Novel strategy to improve vanillin tolerance and ethanol fermentation performances of *Saccharomycere cerevisiae* strains

Dao-Qiong Zheng, Xin-Na Jin, Ke Zhang, Ya-Hong Fang, Xue-Chang Wu

PII: S0960-8524(17)30069-X
DOI: http://dx.doi.org/10.1016/j.biortech.2017.01.040
Reference: BITE 17535

To appear in: *Bioresource Technology*

Received Date: 30 November 2016
Revised Date: 18 January 2017
Accepted Date: 20 January 2017

Please cite this article as: Zheng, D-Q., Jin, X-N., Zhang, K., Fang, Y-H., Wu, X-C., Novel strategy to improve vanillin tolerance and ethanol fermentation performances of *Saccharomycere cerevisiae* strains, *Bioresource Technology* (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.01.040

Novel strategy to improve vanillin tolerance and ethanol fermentation performances of *Saccharomycere cerevisiae* strains

Dao-Qiong Zheng[a,b,1], Xin-Na Jin[a, 1], Ke Zhang[a], Ya-Hong Fang[a], and Xue-Chang Wu[a] *

[a] College of Life Science, Zhejiang University, Hangzhou 310058, Zhejiang Province, China

[b] Ocean College, Zhejiang University, Zhoushan, Zhejiang Province, China

[1] These authors contributed equally to this work.

*Corresponding author. Tel: +86 571 88206636; Fax: +86 571 88206627; E-mail address: mblab@163.com.

Abstract

The aim of this work was to develop a novel strategy for improving the vanillin tolerance and ethanol fermentation performances of *Saccharomyces cerevisiae* strains. Isogeneic diploid, triploid, and tetraploid *S. cerevisiae* strains were generated by genome duplication of haploid strain CEN.PK2-1C. Ploidy increments improved vanillin tolerance and diminished proliferation capability. Antimitotic drug methyl benzimidazol-2-ylcarbamate (MBC) was used to introduce chromosomal aberrations into the tetraploid *S. cerevisiae* strain. Interestingly, aneuploid mutants with DNA contents between triploid and tetraploid were more resistant to vanillin and showed faster ethanol fermentation rates than all euploid strains. The physiological characteristics of these mutants suggest that higher bioconversion capacities of vanillin and ergosterol contents might contribute to improved vanillin tolerance. This study

demonstrates that genome duplication and MBC treatment is a powerful strategy to improve the vanillin tolerance of yeast strains.

Keywords: Bioethanol, vanillin, *Saccharomyces cerevisiae*, tolerance, ploidy

1. Introduction

Lignocellulosic materials, such as wood chips and agricultural residues, have been considered as potential sources for bioethanol production (Koutinas et al., 2016, Sarkar et al., 2012 and Srivastava et al., 2015). During lignocellulosic biomass pretreatment (e.g., dilute-acid hydrolysis), numerous by-products, including weak acids, furans, and phenolic compounds, are formed that can greatly inhibit yeast cell viability (Jönsson et al., 2013, Ravindran and Jaiswal, 2016 and Sindhu et al., 2016). Vanillin, a major phenolic aldehyde compound, has been reported to be a more effective inhibitor of ethanol fermentation than other lignocellulose-derived compounds (Klinke et al., 2004). An effective strategy for generating vanillin-tolerant yeast strains is a prerequisite to efficient yeast-based bioethanol production from lignocellulosic biomass (Ji et al., 2011 and Shen et al., 2014).

Vanillin can suppress translation by affecting the ribosome assembly process, causing accumulation of cytoplasmic mRNP granules and processing bodies (Iwaki et al., 2013). Furthermore, vanillin induces the accumulation of reactive oxygen species and mitochondrial fragmentation in *Saccharomyces cerevisiae* (Kim et al., 2014 and Nguyen et al., 2014). Multiple biological processes, such as chromatin remodeling, vesicle transport, and ergosterol biosynthesis, have been related to vanillin tolerance in *S. cerevisiae* (Endo et al., 2008 and Endo et al., 2009). Given that complex intracellular

processes are involved in vanillin-tolerant phenotypes, limited rational breeding strategies have been proposed to construct vanillin-tolerant yeast strains (Ji et al., 2011).

Previously, ours and other studies have shown that whole-chromosome or segmental aneuploidy phenomena are ubiquitous in industrial *S. cerevisiae* strains (Borneman et al., 2011, Dunn et al., 2012 and Zhang et al., 2016). Copy-number variations in certain DNA regions have been shown to improve fitness under specific conditions, such as ethanol, high salt concentration, lactic acid, and high temperature (Natesuntorn et al., 2015). Recently, Selmecki et al. (2015) described that polyploidy can accelerate evolutionary adaptation in yeast because it drives genomic instability and is inclined towards chromosome loss. In the present work, we developed a novel breeding strategy based on genome duplication and large-scale chromosomal variations to improve the vanillin tolerance and ethanol fermentation performance of *S. cerevisiae*.

2. Materials and methods

2.1. Yeast strains and medium

All *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. Isogeneic homozygous diploid (CEN-D), triploid (CEN-TR), and tetraploid (CEN-TE) strains were generated by cycles of mating-type switching and mating with haploid strain CEN.PK2-1C (CEN-H) (Fig. 1a). Yeast cells were cultured in yeast extract peptone dextrose (YPD) medium (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract; pH 5.0) at 30 °C. Yeast transformation was performed using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007).

2.2. Methyl benzimidazol-2-ylcarbamate (MBC) treatment and screening of vanillin-tolerant mutants

Tetraploid strain CEN-TE was cultured in YPD (5 mL) for 18 h at 30 °C and then resuspended in fresh YPD medium (25 mL) containing MBC (80 mg/L). The cells were incubated for 12 h and then plated onto YPD plates containing vanillin (10 mM). The colonies (~80) that appeared first were selected for further analysis.

To determine the vanillin tolerance of mutants, yeast cells were grown in YPD medium (25 mL) for 18 h. Tenfold serial dilutions of each sample (4 μL) were spotted onto YPD plates with different concentrations of vanillin. The plates were incubated at 30 °C for 2–4 days.

2.3. Microarray-based comparative genomic hybridization (aCGH)

Agilent yeast DNA 8×15K microarrays (AMID 028943) were used to determine variations in chromosomal numbers in aneuploidy yeast strains. Genomic DNA was extracted from the control strain and mutants, and sheared to fragments with an average size of 500 bp by sonication. DNA fragments were separated by agarose gel electrophoresis and the average size was then determined by software Quantity One (Bio-Rad, Hercules, CA, USA). Control DNA and experimental DNA were labeled as dUTP-Cy3 and dUTP-Cy5, respectively, using the Invitrogen BioPrime array CGH labeling system. The labeled DNAs were cohybridized onto microarray slides. The hybridized microarray slides were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and the hybridization signal was quantified using GenePix 6.0 software. The aCGH experiment was previously described by Zhang et al. (2013).

2.4. Ethanol fermentation

The normal ethanol fermentation medium contained glucose (100 g/L), peptone (10 g/L), and yeast extract (5 g/L) at pH 4.5. Vanillin (9 mM) was added into the

fermentation medium as required. Yeast cells were pre-cultured in YPD (25 mL) for 24 h at 30 °C [initial OD_{600} of 0.05; measured by a Ultrospec 3300 UV/VIS spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA) using a cuvette that is 1 cm in path length]. Yeast cells were then collected by centrifugation (6,000 rpm for 5 min) and transferred to 250-mL conical flasks containing fermentation medium (80 mL; initial OD_{600} of 1). Fermentation was performed under anaerobic conditions at 33 °C at 110 rpm for 50 h. Glucose and ethanol in the fermentation medium were measured by high-performance liquid chromatography (HPLC), as described previously (Wang et al., 2012).

2.5. HPLC analysis of vanillin and vanillin derivatives

Vanillin, vanillyl alcohol, and vanillic acid were detected by HPLC (Agilent 1200, USA) equipped with an Eclipse C18 column (250 mm × 4.6 mm, 5 μm) and a UV detector (Agilent Technologies, Santa Clara, CA, USA). Samples were filtered through a 0.45-μm Millipore membrane before loading. The mobile phase was a mixture of methanol, water, and acetic acid (35:65:0.05) at a flow rate of 0.8 mL/min. The C18 column was kept at 30 °C and the detection wavelength was 280 nm.

2.6. Measurement of ergosterol content

Yeast cells cultured overnight were transferred into 25 mL of fresh YPD medium and YPD medium containing 9 mM vanillin (initial OD_{600} of 2), and harvested after incubation for 12 h. Ergosterol was extracted and measured using an HPLC method, as previously described (Endo et al., 2009).

3. Results and discussion

3.1. Relationship between ploidy and vanillin tolerance in S. cerevisiae

Although ploidy modification is an effective strategy to improve the ethanol production or fermentation product quality in industrial yeast stains (Hashimoto et al., 2006, Higgins et al., 2001 and Yamada et al., 2010), the relationship between ploidy and vanillin resistance in *S. cerevisiae* is unknown. To generate polyploidy *S. cerevisiae* strains, plasmid (pSH-HO)-carrying gene *HO*, which functions as a site-specific endonuclease, was used to switch the mating type of the *S. cerevisiae* strains (Table 1). By cycling mating-type switching and mating, as shown in Fig. 1a, *S. cerevisiae* strains ranging from diploidy to tetraploidy were obtained. The ploidies of these constructed strains were confirmed by flow cytometry (data not shown).

The growth capabilities of haploid (CEN-H), diploid (CEN-D), triploid (CEN-TR), and tetraploid (CEN-TE) strains were determined by measuring the biomass formation (OD_{600}) of the yeast cells. In liquid YPD medium, haploid and diploid strains showed similar growth rates in the exponential phase, but behaved diversely after entering diauxic shift (Fig. 1b). Polyploid (triploid and tetraploid) strains had lower cell proliferation abilities and formed less biomass than haploid and diploid strains (Fig. 1b and Table 1). In the presence of vanillin, yeast cell growth was significantly inhibited. The lag phases of these four strains were extended to more than 22 h (Fig. 1c and Table 1). The triploid strain had the shortest lag phase and formed most biomass within 75 h, while the haploid and tetraploid strains were the last to enter exponential growth (Fig. 1c). With the extension of incubation time, haploid and diploid strains still formed more biomass and thus showed higher growth rates than polyploid strains (Table 1). These observations indicated that changes in ploidy dramatically affected the proliferation ability and the vanillin resistance of yeast cells.

3.2. Generation of aneuploid S. cerevisiae strains from CEN-TE

Aneuploidy is a phenomenon commonly observed in *S. cerevisiae* strains, but it is unclear whether certain chromosomal aberrations affect vanillin tolerance. To determine this, multiple aneuploidy strains were generated by MBC treatment, using tetraploid CEN-TE as the parental strain. MBC was shown to have a unique affinity for tubulin and inhibited microtubule polymerization, thus eliciting enhanced levels of aneuploidy (Whittaker et al., 1988). In addition, the effects of MBC on small DNA mutations, such as point mutations, and small insertions and deletions, have not been reported. After exposure to MBC, the yeast mutants that appeared first on YPD plates containing 10 mM vanillin were selected. Eight mutants (labeled TE3, TE7, TE8, TE9, TE12, TE13, TE14, and TE15) showed significantly improved vanillin tolerance over the euploid strains, and were selected for further analysis.

Using the aCGH array, genomic structural variations in the eight mutants were identified. As expected, at least one chromosomal number variation event occurred in an individual mutant (Fig. 2a). Most of the aneuploid events were copy-number changes of whole chromosomes, except for one internal duplication event that occurred on chromosome X in mutant TE14 (Fig. 2b). This duplication event resulted in the copy number increasing (from 4 to 5) for genes located in the DNA region between 353 kb and 537 kb in this chromosome (Fig. 2b). Interestingly, the loss of one copy of chromosome IV, the longest chromosome in the yeast genome, was observed in all eight mutants. Additionally, five of the eight mutants lost one copy of chromosome I. Strain TE13 had the most chromosomal aberration events, with the copy numbers of chromosomes VII, XIV, and XV reduced to three in this strain. These results demonstrated MBC treatment would elicit a high incidence of aneuploidy events in a polyploid strain. Previously, our studies suggested that both “general aneuploid

response" and copy-number variations in certain genes would contribute to phenotypic changes in the industrial aneuploidy of *S. cerevisiae* strains (Zhang et al., 2016 and Zheng et al., 2014). It was assumed that certain aneuploidy events, such as loss of one copy of chromosome IV, would modify the general physiological status and/or change the expression levels of specific genes associated with vanillin tolerance. However, the detailed mechanisms causing the improved vanillin tolerance in the mutants in this work still need further investigation.

3.3. Ethanol fermentation in aneuploid strains in the presence of vanillin

In the normal fermentation medium, euploid strains (CEN-H, CEN-D, CEN-TR, and CEN-TE) and aneuploid mutants both consumed all glucose and produced similar amounts of ethanol (~43 g/L) within 36 h (Fig. 3a). In the presence of vanillin, the fermentation rates of these strains were diminished (Fig. 3b), which was consistent with the observation that cell growth was greatly inhibited by vanillin (Fig. 1c). Among the euploid strains, triploid strain CEN-TR showed the fastest ethanol production rate. At the 36-h time point, the triploid strain had produced 37 g/L of ethanol, which was 13% lower than in the absence of vanillin (Fig. 3b, $p < 0.05$, t-test). In contrast, all aneuploid mutants (TE) achieved complete fermentation within 36 h and produced more ethanol than euploid strains (Fig. 3c, $p < 0.05$, Tukey test). These results suggested that the breeding strategy developed in this work could efficiently improve the ethanol fermentation rates of yeast strains in the presence of vanillin.

3.4. Bioconversion of vanillin in euploid and aneuploid strains

Bioconversion is one of the most important methods for eliminating cellular damage by toxic compounds. In *S. cerevisiae*, the major bioconversion products of vanillin catalyzed by non-specific dehydrogenase enzymes were vanillyl alcohol and vanillic

acid, which are both less toxic than vanillin (Fitzgerald et al., 2003). The vanillin bioconversion capabilities of the euploid and aneuploid mutants were determined in YPD medium containing 9 mM of vanillin (initial OD_{600} of 2; cell numbers and viabilities were stable during this period for all strains) by detecting residual vanillin and vanillin derivatives after 12 h. The triploid strain showed a greater ability to convert vanillin to vanillyl alcohol and vanillic acid than the other euploid strains (Table 3, $p < 0.05$, Tukey test). At 12 h, the triploid strain consumed 23%, 14%, and 28% more vanillin than that of haploid, diploid, and tetraploid strains, respectively (Table 3; $p < 0.05$, Tukey test). Accordingly, the vanillyl alcohol concentration in the CEN-TR culture was higher than those in other euploid strains (Table 3; $p < 0.05$, Tukey test). However, the vanillic acid concentrations were similar for all euploid strains. Compared to triploid strain, certain aneuploid mutants were found to convert even more vanillin after 12 h (Table 3). For example, residual vanillin in the mutant TE15 culture was 40% lower than that in triploid strain CEN-TR. In contrast, TE15 formed 66% more vanillyl alcohol than CEN-TR (Table 3; $p < 0.05$, Tukey test). No differences in vanillic acid formation were observed among the aneuploid mutants (Table 3; $p < 0.05$, Tukey test). These results demonstrated that certain aneuploid mutants had a significantly higher capacity to convert vanillin to vanillyl alcohol. Previously, certain dehydrogenases (such as Adh1p, Adh6p, Ald6p, and Ari1p) and reductases (Ynl134cp and Yjr096wp) have been identified contributing to the vanillin reduction capability in yeast (Wang et al, 2016). It was probably that the higher ability of vanillin bioconversion in the aneuploidy strains was explained by the enhanced activities of these dehydrogenases and/or reductases.

3.5. Ergosterol contents in euploid strains and aneuploid mutants

Ergosterol is a major constituent of cellular membranes in *S. cerevisiae*. Null mutation of the genes involved in the ergosterol biosynthesis pathway results in vanillin-sensitive phenotypes (Endo et al., 2008). When grown in liquid YPD medium, the ergosterol contents in the haploid and triploid strains were slightly lower than those in the diploid and tetraploid strains (Fig. 4, $p < 0.05$, Tukey test). Vanillin in the YPD medium significantly decreased the ergosterol contents of the four euploid strains, but to varying degrees (21%–42%) in different strains (Fig. 4, $p < 0.05$, Tukey test). Among the euploid strains, the tetraploid strain maintained the highest ergosterol content when treated with vanillin (Fig. 4). In the YPD medium, only aneuploid mutant TE14 had higher ergosterol content than that of the euploid strain (Fig. 4, $p < 0.05$, Tukey test). Nevertheless, all aneuploid mutants had higher ergosterol contents than any euploid strain in the presence of vanillin (Fig. 4, $p < 0.05$, Tukey test). Interestingly, in contrast to the euploid strains, in which vanillin decreased the ergosterol content, TE3 and TE12 showed ergosterol contents increased by 20% and 29%, respectively ($p < 0.05$, Tukey test). Therefore, the ergosterol contents of these two mutants were ~1.5-fold that of tetraploid strain CEN-TE in the vanillin-containing medium. These results suggested that the ability to maintain ergosterol content might, at least in part, explain improved vanillin tolerance in the selected aneuploid strains. Since there are multiple genes involved in ergosterol biosynthesis pathway and the altered expression of an individual gene had a limited effect on ergosterol content (Zhang et al, 2016), the breeding strategy in this work might modify the flux through the whole pathway.

4. Conclusions

The effects of changing yeast cell ploidy on vanillin tolerance have been reported for the first time, with the triploid strain confirmed to be the most tolerant to vanillin. A

novel strategy was developed, in which genome duplication and MBC treatment were integrated to improve vanillin resistance in *S. cerevisiae*. In the aneuploid mutants obtained using this method, both growth and ethanol fermentation rates were enhanced in the presence of vanillin. Higher vanillin bioconversion abilities and ergosterol contents were possible physiological mechanisms causing the improved phenotypes in the vanillin-resistant mutants.

Acknowledgement

We thank Thomas D. Petes for technological supports of aCGH analysis. This work was supported by National Natural Science Foundation of China (31370132 and 31401058) and State Key Laboratory of Motor Vehicle Biofuels Technology of China.

References

Borneman A.R., Desany B.A., Riches D., Affourtit J.P., Forgan A.H., Pretorius I.S., Egholm M., Chambers P.J. 2011. Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. PLoS Genet. 7, e1001287.

Dunn B., Richter C., Kvitek D.J., Pugh T., Sherlock G. 2012. Analysis of the Saccharomyces cerevisiae pan-genome reveals a pool of copy number variants distributed in diverse yeast strains from differing industrial environments. Genome Res. 22, 908-924.

Endo A., Nakamura T., Ando A., Tokuyasu K., Shima J. 2008. Genome-wide screening of the genes required for tolerance to vanillin, which is a potential inhibitor of bioethanol fermentation, in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 1, 1-6.

Endo A., Nakamura T., Shima J. 2009. Involvement of ergosterol in tolerance to

vanillin, a potential inhibitor of bioethanol fermentation, in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 299, 95-99.

Fitzgerald D.J., Stratford M., Narbad A. 2003. Analysis of the inhibition of food spoilage yeasts by vanillin. Int. J. Food Microbiol 86, 113-122.

Gietz R.D., Schiestl R.H. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2, 31-34.

Gueldener U., Heinisch J., Koehler G.J., Voss D., Hegemann J.H. 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic. Acids. Res. 30, e23.

Iwaki A., Ohnuki S., Suga Y., Izawa S., Ohya Y. 2013. Vanillin inhibits translation and induces messenger ribonucleoprotein (mRNP) granule formation in *Saccharomyces cerevisiae*: application and validation of high-content, image-based profiling. PLoS ONE 8, e61748.

Ji L., Shen Y., Xu L., Peng B., Xiao Y., Bao X. 2011. Enhanced resistance of *Saccharomyces cerevisiae* to vanillin by expression of lacA from *Trametes sp*. AH28-2. Bioresour. Technol. 102, 8105-8109.

Jönsson L.J., Alriksson B., Nilvebrant N.-O. 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels 6: 1-10.

Kim J.H., Lee H.O., Cho Y.J., Kim J., Chun J., Choi J., Lee Y., Jung W.H. 2014. A vanillin derivative causes mitochondrial dysfunction and triggers oxidative stress in cryptococcus neoformans. PLoS ONE 9, e89122.

Klinke H.B., Thomsen A.B., Ahring B.K. 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biot. 66, 10-26.

Koutinas A., Kanellaki M., Bekatorou A., Kandylis P., Pissaridi K., Dima A., Boura K., Lappa K., Tsafrakidou P., Stergiou P.-Y. 2016. Economic evaluation of technology for a new generation biofuel production using wastes. Bioresour. Technol. 200, 178-185.

Natesuntorn W., Iwami K., Matsubara Y., Sasano Y., Sugiyama M., Kaneko Y., Harashima S. 2015. Genome-wide construction of a series of designed segmental aneuploids in *Saccharomyces cerevisiae*. Sci Rep 5, 12510.

Nguyen T.T.M., Iwaki A., Ohya Y., Izawa S. 2014. Vanillin causes the activation of Yap1 and mitochondrial fragmentation in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 117, 33-38.

Ravindran R., Jaiswal A.K. 2016. A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: challenges and opportunities. Bioresour. Technol. 199, 92-102.

Sarkar N., Ghosh S.K., Bannerjee S., Aikat K. 2012. Bioethanol production from agricultural wastes: An overview. Renew. Energ. 37, 19-27.

Selmecki A.M., Maruvka Y.E., Richmond P.A., Guillet M., Shoresh N., Sorenson A.L., De S., Kishony R., Michor F., Dowell R., Pellman D. 2015. Polyploidy can drive rapid adaptation in yeast. Nature 519, 349-352.

Shen Y., Li H., Wang X., Zhang X., Hou J., Wang L., Gao N., Bao X. 2014. High vanillin tolerance of an evolved *Saccharomyces cerevisiae* strain owing to its enhanced vanillin reduction and antioxidative capacity. J. Ind. Microbiol. Biot 41, 1637-1645.

Sindhu R., Binod P., Pandey A. 2016. Biological pretreatment of lignocellulosic biomass–An overview. Bioresour. Technol. 199, 76-82.

Srivastava N., Rawat R., Singh Oberoi H., Ramteke P.W. 2015. A review on fuel ethanol production from lignocellulosic biomass. Int. J. Green Energy 12, 949-960.

Wang P.M., Zheng D.Q., Liu T.Z., Tao X.L., Feng M.G., Min H., Jiang X.H., Wu X.C. 2012. The combination of glycerol metabolic engineering and drug resistance marker-aided genome shuffling to improve very-high-gravity fermentation performances of industrial *Saccharomyces cerevisiae*. Bioresour. Technol. 108, 203-210.

Wang, X., Liang, Z., Hou, J., Bao, X., Shen, Y. 2016. Identification and functional evaluation of the reductases and dehydrogenases from *Saccharomyces cerevisiae* involved in vanillin resistance. BMC Biotechnol. 16, 31.

Whittaker S.G., Rockmill B.M., Blechl A.E., Maloney D.H., Resnick M.A., Fogel S. 1988. The detection of mitotic and meiotic aneuploidy in yeast using a gene dosage selection system. Mol. Gen. Genet. 215, 10-18.

Zhang H., Zeidler A.F.B., Song W., Puccia C.M., Malc E., Greenwell P.W., Mieczkowski P.A., Petes T.D., Argueso J.L. 2013. Gene copy-number variation in haploid and diploid strains of the yeast *Saccharomyces cerevisiae*. Genetics 193, 785-801.

Zhang K., Zhang L.J., Fang Y.H., Jin X.N., Qi L., Wu X.C., Zheng D.Q. 2016. Genomic structural variation contributes to phenotypic change of industrial bioethanol yeast *Saccharomyces cerevisiae*. FEMS Yeast Res. 16, fov118.

Zheng D.Q., Chen J., Zhang K., Gao K.H., Li O., Wang P.M., Zhang X.Y., Du F.G., Sun P.Y., Qu A.M., Wu S., Wu X.C. 2014. Genomic structural variations contribute to trait improvement during whole-genome shuffling of yeast. Appl. Microbiol. Biot. 98, 3059-3070.

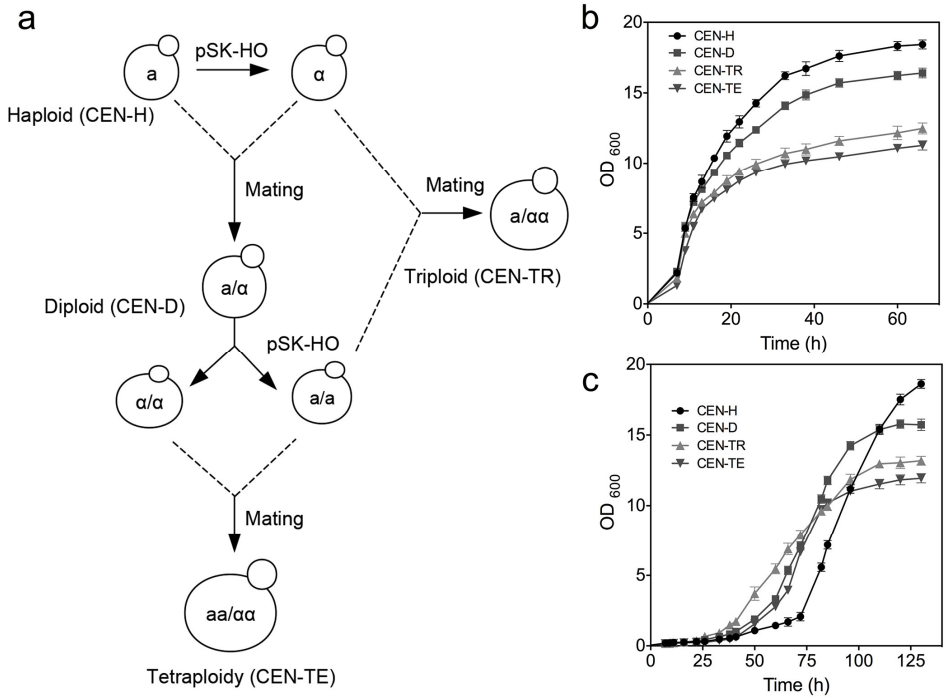
Figure captions

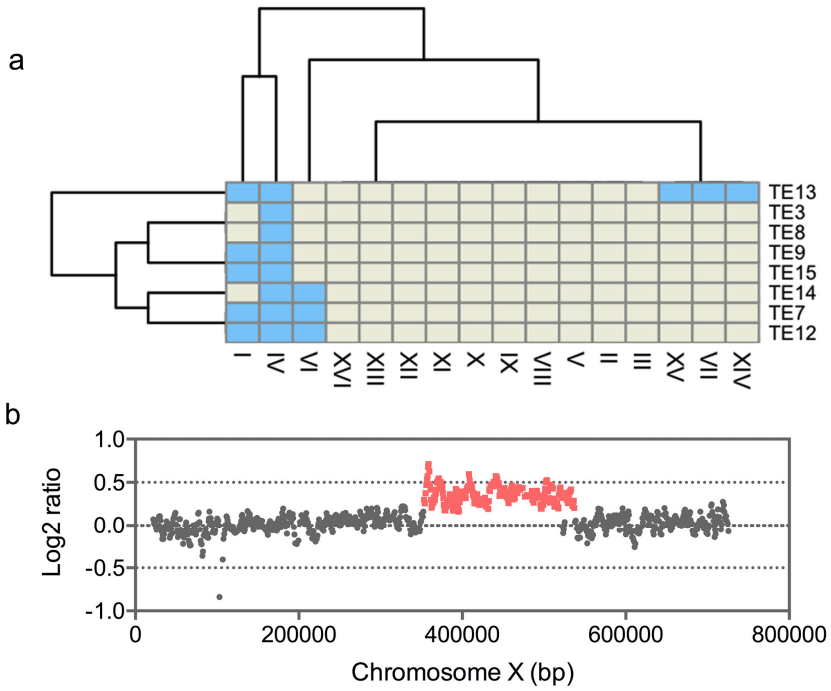
Fig. 1. Different phenotypes of *S. cerevisiae* strains with different ploidies. (a) Strategies for the generation of isogeneic homozygous diploid, triploid, and tetraploid strains from haploid strain CEN-H. (b) Growth of haploid (CEN-H), diploid (CEN-D), triploid (CEN-TR), and tetraploid (CEN-TE) strains in YPD medium (25 mL) at 30 °C with an initial OD_{600} of 0.05. (c) Growth of four euploid strains in YPD (25 mL) with vanillin (9 mM) at 30 °C. Data from three biological repetitions are shown as mean±standard deviation (SD).

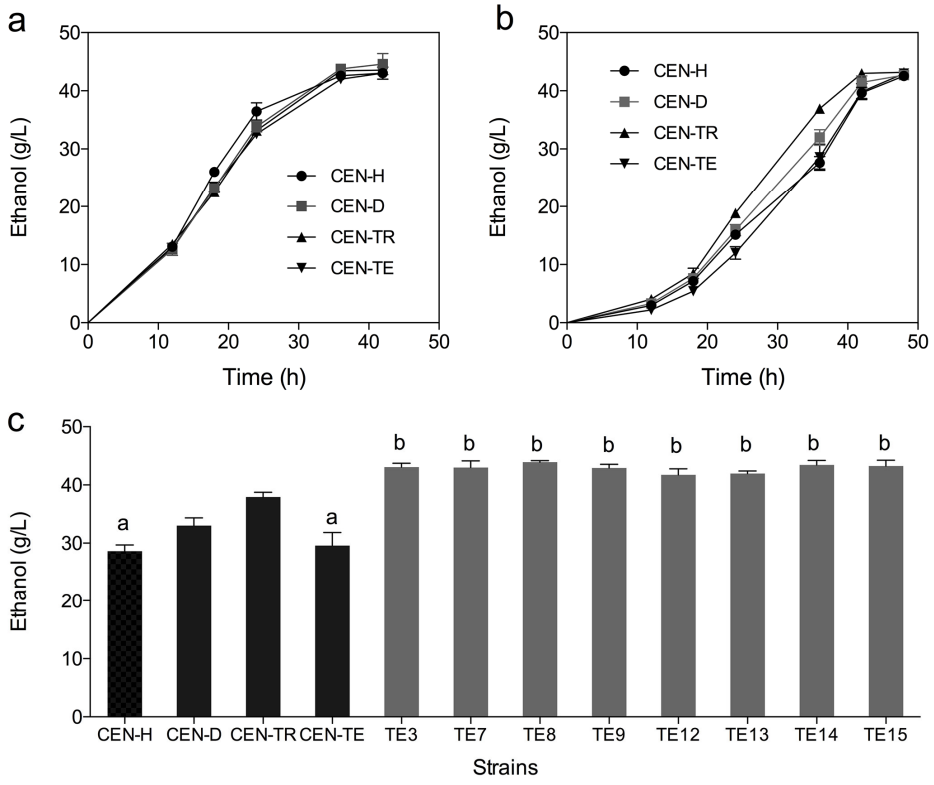
Fig. 2. Genomic structural variations in vanillin-resistant mutants. (a) Two-way hierarchical clustering analysis of aneuploidy events performed using an R package "pheatmap" based on Euclidean distance. Horizontal clustering indicates colonies with similar aneuploidy patterns, while vertical clustering indicates chromosomes with similar events. Blue and ivory rectangles represent three or four copies of certain chromosomes in an individual mutant. (b) Segmental duplication event (pink) that occurred on chromosome X in mutant TE14. Gray points represent normal DNA regions without copy-number variations.

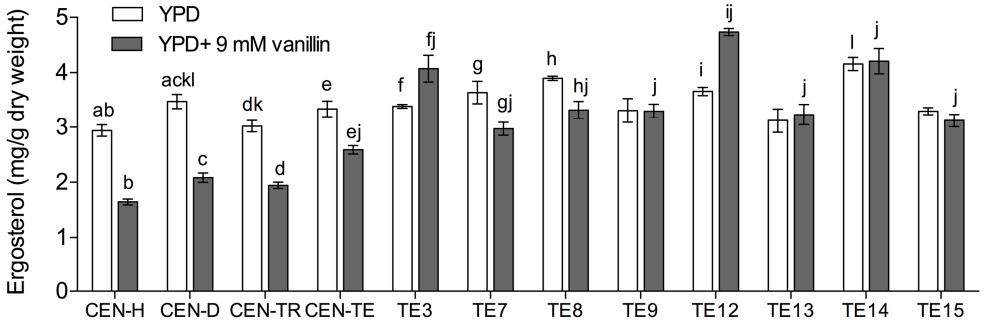
Fig. 3. Ethanol fermentation performances of euploid and aneuploid strains. (a) Ethanol fermentation of euploid strains CEN-H, CEN-D, CEN-TR, and CEN-TE in normal fermentation medium. (b) Ethanol productions of euploid strains in vanillin-containing medium. (c) Comparison of ethanol productions of euploid and aneuploid strains within 36 h in the fermentation medium containing 9 mM vanillin. Data from three biological repetitions are shown as mean ± standard deviation (SD). Mean values labeled with the same letter were not significantly different (Tukey test, $p > 0.05$).

Fig. 4. Ergosterol contents of CEN-H, CEN-D, CEN-TR, CEN-TE, and eight evolved mutants from CEN-TE. Data from three biological repetitions are shown as mean ± SD. Mean values labeled with the same letter were significantly different (Tukey test, $p < 0.05$). For example, "a" indicates the ergosterol content of strain CEN-D was higher than that of CEN-H.









Tables

Table 1 *Saccharomyces cerevisiae* strains and plasmids used in this study.

Strains or plasmids	Description	Reference
CEN-H (CEN.PK2-1C)	*MAT*a*; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2*	EUROSCARF
CEN-D	*MAT*a/α*; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2*	This study
CEN-TR	*MAT*aa/α*; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2*	This study
CEN-TE	*MAT*aa/αα*; ura3-52; trp1-289; leu2-3_112; his3Δ1; MAL2-8C; SUC2*	This study
pSH47	*URA3; Amp*^{R}	(Gueldener et al., 2002)
pSH-HO [a]	p*GAL-HO; URA3; Amp*^{R}	This study

[a] Plasmid constructed by inserting *HO* gene after the promoter *GAL1* into plasmid pSH47.

Table 2 Comparison of growth rate, lag time, and maximum biomass formation of euploid strains in YPD and vanillin containing YPD medium

Strains	YPD			YPD + Vanillin		
	GR[a]	LP (h) [b]	MaxOD[c]	GR	LP (h)	MaxOD
CEN-H	0.28	3.5[d]	18.44	0.14	38.0[f]	18.54
CEN-D	0.25	3.5[d]	16.31	0.13	31.0	15.83
CEN-TR	0.19	3.7[d]	12.49	0.11	22.3	13.18
CEN-TE	0.17	4.0[d]	11.28	0.10	39.0[f]	11.94
Pooled SD	0.005	0.289	0.344	0.003	0.289	0.331

[a-c] GR, LP, and MaxOD represents growth rate (defined as increased value of OD_{600} per hour), lag time (defined as the period between incubation and the time point with an OD_{600} of 0.5), and maximum biomass formation (measured by OD_{600} after 66 h for YPD and after 130 h for YPD + vanillin medium), respectively. The incubation conditions of yeast cells in this experiment were the same as described in Fig. b and Fig. c. Sampling interval time is 0.5 h and 1 h for YPD and YPD + vanillin medium, respectively.

[e-f] Mean values labeled with the same letter were not significantly different ($p > 0.05$, Tukey test).

Table 3 Comparison of vanillin bioconversion in euploid and aneuploid mutants.

Strains	Metabolites (mM) *		
	Consumed vanillin	Vanillyl alcohol	Vanillic acid
CEN-H	3.37[a]	2.49[a]	1.03[a]
CEN-D	3.78	2.95	1.07[a]
CEN-TR	4.39[b]	3.52	1.01[a]
CEN-TE	3.15[a]	2.32[a]	0.98[a]
TE3	5.76[c]	5.40	1.11[a]
TE7	5.67[bd]	5.15[b]	1.02[a]
TE8	4.80[be]	4.10[c]	1.04[a]
TE9	4.77[bef]	4.21[cd]	1.01[a]
TE12	4.98[befg]	4.37[d]	1.02[a]
TE13	4.48[befg]	3.86	1.03[a]
TE14	5.85[cd]	5.11[b]	1.08[a]
TE15	6.71	5.84	1.07[a]
Pooled SD	0.276	0.319	0.074

* Cells were grown in the YPD containing vanillin (9 mM) in 250-mL shake flasks with medium (50 mL) at 30 °C with an initial OD_{600} of 2.

[a-g] Mean values labeled with the same letter were not significantly different ($p > 0.05$, Tukey test).

Highlights:

- A novel strategy to improve vanillin tolerance was proposed.
- Triploid yeast strain is more tolerant to vanillin than other euploid strains.
- MBC treatment of polyploidy strains generated aneuploidy mutants.
- Certain aneuploid mutants showed faster fermentation rates than euploid strains.
- Bioconversion ability and ergosterol content contribute to vanillin tolerance.