Antonie van Leeuwenhoek (2021) 114:1373–1385
https://doi.org/10.1007/s10482-021-01607-6

ORIGINAL PAPER

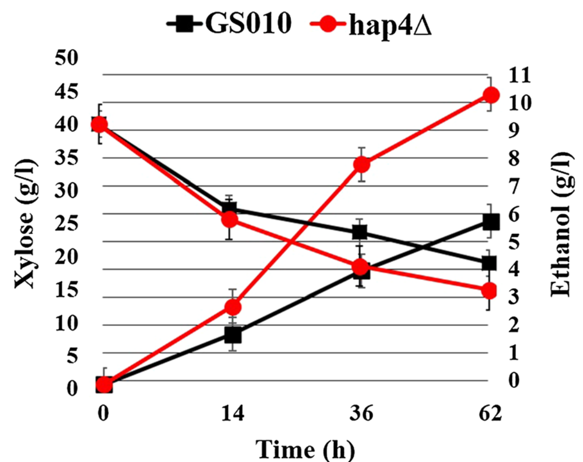
The impact of transcription factors Znf1, Sip4, Adr1, Tup1, and Hap4 on xylose alcoholic fermentation in the engineered yeast *Saccharomyces cerevisiae*

Ljubov Dzanaeva · Barbara Kruk · Justyna Ruchala · Andriy Sibirny · Kostyantyn Dmytruk

Received: 9 February 2021 / Accepted: 18 June 2021 / Published online: 25 June 2021
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2021

Abstract Lignocellulosic biomass is an attractive sustainable platform for fuel ethanol production. Xylose is a second after glucose most abounded sugar in lignocellulosic hydrolysates. Effective conversion of xylose to ethanol is one of key prerequisite for the development of an efficient conversion of biomass to ethanol. Engineered *Saccharomyces cerevisiae* strains are able to xylose fermentation. However, the yield and productivities of xylose fermentation remains lower in comparison with glucose fermentation. In this work, we studied impact of transcription factors Znf1, Sip4, Adr1, Tup1, and Hap4 on xylose catabolism. We have isolated *znf1Δ*, *adr1Δ, tup1Δ* and *hap4Δ* mutants, and strains overexpressing *SIP4, ADR1* and *HAP4* genes on the background of xylose-fermenting strain of *S. cerevisiae* aiming to explore involvement of these transcription factors in regulation of xylose growth and fermentation. It was shown that *hap4Δ* reveal 1.8-fold increase of ethanol production from xylose as compared to that of parental strain. The *hap4Δ* mutant accumulates 10.38 g l^{-1} of ethanol with an overall ethanol yield reaching 0.41 g g^{-1} of consumed xylose. While the other constructed strains revealed a decrease in ethanol production from this pentose.

Graphical Abstract



Keywords Alcoholic fermentation · *S. cerevisiae* · Transcription factors · Xylose

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10482-021-01607-6.

L. Dzanaeva · A. Sibirny · K. Dmytruk (✉)
Department of Molecular Genetics and Biotechnology, Institute of Cell Biology, NAS of Ukraine, Drahomanova Street, 14/16, Lviv 79005, Ukraine
e-mail: dmytruk77@gmail.com

B. Kruk · J. Ruchala · A. Sibirny
Department of Biotechnology and Microbiology, University of Rzeszow, Cwiklinskiej 2, 35-601 Rzeszow , Poland

Introduction

An intensive search for alternative energy sources in the world is conditioned by reduction of crude oil reserves, fluctuations in its price and dependence on energy suppliers. Despite current global drop in oil price and hence in price gasoline, bioethanol remains the most dominant biofuel employed in the transportation sector. According to the Renewable Fuels Association, global fuel ethanol production hit a new record of 29 billion gallons in 2019 with tendency to further increase (https://ethanolrfa.org/statistics/annual-ethanol-production/). Ethanol integrates several advantages: high octane number, ability to mix with gasoline, relatively low prime cost, reduction greenhouse gas emissions compared to conventional gasoline and other (Wang et al. 2007).

Today fuel ethanol is mainly produced from corn and sugarcane, racing competition with starch and sucrose as a food resources. Non-edible lignocellulosic biomass (mainly agricultural and industrial by-products) is an alternative, potentially valuable feedstock for fuel ethanol production. The pentose sugar xylose is the second most abundant monosaccharide in nature following glucose. Xylose is the main pentose in lignocellulosic hydrolysates containing around 30% of sugars released. Yeast *Saccharomyces cerevisiae* is predominant organism for ethanol production due to robustness under harsh industrial conditions. However, the great majority of *S. cerevisiae* wild-type strains cannot utilize xylose owing to lacks pentose assimilation pathways. Genetic engineering approaches have been applied to introduce heterologous xylose utilization pathways and rational metabolic engineering and evolutionary engineering have been performed to develop xylose-fermenting *S. cerevisiae* strains (reviewed by Kwak and Jin 2017; Moysés et al. 2016). Even though significant progress has been made in engineering of *S. cerevisiae* strains, xylose fermentation rate remains lower than for glucose. Thus, study the mechanisms of regulation of xylose utilization and fermentation in engineered *S. cerevisiae* strains is required.

Transcription factors play a central role in the regulation of gene expression via interaction with promoters of corresponding target genes. More than 200 transcription factors have been identified in yeast (Harbison et al. 2004). Transcription factors are an attractive target for studying the regulation of catabolism of artificial substrates. For instance, deletion of general corepressor Cyc8 increased uptake rates of xylose in the presence of glucose in *S. cerevisiae* due to upregulation of panel of HXTs transporters (Nijland et al. 2017). Acetic acid-responsive transcriptional activator, Haa1, was successfully overexpressed to increase xylose fermentation in the presence of acetic acid (Sakihama et al. 2015; Cunha et al. 2018). Overexpression of transcription factor Sut1 and deletion of Hap4 improved cellobiose fermentation in recombinant *S. cerevisiae* (Lin et al. 2014a, b). Introduction of the mutations to the general transcription factor Spt15 improved growth of recombinant *S. cerevisiae* on the corn cob acid hydrolysate (Liu et al. 2011).

Several transcription factors that could potentially be involved in the regulation of xylose alcoholic fermentation by engineered strain of *S. cerevisiae* were selected for the study. Znf1 is involved in regulation of cellular respiration, gluconeogenesis, tricarboxylic acid cycle, glyoxylate shunt; Sip4 activates the transcription of gluconeogenesis genes; Adr1 activates expression of genes involved in peroxisome organization, ethanol, glycerol, and fatty acid utilization; Tup1 is general repressor of transcription; Hap4 is global regulator of respiratory gene expression, are among them. The impact of Znf1, Sip4, Adr1, Tup1 on xylose alcoholic fermentation was not studied. We have isolated *znf1Δ*, *adr1Δ*, *tup1Δ* and *hap4Δ* mutants, and strains overexpressing *SIP4, ADR1* and *HAP4* genes on the background of xylose-fermenting strain of *S. cerevisiae* aiming to explore involvement of these transcription factors in regulation of xylose growth and fermentation. It was shown that *hap4Δ* reveal 1.8-fold increase of ethanol production from xylose as compared to that of parental strain. While the other constructed strains revealed a decrease in ethanol production from this pentose.

Materials and methods

Strains and cultivation conditions

The reference *S. cerevisiae* strain S288C and engineered xylose-utilizing *S. cerevisiae* strain CMB.GS010 (GS010) (Scalcinati et al. 2012) were used through this study. Yeast cells of *S. cerevisiae* were cultured on YPD (10 g yeast extract l^{-1}, 10 g

peptone l^{-1}, 20 g glucose l^{-1}), YPX (10 g yeast extract l^{-1}, 10 g peptone l^{-1}, 20 g xylose l^{-1}) or mineral medium (6.7 g YNB without amino acids l^{-1}, 20 g xylose l^{-1}) at 30 °C. The strain *Escherichia coli* DH5α (Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(rK$^-$, mK +), *supE*44, *relA*1, *deoR*, Δ(*lac-ZYA-argF*) U169) was used as a host for plasmid propagation. The bacterial strain was grown at 37 °C in LB medium as described by (Sambrook et al. 1989). The transformed *E. coli* cells were grown on medium containing 0.1 g ampicillin l^{-1}.

Alcoholic fermentation of *S. cerevisiae* was performed as described previously (Dzanaeva et al. 2020a) with slight modifications. The cells were pre-grown in 50 ml of liquid YPX or YPD media in 100 ml Erlenmeyer flasks at 220 rpm during 24 h with initial biomass 0.03 g l^{-1}. Then the cells were precipitated by centrifugation, washed by water and inoculated into 25 ml of the YNB medium supplemented with 40 g l^{-1} xylose or 80 g l^{-1} glucose in 50 ml Erlenmeyer flasks covered with cotton plugs. The initial biomass concentrations for xylose and glucose fermentation experiments were 0.3 and 0.1 g l^{-1}, respectively. Fermentations were performed at 30 °C and 100 rpm. Alternatively, aerobic alcoholic fermentation was performed as described by (Matsushika and Hoshino 2015). 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.

Molecular biology techniques

Standard cloning techniques were carried out as described (Sambrook et al. 1989). Genomic DNA of *S. cerevisiae* was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases, DNA ligase (Fermentas, Vilnius, Lithuania) and Gibson Assembly master mix (New England Biolabs, Warsaw, Poland) were used according to the manufacturer specifications. Plasmid isolation from *E. coli* was performed with the Wizard® 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 Phusion High-Fidelity DNA Polymerase (Thermo Scientific, 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 (Gietz and Woods 2002). Yeast transformants were selected on the solid YPX medium supplemented with 0.2 g geneticin l^{-1}.

Plasmids and strains construction

For knock out of *ZNF1*, *ADR1*, *TUP1* and *HAP4* genes two linear fragments with a homologous overlap region were used (Wenning et al. 2017). The first linear fragment contained a 50 first nucleotides of the target gene ORF and part of the *KanMX4* marker. First fragment was amplified from deletion cassette 1 using Ko865/Ko808 (*ZNF1*); Ko861/Ko808 (*ADR1*); Ko930/Ko808 (*TUP1*) and Ko853/Ko808 (*HAP4*) pair of primers (Sequences of all primers represented in Table S1, Fig. 1a). The second linear fragment contained part of the *KanMX4* marker, the Cre recombinase gene under control of the *S. cerevisiae GAL1* promoter and a 50 last nucleotides of the target gene ORF was amplified from deletion cassette 2 with a pair of primers Ko866/Ko809 (*ZNF1*); Ko862/Ko809 (*ADR1*); Ko931/Ko809 (*TUP1*) and Ko854/Ko809 (*HAP4*) (Fig. 1a). Both fragments were co-transformed into GS010. Selected strains *znf1Δ; adr1Δ; tup1Δ* and *hap4Δ* were examined by PCR. Fragments with predicted size were amplified using pairs of primers Ko867/Ko811 (601 bp) and Ko873/Ko868 (420 bp) for *znf1Δ*; Ko863/Ko811 (643 bp) and Ko873/Ko864 (420 bp) for *adr1Δ*; Ko932/Ko811 (640 bp) and Ko873/Ko933 (450 bp not shown) for *tup1Δ*; Ko855/Ko811 (613 bp) and Ko873/Ko856 (420 bp) for *hap4Δ* homologous to the sequence of *KanMX4* marker and the Cre recombinase and regions outside from the 5′ and 3′ parts from the target gene ORF used for recombination, respectively (Fig. 1b).

The *HAP4* ORF with own terminator was amplified with primers Ko912/Ko913 from the genomic DNA of *S. cerevisiae* S288C. After that, *HAP4* gene was NdeI digested and cloned into NdeI treated, gel purified and dephosphorylated vector pX-2-Lox-KanMX-pADH1-ZNF1 (Dzanaeva et al. 2020b) instead of *ZNF1* ORF with own terminator. As a result, recombinant plasmid pADH1-HAP4 was constructed (Fig. 2a). Genomic

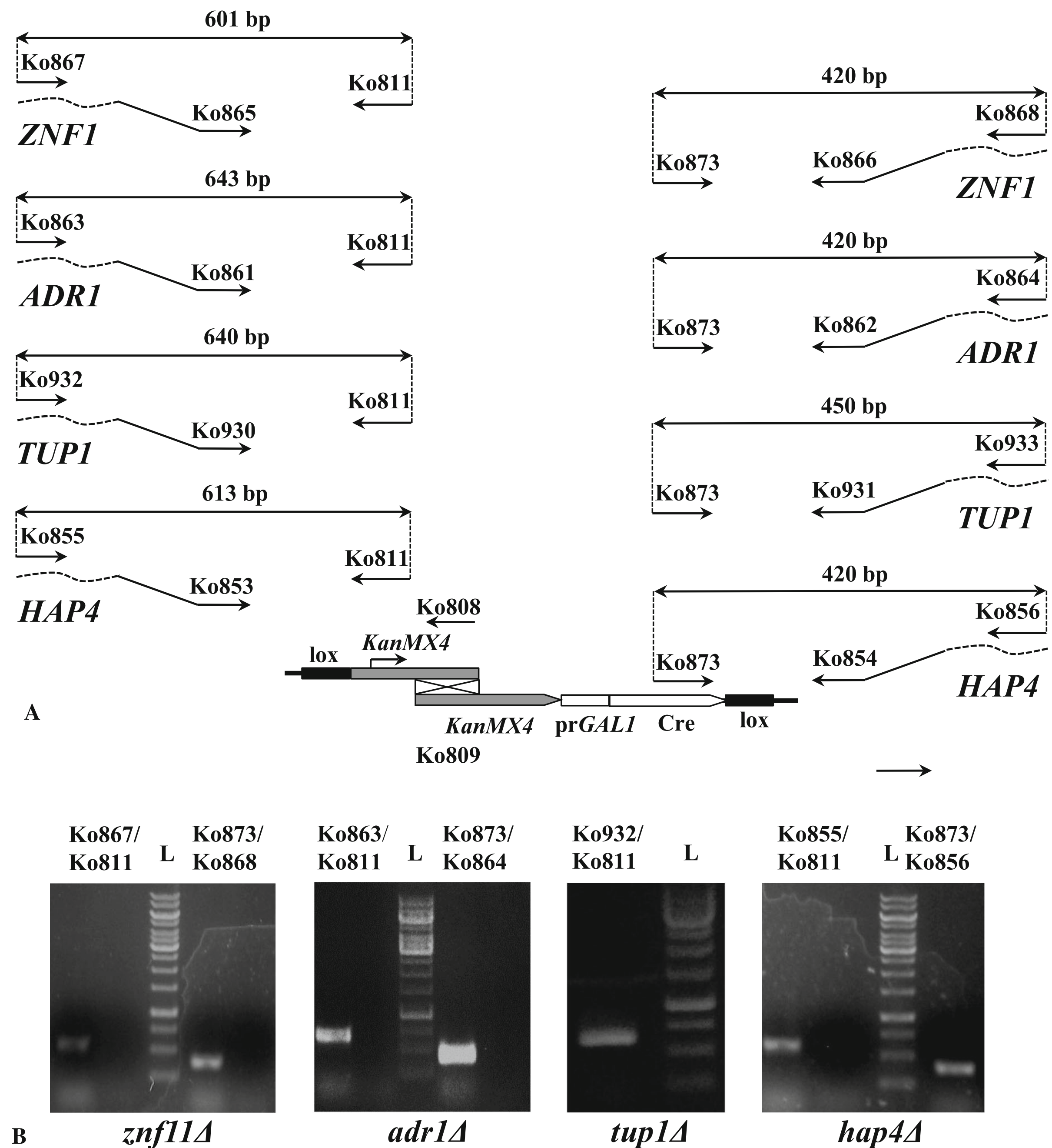


Fig. 1 **a** Scheme of two component *ZNF1*, *TUP1, ADR1* and *HAP4* deletion cassette integrated into the genome of *S. cerevisiae* with primers for PCR verification of the correct cassette integration, lox sequences indicated by black boxes, the Cre recombinase under control of the *GAL1* promoter indicated by white narrow. **b** PCR products with pairs of primers (Ko867/ Ko811 and Ko873/Ko868 and genomic DNA of *znf1Δ* strain as a template); (Ko863/Ko811 and Ko873/Ko864 and genomic DNA of *adr1Δ* strain as a template); (Ko932/Ko811 and genomic DNA of *tup1Δ* strain as a template) and (Ko855/Ko811 and Ko873/Ko856 and genomic DNA of *hap4Δ* strain as a template) confirming correct deletion of the genes *ZNF1*; *ADR1*; *TUP1* and *HAP4*; L–1 kb ladder

DNA of *S. cerevisiae* S288C strain was used as template for isolation of *SIP4* and *ADR1* ORF's with own terminators by PCR amplifications using primers Ko922/Ko923 and Ko927/Ko928 respectively. Backbone plasmid was amplified with primers Ko924/Ko925 from the plasmid pADH1-HAP4. Fragments were then Gibson Assembled generating plasmids pADH1-SIP4; pADH1-ADR1 (Fig. 2a). All constructed plasmids were validated by sequencing. Constructed plasmid with the *ADH1* promotor driving expression of *SIP4, ADR1* and *HAP4* were digested with NotI and integrated into the neutral locus on chromosome X of GS010. Transformants were selected on the solid YPX medium supplemented with 0.2 g l^{-1} of geneticin. Selected strains SIP4, ADR1 and HAP4 were confirmed by PCR using pair of primers Ko914/Ko926, Ko914/Ko929 and Ko914/Ko915, respectively (Fig. 2b). Overexpression of *SIP4, ADR1* and *HAP4* was proved by qRT-PCR. It was found that indeed, the analyzed strains SIP4, ADR1 and HAP4 showed increase in expression of corresponding genes for 3.65, 2.70 and 1.75 times, respectively (Table S2). Constructed strains were subjected for further analysis.

Quantitative real-time PCR (qRT-PCR)

Expression of the *ICL1*, *ACO1*, *MAE1*, *FBP1*, *PCK1*, *CIT1*, *FUM1*, *MDH1*, *PYC1*, *ADH1*, *PDC1*, *TAL1*, *TKL1*, *RKI1* and *RPE1* genes was analysed by qRT-PCR. Total RNA was extracted from yeast cells using the GeneMATRIX Universal RNA Purification Kit with DNAseI (EURx Ltd., Gdansk, Poland). The qRT-PCR was performed by 7500 Fast Real-Time PCR System (The Applied Biosystems, USA) with SG OneStep qRT-PCR kit (EURx Ltd., Gdansk, Poland) using gene-specific pairs of primers (Table S1), RNA as a template and ROX reference passive dye according to the manufacturer's instructions as previously described (Ruchala et al. 2017). Sequences of tested genes were taken from *S. cerevisiae* genome database (www.yeastgenome.org).

Analyses

The biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 600 nm; cuvette, 10 mm) with gravimetric calibration. Concentrations of analytes from fermentation in medium broth were analysed by HPLC (PerkinElmer, Series 2000, USA) with an Aminex HPX-87H ion-exchange

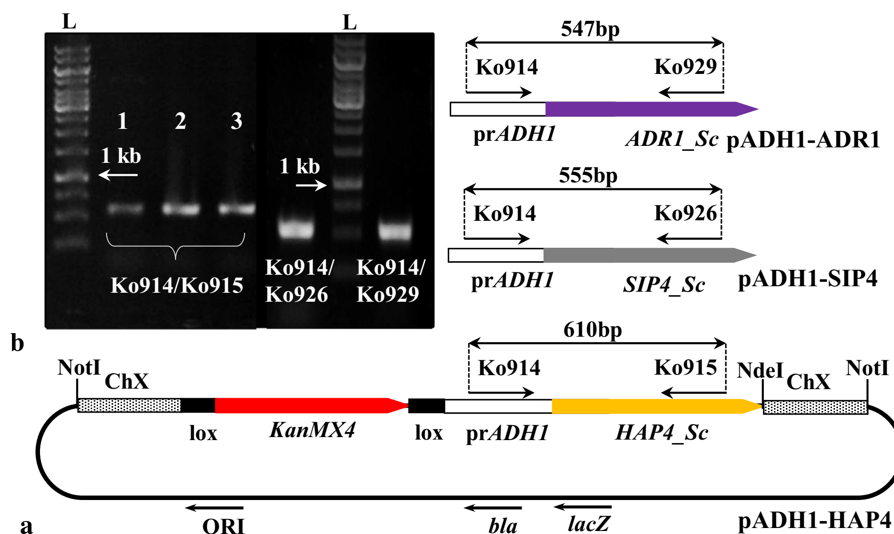


Fig. 2 **a** Circular schemes of plasmids pADH1-HAP4; pADH1-SIP4; pADH1-ADR1, (*KanMX4*–gene conferring resistance to geneticin), lox sequences indicated by black boxes, *HAP4*; *SIP4*; *ADR1* under control of the *TEF1* promoter indicated by orange; gray; violet and white boxes respectively, ChX—neutral locus on chromosome X for integration indicated by dotted boxes, origin of replication (ORI) and ampicillin resistance gene (bla) are shown as arrows. **b** PCR verification of HAP4 strain using pair of primers Ko914/Ko915 (Line 1; 2; 3); SIP4—Ko914/Ko926 (Lines 4); ADR1—Ko914/Ko929 (Line 5); L–1 kb ladder

column (Bio-Rad, Hercules, USA). A mobile phase of 4 mM H_2SO_4 was used at a flow rate 0.6 ml/min and the column temperature was 35 °C. Experiments were performed at least twice.

Statistical analysis

All the experimental data shown in this manuscript were collected from three independent samples to ensure reproducibility of the trends and relationships observed in the cultures. Each error bar indicates the standard deviation (SD) from the mean obtained from triplicate samples. The significance level 5% were used in the statistical analyses.

Results and discussion

Deletion strains *znf1Δ*, *adr1Δ, tup1Δ, hap4Δ* and strains overexpressing *SIP4*, *ADR1* and *HAP4* were constructed on the background of xylose-fermenting strain of *S. cerevisiae* GS010. Constructed strains were subjected to the growth and fermentation experiments.

The growth of *znf1Δ* mutant was tested on xylose containing medium. We observed no difference in growth between *znf1Δ* and parental strain during aerobic flask cultivation (Fig. 3a), however the mutant revealed slight increase in biomass accumulation according to the results of growth drop test (Fig. 3b). Deletion of *ZNF1* did not affect ethanol production from xylose (Fig. 4, Table 1) and glucose (Fig. 5). Mutant *znf1Δ* revealed twofold reduction of *ICL1* expression, while the expression of other tested genes remains on the similar level as that of parental strain (Table S3). It is important to note that overexpression of *ZNF1* on the background of GS010 also did not affect both xylose and glucose fermentation (Dzanaeva et al. 2020b).

Znf1 belongs to transcription activator from the zinc cluster regulator super-family (MacPherson et al. 2006; Soontorngun 2017). Znf1 regulates expression of genes, involved in gluconeogenesis, the glyoxylate shunt, the TCA cycle (Tangsombatvichit et al. 2015), glycolysis, pyruvate metabolism, and alcoholic fermentation (Songdech et al. 2020). It was shown that overexpression of *ZNF1* under control of strong constitutive *TPI1* promoter increased ethanol production by 14–24% under fermentation condition with high glucose concentration (Songdech et al. 2020), more probably due to involvement in tolerance to osmotic stress. In our experiments, we did not noticed impact of *ZNF1* deletion on xylose and glucose alcoholic fermentation in engineered xylose metabolizing strain of *S. cerevisiae*.

Sip4 is zinc cluster protein mediating reprogramming of gluconeogenic gene expression. Strain overexpressing gene *SIP4* possessed insignificant decrease in biomass accumulation during aerobic flask cultivation, drop test on plates and fermentation cultivation in xylose containing medium (Figs. 3a and b and 4). Ethanol production by SIP4 strain was 2.1-fold reduced as compared to that of parental strain (Fig. 4, Table 1). Ethanol yield decrease was less pronounced (1.6-fold) since SIP4 consumed less xylose than GS010 (16.4 g l^{-1} for SIP4 versus 21 g l^{-1} for GS010). The SIP4 produced 1.3-fold less glycerol and 1.25-fold more xylitol than GS010 (Fig. 4), while acetate production remained on the same level. Decrease of ethanol production correlated with reduced substrate consumption and increased accumulation of xylitol. Expressions of *ACO1* and *FBP1* were increased in 1.9- and 3.2-fold while the expression of other genes altered insignificantly (Table S3). Activation the expression of TCA gene *ACO1* and gluconeogenic gene *FBP1* can somehow explain decrease in ethanol production due to xylose redirection to respiratory metabolism and gluconeogenesis. At the same time, no difference in biomass accumulation and ethanol production was detected between SIP4 and parental strain during glucose fermentation (Fig. 5). Deletion of *SIP4* described in our previous work (Dzanaeva et al. 2020b) also did not affect glucose fermentation. The *sip4Δ* possessed 1.4-fold decrease in ethanol production from xylose (Dzanaeva et al. 2020b), while overexpression of the gene resulted in more pronounced (2.1-fold) reduction of ethanol production during xylose alcoholic fermentation.

The role of transcription activator Adr1 on xylose metabolism and fermentation was studied. Biomass accumulation by *adr1Δ* was slightly reduced according to the results of aerobic cultivation and growth drop test on xylose (Fig. 3a and b). The *adr1Δ* displayed decreased growth on glycerol (Fig. 3c). Overexpression of *ADR1* did not affect growth on both xylose and glycerol containing media (Fig. 3a–c). During xylose fermentation, the biomass for *adr1Δ* and ADR1 did not differ from the parental strain

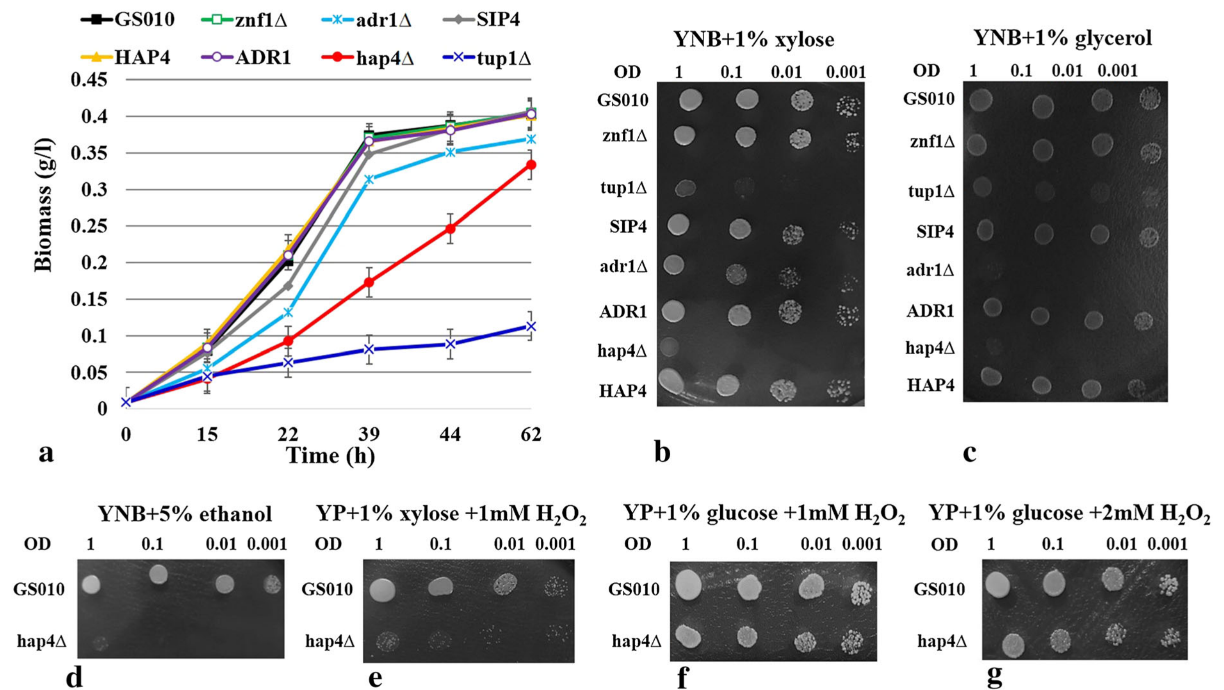


Fig. 3 **a** Time course of biomass accumulation by *S. cerevisiae* GS010, *znf1Δ*, SIP4, *adr1Δ*, ADR1, *tup1Δ*, *hap4Δ* and HAP4 strains during aerobic cultivation (200 rpm) on YNB medium with xylose (1%), initial biomass 0.009 g l^{-1} at 30 °C. Data are shown as mean of three independent experiments. **b** Growth of the strains on YNB medium with xylose (1%) on the third day of cultivation. **c** Growth of the strains on YNB medium with glycerol (1%) on the third day of cultivation. **d** Growth of the strains on YNB medium with ethanol (5%) on the fifth day of cultivation. **e** Growth of the strains on YP medium with xylose (1%) and 1 mM H_2O_2, glucose (1%) and 1 mM H_2O_2 **f**, glucose (1%) and 2 mM H_2O_2 **g** on the third day of cultivation

(Fig. 4). Ethanol production from xylose by *adr1Δ* and ADR1 was 1.3- and 1.4-fold decreased relative to the parental strain (Fig. 4). Decrease in ethanol production is concomitant with decrease in xylose consumption; since both strains consumed 1.3-fold less substrate as compared to that of parental strain (Fig. 4). The *adr1Δ* and ADR1 possessed slight decrease in glycerol production, while *adr1Δ* and ADR1 produced 1.4- and 1.15-fold higher amount of acetate and xylitol, respectively (Fig. 4). Glucose fermentation of the *adr1Δ* strains was the same as in the parental strain, while ethanol production by ADR1 was 1.1-fold reduced as compared to that of parental strain (Fig. 5). The *adr1Δ* revealed 2.4-time increase in expression of *FBP1* suggesting regulatory effect on gluconeogenesis (Table S3). We suppose that activation of key enzyme of gluconeogenesis Fbp1 may reduce ethanol production.

Despite slight increase of ethanol production from glucose reach medium in double deletion strain *adr1Δ cat8Δ* of sake yeast, single deletion of *ADR1* gene did not improve glucose fermentation (Watanabe et al. 2013) similar to that described in work presented here. In contrast to that, both deletion and overexpression of *ADR1* gene significantly decrease ethanol production from xylose.

Tup1 is general transcription repressor. Tup1 is involved in repression of gene families responsible for DNA damage repair, mating type, stress response, glucose utilization and other (Malavé and Dent 2006). We studied the role of Tup1 in xylose and glucose alcoholic fermentation. Biomass accumulation for *tup1Δ* was 3.6-fold reduced under condition of full aeration in xylose-containing medium (Fig. 3a). The *tup1Δ* mutant was impaired in growth on xylose and glycerol containing media according to the drop test analysis (Fig. 3b and c). The ethanol production from xylose was 1.6-fold decreased (Table 1), while xylitol production revealed 1.7-fold increase as compared to parental strain (Fig. 4). Biomass for *tup1Δ* was 1.2-fold decreased during xylose fermentation, which is consistent with slight decrease in xylose consumption

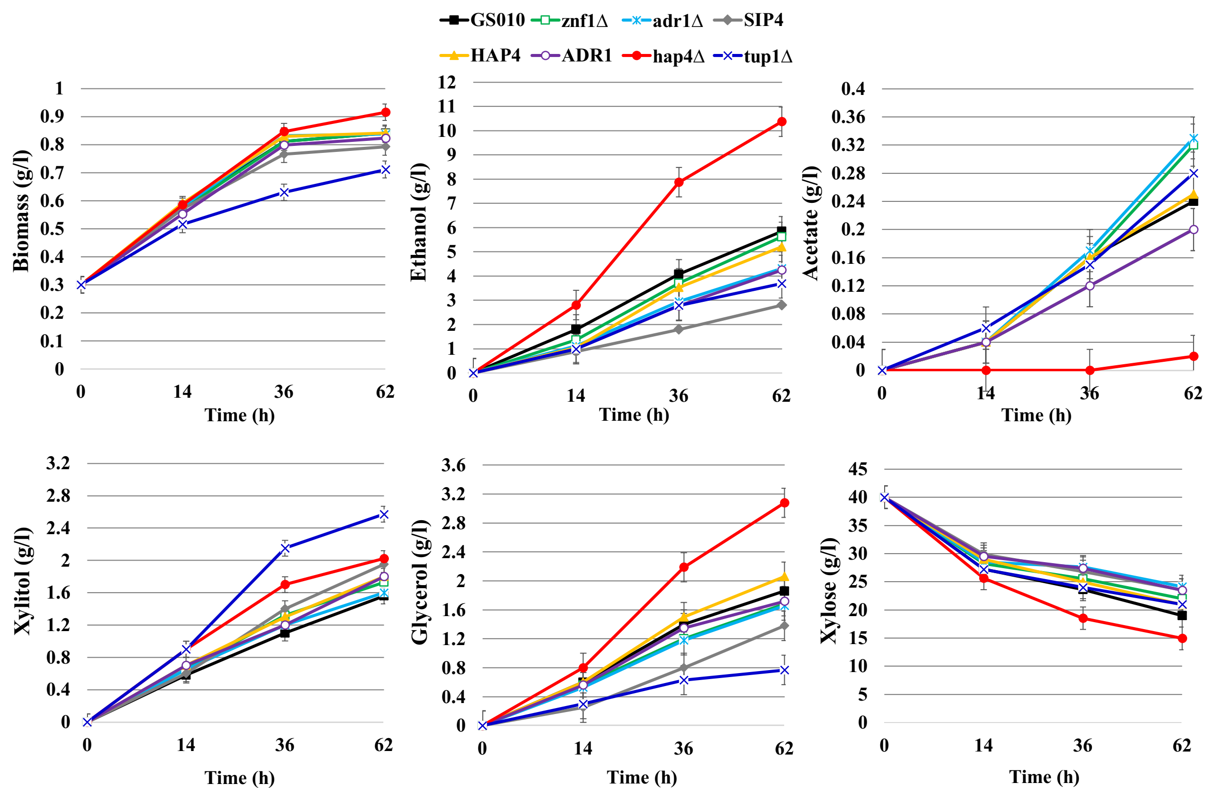


Fig. 4 Time courses of biomass accumulation, ethanol, acetate, xylitol, glycerol production and xylose consumption by *S. cerevisiae* GS010, *znf1Δ*, SIP4, *adr1Δ*, ADR1, *tup1Δ*, *hap4Δ* and HAP4 strains during xylose fermentation. Data are shown as mean of three independent experiments

Table 1 Main parameters of xylose alcoholic fermentation of *S. cerevisiae* GS010, *znf1Δ*, SIP4, ADR1, *adr1Δ*, *tup1Δ*, HAP4 and *hap4Δ* strains under oxygen-limited conditions at 30 °C

Strain	Ethanol (g l^{-1})*	Ethanol yield (g g^{-1} consumed xylose)*	Rate of ethanol production (g g^{-1} biomass h^{-1})**	Productivity of ethanol synthesis (g l^{-1} h^{-1})**
GS010	5.85 ± 0.17	0.278 ± 0.004	0.222 ± 0.008	0.128 ± 0.005
znf1Δ	5.62 ± 0.18	0.311 ± 0.005	0.171 ± 0.009	0.097 ± 0.006
SIP4	2.8 ± 0.17	0.170 ± 0.004	0.110 ± 0.008	0.063 ± 0.005
ADR1	4.25 ± 0.17	0.256 ± 0.004	0.128 ± 0.008	0.070 ± 0.005
adr1Δ	4.32 ± 0.17	0.272 ± 0.004	0.137 ± 0.0 08	0.079 ± 0.005
tup1Δ	3.70 ± 0.17	0.194 ± 0.004	0.137 ± 0.008	0.070 ± 0.005
HAP4	5.20 ± 0.17	0.272 ± 0.004	0.126 ± 0.008	0.074 ± 0.005
hap4Δ	10.38 ± 0.17	0.414 ± 0.004	0.341 ± 0.008	0.200 ± 0.005

*Data are represented on the 62 h of fermentation; **14 h of fermentation

(Fig. 4). Glycerol production for *tup1Δ* was 2.4-fold reduced as compared to GS010 (Fig. 4). During glucose fermentation, ethanol production and biomass accumulation for *tup1Δ* was 1.3-fold decreased, while glycerol and acetate production increased in the same order of magnitude over parental strain (Fig. 5). Glucose consumption remained unimpaired (Fig. 5). The obtained results suggest that the deletion of *TUP1*

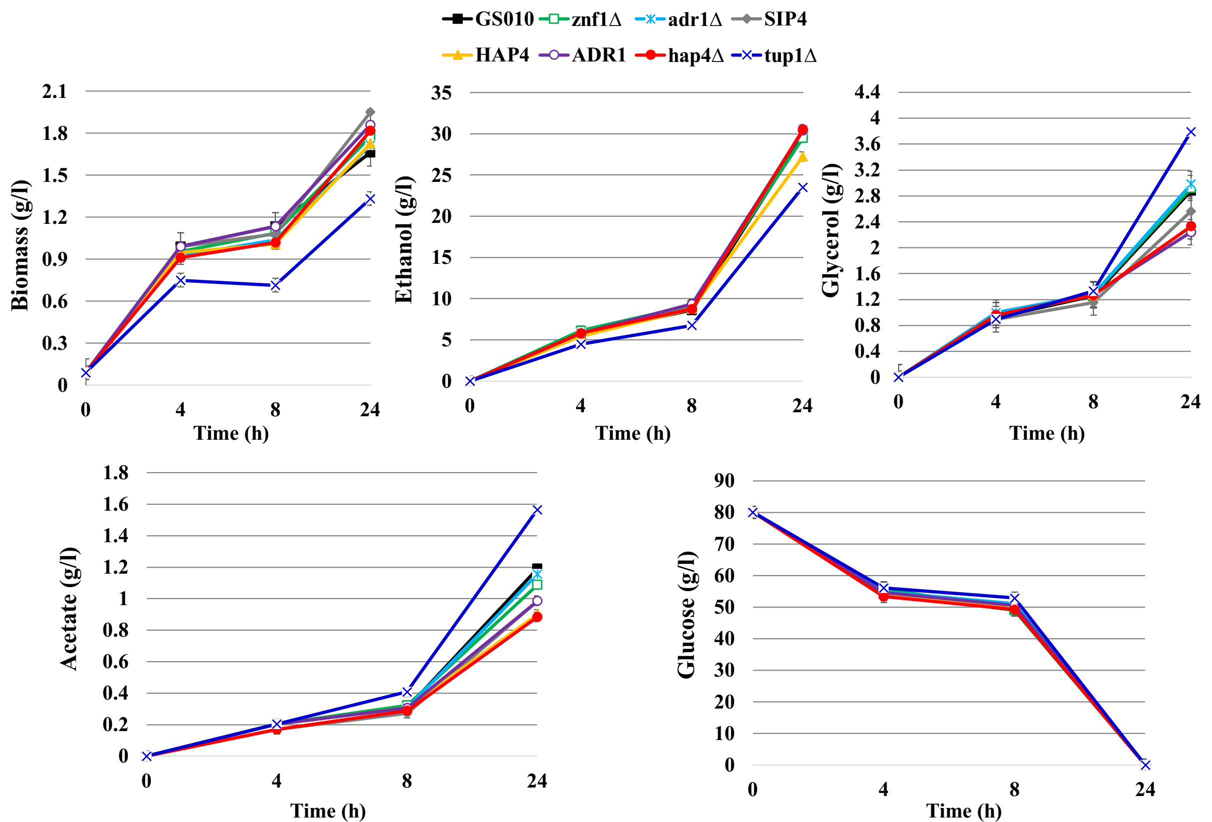


Fig. 5 Time courses of biomass accumulation, ethanol, glycerol, acetate, production and glucose consumption by *S. cerevisiae* GS010, *znf1Δ*, SIP4, *adr1Δ*, ADR1, *tup1Δ*, *hap4Δ* and HAP4 strains during glucose fermentation. Data are shown as mean of three independent experiments

has negative effect on xylose and glucose alcoholic fermentation.

It was previously shown that the deletion of *TUP1* of *S. cerevisiae* did not facilitate the maltose metabolism during co-fermentation glucose and maltose (Lin et al. 2014a, b). The *tup1Δ* did not enhance galactose fermentation from hydrolysates of the red seaweed (Sunwoo et al. 2020). However, overexpression of the truncated *TUP1* gene (without C-terminal repression domain), resulting in 2.5-fold increase of ethanol productivity from galactose compared to the control strain (Lee et al. 2011). Last approach can be used to study regulation of xylose metabolism in xylose-fermenting strains of *S. cerevisiae.*

Hap4 is well studied transcription factor playing pivotal role in balancing between respiration and fermentation metabolism of glucose through activation of respiratory gene expression (Forsburg and Guarente 1989; van Maris et al. 2001). In this work, we studied the impacts of *HAP4* gene overexpression and deletion on ethanol production by a xylose-utilizing *S. cerevisiae* strain. Overexpression of *HAP4* did not influence the growth of the strain in xylose containing medium under condition of full aeration as well as on the plates with xylose and glycerol as sole carbon sources (Fig. 3). Deletion of *HAP4* resulted in 1.2-fold reduce of biomass accumulation in flasks. It was shown significant growth retardation on xylose and glycerol according results of drop test (Fig. 3). Growth deficient on glycerol is additional confirmation of *HAP4* deletion which is in agreement with previously published data (Dudley et al. 2005). The growth drop test of *hap4Δ* was performed on medium supplemented with 5% of ethanol. It was shown that *hap4Δ* is unable to growth on ethanol as compared to parental strain (Fig. 3), which is in line with results described by (Raghevendran et al. 2006), since Hap4 is required for activation of genes essential for ethanol catabolism. It was shown that deletion of *HAP4* leads to increased sensitivity to oxidative stress, which is

connected with down-regulation of *SOD2* encoding a superoxide dismutase (Raghevendran et al. 2006). In similar way *hap4Δ* display increased sensitivity to hydrogen peroxide (Fig. 3). It is important to emphasize that sensitivity was more pronounced on xylose than that on glucose. Such phenomenon can be explained by increased production of endogenous ROS during cultivation on xylose containing medium (Dzanaeva et al. 2020a). The *HAP4* overexpression 1.1-fold decreased ethanol production and xylose consumption and 1.15-fold increased xylitol accumulation relative to that of parental strain (Fig. 4). HAP4 revealed 1.8- and 1.7-fold decrease of rate of ethanol production and productivity of ethanol synthesis than GS010, respectively (Table 1). Reduction of ethanol production from xylose by HAP4 is similar to that for decrease of ethanol production from glucose by *HAP4* overexpressing *S. cerevisiae* strains described elsewhere (van Maris et al. 2001). The *HAP4* strain characterised with 2.1- and 1.5-fold increased expression of *FBP1* and *RKI1*, respectively (Table S3). Notably, deletion of *HAP4* improved xylose alcoholic fermentation by 1.8-fold. The *hap4Δ* mutant accumulates 10.38 g l^{-1} of ethanol with an overall ethanol yield reaching 0.41 g g^{-1} of consumed xylose (Table 1, Fig. 4). The ethanol yield by *hap4Δ* showed a 1.5-fold increase, since xylose consumption by this strain was 1.2-fold increased compared to its parental strain (Table 1, Fig. 4). The rate of ethanol production and productivity of ethanol synthesis by *hap4Δ* were 1.5–1.6-fold increased versus parental strain (Table 1). The *hap4Δ* revealed 1.7-fold increase in glycerol accumulation and significant (12-fold) decrease in acetate production during xylose fermentation with respect to the GS010 strain (Fig. 4). Acetate generated from acetaldehyde by oxidation reaction catalyzed by acetaldehyde dehydrogenases, Ald4. This enzyme plays a major role in generating mitochondrial NADPH (Miyagi et al. 2009). Decrease in acetate generation in *hap4Δ* during fermentation can be explained by deregulation the expression of genes involved in mitochondrial respiration and reductive pathways (Raghevendran et al. 2006). Despite the *hap4Δ* growth retardation during xylose aerobic cultivation and plate assay, biomass accumulation was not affected under fermentation conditions, more probably due to high initial biomass inoculation (Fig. 4).

The *hap4Δ* possessed reduced expression level of key genes of TCA, e.g. *ICL1* (3.8-fold), *CIT1* (2.2-fold), *FUM1* (fivefold), *MDH1* (2.5-fold) confirming deletion of *HAP4*. Expression of *PDC1* was also 2.3-fold reduced under conditions of xylose fermentation (Table S3).

The HAP4 produced 1.1-fold less ethanol from glucose relative to that of GS010 (Fig. 5). There were no significant differences between *hap4Δ* and parental strain in biomass accumulation, and ethanol production during glucose fermentation. Both *hap4Δ* and HAP4 produced 1.2- and 1.3-fold lower amount of glycerol and acetate than GS010, respectively (Fig. 5).

It is wildly accepted that xylose in xylose-consuming strains of *S. cerevisiae* is recognized as more respiratory substrate rather than fermentative substrate (Jin et al. 2004; Souto-Maior et al. 2009). It was shown that natural xylose-fermenting yeast strains of *Scheffersomyces stipitis* and *Ogataea polymorpha* with reduced respiration produce increased amount of ethanol from xylose (Shi et al. 1999; Ruchala et al. 2017). Xylose consumption and ethanol yield was improved in respiratory-deficient xylose-consuming strain of *S. cerevisiae* after the adaptive evolution (Peng et al. 2012). Therefore, reduction of respiratory metabolism to increase xylose alcoholic fermentation appears to be useful and logic approach.

Hap4 is global regulator of respiratory gene expression. Hap4 is therefore very attractive target to study redirection of metabolic flux between respiration and fermentation metabolism. Transcript level of *HAP4*, was around sevenfold higher with xylose than with glucose (Matsushika et al. 2014). This data additionally supports the thesis regarding the xylose as respiratory substrate in recombinant xylose-consuming *S. cerevisiae* strain. The effects of *HAP4* deletion was previously examined on the background of xylose-utilizing strain of *S. cerevisiae*. It was shown, that ethanol yield for *hap4Δ* reached 0.23 g g^{-1} of consumed xylose, while the parental strain was unable to produce ethanol from xylose at all under aerobic conditions (Matsushika and Hoshino 2015). We were able to achieve higher ethanol yield reaching 0.41 g g^{-1} under oxygen-limited conditions using higher initial concentration of xylose (40 g l^{-1}) (Table 1). Approximately 40% of xylose remained unconsumed, which is similar to that described by (Matsushika and Hoshino 2015). The *hap4Δ* produced 1.3-fold more xylitol than that of GS010, reaching

2 g l^{-1} which can be explained by excess of NADH due to blocking of oxidation of this cofactor by respiration. The higher ethanol production by *hap4Δ* is apparently directly correlated with dramatic decrease of acetate production (Fig. 4). We have performed series of xylose and glucose fermentations by *hap4Δ* and parental strain GS010 under aerobic conditions described by (Matsushika and Hoshino 2015). Fermentation profiles on xylose and glucose revealed no significant difference in biomass accumulation, ethanol production, and substrate consumption between analyzed strains (Fig. 1S, Fig. 2S). At the same time, it was shown increase of acetate production during xylose fermentation by *hap4Δ* similar to that described by (Matsushika and Hoshino 2015). Acetate production also increased during fermentation of glucose (Fig. 2S) in contrast to the data presented in (Matsushika and Hoshino 2015). Glycerol production by *hap4Δ* during aerobic xylose fermentation was 1.6-fold increased as compared to GS010 (Fig. 1S) opposing to the data from (Matsushika and Hoshino 2015). Glycerol production by both strains under aerobic glucose fermentation was the same (Fig. 2S), which is in agreement with Matsushika and Hoshino 2015. Taking together we can conclude that the obtained results are strain-specific, since parental strain MA-B42 (Matsushika and Hoshino 2015) was unable to ferment xylose at all in contrast to the GS010. In addition, ethanol production largely depends on the fermentation conditions, including the medium composition, the amount of carbon source, aeration, and the initial biomass.

A *S. cerevisiae* strain overexpressing *HAP4* has been reported to display a 17% reduction in ethanol production and a 10% increase in biomass production compared to the wild type in glucose containing medium (van Maris et al. 2001; Raghevendran et al. 2006). According our results, we also observed decrease in ethanol production; however, biomass accumulation was not statistically distinguishable between HAP4 and GS010 (Fig. 5). In contrast, the *hap4Δ* mutant of *S. cerevisiae* revealed increased ethanol production and decrease of biomass accumulation during microaerated cultivation in chemostats with glucose as sole carbon source (Dikicioglu et al. 2008). Similarly, the lower biomass yield and 10% higher ethanol yield were observed on glucose with *hap4Δ* strain of *S. cerevisiae* under aerobic batch cultivation conditions (Matsushika and Hoshino 2015). Disruption of *HAP4* in *Meyerozyma guilliermondii* threefold increased the ethanol yield under conditions of aerobic glucose fermentation (Qi et al. 2014). However, in this work deletion of *hap4* had no effect on biomass and ethanol yields during glucose fermentation under conditions used here.

Here, for the first time we have studied impact of deletion (*ZNF1, ADR1, TUP1, HAP4*) and overexpression (*SIP4, ADR1, HAP4*) a set of genes coding for transcription factors on xylose alcoholic fermentation by the engineered xylose-utilizing *S. cerevisiae* strain. Among all tested strains only *hap4Δ* revealed increase of ethanol production with ethanol yield 0.41 g g^{-1}, while the other strains characterised with reduced ethanol production to varying degrees. Therefore, manipulation with Hap4 that is one of the key regulator of respiration could be used for further improve of xylose alcoholic fermentation performance of xylose-fermenting *S. cerevisiae* strains.

Acknowledgements This study was supported by Polish National Science Center, grant Opus UMO-2016/21/B/NZ1/00280, DEC-2020/37/B/NZ1/02232 and by National Academy of Sciences of Ukraine (Grant 2-21 and 17-21).

Author contributions All authors contributed to the study conception and design. LD, BK and JR performed the experiments. LD, BK, JR, AS and KD analysed the data. LD, AS and KD drafted the manuscript. All authors have read and approved the manuscript.

Funding This study was supported by Polish National Science Center, grant Opus UMO-2016/21/B/NZ1/00280, DEC-2020/37/B/NZ1/02232 and by National Academy of Sciences of Ukraine (Grant 2–21 and 17–21).

Data availability All data generated during this study are included in this published article and its supplementary files.

Declarations

Conflict of interest The authors declare not to have any conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Cunha JT, Costa CE, Ferraz L, Romaní A, Johansson B, Sá-Correia I, Domingues L (2018) *HAA1 PRS3*

overexpression boosts yeast tolerance towards acetic acid improving xylose or glucose consumption: unravelling the underlying mechanisms. Appl Microbiol Biotechnol 102:4589–4600. https://doi.org/10.1007/s00253-018-8955-z

Dikicioglu D, Pir P, Onsan ZI, Ulgen KO, Kirdar B, Oliver SG (2008) Integration of metabolic modeling phenotypic data in evaluation improvement of ethanol production using respiration-deficient mutants of *Saccharomyces cerevisiae*. Appl Environ Microbiol 74:5809–5816. https://doi.org/10.1128/AEM.00009-08

Dudley AM, Janse DM, Tanay A, Shamir R, Church GM (2005) A global view of pleiotropy phenotypically derived gene function in yeast. Mol Syst Biol 1(2005):0001. https://doi.org/10.1038/msb4100004

Dzanaeva L, Kruk B, Ruchala J, Nielsen J, Sibirny A, Dmytruk K (2020a) The role of peroxisomes in xylose alcoholic fermentation in the engineered *Saccharomyces cerevisiae*. Cell Biol Int 44:1606–1615. https://doi.org/10.1002/cbin.11353

Dzanaeva L, Ruchala J, Sibirny A, Dmytruk K (2020b) The impact of transcriptional factors Znf1 Sip4 on xylose alcoholic fermentation in recombinant strains of yeast *Saccharomyces cerevisiae*. Cytol Genet 54:386–392. https://doi.org/10.3103/S0095452720050035

Forsburg SL, Guarente L (1989) Identification characterization of *HAP4*: a third component of the CCAAT-bound *HAP2*/*HAP3* heteromer. Genes Dev 3:1166–1178. https://doi.org/10.1101/gad.3.8.1166

Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350:87–96. https://doi.org/10.1016/s0076-6879(02)50957-5

Harbison CT, Gordon DB, Lee TI, Rinaldi NG, Macisaac KD, Danford TD, Young AR (2004) Transcriptional regulatory code of a eukaryotic genome. Nature 431:99–104. https://doi.org/10.1038/nature02800

Jin YS, Laplaza JM, Jeffries TW (2004) *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. Appl Environ Microbiol 70:6816–6825. https://doi.org/10.1128/AEM.70.11.6816-6825.2004

Kwak S, Jin YS (2017) Production of fuels chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review perspective. Microb Cell Fact 16:82. https://doi.org/10.1186/s12934-017-0694-9

Lee KS, Hong ME, Jung SC, Ha SJ, Yu BJ, Koo HM, Jin YS (2011) Improved galactose fermentation of *Saccharomyces cerevisiae* through inverse metabolic engineering. Biotechnol Bioeng 108:621–631. https://doi.org/10.1002/bit.22988

Lin X, Zhang CY, Bai XW, Song HY, Xiao DG (2014a) Effects of *MIG1*, *TUP1 SSN6* deletion on maltose metabolism leavening ability of baker's yeast in lean dough. Microb Cell Fact 13:93. https://doi.org/10.1186/s12934-014-0093-4

Lin Y, Chomvong K, Acosta-Sampson L, Estrela R, Galazka JM, Kim SR, Cate JH (2014b) Leveraging transcription factors to speed cellobiose fermentation by *Saccharomyces cerevisiae*. Biotechnol Biofuels 7:126. https://doi.org/10.1186/s13068-014-0126-6

Liu H, Liu K, Yan M, Xu L, Ouyang P (2011) g*TME* for improved adaptation of *Saccharomyces cerevisiae* to corn cob acid hydrolysate. Applied Biochem Biotechnol 164:1150–1159. https://doi.org/10.1007/s12010-011-9201-7

MacPherson S, Larochelle M, Turcotte B (2006) A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev 70:583–604. https://doi.org/10.1128/MMBR.00015-06

Malavé TM, Dent SY (2006) Transcriptional repression by Tup1-Ssn6. Biochem Cell Biol 84:437–443. https://doi.org/10.1139/o06-073

Matsushika A, Hoshino T (2015) Increased ethanol production by deletion of *HAP4* in recombinant xylose-assimilating *Saccharomyces cerevisiae*. J Ind Microbiol Biotechnol 42:1623–1631. https://doi.org/10.1007/s10295-015-1695-5

Matsushika A, Goshima T, Hoshino T (2014) Transcription analysis of recombinant industrial laboratory *Saccharomyces cerevisiae* strains reveals the molecular basis for fermentation of glucose xylose. Microb Cell Fact 13:16. https://doi.org/10.1186/1475-2859-13-16

Miyagi H, Kawai S, Murata K (2009) Two sources of mitochondrial NADPH in the yeast *Saccharomyces cerevisiae*. J Biol Chem 284:7553–7560. https://doi.org/10.1074/jbc.M804100200

Moysés DN, Reis VCB, de Almeida JRM, de Moraes LMP, Torres FAG (2016) Xylose fermentation by *Saccharomyces cerevisiae*: challenges prospects. Int J Mol Sci 17:207. https://doi.org/10.3390/ijms17030207

Nijland JG, Shin HY, Boender LGM, de Waal PP, Klaassen P, Driessen AJM (2017) Improved xylose metabolism by a *CYC8* mutant of *Saccharomyces cerevisiae*. Appl Environ Microbiol 83:e00095-e117. https://doi.org/10.1128/AEM.00095-17

Peng B, Shen Y, Li X, Chen X, Hou J, Bao X (2012) Improvement of xylose fermentation in respiratory-deficient xylose-fermenting *Saccharomyces cerevisiae*. Metab Eng 14:9–18. https://doi.org/10.1016/j.ymben.2011.12.001

Qi K, Zhong JJ, Xia XX (2014) Triggering respirofermentative metabolism in the crabtree-negative yeast *Pichia guilliermondii* by disrupting the *CAT8* gene. Appl Environ Microbiol 80:3879–3887. https://doi.org/10.1128/AEM.00854-14

Raghevendran V, Patil KR, Olsson L, Nielsen J (2006) Hap4 is not essential for activation of respiration at low specific growth rates in *Saccharomyces cerevisiae*. J Biol Chem 281:12308–12314. https://doi.org/10.1074/jbc.M512972200

Ruchala J, Kurylenko OO, Soontorngun N, Dmytruk KV, Sibirny AA (2017) Transcriptional activator Cat8 is involved in regulation of xylose alcoholic fermentation in the thermotolerant yeast *Ogataea* (*Hansenula*) *polymorpha*. Microb Cell Fact 16:36. https://doi.org/10.1186/s12934-017-0652-6

Sakihama Y, Hasunuma T, Kondo A (2015) Improved ethanol production from xylose in the presence of acetic acid by the overexpression of the *HAA1* gene in *Saccharomyces cerevisiae*. J Biosci Bioeng 119:297–302. https://doi.org/10.1016/j.jbiosc.2014.09.004

Sambrook J, Fritsh EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor. Cold Spring Harbor Laboratory Press, NY

Scalcinati G, Otero JM, Van Vleet JR, Jeffries TW, Olsson L, Nielsen J (2012) Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption. FEMS Yeast Res 12:582–597. https://doi.org/10.1111/j.1567-1364.2012.00808.x

Shi NQ, Davis B, Sherman F, Cruz J, Jeffries TW (1999) Disruption of the cytochrome c gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. FEMS Yeast Res 15(11):1021–1030. https://doi.org/10.1002/(SICI)1097-0061(199908)15:11%3c1021::AID-YEA429%3e3.0.CO;2-V

Songdech P, Ruchala J, Semkiv MV, Jensen LT, Sibirny A, Ratanakhanokchai K, Soontorngun N (2020) Overexpression of transcription factor *ZNF1* of glycolysis improves bioethanol productivity under high glucose concentration enhances acetic acid tolerance of *Saccharomyces cerevisiae*. Biotechnology 15(7):e1900492. https://doi.org/10.1002/biot.201900492

Soontorngun N (2017) Reprogramming of nonfermentative metabolism by stress-responsive transcription factors in the yeast *Saccharomyces cerevisiae*. Curr Genet 63:1–7. https://doi.org/10.1007/s00294-016-0609-z

Souto-Maior AM, Runquist D, Hahn-Hägerdal B (2009) Crabtree-negative characteristics of recombinant xylose-utilizing *Saccharomyces cerevisiae*. Biotechnology 143:119–123. https://doi.org/10.1016/j.jbiotec.2009.06.022

Sunwoo IY, Sukwong P, Park YR, Jeong DY, Kim SR, Jeong GT, Kim SK (2020) Enhancement of galactose uptake from *Kappaphycus alvarezii* using *Saccharomyces cerevisiae* through deletion of negative regulators of *GAL* genes. Appl Biochem Biotechnol. https://doi.org/10.1007/s12010-020-03434-3

Tangsombatvichit P, Semkiv MV, Sibirny AA, Jensen LT, Ratanakhanokchai K, Soontorngun N (2015) Zinc cluster protein Znf1, a novel transcription factor of non-fermentative metabolism in *Saccharomyces cerevisiae*. FEMS Yeast Res 15:002. https://doi.org/10.1093/femsyr/fou002

van Maris AJ, Bakker BM, Brandt M, Boorsma A, de Mattos MJT, Grivell LA, Blom J (2001) Modulating the distribution of fluxes among respiration fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*. FEMS Yeast Res 1:139–149. https://doi.org/10.1111/j.1567-1364.2001.tb00025.x

Wang M, Wu M, Huo H (2007) Life-cycle energy greenhouse gas emission impacts of different corn ethanol plant types. Environ Res Lett 2:24001–24013. https://doi.org/10.1088/1748-9326/2/2/024001

Watanabe D, Hashimoto N, Mizuno M, Zhou Y, Akao T, Shimoi H (2013) Accelerated alcoholic fermentation caused by defective gene expression related to glucose derepression in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 77:2255–2262. https://doi.org/10.1271/bbb.130519

Wenning L, Yu T, David F, Nielsen J, Siewers V (2017) Establishing very long-chain fatty alcohol wax ester biosynthesis in *Saccharomyces cerevisiae*. Biotechnol Bioeng 114:1025–1035. https://doi.org/10.1002/bit.26220

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.