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Novel Approach in the Construction of Bioethanol-Producing *Saccharomyces cerevisiae* Hybrids[§]

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SUMMARY

Bioethanol production from lignocellulosic hydrolysates requires a producer strain that tolerates both the presence of growth and fermentation inhibitors and high ethanol concentrations. Therefore, we constructed heterozygous intraspecies hybrid diploids of *Saccharomyces cerevisiae* by crossing two natural *S. cerevisiae* isolates, YIIc17_E5 and UWOPS87-2421, a good ethanol producer found in wine and a strain from the flower of the cactus *Opuntia megacantha* resistant to inhibitors found in lignocellulosic hydrolysates, respectively. Hybrids grew faster than parental strains in the absence and in the presence of acetic and levulinic acids and 2-furaldehyde, inhibitors frequently found in lignocellulosic hydrolysates, and the overexpression of *YAP1* gene increased their survival. Furthermore, although originating from the same parental strains, hybrids displayed different fermentative potential in a CO_2 production test, suggesting genetic variability that could be used for further selection of desirable traits. Therefore, our results suggest that the construction of intraspecies hybrids coupled with the use of genetic engineering techniques is a promising approach for improvement or development of new biotechnologically relevant strains of *S. cerevisiae*. Moreover, it was found that the success of gene targeting (gene targeting fidelity) in natural *S. cerevisiae* isolates (YIIc17_E5α and UWOPS87-2421α) was strikingly lower than in laboratory strains and the most frequent off-targeting event was targeted chromosome duplication.

Key words: yeast *Saccharomyces cerevisiae*, intraspecies hybrids, lignocellulosic hydrolysates, growth and fermentation inhibitors, gene targeting

INTRODUCTION

The requirement for the fuel is constantly increasing and the production of biofuels (bioethanol, biodiesel and biobutanol) from renewable sources has become more frequent in the last several decades. Biofuels are usually classified depending on the source of material used for production (*1*). Biofuels of the first generation are made by fermentation of raw starch-containing material that can be also used for human consumption, resulting in both expensive and unethical production. Biofuels of the second generation are made using renewable lignocellulosic waste, while third generation biofuels are made from algae and microbial biomass (*1*).

Although lignocellulosic waste consists of cellulose (35-50 %), hemicellulose (20-35 %) and lignin (5-30 %), its exact composition depends on the origin of the material (*2*). Cellulose is a linear polymer of glucose, hemicellulose consists of D-xylose, L-arabinose, D-glucose, D-galactose and D-glucuronic acid, while lignin consists of *p*-coumaryl, conypheryl and synapyl alcohol (*3*). Since microorganisms are not able to ferment raw lignocellulosic material directly, it is first pretreated to release compounds that can be further hydrolysed and then used by yeasts for fermentation. Although there are different methods of pretreatment (chemical, physical, physicochemical and enzymatic), the choice of method is still one of the main challenges resulting in hydrolysates whose chemical composition depends both on the type of starting material and the type of pretreatment. The pretreatment does not result in a complete degradation of a starting material releasing

fermentable sugars, it also generates different compounds that act as growth and fermentation inhibitors. Hydrolysis of cellulose releases glucose, while hydrolysis of hemicellulose releases xylose, arabinose, galactose and glucose. Further degradation of xylose results in the formation of 2-furaldehyde (furfural), and the degradation of mannose, galactose and glucose releases 5-hydroxymetylfurfural (HMF). Acetyl groups of hemicellulose form acetic acid, while formic and levulinic acid are released during the pretreatment of 5-hydroxymethylfurfural and 2-furaldehyde. Although aliphatic acids can be used by yeast as a carbon source if their concentration is below 100 mM (*4*), when present in higher concentrations they enter cells, dissociate and decrease intracellular pH resulting in the increase of a doubling time (*3*). Phenols released during the pretreatment have different effects on yeast because their inhibitory activity depends on functional side groups and the number of unsaturated bonds between carbon atoms (*5*). The 2-furaldehyde decreases both the growth rate and ethanol yield, but its inhibitory effect also depends on cell density, conditions of cultivation and aeration (*6*). Comparative analysis of four different lignocellulose hydrolysates showed that 2-furaldehyde was the most toxic compound (*7*). Ethanol is the main product of fermentation and it changes the expression of genes involved in ionic homeostasis, trehalose synthesis and antioxidant defence, and of those encoding heat shock proteins (*8*). Moreover, each inhibitor alone negatively influences yeast cell growth but the combination of acetic acid, aldehydes and furan compounds or acetic acid and 2-furaldehyde synergistically decreases the growth rate (*9,10*).

Since the fermentation inhibitors are released regardless of the type of pretreatment, there have been different approaches to increase the resistance of producer strains. These approaches range from the use of different industrial strains (*11*) to the adaptation of the strains to the presence of inhibitors (*12,13*) or by different genetic modifications of the producer strains. Both endogenous yeast genes and genes from different species have been expressed in *S. cerevisiae* in order to increase the resistance of producer strains. Expression of *LCC2* gene from *Trametes versicolor* in *S. cerevisiae* allowed simultaneous detoxification and fermentation (*14*), while overexpression of genes from pentose phosphate pathway conferred tolerance to weak acids (*15*). Alriksson *et al.* (*16*) constructed strains that overexpressed endogenous *ATR1, FLR1* or *YAP1* gene, involved in multidrug resistance or stress tolerance, and the most positive effect was observed after overexpression of *YAP1* gene that encodes transcription factor involved in stress response. Ask *et al.* (*17*) overexpressed genes involved in glutathione synthesis resulting in the resistance to inhibitors and an increase in ethanol production.

In this work, we constructed intraspecies hybrids of *S. cerevisiae* strains by mating haploids of two natural isolates having different properties desirable for bioethanol producer, such as high ethanol production and resistance to several growth and fermentation inhibitors. The constructed hybrid diploids survived better than both parental haploids and diploids in the presence of acetic acid, levulinic acid and 2-furaldehyde. In order to further increase the survival of constructed strains, the influence of the overexpression of *ATR1, FLR1, YAP1* and *GSH1* genes was tested. Although there was a difference in the increase of the survival of different strains, the overexpression of *YAP1* gene had a more positive overall effect than the overexpression of *ATR1* gene, whereas the overexpression of *FLR1* or *GSH1* gene did not have any significant influence. Moreover, constructed intraspecies hybrids displayed genetic variability that could be used for further selection of desirable traits. Therefore, construction of intraspecies hybrids is a promising approach for an improvement or development of new biotechnologically relevant *S. cerevisiae* strains.

MATERIALS AND METHODS

Plasmids

Plasmids used in this study are listed in **Table S1**. The plasmid pRED150 (*18*) was used to change the genotype of parental YIIc17_E5α and UWOPS87-2421α strains from *ADE2 ura3* (phenotype Ade$^+$ Ura$^-$) to *ade2 URA3* (phenotype Ade$^-$ Ura$^+$) by plasmid integration in *ADE2* locus (**Fig. 1**). This plasmid contains a 150 bp long perfect palindrome which strikingly enhances plasmid pop-out (*19*) and is used to restore original *ADE2 ura3* genotype in diploids constructed by mating (**Fig. 2** (*19, 20*)). Plasmid pRED150 is constructed from pAB9-150 (*19*) by replacing *CYC1* region with 1.1-kb central part of the *ADE2* gene (from EcoRV to DraI cut site), whereas the approach for a perfect palindrome construction is described by Svetec *et al.* (*21*). All other plasmids used in this work were constructed by PCR amplification, using Q5 Polymerase (New England Biolabs (NEB), Ipswich, MA, USA), of genes from UWOPS87-2421 genome and were cloned in MCS of pSP-G2 (*22*) or pSP-AC (complete list of primers with restriction sites is listed in **Table S2**. Standard media and procedures were used for the cultivation of the *Escherichia coli* strains (DH5α and XL1blue) and all DNA manipulations (*23*).

Yeast strains

Saccharomyces cerevisiae strains used in this study are listed in **Table 1** (*24,25*), while schematic representation of the construction of all strains is shown in **Fig. 2** (*19,20*). Throughout the work, standard methods for yeast cultivation and manipulation were used (*26*). Strains 2421REDα and E5REDα were constructed by integration of pRED150 in *ADE2* locus of UWOPS87-2421α and YIIc17_E5α, respectively (**Fig. 1**). Transformants obtained by transformation with pRED150 were patched on synthetic complete (SC) without uracil (SC-Ura) plates and yeast extract-peptone-dextrose (YPD) medium and replica plated to SC-Ade plates to verify *ade2 URA3* genotype. Ade$^-$ Ura$^+$ transformants were analysed by Southern

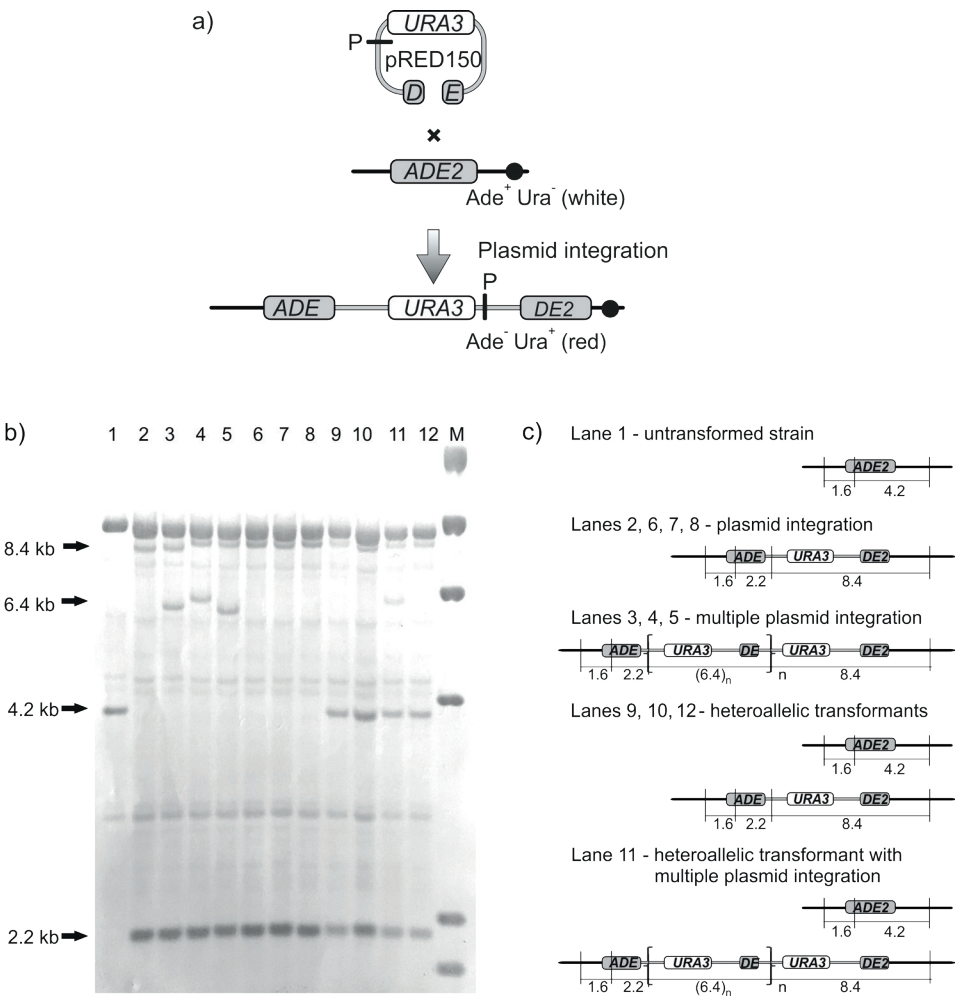

Fig. 1. Construction of 2421_REDα and E5_REDα strains by transformation of UWOPS87-2421α and YIIc17_E5α strains, respectively, by pRED150 plasmid: a) schematic representation of the plasmid integration assay, P denotes 150 bp palindrome, b) typical results of the molecular analysis of transformants, and c) schematic representation of the transformation events observed during Southern blotting. To allow better separation of longer DNA fragments, 1.6-kb band that would have been hybridized with *ADE2* gene in all samples was allowed to exit the gel. Genomic DNA of the transformants was cut with AseI

blotting to confirm plasmid integration in *ADE2* region. Two Ade$^-$ Ura$^+$ transformants (strains 2421REDα and E5REDα), containing a single pRED150 molecule integrated in *ade2* locus, were mated with haploids (UWOPS87-2421a and YIIc17_E5a) with Ade$^+$ Ura$^-$ phenotype to construct Ade$^+$ Ura$^+$ diploid heterozygous hybrids (H1, H2, H3 and H4) and homozygous controls (2421_C1 and E5_C2) (**Fig. 2** (*19,20*)). Hybrids H1 and H4 were isolated as single colonies from a cross of α-mating 2421REDα and a-mating YIIc17_E5a, while H2 and H3 were taken as single colonies from a cross of α-mating E5REDα and a-mating UWOPS87-2421a. In order to restore the genotype of parental strains (Ade$^+$ Ura$^-$), six constructed Ade$^+$ Ura$^+$ diploids (H1-H4, 2421_C1 and E5_C2) were grown in complete YPD medium to allow for pop-out of the plasmid pRED150 integrated in *ade2* region, resulting in *ADE2 ura3* genotype (**Fig. 2** (*19,20*)).

Karyotype of parental α-mating type haploid strains (UWOPS87-2421α and YIIc17_E5α) was confirmed by quantitative polymerase chain reaction (qPCR) copy number analysis for all 16 yeast chromosomes as described previously (*27*).

Yeast transformation and molecular analysis of transformants by Southern blotting

Lithium acetate transformation was done as described previously (*28*) but since it was observed that the efficiency of transformation in natural isolates was lower than in commonly used laboratory strains, cells were first allowed to recover for 30 min in rich YPD medium and then plated on selective SC-Ura plates. Isolation of the genomic DNA was performed as described previously (*29*) and molecular analysis of transformants was done by Southern blotting (*30*) using dioxigenin (DIG)-labelled *ADE2* gene (Roche, Darmstadt,

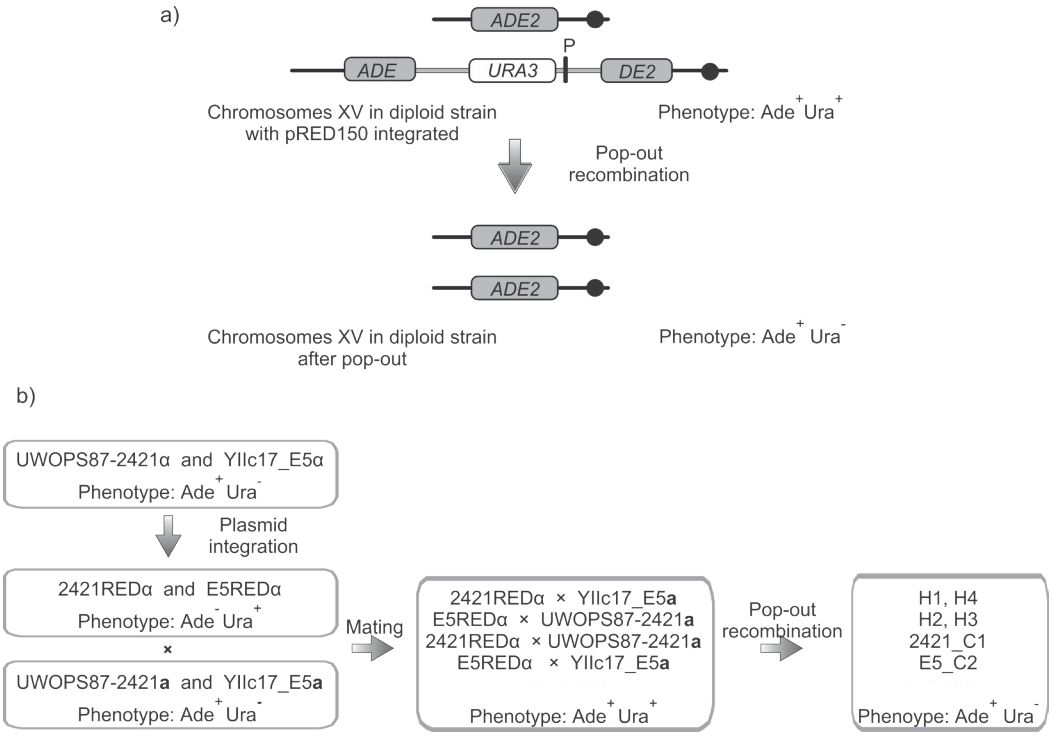

Fig. 2. Pop-out (loss) of the pRED150 plasmid and construction of hybrid and control diploid strains: a) plasmid pRED150 was previously integrated in the *ade2* locus on chromosome XV in order to facilitate diploid construction. P represents 150 bp palindrome that stimulates pop-out recombination resulting in the loss of the plasmid containing *URA3* gene (*19*) and restoration of *ADE2* gene; pop-out recombinants were selected on 5-FOA plates (*20*), and b) strains UWOPS87-2421α and YIIc17_E5α were transformed with plasmid pRED150 (see Fig. 1) to construct 2421RADα and E5REDα strains which were mated with UWOPS87-2421a and YIIc17_E5a strains in order to construct Ade^+ Ura^+ diploids. Afterwards, the constructed diploids were grown under non-selective conditions to allow pop-out (loss) of pRED150 in order to construct Ade^+ Ura^- hybrids (H1, H2, H3 and H4) and homozygous controls (2421_C1 and E5_C2)

Germany). Statistical analysis of the spectra of transformation events was done using two-tailed Pearson's chi-squared test (χ^2).

Semi-quantitative test of fermentative activity

Assessment of fermentation ability was done using YPD and SC plates containing 130.0 mg/L bromothymol blue (Kemika, Zagreb, Croatia, BTB plates (*31,32*)). Yeast cultures were grown to the stationary phase in YPD or SC medium, decimal dilutions were prepared and spotted on BTB plates. Additionally, 100 µL of sixth decimal dilution of yeast cultures were plated on BTB plates to allow for the growth of single colonies.

Analysis of the growth in the presence of inhibitors

Growth in the presence of acetic and levulinic acids and ethanol (Carlo Erba, Barcelona, Spain), and 2-furaldehyde (Acros Organics, New Jersey, USA) was determined in liquid media and/or on solid plates. For the analysis of growth in liquid media, strains were grown in YPD to the stationary phase, diluted in YPD to $A_{600\,nm}$=0.01, and 4-mL aliquots were put in test tubes containing a specific volume of 1 M inhibitor stock. If the volume of the inhibitor stock was bigger than 0.5 mL, additional control samples that contained the same volume of water were prepared to correct for the medium dilution. Cultures were grown in an orbital shaker at 150 rpm and 28 °C, and all absorbance values were measured at time points 0, 3, 6, 12 and 24 h. For each strain/inhibitor pair, two to four independent experiments were performed and all absorbance measurements were done by mixing the culture with 0.1 M sodium citrate in 1:1 volume ratio to disperse any floccules that might have formed during growth. In all experiments in liquid media, yeast strain CEN.PK2 was used as an additional control.

To determine growth inhibition on solid media, complex complete (YPD), synthetic complete (SC) or synthetic complete without uracil (SC-Ura) plates with the addition of inhibitors were prepared. Strain precultures were grown to stationary phase in YPD or SC-Ura medium and the first decimal dilution was prepared in 0.1 M sodium citrate to disperse all formed floccules. All other serial dilutions were prepared using sterile water and 5 µL of each serial dilution were spotted on a plate. Plates were incubated for 4 days at 28 °C and colonies were counted on the second and fourth day to calculate the percentage of survival.

Table 1. Yeast strains used in this study

Strain name	Genotype	Reference
Parental haploid strain (Ade$^+$ Ura$^-$)		
UWOPS87-2421a (NCYC 3582)	*MAT**a** ura3::KanMX ho::HygMX*	(*24*)
UWOPS87-2421α (NCYC 3609)	*MATα ura3::KanMX ho::HygMX*	
YIIc17_E5a* (NCYC 3586)	*MAT**a** ura3::KanMX ho::HygMX*	
YIIc17_E5α* (NCYC 3612)	*MATα ura3::KanMX ho::HygMX*	
Parental haploid strain transformed with pRED150 (Ade$^-$ Ura$^+$)		
2421REDα	*MAT**a** ura3::KanMX ho::HygMX ade2::pRED150*	This study
E5REDα	*MATα ura3::KanMX ho::HygMX ade2::pRED150*	
Heterozygous hybrid diploid strain (Ade$^+$ Ura$^-$)		
H1 (2421REDα × YIIc17_E5a)**	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX*	This study
H4 (2421REDα × YIIc17_E5a)**		
H2 (E5REDα × UWOPS87-2421a)***		
H3 (E5REDα × UWOPS87-2421a)***		
Control homozygous diploid strain (Ade$^+$ Ura$^-$)		
2421_C1 (2421REDα × UWOPS87-2421a)	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX*	This study
E5_C2 (E5REDα × YIIc17_E5a)		
Control laboratory strain (Ade$^+$ Ura$^-$)		
CEN.PK2	*MAT**a**/α his3Δ1/his3Δ1 leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-289/trp1-289 MAL2-8^c/MAL2-8^c SUC2/SUC2*	(*25*)
Diploid strain transformed with plasmids pSP-G2		
H1-pSP	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-G2	This study
H2-pSP		
H3-pSP		
H4-pSP		
2421_C1-pSP		
E5_C2-pSP		
Diploid strain transformed with plasmids pSP-ATR1		
H1-ATR	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-ATR1	This study
H2-ATR1		
H3-ATR1		
H4-ATR1		
2421_C1-ATR1		
E5_C2-ATR1		
Diploid strain transformed with plasmids pSP-YAP1		
H1-YAP1	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-YAP1	This study
H2-YAP1		
H3-YAP1		
H4-YAP1		
2421_C1-YAP1		
E5_C2-YAP1		
Diploid strain transformed with plasmids pSP-FLR1		
H1-FLR1	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-FLR1	This study
H2-FLR1		
H3-FLR1		
H4-FLR1		
2421_C1-FLR1		
E5_C2-FLR1		
Diploid strain transformed with plasmids pSP-AC		
H1-AC	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-AC	This study
H2-AC		
H3-AC		
H4-AC		
2421_C1-AC		
E5_C2-AC		
Diploid strain transformed with plasmids pSP-AC-GSH1		
H1-GSH1	*MAT**a**/α ura3::KanMX/ura3::KanMX ho::HygMX/ho::HygMX* pSP-AC-GSH1	This study
H2-GSH1		
H3-GSH1		
H4-GSH1		
2421_C1-GSH1		
E5_C2-GSH1		

*During research we noticed that starting YIIc17_E5 strains were also histidine auxotrophs that could not be complemented with a functional *HIS3* gene
**H1 and H4 were isolated as separate random colonies that grew on selective media after mating of 2421REDα and with YIIc17_E5a
***H2 and H3 were isolated as separate random colonies that grew on selective media after mating of E5REDα and with UWOPS87-2421a

Plasmid retention under non-selective conditions

To determine the retention of plasmids in non-selective conditions, strains transformed with replicative plasmid pSP-YAP1 containing *URA3* gene as a selectable marker (**Table S1**) were first grown in selective SC-Ura liquid medium until the stationary phase, and then they were diluted and inoculated in four test tubes containing rich YPD medium. One test tube was used as a control, while in the other test tubes growth inhibitors were added to the final concentration of 100 mM (acetic and levulinic acids) or to final concentration of 20 mM (2-furaldehyde). After two days in the shaker at 28 °C, cultures were diluted and fifth and sixth decimal dilutions were plated on YPD medium. Colonies that grew on YPD medium were replica plated on SC-Ura medium and the percentage of Ura^+ cells that retained replicative plasmid was calculated.

RESULTS AND DISCUSSION

Lignocellulosic waste is abundant but, due to its complex chemical composition, yeasts cannot use it directly for fermentation and bioethanol production. Therefore, lignocellulosic materials need to be pretreated to release fermentable sugars, but during pretreatment aliphatic acids and furan derivates, which inhibit growth and fermentation, are also formed. Therefore, apart from high ethanol concentration, bioethanol producer needs to be resistant to growth and fermentation inhibitors.

The aim of this study was to investigate whether the construction of *Saccharomyces cerevisiae* intraspecies hybrids by mating parental haploids having desirable traits is a promising approach for construction of bioethanol producer. To achieve this, we used two natural strains of *S. cerevisiae*, UWOPS87-2421 that tolerates acetic and levulinic acids and was found in the flower of the cactus *Opuntia megacantha* in Hawaii, and YIIc17_E5, isolated from wine in France that has been shown to be sensitive to formic acid, 2-furaldehyde and 5-hydroxymethylfurfural (*33,34*).

To expedite the construction of hybrid (H1-H4) and control (2421_C1 and E5_C2) diploids by mating of haploids, UWOPS87-2421α and YIIc17_E5α strains were first modified by plasmid integration. Afterwards, constructed diploids were analysed for their ability to produce CO_2 by fast fermentation test on BTB plates and to grow in the presence of growth and fermentation inhibitors. Additionally, the strains were transformed with replicative plasmids carrying yeast *ATR1*, *FLR1* and *YAP1* genes previously shown to increase the resistance of strains to growth and fermentation inhibitors.

Low gene targeting fidelity in UWOPS87-2421α and YIIc17_E5α S. cerevisiae strains

Starting **a**- and α-mating parental haploids had the same Ade^+ Ura^- phenotype (**Fig. 1** and **Table 1** (*24,25*)). To facilitate the construction of diploid strains by mating, α-mating strains were first modified by targeted integration of the plasmid pRED150 in *ADE2* gene, changing their phenotype to Ade^- Ura^+. Furthermore, such transformants can be easily recognised because inactivation of *ADE2* gene produces red colonies (*35*).

Surprisingly, during construction of 2421REDα and E5REDα strains, phenotypic analyses of transformants revealed significantly lower percentage of transformants carrying targeted plasmid integration than in standard *S. cerevisiae* laboratory strains. Thus, the molecular analysis by Southern blotting confirmed that success of targeted plasmid integration (gene targeting fidelity) is only 25 and 1.7 % in UWOPS-2421α and YIIC_E5α strains, respectively ($p<0.0001$, **Fig. 1** and **Table 2**), whereas in standard *S. cerevisiae* it is as high as 98 % (*28*). Apart from low gene targeting fidelity in UWOPS-2421α and YIIC_E5α strains, the percentage of multiple targeted plasmid integration also seems to be high (around 50 % in comparison to 10 % in standard *S. cerevisiae* laboratory strains during spheroplast transformation (*36*)). In addition, molecular analysis of white Ade^+ Ura^+ transformants revealed spectra of aberrant (off-targeted) genetic events (**Table 2**). We have previously shown that aberrant (off-targeting) transformation events in plasmid integration assays are: (*i*) integration of plasmid molecule in the random position of the host genome (illegitimate integration), and (*ii*) targeted chromosome duplication (TCD) resulting in heteroallelic transformants – aneuploids having at least two copies of targeted chromosome, one carrying untransformed allele and the other carrying the allele expected after successful gene targeting (*28*).

Concerning spectra of off-targeted events in UWOPS-2421α and YIIC_E5α strains (3.6-16.7 % of illegitimate integration and 83.3-96.4 % of TCD), they are similar to the spectra in standard *S. cerevisiae* laboratory strains. However, due to low gene targeting fidelity, and considering all analysed transformants, TCD is the most frequent genetic event, being higher than 60 and 90 % for UWOPS-2421α and YIIC_E5α strains, respectively. This could be a consequence of chromosome duplication during gene targeting (TCD) but also the existence of at least two copies of chromosome XV carrying *ADE2* gene in UWOPS-2421α and YIIC_E5α strains prior to transformation. However, a qPCR analysis of chromosome copy number

Table 2. Spectra of genetic events in strains UWOPS87-2421α and YIIc17_E5α during gene targeting with pRED150 plasmid

Genetic event	UWOPS87-2421α	YIIc17_E5α	Genetic event	UWOPS87-2421α	YIIc17_ E5α
Successful gene targeting (percentage of red, Ade^- Ura^+ transformants)	22/88 (25.0 %)	2/115 (1.7 %)	Single plasmid integration	12/22 (54.5 %)	1/2 (50.0 %)
			Multiple plasmid integration	10/22 (45.4 %)	1/2 (50.0 %)
Off-targeted events (percentage of white, Ade^+ Ura^+ transformants)	66/88 (75.0 %)	113/115 (98.3 %)	Illegitimate plasmid integration	1/6 (16.7 %)	1/28 (3.6 %)
			Targeted chromosome duplication	5/6 (83.3 %)	27/28 (96.4 %)
Analysed transformants	88	115	Analysed transformants	28	30

in these two strains revealed haploid DNA content (data not shown). Therefore, frequent TCD in these strains could be a consequence of some mutations, change in gene expression and/or protein activity that remains to be identified. From this point of view, it should be noted that fidelity of gene targeting and spectrum of genetic events strongly depend on the transformation procedure used to deliver the transforming DNA to the yeast cell (*30*) as well as on the influence of *e.g. EXO1* and *SGS1* genes on both transformation efficiency and gene targeting fidelity (*28,37*).

Isogenic hybrids display phenotypic diversity and different fermentation activity

First, by crossing α-mating Ade$^-$ Ura$^+$ cells with **a**-mating Ade$^+$ Ura$^-$ cells, we constructed hybrid and control diploids with Ade$^+$ Ura$^+$ phenotype. In the second step, a loss of pRED150 plasmid from the *ade2* region in the genome resulted in restoring parental Ade$^+$ Ura$^-$ genotype, enabling comparison of parental haploids and constructed diploids (**Fig. 2** (*20,21*)).

Constructed diploids (H1-H4, 2421_C1 and E5_C2) and parental haploids (UWOPS87-2421a and YIIc17_E5a) were subjected to semi-quantitative test for ethanol production on bromothymol blue (BTB) plates. As described previously (*31,32*), the plates containing an indicator BTB can be used for semi-quantitative monitoring of fermentation activity of a strain because the production of CO_2, due to fermentation, decreases pH. As the pH decreases, the colour of BTB and media containing it changes from green to yellow or even deep orange/red. Moreover, deep orange or red colour of the BTB medium and even colonies was observed with yeast strains that are frequently used for the production of wines, indicating their better fermentation ability (*31,32*). As a continuation of this research, batch ethanol production in fermentation flasks or laboratory scale bioreactors will be performed to quantify ethanol titres and yields obtained by newly constructed intraspecies hybrid strains.

The test of fermentative potential showed that hybrids H1--H4, although isogenic and originating from the same parental strains, have different fermentative ability, whereas, as expected, control haploid YIIc17_E5a and diploid E5_C2 strains (isolated from wine) have the highest fermentation activity (**Fig. 3**). Additionally, when different hybrid cultures were diluted and plated on BTB plates to allow for the growth of single colonies, it was observed that hybrids H1 and H4 formed colonies of uniform colour and size, while hybrids H2 and H3 formed colonies of different size and colour indicating phenotypic variability among descendants of a single heterozygous diploid cell (**Fig. 3**). This suggested that hybrids that originated from a cross of E5REDα and UWOPS87-2421a experience genome instability and it is in agreement with the already observed genetic instability of intraspecies hybrids used for wine production, which could explain differences in both the amount of produced ethanol and strain stability (*38-40*). More importantly, phenotypic diversity of constructed intraspecies hybrids could allow further selection of properties useful for bioethanol production on different lignocellulosic hydrolysates.

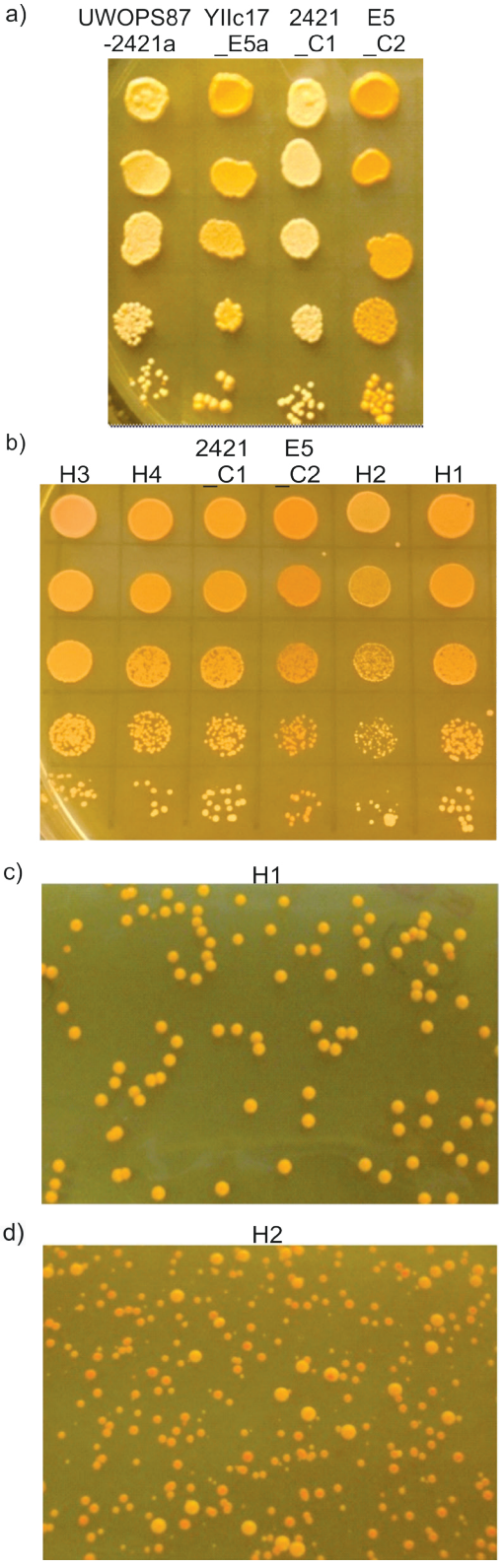

Fig. 3. Fermentative potential of yeast strains assessed by semi-quantitative method using bromothymol blue (BTB) plates. Comparison of: a) parental haploids and parental diploids, b) hybrids (H1-H4) and control diploid strains (2421_C1 and E5_C2), colonies obtained from the single colony of c) H1 (2421REDα × YIIc17_E5a) and d) H2 (E5REDα × UWOPS87-2421a) diploids, respectively

Growth and survival of strains in the presence of growth and fermentation inhibitors

Growth of the constructed heterozygous hybrids H1--H4 and control diploids 2421_C1 and E5_C2 and their performance in media containing inhibitors frequently found in lignocellulosic hydrolysates were analysed in liquid and on solid media. First, single growth and fermentation inhibitors (acetic and levulinic acids, 2-furaldehyde and ethanol) were added to liquid complex complete YPD medium (**Fig. 4**). The obtained results clearly show that hybrids H1 and H2 grew faster both in the presence and in the absence of acetic and levulinic acids than controls (2421_C1 and E5_C2, **Fig. 4**). At lower concentrations of 2-furaldehyde (10 mM), hybrid H1 grew the best, while further increase of the concentration equally inhibited the growth of all strains (**Fig. 4**). Ethanol, in volume fractions used here, severely affected all tested strains, although at 10 % the best growth was observed of control 2421_C1 and hybrid H2 strains (**Fig. 4**). The growth of the other two hybrids (H3 and H4) in the presence of growth and fermentation inhibitors was also tested, but these strains did not perform as well as H1 and H2 and were excluded from further studies.

Additionally, it was noticed that control 2421_C1 and hybrid H2 always form big floccules, aggregates that have been shown to protect cells from harsh environment (*41*). Although flocculation is still not completely understood, it is influenced by temperature, pH (*42,43*), osmotic stress, ethanol concentration, nutrient availability and the presence of calcium and zinc ions (*41*). While the flocculation of laboratory strains is unwanted, it positively influences production of bioethanol, heterologous proteins and bioremediation of heavy metals. Moreover, in the industrial setting, flocculation usually starts at a specific phase of culture growth or fermentation and allows easier separation of the producer strain at the end of the process.

Survival in the presence of inhibitors was also tested on YPD, SC or SC-Ura media that contained acetic and levulinic acids and 2-furaldehyde (**Fig. 5**). Hybrid H1 had the highest survival on rich YPD plates containing 80 mM acetic acid and 5 mM 2-furaldehyde, while on the plates containing 140 mM levulinic acid the survival of hybrids H1 and H2 was almost the same (**Fig. 5**). As expected, the change of medium composition significantly influenced the growth and survival of all strains. Thus, the survival of all strains on SC plates containing 80 mM acetic and levulinic acids significantly decreased, while the change of medium had only a minor effect on the survival of strains on the SC plates containing 5 mM 2-furaldehyde, and hybrid H1 again had the highest survival (**Fig. 5**).

Although concentrations of acetic and levulinic acids and 2-furaldehyde in lignocellulosic hydrolysates vary (*4,5,7*), they are lower than those used here. The presented results show that levulinic acid has lower inhibitory effect on the growth of yeast strains than acetic acid (**Fig. 4** and **Fig. 5**). This is in contrast to higher toxic effect of levulinic acid reported by Jönsson *et al.* (*4*), explained by easier entrance to cells (*44*). Still, Mirisola and Longo (*45*) suggested that the pH and the exact composition of the medium have a significant impact on yeast growth and ageing, and that intracellular drop of pH results in

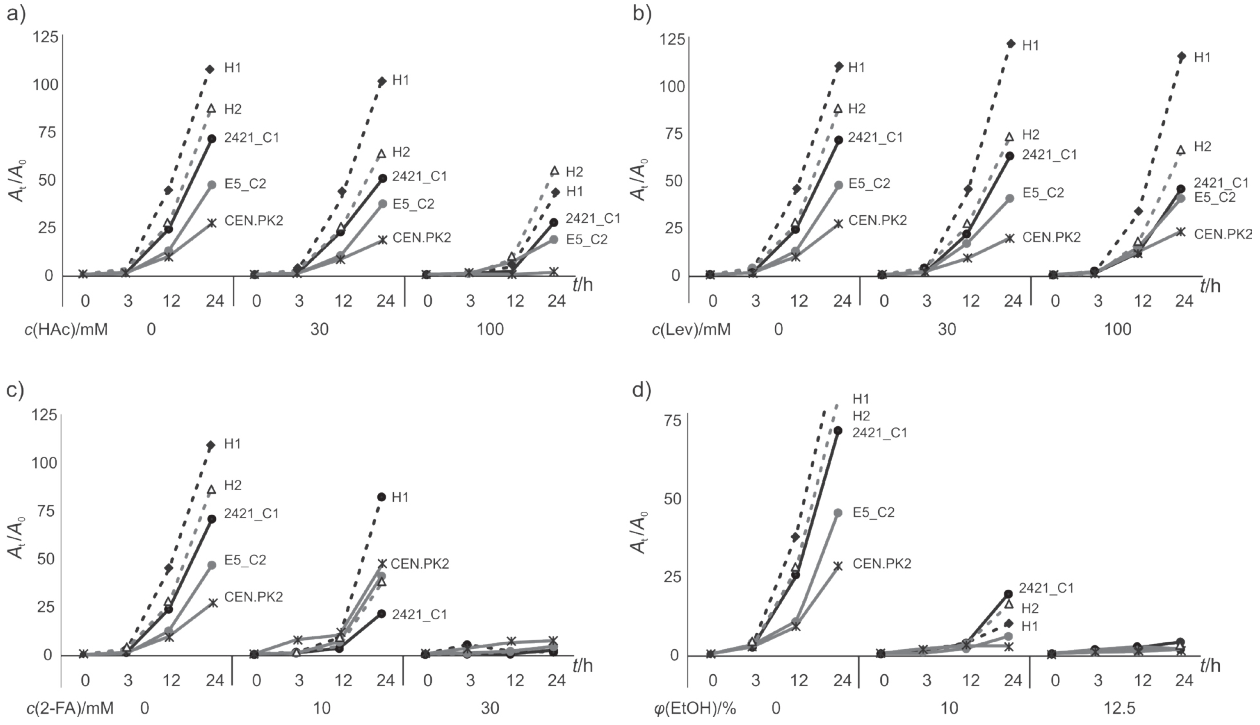

Fig. 4. Growth of heterozygous hybrid (H1 and H2) and homozygous control (2421_C1 and E5_C2) strains in liquid YPD medium containing: a) acetic acid, b) levulinic acid, c) 2-furaldehyde and d) ethanol. The results are expressed as the ratio of the absorbance (A_t) at a particular time (t=3, 12 and 24 h) and the absorbance (A_0) at the beginning of the experiment (t=0 h). CEN.PK2 strain was used as an additional control

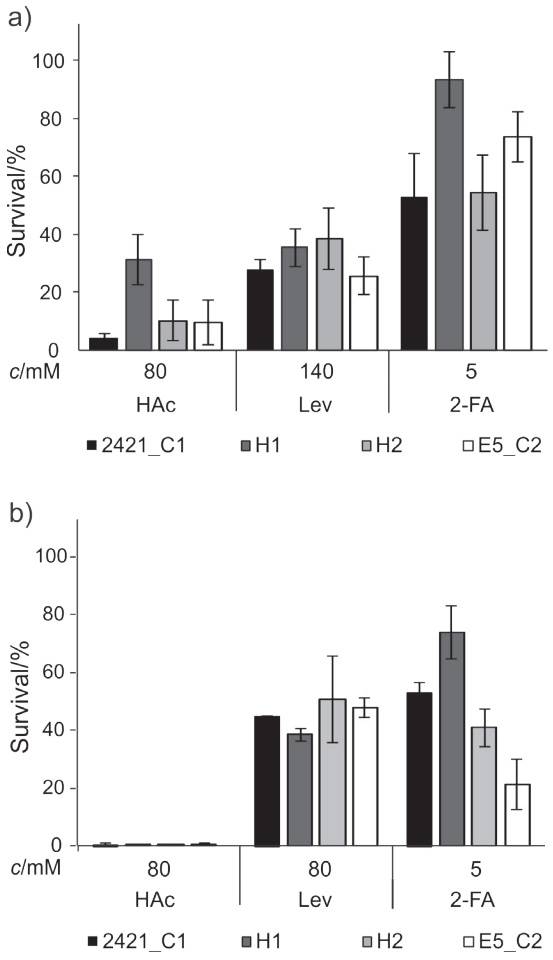

Fig. 5. Survival of heterozygous hybrid (H1 and H2) and homozygous control (2421_C1 and E5_C2) strains on: a) complex complete (YPD) and b) synthetic complete (SC) solid media containing acetic (HAc) and levulinic (Lev) acids and 2-furaldehyde (2-FA). Results are expressed according to the 100 % strain survival on the corresponding medium (YPD or SC) without inhibitors. Error bars represent standard deviation

the production of reactive oxygen species. In the scope of this work, it is important to mention that Burtner *et al.* (*46*) suggested that acetic acid is one of the most important factors that induce yeast ageing and cell death, while Heer and Sauer (*7*) identified 2-furaldehyde as a main toxic compound that inhibits both cell growth and ethanol production, and this has been confirmed by the results presented here (**Fig. 4** and **Fig. 5**). Although yeasts can tolerate up to 18 % of ethanol, ethanol volume fractions higher than 10 %, especially in the presence of 2-furaldehyde, have a significant inhibitory effect (*4*).

To investigate the influence of overexpression of yeast *ATR1, YAP1, FLR1* and *GSH1* genes on the survival in the presence of inhibitors, all 36 strains transformed with replicative plasmids carrying *URA3* gene as selectable marker and either *ATR1*, *YAP1*, *FLR1* or *GSH1* gene under the regulation of *TEF1* or *PGK1* promoter (**Table S1**) were tested on SC-Ura plates (to prevent the loss of replicative plasmids) containing 60 mM acetic acid, 100 mM levulinic acid or 5 mM 2-furaldehyde (**Fig. 6**).

To assure the overexpression, *ATR1, YAP1* and *FLR1* genes were cloned in pSP-G2 plasmid which is present in approx. 50 copies per cell because it contains 2μ origin of replication (*47,48*), whereas *GSH1* gene was cloned in centromeric pSP-AC plasmid since the positive effect of the *GSH1* gene was observed only if it was present in several copies per cell (*17*). However, in this work overexpression of *FLR1* and *GSH1* genes did not have any significant influence (data not shown), while overexpression of *YAP1* gene had overall more positive effect on the survival of the strains in the presence of growth inhibitors than the overexpression of *ATR1* gene (**Fig. 6**). Beneficial effect of overexpression of *YAP1* gene can be explained by the fact that it encodes a transcription factor for various genes involved in the response to oxidative and heavy metal stress (*49-52*). Positive but less pronounced effect of the

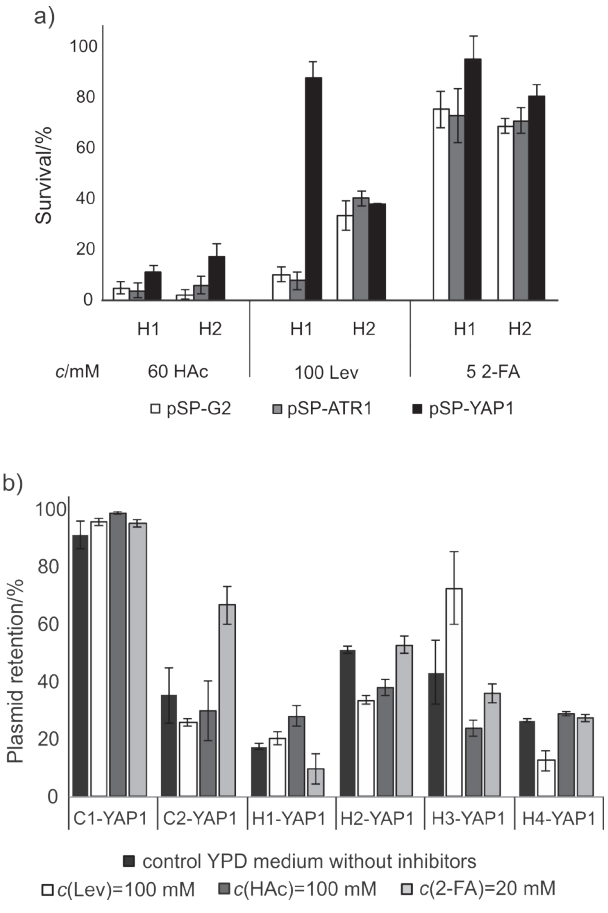

Fig 6. Survival of the hybrid strains overexpressing *YAP1* and *ATR1* genes and retention of the plasmid pSP-YAP1 in diploid strains in nonselective conditions: a) survival of the hybrid strains H1 and H2 containing vector pSP-G2 or plasmids pSP-ATR1 and pSP-YAP1 overexpressing *ATR1* and *YAP1* genes on solid SC-Ura medium in the presence of acetic (HAc) and levulinic (Lev) acids and 2-furaldehyde (2--FA). Results are expressed according to the 100 % strain survival on the SC-Ura medium without inhibitors; error bars represent standard deviation; and b) retention of the plasmid pSP-YAP1 overexpressing *YAP1* gene in heterozygous hybrid (H1- H4) and homozygous control (2421_C1 and E5_C2) diploid strains grown in YPD medium containing acetic and levulinic acids and 2-furaldehyde. The percentage of cells which retained the plasmid after 48 h of cultivation is shown; error bars represent standard deviation

overexpression of *ATR1* gene (**Fig. 6**) could be a consequence of a direct involvement of Atr1 protein in the formation of transmembrane pumps (*53*).

Since the *YAP1* gene had the most positive influence on the survival of yeast strains in the presence of inhibitors in SC-Ura medium (see above), it was investigated if the growth inhibitor can be used as the only selective pressure for the retention of the replicative plasmid carrying *YAP1* gene (**Fig. 6**). The results showed that the presence of the inhibitors did not result in higher frequency of plasmid retention. However, it is interesting that retention of the pSP-YAP1 plasmid in the 2421_C1 strain was as high as 90 % both in the presence and absence of inhibitors.

The newly constructed intraspecies hybrids described here survive better than the parental strains in complex and synthetic media containing different single inhibitors of growth and fermentation. Since lignocellulosic hydrolysates used for bioethanol production contain several growth and fermentation inhibitors that frequently have synergistic effect on a producer yeast strain, we plan to challenge hybrid strains by inoculating them both in complex and synthetic media containing acetic and levulinic acids and 2-furaldehyde, and in lignocellulosic hydrolysates.

CONCLUSIONS

Construction of intraspecies hybrids by mating of natural isolates having desirable traits is a promising approach in the development of biotechnologically applicable *Saccharomyces cerevisiae* strains. In this work such approach was used during development of bioethanol producer and it was found that the constructed heterozygous diploids display genetic instability, resulting in different fermentation ability, allowing further improvement by selection of desirable biotechnological characteristics. Some of the constructed hybrids showed better performance in the presence of several growth and fermentation inhibitors, frequently present in lignocellulosic hydrolysates, which was further increased by the overexpression of the *YAP1* gene.

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SUPPLEMENTARY MATERIAL

All supplementary material is available at www.ftb.com.hr.

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