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TRANSCRIPTIONAL FACTOR Cat8 IS INVOLVED IN REGULATION OF XYLOSE FERMENTATION IN ENGINEERED SACCHAROMYCES CEREVISIAE

Aim. The aim of this work is the construction of cat8Δ strain on the base of xylose-fermenting S. cerevisiae strain and evaluation of the xylose fermentation rate. Methods. The CAT8 deletion cassette harboring natNT2 gene flanking 5’ and 3’ non-coding regions of CAT8 gene has been constructed. After transformation by the cassette the cat8Δ strain was selected on the nourseothricin containing medium. Xylose fermentation experiments of constructed strain was performed in mineral medium supplemented with xylose under oxygen-limited conditions. Results. Xylose-fermenting cat8Δ S. cerevisiae strain has been constructed by homologous recombination of the CAT8 deletion cassette with target sequences in the genome of GS010 strain. The cat8Δ strain possessed increase in ethanol accumulation, ethanol yield, rate of ethanol production and productivity of ethanol synthesis relative to the parental GS010 strain for 9.5, 6, 20 and 12 %, respectively. Conclusions. The mutant of the xylose-fermenting S. cerevisiae strain with knock out of the CAT8 gene coding for transcriptional activator, has been constructed. The cat8Δ mutant showed 9.5 % increase in ethanol production from xylose relative to parental strain. Keywords: alcoholic fermentation, xylose, S. cerevisiae, Cat8.

Fluctuation in oil and oil products prices and the prospect of global warming are driving the development of technologies for the production of alternative liquid fuels such as ethanol [1]. Ethanol can be blended with gasoline or used as a neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization; furthermore, it is an excellent fuel for future advanced flexi-fuel hybrid vehicles.

Today biofuel industry primarily produces ethanol from a corn or sugarcane. However, this so-called first generation ethanol produced from starch and sugar, is in competition with a food and animal feed industry. In contrast, lignocellulosic biomass (crop wastes, agricultural and forestry residues, and municipal waste) offers a high potential as feedstock for biofuels, because it is the most abundant sustainable raw material worldwide and occurs as byproduct without competing uses. The following are other advantages of lignocellulose-based ethanol: higher per hectare productivities, generation low net greenhouse gas emissions, reducing environmental impacts, particularly climate change as well as possibility to provide employment in rural areas.

Studies on conversion of lignocellulosics to ethanol focused on the searching for natural microbial strains and construction of recombinants able to ferment efficiently all sugars of lignocellulosic hydrolysates. Effective alcoholic fermentation of xylose, the second abundant after glucose sugar of lignocellulose hydrolysates (consists approx. 30 % of hydrolyzate sugars) is one of the main unresolved problems.

Although there are many bacterial and yeast strains capable of naturally utilizing xylose, Saccharomyces cerevisiae has advantages over the innate xylose-utilizing microorganisms regarding robustness against various stresses in industrial environments, such as low pH, high osmotic pressure, high alcohol concentration, and phage contamination [2].

S. cerevisiae cannot naturally utilize xylose. Metabolic engineering approaches for introducing heterologous xylose utilization pathways and optimizing internal metabolisms have been undertaken to develop efficient xylose-fermenting S. cerevisiae strains.

Bacterial or fungal xylose isomerase (XI) pathway catalyzing the isomerization of xylose to xylulose, and heterologous yeast xylose reductase (XR) / xylitol dehydrogenase (XDH) pathway, where xylose is first reduced to xylitol, which is then oxidized to xylulose, have been successfully
introduced into \textit{S. cerevisiae} [3]. The main drawback of the XR/XDH pathway consisting in dual cofactor preference for XR and XDH, was minimized by protein engineering, controlling activity ratio of XR/XDH, and adding heterologous electron sink reactions [reviewed by 4]. Additional overexpression of the native \textit{S. cerevisiae} genes encoding xylulokinase (XK) and non-oxidative pentose phosphate pathway (PPP) enzymes with subsequent evolutionary engineering enabled anaerobic growth of resulted strains on xylose [5–7].

A positive effect of mutations in the \textit{PHO13} phosphatase gene on xylose fermentation rates in XI- and XR/XDH-based strains has been attributed to transcriptional upregulation of PPP-related genes [8, 9]. Unspecific phosphatase activity of Pho13 on xylulose-5-phosphate might generate a futile cycle with XK overexpression [10]. Additionally, Pho13 has been implicated in dephosphorylation of the PPP intermediate sedoheptulose-7-phosphate [6].

Xylose enters the yeast cell via hexose transporters, as it does not have specific pentose transporters. Expression of heterologous xylose/hexose transporters has been reported to improve xylose uptake rate in engineered \textit{S. cerevisiae} strains [reviewed by 11]. Also, favorable mutants of homologous hexose transporters Hxt7 (N370S) and Gal2 (N376F) have been reported [12].

Despite of intensive engineering of \textit{S. cerevisiae} strains, xylose fermentation rate still remains lower than for glucose. Therefore, xylose fermentation performance requires improvement.

\textit{S. cerevisiae} Cat8 is a Zn-cluster transcriptional activator necessary for expression of genes involved in gluconeogenesis, ethanol utilization and diauxic shift from fermentation to respiration [13, 14]. It was shown that knock out of \textit{CA78} slightly improved glucose fermentation rate in \textit{S. cerevisiae} [15]. Xylose-utilizing \textit{S. cerevisiae} strains recognize xylose as a respiratory carbon source. Xylose did not fully repress the genes encoding enzymes of the tricarboxylic acid and respiratory pathways, even under anaerobic conditions [16, 17]. As Cat8 responsible for switching from fermentation to respiration, it could be expected that deletion of the gene in the xylose-utilizing \textit{S. cerevisiae} strain will increase of xylose fermentation rate, due to shifting of respiration metabolism to fermentation one. In addition, previous investigations in our laboratory have shown that the deletion on \textit{CA78} gene in naturally xylose-fermenting yeast \textit{Ogataea polymorpha} increased ethanol production from xylose for 30–50% as compared to parental strains [18].

In this study, we report the isolation of \textit{cat8A} mutant on the background of xylose-utilizing \textit{S. cerevisiae} strain. Constructed strain possessed 20% increase in xylose fermentation rate when compared to initial strain.

**Materials and methods**

The reference \textit{S. cerevisiae} strain S288C and xylose-utilizing \textit{S. cerevisiae} strain CMB.GS010 (GS010) [17] were used through this study. Yeast cells of \textit{S. cerevisiae} were grown on YPD (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose), YPX (10 g/L yeast extract, 10 g/L peptone, 20 g/L xylose) or mineral medium (6.7 g/L YNB without amino acids, 20 g/L xylose) at 30°C. The strain \textit{Escherichia coli} DH5\textsubscript{α} (Φ80lacZΔM15, recA1, endA1, gyr96, thi-1, hsdR17(rk-,mr+), supE44, relA1, deoR, Δ(lacZYA-argF) U169) was used in the part of the experiments that required a bacterial host. The bacterial strain was grown at 37°C in LB medium as described by [19]. The recombinant \textit{E. coli} strains were grown on medium containing 0.1 g/L of ampicillin and agar (20 g/L) added to solidify the media.

Alcoholic fermentation of \textit{S. cerevisiae} was fulfilled by cultivation in liquid mineral medium at oxygen-limited conditions at 30°C. The conditions were provided by agitation at 100 rpm. 40 g/L xylose was added into the medium used for the fermentation. The cells were pre-grown in 50 mL of liquid YPX media in 150 mL Erlenmeyer flasks at 200 rpm during 24 hours with initial biomass 0.03 g/L. Then the cells were precipitated by centrifugation, washed by water and inoculated into 25 mL of the fermentation medium in 150 mL. Erlenmeyer flasks were covered with cotton plugs. The initial biomass concentration for fermentation experiments was 0.3 g/L. Fermentations were repeated at least in three independent experiments, each performed in triplicate. The bars in the figures indicate the ranges of the standard deviation.

Standard cloning techniques were carried out as described [19]. Genomic DNA of \textit{S. cerevisiae} was isolated using the Wizard\textsuperscript{®} Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases and DNA ligase (Fermentas, Vilnius, Lithuania) were used according to the manufacturer specifications. Plasmid isolation from \textit{E. coli} was performed with the Wizard\textsuperscript{®} Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). DNA fragments were separated on a 0.8 % agarose (Fisher Scientific, Fair Lawn, NJ, USA) gel. Isolation of fragments
from the gel was carried out with a DNA Gel Extraction Kit (Millipore, Bedford, MA, USA). PCR-amplification of the fragments of interest was done with Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) according to the manufacturer specification. PCRs were performed in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Transformation of the yeast S. cerevisiae was carried out as described previously [20].

The biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. Concentrations of xylose and ethanol from fermentation in medium broth were 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 H₂SO₄ was used at a flow rate 0.6 mL/min and the column temperature was 35°C. Experiments were performed at least twice.

Results and discussion
Genomic DNA of S. cerevisiae strain S288C strain was used as a template for isolation of 5’ and 3’ non-coding regions of CAT8 gene by PCR amplifications using primers Ko776 (CCG GAA TTC TGA ATG TGA AAA GGA TCA GAG GCA G) / Ko777 (CAG ATA CAT TAT CTG TGT TGG AAC GGA TCC ATT ATT TGC CAT AAT TTT GTG TCT TG) and Ko778 (CAA GAC ACA AAA TTA TGG CAA ATA ATG GAT CCG TTC CAA CAC AGA TAA TGT ATC TG) and Ko787 (CCC AAG CTT TCA TTT GTC ACT TGA GGT GAT TFG). The resulted 5’CAT8 (762 bp) and 3’CAT8 (922 bp) fragments were combined by overlap PCR with primers Ko776 / Ko779, EcoRI/HindIII double digested and cloned into corresponding sites of vector pUC57 (Fermentas, Vilnius, Lithuania). The resulted recombinant plasmid was named pUC57-Sc_cat8Δ. Gene natNT2 (1318 bp) conferring resistance to nourseothricin was amplified using vector pRS41N [21] as a template and primers OK19 (CCC AAG CTT GGC GCG CCA GAT CTA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT CTT AAC TAT GCG GCA TCA GAG) / OK20 (CCC AAG CTT GGC GCG CCA GAT CTA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT CCG AGA TTC ATC AAC TCA TTT C). Obtained fragment was BglII-digested and subcloned into BamHI-linearized plasmid pUC57-Sc_cat8Δ. As a result of further genetic manipulations recombinant plasmid pUC57-Sc_cat8Δ-natNT2 was constructed (Fig. 1). After that, deletion cassette was EcoRI/HindIII cut out from plasmid pUC57-Sc_cat8Δ-natNT2 and transformed into S. cerevisiae strain CMB.GS010. Transformants were selected on the solid YPD medium supplemented with 0.1 g/L of nourseothricin after three days of incubation at 30°C. Obtained transformants were examined by PCR using genomic DNA of recombinant strains as a template. Transformants with confirmed deletion of CAT8 were stabilized by altering cultivation in nonselective and selective media and once again examined by PCR. Fragments with predicted size 977 bp and 1078 bp were amplified using pairs of primers (Ko780 (ACA AAC ACA TTG ACA CAT TC) / Ko781 (GAT GCA AAT GAT TAT ACA TG)) and Ko782 (CTC TTA TTG ACC ACA CCT C) / Ko783 (CAA AGG AAC TGA AGT AAG TG)) homologous to the sequence of selective marker and regions outside from the 5’ and 3’ fragments used for recombination, respectively (Fig. 1).

Xylose fermentation of the isolated cat8Δ strain was studied under oxygen-limited conditions (see “Materials and methods” section). It was found that defects of CAT8 gene leads to 9.5 % increase in ethanol accumulation versus parental strain reaching 6.45 g/L (Fig. 2; Table). Data of Table show that the strain cat8Δ possessed increase in ethanol yield, rate of ethanol production and productivity of ethanol synthesis relative to the parental GS010 strain for 6, 20 and 12 %, respectively. Increased ethanol production from xylose was accompanied by decreased xylitol accumulation, while difference in amount of other byproducts of alcoholic fermentation such as glycerol and acetate was insignificant (Fig. 2). More probably, the increase of xylose fermentation rate is connected with restriction of a respiratory metabolism in cat8Δ strain. Cell respiration of cat8Δ cells in xylose-containing media will be studied.

Thus, obtained results supported our hypothesis that Cat8 transcription factor is involved in the regulation of xylose alcoholic fermentation in engineered xylose-fermenting S. cerevisiae strain and the deficiency of this protein activates ethanol production from xylose. These results are in good agreement with our previous investigations that postulated involvement of Cat8 in regulation of xylose alcoholic fermentation in naturally xylose-fermenting yeast O. polymorpha [18].
Fig. 1. A) scheme of plasmid pUC57-Se_cat8Δ-natNT2; B) scheme of CAT8 deletion cassette for S. cerevisiae; C) PCR verification of the correct cassette integration into genome of the GS010 using primers Ko780/Ko781 or Ko782/Ko783 and genomic DNA of constructed deletion strain cat8Δ of S. cerevisiae as a template; L – 1kb ladder.

Fig. 2. Time courses of biomass accumulation, ethanol, glycerol, acetate, xylitol production and xylose consumption by S. cerevisiae cat8Δ and GS010 during xylose fermentation. Represented data of typical fermentation experiment.

Table. Main parameters of xylose fermentation the S. cerevisiae GS010 and cat8Δ strains under oxygen-limited conditions at 30°C

| Strain  | Ethanol (g/L)* | Ethanol yield (g/g consumed xylose)** | Rate of ethanol production (g/g biomass/h)** | Productivity of ethanol synthesis (g/L/h)** |
|---------|----------------|-------------------------------------|---------------------------------------------|---------------------------------------------|
| GS010   | 5.89±0.17      | 0.240±0.004                         | 0.203±0.008                                 | 0.197±0.005                                 |
| cat8Δ   | 6.45±0.18      | 0.254±0.005                         | 0.244±0.009                                 | 0.221±0.006                                 |

Notes: * – Data are represented on the 41 h of fermentation; ** – 16 h of fermentation.
Transcriptional factor Cat8 is involved in regulation of xylose fermentation in engineered Saccharomyces cerevisiae

Conclusions
The mutant of the xylose-fermenting S. cerevisiae strain with knock out of the CAT8 gene coding for transcriptional activator, has been constructed. The cat8Δ mutant showed 9.5% increase in ethanol production from xylose relative to parental strain.

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ТРАНСКРИПЦІЙНИЙ ФАКТОР Cat8 ЗАЛУЧЕНИЙ В РЕГУЛЯЦІЮ ФЕРМЕНТАЦІЇ КСИЛОЗИ В РЕКОМБІНАНТНОМУ ШТАМІ SACCHAROMYCES CEREVISIAE

Мета. Мета даної роботи полягає в конструюванні cat8Δ на основі ксилозоферментуючого штаму S. cerevisiae та аналіз алкогольної ферментації ксилози. 

Методи. Було сконструйовано касету для делеції гена CAT8, що містить маркерний ген natNT2, флANKований 5’ та 3’ некодуючими ділянками цільового гена. Штам cat8Δ було відібрано після трансформації сконструйованою касетою на середовищі з антибіотиком норзеотрицином. Алкогольну ферментацію проводили на мінеральному середовищі з додаванням ксилози за умов обмеженої аератації. 

Результати. Ксилозоферментуючий cat8Δ штам S. cerevisiae було сконструйовано за допомогою гомологічної рекомбінації делеційної CAT8 касети з цільовими послідовностями геному штаму GS010. У порівнянні з батьківським штамом, cat8Δ характеризувався підвищеною продукцією етанолу, виходом етанолу, питомою продукцією та продуктивністю етанолу на 9,5; 6; 20 та 12 %, відповідно. 

Висновки. Було сконструйовано ксилозоферментуючий мутант S. cerevisiae з делецією гена CAT8, що кодує транскрипційний активатор. Штам cat8Δ характеризувався підвищеною на 9,5 % продукцією етанолу з ксилози у порівнянні з батьківським штамом.

Ключові слова: алкогольна ферментація, ксилоза, S. cerevisiae, Cat8.

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