycerol production has been established to have the highest osmotolerance. The BY/TPI25/gpd1gpp2f, 563/TSL1, 563/TPS1/2 and 563/TPS1/2/TSL1 strains have shown higher viability after freeze-thaw as compared with the corresponding parental strains, but not higher resistance to desiccation. The recombinant strain 563/TPS1/2/TSL1 with a high trehalose content have been established to have higher activity during fermentation of sugar in sweet dough and to longer keep stable at 35 °C as compared with the initial strain Y-563.*

Conclusions. *Constructed recombinant strains of S. cerevisiae with higher osmotolerance or freeze-thaw resistance can be implemented in industrial processes accompanied with these types of stresses. Baker's yeast made of high trehalose-containing biomass will have prolonged shelf life.*

Key words: baker's yeasts, trehalose, glycerol, freeze-thaw, desiccation, and osmotolerance.

INTRODUCTION

Baker's yeast *Saccharomyces cerevisiae* is used in bread baking, manufacture of soft and strong alcoholic beverages, production of commercial ethanol and fuel, preparation of food/feed supplements (inactivated flaked yeast, yeast autolysates, yeast hydrolysates, yeast extracts, vitamin supplements, etc.). To be used in these industrial processes, yeast cells have to efficiently resist

various external stresses. This research deals with some stress factors to which yeast cells are exposed during the production of different yeast preparations for baking (compressed yeast, crumbled yeast, liquid yeast, active dry yeast etc.) and dough fermentation. Such baking-associated stresses include, for instance, desiccation, freeze-thaw, and osmotic stress (Fig. 1).

Baker's yeast preparations made mainly of *S. cerevisiae* biomass are indispensable element of bread baking (Randez-Gil, Sanz, & Prieto, 1999). Annually, about 2 million ton baker's yeast is produced worldwide (Attfield, 1997). Baker's yeast is produced as suspension that contains about 20% of dry cells biomass; as compressed yeast that contains 30% of dry cells biomass; and dry yeast with a moisture content of only 5%. In Ukraine, mainly compressed yeast or yeast suspension is used for baking. Dry yeast is also prepared in small amount or imported from other countries and is used for bread baking in small bakeries or households.

The yeast suspensions should be stored and transported at a temperature of 4 °C. They have a limited shelf life and as it expires undergo cell autolysis. For such yeast, extension of shelf life is very important. The yeast cells used for dried yeast preparation must be resistant to desiccation and retain a high fermentation activity after rehydration. If dough recipe requires a lot of sugar (the so called sweet dough), the yeast cells are exposed to high osmotic pressure. This leads to water loss and shrinkage of cells, so yeast cells have to be able to retain water and in such way to resist osmotic stress. When bread is baked from frozen dough (which often happens), yeast cells have to keep viable and active after freeze-thaw procedure. Bread baking and other industrial processes involving *S. cerevisiae* will massively benefit from obtaining robust *S. cerevisiae* strains resistant to all of the mentioned stresses.

These different stress factors are considered to induce oxidative stress accompanied by the formation of reactive oxygen species (ROS) due to protein denaturation and destruction of mitochondrial and electron transport chain components (Ando, Nakamura, Murata, Takagi, & Shima, 2007). In the presence of high amounts of ROS the yeast cells fermentation activity is severely suppressed, which impairs their performance in the industrial processes.

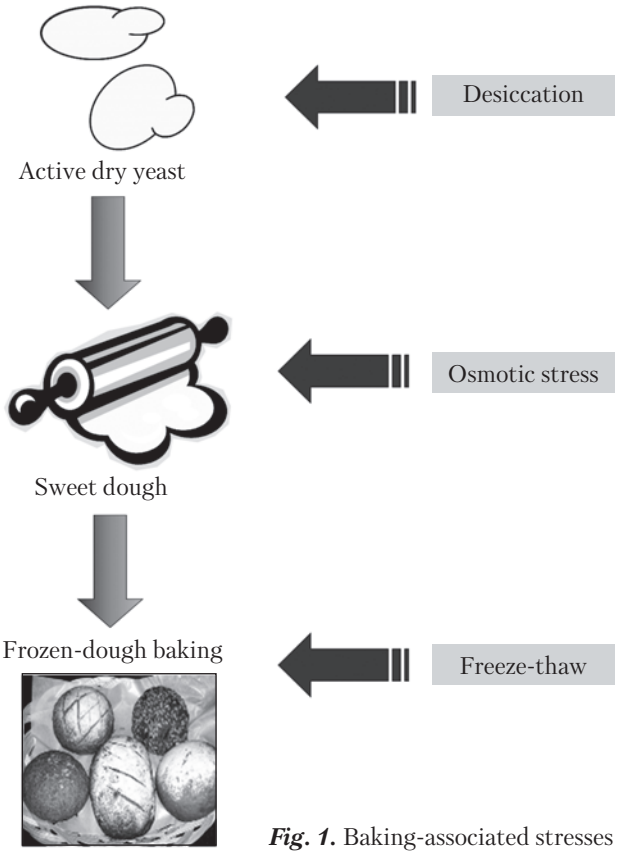


Fig. 1. Baking-associated stresses

The yeast cells have multiple mechanisms of adaptation to stress factors, e.g. synthesis of heat-shock proteins, compounds with antioxidant properties and compatible solutes, modification of plasma membrane components and suppression of translation, etc. In this research, the role of trehalose and glycerol as stress-protectors is studied.

Disaccharide trehalose is one of the most comprehensively studied factors of cell protection in unfavorable environments caused by desiccation, freezing, high temperature, ethanol, and osmotic stresses (Crowe, 2007). Trehalose protects membrane structures by decreasing temperature of

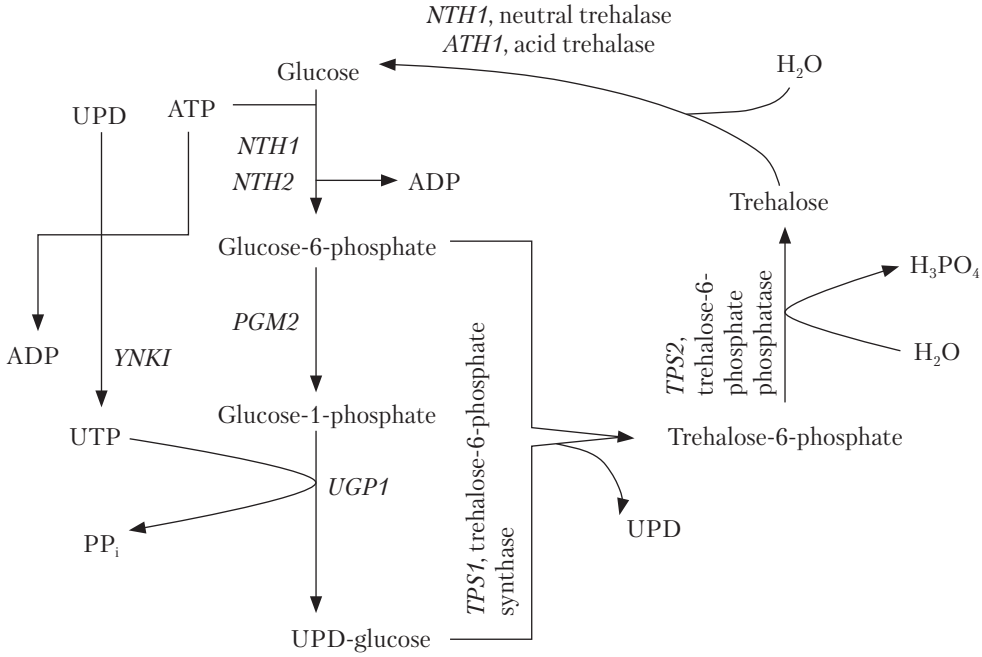


Fig. 2. Trehalose metabolism in *S. cerevisiae*. *HXK1*, *HXK2* – hexokinases; *UGP1* — UDP-glucose pyrophosphorylase; *PGM2* — phosphoglucomutase; *YNK1* — nucleoside diphosphate kinase; *NTH1* — neutral trehalase; *ATH1* — acid trehalose; *TPS1* — trehalose-6-phosphate synthase; *TPS2* — trehalose-6-phosphate phosphatase

membrane lipids thawing, stabilizes the process of protein folding, and prevents proteins from aggregation (Tapia & Koshland, 2014). The increased level of intracellular trehalose has been found to be necessary for yeast cells to survive after desiccation-rehydration (Tapia & Koshland, 2014). It is known, that during alcoholic fermentation the yeast cells accumulate trehalose and its level can reach 15% of dry cell mass (Francois & Parrou, 2001). However, the most thoroughly studied function of trehalose is protection of cells against heat shock (Wiemken, 1990).

Trehalose metabolism in *S. cerevisiae* has been studied in detail and represents a precise balance between the substance synthesis and hydrolysis (Fig. 2) (Kim, Alizadeh, Harding, Hefner-Gravink, & Klionsky, 1996). Trehalose synthesis is performed by big enzymatic complex that consists of trehalose-6-phosphate synthase encoded by *TPS1* gene and trehalose-6-phosphate phosphatase encoded by *TPS2* gene. Trehalose-6-phosphate synthase catalyzes condensation between glucose-6-phospate and UDP-glucose to form trehalose-6-phosphate. Subsequently, trehalose-6-phosphate is converted into trehalose by the action of trehalose-6-phosphate phosphatase. Overexpression of, at least, one of these genes — *TPS1* — causes an increase in intracellular trehalose concentration and an enhanced heat tolerance of the corresponding recombinant *S. cerevisiae* strains. Trehalose hydrolysis is catalyzed by two isoenzymes: acid trehalase (encoded by *ATH1* gene) and neutral trehalase (encoded by *NTH1* gene) (Londesborough & Varimo, 1984). Acid trehalase is necessary for *S. cerevisiae* growth in the medium with trehalose as a sole source of carbon and energy (Nwaka, Mechler, & Holzer, 1996). The deletion of *ATH1* gene in *S. cerevisiae* causes a more noticeable increase in cellular trehalose level, than that of *NTH1* gene (Kim et al., 1996). It has been shown, that *ath1Δ* strain of *S. cerevisiae* is more resistant to desiccation, low-temperature incubation, ethanol, and osmotic stresses as compared with the homogenic strain (Kim et al., 1996). A decrease in acid trehalase activity in *S. cerevisiae* enhances its tolerance to

ethanol and productivity of alcoholic fermentation (Jung & Park, 2005).

So, the enhanced trehalose level ensures the resistance of yeast cells to high temperature, freeze-thaw stress, high osmotic pressure in the medium, high ethanol concentration etc. (Guo, Zhang, Ding, & Shi, 2011). Recently, trehalose has been suggested to be very efficient in the medical treatment of some human diseases (Byun, Lee, & Lee, 2017). Therefore, the prospects for obtaining trehalose-overproducing *S. cerevisiae* recombinant strains are very promising.

Sustainable water balance is an obligatory condition of cell existence. Rapid changes in environmental osmolarity and moisture content can be detrimental for the living cells, for example, increased osmolarity of the cellular environment causes water efflux from the cells and, as a result, their shrinkage. Universal strategy of cell survival in these conditions is a synthesis of compatible solutes to compensate for a moisture decrease (Yancey, Clark, Hand, Bowlus, & Somero, 1982). Such molecules are compatible with intracellular processes and either replace water or/and revert the water concentration gradient and drive water back into cells. The most abundant compatible solutes in microorganisms are small uncharged molecules such as: (1) polyols (glycerol, arabitol, trehalose or sucrose); (2) amino acids (proline, glutamate or glutamine); and (3) ectoines, (ectoine or β-hydroxyectoine) (Grant, 2004). In the yeasts, glycerol is one of the most widespread compatible solute that ensures an acceptable cell turgor under high extracellular osmolarity (Blomberg & Adler, 1992; Brown, 1978). For example, during the initial phase of alcoholic fermentation, *S. cerevisiae* cells accumulate an enhanced amount of glycerol in response to osmotic stress caused by a high sugar concentration in grape must. Also, *S. cerevisiae* produces a lot of glycerol while growing in the medium with a high salt (NaCl) concentration (Blomberg & Adler, 1989). Adaptation of *S. cerevisiae* to hyper-osmotic stress is accomplished via increased glycerol accumulation and its retention inside the cells. The mutants blocking the synthesis of glycerol (e.g. *gpd1Δgpd2Δ*) or causing the leakage of glycerol out of the cell (e.g. with hyperactive membrane channel Fps1), have an osmo-sensitive phenotype (Hohmann, 2002).

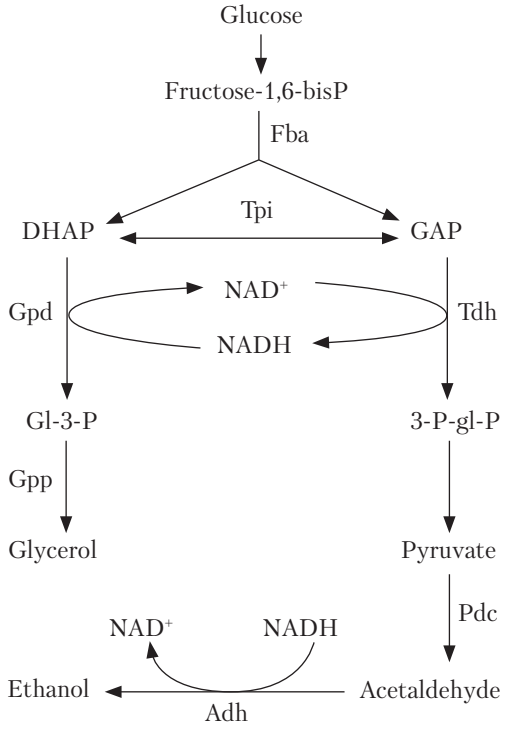


Fig. 3. Central metabolism and glycerol production by *S. cerevisiae*. DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; Gl-3-P, glycerol 3-phosphate; 3-P-gl-P, 1,3-bisphosphoglycerate; Fba, aldolase; Tpi, triose phosphate isomerase; Gpd, glycerol 3-phosphate dehydrogenase; Gpp, glycerol 3-phosphate phosphatase; Pdc, pyruvate decarboxylase; Adh, alcohol dehydrogenase

In *S. cerevisiae*, glycerol is synthesized from dihydroxyacetone phosphate (DHAP) in two consequential reactions catalyzed by cytosolic enzymes glycerol-3-phosohate dehydrogenase (Gpd) and glycerol-3-phosphate phosphatase (Gpp) (Fig. 3). DHAP can also be converted to glyceraldehyde-3-phosphate and vice versa under the action of triose phosphate isomerase (Tpi). The recombinant *S. cerevisiae* strain with *TPI1* gene deletion produces high amounts of glycerol, but is unable to grow on the glucose as a sole carbon source (Overkamp et al., 2002).

Cytosolic NAD^+-depended Gpd catalyzes the DHAP reduction to glycerol-3-phosphate, which is accompanied by oxidation of NADH to NAD^+. The Gpd activity determines the overall rate of glycerol synthesis in the cells (Remize, Barnavon, & Dequin, 2001). In *S. cerevisiae,* Gpd is encoded by two isogenes: osmotically induced *GPD1* gene (Albertyn, Hohmann, Thevelein, & Prior, 1994; Larsson, Ansell, Eriksson, & Adler, 1993) and *GPD2* gene which translation is activated during cell growth in anaerobic conditions (Eriksson, Andre, Ansell, Blomberg, & Adler, 1995; Larsson et al., 1993). During osmotic stress, the activity of Gpd1 isoform is also regulated on post-translational level by dephosphorylation (Lee, Jeschke, Roelants, Thorner, & Turk, 2012; Oliveira et al., 2012).

Like for Gpd, two isoforms of Gpp have been identified: the first one that is encoded by *GPP1* gene is induced by a shift to anaerobic conditions (Pahlman, Granath, Ansell, Hohmann, & Adler, 2001) and the second one that is encoded by *GPP2* gene is activated during hyperosmotic stress (Norbeck, Pahlman, Akhtar, Blomberg, & Adler, 1996; Pahlman et al., 2001). The evolutionary engineering of bicistronic artificial operon containing the yeast *GPD1* and *GPP2* genes in a heterologous system of *Escherichia coli* has resulted in obtaining *GPD1-GPP2* fused gene (Meynial Salles, Forchhammer, Croux, Girbal, & Soucaille, 2007). The product of this gene has both the catalytic sites of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase and is able to convert DHAP into glycerol faster than separated glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase, which is likely explained by substrate channeling between the two active sites.

In *S. cerevisiae,* glycerol is exported from cells through the membrane channel formed with the protein Fps1 (Luyten et al., 1995). If the yeast cells are exposed to a high osmotic pressure, the channel would be shut down and the synthesized glycerol would be retained inside the cells (Luyten et al., 1995; Tamas et al., 1999). In anaerobic conditions, the expression level of *FPS1* gene is higher, while in aerobic conditions, the transporter is needed to remove excessive glycerol (ter Linde et al., 1999). Fps1 protein has long N- and C-terminal domains that are necessary to shut down the channel. The Fps1 modified protein that lacks N- or C-terminal domain becomes constantly open and exporting glycerol into cultivation medium (Ahmadpour, Geijer, Tamas, Lindkvist-Petersson, & Hohmann, 2014). It is an interest fact that such intricate mechanism of Fps1 protein opening and closing occurs only in the yeasts closely related to *S. cerevisiae* (Pettersson, Filipsson, Becit, Brive, & Hohmann, 2005). Supposedly it is the way to adapt itself to quickly changing environment osmotic pressure. To conclude, an increase in intracellular glycerol concentration in *S. cerevisiae* improves its resistance to osmotic stress. So the research aims at obtaining glycerol-overproducing recombinant strains to see how high osmotolerance they can have.

MATERIALS AND METHODS

In this research, the following microbial strains are used: *S. cerevisiae*: BY4742 (MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) — WT strain; BY/gpd1 — recombinant strain with *GPD1* gene overexpression; BY/gpd1gpp2f — recombinant strain with artificial fused *GPD1-GPP2fus* gene overexpression; BY/TPI25/gpd1gpp2f — recombinant strain with *TPI1* promoter region shortened to 25 bp and overexpression of *GPD1-GPP2fus* gene; BY/TPI25/gpd1gpp2f/fps1m — recombinant strain with *TPI1* promoter region shortened to 25 bp and overexpression of genes *GPD1-GPP2fus* and *FPS1m*; Y-563 — a triploid hybrid yeast strain obtained by crossing the osmophilic ethanol-producing strain *S. cerevisiae* SH-1 with the bakery strain *S. cerevisiae* 2—10, which are able to effectively ferment raffinose; 563/TSL1 — recombinant strain with *TSL1* gene overexpression, constructed based on Y-563 strain; 563/TPS1/2 — recombinant strain with overexpression of the genes *TPS1* and *TPS2*, constructed based on Y-563 strain; 563/TPS1/2/TSL1 — recombinant strain

with overexpression of the genes *TPS1*, *TPS2*, and *TSL1*, constructed based on Y-563 strain. The *Escherichia coli* DH5α strain (80d*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) is used as a host for propagation of plasmids. Strain DH5α is grown at 37 °C in LB medium. The transformed *E. coli* cells are maintained in a medium containing 100 mg/L ampicillin.

The *S. cerevisiae* strains are incubated at 30 °C and maintained in rich YPD (10 g/L yeast extract, 10 g/L peptone and 20 g/L glucose) or in mineral YNB (1.7 g/L yeast nitrogen base without amino acids, DIFCO, 5 g/L ammonium sulfate, 20 g/L glucose) media. Histidine (20 mg/L), leucine (60 mg/L), lysine (20 mg/L), or uracil (20 mg/L) are added when required. For ethanol fermentation, YNB medium with 100 g/L glucose is used.

The *S. cerevisiae* transformation is performed using Li-Ac-PEG method (Kawai, Hashimoto, & Murata, 2010). For selection of yeast transformants in YPD, 100 mg/L nourseothricin and 150 mg/L hygromycin B or 200 mg/L geneticin are added.

For alcoholic fermentation, the cells of studied yeast strains are grown in 50 mL YPD medium, in Erlenmeyer flasks (100 mL bottles), for 24 hours and then inoculated into 20 mL YNB medium with 100 g/L glucose, in 50 mL Erlenmeyer flasks. An initial biomass concentration of 0.9—1.15 g (dry weight)/L is used for fermentation. The fermentation is carried out at a temperature of 30 °C with a stirring rate of 120 rpm. The samples are taken daily.

The ethanol concentration in the fermentation broth is determined using alcohol oxidase/peroxidase-based *Alcotest* enzymatic kit (Gonchar, Maidan, Pavlishko, & Sibirny, 2001). Alternatively, the concentrations of glycerol, glucose, and ethanol in the medium broth are analyzed by HPLC (PerkinElmer, Series 2000, USA) with an *Aminex* HPX-87H ion-exchange column (Bio-Rad, Hercules, USA). A mobile phase of 4 mM H_2SO_4 is used at a flow rate 0.6 mL/min and at a column temperature of 35 °C. The intracellular trehalose level is assayed as described earlier (Ishchuk, Voronovsky, Abbas, & Sibirny, 2009).

RESULTS AND DISCUSSION

In the previous research, a set of glycerol-overproducing *S. cerevisiae* recombinant strains was constructed (Semkiv, Dmytruk, Abbas, & Sibirny, 2017): BY/gpd1; BY/gpd1gpp2f; BY/TPI25/gpd1gpp2f; BY/TPI25/gpd1gpp2f/fps1m (see *Materials and Methods*). These strains produce gradually increasing amounts of glycerol under semi-aerobic and anaerobic conditions (Fig. 4).

To construct the recombinant *S. cerevisiae* with increased intracellular trehalose level, a triploid hybrid yeast strain Y-563 is used. To overexpress

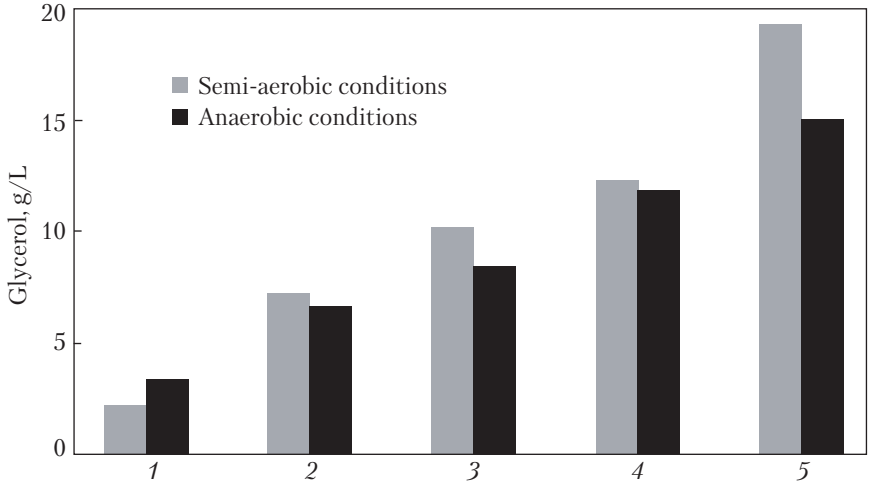


Fig. 4. Glycerol production (g/L) at the 48th hour of fermentation in semi-aerobic conditions (gray bars of the graph) or at the 72nd hour of fermentation in anaerobic conditions (black bars of the graph). BY4742 (*1*) — WT strain; BY/gpd1 (*2*); BY/gpd1gpp2f (*3*); BY/TPI25/gpd1gpp2f (*4*); BY/TPI25/gpd1gpp2f/fps1m (*5*) — studied recombinant strains

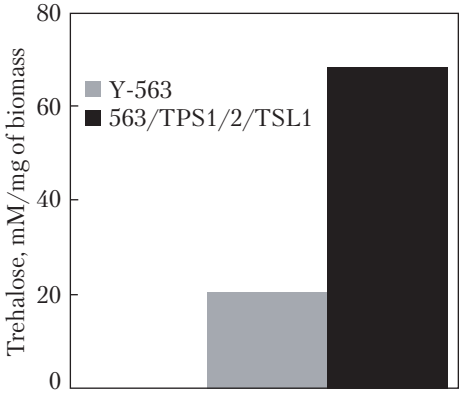


Fig. 5. Intracellular trehalose content (mM/mg) of cellular biomass for the Y-563 strain and its derivative recombinant strain 563/TPS1/2/TSL1 with the overexpression of *TPS1*, *TPS2*, and *TSL1* genes

the genes that encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, there is constructed a vector for multicopy integration, in which ORF of *TPS1* and *TPS2* genes are placed under control of strong constitutive promoter of *ADH1* gene (coding for alcohol dehydrogenase) (Luzhetskyi, Semkiv, Dmytruk, & Sibirny, 2015). The plasmid obtained is named pdelta-TPS1-TPS2 and used for transformation of Y-563 strain. One of the selected recombinant strains is named 563/TPS1/2 and used for the further analysis.

For overexpression of *TSL1* gene, pdelta-TSL1 vector is constructed (Luzhetskyi et al., 2015) that contains ORF of the gene under the control of *ADH1* promoter and *hphNT1* selection marker gene conferring resistance to hygromycin B. The plasmid obtained is linearized with AhdI restriction endonuclease and used for transformation of *S. cerevisiae* strains Y-563 and 563/TPS1/2. Among the selected transformants, a strain with *TSL1* gene overexpression and a strain with *TPS1*, *TPS2*, and *TSL1* genes overexpression are chosen and named 563/TSL1 and 563/TPS1/2/TSL1, respectively. The intracellular trehalose content in the recombinant strain 563/TPS1/2/TSL1 has shown a 3.3-fold increase as compared with the initial strain Y-563 (Fig. 5).

Alcoholic fermentation with strains Y-563 and 563/TPS1/2/TSL1 is performed in 1 L fermenter in YPD medium at a stirring rate of 200 rpm, a temperature of 40 °C, and pH = 5.5. Glucose in the medium is completely consumed at the 16th hour of fermentation. The highest production of ethanol in the studied strains is also observed at the 16th hour of fermentation (Fig. 6). Ethanol production by 563/TPS1/2/TSL1 strain reaches 40 g/L, whereas the parental strain Y-563 produces only up to 30 g/L ethanol. Therefore, an increase in ethanol production makes up 33% for the recombinant strain during high-temperature fermentation (at 40 °C).

The obtained glycerol and trehalose overproducing strains are tested to estimate their resistance to various stresses.

To check the strains resistance to high osmotic pressure, cell suspensions of the corresponding yeast strains with optical densities of 1.0, 0.1, 10^{-2}, 10^{-3}, 10^{-4} (wave length of 600 nm) are prepared; then 5 μL suspension is plated onto YPD medium

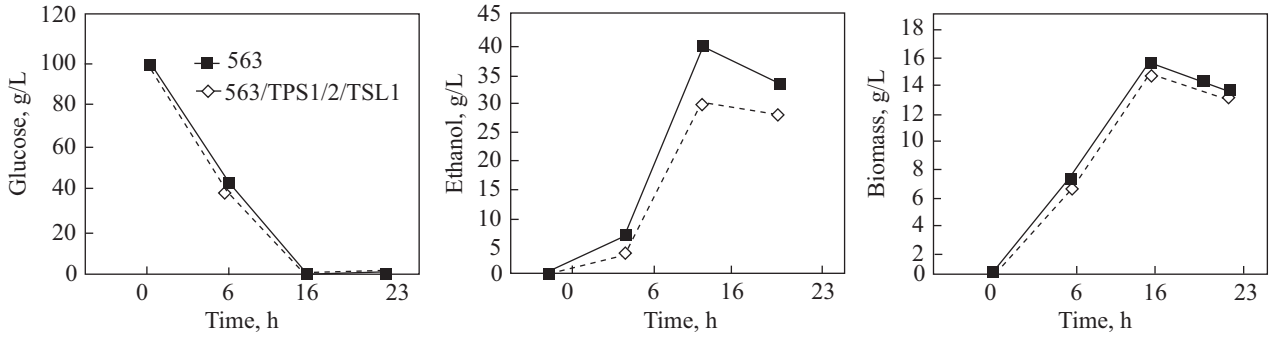


Fig. 6. Kinetics of glucose consumption, biomass accumulation, and ethanol production (g/L) by Y-563 and 563/TPS1/2/TSL1 strains during fermentation in 1 L fermenter in YPD medium at a stirring rate of 200 rpm, a temperature of 40 °C and pH = 5.5

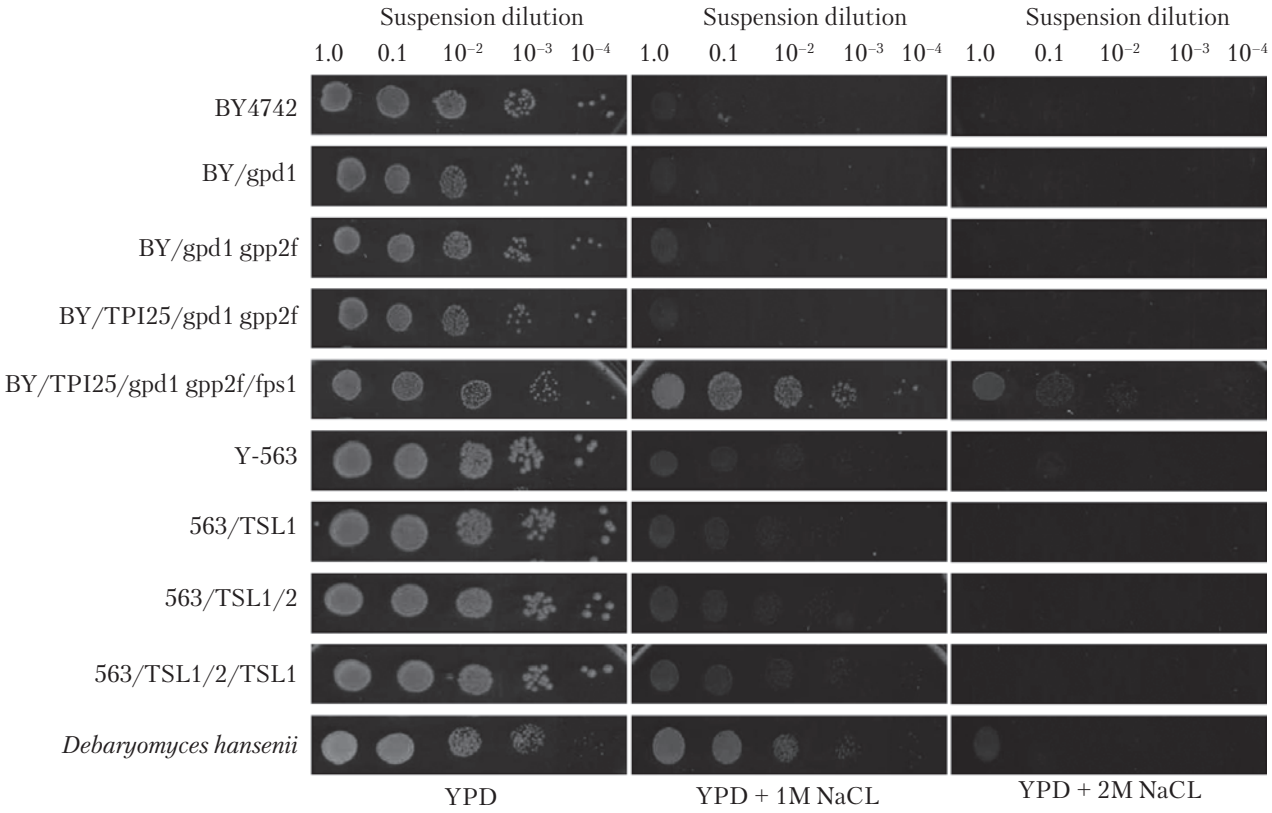


Fig. 7. Growth of studied recombinant *S. cerevisiae* strains on the media with different salt concentrations (1M or 2M NaCl)

and YPD with 1M NaCl or 2M NaCl added. For comparison, cell suspension of osmotolerant yeast *Debaryomyces hansenii* is used. Strain BY/TPI25/gpd1gpp2f/fps1 with the highest level of glycerol synthesis has been shown to have the highest osmotolerance, even higher than in *D. hansenii* (Fig. 7).

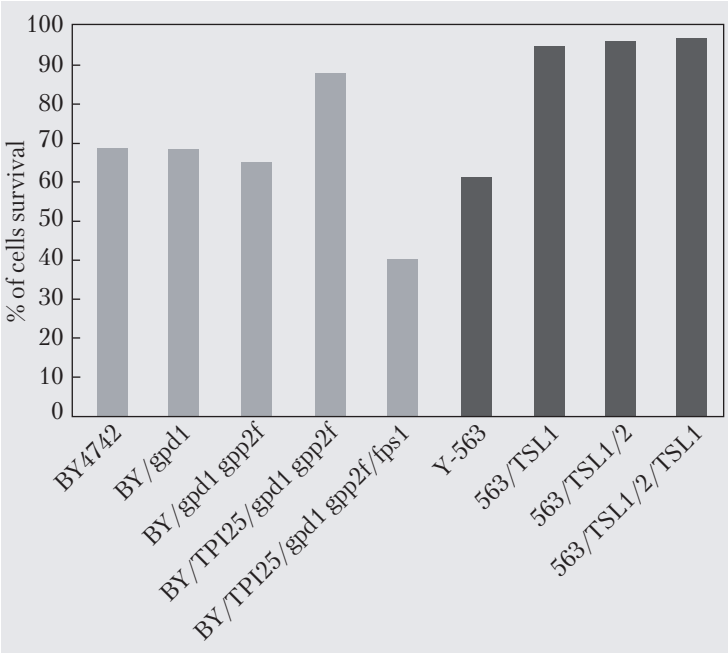
In order to check the recombinant strains resistance to freeze-thaw, cells suspensions with optical densities 2.0, 1.0, and 0.5 are prepared and frozen at −20°C for 72 hours. To estimate the amount of living cells, the suspensions are diluted before and after freezing, plated onto the YPD medium and incubated for 2—3 days. The yeast colonies obtained after incubation are analyzed. The percentage of cells survived is expressed as the amount of colonies obtained on the plates from suspension after freezing, divided by the amount of colonies obtained on the plates from suspension before freezing and multiplied by 100. Fig. 8 features the averaged result of three individual experiments.

Fig. 8 shows that strains BY/TPI25/gpd1gpp2f, 563/TSL1, 563/TPS1/2, and 563/TPS1/2/TSL1 possess higher resistance to freeze-thaw in comparison with the respective parental strains.

The recombinant strains resistance to desiccation are calculated in similarly, i.e. by counting living cells before and after freeze desiccation in Cryo Dryer. Unfortunately, the studied recombinant strains do not show higher resistance to freeze desiccation than the parental strains (no data are shown).

The experiments with the recombinant strain 563/TPS1/2/TSL1 and parental strain Y-563 in conditions close to industrial processing are performed using the facilities of partner *ENZYM COMPANY* PJSC (Lviv).

The recombinant strain ability to grow and to ferment is tested using a pilot small-scale fer-



◂***Fig. 8.*** Percentage of cells survived after freezing at –20°C for 72 hours

Fig. 9. Pilot fermenter (7 L) used for semi-industrial cultivation of the studied *S. cerevisiae* strains

menter (Fig. 9) with a total volume of 7 L, filled with 4 L industrially-used medium with molasses, for 16 hours with sterile air aeration (at a purging rate of 10 L/min). The incubation temperature is 30 °C. The medium contains as follows: tap water; 10% molassess as sugars source; 0.5% ammonia water as nitrogen source; 0.3% KH_2PO_4 as phosphorous source; 0.1% Na_2SO_4 as sulfur source, antifoam solution to prevent excessive foam formation and leakage of the culture medium through the valve of the fermenter. The microelements are introduced to the medium by adding the following salts: $MgSO_4$ — 0.5 g/L; $ZnSO_4$ — 0.05 g/L; and $CuSO_4$ — 0.01 g/L. The medium is sterilized at a temperature of 121°C and a pressure of 1 atmosphere, for 30 min. Vitamin mix including biotin and vitamins B1, B5, B6 is added after the sterilization.

The Y-563 and 563/TPS1/2/TSL1 strain biomass obtained after incubation in the fermenter is used for determination of some yeast properties important for bread baking, in particular, productivity (ethanol production in g/L), humidity in %, stability at 35 °C, and lifting force during the kneading of lean and sweet dough (Table). The lean dough contains high-grade flour, 2.5% saline solution, and yeasts. The sweet dough is prepared similarly to the lean one, but additionally contains 15% sugar and margarine, which create a medium with a high osmotic pressure.

The recombinant strain with high intracellular trehalose content (563/TPS1/2/TSL1) has been shown to longer keep its fermentation activity at 35°C in comparison with the initial strain. Although the recombinant strain ferments the lean dough worse than the initial strain, it better fer-

Properties of Strains Y-563 and 563/TPS1/2/TSL1 Biomass Produced in Semi-Industrial Conditions in Pilot Fermenter

Strain	Productivity, g/L ethanol	Humidity, %	Lifting force (lean dough) CO_2 ml/g dough	Lifting force (sweet dough) CO_2 ml/g dough	Stability at 35 °C, hours
Y-536	53	75.2	339	331	94
563/TPS1/2/TSL1	52	74.6	295	375	100

ments sugar in the sweet dough, therefore it is more resistant to high osmotic pressure than the Y-563 strain.

CONCLUSIONS

The *S. cerevisiae* recombinant strains with higher glycerol production (up to 19 g/L) have been constructed based on BY4742. The industrial ethanol-producing strain Y-563 has been used as parental one for construction of recombinant strains with up to 3.3-fold increase in the intracellular trehalose level. The resistance of obtained recombinant strains to different stress factors has been evaluated. The strain BY/TPI25/gpd1gpp2f/fps1 has shown the highest osmotolerance. The strains BY/TPI25/gpd1gpp2f, 563/TSL1, 563/TPS1/2, and 563/TPS1/2/TSL1 have shown higher viability after freeze-thaw as compared with the corresponding parental strains. The recombinant strain 563/TPS1/2/TSL1 with a high trehalose content and the corresponding initial strain Y-563 have been grown in the commercial medium in 7 L pilot fermenter and tested. The recombinant strain cells have been established to have higher activity during fermentation of sugar in the sweet dough and to longer keep their stability at 35°C. Therefore, this newly constructed strain can be used for commercial production of baker's yeast having extended shelf life and better suitability for sweet dough leavening. Also, this strain can be used as a platform for implementing other authors' offerings (for example, for metabolic engineering that increases glycerol production) and for obtaining *S. cerevisiae* with higher robustness.

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ВПЛИВ ТРЕГАЛОЗИ ТА ГЛІЦЕРИНУ НА СТІЙКІСТЬ ДО ВИСУШУВАННЯ, ЗАМОРОЖУВАННЯ-РОЗМОРОЖУВАННЯ ТА ОСМОТИЧНОГО СТРЕСУ У РЕКОМБІНАНТНИХ ШТАМІВ *SACCHAROMYCES CEREVISIAE*

Вступ. Пекарські дріжджі *Saccharomyces cerevisiae* використовують у виробництві хлібобулочних виробів, харчових та кормових добавок, алкогольній ферментації тощо. У біотехнологічних процесах клітини дріжджів зазнають дії значної кількості стресових факторів (висока концентрація цукру та етанолу, підвищена температура, висушування або заморожування тощо), що негативно впливає на їх життєздатність. Дріжджі володіють певними системами захисту від стресу, зокрема накопичення дисахариду трегалози та продукування гліцерину.

Проблематика. Посилення дії захисних систем дріжджів шляхом збільшення концентрації гліцерину або трегалози може надати більшої стресостійкості штамам *S. cerevisiae*.

Мета. Конструювання рекомбінантних штамів *S. cerevisiae* з підвищеним рівнем накопичення трегалози або продукування гліцерину та оцінка стійкості отриманих штамів до низки стресових факторів.

Матеріали й методи. Трансформацію *S. cerevisiae* здійснювали методом Li-Ac-PEG. Алкогольну ферментацію здійснювали при температурі 30 °С при перемішуванні зі швидкістю 120 об/хв.

Результати. На основі штаму *S. cerevisiae* BY4742 було сконструйовано рекомбінантні штами з підвищеним рівнем продукування гліцерину (до 19 г/л). На основі промислового штаму Y-563 як продуцента етанолу було сконструйовано рекомбінантні штами з підвищеним в 3,3 рази внутрішньоклітинним вмістом трегалози. Визначено резистентність отриманих рекомбінантних штамів до різних стресових факторів. Штам BY/TPI25/gpd1gpp2f/fps1 з найвищим рівнем продукції гліцерину виявляв найвищу осмотолерантність. Штами BY/TPI25/gpd1gpp2f, 563/TSL1, 563/TPS1/2 та 563/TPS1/2/TSL1 характеризувалися підвищеною життєздатністю після заморожування-розморожування порівняно з батьківськими штамами, проте не виявляли вищої стійкості до висушування. Рекомбінантний штам 563/TPS1/2/TSL1 з високим вмістом трегалози виявляв вищу активність при зброджуванні цукру в здобному тісті та довше зберігав життєвість при 35 °С, порівняно з батьківським штамом Y-563.

Висновки. Сконструйовані рекомбінантні штами *S. cerevisiae* можуть бути використані в промислових процесах, що супроводжуються заморожуванням-розморожуванням клітин дріжджів або високим осмотичним тиском у культуральному середовищі. Хлібопекарські дріжджі з підвищеним внутрішньоклітинним вмістом трегалози мають більш тривалий термін зберігання.

Ключові слова: пекарські дріжджі, трегалоза, гліцерол, заморожування-розморожування, висушування, осмотолерантність.

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ВЛИЯНИЕ ТРЕГАЛОЗЫ И ГЛИЦЕРИНА НА УСТОЙЧИВОСТЬ К ВЫСУШИВАНИЮ, ЗАМОРАЖИВАНИЮ-РАЗМОРАЖИВАНИЮ И ОСМОТИЧЕСКОМУ СТРЕССУ У РЕКОМБИНАНТНЫХ ШТАММОВ *SACCHAROMYCES CEREVISIAE*

Введение. Пекарские дрожжи *Saccharomyces cerevisiae* используют при изготовлении хлебобулочных изделий, пищевых и кормовых добавок, алкогольной ферментации и т.д. В биотехнологических процессах клетки дрожжей подвергаются действию значительного количества стрессовых факторов (высокая концентрация сахара и этанола, повышенная температура, высушивание или замораживание и прочие), что отрицательно влияет на их жизнеспособность. Дрожжи владеют определенными системами защиты от стресса, в частности накопление дисахарида трегалозы и продуцирование глицерина.

Проблематика. Усиление действия защитных систем дрожжей путем увеличения концентрации глицерина или трегалозы может способствовать большей стрессоустойчивости штаммов *S. cerevisiae*.

Цель. Конструирование рекомбинантных штаммов *S. cerevisiae* с повышенным уровнем накопления или продуцирования глицерина, а также оценка устойчивости полученных штаммов к ряду стрессовых факторов.

Материалы и методы. Трансформацию *S. cerevisiae* выполняли методом Li-Ac-PEG. Алкогольную ферментацию проводили при температуре 30 °С при перемешивании со скоростью 120 об/мин.

Результаты. На основании штамма *S. cerevisiae* BY4742 были сконструированы рекомбинантные штаммы с повышенным уровнем продуцирования глицерина (до 19 г/л). На основании промышленного штамма Y-563 как продуцента этанола было сконструировано рекомбинантные штаммы с повышенным в 3,3 раза внутриклеточным содержанием трегалозы. Определено резистентность полученных рекомбинантных штаммов к различным стрессовым факторам. Штамм BY/TPI25/gpd1gpp2f/fps1 с наивысшим уровнем продуцирования глицерина, имел наивысшую осмотолерантность. Штаммы BY/TPI25/gpd1gpp2f, 563/TSL1, 563/TPS1/2 и 563/TPS1/2/TSL1 имели повышенную жизнеспособность после замораживания-размораживания по сравнению с родительскими штаммами, но не проявляли большей стойкости к высушиванию. Рекомбинантный штамм 563/TPS1/2/TSL1 с высоким содержанием трегалозы проявлял более высокую активность при брожении сахара в сдобном тесте и дольше сохранял жизнеспособность при 35 °С, по сравнению с родительским штаммом Y-563.

Выводы. Сконструированные рекомбинантные штаммы *S. cerevisiae* могут использоваться в промышленных процессах, которые сопровождаются замораживанием-размораживанием клеток дрожжей или высоким осмотическим давлением в культуральной среде. Хлебопекарские дрожжи с повышенным внутриклеточным содержанием трегалозы имеют более длительный срок хранения.

Ключевые слова: пекарские дрожжи, трегалоза, глицерол, замораживание-размораживание, высушивание, осмотолерантность.