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BIOENERGY AND BIOFUELS

Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains

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Abstract Enzyme cost is a major impediment to second-generation (2G) cellulosic ethanol production. One strategy to reduce enzyme cost is to engineer enzyme production capacity in a fermentative microorganism to enable consolidated bioprocessing (CBP). Ideally, a strain with a high secretory phenotype, high fermentative capacity as well as an innate robustness to bioethanol-specific stressors, including tolerance to products formed during pre-treatment and fermentation of lignocellulosic substrates should be used. *Saccharomyces cerevisiae* is a robust fermentative yeast but has limitations as a potential CBP host, such as low heterologous protein secretion titers. In this study, we evaluated natural *S. cerevisiae* isolate strains for superior secretion activity and other industrially relevant characteristics needed during the process of lignocellulosic ethanol production. Individual cellulases namely *Saccharomycopsis fibuligera* Cel3A (β-glucosidase), *Talaromyces emersonii* Cel7A (cellobiohydrolase), and *Trichoderma reesei* Cel5A (endoglucanase) were utilized as reporter proteins. Natural strain YI13 was identified to have a high secretory phenotype, demonstrating a 3.7- and 3.5-fold higher Cel7A and Cel5A activity, respectively, compared to the reference strain S288c. YI13 also demonstrated other industrially relevant characteristics such as growth vigor, high ethanol titer, multi-tolerance to high temperatures (37 and 40 °C), ethanol (10 % *w*/*v*), and towards various concentrations of a cocktail of inhibitory compounds commonly found in lignocellulose hydrolysates. This study accentuates the value of natural *S. cerevisiae* isolate strains to serve as potential robust and highly productive chassis organisms for CBP strain development.

Keywords Bioethanol · Cellulolytic enzymes · Industrial tolerance · *Saccharomyces cerevisiae* · Secretion

Introduction

Genomic analysis studies of the microbiome found in industrial ethanol fermentation processes have demonstrated that among the vast diversity of yeast species, *Saccharomyces cerevisiae* is the most dominant species (Pretorius 2000; Basso et al. 2008; Steensels et al. 2014). This dominance has been studied, and the traits that make *S. cerevisiae* an ideal candidate for industrial production of ethanol and other commodity products include its rapid growth rate, high cell density fermentation capabilities, microbial safety, and eukaryotic post-translational processing (Den Haan et al. 2015). Furthermore, its ability to produce high ethanol yields and its high ethanol tolerance make this yeast especially useful in biomass bio-processing (Favaro et al. 2015). However, the fermentation environment in second-generation (2G) cellulosic bioethanol production is significantly different to the classical fermentation set up (Lambertz et al. 2014). Common features of the 2G bioethanol production process include (1)

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the presence of inhibitory molecules as a result of pretreatment of biomass, and (2) the inhibitory nature of the desirable products (ethanol) and by-products. The ideal host strain would not only have to tolerate the complex and challenging fermentation medium presented by lignocellulosic hydrolysates, but also display high levels of recombinant cellulase activity (La Grange et al. 2010).

S. cerevisiae lacks cellulolytic enzyme activity, a desirable trait which has the benefit of lowering production costs in biomass bioprocessing industry (Idiris et al. 2010). Consolidated bio-processing (CBP) requires the production of high titers of key cellulases including endoglucanases (EGs), exoglucanases such as cellobiohydrolases (CBHs) and β-glucosidases (BGLs) to break down lignocellulosic substrates to fermentable sugars (Lambertz et al. 2014). If fermentative microorganisms can be engineered to produce high cellulase production capabilities, the subsequent steps of biomass processing can be carried out simultaneously in one bioreactor in a CBP (Den Haan et al. 2015). However, *S. cerevisiae* cannot secrete large amounts of heterologous protein. Low to moderate secreted activity levels of heterologous cellulases have been found in domesticated ("laboratory" and "industrial") *S. cerevisiae* strains (Ali et al. 2014). In particular, high cellulase secretion from *S. cerevisiae* has been hampered due to secretory bottlenecks that form as a result of overproduction and misfolding of heterologous proteins (Mattanovich et al. 2004). It has been assumed that heterologous proteins stimulates the unfolded protein response (UPR), and are degraded by inducing the endoplasmic reticulum associated degradation machinery (ERAD). Thus, efficient degradation of cellulosic materials requires enhanced heterologous protein production in yeast.

Several studies aimed to improve *S. cerevisiae* strains for first-generation (1G) and second-generation (2G) bioethanol production by targeting ideal traits, such as tolerance to inhibitors, secretory capacity of heterologous proteins and ethanol yields, e.g., by quantitative trait locus (QTL) analysis, evolutionary and genetic engineering, and mutagenesis (Liu 2012; Swinnen et al. 2012; Koppram et al. 2012; Hubmann et al. 2013; Kroukamp et al. 2013; Demeke et al. 2013). Several recent studies have focused on evaluating natural and industrial *S. cerevisiae* isolates for a range of desirable phenotypes required in biomass bioprocessing production. Since the desired traits for strains used in wine fermentation are similar to the traits desired for industrial strains in bioethanol fermentations, e.g., tolerate and produce high ethanol yields (Zakrzewska et al. 2011), it is logical to search for superior industrial strains in the same environment. Several studies have been performed on the biodiversity of *S. cerevisiae* strains found in this particular niche, i.e., vineyards, with findings of large phenotypic variability existing in terms of tolerance profiles (Kvitek et al. 2008), sensitivity to copper sulfate (Fay et al. 2004) and ethanol yields (Carreto et al. 2008). A recent survey of genetic polymorphisms demonstrated that vineyard isolates of yeast represent a diverse, natural population that was genetically different from domesticated strains (Cavalieri et al. 2000; Schuller and Casal 2007; Kvitek et al. 2008). Vineyard isolate strains therefore provide an untapped resource of natural genetic polymorphisms resulting from environmental selective pressures distinct from those of laboratory strains. Initially, Brazilian bioethanol fermentations used baker's yeast strains in starter cultures for yeast recycling; however, these were quickly outcompeted by dominant and persistent natural *S. cerevisiae* strains (Da Silva-Filho et al. 2005; Basso et al. 2008). This demonstrated the potential of phenotyping the natural biodiversity of *S. cerevisiae* to find superior industrial strains. Unfortunately, many natural strains may not be suitable for direct industrial fermentation, but industrially relevant traits can potentially be transferred to industrial strains, thereby creating novel yeast strain(s) with desirable features.

Examples of traits evaluated among *S. cerevisiae* isolates include high tolerance to various inhibitors (Jin et al. 2013; Steensels et al. 2014; Ruyters et al. 2014) varying fermentation profiles (Mukherjee et al. 2014; Ruyters et al. 2014), as well as tolerance to other environmental stresses including fluctuating osmolarity and high temperatures (Favaro et al. 2013). Few studies have evaluated *S. cerevisiae* isolates for protein secretion capacities. An example of the difference in secretion capacity was the significant differences in extracellular activity levels of *Saccharomycopsis fibuligera* Cel3A among seven diverse *S. cerevisiae* strains, with secreted enzyme activities in the range of 73 to 250 mU/mL (Gurgu et al. 2011). Similarly, De Baetselier et al. (1991) demonstrated a 100-fold difference in enzyme activities between the lower and higher performances after screening several recombinant *S. cerevisiae* strains producing *Aspergillus niger* glucose oxidase. Therefore, strain choice is a critical factor to ensure maximum recombinant enzyme production. To date, no study has evaluated the secretory phenotypes of natural *S. cerevisiae* strain isolates for the expression of heterologous cellulase genes. The same variation observed between isolates for tolerance and fermentation capabilities is expected for the production and secretion of heterologous cellulases.

In this study, a comprehensive phenotypic evaluation of a collection of natural *S. cerevisiae* isolate strains was performed for the evaluation of heterologous cellulase secretion in aerobic and oxygen-limited conditions. In order to identify natural strains with good general secretion phenotypes, we screened for the individual, episomal expression of the cellulase genes *S. fibuligera cel3A* (*S.f.cel3A*), *Trichoderma reesei cel5A* (*T.r.cel5A*), and *Talaromyces emersonii* (*T.e.cel7A*). Three plasmids were transformed separately into each of the natural strains and the extracellular activities of the corresponding enzymes coded by the plasmids were subsequently assayed separately. In addition, the strains were evaluated

based on industrially relevant characteristics such as fermentation and tolerance characteristics to CBP process relevant inhibitors, ploidy state and growth vigor. Protein and strain-specific variances to heterologous secretion, fermentation, and tolerance profiles were observed. The information obtained in this study will be valuable to select natural isolate strains as platforms for future yeast engineering experiments directed to convert cellulosic substrates to ethanol in a CBP configuration.

Materials and methods

Strains, media, and analysis

The strains utilized in this study were obtained from a culture collection of strains isolated from the various vineyards along the coastal region of the Western Cape, South Africa by the Agricultural Research Council (ARC) Infruitec - Nietvoorbij Wine Research Centre (Van Der Westhuizen et al. 2000). The most significant strains of this study were deposited into the culture collection at the Plant Protection Research Institute (PPRI-ARC, Queenswood, Pretoria). Thirty *S. cerevisiae* strains were selected based on fermentative vigor in stressed conditions as reported by Blaauw (2015). The industrial benchmark strain MH1000 (distillery yeast, Stellenbosch University, South Africa), Hoeg (brewing yeast, Stellenbosch University, South Africa), and laboratory strains S288c (ATCC 204508) and Y294 (ATCC 201160) were included in this study for comparison of relatively diverse backgrounds and used as reference strains. For the purposes of this study, reference laboratory strains S288c and Y294 were constructed to be diploid.

All strains were stored at −80 °C in 15 % glycerol-based standard storage medium. Yeast cells were routinely cultivated at 30 °C in YPD (yeast peptone dextrose) (1 % yeast extract [Merck, Darmstadt, Germany]), 2 % peptone [Merck], 2 % glucose [Merck]) medium. All *S. cerevisiae* transformants were selected on YPD agar supplemented with 400 μg/ml G418 disulphate [Melford laboratories, Ipswich, UK], whilst liquid cultures were cultivated on a rotary shaker (200 rpm) at 30 °C. Superior isolates were characterized at a strain level by genotyping by employing the primers to amplify delta12 (D1) and delta 21 (D2) regions of 26S rDNA (Table 1). Genomic DNA was extracted as described previously by Hoffman et al. (1987). PCR amplification was performed using a C1000 thermal cycler (BioRad, Hercules, California, USA). Amplification was achieved with an initial denaturation step at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min. Sequence verification was carried out using the dideoxy chain termination method, with an ABI PRISM™ 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) (Central Analytical Facility, Stellenbosch University). Thereafter, the identities of isolate strains were confirmed as *S. cerevisiae* by comparing their D1/D2 region sequences to known sequences available on GenBank using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast). *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used for general cloning procedures and strains were routinely cultivated in Luria Bertini (LB) broth (0.5 % yeast extract [Merck], 1 % tryptone [Merck], 1 % NaCl [Merck]) supplemented with 100 μg/ml ampicillin (Roche, Johannesburg, South Africa) at 37 °C.

Fermentations analyses were performed in triplicates in serum bottles containing 20 mL YPD. Samples were inoculated to an initial A_{600} of 0.1 and incubated on a shaker at 250 rpm for 96 h at 30 °C. The shake flasks were sealed with rubber plugs to maintain the oxygen-limited conditions. A 0.8 × 25-mm needle was pierced into the rubber plugs to be used as a CO_2 outlet. Ethanol, glycerol, acetic acid and glucose concentrations were quantified with high performance liquid chromatography (HPLC) (Finnigan Survey UV–VIS Plus detector, Thermo-Scientific, Waltham, MA, USA) consisting of a LC pump (Thermo-Scientific, Waltham, MA, USA), autosampler (Thermo-Scientific), and Refractive Index Detector (Thermo-Scientific). The compounds were separated on a Rezex RHM Monosaccharide 7.8 × 300 mm column (00H0132-K0, Phenomenex, Torrance, CA, USA) at 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml min^{-1}.

Growth analyses were performed by inoculating strains in triplicate at a starting point A_{600} in 20 mL YPD in 125-mL Erlenmeyer flasks. Flasks were incubated on a rotary shakers (200 rpm) at 30 °C for the duration of the analysis. Samples were diluted (1:10) after which A_{600} readings were taken using BioRad xMark™ Microplate Spectrophotometer. Samples were taken periodically until growth had either cessated or reached stationary phase and data was normalized.

DNA manipulation and yeast transformation

Standard molecular biology techniques were used as described by Sambrook and Russel (2001). Initial PCR products were amplified using the Phusion™ High-Fidelity DNA polymerase (Thermo-Scientific) on an Applied BioSystems 2720 thermocycler, as instructed by manufacturer, using forward and reverse primers indicated in Table 2. All kits and enzymes were used as recommended by manufacturer. Restriction endonucleases and T4 DNA ligase were purchased from Thermo-Scientific or New England Biolabs (Ipswich, MA, USA). PCR products and DNA fragments were routinely separated on 1 % (*w*/*v*) agarose (Lonza, Rockland, ME, USA) gels and fragments of appropriate sizes isolated using the

Table 1 Plasmids and primers used in this study

Gene of interest	Plasmid	Strain abbrev.	Primers used for verification (5′-3′)
*S. fibuligera cel*3A[a]	pMUSD1	[Cel3A]	cel3A-L GACTCGCGAGTCCCAATTCAAAACTATACC
			cel3A-R CCGCTCGAGCGGTCAAATAGTAAACAGGACAGATG
T. reesei cel5A[b]	pMUSD2	[Cel5A]	cel5A-L GTTAACAACAATTTGGGTGG
			cel5A-R CAATGGAGAAAAAGCACC
T. emersonii cel7A[c]	pMUSD3	[Cel7A]	cel7A-L GACTTTAATTAAAATGCTAAGAAGAGCTTTACTATTG
			cel7A-R GACTGGCGCGCCTTACAAACATTGAGAGTAGTATGGG
D1/D2 26S rDNA regions	n/a	D1/D2 regions	F63-GCATATACAATAAGCGGAGGAAAAG
			LR3-GGTCCGTGTTTCAAGACGG
S. cerevisiae Mata/α[d]	n/a	*Mata/α*	Mata-L ACTCCACTTCAAGTAAGAGTTTG
			Matα-L GCACGGAATATGGGACTACTTCG
			Mat locus-R GCA CGG AAT ATG GGA CTA CTT CG
S. cerevisiae ALG9[e]	n/a	*ALG9*	*ALG9*-L TGCATTTGCTGTGATTGTCA
			ALG9-R GCCAGATTCCTCACTTGCAT
kanMX	n/a	G418	*kanMX*-L CCGCGATTAAATTCCAACAT
			kanMX-R CGATAGATTGTCGCACCTGA

[a] *T. reesei xyn2* secretion signal (Van Rooyen et al. 2005)

[b] Native secretion signal (GenBank accession nr. P07982.1)

[c] Native secretion signal, contains catalytic domain from *T. emersonii* Cel7A and modified carbohydrate binding module (CBM) from *T. reesei* Cel7A (Ilmén et al. 2011)

[d] Blaauw (2015)

[e] A single reference gene encoding α-1,2-mannosyltransferase in *S. cerevisiae* genome (Van Zyl et al. 2014)

Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA).

All plasmids constructed and utilized in this study are summarized in Table 2 and Electronic Supplementary Material (ESM) Fig. S1. Preliminary screening was performed with the reporter protein *S.f.*Cel3A on a high-copy plasmid pMUSD1, before further screening with high-copy plasmids pMUSD2 and pMUSD3 expressing *T.r.cel5A* and *T.e.cel7A* with a modified carbohydrate binding module, respectively (Ilmén et al. 2011). For the construction of plasmids pMUSD1/2/3, the DNA fragments containing *S.f.cel3A*, *T.r.cel5A*, and *T.e.cel7A* open reading frames were released from their respective plasmids namely pBKD1-BGL1, pRDH147, pRDH226 by digesting with *Pac*I and *Asc*I, and subsequently ligating the individual fragments into the same restriction sites on the yeast expression vector pMU1531. In this way, three distinct plasmids encoding different reporter proteins were constructed. The pRDH147 plasmid is a yENO1 yeast expression vector containing a *URA3* selection marker; 2-μm sequence for autonomous replication, as well as the synthetic *T. reesei cel5A* gene under the transcriptional control of the *S. cerevisiae* enolase gene (*ENO1*) promoter and terminator. The synthetic gene was based on an amino acid sequence retrieved from GenBank (accession no. P07982.1) and codon optimized for expression in *S. cerevisiae* by proprietary gene design software from GeneArt Gene Synthesis (Burlingame, CA, USA) (GenBank accession nr. KX255673). The zeocin resistance marker was

Table 2 All plasmid genotypes utilized in this study

Plasmid	Relevant genotype	Reference
pMU1531	*bla* $ENO1_P$-$ENO1_T$ *sh ble*	This laboratory
pBKD1-BGL1	*bla URA3* $PGK1_P$ *xyn2 s.f.cel3A* $PGK1_T$	McBride et al. (2007)
pBKD2	*bla δ-site* $ENO1_P$-$ENO1_T$ *kanMX δ-site*	McBride et al. (2007)
pRDH147	*Bla URA3* $ENO1_P$ *T.r.cel5A* $ENO1_T$	This laboratory
pRDH226	*bla δ-site* $ENO1_P$ *T.e.cel7A* $ENO1_T$ *sh ble δ-site*	This laboratory
pMUSD1	*bla* $ENO1_P$ *xyn2 S.f.cel3A* $ENO1_T$ *kanMX*	This work
pMUSD2	*bla* $ENO1_P$ *T.r.cel5A* $ENO1_T$ *kanMX*	This work
pMUSD3	*bla* $ENO1_P$ *T.e.cel7A* $ENO1_T$ *kanMX*	This work

removed from pMU1531 by restricting the plasmid with *Bam*HI and *Spe*I and replaced by the *kan*MX resistance marker from plasmid pBKD2 backbone, to yield plasmids pMUSD1/2/3 expressing *S.f.cel3A*, *T.r.cel5A*, and *T.e.cel7A*, respectively.

Plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russel 2001). Yeast transformation was carried out using an adapted electroporation method (Cho et al. 1999). Transformants were plated out on 200 μg/ml G418-containing plates after an expression step of 3 h in YPD medium containing 1 M sorbitol. Up to ten colonies from each transformant strain were screened in 5 mL YPD based on total cell-specific activity, to account for clonal variance. The transformants with the highest cell-specific activity were chosen for triplicate assays. The total DNA of transformants was isolated and the presence of the cellulase genes in strains was confirmed through PCR using enzyme specific primers (Table 1) and with esculin and carboxymethyl cellulose (CMC) plate assays (data not shown) (Njokweni et al. 2012).

Enzyme activity assays

Each of the pMUSD1/2/3 plasmid containing strains were cultured separately and enzyme activities subsequently assayed in triplicate. Transformants were inoculated to an A_{600} of 1 into 20 mL YPD (supplemented with 400 μg/mL G418) in 125-mL Erlenmeyer flasks and were grown up separately for 72 h, in order to assay three individual, extracellular enzyme activities namely *S.f.*Cel3A, *T.r.*Cel5A, and *T.e.*Cel7A. To evaluate β-glucosidase activities produced by transformants expressing the gene *S.f.cel*3A, enzyme assays were performed in triplicate at 24-h intervals with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Sigma-Aldrich, St. Louis, MO, USA) as a substrate, with reactions times of 5 min at 50 °C (Kroukamp et al. 2013). Cellobiohyrolase activity from transformants expressing the gene *T.e.cel*7A was evaluated at 24-h intervals according to an adapted method described by Ilmén et al. (2011), using methyllumberiferyl β-D-lactopyranoside (MULac) (Sigma) as a substrate, with reactions carried out for 15 min at 50 °C. To evaluate the endoglucanase activity from transformants expressing the gene *T.e.cel*5A, enzyme assays were performed at 24-h intervals using the substrate Cellazyme C (Megazyme, Bray, Ireland) (according to manufacturer's instructions) and an adapted CMC/DNS method for 15 min at 50 °C (Den Haan et al. 2007).

For the *p*NPG assays, a *p*NP standard curve in the range of 1.5–3 mM was used. The DNS standard curve ranged between 0.5–1.5 mM glucose and the MU standard curve ranged between 0.63–20 μM. Enzyme activities were expressed as units/mg or units/g DCW, where one unit was defined as the amount of enzyme required to release 1 μmol of reducing sugar or equivalent per minute. All volumetric values were normalized with dry cell weight (DCW) of the corresponding yeast cultures in milligram per milliliter (Meinander et al. 1996). Native invertase activity was measured by adapting the protocol of Harkness and Arnason (2014). The data sets for enzyme activities were tested for statistical significance using the Student's *T* test and ANOVA. The p values < 0.05 were deemed significant.

Quantification of gene copy number using quantitative PCR

Real-time quantitative PCR was used to enumerate the *kan*MX antibiotic selection marker that had been used to facilitate plasmid selection during transformation, allowing us to elucidate the copy numbers of each of the cellulase expression cassettes. The gene encoding α-1,2-mannosyltransferase (*ALG*9) was selected to normalize the copy number of our genes of interest, as it is present as a single copy in the haploid complement *S. cerevisiae* genome (Teste et al. 2009). All DNA concentrations measurements were carried out using the ND-1000 Spectrophotometer NanoDrop (ThermoFischer Scientific). Real-time quantitative PCR was carried out using KAPA™ HRM Fast PCR kit and the Applied Biosystems StepOne™ Real-Time PCR System, whilst quantifications of gene copy number were carried out using relative standard curve method (Applied Biosystems: Guide to Performing Quantification of Gene Expression Using RT qPCR; 2008). The copy numbers of the cellulase genes were determined using the *kan*MX marker gene (present downstream of the cellulase genes on the plasmids) relative to the *ALG9* reference gene found as a single copy in the genome. The efficiency of amplification for each primer set was determined from a plot of C_t values of serial dilutions of the template DNA. The efficiency of amplification of the *ALG9* gene was 2.08, while that of the *kan*MX gene was 1.98.

Ploidy determination

Flow cytometry and PCR using primers detailed in Table 1 were used to verify the ploidy of each wild-type strain. Protocols for ploidy determination were adapted from published methods and cells were prepared for flow cytometry analysis accordingly (Dumortier et al. 2006). A haploid strain of S288c was used as a reference for a haploid complement of genomic DNA. Samples of *S. cerevisiae* cells (2×10^6 cells/mL) were fixed in 70 % ethanol (*v*/*v*) at −20 °C for 24 h and the cells were subsequently washed with Tris-buffer (pH 7.7) and re-suspended with 1 mg/mL RNase A (ThermoScientific) and 50 μl propidium iodide (Life Technologies, Waltham, MA, USA). After 48 h at 4 °C, cells were resuspended in buffer. DNA histograms were recorded with a

Table 3 Total cell and extracellular *S.f.*Cel3A activity obtained from the transformants generated in this work

Ranking[a]	Host strain[b]	*S.f.*Cel3A activity[c] (U/mg DCW)	
		Extracellular activity	Total cell activity
1	FIN1	2.54[d] ± 1.38	7.07 ± 2.49
2	MF15	1.22[e] ± 0.57	2.12 ± 0.96
3	YI19	1.19[e] ± 0.24	1.72 ± 1.45
4	YI27	1.05 ± 0.81	2.18 ± 1.46
5	HR4	0.90[e] ± 0.26	0.86 ± 0.81
6	YI59	0.88[e] ± 0.15	1.89 ± 1.32
7	W21	0.87 ± 0.28	1.45 ± 1.07
8	YI9	0.74 ± 0.39	1.38 ± 1.06
9	MF1	0.59 ± 0.16	1.75 ± 1.24
10	YI14	0.56 ± 0.17	2.09 ± 1.38
11	B25	0.53 ± 0.25	1.24 ± 0.70
12	C11	0.50 ± 0.09	3.93 ± 1.72
13	YI64	0.44 ± 0.08	1.04 ± 0.92
14	YI2	0.32 ± 0.23	1.17 ± 0.90
15	W13	0.31 ± 0.09	1.11 ± 0.84
16	YI57	0.30 ± 0.04	1.08 ± 0.73
17	YI13	0.27[e] ± 0.10	2.01 ± 1.45
18	YI52	0.26 ± 0.13	1.84 ± 1.47
19	YI38	0.25 ± 0.05	2.52 ± 1.26
20	W12	0.24 ± 0.05	2.21 ± 1.33
21	W24	0.23 ± 0.04	1.79 ± 1.41
22	Y11	0.20[e] ± 0.07	1.50 ± 1.15
23	V3	0.20 ± 0.14	1.33 ± 1.04
24	F11	0.18 ± 0.25	2.25 ± 1.04
25	YI46	0.18 ± 0.31	1.18 ± 0.98
26	W5	0.18 ± 0.14	0.26 ± 0.60
27	YI56	0.13 ± 0.17	2.08 ± 1.77
28	W1	0.11 ± 0.04	1.43 ± 1.23
29	YI40	0.04 ± 0.18	1.16 ± 0.95
30	YI32	0.01 ± 0.33	4.99 ± 2.87
Ref.	HOEG[f]	0.61 ± 0.05	0.67 ± 0.92
Ref.	MH1000[g]	0.10 ± 0.21	1.05 ± 0.91
Ref.	Y294[h]	0.42 ± 0.22	4.83 ± 0.21
Ref.	S288c[i]	0.12 ± 0.05	1.15 ± 0.95

[a] Enzyme ranking according to extracellular activity level

[b] Strains are from microbial collection of *S. cerevisiae* strains, Stellenbosch University, Microbiology Department, collected by the ARC Infruitec-Nietvoorbij wine research centre (Van Der Westhuizen et al. 2000)

[c] Standard deviation of triplicates is indicated with ±

[d] Cell-specific *S.f.*Cel3A activities significantly higher than the reference strain S288c with 95 % confidence

[e] Cell-specific *S.f.*Cel3A activities significantly higher than the reference strain S288c with 99 % confidence

[f] Reference industrial strain (brewing yeast, Stellenbosch University, South Africa)

[g] Reference industrial strain (distillery yeast, Stellenbosch University, South Africa)

[h] Reference laboratory strain (ATCC 201160; diploid version)

[i] Reference laboratory strain (ATCC 204508; diploid version)

FACS Diva Version 6.1.3 flow cytometer (BD BioSciences, Franklin Lakes, New Jersey, USA). The DNA histograms were analyzed with a mathematical model to determine the distribution of cells in the various ploidy classes.

Screening for inhibitor tolerance

In order to screen for innate tolerance capabilities between the wild-type and transformed strains, the yeasts were grown at 30 °C to an A_{600} of 0.3 in 5 mL YPD (supplemented with 400 μg/mL G418 for the transformed strains). Tenfold serial dilutions were spotted onto YPD agar plates supplemented with the appropriate inhibitors as detailed below, or incubated at different temperatures, to determine the tolerance capabilities of the transformed and wild-type strains. Cells were grown for 2–3 days at 30 °C, unless otherwise noted, and viability of each dilution was scored relative to the unchallenged control for each strain. Final resistance scores were summed over the three serial dilutions then averaged over replicates and stress doses (data not shown), providing a score ranging from no growth, initial growth, medium growth to complete growth for each strain and for each stress factor (Kvitek et al. 2008). The scores were gray-scale coded to create a tolerance map as demonstrated by Kvitek et al. (2008). To evaluate inhibitor tolerance, a concentrated inhibitor cocktail was prepared as described by Martin and Jönsson (2003) containing inhibitors commonly found in lignocellulosic hydrolysates. These were: hydroxymethylfurfural (HMF) (Sigma), cinnamic acid (Sigma), and coniferyl aldehyde (Sigma) dissolved in redistilled water, as well as formic acid (Sigma), acetic acid (Sigma), and finally furfural (Sigma), resulting in a pH range 2–4 (ESM Table S1). In order to evaluate the strains' resistance to endoplasmic reticulum (ER) stress and detoxifying abilities, two antibiotics namely tunicamycin, which inhibits glycosylation (Bull and Thiede 2012), and sodium orthovanadate, which inhibits activity of phosphate metabolism (Kanik-Ennulat et al. 1995), were used respectively.

Results

Preliminary screening for superior heterologous cellulase activity from natural isolate transformants

The strains utilized in this study were obtained from a culture collection of strains isolated from the various vineyards along the coastal region of the Western Cape, South Africa (Van Der Westhuizen et al. 2000). Genotyping of PCR-based fingerprinting (sequence analysis of D1/D2 region) identified the strains as *S. cerevisiae*. We found that the transformation frequency varied with strain background and the standard laboratory strains (S288c and Y294) gave much higher transformation efficiencies than the natural isolate strains. Also, the natural isolate strains were more sensitive to G418 (Melford Laboratories) than other antibiotics such as zeocin (Melford Laboratories) and hygromycin (Calbiochem, San Diego, CA,

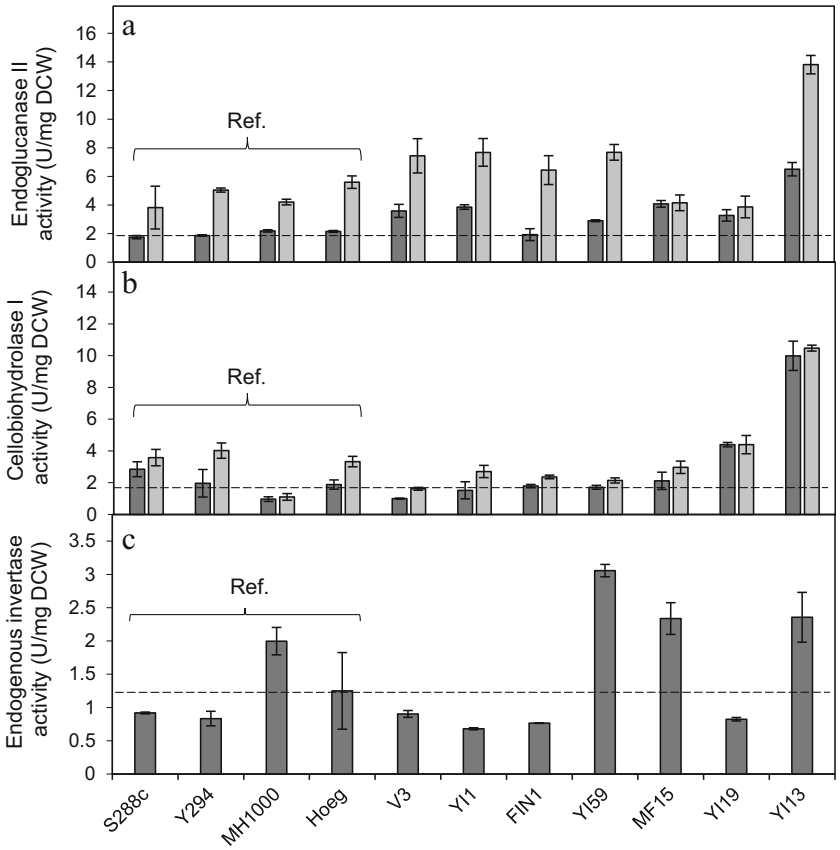

Fig. 1 Comparison between extracellular (*dark gray bars*) and total cell (*light gray bars*) **a** *T.r.*Cel5A and **b** *T.e.*Cel7A cell-specific activities of episomal *S. cerevisiae* transformants compared to reference strains (indicated as *Ref.* on the graphs). **c** Extracellular endogenous invertase activities. The *error bars* represent standard deviations from the mean of biological triplicates. Values obtained were normalized with the dry cell weight (DCW) of the yeast after 72-h incubation. The *dotted line* shown in **a–c** represents the average extracellular activity levels of the four reference strains

USA). The wild-type strains displayed no measurable activity for the cellulases tested (ESM Fig. S2).

Preliminary screening was carried out on 30 natural *S. cerevisiae* strains episomally expressing *S.f.cel3A*, with the strains illustrating the highest extracellular *S.f.*Cel3A activity selected for further study (Table 3 and ESM Fig. S2). A clear variation in heterologous secretion capacity of *S.f.cel3A* was observed between the transformants ranging between 0.04–2.54 U/mg DCW, even though the same regulatory sequence (*ENO*1 promoter and terminator) and the same vector (pMUSD1) was used (Table 3). Based on enzyme activity rankings of the transformants, the FIN1[*S.f.*Cel3A] strain was noted to have highest total cell (7.07 U/mg DCW) and extracellular (2.54 U/mg DCW) activity compared to the transformants, with a significantly higher extracellular enzyme activity that was 21-fold (p value 0.005) higher than that of the reference strain S288c. Four superior strains (FIN1, MF15, and YI19 and YI59) with promising secretion phenotypes were selected to act as host strains for further cellulase activity studies. A further three strains with moderate extracellular enzyme activity levels (V3, YI1, and YI13) were included based on their high ethanol tolerance performance (data not shown). The genes representing each of the other two classes of cellulolytic enzymes, namely *T.e.cel*7A and *T.r.cel*5A, as well as endogenous invertase activity were chosen as reporter enzymes for further analysis of extracellular enzyme activity.

While the FIN1[*S.f.*Cel3A] transformant demonstrated a significantly higher extracellular activity level (p value 0.0029) compared to the reference transformant S288c[*S.f.*Cel3A] (Table 3), the FIN1 strain demonstrated low to moderate total cell and extracellular enzyme activity levels for *T.r.cel5A* and *T.e.cel7A*, respectively (Fig. 1a and b). In contrast, natural isolate strain YI13 containing either pMUSD3 or pMUSD2, demonstrated high extracellular enzymatic activities for *T.e.*Cel7A (9.99 U/mg DCW; p value 0.041), and *T.r.*Cel5A (6.50 U/mg DCW; p value 0.0296). An impressive 3.7- and 3.5-fold higher *T.r.*Cel5A and *T.e.*Cel7A extracellular enzyme activities were observed in this strain compared to reference strain S288c, respectively. Interestingly, while the YI13 strain showed good extracellular activity levels of *T.r.*Cel5A and *T.e.*Cel7A, the *S.f.*Cel3A extracellular activity levels remained relatively low in this strain, similar to those of the reference strains (Table 3). This data coincided with the native invertase extracellular activity levels which acted as a comparative secretion reference (Fig. 1c). The native invertase activity was 2.6-fold higher in natural isolate strain YI13 compared to reference strain S288c. However, the YI59 strain was noted to have highest invertase activity (3.06 U/mg DCW), despite displaying moderate to low extracellular cellulase activity levels, while the FIN1 strain demonstrated comparatively low activity compared to the other transformants.

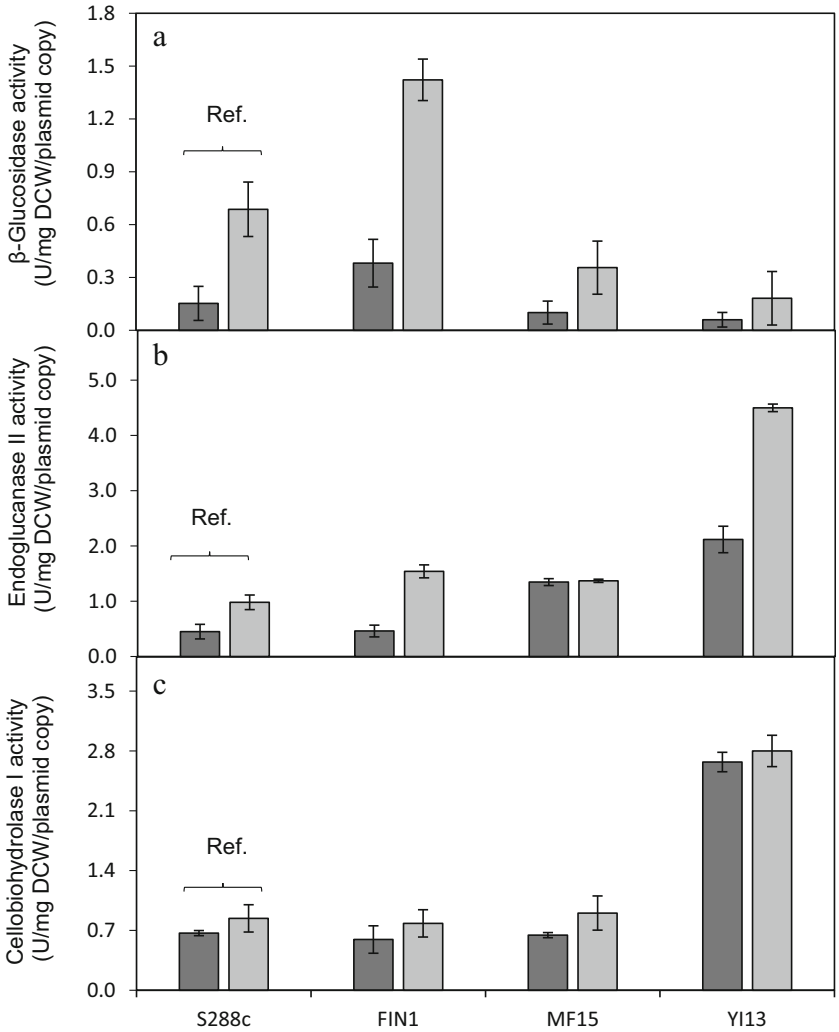

Fig. 2 Extracellular (*dark gray bars*) and total (*light gray bars*) recombinant cellulolytic activities normalized per relative plasmid copy number as determined by real-time PCR (qPCR). Transformants are ranked against the reference strain (indicated as *Ref.* on the graphs) expressing **a** *S.f.cel3A*, **b** *T.r.cel5A*, and **c** *T.e.cel7A* genes from episomal plasmids. Each enzymatic assay and qPCR was performed in triplicate. The *error bars* represent standard deviations from the mean from three biological repeats

In order to determine the relative cellulase gene copy number, real-time PCR was used to enumerate *ALG9* and *kan*MX genes. The PCR revealed that the episomal transformants had no more than a 1.7-fold difference in the copy number across all strains (ESM Table S2), therefore this parameter alone could not account for the observed significant differences in the extracellular cell-specific enzyme activities between the reference strain S288c, natural isolate strains YI13 and FIN1 transformants. However, in order to account for this variation, the cellulolytic enzyme activity levels were normalized relative to plasmid copy number (Fig. 2). After normalizing the relative plasmid copy number, the enzyme ranking of the strains did not change, with the FIN1 and YI13 continuing to be the superior strains. It is important to note that in the selection process, we selected only the highest secreting transformants and could have excluded a number of strains that had higher plasmid copy numbers which could have had an inverse effect on the secretion of the particular enzyme as suggested by Ilmén et al. (2011).

Fermentation profiles and *S.f.*Cel3A activity levels in oxygen-limited conditions

To validate the potential of the natural isolate strains expressing heterologous cellulases under oxygen-limited conditions, six transformants expressing *S.f.cel3A* were chosen based on promising cellulase activity levels (Fig. 1) for fermentation assays in simulated classical fermentation conditions to compare their fermentation profiles and secretion efficiencies simultaneously. Cell ploidy was also determined using flow cytometry and the five of the six natural isolate strains, including the superior strains YI13 and FIN1, were confirmed to be diploid (Table 4 and ESM Fig. S3). The V3 natural strain demonstrated a very large spread of fluorescence ~3–6 n) which could be indicative of aneuploidy". MH1000 yielded the most ethanol and produced 9.09 g/L (88 % theoretical ethanol yield) after 96-h fermentation, followed by YI19 and V3 which produced 88 and 84 % of the theoretical yield, respectively. The multi-tolerant strain YI13 had comparable levels (9.02 g/L and 88 % theoretical ethanol yield) and consumed glucose the fastest with

Table 4 Identities of the wild-type *S. cerevisiae* isolate strains and the fermentation performances of the strains episomally expressing *S.f.cel*3A in oxygen-limited conditions in YPD medium

Strains	Culture collection number[a]	GenBank accession number[b]	Origin	Ploidy[c]	Product yields (g/L)[d] of *S.f.*Cel3A transformants			
					Ethanol[e]	Residual glucose	Acetate	Glycerol
MH1000[f]	n/a	KX428525	Distillery	Diploid	9.09 ± 0.02 (88 %)	0.26 ± 0.08	0.81 ± 0.11	1.16 ± 0.81
S288c[g]	n/a	n/a	Laboratory	Diploid	8.98 ± 0.01 (89 %)	0.17 ± 0.00	0.89 ± 0.02	1.14 ± 0.89
V3	21,381	KX428526	Vineyard	Unknown[h]	9.03 ± 0.03 (84 %)	0.21 ± 0.08	0.68 ± 0.03	0.97 ± 0.68
FIN1	21,379	KX428522	Vineyard	Diploid	8.55 ± 0.03 (86 %)	0.52 ± 0.53	0.72 ± 0.14	1.41 ± 0.08
YI19	21,384	KX428529	Vineyard	Diploid	9.03 ± 0.12 (88 %)	0.54 ± 0.01	0.87 ± 0.12	1.08 ± 0.007
YI59	21,382	KX428530	Vineyard	Diploid	8.78 ± 0.01 (89 %)	0.21 ± 0.36	0.78 ± 0.11	1.44 ± 0.07
YI1	21,383	KX428527	Vineyard	Diploid	8.83 ± 0.01 (87 %)	0.50 ± 0.14	0.74 ± 0.18	1.19 ± 0.08
YI13	21,378	KX428528	Vineyard	Diploid	9.02 ± 0.04 (89 %)	0.17 ± 0.30	0.93 ± 0.08	1.09 ± 0.00

[a] Cultures of natural strain isolates were deposited at the Plant Protection Research Institute (PPRI), Agricultural Research Council (ARC), Queenswood, Pretoria, South Africa

[b] Identified directly with D1/D2 sequencing

[c] Ploidy was determined by flow cytometry by staining the DNA content of *S. cerevisiae* strains. Laboratory strains were constructed to be diploid

[d] Accumulated product yields after 96 h in YPD under oxygen-limited conditions. Product yield was calculated at the time of maximal ethanol concentration determined by high-performance liquid chromatography (HPLC)

[e] The percentages are the calculated theoretical ethanol yields

[f] Benchmark industrial strain (distillery yeast, Stellenbosch University, South Africa)

[g] Common laboratory strain (ATCC 204508)

[h] Possible aneuploid

residual glucose concentrations of 0.30 g/L and 0.29 (Table 4), respectively compared to the rest of the transformants. The YI13 strain demonstrated the highest acetate production levels (0.93 g/L) and YI59 the highest glycerol yields (1.44 g/L) compared to the other transformants after 96 h. The FIN1 strain demonstrated the lowest ethanol yields (8.55 g/L) and highest residual glucose concentrations (0.52 g/L). However, this strain demonstrated the highest extracellular *S.f.*Cel3A activity (3.69 U/mg DCW) (Fig. 3a) and total cell activity (15.95 U/mg DCW) under oxygen-limited conditions (Fig. 3b). With the exception of one strain, an improvement in total cell *S.f.*Cel3A enzyme activity levels was detected in all transformants under oxygen-limited conditions in comparison to aerobic conditions, with an average total cell *S.f.*Cel3A activity increase of 2.1-fold (Fig. 3a). Interestingly, this increase did not always translate to an increase in extracellular activity. The FIN[*S.f.*Cel3A] and S288c[*S.f.*Cel3A] strains had an increase in extracellular activity 2.2- and 10.3-fold respectively, in comparison to aerobic conditions, suggesting that the cell-specific secretion of these strains was improved under oxygen-limited conditions.

Growth analysis of natural isolate strains

Since the transformants of YI13 demonstrated relatively high extracellular cellulase activity levels, the effect of recombinant enzyme production on growth kinetics was determined and compared to reference strain S288c. The YI13 transformants reached higher OD_{600nm}, in the range of 12.19–9.65, compared to S288c transformants which had a range of OD_{600nm} 10.00–9.50 (Fig. 4). The cellulase expression had no significant deleterious effect on the growth capability of most of the yeast strains. However, the strain YI13[Cel5A] illustrated a notable decrease in growth rate (0.43 $\mu max/h^{-1}$) relative to control strain YI13[empty] (0.53 $\mu max/h^{-1}$) expressing an empty plasmid pBKD2 (Table 5). The diminished growth rate became prevalent after 23 h, after the cultures had entered diauxic growth, a period where the yeast switched its metabolism from utilizing glucose though glycolysis to utilization of ethanol (Teste et al. 2009). Although *T.r.*Cel5A seemed to have a significant impact on growth rate, YI13 [Cel5A] reached the same high optical densities after 62 h compared to the other YI13 transformants.

Stress tolerance characteristics of natural isolate strains

The gray-scale gradient in the phenotypic stress assays in Fig. 5 shows the tolerance of each *S. cerevisiae* strain to a given stress, with black being most tolerant and light gray as the least tolerant. The variation in gray-scale within each column in Fig. 5 demonstrates the vast phenotypic diversity among the *S. cerevisiae* strains for each of the stress concentrations tested. Relative scoring of growth of all strains on all parameters is given in ESM Table S2. The effect of genetic manipulation on the innate tolerance capabilities of the natural

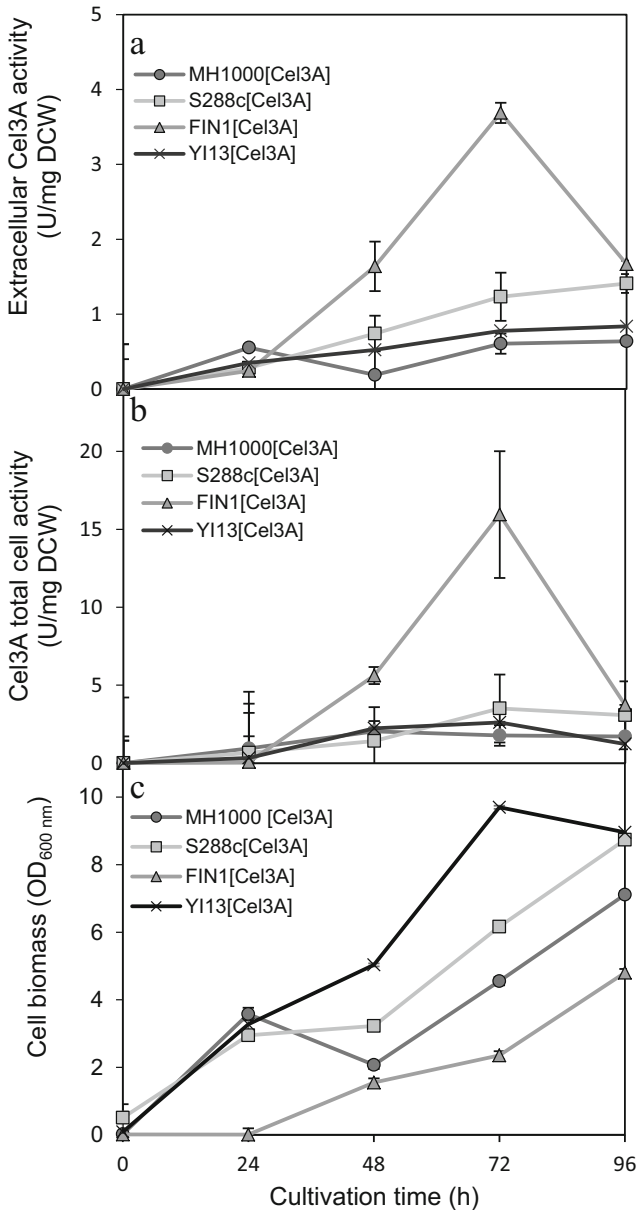

Fig. 3 Recombinant *S.f.*Cel3A activity profiles of natural strains FIN1 and YI13 compared to the reference strains MH1000 and S288c cultured in 100 mL YPD under oxygen-limited conditions. Graphs display the **a** extracellular *S.f.*Cel3A activity, **b** total-cell *S.f.*Cel3A activity, and **c** cell biomass over the 96-h period in shake flasks. Error bars are standard deviations from the mean calculated from three biological triplicates

isolate strains was also examined. Significant differences were observed in the overall phenotypic diversity between the *S. cerevisiae* strains. For specific strains, such as superior strain YI13 and industrial strain MH1000, multi-tolerance characteristics were observed for NaCl, ethanol, heat, and inhibitor stress. Additionally, the overall tolerance capabilities were lower among laboratory S288c strains and superior strain FIN1. Since a highly active pathway is important for the cell to tolerate environmental stresses (Mattanovich et al. 2004), the strains' tolerance to two secretion stresses namely tunicamycin and sodium orthovanadate were chosen to provoke diverse physiological responses. Interestingly, the multi-tolerant YI13 strain and its transformants were identified as being resistant to varying concentrations of secretion stressor tunicamycin (0.8–1.0 μg/ml). In comparison, the reference strain S288c was more sensitive to tunicamycin, with growth inhibited at a relatively low concentration of 0.5 μg/ml.

Discussion

The main aim of this study was to exploit the natural biodiversity of *S. cerevisiae* in order to find strains with combined desirable characteristics for CBP, i.e. high secretory phenotype and tolerance to various stresses associated with 2G bioethanol productions, in comparison to reference laboratory and industrial strains. Our results demonstrate a remarkably large phenotypic diversity among *S. cerevisiae* isolates, extending the results of Mukherjee et al. (2014); Kvitek et al. (2008); Skelly et al. (2013), and Warringer et al. (2011). However, to date, no studies have evaluated the potential of natural *S. cerevisiae* strains with regards to secreted recombinant cellulase activity. The multi-tolerant natural YI13 strain was superior to the reference laboratory strain S288c and other transformants with respect to cell-specific extracellular activity levels of *T.r.*Cel5A and *T.e.*Cel7A (Fig. 1a and b). However, this strain displayed comparatively lower invertase expression signifying that this native extracellular enzyme was a poor indicator to heterologous secretion potency (Fig. 1c). The natural isolate strain FIN1 outperformed the YI13 strain with regards to *S.f.*Cel3A secreted cell-specific activity in aerated (Table 3) and oxygen-limited conditions (Fig. 3a) suggesting that cell-specific activity levels were dependent on the genetic background of the host. To our knowledge, this is the first reported case demonstrating high secretory capacity among natural isolate strains compared to domesticated strains, indicating that the inherent genetic background influences heterologous protein secretion levels.

This study substantiates previous reports that recombinant enzyme activity levels are distinctly protein-specific as observed by Idiris et al. (2010),; Kroukamp (2015), and Van Zyl et al. (2014). In our study, no single strain demonstrated the highest secreted activity for all enzymes evaluated, further suggesting a compatibility factor in terms of the properties of the protein itself and the host which may influence the cell-specific enzyme activity levels (Kroukamp et al. 2013; Van Zyl et al. 2014). However, different proteins will cause different categories and different levels of cellular stress, and will hence result in different levels of final product (Liu et al. 2013). In particular, the expression of cellobiohydrolases and β-glucosidases in *S. cerevisiae* has consistently proven to be difficult (Njokweni et al. 2012; Den Haan et al. 2013). High heterologous cellulase expression causes endoplasmic

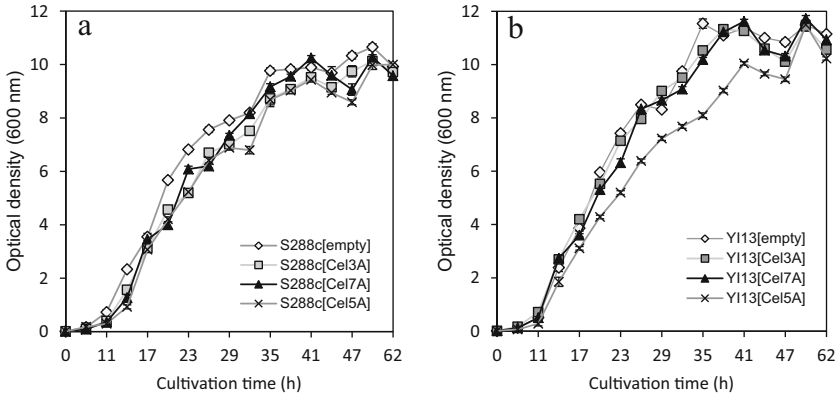

Fig. 4 Growth curves of the **a** S288c reference transformants and **b** YI13 transformants episomally expressing *S.f.cel3A*, *T.r.cel5A*, and *T.e.cel7A* genes or an empty plasmid during the cultivation period. Absorbance was measured at 600 nm. Mean values from triplicate experiments are shown and error bars indicate the standard deviation from the mean

reticulum (ER) stress which subsequently activates the unfolded protein response (UPR), inducing genes needed to alleviate stress in secretory pathway to improve protein folding capacity (Ilmén et al. 2011). In our study, strains demonstrated different tolerance thresholds for secretion stresses induced by tunicamycin and sodium orthovanadate. The multi-tolerant YI13 strain demonstrated higher tolerance to tunicamycin, suggesting that this strain possesses a greater ER protein folding capacity and, subsequently, was able to tolerate higher protein processing which likely resulted in a better secretory and tolerance phenotype simultaneously (Mattanovich et al. 2004; Gasser et al. 2008). A highly active secretory pathway is important for the cell to tolerate different environmental conditions since the secretory pathway is implicated in other processes such as lipid biosynthesis, protein targeting and secretion as reviewed by Mattanovich et al. (2004).

Table 5 Maximum specific growth rate of the plasmid-containing reference strain S288c and the natural strain YI13

Strain	Heterologous protein[a]	μmax[bc] (h^{-1})
S288c	Empty vector	0.51 ± 0.01
	*S.f.*Cel3A	0.52 ± 0.05
	*T.r.*Cel5A	0.48 ± 0.02
	*T.e.*Cel7A	0.55 ± 0.05
YI13	Empty vector	0.53 ± 0.02
	*S.f.*Cel3A	0.49 ± 0.03
	*T.r.*Cel5A	0.43[d] ± 0.02
	*T.e.*Cel7A	0.52 ± 0.01

[a] The transformants carrying either pMUSD1/2/3 are denoted *S.f.*Cel3A/ *T.r.*Cel5A/ *T.e.*Cel7A, respectively

[b] Standard deviations from biological triplicate experiments are indicated by ±

[c] Maximum velocity was obtained from the slope of the growth curves from time period of 8–29 h

[d] Specific growth rates significantly lower than the control empty vector strains

Increased enzyme activity was reported to correlate with the DNA content of yeast cells and gene copy number with diploid states having higher levels compared to haploid states, and even greater levels produced in tetraploid species (Yamada et al. 2010). In our study, similar ploidy states (Table 4) and low variation in plasmid copy numbers (Fig. 2) were observed between the superior transformants and reference strains suggesting that differential enzyme activity most likely resulted from differences in other factors including post-translational processing (Idiris et al. 2010), protein or RNA stability (Kricka et al. 2015) and/or export (Idiris et al. 2010). Although many studies have researched the secretion pathway of *S. cerevisiae* as reviewed by Idiris et al. (2010), few show the secretion abilities of the strains in anaerobic growth conditions. From a CBP perspective, a host cell capable of high secretion and utilization of substrates under anaerobic conditions is ideal since bioethanol production processes are carried out under oxygen-limited conditions (Olson et al. 2012). The cell-specific extracellular *S.f.*Cel3A activity levels were higher in oxygen-limited conditions (Fig. 3a) compared to aerated conditions (ESM Fig. S2). Recent research demonstrated that α-amylase enzyme production from *S. cerevisiae* in aerobic conditions was limited by the protein folding capacity and that higher productivity was obtained under anaerobic conditions (Liu et al. 2013). The amylase volumetric yield increased 2-fold in anaerobic cultivation compared to aerobic conditions suggesting that amylase tended to gain higher production and reduced rates of ER misfolding in these conditions. Based on Fig. 3d, the decrease in biomass resulted in the cell-specific *S.f.*Cel3A activities being higher in oxygen-limited conditions compared to aerobic conditions. While the FIN1[*S.f.*Cel3A] strain produced the highest total and extracellular *S.f.*Cel3A activity under oxygen-limited conditions (Fig. 3a), the strain consumed glucose slowly (Fig. 3c) and produced the highest glycerol yields compared to the rest of the transformants indicating that the strain was stressed (Table 4). The difference in growth rates

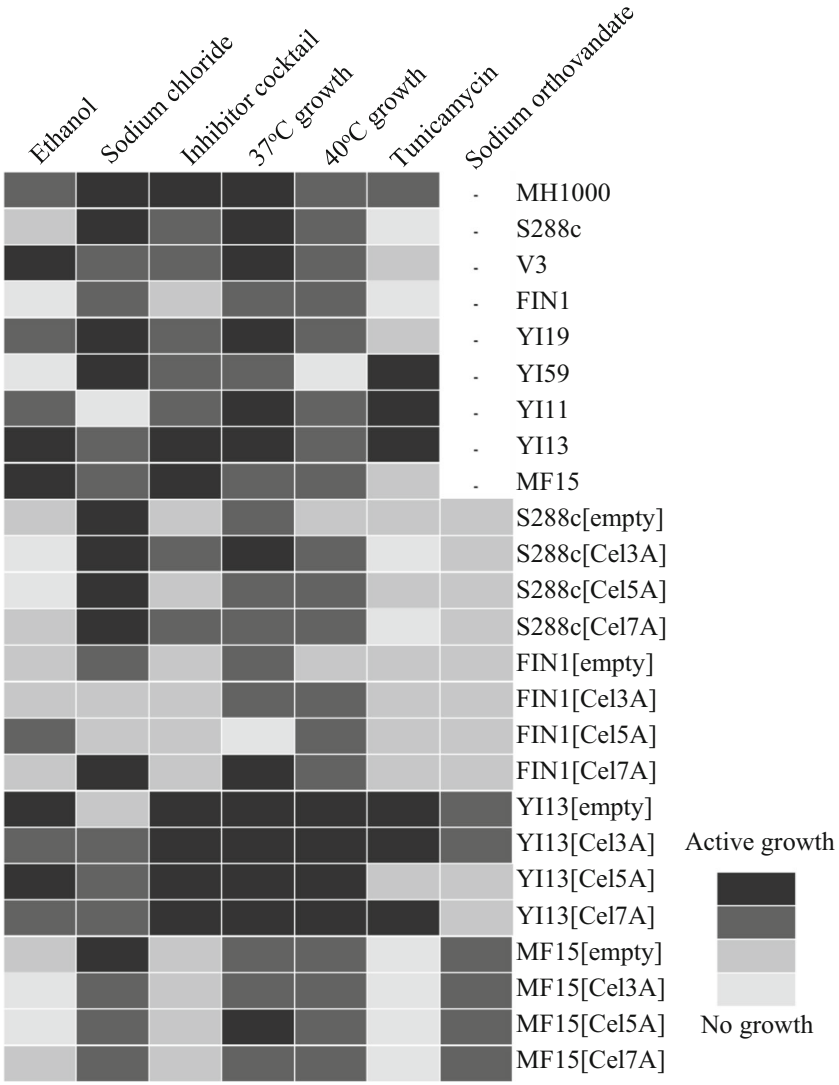

Fig. 5 Inter-strain diversity of tolerance between wild-type natural isolates, transformed strains, and reference strains. The viability of seven natural strains, two reference strains MH1000 and S288c, and 16 recombinant strains cultivated under seven different environmental conditions was measured. *Each row* on the plot represents a different strain and *each column* indicates a given environment. *Gray-scale boxes* represent the average growth rate score of each strain cultivated in each environment, according to the key shown at the *lower right* as adapted from Kvitek et al. (2008)

possibly resulted in lower ER throughput, therefore less secretion stress, resulting in higher *S.f.*Cel3A secreted activity (Ilmén et al. 2011).

In addition to this phenotypic variability analysis, we assessed the fermentation profiles and tolerance capabilities of natural isolate strains which may be suited to bioethanol production. Although the natural isolate strains resulted in similar ethanol titers ranging from 8.6–9.1 g/L under oxygen-limited conditions, the concentrations of the secondary products differed considerably after 96 h (Table 4). The YI13[*S.f.*Cel3A], S288c[*S.f.*Cel3A] and YI19[*S.f.*Cel3A] strains demonstrated the highest acetate production titers ranging from 0.93–0.87 g/L after 96 h compared to all the transformants which is undesirable since this compound is inhibitory (Den Haan et al. 2015) and directs the carbon flow push away from the desired ethanol production (Idiris et al. 2010). The MH1000, originally used as a distillery yeast (Favaro et al. 2013), outcompeted all other strains in terms of ethanol yield (9.1 g/L) and was clearly superior in conditions mimicking 1G bioethanol production. Nevertheless, the 2G bioethanol production contains harsh conditions in lignocellulosic hydrolysates.

Based on the findings of the plate assays (ESM Table S3), a 'tolerance map' (Fig. 5) was created, providing an overview on the innate tolerance capabilities of wild-type and transformed strains to a number of environmental stresses specific to 2G bioethanol industry. Not surprisingly, currently used MH1000 industrial strain was, in general, considerable tolerant to multiple stress factors (Fig. 5). However, we also identified multi-tolerant natural isolate strains that sometimes outperformed the industrial strain for certain traits. Therefore, we hypothesize that natural isolate strains might have great potential for commercial bioethanol production. The natural isolate strain YI13 demonstrated high resistance to osmo-tolerance, varying concentrations of ethanol and a cocktail of hydrolysate inhibitors as well as high temperatures. Furthermore, no difference in sensitivity to environmental stresses could be detected between the wild-type and

recombinant strains indicating that the YI13 strain could be an industrially attractive production organism with the advantage of facilitating controlled genetic manipulations.

In summary, this screening and analysis allowed us to identify natural *S. cerevisiae* strains with desirable traits for 2G bioethanol fermentations. Additionally, it provides new insights into the overlap between enhanced tolerance and enhanced secretory pathways. Our results illustrated the potential of evaluating the natural biodiversity between *S. cerevisiae* strains to find superior hosts that may be useful in 2G bioethanol production. This approach provides excellent candidates for further strain improvement through strain breeding to combine useful traits.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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