Enzyme and Microbial Technology 131 (2019) 109382

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

# Improved cellulase expression in diploid yeast strains enhanced consolidated bioprocessing of pretreated corn residues

Steffi A Davison[a], Nadine T Keller[b], Willem H van Zyl[a], Riaan den Haan[c,*]

[a] Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa
[b] Department of Molecular Biotechnology, Goethe-University, Germany
[c] Department of Biotechnology, University of the Western Cape, South Africa

ARTICLE INFO

*Keywords:*
Corn residues
Cellulases
Fermentation
Enzyme ratio
*S. cerevisiae*
Consolidated bioprocessing

ABSTRACT

In an effort to find a suitable genetic background for efficient cellulolytic secretion, genetically diverse strains were transformed to produce core fungal cellulases namely, β-glucosidase (BGLI), endoglucanase (EGII) and cellobiohydrolase (CBHI) in various combinations and expression configurations. The secreted enzyme activity levels, gene copy number, substrate specificities, as well as hydrolysis and fermentation yields of the transformants were analysed. The effectiveness of the partially cellulolytic yeast transformants to convert two different pre-treated corn residues, namely corn cob and corn husk was then explored. Higher secretion titers were achieved by cellulolytic strains with the YI13 genetic background and cellulolytic transformants produced up to 1.34 fold higher glucose concentrations (g/L) than a control composed of equal amounts of each enzyme type. The transformant co-producing BGLI and EGII in a secreted ratio of 1:15 (cellulase activity unit per gram dry cell weight) converted 56.5% of the cellulose present in corn cob to glucose in hydrolysis experiments and yielded 4.05 g/L ethanol in fermentations. We demonstrate that the choice of optimal genetic background and cellulase activity secretion ratio can improve cellulosic ethanol production by consolidated bioprocessing yeast strains.

## 1. Introduction

Utilisation of cellulosic feedstocks for the production of bioethanol is gaining attention for its potential advantages in a global market including balance of trade, rural employment benefits and meaningful energy security [1]. Annually, about 1.5 Pg of dry lignocellulosic biomass from agricultural crops is available for conversion to bioethanol. Corn residues, in particular, are a favorable feedstock for industrial cellulosic ethanol production and contain a high content of cellulose (32-36% dry weight) and low content of lignin (16-17% dry weight) [2]. However, due to variation in cell wall composition between different corn residues [3], it is important to determine conversion efficiencies on different pretreated corn residues such as corn cob and corn husk with any conversion methodology used. Current commercial cellulosic ethanol plants employ separate hydrolysis and fermentation or simultaneous saccharification and fermentation conversion methods [4]. However, a consolidated bioprocess (CBP) configuration, defined as the combination of saccharolytic enzyme production and secretion, hydrolysis of polysaccharides and fermentation of available sugars within a single unit is envisaged for improved process economics.

One favored strategy for CBP organism development is engineering *Saccharomyces cerevisiae* with the ability to utilise cellulose by expressing heterologous cellulase encoding genes (as reviewed by Den Haan and co-workers [5]). A minimal or 'core' combination of cellulases needs to be produced to achieve significant hydrolysis of cellulosic substrates. Several combinations of genes such as *Saccharomycopsis fibuligera* β-glucosidase (*Sf-BGLI*), *Trichoderma reesei* endoglucanase (*Tr-EGII*) and *Talaromyces emersonii* cellobiohydrolase (*Te-CBHI*) have been expressed in yeast and shown to partially hydrolyse lignocellulose [6–8]. In addition, engineered strains with genetically different backgrounds have demonstrated ranging cellulolytic secretion capabilities [9–12].

Past research has demonstrated that excessively high cellulase (20 FPU/g biomass) and β-glucosidase (20 U/g biomass) loadings significantly decrease glucose concentrations [13–16], suggesting that a fine balance of cellulase activity (or "cellulase ratio") is required. The efficient conversion of lignocellulosic feedstocks, such as pretreated corn residues, to fermentable sugars and subsequently ethanol thus requires an in depth understanding of the relationship between the recalcitrant, complex substrate and the combinations of cellulases which need to be secreted at specific enzyme ratios [17]. In biotechnological industry, increased expression cassette stability and copy

* Corresponding author.
*E-mail address:* rdenhaan@uwc.ac.za (R. den Haan).

https://doi.org/10.1016/j.enzmictec.2019.109382
Received 29 May 2019; Received in revised form 27 June 2019; Accepted 12 July 2019
Available online 15 July 2019

**Table 1**
Cellulases and primers used in this study.

| Primer name | Source | Family | Function | GenBank accession no. | Primers used for verification (5′-3′) |
|---|---|---|---|---|---|
| cel3A | *S. fibuligera BGLI* | GH3 | Beta-glucosidase | [GenBank:AEV40916.1] | F-GACTCGCGAGTCCCAATTCAAAACTATACC<br>R-CCGCTCGAGCGGTCAAATAGTAAACAGGACAGATG |
| cel7A | *T. emersonii CBHI* | GH7 | Cellobiobiohydrolase I | [GenBank:AAL89553] | F-GACTTTAATTAAAATGCTAAGAAGAGCTTTACTATTG<br>R-GACTGGCGCGCCTTACAAACATTGAGAGTAGTATGGG |
| cel5A | *T. reesei EGII* | GH5 | Endo-1,4-glucanase | [GenBank:KX255673] | F-GTTAACAACAATTTGGGTGG<br>R-CAATGGAGAAAAAGCACC |
| Qcel3A | *S. fibuligera BGLI* | GH3 | Beta-glucosidase | [GenBank:AEV40916.1] | F-TTTGGTAAAGCGAACCCATC<br>R-AGGTTCACCACTCGATGGAC |
| Qcel7A | *T. emersonii CBHI* | GH7 | Cellobiobiohydrolase I | [GenBank:AAL89553] | F-CTGACGTCGAATCCCAATCT<br>R-GACCTGGAGGGTTAGAAGCA |
| Qcel5A | *T. reesei EGII* | GH5 | Endo-1,4-glucanase | [GenBank:KX255673] | F-TCAATGTATTCCAGGTGCT<br>R-GGTGGAGTAGAAGAAGATG |
| ALG9 | *S. cerevisiae* | GT71 | α-1,2-mannosyltransferase | [GenBank: Z7149.1] | F-TGCATTTGCTGTGATTGTCA<br>R-GCCAGATTCCTCACTTGCAT |

number serve as important means of maintaining consistently high production levels of heterologous proteins in *S. cerevisiae* [17]. With the advancement of techniques which allow stable, high copy numbers in yeasts such as *POT1*-mediated delta (δ) integration [18], it is important to understand the effect of copy number on protein production ratio's and the influence this has on hydrolysis and fermentation. In nature, the genomes of cellulolytic organisms encode a wide array of catalytic subunits evolved to address the challenges presented by chemical heterogeneity and structural complexity of natural lignocellulosic substrates. Furthermore, the ratio of each of the cellulases are fine-tuned via regulation of the expression of the cellulase encoding genes to achieve the maximum hydrolysis in response to the environment (as reviewed by Kunitake and co-workers [19]). A combination of genetic background, transcription machinery, selecting the optimal cellulase encoding gene as well as gene copy number have been shown to be the most significant factors influencing the conversion of cellulosic substrates by recombinant strains [9,20,21]. It has proved challenging to adjust the specific concentration and ratios of each cellulase in a heterologous system in order to achieve a more efficient hydrolysis process utilising lower enzyme dosages [21,22]. As a result, improving the efficiency of cellulolytic enzymes has been an active area of research, with efforts dedicated towards understanding the synergy displayed by combinations of cellulases and optimisation of cellulase ratios [16,17]. All of these elements are aimed at reducing enzyme loading for efficient cellulose hydrolysis, and ultimately reducing production cost.

It is clear that variation in cellulase secretion capabilities of *S. cerevisiae* can be explained in terms of many factors including impact of genetic background [9–12,23,24]. Since external and internal stresses can impact the yield of secreted recombinant protein in *S. cerevisiae* [23–26], utilising a stress-tolerant strain background may make a significant difference in the feasibility and profitability of the cellulosic bioethanol production process. Previously, we evaluated thirty natural *S. cerevisiae* isolates for superior secretion activity and other industrially relevant characteristics needed during the process of lignocellulosic ethanol production [9]. Natural strain YI13 was identified to have a high secretory phenotype, demonstrating a 3.7- and 3.5-fold higher Cel7A (CBHI) and Cel5A (EGII) secreted enzyme activity respectively, compared to a reference laboratory strain. YI13 also demonstrated other industrially relevant characteristics such as growth vigor, high ethanol titer, tolerance to high temperatures (37 °C and 40 °C), ethanol (10% w/v), and towards various concentrations of a cocktail of inhibitory compounds commonly found in lignocellulose hydrolysates. Other studies have identified *S. cerevisiae* strains capable of effectively fermenting glucose from pretreated biomass (13,27-29). However, no work has been published reporting the engineering of an *S. cerevisiae* strain, with a natural strain isolate background, with partial cellulolytic capabilities that can ferment glucose from pretreated biomass, a requirement for a CBP process.

In this study, we evaluated the expression of a combination of cellulases in different strain backgrounds namely the natural strain isolate YI13, a diploid version of the laboratory strain S288C a/α and industrial strain Ethanol Red, and investigated different configurations of partially cellulolytic *S. cerevisiae* strains using a combined strategy of δ-integration of the *Sf-BGLI* (*cel3A*) and subsequent transformation with high-copy plasmids containing either *Tr-EGII* (*cel5A*) or *Te-CBHI* (*cel7A*), respectively. The enzyme hydrolysis activity of the cellulolytic strains were compared to a control composed of equal enzyme activity units (U/g DCW) of each enzyme type. We evaluated the efficiency of these strains for enzymatic hydrolysis on different corn residues for the release of fermentable sugars and the importance of different cellulases ratios. Furthermore, the fermentation ability of cellulolytic strains were compared to control fermentations that were supplemented with a commercial cellulase Cellic® CTec2 (Novozymes, Bagsværd, Denmark). This study combined the advantages of using a robust fermentative yeast strain, with improved cellulase ratios to convert corn residues to bioethanol, demonstrating a reduced requirement for externally supplied enzyme. Furthermore, we explored how the heterogeneity of agricultural feedstocks influenced ethanol concentrations in a CBP.

## 2. Materials and Methods

### 2.1. DNA manipulation and construction of recombinant strains

Standard molecular biology techniques were used as described by Sambrook and Russel [30]. *E. coli* was grown in LB medium (0.5% yeast extract, 1% tryptone, 1% sodium chloride; Merck, Darmstadt) containing 100 μg/mL ampicillin. Techniques for manipulation of *S. cerevisiae* were described previously [31,32]. Diploid *Saccharomyces cerevisiae* strains Ethanol Red (Fermentis, a division of S. I. Lesaffre, Lille, http://www.fermentis.com), natural strain isolate YI13 (KX428528.1) and the diploid version of S288c (ATCC 204508) were used as host strains for the expression of multiple cellulase genes namely *S. fibuligera cel3A* (called *Sf-BGL*), *T. reesei cel5*A (called *Tr-EGII*) and *T. emersonii cel7A* (called *Te-CBHI*) (Table 1 and Table 2).

PCR products were amplified from pMUSD1 using the Phusion High Fidelity DNA polymerase (Thermo Scientific-Waltham, USA) used on an Applied Biosystems 2720 thermocycler (Life Technologies-CA, USA) as instructed by the manufacturer, using forward and reverse primers (Table 1) that included *Pac*I and *Asc*I restriction sites for subsequent directional cloning of *Sf-BGLI* from pMUSD1 [9] into the pBCD1 [7] to create the pRDH234 yeast integration vector (Table 2). High-copy plasmids namely pMUSD1, pMUSD2 and pMUSD3 were previously constructed in this laboratory [9] (Supplementary Fig. S1) and plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method [30]. PCR products and DNA fragments were routinely separated on 1% (w/v) agarose (Lonza, Rockland, ME, USA) gels and

**Table 2**
Plasmid and strain constructs used in this study.

| Components | Genotype | Reference |
|---|---|---|
| *S. cerevisiae strains* | | |
| S288c | *MATa/α, α leu2-3,112 ura3-52 his3 trp1-289LEU3* | ATCC 204508 |
| S288c[cel3A] | *MATa/α, α leu2-3,112 ura3-52 his3 trp1-289LEU3, ENOp-cel3A-ENOt-kanMX* | [9] |
| S288c[cel7A] | *MATa/α, α leu2-3,112 ura3-52 his3 trp1-289LEU3, ENOp-cel7A-ENOt-kanMX* | [9] |
| S288c[cel5a] | *MATa/α, α leu2-3,112 ura3-52 his3 trp1-289LEU3, ENOp-cel5A-ENOt-kanMX* | [9] |
| Ethanol Red | *MATa/α* | This study |
| YI13 | *MATa/α* | KX428528 |
| YI13[cel3A] | *MATa/α, ENOp-cel3A-ENOt-kanMX* | [9] |
| YI13[cel7A] | *MATa/α, ENOp-cel7A-ENOt-kanMX* | [9] |
| YI13[cel5A] | *MATa/α, ENOp-cel5A-ENOt-kanMX* | [9] |
| Ethanol Red[cel3A] | *MATa/α, ENOp-cel3A-ENOt-kanMX* | This study |
| Ethanol Red[cel7A] | *MATa/α, ENOp-cel7A-ENOt-kanMX* | This study |
| Ethanol Red[cel5A] | *MATa/α, ENOp-cel5A-ENOt-kanMX* | This study |
| YI13_cel3A | *MATa/α, δ-site PGKp-cel3A-PGKt-natMX δ-site* | This study |
| YI13_cel3A[cel7A] | *MATa/α, δ-site PGKp-cel3A-PGKt-natMX δ-site / ENOp-cel7A-ENOt-kanMX* | This study |
| YI13_cel3A[cel5A] | *MATa/α, δ-site PGKp-cel3A-PGKt-natMX δ-site / ENOp-cel5A-ENOt-kanMX* | This study |
| *Plasmids* | | |
| pRDH234 | *bla URA3 δ-site PGKp-cel3A-PGKt-natMX δ-site* | This study |
| pBCD1 | *bla URA3 δ-site PGKp-PGKt-natMX δ-site* | [33] |
| pMUSD1 | *bla URA3 ENO1p-cel3A-ENO1t-kanMX* | [9] |
| pMUSD2 | *bla URA3 ENO1p-cel5A-ENO1t-kanMX* | [9] |
| pMUSD3 | *bla URA3 ENO1p-cel7A-ENO1t-kanMX* | [9] |

fragments of appropriate sizes were isolated using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA).

For yeast transformation, an electroporation method was used [31]. After an expression step of 2 h in YPD medium, the transformants were plated out on YPD agar plates with the respective antibiotic (50 µg/mL cloNAT [Werner BioAgents, Jena, Germany] and 200 µg/mL G418 [Merck]). The transformed yeast strains used for enzyme assays and fermentations were cultured at 30 °C and 200 rpm in YPD (1% yeast extract, 2% peptone, 2% glucose; Merck, Darmstadt) supplemented with the appropriate antibiotics. Recombinant strains were created through δ-integration with gene cassettes containing *Sf-BGLI* under control of the *S. cerevisiae PGK1* promoter and terminator sequences, creating cellulolytic yeast strains listed in Table 2. High copy plasmids pMUSD2 and pMUSD3, containing *Tr-EGII* and *Te-CBHI* genes respectively, under the control of the *S. cerevisiae ENO1* promoter and terminator sequences, were transformed into *Sf-BGLI*-integrated strains creating co-expressing strains (Table 2). The presence of cellulase genes in the colonies were confirmed through colony PCR using enzyme specific primers (Table 1) and with esculin and carboxymethyl cellulose (CMC) plate assays (data not shown) [34].

### 2.2. Enzyme liquid assays

The enzyme activity profiles of three clonal transformants with an Ethanol Red background were compared to the best performing YI13 and S288c transformants constructed in an earlier study [9]. Yeast cells were grown in 100 mL Erlenmeyer flasks with 10 mL YPD media for 72 h at 30 °C at 200 rpm. Cellulase activity assays for β-glucosidase (Cel3A) and cellobiohydrolase (Cel7A) were performed according to the protocols in Davison and co-workers [9]. All liquid enzyme activity plate assays were performed in 96 well plate formats. The β-glucosidase and cellobiohydrolase activities were monitored using *p*-nitrophenyl-β-D-glucoside (Sigma-Aldrich, St. Louis, MO, USA) and the fluorescent substrate 4-methylumbelliferyl β-D-lactoside (MULac) (Sigma-Aldrich) as substrates, respectively.

Endoglucanase activity of the samples was measured using AZO-CM cellulose (Megazyme, Wicklow, Ireland) as a substrate. The substrate solution contained 1 g AZO-CM-cellulose mixed with 100 mL 50 mM sodium acetate buffer, pH 4.8. Precipitation solution contained 40 g sodium acetate trihydrate and 4 g zinc acetate diluted in 200 mL of deionised water, which was mixed with 800 ml 96 % ethanol (v/v) as described by Megazyme. A total of 100 µl of diluted sample or standard was added to a 1.5-ml Eppendorf tube and pre-equilibrated to 50 °C. A 100 µl amount of substrate solution was added to the tubes and mixed well. After 10 minutes of incubation, the reaction was terminated by the addition of 500 µl precipitation solution. Samples were cooled for 5 min before centrifugation for 10 min, 3,300 rpm, 1,000 × g. Absorbance was measured at 595 nm.

Plate activity screenings were done for a qualitative evaluation of enzyme activity (data not shown). Cultures were spot inoculated to screen for endoglucanase and β-glucosidase enzyme activity. The endoglucanase activity was monitored on 2% agar plates containing 1% (w/v) carboxymethylcellulose (Sigma-Aldrich) as the only carbohydrate source [35]. Plates were incubated for 72 h at 30 °C and zone formation was visualised by staining with 0.1% (w/v) Congo red (Sigma-Aldrich) for 15 min and destaining with 1 M NaCl for 30 min. The enzyme activity of β-glucosidase was measured on esculin screening plates which contained 0.1% (w/v) esculin (Sigma-Aldrich) and 0.05% (w/v) ferric citrate (Sigma-Aldrich) [34]. Plates were incubated for 72 h at 30 °C after which they were observed for black zone formation.

### 2.3. Quantitative PCR

Copy number was quantified for the best performing transformants, based on enzyme activity, by comparing the cycle threshold (*Ct*) values of target and reference genes using a previously described method [9]. Plasmids pMUSD1, pMUSD2 and pMUSD3 (described in Table 2), containing one copy of each cellulase gene, was used as template for the *Sf-BGL1*, *Tr-EGII* and *Te-CBHI* quantitative PCR (qPCR) standard curve analysis. The *ALG9* gene was selected to normalise the copy number of the gene of interest, as it is present as a single copy in the haploid complement of the *S. cerevisiae* genome [36]. The target genes were amplified using the primer pairs Qcel3A-F/Qcel3A-R, Qcel5A-F/Qcel5A-R and Qcel7A-F/Qcel7A-R, and reference gene, *ALG9*, was amplified using primer pair ALG9-F/ALG9-R (Table 1). Cycling conditions were set up according to manufacturer's instructions using KAPA™ HRM Fast PCR kit (Sigma-Aldrich) and the Applied Biosystems StepOne™ Real-Time PCR system was used for melting curve and qPCR analysis.

### 2.4. Pretreatment methods

Corn residues were kindly provided by Dr. Danie la Grange (North West University, Potchefstroom, South Africa). In brief, corn cobs and husks were milled using a Model 4 Wiley mill and sieved with a 0.5 mm screen to obtain particles ranging in size from 250 to 850 µm. The feedstock was pretreated with an alkali and autoclaving method developed by Latif and Rajoka [37]. The fiber material was treated with 2% sodium hydroxide (Merck) in a ratio of 1:5 (w/v) and autoclaved at 120 °C for 15 min. The pretreated corn residue was washed with water and the solids were used for fermentation and chemical composition analysis. Corn stover hydrolysates were stored at 4 °C before its use for subsequent ethanol and sugar production.

### 2.5. Substrate and chemical analysis

The composition of the substrates is detailed in Table 3. The carbohydrate, lignin and protein contents were determined according to the analytical procedure recommended by the National Renewable Energy Laboratory (NREL) (Colarado, USA). The cellulose,

**Table 3**
Chemical composition of the sodium alkaline untreated and pre-treated corn residues (% w/w).

| Substrate | Hemicellulose | Cellulose | Xyl[1] | AIL[2] | ASL[3] | Ash |
|---|---|---|---|---|---|---|
| Corn husk | 47 ± 2.290 | 36 ± 3.858 | 11 ± 3.299 | 0.5 ± 0.001 | 5.7 ± 0.074 | 1.0 ± 0.056 |
| Pre-treated corn husk | 43 ± 2.372 | 44 ± 7.250 | 7 ± 1.1450 | 0.4 ± 0.002 | 5.0 ± 0.020 | 0.5 ± 0.024 |
| Corn cob | 51 ± 4.241 | 32 ± 4.242 | 11 ± 5.3201 | 1.0 ± 0.267 | 4.7 ± 0.303 | 2.6 ± 0.0.12 |
| Pre-treated corn cob | 45 ± 4.776 | 43 ± 2.297 | 5 ± 1.8201 | 1.0 ± 0.009 | 4.3 ± 0.075 | 1.0 ± 0.051 |

[1] Xyl, xylose.
[2] AIL, acid-insoluble lignin.
[3] ASL, acid-soluble lignin.

hemicellulose and lignin content of the corn cob and corn husk before and after pretreatment were determined according to the laboratory analysis protocol of the NREL. Analysis was performed in triplicate. Fermentation and hydrolysis products were determined by HPLC as previously described in Davison and co-workers [9]. Samples were centrifuged and supernatant was filtered through a 0.22 µm filter. The concentrations of ethanol, glucose, cellobiose, xylose, lactic acid, acetic acid and glycerol were estimated.

### 2.6. Enzymatic hydrolysis

Enzymatic hydrolysis experiments were conducted in 100 mL total volume in 100 mL sealed serum bottles at 4% (w/v) substrate loading at 30 °C with stirring at 200 rpm. The enzyme hydrolysis medium contained corn stover, consisting of either corn cob or corn husk. Serum bottles with substrates were pre-incubated at 30 °C for 20 min before the addition of the supernatant of the constructed cellulolytic yeast transformants and the control enzyme ratio composed of equal enzyme units (U/g DCW) of each enzyme type. Enzyme activity ratio was reported as the ratio of secreted enzyme activity levels (U) per dry cell weight (DCW) for each enzyme type. Strains were cultured for 72 h in YPD media supplemented with the appropriate antibiotics at 30 °C and 200 rpm. The supernatant of each sample was collected by first centrifuging then filtering samples using 0.45 µm filters (Millipore, Sigma-Aldrich). Three different enzyme applications were examined in this study by examining the single and co-expression strains producing the following enzymes: i) individual Cel3A (BGLI); ii) Cel3A (BGLI) co-expressed with Cel5A (EGII) or iii) Cel3A (BGLI) co-expressed with Cel7A (CBHI). A control enzyme ratio was used with equal enzyme activity (in U/g DCW) of all three enzymes (1:1:1).

### 2.7. Fermentation of the pretreated corn

The yeast seed culture for the fermentation inoculum was prepared by culturing cells for 72 h under aerobic conditions in 50 mL YPD supplemented with the appropriate antibiotics in 200 mL flasks at 30 °C and 200 rpm. Fermentation analysis was performed under micro-aerobic conditions. The fermentation medium contained pretreated corn stover, consisting of either corn cob or corn husk. The fermentations were conducted with a final volume of 50 mL in 100 mL sealed serum bottles at 2% (w/v) substrate loading at pH 7.0 and supplemented with 100 µg/mL streptomycin and ampicillin to suppress bacterial growth. Serum bottles with substrates were pre-incubated at 30 °C for 20 min before the addition of the strain inoculums at $OD_{600}$ = 0.5. Fermentations were performed at 30 °C on a magnetic stirrer at the speed of 200 rpm. A syringe needle was used to act as a $CO_2$ outlet. Aliquots of 1 mL were taken at various times points and analysed with HPLC. Control fermentations were supplemented with 5 FPU/g Cellic® CTec2 (Novozymes) and Novozyme-188 (Novozymes).

### 2.8. Data analysis

The conversion yields were reported as a percentage of the theoretical yield. The theoretical yields were calculated assuming that 1 g of cellulose in the solid fraction yields 1.11 g of glucose and that 1 g glucose would theoretically yield 0.511 g ethanol according to equations as (Eq.(1)) and (Eq.(2)) respectively:

$$\textit{Cellulose converison yield}\ (\%) = \frac{\textit{glucose}\ (g)}{1.111\text{x}\ \textit{cellulose in substrate}\ (g)}\text{x}\ 100\% \quad (1)$$

$$\textit{Ethanol conversion yield}\ (\%) = \frac{\textit{ethanol}\ (g)}{0.511\text{x}\ \textit{cellulose in substrate}\ (g) x\ 1.111}\ \text{x}\ 100\% \quad (2)$$

The method for quantifying yield was performed according to García-Aparicio and co-workers [38]. A correction factor of 0.9 was used to compensate for the addition of a water molecule during hydrolysis according to the following equation as (Eq.(3)):

$$\textit{Glucose yield}\ (\%) = \frac{\textit{glucose}\ (g) \times\ 0.9}{\textit{cellulose} + \textit{hemicellulose}\ (\textit{polysaccharides in substrate})\ (g)} \times\ 100\% \quad (3)$$

The data sets for enzyme and fermentation activities were tested for statistical significance using ANOVA and Student's T test. The $p$ values $< 0.05$ were deemed significant.

## 3. Results and Discussion

### 3.1. Comparison of the expression of individual and multiple recombinant cellulases in S. cerevisiae strains

Although there are a number of reports on cellulolytic *S. cerevisiae* development (as reviewed by Den Haan and co-workers [5]), studies on heterologous expression of cellulases and cellulosic ethanol fermentations in native yeast isolates are limited. It is well known that the phenotypic expression of desirable traits is impacted by the genetic background and a range of recombinant cellulase secretory capacities in natural and industrial *S. cerevisiae* strains has been demonstrated [9–12]. In this study, yeast strains with diverse genetic backgrounds namely the industrial strain Ethanol Red, laboratory strain S288c and natural strain isolate YI13 were engineered to produce core cellulases namely; BGLI (Cel3A), EGII (Cel5A) and CBHI (Cel7A). The recombinant strains, expressing different combinations of *Sf-BGLI*, *Tr-EGII* and *Te-CBHI* genes, were used to compare the variation in enzyme secretion phenotypes between transformants as well as to obtain as near complete hydrolysis of a lignocellulosic substrate as possible.

We evaluated the heterologous enzyme activities of transformed strains with varying genetic backgrounds, individually expressing *Sf-BGLI*, *Tr-EGII* or *Te-CBHI* genes in different expression configurations by using a combination of high copy plasmids and integration cassettes (Table 2) (Fig. 1). A range of secreted enzyme activity was observed between the cellulolytic yeast transformants tested. As expected, all the *Sf-BGLI* transformants displayed low activity ranging in activity levels of 7.23-16.0 U/g DCW (Fig.1a). However, it is important to highlight that no significant differences ($p$ value $> 0.05$) in extracellular activity levels of *Sf-BGLI* were observed between different genetic backgrounds, aligning with previous findings regarding the difficulty in secretion of this particular enzyme [7,11]. The YI13[Cel7A] continued to

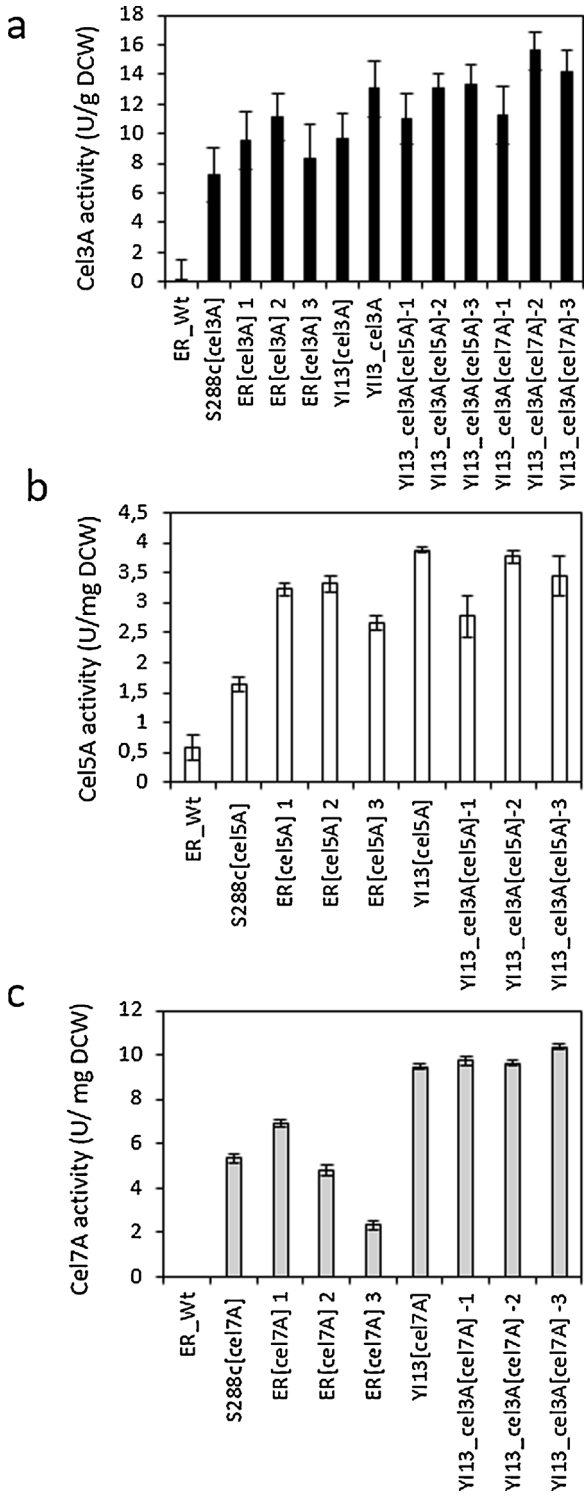


**Fig. 1.** Extracellular enzyme activity of yeast transformants based on Ethanol Red, S288c a/α and YI13 expressing individual cellulases. (a) β-Glucosidase (Cel3A) activity of single-copy integrated *Sf-BGLI* transformants. (b) Endoglucanase (Cel5A) and (c) cellobiohydrolase (Cel7A) activity of *Tr-EGII* and *Te-CBHI* high-copy expressing transformants, respectively. Data presented as means and standard deviations of biological triplicates.

demonstrate significantly higher secreted activity levels of *Te*-CBHI (Fig. 1c) ($p$ value $< 0.05$) compared to the Ethanol Red transformants. In contrast, the three transformants from the Ethanol Red strain (1-3) demonstrated low *Te*-CBHI activity (in the range of 2.33-7.32 U/mg DCW) after 72 h (Fig.1c). Although the YI13[Cel5A] strains also demonstrated higher *Tr*-EGII secreted activity levels (3.87 U/mg DCW) than the Ethanol Red transformants (2.66-3.32 U/mg DCW), not as large a difference was observed as for *Te*-CBHI secreted activity levels.

The YI13 strain not only displayed a good secretory phenotype as shown in Fig. 1c and previous studies [9,10], but also exhibited marked tolerance to various environmental stressors [9]. As a result, a base strain of YI13 with a single copy δ-integrated *Sf-BGLI* gene cassette (called strain YI13_cel3A) was utilised to build a more efficient cellulolytic, fermentative host strains. Subsequently, YI13_cel3A was co-transformed with either pMUSD2 or pMUSD3, expressing either *Tr-EGII* or *Te-CBHI* genes from episomal plasmids, respectively. Enzyme activity measurements presented in Fig. 1a showed that there was no significant difference in the *Sf*-BGLI secreted enzyme activity levels between the single gene expressing strain YI13_cel3A and the best performing co-expression transformants namely YI13_cel3A[cel5A]-3 and YI13_cel3A[cel7A]-3. Similarly, the transformant strains YI13_cel3A[cel7A] 1-3 demonstrated no significant difference in secreted CBHI activity compared to the positive control YI13 individually expressing the high copy plasmid with *Te-CBHI* (namely YI13[cel7A]) (Fig. 1c). The best performing transformant in terms of EGII activity namely YI13_cel3A[cel5A]-2 demonstrated no significant difference in EGII activity levels compared to the positive control YI13[cel5A] (Fig.1b). Furthermore, no significant variation in secreted *Te*-CBHI and *Tr*-EGII activity levels was observed between the three YI13 transformants co-expressing cellulase genes *Sf-BGLI* and *Te-CBHI* as well as between transformants expressing the genes *Sf-BGLI* and *Tr-EGII*, respectively. Therefore, it is speculated that the adverse effects of additional cellulase gene expression was negligible on secreted enzyme activity levels. This is contrasted with previous research which indicated that extracellular endoglucanase activities were generally lower when co-expressed with integrated *Sf-BGLI* in a haploid laboratory yeast strain [39]. However, clonal variation in terms of plasmid copy-number differences may account for the range of enzyme activities observed between transformants.

Quantitative PCR revealed differences in plasmid copy number between genetic backgrounds (Fig. 2). Only one copy of *Sf-BGLI* was integrated into the genome of YI13 and ER12, with minimal fold difference being observed in plasmid copy number between the single and co-expression configurations in a YI13 background (no more than 1.18 fold) (Fig. 2). In contrast, a difference between 5 and 8 copies for the *Tr-EGII* gene and 9 and 12 copies for the *Te-CBHI* gene between the ER and YI13 strains is a significant amount (Fig. 2) and potentially accounts for the observed higher activity in the latter strain (Fig. 1). We previously demonstrated that the YI13 strain could tolerate high levels of tunicamycin [9,10], a chemical stressor known to elicit endoplasmic reticulum stress and activate the conserved unfolded protein response pathway which is intimately linked to the secretion pathway [10,26]. Therefore, the innate high endoplasmic reticulum stress tolerance demonstrated by the natural strain isolate YI13 compared to industrial and laboratory strains [9,10], potentially allowed this strain to maintain higher plasmid copy numbers under cellulase co-expression compared to the industrial strain Ethanol Red (Fig. 2). This is supported by the results shown here, where higher plasmid copy numbers were observed in the YI13 strain. We hypothesize that the YI13 strain is better adapted to secretion stress and, therefore, does not need to down-regulate plasmid copy number as seen in previous cellulase expression studies by van Rensburg and co-workers [40] and Ilmén and co-workers [41], therefore resulting in higher secreted enzyme activity. A study by Ilmén and co-workers [41] evaluated the burden of maintaining a multicopy plasmid and reported that *T. emersonii* CBHI, the same enzyme used in this study, increased in protein production which correlated to an increase in secretion stress, suggesting that the organism lowers its stress burden by lowering the plasmid number.

### 3.2. Enzymatic hydrolysis of corn residues by cellulolytic yeast strains

Agricultural waste such as corn residues could provide a cheap and

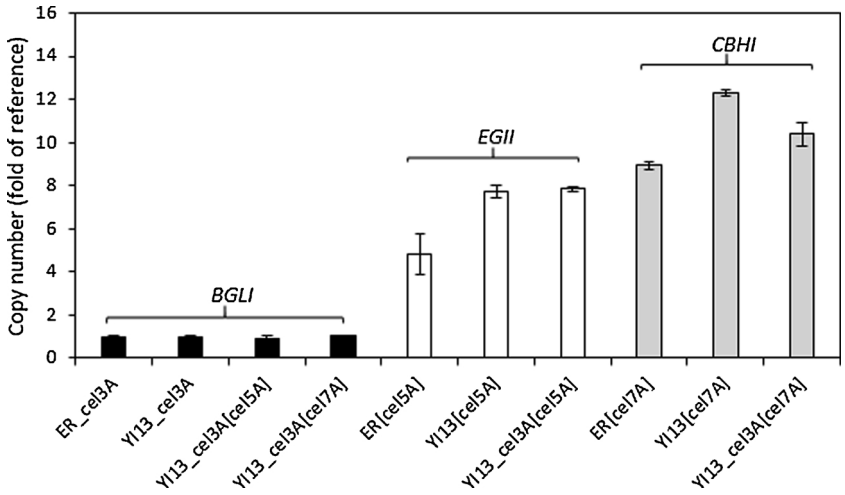


**Fig. 2.** Copy number determination of cellulase genes in the cellulolytic yeast strains as determined by qPCR. Data presented as means and standard deviations of biological triplicates.

sustainable alternative substrate for the production of bioethanol and value-added products [42]. However, recalcitrance and heterogeneity of lignocellulosic feedstocks are key challenges in their enzymatic hydrolysis and fermentation [43]. Several pretreatment methods of corn residues, including the combination of dilute alkaline treatment and milling used in this study, have been reported to increase the amount of amorphous cellulose created from crystalline cellulose in the substrate, lowering overall substrate recalcitrance [44,45]. Therefore, the applicability of cellulolytic yeast strains to converting pretreated corn residues to ethanol was studied through the hydrolysis of two different corn residues with a high cellulose content namely pretreated corn cob and corn husk (42.6% and 44.5% cellulose [w/w]) respectively (Table 3). It is speculated that factors relating to type of raw material and solid content used, affect the enzyme activity and hydrolysis during the fermentation period. For example, the higher lignin content observed in corn husk (Table 3) could contribute to a higher loss of enzyme activity due to the irreversible binding of cellulases to lignin [27].

In this study, the effective enzymatic saccharification of alkali-treated corn cob and corn husk without supplementation with commercial cellulase cocktails by a natural *S. cerevisiae* isolate engineered with different cellulase expression configurations was demonstrated for the first time. From Fig. 3a and Fig. 3b, it is observed that the amounts of sugars released from both residues by the enzymes in the supernatants of transformants increased over time from 24 h to 168 h. In the saccharification of corn cob residue, the enzyme activities from the co-expression of *Sf-BGLI* and *Tr-EGII* demonstrated higher glucose concentrations and yields (10.8 g/L, equivalent to 56.5% cellulose conversion) compared to the expression of *Sf-BGLI* and *Te-CBHI* (7.08 g/L, equivalent to 37.1% conversion) and was significantly higher than the 1:1:1 cellulase ratio activity levels (8.03 g/L, equivalent to 42.0% cellulose conversion) after 168 h incubation (Table 4). These results demonstrate that both *Te-CBHI* and *Tr-EGII* multicopy expression, with plasmid copy numbers of 10 and 8 respectively, in a *S. cerevisiae* host containing one δ-integrated *Sf-BGlI* gene cassette could efficiently promote saccharification of different corn residue substrates. While many reports have suggested that amorphous cellulose such as β-glucan or PASC can be degraded into glucose by BGL and EG activity without CBHI [28,29], our study suggests that the optimum recombinant expression ratio of cellulases required may also be dependent on the fraction of the corn residue used. For efficient degradation of crystalline cellulose such as Avicel, CBH activity is considered paramount, while for degradation of amorphous cellulose such as PASC, the activity of EG is considered more important [46]. This has implications on all different lignocellulose sources used and different pretreatment methodologies applied. However, both substrates in this study demonstrated that co-expression with genes *Te-EGII* and *Sf-BGLI* from a single strain produced higher glucose yields and subsequently higher cellulose conversion yields, potentially due to large amorphous regions contained within the cellulose component, to allow the endoglucanase to have such a large impact.

The transformants which co-expressed the genes *Te-EGII* with *Sf-BGLI*, which produced an enzyme activity ratio (U/g DCW) of ~15:1, demonstrated higher hydrolysis yields on both substrates compared to the transformants co-expressing *Te-CBHI* and *Sf-BGLI*, as well as the control enzyme ratio (Table 4). This aligned with the observations of past studies whereby lower ratio's of β-glucosidase to the total cellulase activity generated higher glucose yields from cellulosic substrates [13,47], and that the specific enzyme activities of cellulases can have different hydrolysis effects on pretreated corn stover [48]. These results also align with a study by Yamada and co-workers [49] whereby the best performing strain, based on degradation activity of PASC, contained BGL, EG and CBH encoding genes in copy numbers 1:8:2 and outperformed the conventional control strain which contained one copy of each gene.

### 3.3. Fermentation of corn residues

Fermentations were performed on both corn residues substrates using the wildtype strain with added commercial enzyme, Cellic® CTec2 (Novozymes), and partially cellulolytic strains YI13_cel3A, YI13_cel3A[cel5A] and YI13_cel3A[cel7A] in order to investigate the effect of different combinations of cellulases and the effect of different corn residues substrates (Table 5). High ethanol yields were achieved by control fermentations with wildtype YI13 supplemented with 5 FPU/g Cellic® CTec2 (Novozymes), which resulted in 5.12 g/L ethanol from the corn husk fermentation and 4.53 g/L ethanol from corn cob fermentation after 168 h (Table 5). For the single *Sf-BGLI* expression strain YI13-cel3A, minimal ethanol production of less than 18.2% ethanol conversion yields was observed on both substrates after 168 h as expected. During corn cob fermentations YI13_cel3A[cel5A] outcompeted the other cellulase producing strains by yielding significantly higher ethanol levels (4.05 g/L) and conversion yield (83.7%) after 168 h, with only a small difference in ethanol yield compared to the control fermentation of YI13 supplemented with Cellic® CTec2 (Novozymes), which reached conversion yields of 93.5% (Fig. 4 and Table 5). Furthermore, the results of the fermentation of corn cob residues (Fig. 4b) correspond to the enzymatic hydrolysis results in Fig. 3b.

To date, few cellulolytic yeast strains have been shown to

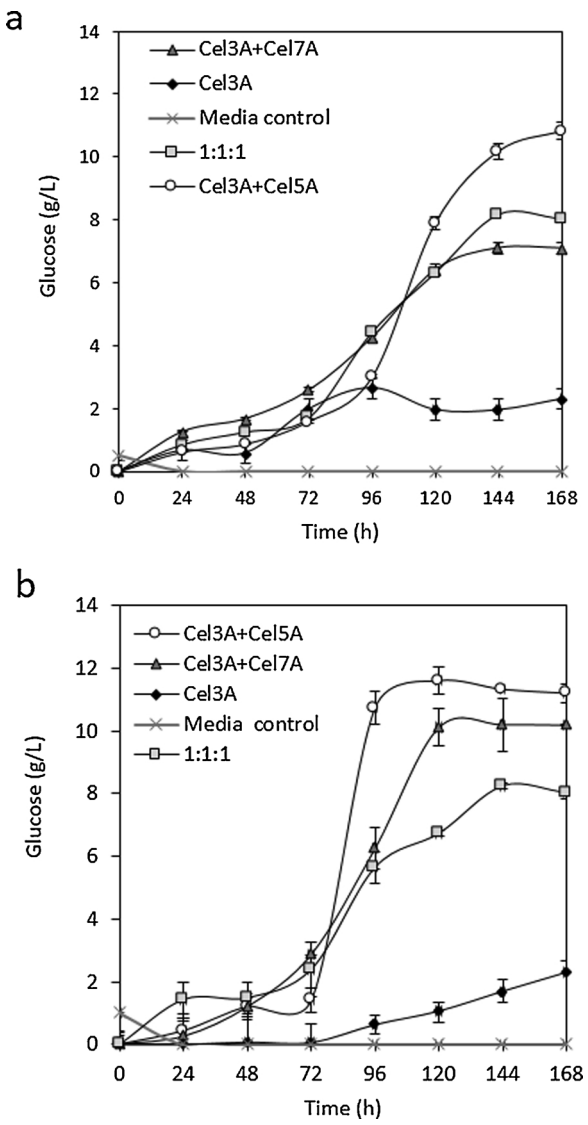


**Fig. 3.** Time-course hydrolysis assay of (a) corn husk and (b) corn cob using supernatant of cellulolytic YI13 yeast strains and a control made by mixing the supernatants of strains producing one cellulase (BGL, EG or CBH), resulting in an equal enzyme activity ratio of 1:1:1 based on U/mg DCW. Glucose concentrations after 168 h from 4% alkaline pre-treated corn residues are presented. Data presented are means and standard deviations of biological triplicates.

significantly degrade a 'real world' cellulosic substrate to ethanol without the additional of exogenous enzymes [28,29,50,51]. While Lee and co-workers [52] demonstrated high ethanol conversions yields of ~71% from 3% (w/v) rice straw using a mixed culture of four strains individually expressing essential cellulases, this was with supplementation with the commercial cocktail mix of 10 FPU Cellic® CTec2 mix/g glucan. In contrast, Khramtsov and co-workers [50] demonstrated lower ethanol conversion yields of 36.15% albeit at a higher substrate loading of 10% (w/v) of the cellulose fraction of corn residues by utilising *S. cerevisiae* with delta integrated *T. reesei* EG, *Aspergillus aculeatus* BGLI and *T. reesei* CBH encoding genes, without supplementation. It is important to note that at higher substrate loadings, similar high substrate conversion levels were not obtained by the cellulolytic transformants in this study (data not shown). Inefficient mixing or inactivation of enzymes at higher substrate loadings may be pivotal in this, highlighting the challenges which still exist in developing recombinant host strains with optimal cellulase secretion capacity, to effectively hydrolyse cellulosic biomass at higher loadings. Here we report a range of ethanol conversion yields, between 59.5%-83.7%, from 2% (w/v) corn husk and corn cob (Table 5) by simultaneously utilising high and low gene copy expression methods in a secretion stress-tolerant strain which resulted in higher hydrolysis and fermentation performance in corn residues.

## 4. Conclusion

In this study we confirmed that the choice of strain background was among the most important considerations when developing CBP yeasts and the natural strain YI13 demonstrated higher heterologous cellulase secretion compared to industrial and laboratory counterparts. The recombinant YI13 cellulolytic strains successfully hydrolysed and fermented alkali-pretreated corn cob and corn husk, without the addition of exogenous enzymes, potentially due to improved ratio of enzymes secreted by the constructed cellulolytic yeast strains. This study presents a novel comparison of various cellulolytic strain configurations in different yeast backgrounds as well as comparisons of subsequent hydrolysis efficiency and fermentation yields on different corn residues. Although the current strains can be used to partially displace commercial cellulase in substrate conversions, understanding the genetic background and genetic determinants involved in good secretion phenotypes complemented with good tolerance capabilities will be required for engineering improved industrial strains for biomass degradation in future.

**Table 4**
The product yields of substrate-enzyme hydrolysis assays on 4% corn residues after 168 h using supernatants of cellulolytic YI13 yeast strains and a control with a 1:1:1 ratio of enzyme activity on a U/gDCW basis. Data are presented as means and standard deviations of biological triplicates.

| Substrate | Glucose (g/L) | Acetic acid (g/L) | Glucose yield (%) | Cellulose conversion (%) |
|---|---|---|---|---|
| *Corn cob* | | | | |
| [cel3A] | 2.31 ± 0.598 | ND[1] | 5.88 ± 0.301 | 12.0 ± 0.498 |
| cel7A + cel3A | 7.08 ± 0.728 | 0.780 ± 0.336 | 25.8 ± 1.19 | 37.1 ± 0.238 |
| cel5A + cel3A | 10.8 ± 0.356 | 2.50 ± 0.897 | 27.6 ± 1.98 | 56.5 ± 0.298 |
| 1:1:1 | 8.03 ± 0.256 | 0.201 ± 0.279 | 20.5 ± 2.59 | 42.0 ± 0.138 |
| *Corn husk* | | | | |
| [cel3A] | 1.89 ± 0.269 | ND[1] | 4.88 ± 3.870 | 9.69 ± 0.289 |
| cel7A + cel3A | 10.2 ± 0.598 | 0.521 ± 0.087 | 26.4 ± 1.28 | 52.2 ± 0.398 |
| cel5A + cel3A | 11.5 ± 0.953 | 1.62 ± 0.199 | 29.7 ± 2.89 | 58.8 ± 0.897 |
| 1:1:1 | 8.64 ± 0.295 | 1.50 ± 0.308 | 22.3 ± 3.98 | 44.2 ± 0.597 |

[1] ND, not detected.

**Table 5**
Fermentation product yields of wild type YI13 supplemented with Cellic® CTec2 (Novozymes) and the cellulolytic YI13 yeast strains after 168 h on 2% corn residue substrates. Data presented as means and standard deviations of biological triplicates.

| Components | Enzyme activity levels (U/mg DCW) | | | Glucose (g/L) | Glycerol (g/L) | Acetic acid (g/L) | Ethanol (g/L) | Ethanol conversion yield (%) | Ethanol yield (g/g substrate) |
|---|---|---|---|---|---|---|---|---|---|
| | cel3A | cel5A | cel7A | | | | | | |
| *Corn husk* | | | | | | | | | |
| YI13 + CTec2 | 0.005[1] | | | 1.01 ± 0.187 | 0.803 ± 0.093 | 0.755 ± 0.133 | 5.12 ± 0.063 | 99.5 ± 5.82 | 0.251 ± 0.001 |
| YI13_cel3A[cel7A] | 0.28 ± 0.01 | ND[2] | 9.37 ± 1.01 | 1.20 ± 0.007 | 0.445 ± 0.011 | 0.504 ± 0.006 | 3.38 ± 0.022 | 66.9 ± 4.48 | 0.163 ± 0.007 |
| YI13_cel3A[cel5A] | 0.25 ± 0.04 | 3.76 ± 0.32 | ND[2] | 0.99 ± 0.029 | 0.443 ± 0.025 | 0.583 ± 0.090 | 3.00 ± 0.150 | 59.5 ± 1.70 | 0.156 ± 0.004 |
| YI13_cel3A | 0.27 ± 0.01 | ND[2] | ND[2] | 1.08 ± 0.026 | 0.434 ± 0.013 | 1.622 ± 0.075 | 0.50 ± 0.072 | 9.90 ± 9.66 | 0.031 ± 0.004 |
| *Corn cob* | | | | | | | | | |
| YI13 + CTec2 | 0.005[1] | | | 1.09 ± 0.034 | 0.554 ± 0.026 | 0.513 ± 0.273 | 4.53 ± 0.182 | 93.5 ± 3.49 | 0.235 ± 0.009 |
| YI13_cel3A[cel7A] | 0.28 ± 0.01 | ND[2] | 9.37 ± 1.01 | 1.10 ± 0.006 | 0.463 ± 0.093 | 0.371 ± 0.027 | 3.26 ± 0.066 | 67.4 ± 14.1 | 0.161 ± 0.003 |
| YI13_cel3A[cel5A] | 0.25 ± 0.04 | 3.76 ± 0.32 | ND[2] | 1.00 ± 0.017 | 0.444 ± 0.011 | 0.472 ± 0.087 | 4.05 ± 0.139 | 83.7 ± 2.12 | 0.204 ± 0.007 |
| YI13_cel3A | 0.27 ± 0.01 | ND[2] | ND[2] | 1.02 ± 0.033 | 0.455 ± 0.001 | 0.281 ± 0.148 | 0.88 ± 0.095 | 18.2 ± 7.16 | 0.052 ± 0.005 |
| *YPD* | | | | | | | | | |
| YI13wt | ND[2] | ND[2] | ND[2] | 0.058 ± 0.029 | 0.681 ± 0.026 | 1.132 ± 0.357 | 5.746 ± 0.387 | n/a[3] | 0.287 ± 0.026 |
| YI13_cel3A[cel7A] | 0.28 ± 0.01 | ND[2] | 9.37 ± 1.01 | 0.041 ± 0.016 | 0.052 ± 0.093 | 0.741 ± 0.116 | 5.103 ± 0.525 | n/a[3] | 0.255 ± 0.017 |
| YI13_cel3A[cel5A] | 0.25 ± 0.04 | 3.76 ± 0.32 | ND[2] | 0.011 ± 0.012 | 0.073 ± 0.013 | 0.635 ± 0.004 | 4.878 ± 0.332 | n/a[3] | 0.249 ± 0.007 |

[1]FPU/mg, [2], not detected, [3], not applicable.

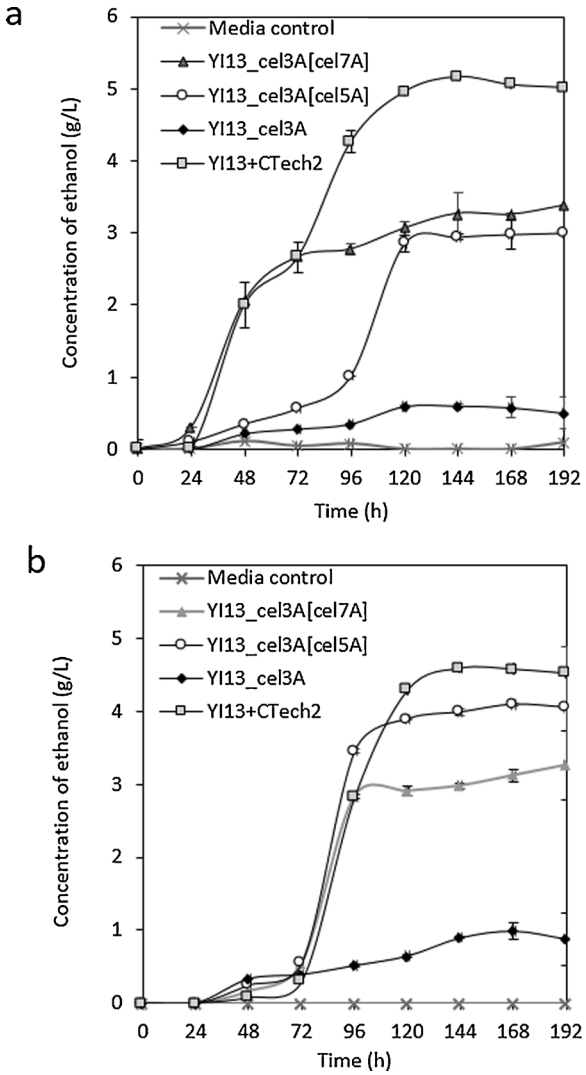


**Fig. 4.** Fermentation performance of the YI13 cellulolytic yeast strains on different corn residue substrates. Time course of ethanol concentrations from fermentation of 2% (a) corn cob and (b) corn husk by cellulolytic yeast strains. Data presented as means and standard deviations of biological triplicates.

## Acknowledgements

This work was supported by the National Research Foundation (NRF) through the Chair of Energy Research: Biofuels and other clean alternative fuels (NRF grant 86423).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2019.109382.

## References

[1] S. Kim, B.E. Dale, Global potential bioethanol production from wasted crops and crop residues, Biomass and Bioenergy. 26 (2004) 361–375, https://doi.org/10.1016/j.biombioe.2003.08.002.
[2] C. Zhao, Z. Zou, J. Li, H. Jia, J. Liesche, H. Fang, S. Chen, A novel and efficient bioprocess from steam exploded corn stover to ethanol in the context of on-site cellulase production, Energy. 123 (2017) 499–510, https://doi.org/10.1016/j.energy.2017.02.041.
[3] A.J. Sindelar, C.C. Sheaffer, J.A. Lamb, H.J.G. Jung, C.J. Rosen, Maize stover and cob cell wall composition and ethanol potential as affected by nitrogen fertilization, Bioenergy Res. 8 (2015) 1352–1361, https://doi.org/10.1007/s12155-015-9595-0.
[4] L.R. Lynd, X. Liang, M.J. Biddy, A. Allee, H. Cai, T. Foust, M.E. Himmel, M.S. Laser, M. Wang, C.E. Wyman, Cellulosic ethanol: status and innovation, Curr. Opin. Biotechnol. 45 (2017) 202–211, https://doi.org/10.1016/j.copbio.2017.03.008.
[5] R. den Haan, E. van Rensburg, S.H. Rose, J.F. Görgens, W.H. van Zyl, Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing, Curr. Opin. Biotechnol. 33 (2015) 32–38.
[6] C. Lambertz, M. Garvey, J. Klinger, D. Heesel, H. Klose, R. Fischer, U. Commandeur, Challenges and advances in the heterologous expression of cellulolytic enzymes : a review, Biotechnol. Biofuels. (2014) 1–15.
[7] J.E. McBride, J.J. Zietsman, W.H. van Zyl, L.R. Lynd, Utilization of cellobiose by recombinant β-glucosidase-expressing strains of *Saccharomyces cerevisiae*: characterization and evaluation of the sufficiency of expression, Enzyme Microb. Technol. 37 (2005) 93–101, https://doi.org/10.1016/j.enzmictec.2005.01.034.
[8] D.G. Olson, J.E. McBride, A. Joe Shaw, L.R. Lynd, Recent progress in consolidated bioprocessing, Curr. Opin. Biotechnol. 23 (2012) 396–405, https://doi.org/10.1016/j.copbio.2011.11.026.
[9] S.A. Davison, R. den Haan, W.H. van Zyl, Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains, Appl. Microbiol. Biotechnol. 100 (2016) 8241–8254, https://doi.org/10.1007/s00253-016-7735-x.
[10] S.A. Davison, R. den Haan, W.H. van Zyl, Identification of superior cellulase secretion phenotypes in haploids derived from natural *Saccharomyces cerevisiae* isolates, FEMS Yeast Res. (2019) 1–13, https://doi.org/10.1093/femsyr/foy117.
[11] L. Gurgu, Á. Lafraya, J. Polaina, J. Marín-Navarro, Fermentation of cellobiose to ethanol by industrial *Saccharomyces* strains carrying the β-glucosidase gene (*BGL1*) from *Saccharomycopsis fibuligera*, Bioresour. Technol. 102 (2011) 5229–5236, https://doi.org/10.1016/j.biortech.2011.01.062.
[12] M. de Baetselier, A. Vasavada, P. Dohet, V. Ha-This, M. de Beukelaer, T. Erpicum, L. de Clerck, J. Hanotier, S. Rosenberg, Fermentation of a yeast producing *A. niger* glucose oxidase: Scale up, Purification and characterization of the recombinant enzyme, Nat. Biotechnol. 9 (1991) 1067–1072, https://doi.org/10.1038/nm0798-

822.
[13] G. Wang, C. Liu, J. Hong, Y. Ma, K. Zhang, X. Huang, S. Zou, M. Zhang, Comparison of process configurations for ethanol production from acid- and alkali-pretreated corncob by *Saccharomyces cerevisiae* strains with and without β-glucosidase expression, Bioresour. Technol. 142 (2013) 154–161, https://doi.org/10.1016/j.biortech.2013.05.033.
[14] V.R. Pallapolu, Y.Y. Lee, R.J. Garlock, V. Balan, B.E. Dale, Y. Kim, N.S. Mosier, M.R. Ladisch, M. Falls, M.T. Holtzapple, R. Sierra-Ramirez, J. Shi, M.A. Ebrik, T. Redmond, B. Yang, C.E. Wyman, B.S. Donohoe, T.B. Vinzant, R.T. Elander, B. Hames, S. Thomas, R.E. Warner, Effects of enzyme loading and β-glucosidase supplementation on enzymatic hydrolysis of switchgrass processed by leading pretreatment technologies, Bioresour. Technol. 102 (2011) 11115–11120, https://doi.org/10.1016/j.biortech.2011.03.085.
[15] G. Banerjee, J.S. Scott-Craig, J.D. Walton, Improving enzymes for biomass conversion: A basic research perspective, Bioenergy Res. 3 (2010) 82–92, https://doi.org/10.1007/s12155-009-9067-5.
[16] K. Olofsson, M. Bertilsson, G. Lidén, A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks, Biotechnol. Biofuels. 1 (2008) 1–14, https://doi.org/10.1186/1754-6834-1-7.
[17] R. den Haan, J.M. van Zyl, T.M. Harms, W.H. van Zyl, Modeling the minimum enzymatic requirements for optimal cellulose conversion, Environ. Res. Lett. 8 (2013) 025013, , https://doi.org/10.1088/1748-9326/8/2/025013.
[18] X. Song, Q. Liu, J. Mao, Y. Wu, Y. Li, K. Gao, X. Zhang, Y. Bai, H. Xu, M. Qiao, POT1-mediated δ-integrated strategy for high-copy, stable expression of heterologous proteins in *Saccharomyces cerevisiae*, FEMS Yeast Res. (2017) 1–9, https://doi.org/10.1093/femsyr/fox064.
[19] E. Kunitake, T. Kobayashi, Conservation and diversity of the regulators of cellulolytic enzyme genes in *Ascomycete* fungi, Curr. Genet. 63 (2017) 951–958, https://doi.org/10.1007/s00294-017-0695-6.
[20] W. Zhang, C. Liu, G. Wang, K. Zhang, S. Zou, M. Zhang, Comparison of the expression in *Saccharomyces cerevisiae* of endoglucanase II from *Trichoderma reesei* and endoglucanase I from *Aspergillus aculeatus*, Bioresources. 7 (2012) 4031–4045.
[21] Z. Li, G. Liu, Y. Qu, Improvement of cellulolytic enzyme production and performance by rational designing expression regulatory network and enzyme system composition, Bioresour. Technol. (2017), https://doi.org/10.1016/j.biortech.2017.06.120.
[22] Y.H.P. Zhang, L.R. Lynd, Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, Biotechnol. Bioeng. 88 (2004) 797–824, https://doi.org/10.1002/bit.20282.
[23] J. Marin-Navarro, L. Gurgu, S. Alamar, J. Polaina, Structural and functional analysis of hybrid enzymes generated by domain shuffling between *Saccharomyces cerevisiae* (var. diastaticus) Sta1 glucoamylase and *Saccharomycopsis fibuligera* Bgl1 beta-glucosidase, Appl Microbiol Biotechnol 89 (2011) 121–130, https://doi.org/10.1007/s00253-010-2845-3.
[24] D. Wang, F.L. Li, S.A. Wang, Engineering a natural *Saccharomyces cerevisiae* strain for ethanol production from inulin by consolidated bioprocessing, Biotechnol. Biofuels. 9 (2016) 96, https://doi.org/10.1186/s13068-016-0511-4.
[25] B. Gasser, M. Saloheimo, U. Rinas, M. Dragosits, E. Rodríguez-Carmona, K. Baumann, M. Giuliani, E. Parrilli, P. Branduardi, C. Lang, D. Porro, P. Ferrer, M.L. Tutino, D. Mattanovich, A. Villaverde, Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview, Microb. Cell Fact. 7 (2008) 11, https://doi.org/10.1186/1475-2859-7-11.
[26] D. Mattanovich, B. Gasser, H. Hohenblum, M. Sauer, Stress in recombinant protein producing yeasts, J. Biotechnol. 113 (2004) 121–135, https://doi.org/10.1016/j.jbiotec.2004.04.035.
[27] J.M. Yarbrough, A. Mittal, E. Mansfield, L.E. Taylor, S.E. Hobdey, D.W. Sammond, Y.J. Bomble, M.F. Crowley, S.R. Decker, M.E. Himmel, T.B. Vinzant, New perspective on glycoside hydrolase binding to lignin from pretreated corn stover, Biotechnol. Biofuels. 8 (2015) 1–14, https://doi.org/10.1186/s13068-015-0397-6.
[28] R. den Haan, S.H. Rose, L.R. Lynd, W.H. van Zyl, Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*, Metab. Eng. 9 (2007) 87–94, https://doi.org/10.1016/j.ymben.2006.08.005.
[29] Y. Fujita, J. Ito, M. Ueda, H. Fukuda, A. Kondo, Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme, Appl. Environ. Microbiol. 70 (2004) 1207–1212, https://doi.org/10.1128/AEM.70.2.1207-1212.2004.
[30] J. Sambrook, D. Russel, Molecular cloning: a laboratory manual, Cold Spring Harb, Lab. Press, 2001.
[31] K.M. Cho, Y.J. Yoo, H.S. Kang, δ-Integration of endo/exo-glucanase and β-glucosidase genes into the yeast chromosomes for direct conversion of cellulose to ethanol, Enzyme Microb. Technol. 25 (1999) 23–30, https://doi.org/10.1016/S0141-0229(99)00011-3.
[32] H.P. Cho, M.T. Nakamura, S.D. Clarke, Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase, J. Biol. Chem. 274 (1999) 471–477, https://doi.org/10.1074/jbc.274.1.471.
[33] J.E.E. McBride, K.M. Delault, L.R. Lynd, J.T. Pronk, Recombinant yeast strains expressing tethered cellulase enzymes, (2010). U.S. Patent Application 12/516,175.
[34] A.P. Njokweni, S.H. Rose, W.H. van Zyl, Fungal β-glucosidase expression in *Saccharomyces cerevisiae*, J. Ind. Microbiol. Biotechnol. 39 (2012) 1445–1452, https://doi.org/10.1007/s10295-012-1150-9.
[35] Z.L. Liu, H.N. Li, H.T. Song, W.J. Xiao, W.C. Xia, X.P. Liu, Z.B. Jiang, Construction of a trifunctional cellulase and expression in *Saccharomyces cerevisiae* using a fusion protein, BMC Biotechnol. 18 (2018) 43, https://doi.org/10.1186/s12896-018-0454-x.
[36] M.A. Teste, M. Duquenne, J.M. François, J.-L. Parrou, Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*, BMC Mol. Biol. 10 (2009) 99, https://doi.org/10.1186/1471-2199-10-99.
[37] F. Latif, M.I. Rajoka, Production of ethanol and xylitol from corn cobs by yeasts, Bioresour. Technol. 77 (2001) 57–63, https://doi.org/10.1016/S0960-8524(00)00134-6.
[38] M.P. García-Aparicio, M. Ballesteros, P. Manzanares, I. Ballesteros, A. González, M. José Negro, Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw, Appl. Biochem. Biotechnol. 137–140 (2007) 353–365, https://doi.org/10.1007/s12010-007-9064-0.
[39] L. du Plessis, S.H. Rose, W.H. van Zyl, Exploring improved endoglucanase expression in *Saccharomyces cerevisiae* strains, Appl. Microbiol. Biotechnol. 86 (2010) 1503–1511, https://doi.org/10.1007/s00253-009-2403-z.
[40] E. van Rensburg, R. den Haan, J. Smith, W.H. van Zyl, J.F. Görgens, The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture, Appl. Microbiol. Biotechnol. 96 (2012) 197–209, https://doi.org/10.1007/s00253-012-4037-9.
[41] M. Ilmén, R. den Haan, E. Brevnova, J. McBride, E. Wiswall, A. Froehlich, A. Koivula, S.P. Voutilainen, M. Siika-Aho, D.C. la Grange, N. Thorngren, S. Ahlgren, M. Mellon, K. Deleault, V. Rajgarhia, W.H. van Zyl, M. Penttilä, High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*, Biotechnol. Biofuels. 4 (2011) 30, https://doi.org/10.1186/1754-6834-4-30.
[42] J.K. Saini, R. Saini, L. Tewari, Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments, 3 Biotech 5 (2015) 337–353, https://doi.org/10.1007/s13205-014-0246-5.
[43] C. Lei, J. Zhang, L. Xiao, J. Bao, An alternative feedstock of corn meal for industrial fuel ethanol production: Delignified corncob residue, Bioresour. Technol. 167 (2014) 555–559, https://doi.org/10.1016/j.biortech.2014.06.005.
[44] S. Sharma, V. Sharma, A. Kuila, Thermochemical pretreatment of corn husk and enzymatic hydrolysis using mixture of different cellulases, Biomass Conv. Bioref. (2017), https://doi.org/10.1007/s13399-017-0255-9.
[45] K. Karimi, M.J. Tahercadeh, A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity, Bioresearouce Technology. 200 (2016) 1008–1018, https://doi.org/10.1061/j.biortech.2015.11.022.
[46] M. Kostylev, D. Wilson, Synergistic interactions in cellulose hydrolysis, Biofuels. 3 (2012) 61–70, https://doi.org/10.4155/bfs.11.150.
[47] C. Feng, S. Zou, C. Liu, H. Yang, K. Zhang, Y. Ma, J. Hong, M. Zhang, Ethanol production from acid- and alkali-pretreated corncob by endoglucanase and β-glucosidase co-expressing *Saccharomyces cerevisiae* subject to the expression of heterologous genes and nutrition added, World J. Microbiol. Biotechnol 32 (2016), https://doi.org/10.1007/s11274-016-2043-2.
[48] A. Pribowo, V. Arantes, J.N. Saddler, The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulase/xylanase components when hydrolysing steam pretreated corn stover, Enzyme Microb. Technol. 50 (2012) 195–203, https://doi.org/10.1016/j.enzmictec.2011.12.004.
[49] R. Yamada, N. Taniguchi, T. Tanaka, C. Ogino, H. Fukuda, A. Kondo, Cocktail delta-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains, Microb. Cell Fact. 9 (2010) 32, https://doi.org/10.1186/1475-2859-9-32.
[50] N. Khramtsov, L. McDade, A. Amerik, E. Yu, K. Divatia, A. Tikhonov, M. Minto, G. Kabongo-Mubalamate, Z. Markovic, M. Ruiz-Martinez, S. Henck, Industrial yeast strain engineered to ferment ethanol from lignocellulosic biomass, Bioresour. Technol. 102 (2011) 8310–8313, https://doi.org/10.1016/j.biortech.2011.05.075.
[51] Z. Liu, K. Inokuma, S.H. Ho, R. den Haan, W.H. van Zyl, T. Hasunuma, A. Kondo, Improvement of ethanol production from crystalline cellulose via optimizing cellulase ratios in cellulolytic *Saccharomyces cerevisiae*, Biotechnol. Bioeng. 114 (2017) 1201–1207, https://doi.org/10.1002/bit.26252.
[52] C.R. Lee, B.H. Sung, K.M. Lim, M.J. Kim, M.J. Sohn, J.H. Bae, J.H. Sohn, Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol, Sci. Rep. 7 (2017) 1–14, https://doi.org/10.1038/s41598-017-04815-1.