ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2016. Випуск 73. С. 197–202
Visnyk of the Lviv University. Series Biology. 2016. Issue 73. P. 197–202

УДК 576.343:582.282.23

REGULATED EXPRESSION OF *TPI1* GENE FOR IMPROVEMENT OF GLYCEROL FORMATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

M. Semkiv[1], K. Dmytruk[1], A. Sibirny[1,2*]

[1]Department of Molecular Genetics and Biotechnology
Institute of Cell Biology, NAS of Ukraine
14/16, Drahomanov St., Lviv 79005, Ukraine
[2]Department of Biotechnology and Microbiology, University of Rzeszow
2, Cwiklinskiej St., Rzeszow 35-601, Poland
e-mail: sibirny@cellbiol.lviv.ua

Increase of glycerol production during fermentation of grape musts by *Saccharomyces cerevisiae* can improve wine taste and quality. It is known, that the absence of triose phosphate isomerase (Tpi1) activity in the cells of yeasts *S. cerevisiae* leads to increase of glycerol production during alcoholic fermentation, however, it also severely impairs growth and robustness of such strains. In the present work, we describe the construction and evaluation of *S. cerevisiae* recombinant strain, characterized by oxygen regulated expression of *TPI1* gene, in particular, the high expression during aerobic growth and the low expression under conditions of anaerobic or micro-aerobic fermentation. Promoter of *TPI1* gene was substituted by oxygen and glucose-regulated promoter of *CYC1* gene, coding for cytochrome *c*. Such substitution led to substantial suppression of triose phosphate isomerase activity, when sufficient amount of glucose (2% or more) was added to the cultivation medium. Recombinant *S. cerevisiae* strain with *TPI1* gene controlled by *CYC1* promoter produced two times more glycerol as compared to the wild-type strainunder fermentation conditions.This newly constructed strain could be useful for production of wine with improved organoleptic characteristics.

Keywords: S. cerevisiae, glycerol synthesis, triose phosphate isomerase.

The flourishing area of biotechnology – wine production – uses process of fermentation of grape musts by the yeast *Saccharomyces cerevisiae*. Outcome of this process in terms of final metabolites composition has crucial effect on wine taste. For example, it was shown that enhanced content of glycerolin wine improves itssweetness, smoothness and overall mouth feel [2]. Also glycerol plays a role in the formation of the aroma profile of a wine by interaction with the various flavor compounds. The relationship between the concentration of glycerol and the perceived quality of wine is statistically significant [7]. Increase of glycerol production during alcoholic fermentation by *S. cerevisiae*can be achieved via manipulations of environmental factors, mainly aeration, pH or sulfite ions addition [10] or by genetic engineering of yeast strains which are used for wine production [5].

In the yeast *S. cerevisiae*, glycerol is synthesized from dihydroxyacetone phosphate in two consecutive reactions catalyzed by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase. But this reactions have only additional role for the *S. cerevisiae* metabolism (they are activated when NAD pool refilling is needed), and the most of the formed dihydroxyacetone phosphateis converted to glyceraldehyde-3-phosphate and, subsequently, to ethanol. *S. cerevisiae* gene *TPI1* encodes enzyme triose phosphate isomerase that catalyzes dihydroxyacetone phosphate / glyceraldehyde-3-phosphate interconversions. *S. cerevisiae* strains deleted in the *TPI1* gene showed significant increase in glycerol production ratio [8]. However, such mutants fail to grow on glucose [1], apparently due to an accumulation of toxic derivatives such as dihydroxyacetone

phosphate or methyl glyoxal [6], providing limitation for industrial applications. In fermentation processes applying *S. cerevisiae*, firstly yeasts are cultivated under aerobic conditions to accumulate sufficient amount of biomass and then aeration is switched off in part with addition of excess amounts of carbon source, starting fermentation [5]. Repression of *TPI1* gene under fermentation conditions would allow generating strains preferably producing glycerol, which could be promising for industrial applications. Such hypothesis was tested by substitution of the *TPI1* gene promoter with the *MET25* gene promoter which is repressed in the presence of methionine in the growth medium [11]. Exogenous methionine was shown to cause a drop in triose phosphate isomerase activity and increased glycerol production in the constructed recombinant strains. However, the requirement for of methionine supplementation is a drawback for application of the constructed strain for the industrial glycerol production. Cytochrome *c* (Cyc1) is an electron carrier of mitochondrial intermembrane space that transfers electrons from ubiquinone-cytochrome *c* oxidoreductase to cytochrome *c* oxidase during cellular respiration. Expression of *CYC1* gene is repressed by high glucose concentration and low oxygen supply [12]. In this work, promoter of *TPI1* gene was substituted by promoter of *CYC1* gene. Constructed strain of *S. cerevisiae* revealed sufficient activity of Tpi1 under conditions of respiratory growth (low glucose concentration, available oxygen) and reduced activity of the enzyme under conditions of alcoholic fermentation (high glucose concentration, low oxygen supply). Recombinant strain is characterized by two-fold increase of glycerol formation as compared to the wild-type strain.

Material and methods

Strains, media, growth and fermentation conditions. The *S. cerevisiae* strain BY4742 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; [3] was used for construction of recombinant strains in this work. When required, histidine (20 mg L^{-1}), leucine (60 mg L^{-1}), lysine (20 mg L^{-1}), or uracil (20 mg L^{-1}) were added.

S. cerevisiae strains were incubated at 30°C. Yeast strains were maintained in rich YPD (1% yeast extract, 1% peptone and 2% glucose), YPE (1% yeast extract, 1% peptone, 0.2% glucose, 3% ethanol) or mineral YNB (0.67%, yeast nitrogen base without amino acids, DIFCO, 0.5% ammonium sulfate, 2% glucose and growth factors – 40 mg L^{-1} histidine, 20 mg L^{-1} leucine, 20 mg L^{-1} lysine, 20 mg L^{-1} uracil) media.

For alcoholic fermentation cells of WT BY4742 strain and its recombinant derivative were pre-grown in 50 mL of YPD (1% yeast extract, 1% peptone, 2% glucose) medium in Erlenmeyer flasks (bottle size – 100 mL) during 24 hours and then after the washing with sterile water twice inoculated into the 20 mL of YNB medium with 10% glucose as Carbon source in 50 mL Erlenmeyer flasks. An initial biomass concentration of 1.2 g (dry weight)/L was used for fermentation. Fermentation was carried out at the temperature of 30°C under micro-aerobic condition with mixing (120 revolutions/min). Samples were taken daily.

The *Escherichia coli* DH5α strain (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. Strain DH5α was grown at 37 °C in LB medium. Transformed *E. coli* cells were maintained on a medium containing 100 mg L^{-1} of ampicillin.

Plasmid construction. Plasmid vector for native *TPI1* gene promoter substitution with regulated promoter of gene *CYC1* was constructed as follows. 803 bp DNA fragment corresponding to the promoter of gene *CYC1* was amplified from the genomic DNA of *S. cerevisiae* strain BY4742 using primers SM74 (CGC **GAG CTC** GTA CAG TAA ATT GAC CTG AAT ATA TC) and SM75 (CGC **GGA TCC** TAT TAA TTT AGT GTG TGT ATT TGT GTT TG), SacI/BamHI digested and cloned instead of *MET25* promoter into the corresponding sites of the plasmid pUC57-ZeoR-MET25pr-TPI1fragm [11]. The resulted vector was named pUC57-ZeoR-CYC1pr-TPI1fragm (Fig. 1).

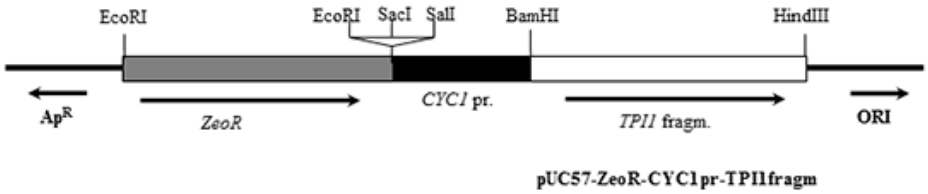

Fig. 1. Linear scheme of plasmid pUC57-ZeoR-CYC1pr-TPI1 fragm

Selection of strain with *TPI1* to *CYC1* promoter substitution. Plasmid pUC57-ZeoR-CYC1pr-TPI1 fragm was digested with HindIII endonuclease and used for transformation of *S. cerevisiae* strain BY4742. The transformants were selected on the solid YPD medium containing 200 mg/L zeocin as a selective agent and 2% glucose as the Carbon source. Recombinant strains with *TPI1* to *CYC1* promoter substitution (BY-CYC-TPI) were selected via PCR genotyping with pair of primers SM74 and SM38 (ACC GGT ACC AAT GGC CCA GAC TG). SM38 primer is homologous to the part of *TPI1* gene ORF outside from the *TPI1* fragment cloned in frame of the plasmid pUC57-ZeoR-CYC1pr-TPI1 fragm.

Biochemical methods and analyses. Cell-free extracts for triose phosphate isomerase activity measurements were prepared from 1 day cultures grown on synthetic medium with 2% glucose or 2 days cultures grown on synthetic medium with 0.2% glucose and 3% ethanol as a Carbon sources.

Triose phosphate isomerase activity was measured as described in [9].

Glycerol was measured using BioVision Free Glycerol Colorimetric/Fluorometric Assay Kit (BioVision Incorporated, USA).Concentrations of ethanol in media were determined using alcohol oxidase / peroxidase-based enzymatic kit «Alcotest» [4]. The biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. Glucose concentration was determined using the «Diagluc» assay kit (UBT, Lviv) [4]. All assay experiments were repeated at least twice.

Results and discussion

Constructed recombinant strain BY-CYC-TPI with *TPI1* to *CYC1* promoter substitution was biochemically characterized.

The Tpi1 activity was assayed in WT and BY-CYC-TPI strains cultivated in YPD or YNB medium supplemented with 2% or 10% glucose as a Carbon source. Cells growing in the medium with 2% glucose were subjected to higher level of aeration (200 rpm) to simulate conditions for biomass accumulation prior alcoholic fermentation. Cells growing in 10% glucose were mixed slower (120 rpm) to reproduce alcoholic fermentation conditions. In addition, cells harvested after two-phase growth including initial biomass accumulation phase on YPD or YNB and high-gravity fermentation phase on YNB with 10% glucose. Tpi1 activities of cells cultivated in mentioned conditions fluctuated a bit, for example, strain BY-CYC-TPI revealed slightly increased triose phosphate isomerase activity after cultivation on 2% glucose with high aeration (Fig. 2, A). In general, strain BY-CYC-TPI revealed much lower triose phosphate isomerase activity than the WT strain in all studied cases (Fig. 2, A). It was assumed that relatively low concentration of glucose (*i.e.* 2%) was sufficient to inhibit *CYC1* promoter expression level. Therefore, in our next experiments Tpi1 activity was assayed in cell-free extracts harvested from 1 day cultures grown on synthetic medium with 2% glucose or 2 days cultures grown on synthetic medium with 0.2% glucose and 3% ethanol as carbon sources. Strain BY-CYC-TPI revealed 3-fold decreased Tpi1 activity comparing to WT strain after cultivation in the medium with 2% glucose as a Carbon source, whereas Tpi1 activity after cultivation in the medium with ethanol was almost the same for both strains (Fig. 2, B).

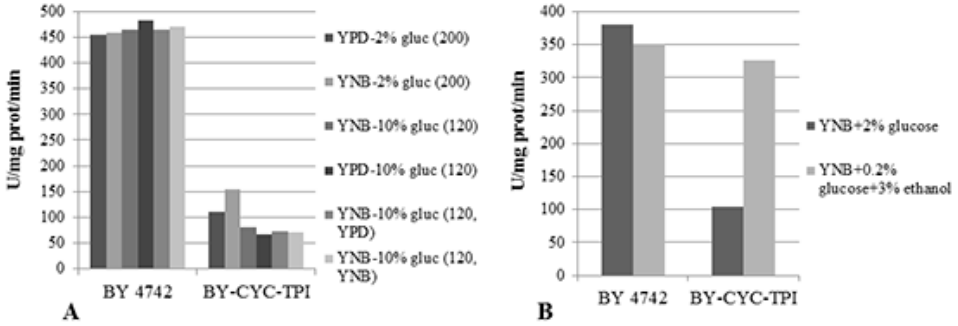

Fig. 2. A. Tpi1 in U/mg of protein/min for WT (BY4742) and recombinant (BY-CYC-TPI) strains after cultivation under different conditions (YPD or YNB with 2% glucose under 200 rpm agitation; YPD or YNB with 10% glucose under 120 rpm agitation; two-phase growth with biomass accumulation phase on YPD or YNB and fermentation phase on YNB with 10% glucose); B. Tpi1 activity of the same strains after cultivation on 2% glucose or 0.2% glucose and 3% ethanol as a Carbon sources

Alcoholic fermentation of the strains studied was performed as described in Material and Methods section. Despite reduced Tpi1 activity in BY-CYC-TPI strain cultivated in YPD medium, biomass accumulation and glucose consumption did not differed for both tested strains (Fig. 3, B, C). Glycerol production by strain BY-CYC-TPI was two-fold increased as compared to that of WT strain, reaching 5 g/L under conditions of alcoholic fermentation (Fig. 3, A). Increase of glycerol formation was achieved most likely due to reduction of Tpi1 activity.The newly constructed strain could be useful for production of wine with improved organoleptic characteristics. It could also serve as a useful platform for further increase in glycerol overproduction, based on the approaches of metabolic engineering and classical selection.

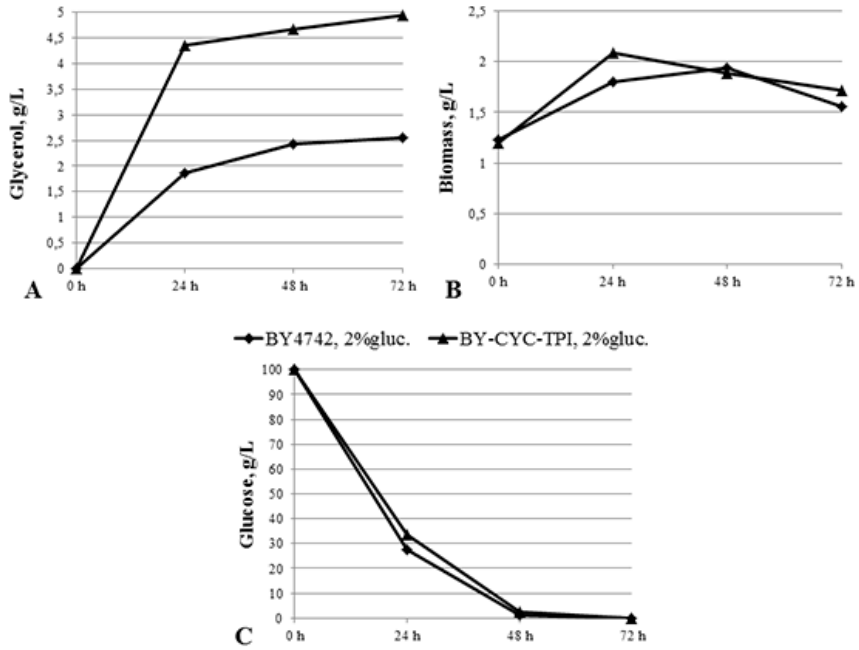

Fig. 3. Glycerol production (A), biomass accumulation (B) and glucose consumption (C) in g of biomass or metabolite per L of the fermentation medium during alcoholic fermentation by strains BY4742 and BY-CYC-TPI after pre-growth on YPD medium

Acknowledgments

This work was supported in part by National Academy of Sciences of Ukraine (Grant 35-16).

REFERENCES

1. *Compagno C., Brambilla L., Capitanio D.* et al. Alterations of the glucose metabolism in a triosephosphate isomerase-negative *Saccharomyces cerevisiae* mutant // Yeast. 2001. Vol. 18. №7. P. 663-670.
2. *Eustace R., Thornton R.J.* Selective hybridization of wine yeasts for higher yields of glycerol // Can. J. Microbiol. 1987. Vol. 33. P. 112–117.
3. *Giaever G., Chu A.M., Ni L.* et al. Functional profiling of the *Saccharomyces cerevisiae* genome // Nature. 2002. Vol.418. P.387-391.
4. *Gonchar M.V.* Sensitive method for quantitative determination of hydrogen peroxide and oxidase substrates in biological samples // Ukr. Biokhim. Zh. 1998. Vol. 70. P. 157–163.
5. *Lin Y., Tanaka S.* Ethanol fermentation from biomass resources: current state and prospects // Appl. Microbiol. Biotechnol. 2006. Vol. 69. P. 627–642.
6. *Martins A.M., Cordeiro C.A., PoncesFreire A.M.* In situ analysis of methyl glyoxal metabolism in *Saccharomyces cerevisiae* // FEBS Lett. 2001. Vol. 499. №1-2. P. 41-44.
7. *Nieuwoudt H., Prior B.A., Pretorius I.S., Bauer F.F.* Glycerol in South African table wines: an assessment of its relationship to wine quality // S. Afr. J. Enol.Vitic. 2002. Vol. 23. P. 22–30.
8. *Overkamp K.M., Bakker B.M., Kötter P. et al.* Metabolic Engineering of Glycerol Production in *Saccharomyces cerevisiae* // Appl. Environ. Microbiol. 2002. Vol. 68. №6. P. 2814-2821.
9. *Plaut B., Knowles J.R.* pH-dependence of the triose phosphate isomerase reaction //Biochem. J. 1972. Vol. 129. P. 311–320.
10. *Scanes K.T., Prior B.A., Hohmann S.* Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review // S. Afr. J. Enol. Viticult. 1998.Vol. 19. P. 17–24.
11. *Semkiv M., Dmytruk K., Sibirny A.* Impact of Triose Phosphate Isomerase Activity Modulation on Glycerol Production by the Yeast Saccharomyces cerevisiae // In: Living Organisms and Bioanalytical Approaches for Detoxification and Monitoring of Toxic Compounds: Monograph. 2015. P. 313-322.
12. *Weinhandl K., Winkler M., Glieder A., Camattari A.* Carbon source dependent promoters in yeasts // Microb. Cell Fact. 2014. Vol. 13. P. 5.

Стаття: надійшла до редакції 11.07.16
доопрацьована 29.08.16
прийнята до друку 31.08.16

РЕГУЛЬОВАНА ЕКСПРЕСІЯ ГЕНА *TPI1* ДЛЯ ПІДВИЩЕННЯ ПРОДУКЦІЇ ГЛІЦЕРИНУ У ДРІЖДЖІВ *SACCHAROMYCES CEREVISIAE*

М. Семків[1], К. Дмитрук[1], А. Сибірний[1,2*]

[1]Відділ молекулярної генетики і біотехнології
Інститут біології клітини НАН України
вул. Драгоманова, 14/16, Львів 79005, Україна
[2] Відділ біотехнології і мікробіології, Жешувський університет
вул. Zelwerowicza, 4, Жешув 35-601, Польща
e-mail:sibirny@cellbiol.lviv.ua

Підвищення продукції гліцерину під час ферментації виноградного сусла дріжджами *Saccharomyces cerevisiae* може покращувати смак і якість виготовленого

вина. Відомо, що відсутність активності ферменту тріозофосфатізомерази (Tpi1) у клітинах дріжджів *S. cerevisiae* приводить до підвищення продукції гліцерину під час спиртового бродіння, проте водночас виявляє негативний вплив на ріст і стійкість відповідних штамів. У даній роботі описано конструювання та біохімічний аналіз штаму *S. cerevisiae* з експресією гена *TPI1*, що активується під час росту при високій аерації та знижується під час ферментації в анаеробних чи напіванаеробних умовах. Промотор гена *TPI1* було заміщено на регульований глюкозою і киснем промотор гена *CYC1*, що кодує цитохром *c*. Така заміна призводила до суттєвого зниження активності тріозофосфатізомерази за умов, коли достатня кількість глюкози (2% і більше) була додана до середовища для культивування дріжджів. Отриманий рекомбінантний штам *S. cerevisiae* продукував удвічі більше гліцерину порівняно з вихідним штамом за умов ферментації. Цей штам може бути використано для виробництва вина з покращеними органолептичними характеристиками.

Ключові слова: S. cerevisiae, продукція гліцерину, тріозофосфатізомераза.

РЕГУЛИРУЕМАЯ ЭКСПРЕССИЯ ГЕНА TPI1 ДЛЯ ПОВЫШЕНИЯ ПРОДУКЦИИ ГЛИЦЕРИНА У ДРОЖЖЕЙ *SACCHAROMYCES CEREVISIAE*

М. Семкив[1], К. Дмитрук[1], А. Сибирный[1,2*]

[1]Отдел молекулярной генетики и биотехнологии
Институт биологии клетки НАН Украины
ул. Драгоманова, 14/16, Львов 79005, Украина
[2]Отдел биотехнологии и микробиологии, Жешувский университет
ул. Zelwerowicza, 4, Жешув 35-601, Польша
e-mail: sibirny@cellbiol.lviv.ua

Повышение продукции глицерина при ферментации виноградного сусла дрожжами *Saccharomyces cerevisiae* может улучшать вкус и качество изготовленного вина. Известно, что отсутствие активности фермента триозофосфатизомеразы (Tpi1) в клетках дрожжей *S. cerevisiae* приводит к повышению продукции глицерина во время спиртового брожения, но в то же время крайне негативно влияет на рост и устойчивость соответствующих штаммов. В данной работе описано конструирование и биохимический анализ штамма *S. cerevisiae* с экспрессией гена *TPI1*, которая активируется во время роста при высоком уровне аэрации и снижается при ферментации в анаэробных или полуанаэробных условиях. Промотор гена *TPI1* был замещен на регулируемый глюкозой и кислородом промотор гена *CYC1*, кодирующего цитохром *c*. Такая замена приводила к существенному угнетению активности триозофосфатизомеразы в условиях, когда достаточное количество глюкозы (2 % и более) было добавлено к среде для культивирования дрожжей. Полученный рекомбинантный штамм *S. cerevisiae* производил вдвое больше глицерина по сравнению с исходным штаммом в условиях ферментации. Этот штамм может быть использован для производства вина с улучшенными органолептическими характеристиками.

Ключевые слова: S. cerevisiae, продукция глицерина, триозофосфатизомераза.