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Applied Microbiology and Biotechnology

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https://doi.org/10.1007/s00253-023-12843-3

Applied Microbiology and Biotechnology (2024) 108:21
https://doi.org/10.1007/s00253-023-12843-3

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Genes controlling hydrolysate toxin tolerance identified by QTL analysis of the natural *Saccharomyces cerevisiae* BCC39850

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Received: 17 April 2023 / Revised: 21 September 2023 / Accepted: 30 September 2023

Abstract

Lignocellulosic material can be converted to valorized products such as fuels. Pretreatment is an essential step in conversion, which is needed to increase the digestibility of the raw material for microbial fermentation. However, pretreatment generates by-products (hydrolysate toxins) that are detrimental to microbial growth. In this study, natural *Saccharomyces* strains isolated from habitats in Thailand were screened for their tolerance to synthetic hydrolysate toxins (synHTs). The *Saccharomyces cerevisiae* natural strain BCC39850 (toxin-tolerant) was crossed with the laboratory strain CEN.PK2-1C (toxin-sensitive), and quantitative trait locus (QTL) analysis was performed on the segregants using phenotypic scores of growth (OD_{600}) and glucose consumption. *VMS1*, *DET1*, *KCS1*, *MRH1*, *YOS9*, *SYO1*, and *YDR042C* were identified from QTLs as candidate genes associated with the tolerance trait. CEN.PK2-1C knockouts of the *VMS1*, *YOS9*, *KCS1*, and *MRH1* genes exhibited significantly greater hydrolysate toxin sensitivity to growth, whereas CEN.PK2-1C knock-ins with replacement of *VMS1* and *MRH1* genes from the BCC39850 alleles showed significant increased ethanol production titers compared with the CEN.PK2-1C parental strain in the presence of synHTs. The discovery of *VMS1*, *YOS9*, *MRH1*, and *KCS1* genes associated with hydrolysate toxin tolerance in *S. cerevisiae* indicates the roles of the endoplasmic-reticulum-associated protein degradation pathway, plasma membrane protein association, and the phosphatidylinositol signaling system in this trait.

Key points

- *QTL analysis was conducted using a hydrolysate toxin-tolerant S. cerevisiae natural strain*
- *Deletion of VMS1, YOS9, MRH1, and KCS1 genes associated with hydrolysate toxin-sensitivity*
- *Replacement of VMS1 and MRH1 with natural strain alleles increased ethanol production titers in the presence of hydrolysate toxins*

Keywords Hydrolysate · Inhibitors · *Saccharomyces cerevisiae* · Industrial traits · QTL analysis

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Published online: 30 December 2023

Introduction

Agricultural lignocellulosic waste can be converted to valorized products such as biofuels. The industrial biorefinery process involves pretreatment, enzymatic hydrolysis, and microbial fermentation steps. Pretreatment of lignocellulosic material is generally required to increase enzymatic digestibility. However, hydrolysis by-products from this step, including weak acids, furan aldehydes, inorganic ions, and phenolic compounds, can inhibit the subsequent steps. The generation of by-product inhibitors is strongly dependent on the feedstock and pretreatment method (Jönsson et al. 2013). Physical, physicochemical, and biological approaches have been established to remove pretreatment by-products from lignocellulosic hydrolysate (Mussatto and Roberto 2004). Although each method is suited to certain types of compounds, better results can be obtained by combining two or more different methods; however, this can increase the production cost. Therefore, one of the major challenges for the economic conversion of lignocellulose in biorefinery is to generate robust and versatile microbial strains that can tolerate pretreatment by-products whilst maintaining high metabolic function for the efficient conversion of raw material to target products.

Saccharomyces cerevisiae is commonly used as a cell factory (production microorganism) for biorefinery (Nielsen 2013). Generally, chemical tolerance and industrial fermentation traits are complex. These traits are influenced by multiple genes working in concert, as shown by quantitative trait locus (QTL) analysis (Ehrenreich et al. 2010; Swinnen et al. 2012; Hubmann et al. 2013; Cubillos et al. 2017; Wang et al. 2019; de Witt et al. 2019; Ho et al. 2021). Several attempts have been applied to construct *S. cerevisiae* cell factories with improved tolerance to inhibitory pretreatment hydrolysate by-products and the harsh conditions used in industrial processes including genetic modification, conventional breeding, and evolutionary engineering (Keasling 2010; Caspeta et al. 2015; Brandt et al. 2021; Hacısalihoğlu et al. 2019; Balaban et al. 2019; Kocaefe-Özşen et al. 2022; Menegon et al. 2022).

QTL analysis of tolerance to inhibitory hydrolysate toxin compounds (Meijnen et al. 2016; Maurer et al. 2017; de Witt et al. 2019) identified genes associated with this trait, including *HAA1* (transcriptional activator for adaptation to weak acid stress), *VMA7* (vacuolar membrane ATPase), *GLO1* (glyoxalase I), *DOT5* (nuclear thiol-peroxidase), *CUP2* (copper-binding transcription factor), *HAP1* (zinc-finger transcription factor), *YGL176C* (uncharacterized open reading frame), and *XRN1/BUD13* (bud-site selection). These genes function in oxidative, osmotic, acidic, and proteotoxic stress responses. Therefore, to construct industrial strains tolerant of various hydrolysate toxins, the collective effects of multiple genes from these pathways must be considered. The understanding of the genes and their interactions controlling this trait may be incomplete as previous QTL studies were performed with selected industrial, laboratory, or natural strains. Natural strains from habitats not sampled previously may harbor variants in other genes controlling this trait that were not considered.

In this study, natural *Saccharomyces* spp. isolated from diverse habitats in Thailand were screened for tolerance to synthetic hydrolysate toxin compounds (synHTs). One isolate of *S. cerevisiae* (BCC39850) displaying superior tolerance and a sensitive laboratory strain (CEN.PK2-1C) was used for QTL analysis to identify genes associated with hydrolysate toxin tolerance, and the functions of candidate genes with respect to the tolerance phenotype were tested by gene knockout and knock-in experiments.

Materials and methods

Strains, cultivation conditions, and reagents

S. cerevisiae laboratory strain CEN.PK2-1C (*MATa*; *his3D1*; *leu2-3_112*; *ura3-52*; *trp1-289*; *MAL2-8c*; *SUC2*) was obtained from EUROSCARF, Frankfurt, Germany. *S. cerevisiae* isolates BCC39850 and other wild strains were obtained from the Thailand Bioresource Research Center (www.tbrcnetwork.org). Yeast strains were grown in YPD liquid medium (20 g/L peptone, 20 g/L glucose, and 10 g/L yeast extract) and stored in YPD broth containing 20% glycerol at – 70 °C.

Yeast mating

S. cerevisiae BCC39850 haploid#17 (*MATalpha*; hydrolysate toxin tolerant) and CEN.PK2-1C (*MATa*; sensitive laboratory strain) strains were crossed on a YPD agar plate and grown overnight at 30 °C. After mating, a loop full of the mated population was suspended in SC broth and streaked on an SC agar plate (without amino acid) and then incubated at 30 °C for 3–5 days until diploid colonies appeared. Diploid colonies were taken and re-streaked on sporulation agar plates (10 g/L potassium acetate, 1 g/L yeast extract, 0.5 g/L dextrose, 20 g/L agar) and incubated at 30 °C for 3–5 days until tetrad formation was observed by microscopy. Sporulation and isolation of haploid segregants were carried out using standard protocols (Sherman and Hicks 1991).

Genome sequencing and mapping of SNPs

Genomic DNA samples were obtained from 93 yeast strains, including the parental strains (BCC39850 haploid#17 and CEN.PK2-1C) and 91 segregants derived from the F1 progeny using a genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 20 ng/μL. Samples were submitted for whole-genome sequencing service (National Omics Center, Pathum Thani, Thailand) using the MGISEQ-2000RS platform (MGI Tech, Guangdong, China). Whole-genome sequencing libraries were constructed using the MGIEasy FS DNA Library Prep Set (MGI Tech, Guangdong, China). Genomic DNA samples were fragmented, end-repaired, and modified to form a single adenine base overhang (A-tailing). Subsequently, the DNA fragments were ligated to barcoded adapters and amplified by PCR. The purified PCR products were then subjected to denaturation, circularization, exonuclease digestion, and cleanup processes. The resulting DNA libraries were quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and sequenced using DNA NanoBalls (DNBs) technology to obtain 150 bp paired-end reads. The quality of the raw reads was assessed using FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing adapters and low-quality reads were trimmed and removed. Reads were mapped to the *S. cerevisiae* S288c reference genome available from the National Center for Biotechnology Information (Engel et al. 2022) using minimap2 with default parameters (Li 2018). Variants were called using Genomic Analysis Toolkit best practice; GATK version 4.1.4.1 with the HaplotypeCaller method (https://gatk.broadinstitute.org/hc/en-us). A total of 71,430 single-nucleotide polymorphisms (SNPs) were identified. After removing monomorphic markers to ensure that only markers segregating among progeny were considered, 67,423 SNPs were filtered using the following criteria: (1) base quality scores > 20, (2) coverage depths between 20× and 1000×, (3) missing data ≤ 20%, and (4) a minor allele frequency ≥ 0.1. A total of 27,977 SNPs were used as the final variant set for genetic analysis. The variants were annotated for their effects on gene function using SnpEff version 3.4 (Cingolani et al. 2012). The accession number for the raw sequence read data in the NCBI Sequence Read Archive (SRA) is PRJNA971766 (BioProject).

Linkage and quantitative trait locus analysis

Linkage analysis was performed with Lep-Map3 (Rastas 2017). The Lep-Map3 module "SeparateChromosomes2" was used to assign markers into linkage groups (LGs) by computing all pair-wise logarithm of odds (LOD) scores between markers with a threshold of 6.0. The "OrderMarkers2" module was used to order the markers for each LG by maximizing the likelihood of the data for alternative orders. Marker distances were converted to map distances in centiMorgans (cM) using the Kosambi mapping function (Kosambi 1944). Quantitative trait locus (QTL) analysis was performed using the R package qtl (Broman et al. 2003). Using the composite interval mapping (CIM) method, the marker-phenotype association and statistical significance were determined for each QTL by combining interval mapping with multiple regression. LOD thresholds ($P < 0.05$) were determined via permutation tests (PT) with 1000 iterations.

Validation of causative genes by gene knockout and knock-in

The validation of candidate genes was carried out in the CEN.PK2-1C background strain. Deletion cassettes were obtained by PCR amplification of the hygromycin deletion cassette (*hygMX6*) with 42 bp flanking homologous regions up- and downstream of the target locus. The knock-in cassettes containing the BCC39850 allele fused with the *hygMX6* module with 42 bp flanking homologous regions were amplified by overlapping extension PCR. The geneticin resistance (*G418MX6*) module was also used as selective markers for double knock-in. Gene knock-in cassettes containing the BCC39850 allele were amplified by overlapping extension PCR. Yeast cells were transformed using the LiAc/SS Carrier DNA/PEG method as described previously (Gietz and Schiestl 2007). Positive transformants were screened on YPD agar plates containing 200 mg/mL hygromycin and/or geneticin. Successful gene deletions and replacements were confirmed by PCR amplification and Sanger sequencing. The schematic diagrams for genomic modifications and all primers used in this experiment are summarized in Supplementary information: Fig. S1 and Table S1.

Phenotypic assays

Sugarcane bagasse after mild alkaline pretreatment is a potential feedstock for yeast fermentation. A mixture of synHTs (furan aldehyde, acids, and phenols) was formulated in a composition to mimic the hydrolysate toxins present in sugarcane bagasse from the alkaline pretreatment process, with minor modifications based on the study by van der Pol et al. (2015). Previous reports on lignocellulosic inhibitory compounds have indicated that coniferyl aldehyde (an aromatic aldehyde) inhibits *S. cerevisiae* growth more than furan aldehydes (Larsson et al. 2000; Adeboye et al. 2015; Jönsson and Martín 2015). However, the concentration of furan aldehydes (derived from carbohydrates) in lignocellulosic hydrolysates is higher than that of lignin-derived aromatic aldehydes (Jönsson and Martín 2015). Phenotypic

assays were performed on yeast cultures for up to 72 h at 30 °C with shaking at 220 rpm in YPD medium. In tests of hydrolysate toxin tolerance, the medium was supplemented with synHTs comprising furfural, 0.80 g/L; acetic acid, 2.0 g/L; formic acid, 1.0 g/L; vanillin, 0.07 g/L; and syringaldehyde 0.06 g/L. For screening of isolates and testing of segregants, pre-cultured inoculum prepared in YPD medium at 30 °C overnight was used to inoculate 2 mL of fresh culture in a 1-mL tube to obtain the starting OD_{600} of 0.05 (approximately 5×10^5 cells/mL). Cell growth (OD_{600}) and glucose consumption (g/L) were monitored at 16-, 24-, and 48-h time points. A batch fermentation experiment was carried out in 250-mL Erlenmeyer flasks containing 50 mL of YPD containing 100 g/L glucose with *S. cerevisiae* cells suspended at an initial density OD_{600} of 0.2 (approximately 2×10^6 cells/mL). One-milliliter samples were taken at 16-, 24-, 48-, and 72-h intervals for analysis of growth (OD_{600}) and ethanol production. Real-time growth monitoring was carried out by microtiter plate cultivation; BioLector (Beckman Coulter GmbH, Krefeld, Germany) in 48 round-bottom well plates. A total volume of 1 mL of YPD (100 g/L glucose) with synHTs was added to each well with the starting OD_{600} of 0.05. The microtiter plate was sealed with gas permeable film (Beckman Coulter GmbH, Krefeld, Germany) and incubated at 30 °C with shaking at 1000 rpm and a relative humidity above 80%. Biomass was monitored every 5 min for 48 h by measuring scattered light signal reflecting the amount of biomass suspended in culture medium.

Product analysis

Samples from batch fermentation were harvested by centrifugation (10,000 × *g* for 5 min at 4 °C) and filtered through 0.2-micron filter cellulose acetate membranes (Millipore, Bedford, MA, USA). The amount of glucose and ethanol was determined using a high-performance liquid chromatographic (HPLC) system (Shimadzu Prominence LC-20; Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector and an Aminex-HPX-87H Column (Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 65 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL/min.

Data analysis

All experiments were performed independently at least three times. Analysis of variance was conducted by one-way analysis of variance (ANOVA) using Tukey's post hoc method on the SPSS statistical package (version 18.0 for Windows, SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P < 0.05$.

Results

Screening and selection of hydrolysate toxin tolerant strain

Wild isolates of *Saccharomyces* spp. from a wide range of ecological niches in Thailand deposited in the Thailand Bioresource Research Center (TBRC; Pathumthani, Thailand) were screened for tolerance to inhibitory hydrolysate compounds using synHTs (Fig. 1a). Seven candidate *S. cerevisiae* strains from screening were selected for further evaluation of ethanol production (Fig. 1b). Strain BCC39850 was selected based on its phenotype (high yield of ethanol) and ability to sporulate. Strain BCC39850 was sporulated, and haploid segregants were tested for growth along with the laboratory strain CEN.PK2-1C, which is also haploid (Fig. 1c). From the five haploids isolated from the natural isolate BCC39850 that were analyzed, the haploid#17 strain showed the greatest synHTs tolerance. This haploid was then crossed with CEN.PK2-1C to generate hybrid strains. The hybrid#31 strain was selected for genetic analysis based on the growth on YPD plates supplemented with synHTs. This hybrid was then sporulated to generate F1 haploid segregants. The selected haploid segregants were analyzed for growth (OD_{600}) and glucose consumption at 16 and 24 h (Fig. 1d). The results were then converted to phenotypic scores for QTL analysis.

Genome sequence mapping and SNP analysis

Genomic DNA samples from the two parental strains (Haploid#17 and CEN.PK2-1C) and 91 selected segregants from the cross were sequenced, and reads were mapped to the *S. cerevisiae* S288c reference genome. Raw read statistics are shown in Supplemental Table S2. A total of 71,430 single-nucleotide polymorphisms (SNPs) were found. The annotation and prediction of the SNP effects showed that most SNPs were found in upstream (43.14%) and downstream (41.40%) of genome regions, with less identified in exon (Supplemental Table S3). After filtering, 27,977 SNPs remained, which were used to construct the linkage map (Fig. 2a). The distributions of SNP markers on the linkage groups are summarized in Supplemental Table S4.

Identification of QTLs associated with hydrolysate toxin tolerance

QTLs were identified from the marker-phenotype association, with one major region passing significance thresholds located on chromosome IV (Fig. 2b). Using the applied phenotypic scores of glucose consumption and OD_{600} from the

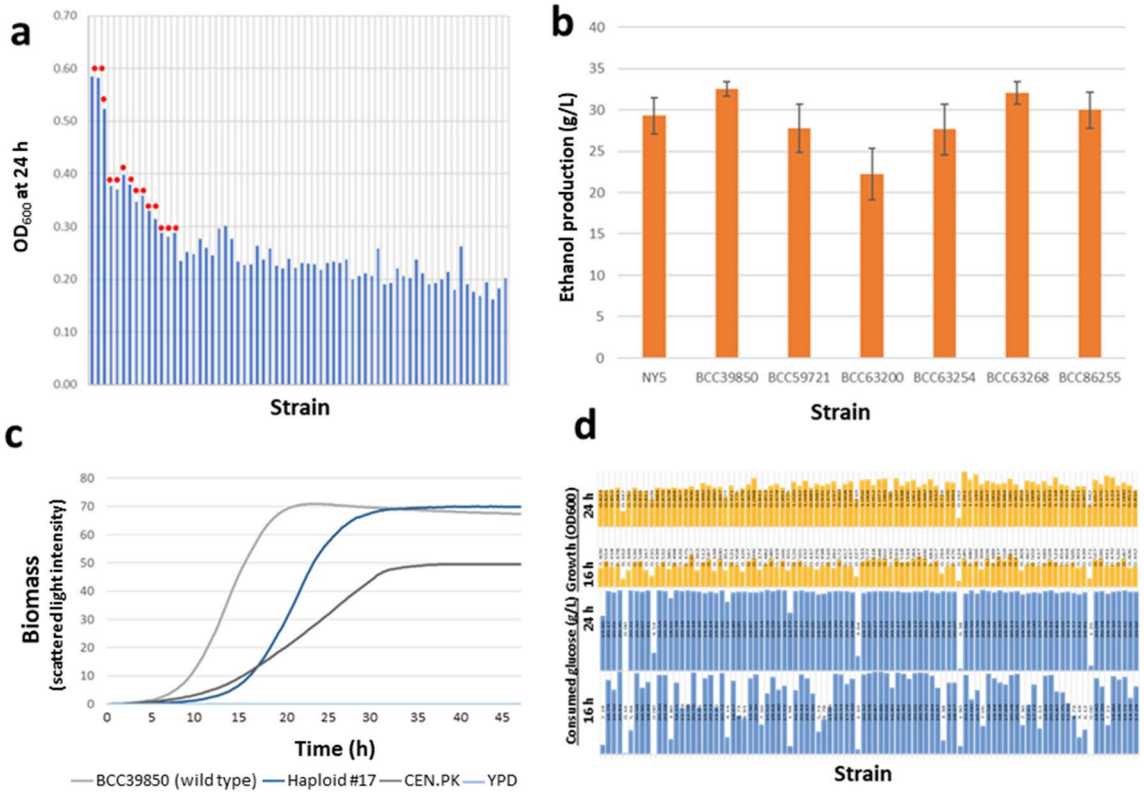

Fig. 1 Screening and phenotyping for hydrolysate toxin tolerance. **a** Screening for hydrolysate toxin tolerance of *Saccharomyces* spp. culture collection isolates. Candidate tolerant strains are indicated by red dots. **b** Ethanol production of selected *S. cerevisiae* candidate tolerant strains in YPD containing 200 g/L glucose. **c** Growth analysis (BioLector) of parental strains used for mating in YPD with synHTs. Haploid#17 is a segregant from BCC39850 sporulation, which was mated with the CEN.PK2-1C reference strain (hydrolysate toxin-sensitive). **d** Assessment of selected segregants from the cross of haploid#17 and CEN.PK2-1C for glucose consumption and growth (OD_{600}) sampled at 16 and 24 h in YPD supplemented with synHTs

16 h time point, analysis of markers (LOD threshold = 3.26) identified six genes (*YOS9*, *VMS1*, *DET1*, *YDR042*, *KCS1*, and *MRH1)*. Using the 24-h time point data (LOD threshold = 2.87), one additional gene was identified (*SYO1*). All of these candidate genes contained at least one non-synonymous missense variant compared with the S288c reference. The candidate genes associated with hydrolysate toxin tolerance and their functions are summarized in Tables 1 and 2.

Testing candidate gene function by gene deletion

To assess whether candidate gene function is required for hydrolysate toxin tolerance, candidate genes from QTL analysis were deleted by *hygMX6* cassette disruption in the CEN.PK2-1C strain. After selection on YPD containing 0.2 mg/mL hygromycin, integration of the deletion cassettes at target genes was validated by PCR. The tolerance phenotype of the knock-out mutants was initially tested by spot assay (Fig. 3a). Four deletion mutants (CEN.PK2-1C_*VMS1*Δ, CEN.PK2-1C_*KCS1*Δ, CEN.PK2-1C_*MRH1*Δ, and CEN.PK2-1C_*YOS9*Δ) demonstrated markedly greater sensitivity to synHTs, whereas CEN.PK2-1C_*DET1*Δ, CEN.PK2-1C_*SYO1*Δ, and CEN.PK2-1C_*YDR042C*Δ did not appear to differ in toxin sensitivity from the CEN.PK2-1C parental strain. In standard YPD medium, deletion of candidate genes was found to have little effect on growth except for *KCS1*, in which the CEN.PK2-1C_*KCS1*Δ strain showed impaired growth (Supplemental Fig. S2). Real-time growth analysis of the parental strains and hydrolysate toxin- sensitive knockout mutants was performed (Fig. 3b), and the data were used for the calculation of specific growth rate (Table 3). Among these deletion strains, CEN.PK2-1C_*VMS1*Δ showed the greatest sensitivity to synHTs, as indicated by the decreased specific growth rate (0.0101 h^{-1}) followed by CEN.PK2-1C_*KCS1*Δ (0.0147 h^{-1}), CEN.PK2-1C_*MRH1*Δ (0.0237 h^{-1}), and CEN.PK2-1C_*YOS9*Δ (0.0258 h^{-1}), respectively. The ethanol production titers were also determined; all deletion variants displayed a delayed ethanol production (Fig. 3c) resulting from extended lag time. At 28 h of growth, CEN.PK2-1C showed a reduced growth rate, and then switched to diauxic shift at 35-h time point (Fig. 3b). While CEN.PK2-1C_*YOS9*Δ strain entered stationary phase at a slower

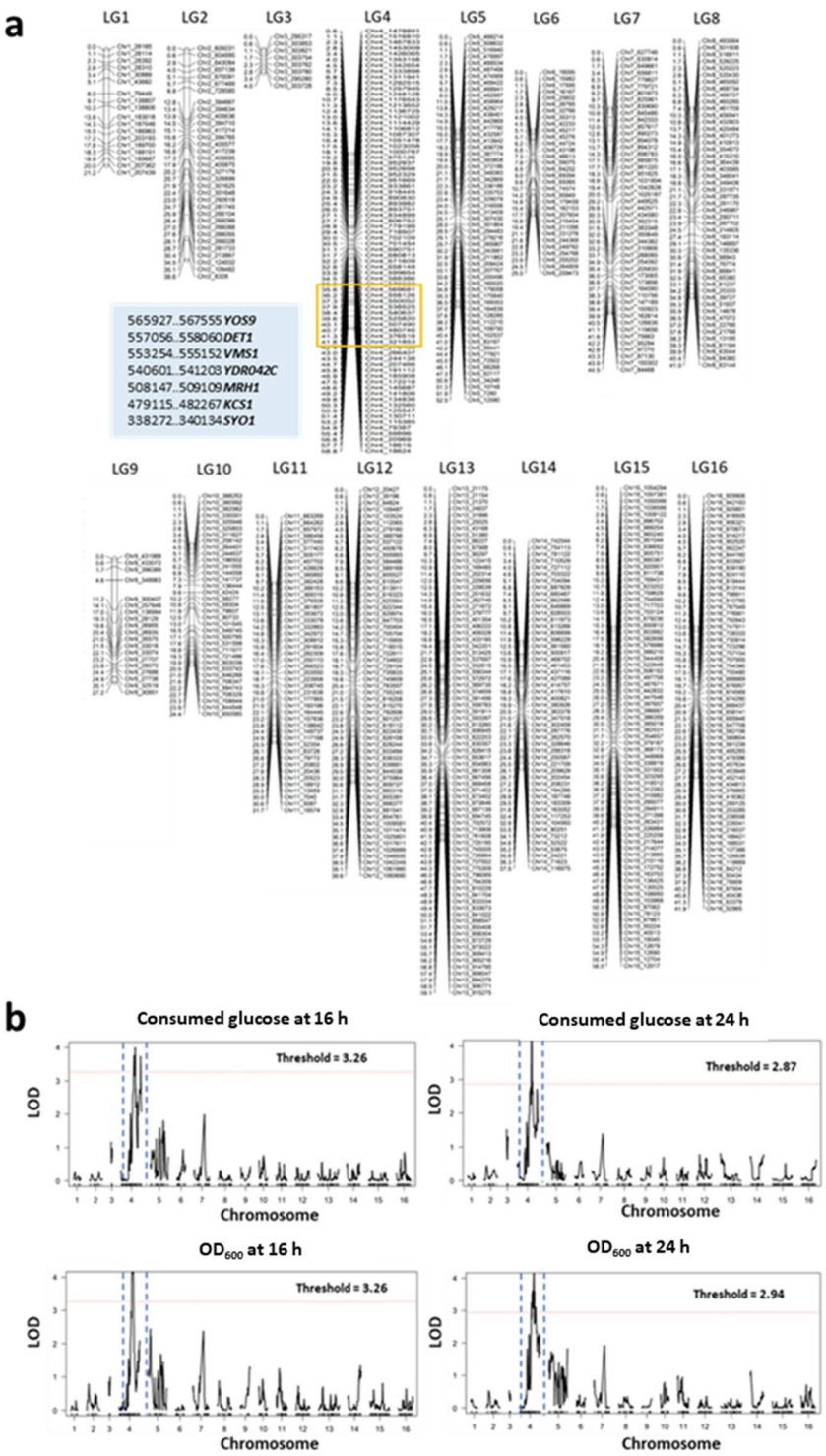

Fig. 2 QTL analysis to identify genes associated with hydrolysate toxin tolerance. **a** Linkage map and distribution of QTLs associated with hydrolysate toxin tolerance (highlighted box). The light blue box indicates the identified QTLs and their positions on the chromosome. **b** QTL mapping of 91 selected segregants using phenotypic scores of glucose consumption (upper panels) and OD_{600} (lower panels) at 16 h and 24 h. The logarithm of the odds (LOD) scores was plotted against the respective chromosomal positions. LOD score thresholds were determined separately for each data type by permutation test, as indicated on each panel

rate than CEN.PK2-1C and consumed less ethanol resulting in higher ethanol concentrations after 40-h fermentation (Fig. 3c). The different growth characteristics are possibly due to differences in transcription regulation during oxidative metabolism. Since the transition from exponential phase to diauxic shift and stationary phase is regulated by multiple signaling pathways such as protein kinase A (PKA), TOR (target of rapamycin), Snf1p, and Rim15p (Galdieri et al. 2010). The knock-out mutants showed varying levels of sensitivity to different hydrolysate compounds. Among these mutants, the most sensitive knock-out strain was CEN.PK2-1C_*VMS1*Δ, which showed a very high sensitivity to furfural but not acetic acid (Fig. 4). Notably, this gene has not been associated with hydrolysate toxin tolerance previously.

Table 1 Genes with nonsynonymous variants in the major QTL region

Gene	Chr	Coordinate	LOD score	Length (aa)	Annotated gene	Amino acid substitution (relative to S288c reference)	
						Haploid #17	CEN.PK2-1C
1	IV	553,254..555152	3.74	632	*VMS1*	L60H D22N N561D K194R S49F	Wild type
2	IV	557,056..558060	3.74	334	*DET1*	H16Q G17C	Wild type
3	IV	479,115..482267	3.99	1050	*KCS1*	Wild type	N508Y
4	IV	508,147..509109	3.67	320	*MRH1*	S299G	Wild type
5	IV	540,601..541203	3.77	200	*YDR042C*	I143V	Wild type
6	IV	565,927..567555	3.85	542	*YOS9*	T524A	Wild type
7	IV	338,272..340134	2.99	620	*SYO1*	I340M	Wild type

Table 2 Functional descriptions of candidate genes

QTL	Gene	Description
1	*VMS1*	Peptidyl-tRNA hydrolase that releases stalled peptides from ribosomes, component of a Cdc48p-complex; involved in protein quality control
2	*DET1*	Acid phosphatase (broad substrate activity); involved in the non-vesicular transport of sterols between endoplasmic reticulum (ER) and plasma membrane
3	*KCS1*	Inositol hexakisphosphate and inositol heptakisphosphate kinase; generation of high energy inositol pyrophosphates
4	*MRH1*	Protein that localizes primarily to the plasma membrane
5	*YDR042C*	Putative protein of unknown function
6	*YOS9*	ER quality-control lectin; integral subunit of the HRD (3-hydroxy-3-methylglutaryl coenzyme A reductase degradation) ligase; participates in efficient ER retention of misfolded proteins
7	*SYO1*	Transport adaptor or symportin; assembly chaperone that co-translationally associates with nascent Rpl5p

MRH1 deletion resulted in acetic acid hyper-sensitivity (Fig. 4), consistent with a previous report by Takabatake and coworkers (2015).

Testing BCC39850 alleles for function by gene knock-in

Next, we tested the *VMS1*, *MRH1*, *EMC10*, *KCS1*, and *YOS9* alleles from the BCC39850 strain for function in the CEN.PK2-1C strain background by gene knock-in. The CEN.PK2-1C_*VMS1*^{rep} and CEN.PK2-1C_*MRH1*^{rep} knock-in strains exhibited growth on YPD containing acetic acid, furfural, or synHTs comparable to the CEN.PK2-1C strain by spot-test (Fig. 4). The growth profiles of the single knock-in strains from real-time measurements were not significantly different from the CEN.PK2-1C strain (Fig. 5a). Additionally, double knock-in mutants were constructed to test the interaction of *VMS1* with the *MRH1* and *YOS9* genes on the hydrolysate toxin tolerance phenotype. Two double knock-in strains were constructed (CEN.PK2-1C_*VMS1*^{rep}*MRH1*^{rep} and CEN.PK2-1C_*VMS1*^{rep}*YOS9*^{rep}) and tested for growth and ethanol production. The growth profiles of the double knock-in strains from real-time measurement were also not significantly different from the CEN.PK2-1C strain (Fig. 5b). The ethanol production of CEN.PK2-1C, knock out, and knock-in strains was investigated at different time points (16, 24, and 48 h) during fermentation. The results showed that CEN.PK2-1C_*MRH1*^{rep} and CEN.PK2-1C_*VMS1*^{rep}*MRH1*^{rep} strains exhibited significantly higher ethanol production compared with CEN.PK2-1C at 16 and 24 h of fermentation (Fig. 5c). At 24-h time point, the ethanol production of CEN.PK2-1C_*VMS1*^{rep} strain was also significantly improved. In this experiment, the improvement of ethanol production of the double knock-in strains was only observed in CEN.PK2-1C_*VMS1*^{rep}*MRH1*^{rep} but not for CEN.PK2-1C_*VMS1*^{rep}*YOS9*^{rep}.

Discussion

In this study, we identified a hydrolysate toxin-tolerant *S. cerevisiae* strain from screening a collection of natural isolates and performed QTL mapping to identify genes that are associated with the tolerance trait. From the seven QTLs

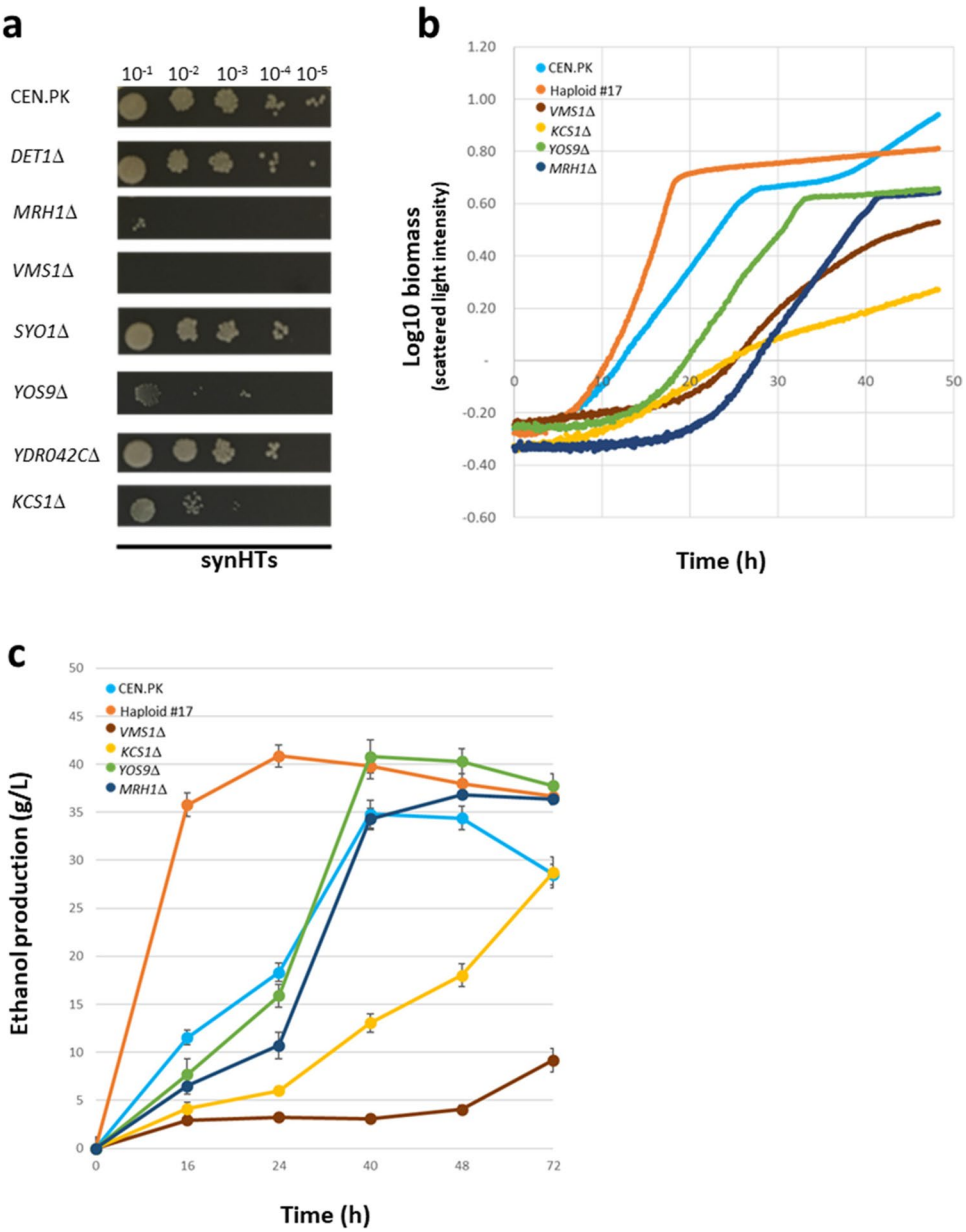

Fig. 3 Hydrolysate toxin tolerance of CEN.PK2-1C (CEN.PK), CEN.PK2-1C_*DET1*Δ (*DET1*Δ), CEN.PK2-1C_*VMS1*Δ (*VMS1*Δ), CEN.PK2-1C_*KCS1*Δ (*KCS1*Δ), CEN.PK2-1C_*MRH1*Δ (*MRH1*Δ), CEN.PK2-1C_*YDR042C*Δ (*YDR042C*Δ), CEN.PK2-1C_*YOS9*Δ (*YOS9*Δ), and CEN.PK2-1C_*SYO1*Δ (*SYO1*Δ). **a** Spot test; all strains were spotted on the same YPD plate supplemented with synHTs. Growth was recorded at day 3 (30 °C). Serial dilutions of each strain were spotted on the plate, as indicated on the top. **b** Growth and **c** ethanol production titers of CEN.PK2-1C and deletion strains. Fermentation was performed at 30 °C in YPD with synHTs and 100 g/L of glucose ($n = 3$)

identified, four candidate genes (*VMS1*, *YOS9*, *MRH*, and *KCS1*) were tested for their involvement in hydrolysate toxin tolerance.

VMS1 and *YOS9* gene functions are important for hydrolysate toxin tolerance, since the knockout mutants of these genes were more sensitive to hydrolysate toxin than wild type (Fig. 3). The *VMS1* product, Vms1p, forms a complex with Cdc48p and Npl4p. The Vms1p-Cdc48p-Npl4p complex acts in the ribosome quality control pathway, in which Vms1p removes tRNA from nascent polypeptides at stalled ribosomes (Verma et al. 2018). The Vms1p-Cdc48p-Npl4p complex functions in retrotranslocation of aberrant mitochondrial proteins (particularly proteins damaged by oxidative stress) to the cytoplasm for ubiquitin–proteasome–dependent degradation (Heo et al. 2010; Nielson et al. 2017). The Vms1p-Cdc48p-Npl4p complex also retrotranslocates aberrant ER (endoplasmic reticulum) proteins for degradation via the ERAD (endoplasmic-reticulum-associated protein degradation) system (Tran et al. 2011). Yos9p is an ER-localized lectin containing a mannose 6-phosphate receptor homology (MRH) domain (Hosokawa et al. 2009). This domain recognizes an *N*-glycan–specific structure (Man7GlcNAc2) on misfolded glycoproteins targeting them for degradation via the ERAD pathway (Szathmary et al. 2005). Vms1p and Yos9p functions are thus linked by the ERAD system for removal of aberrant ER proteins. Recently, Kocaefe-Ozsen and coworkers (2022) also reported that the *UFD1* gene (encodes a cofactor of the

Table 3 Maximum specific growth rate (μMax) of *Saccharomyces cerevisiae* strains cultured in the presence of synthetic hydrolysate toxins

	μMax (h^{-1})*	S.D
CEN.PK2-1C	0.0363c,d	0.001
CEN.PK2-1C_*VMS1*Δ	0.0101a	0.001
CEN.PK2-1C_*KCS1*Δ	0.0147a	0.001
CEN.PK2-1C_*MRH1*Δ	0.0237b	0.002
CEN.PK2-1C_*YOS9*Δ	0.0258b	0.004
CEN.PK2-1C_*SYO1*Δ	0.0294c	0.001
CEN.PK2-1C_*VMS1*^{rep}	0.0389d	0.001
CEN.PK2-1C_KCS*1*^{rep}	0.0334c	0.001
CEN.PK2-1C_*MRH1*^{rep}	0.0361c, d	0.002
CEN.PK2-1C_*YOS9*^{rep}	0.0353c, d	0.003
CEN.PK2-1C_*VMS1*^{rep}*MRH1*^{rep}	0.0335c	0.001
CEN.PK2-1C_*VMS1*^{rep}*YOS9*^{rep}	0.0333c	0.001

*The cell biomass was monitored every 5 min in YPD (100 g/L glucose) at 30 °C, 1000 rpm for 48 h. Specific growth rates were calculated from 0 to 28-h time points. Means with the same letter are not significantly different ($n=5$)

Cdc48p-Npl4p-Ufd1p) and *ATG40* (encodes an autophagy receptor) play important roles in enhanced oxidative stress tolerance in a *S. cerevisiae* strain obtained by evolutionary engineering. The suggested functions of these two genes contribute to the dislocation of misfolded proteins and ER degradation.

The *MRH1* knockout strain demonstrated significantly lower growth and ethanol production titer in the presence of synHTs compared with CEN.PK2-1C (Fig. 3), which is mainly due to acetic acid hyper-sensitivity (Fig. 4). Mrh1p is a membrane-bound protein related to heat shock protein (Hps30p); this protein and its paralog *YRO2* (Yro2p) have been reported to play roles in tolerance to acetic acid (Wu et al. 2000; Mira et al. 2010; Haitani et al. 2012; Takabatake et al. 2015). As suggested by Sklodowski and coworkers (2017), Mrh1p is a pseudokinase as evidenced from mutations in the conserved kinase motifs, which modulate its function in the recognition of chemical signals to recruit other proteins. It has also been reported that the upregulation of *HSP30* and *YRO2* genes is associated with an oxidative stress response in an iron-resistant *S. cerevisiae* strain (Balaban et al. 2019). Similarly, in a coniferyl aldehyde-resistant strain of *S. cerevisiae* that showed cross-resistance to vanillin, ferulic acid, and 4-hydroxybenzaldehyde, the *HSP30* gene has been found to be upregulated (Hacısalihoğlu et al. 2019). These findings suggested important roles of *MRH1*, *HSP30*, and *YRO2* in stress tolerance, particularly in response to acetic acid and other chemical stressors.

KCS1 encodes an inositol hexakisphosphate/heptakisphosphate kinase that synthesizes the 5-diphosphoinositol pentakisphosphate (5-IP7) and inositol octakisphosphate (IP8) (Taylor et al. 2012). These high-energy signaling molecules are involved in diverse cellular processes such as regulation of autophagy, DNA repair, cell wall synthesis, telomere maintenance, and phosphate homeostasis (Bennett et al. 2006; Onnebo and Saiardi 2007; Bhandari et al. 2007). The changes in phosphatidylinositol-mediated signaling pathways and phosphate metabolism caused by mutations in *STT4* (encodes phosphatidylinositol-4-kinase) and *VPS34* (encodes phosphatidylinositol-3-kinase) have

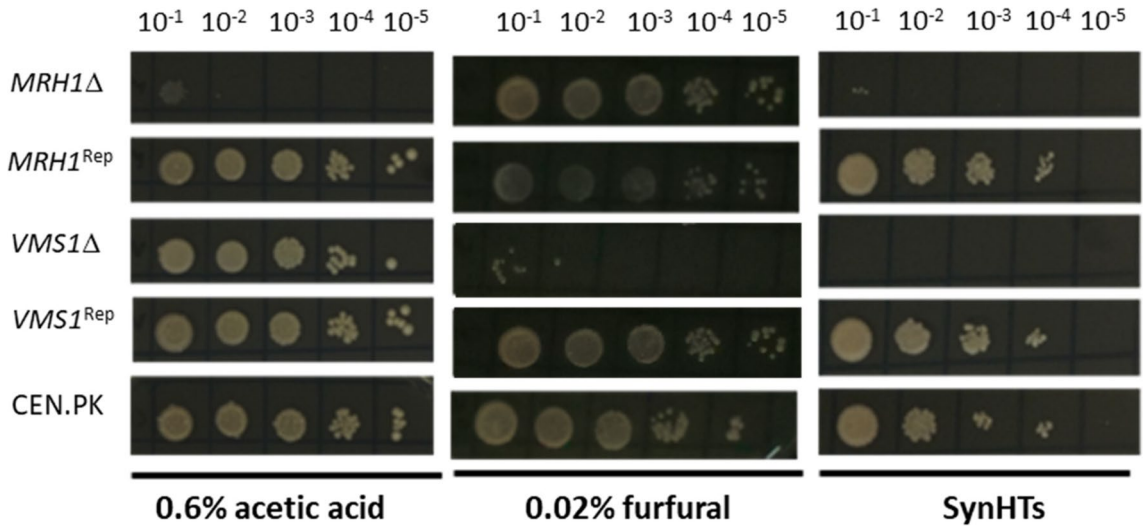

Fig. 4 Characterization of acetic acid, furfural, and synHTs tolerance of CEN.PK2-1C (CEN.PK); CEN.PK2-1C_*MRH1*Δ (*MRH1*Δ); CEN.PK2-1C_*MRH1* knock-in (*MRH1*^{rep}); CEN.PK2-1C_*VMS1*Δ (*VMS1*Δ); and CEN.PK2-1C_*VMS1* knock-in (*VMS1*.^{rep}). All strains were spotted at serial dilutions as indicated on the top of YPD plates containing 0.6% acetic acid, 0.02% furfural, or synHTs. Growth at 30 °C was recorded on day 3

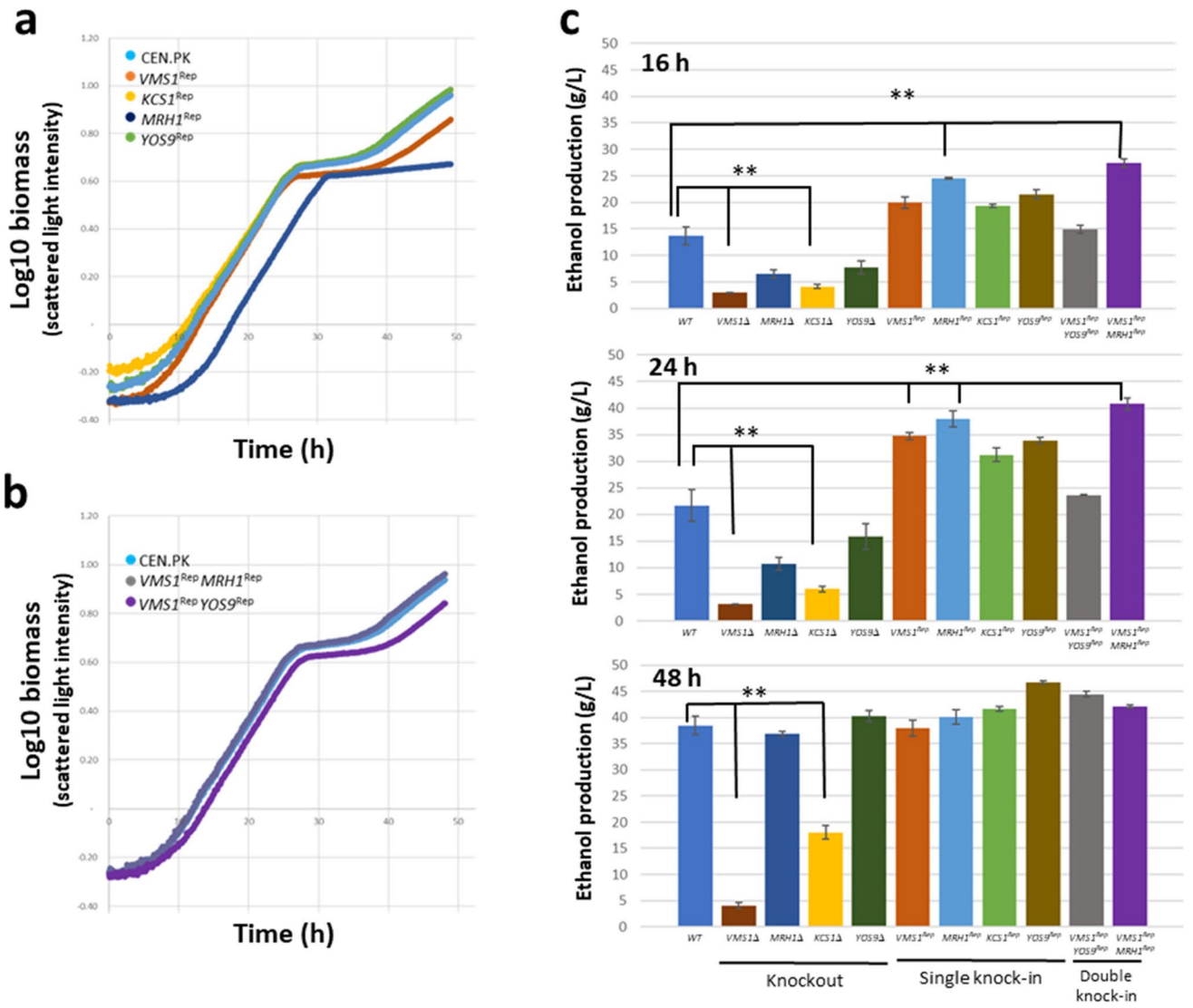

Fig. 5 Real-time growth measurement and ethanol production titers over 48 h. **a** Comparison of growth profiles of CEN.PK2-1C (CEN.PK), CEN.PK2-1C_*VMS1*^{Rep} (*VMS1*^{Rep}), CEN.PK2-1C_*KCS1*^{Rep} (*KCS1*^{Rep}), CEN.PK2-1C_*MRH1*^{Rep} (*MRH* ^{Rep}), and CEN.PK2-1C_*YOS9*^{Rep} (*YOS9*^{Rep}). **b** Comparison of growth profiles of CEN.PK2-1C, CEN.PK2-1C_*VMS1*^{Rep}*MRH1*^{Rep} (*VMS1*^{Rep} *MRH1*^{Rep}) and CEN.PK2-1C_*VMS1*^{Rep}*YOS9*^{Rep} (*VMS1*^{Rep} *YOS9*^{Rep}) strains. **c** Ethanol production titers of CEN.PK2-1C, knock-out, and knock-in strains at 16, 24, and 48 h of fermentation (asterisks indicate significant at 0.05 level). Data shown in **a**, **b**, and **c** are mean values obtained from three independent experiments; error bars represent standard deviations

also been reported in an iron-resistant strain of *S. cerevisiae* (Balaban et al. 2019).

From the available information on the function of the *VMS1*, *YOS9*, *MRH1*, and *KCS1* genes described above, we propose three pathways that are involved in response to hydrolysate toxin: (1) the ERAD pathway; (2) plasma membrane protein association; and (3) the phosphatidylinositol signaling system. The functions of these pathways in hydrolysate toxin sensitivity can be understood by considering the cellular stresses imposed by hydrolysate toxins. The aldehyde hydrolysate toxins furfural and hydroxymethyl furfural can induce the accumulation of reactive oxygen species (ROS) that cause damage to mitochondria, DNA, proteins, lipids, and the cytoskeleton (Allen et al. 2010). *S. cerevisiae* possesses NADPH and/or NADH cofactor-dependent aldehyde reductase/dehydrogenase enzymes that are capable of detoxifying aldehyde hydrolysate toxins. However, the action of these enzymes may be insufficient to prevent the accumulation of ROS-mediated damaged mitochondrial proteins triggered by aldehyde hydrolysate toxins, which must be removed by the action of the ERAD system and autophagy. Hydrolysate toxins, especially weak acids, can trigger osmotic stress (Guo and Olsson 2014). To counter this osmotic stress, *S. cerevisiae* maintains intracellular pH

through the proton efflux action of the plasma membrane H^+-ATPase (Holyoak et al. 1996). The redox imbalance and energy depletion caused by hydrolysate toxins subsequently affect cell growth and other cellular properties such as the cell turgor, amino acid pool, and mitochondrial integrity (López et al. 2021). The phosphatidylinositol signaling system is also vital for *S. cerevisiae* to maintain normal growth, cell wall integrity, and adaptation to hypersaline stress (Dubois et al. 2002). Overall, the oxidative, osmotic, and acid stresses caused by hydrolysate toxins deplete the energy supply to maintain the proper cellular processes, including the level of high-energy inositol-pyrophosphate molecules produced from the phosphatidylinositol signaling system, which are crucial for cell growth, membrane integrity, and homeostatic responses to osmotic stress in *S. cerevisiae* (Dubois et al. 2002). The *VMS1* and *MRH1* knock-in strains demonstrated significantly greater ethanol production titers in the presence of synHTs compared with the CEN.PK2-1C strain (Fig. 5c), suggesting that the variants originating from strain BCC39850 contribute to gain-of-function phenotypes. No marked synergistic effect was observed in the double knock-in experiment (Fig. 5c), suggesting that epistatic interactions controlling the tolerance trait are complex and dependent on the strain genetic background (Gasch et al. 2016; Sardi et al. 2016).

This work demonstrates the use of QTL mapping to identify genes associated with hydrolysate toxin tolerance in the natural *S. cerevisiae* strain BCC39850. *VMS1*, *YOS9*, *MRH*, and *KCS1* were thus identified for the first time as genes controlling hydrolysate toxin tolerance in *S. cerevisiae*. The functions of these genes suggest that the tolerance involves the ERAD pathway, plasma membrane protein association, and the phosphatidylinositol signaling system. The results allow us to better understand the molecular mechanisms of hydrolysate tolerance in *S. cerevisiae.* These can be used for further development of yeast strains with improved chemical tolerance for industrial applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-023-12843-3.

Acknowledgements The authors would like to thank Dr. Philip J. Shaw for critically reading this manuscript.

Author contribution WS conducted experiment; WS and CS analyzed data, ST, SI, WR, LE, VC, ST, PS, and VMS conceived and designed the research; WS wrote the manuscript. All authors have read and approved the manuscript.

Funding This research was funded by Wageningen University & Research and National Science and Technology Development Agency (P1952258), Thailand.

Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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