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Increasing extracellular cellulase activity of the recombinant *Saccharomyces cerevisiae* by engineering cell wall-related proteins for improved consolidated processing of carbon neutral lignocellulosic biomass

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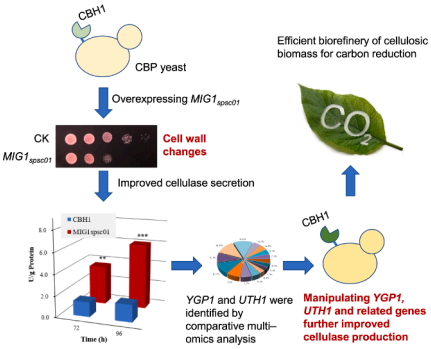
HIGHLIGHTS

- The CBP yeast strains were engineered to benefit conversion of cellulosic biomass.
- Overexpression of *MIG1*$_{spsc01}$ affected expression of proteins related to cell wall.
- Engineering *MIG1*$_{spsc01}$, *UTH1* and *YPG1* are novel strategies for improving CBP.
- Disruption of *YGP1* with overexpressing *SED5* elevated CBH activity of 2.2-fold.
- CBP yeast with improved cellulase production facilitates economic bioproduction.

GRAPHICAL ABSTRACT



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ABSTRACT

Sustainable bioproduction using carbon neutral feedstocks, especially lignocellulosic biomass, has attracted increasing attention due to concern over climate change and carbon reduction. Consolidated bioprocessing (CBP) of lignocellulosic biomass using recombinant yeast of *Saccharomyces cerevisiae* is a promising strategy for lignocellulosic biorefinery. However, the economic viability is restricted by low enzyme secretion levels. For more efficient CBP, *MIG1* $_{spsc01}$ isolated from the industrial yeast which encodes the glucose repression regulator derivative was overexpressed. Increased extracellular cellobiohydrolase (CBH) activity was observed with unexpectedly decreased cell wall integrity. Further studies revealed that disruption of *CWP2, YGP1*, and *UTH1,* which are functionally related to *MIG1* $_{spsc01}$, also enhanced CBH secretion. Subsequently, improved cellulase production was achieved by simultaneous disruption of *YGP1* and overexpression of *SED5*, which remarkably increased extracellular CBH activity of 2.2-fold over the control strain. These results provide a novel strategy to improve the CBP yeast for bioconversion of carbon neutral biomass.

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1. Introduction

In the recent years, the climate change and its social and economic impact have attracted great attention worldwide. Reducing carbon dioxide emission is advocated for all sectors in industries to lower the temperature rise. Biomass has been recognized as a net-zero feedstock for production of fuels and chemicals. Shifting society's dependence from petroleum-based economy towards renewable biomass-based one is critical to tackle the greenhouse gas emissions (Amjith and Bavanish, 2022). Biorefinery of biomass feedstock, particularly lignocellulosic biomass, has gained extensive worldwide attention for environmental protection and carbon neutral economy. Lignocellulosic biomass is abundant in nature, which is renewable and carbon neutral. Sustainable production of biofuels and biochemicals using lignocellulosic biomass has attracted increasing attention in the past decades due to concern over climate change and global requirement of carbon reduction (De Bhowmick et al., 2018; Liu et al., 2019). However, great challenges still remain for economic biorefinery of lignocellulosic biomass, and one of the key challenges is the high production cost of lignocelluolic degrading enzymes (Baral et al., 2022). Therefore, it is imperative to optimize enzyme hydrolysis process by searching for more efficient strategies.

Consolidated bioprocessing (CBP) of lignocellulosic biomass using recombinant budding yeast *Saccharomyces cerevisiae* integrates cellulase production, enzyme hydrolysis and fermentation production into the same strain, which is a promising strategy to reduce the cost for cellulase. However, the economic viability of CBP is restricted by low enzyme secretion levels in *S. cerevisiae*. Typically, endoglucanase (EG; EC 3.2.1.4), cellobiohydrolase, also called exoglucanase (CBH; EC 3.2.1.91) and β-glucosidase (BGL; EC 3.2.1.21) are the three main types of cellulosic enzymes for constructing CBP yeast, and low CBH secretion is especially challenging (Liu et al., 2018; Sharma et al., 2022). Many studies have focused on molecular mechanisms of protein secretion and then engineering the protein secretion pathway to improve heterologous protein production (Kroukamp et al., 2018). Manipulating key genes involved in protein folding, protein trafficking, as well as reducing the glycosylation of the heterologous protein have successfully improved heterologous protein secretion (Hou et al., 2012; Kroukamp et al., 2018). Multi-omics analyses revealed that heterologous protein production affects global gene transcription and expression (Huang et al., 2017). Therefore, novel target genes with various functions can be discovered by multi-omics analysis to improve efficiency of CBP.

Yeast cell wall composition and structure endorse the barrier function of cell wall, subsequently affect the export of the heterologous protein into the medium (Orlean, 2012). It was reported that the cell wall integrity defects caused by disrupting *OCH1* or *MNN9*, encoding key Golgi mannosyltransferases genes, contributed to improved secretion of β-glucosidase, cellobiohydrolase and endoglucanase (Tang et al., 2016). In the previous studies, disruption of *CWP2* and *YGP1*, which encode a cell wall mannoprotein and a cell wall-related secretory glycoprotein, enhanced extracellular CBH secretion and cell surface display activity of BGLs, respectively (Li et al., 2020; Arnthong et al., 2022), suggesting that proteins related to cell wall functions are promising in optimizing CBP yeast. However, how the cell wall proteins are regulated? Is it possible to further improve the strain by synergistic optimization of cell wall proteins and proteins in other pathways? These questions remain unsolved.

The industrial flocculating yeast SPSC01 was developed previously and has been used in large scale fuel ethanol production (He et al., 2012). Further work identified that *MIG1spsc01* encodes a mutant protein of Mig1 which is the transcription regulator for glucose repression (Xu et al., 2018). However, its role in cell wall functions remains unclear. To achieve improved CBP efficiency, it is necessary to engineer the CBP yeast to stimulate cellulase secretion. The novelty of this study is to report the beneficial effect of overexpressing *MIG1spsc01* on extracellular activity of CBH. In addition, *MIG1spsc01* was found to affect the cell wall integrity. Subsequently, the CBP yeast engineered by combing the key cell wall related genes and the other key genes revealed by comparative omics analyses significantly improved enzyme secretion. These results highlight the importance of synergistic optimization to enhance the yeast performance, and provide novel strategy for developing robust CBP to achieve economic bioproduction.

1.1. Media and culture conditions

The strains and plasmids used in this study are listed elsewhere (See supplementary material). *Escherichia coli* DH5α purchased from TransGen Biotech (Beijing, China) used for the construction and propagation of plasmids was cultivated at 37 °C and 200 rpm, in Luria-Bertani medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl), 100 mg/L ampicillin was added when necessary. *S. cerevisiae* Y294 expressing cellulase genes were used as a host strain (Ilmén et al., 2011). Genomic DNA extracted from *S. cerevisiae* SPSC01 and *S. cerevisiae* S288c were used as template for gene cloning. All *S. cerevisiae* strains were cultured in YPD medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) at 30 °C and 150 rpm. Solid agar plates were prepared by adding 20 g/L Bacto agar into the medium.

1.2. Construction of plasmids and yeast strains

The recombinant yeast strains were constructed via CRISPR/Cas9-based genome editing technology following the previously described methods (Zhang et al., 2014, Li et al., 2020). After confirmed by DNA sequencing, the gRNA expression vectors were used for genome editing in *S. cerevisiae* Y294. The open reading frame (ORF) of $MIG1_{SPSC01}$ or $MIG1_{S288c}$ was amplified by polymerase chain reaction (PCR) from the genome of *S. cerevisiae* SPSC01 or *S. cerevisiae* S288c. The *PGK1* promotor and *CYC1* terminator were amplified from the genome of *S. cerevisiae* S288c. These three fragments were assembled into the USER cloning vector (Jensen et al., 2014), resulting in *PGK1-*$MIG1_{SPSC01}$*-CYC1* or *PGK1-*$MIG1_{S288c}$*-CYC1* expression cassette. Linear DNA fragments containing *PGK1-*$MIG1_{SPSC01}$*-CYC1* or *PGK1-*$MIG1_{S288c}$*-CYC1* expression cassette flanking by 40-bp upstream and downstream homologous arms for homologous recombination were generated by PCR as the double strand donor DNA. For gene disruption, double-stranded 90 bp DNA fragments containing a stop codon (TAA) were generated by PCR with primer pairs, and the products were used as donor DNA. DNA donors, together with the plasmid containing gRNA were transformed into *S. cerevisiae* Y294 possessing the Cas9 plasmid via LiAc/ssDNA method. The transformants were selected on YPD medium supplemented with 300 μg/mL G418 and 300 μg/mL HygB, and were subsequently confirmed by sequencing. The elimination of Cas9 and gRNA expression plasmids in the transformants were performed via serial transfer in YPD medium. All the strains and the primers can be found in the file elsewhere (See supplementary material).

1.3. Determination of cell density

The cell density was determined by measuring optical density at 600 nm (OD_{600}) via a spectrophotometer (Thermo-Fisher Scientific™, Multiskan™ GO, MA, USA). Samples were taken from the culture, and diluted with ddH_2O to an OD_{600} within 0.2–0.8, after which 200 μL of the diluted suspension was added into the 96-well plates for analysis.

1.4. Enzyme activity assays

Yeast strains were cultivated in 250-mL Erlenmeyer flasks with 100 mL YPD medium for 96 h. Samples withdrawn from the cell culture were centrifuged at 4 °C and 8,000 rpm for 2 min, the supernatants were collected for enzyme activity assay according to the reference (Li et al., 2020; van Zyl et al., 2016).

1.5. Analysis of cell wall integrity in recombinant strains.

The cell wall integrity of yeast cells was evaluated via spot assays on YPD plates with Congo red as described previously (Li et al., 2020). Briefly, yeast cells were harvested from the overnight culture and the cell density of each sample was adjusted to $OD_{600} \approx 1.0$. Then each sample was diluted in 10-fold gradient (OD_{600} 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}), and 2 μL from the serial dilutions was spotted onto the YPD plate containing Congo red or CFW, respectively.

1.6. SDS-PAGE and N-deglycosylation

The supernatant collected from the 96-h yeast cell culture in YPD medium was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein samples were treated or untreated with Endo H enzyme (New England BioLabs, Hertfordshire, UK) for *N*-deglycosylation. Protein samples (22.5 μL) were analyzed using a 10 % SDS-PAGE gel, according to the method described before (Li et al., 2020). Separated proteins were visualized by silver staining (Kroukamp et al., 2013). The relative protein abundance of each sample on the silver-stained gels was analyzed by AlphaView SA software according to the instructions.

1.7. RT-qPCR analysis

The relative transcription levels of specific genes were determined by reverse transcription quantitative PCR (RT-qPCR). Total RNA of the 24-h and 48-h cell culture were extracted with the HiPure Yeast RNA Kit (Magen, Guangzhou, China). After reverse transcription with the reverse transcription kit (Takara ltd, Dalian, China), the mRNA level was determined by 2 × T5 Fast qPCR Mix (SYBRGreen I) (TsingKe Biotech Co., ltd., Beijing, China). Relative transcription levels were calculated by the $2^{-\Delta\Delta Ct}$ method with *ALG9* as an internal reference gene. All primers used in this study are listed elsewhere (See the supplementary material).

1.8. Statistical analysis

All the experiments were duplicated and the results were expressed as mean value and standard derivations. The significant statistic tests were performed with a one-tailed homoscedastic (equal-variance) *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

2. Results and discussion

2.1. Overexpression of $MIG1_{spsc01}$ improved recombinant protein secretion

Mig1 is well known as a regulator responsible for glucose repression (Kayikci and Nielsen, 2015). However, its role in cell wall organization in *S. cerevisiae* has not been reported. Overexpression of $MIG1_{spsc01}$ did not affect cell growth of the recombinant strain in comparison to the parent strain, and the maximum specific growth rate of the strains was similar (Fig. 1a). In case of enzyme secretion, compared with the parental control strain, $MIG1_{spsc01}$ overexpression remarkably improved the extracellular CBH activity by 122.0 % at 96 h (Fig. 1b). In addition, remarkably improved intracellular CBH activity by three times was detected in the $MIG1_{spsc01}$ overexpressing strain compared to the parent strain (Fig. 1c). However, $MIG1_{S288c}$ overexpression have no obvious effect on growth of the strain or the CBH secretion (data not shown), suggesting that the different protein sequences in $Mig1_{spsc01}$ are responsible for the newly discovered effect. No apparent differences were observed when EG and BGL activity were examined in the $MIG1_{spsc01}$ overexpressing strains, which is in agreement with the previous report that different proteins show specific responses to the same genetic manipulation (Kroukamp et al., 2018; Tang et al., 2016).

When the supernatants from cultures of these strains were analyzed by SDS-PAGE, it was revealed that higher proportion of extracellular cellobiohydrolase in total extracellular protein were secreted by the $MIG1_{spsc01}$ overexpressing strain than that of the control strain, regardless of Endo H treatment (Fig. 1d). The relative amount of

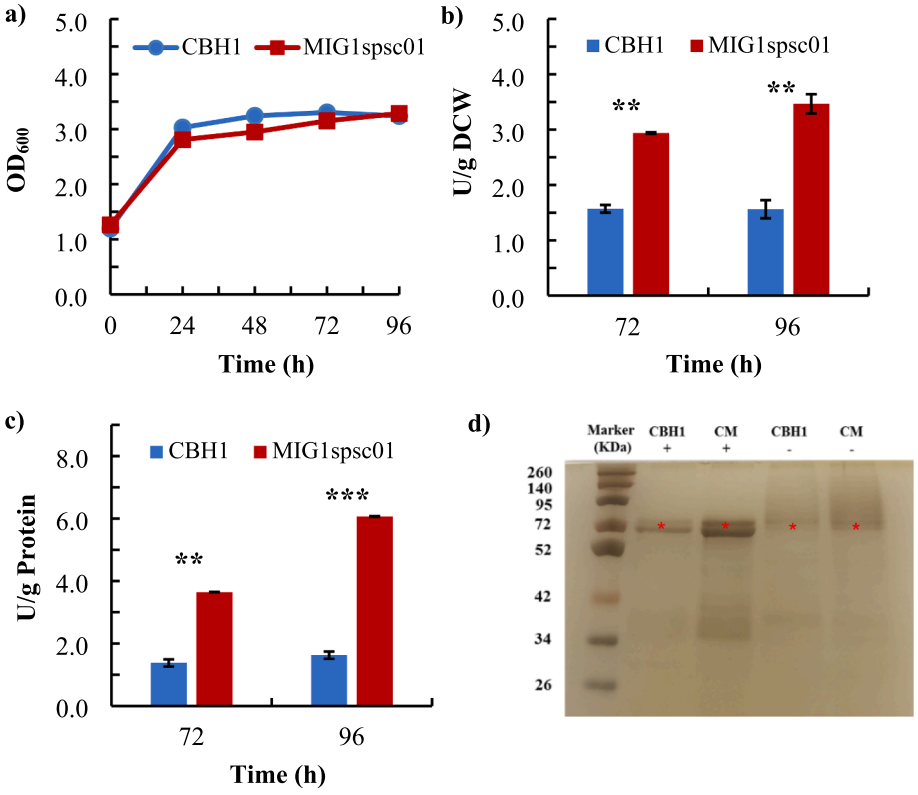

Fig. 1. Effects of $MIG1_{spsc01}$ overexpression on extracellular cellobiohydrolase activity. a) Growth curve of the $MIG1_{spsc01}$ overexpression strain and the control strain. b) and c) Extracellular and intracellular activity of cellobiohydrolase of the $MIG1_{spsc01}$ overexpression strain and the control strain, respectively. d) SDS-PAGE analysis of the supernatant of $MIG1_{spsc01}$ overexpression strain and the control strain. The same amount (22.5 μL) of supernatant was loaded on the gel, CBH1 and MIG1spsc01 represent the control strain Y294-CBH1 and the overexpression strain Y294-CBH1-$MIG1_{spsc01}$, respectively. + and − indicated samples treated with or without Endo H for deglycosylation, and the target protein-CBH1 was marked with red asterisk.. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cellobiohydrolase of the *MIG1*$_{spsc01}$ overexpression strain is increased by 25.3 % after Endo H treatment and 181.5 % from none Endo H treatment than the control strain, respectively.

2.2. Manipulation of *MIG1*$_{spsc01}$ and *UTH1* affected cell wall function

To investigate the changes of extracellular protein secretion by *MIG1*$_{spsc01}$ overexpression, comparative secretome analysis was performed using the mutant strain and the control strain. Interestingly, a number of proteins related to cell wall functions were changed by *MIG1*$_{spsc01}$ overexpression (data not shown), suggesting that the function of this allele of *MIG1* affected cell wall function. Then, growth of the mutant strain and the parent strain was detected in the presence of Congo Red (CR). Indeed, decreased growth of the mutant strain was found by CR treatment, suggesting that the function of cell wall was impaired by *MIG1*$_{spsc01}$ overexpression. Subsequently, disruption of *UTH1*, whose function is implicated in biogenesis of cell wall (Ritch et al., 2010), was also examined, and similar results were found to that of *MIG1*$_{spsc01}$ overexpression. The results of *UTH1* contradicted with the previous report that yeast cells lacking *UTH1* showed higher resistance to yeast cell wall perturbing agents calcofluor white and sodium dodecyl sulfate, as well as higher levels of beta-d-glucan (Ritch et al., 2010). We deduce that the inconsistent results may be due to different strain genetic background, which may be further explored in the future studies.

Subsequently, we also performed comparative transcriptomic analysis to reveal the global effect of *MIG1*$_{spsc01}$ overexpression. Unexpectedly, we only found the down-regulation of two genes encoding proteins related to cell wall functions, namely, *CWP2* and *YGP1*, which was confirmed by RT-qPCR analysis. At the same time, the up-regulated transcriptional level of *CBH1* was confirmed by RT-qPCR in *MIG1*$_{spsc01}$ overexpressing strain, when compared with the control strain. The mRNA levels of *MIG1*$_{spsc01}$ overexpressing strain were 2.01 and 2.17 folds of those of the control strain at 24 h and 48 h, respectively (Fig. 2). These results indicated that overexpression of *MIG1*$_{spsc01}$ not only enhanced the extracellular CBH activities but also improved the synthesis of CBH, both at mRNA level and protein level. This is the first report on the correlation of the Mig1-derivative protein with genes *CWP2* and *YGP1* encoding cell wall functions in budding yeast, and the findings promoted improvement of fermentation performance by manipulating novel derivative proteins from industrial strains. In the previous study, KlMig1p from *Kluvyromyces lactis* was suggested to exert a negative regulatory role on cell wall biosynthesis (Rippert et al., 2017). However, the relate studies have not been performed in *S. cerevisiae*. The results here provide basis for further exploration of the functions related to specific Mig1 structure in regulation of cell wall function.

It was hypothesized that disruption of *CWP2* or *YGP1* might also improve CBH secretion. Therefore, *CWP2* and *YGP1* were disrupted, respectively, via CRISPR-Cas9 by introducing a stop codon (TAA) in the ORF of each gene. It was reported previously that *CWP2* disruption enhanced CBH secretion in the CBP yeast (Li, et al., 2020). Here, disruption of either *CWP2* or *YGP1* on increasing cellobiohydrolase secretion was compared, and slightly higher increase was found by *YGP1* disruption (by 85.9 % and 110.7 % at 96 h, respectively) (Fig. 3).

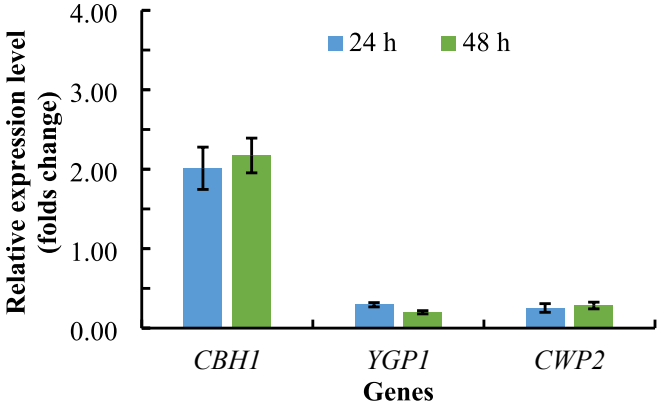

Fig. 2. *MIG1*$_{spsc01}$ overexpression affected transcription of *CBH1* and genes *YGP1* and *CWP2* which are related to cell wall functions in the recombinant yeast.

2.3. Disruption of *UTH1* improved cellulase secretion

The impaired cell wall integrity promoted us to further examine the effect of *UTH1* disruption on cellulase production. Uth1p was reported to act as a mitochondrial membrane protein (Welter et al., 2013), in addition to its role in cell wall biogenesis (Ritch et al., 2010). Based on the finding that similar effects were obtained for *MIG1*$_{spsc01}$ overexpression and *UTH1* disruption in cell wall function (Fig. 2), we subsequently examined whether the *UTH1* disruptant has higher enzyme secretion. Remarkable increase in extracellular CBH activity was achieved by *UTH1* disruption, which is 2.35-fold of that of the parent strain at 96 h (Fig. 3a). Analysis by SDS-PAGE revealed that protein content in the supernatant in the mutant was significantly higher than that of the parent strain (Fig. 3b). The CBH content before and after Endo H treatment in the mutant was 1.64-fold and 2.23-fold, respectively, and improved intracellular CBH activity was also observed by *UTH1* disruption (Fig. 3c). Improved endoglucanase secretion was also detected by *UTH1* disruption (Fig. 3d). In addition, we also found about 2.13-fold of BGL activity by *UTH1* disruption (data not shown). To summarize, disruption of *UTH1* resulted in improved cellulase production. This is the first report that *UTH1* disruption affected protein secretion in budding yeast, which provide a novel target for improving the performance of CBP yeast.

2.4. Disruption of *YGP1* improved cellulase secretion

YGP1 encodes a secreted extensively *N*-glycosylated glycoprotein, involved in biogenesis of the yeast cell wall. *YGP1* also plays a role in biofilm formation, while the deletion of *YGP1* caused a remarkable decrease in the dry weight of flor yeast strain (Moreno-García et al., 2018). We further examined the effect of *YGP1* disruption on BGL production. Disruption of *YGP1* did not affect cell growth (Fig. 4a), but enhanced both the extracellular and intracellular activity of CBH and BGL, by 85 % and 53 %, respectively (Fig. 4b and 4c). However, different from the results of manipulating *UTH1* and *MIG1*$_{spsc01}$, no significant effect was observed by Endo H treatment (Fig. 4d). Then, transcriptome analysis of the *YGP1* disruptant producing CBH and the parent strain was performed. Interestingly, it was found remarkably decreased transcription of *MIG1* by *YGP1* disruption (see supplement material), further supporting the connection of *YGP1* with *MIG1* in cell wall function (Fig. 5).

In another related report, disruption of *YGP1* increased cell surface display activity of BGL and ethanol production (Arnthong et al., 2022). Combing with the current results, it was suggested that *YGP1* is a useful target for engineering heterologous protein secretion in recombinant yeast, and the results here revealed this novel function.

2.5. Simultaneous manipulation of YGP1 with other key genes enhanced cellulase secretion

So far, studies on the function of *YGP1* in *S. cerevisiae* remain limited. It was reported that Ygp1 responses to acid stress (Kawahata et al., 2006). In a recent study, Ygp1 was found to participate in flor formation in wine yeast (Moreno-García et al., 2018). We are interested in that how *YGP1* may affect global gene transcription for cellulase production. Comparative transcriptome analysis of the *YGP1* disruptant producing CBH and the parent strain was then performed. In addition to changes in cell wall related genes and genes related to protein secretion, which is

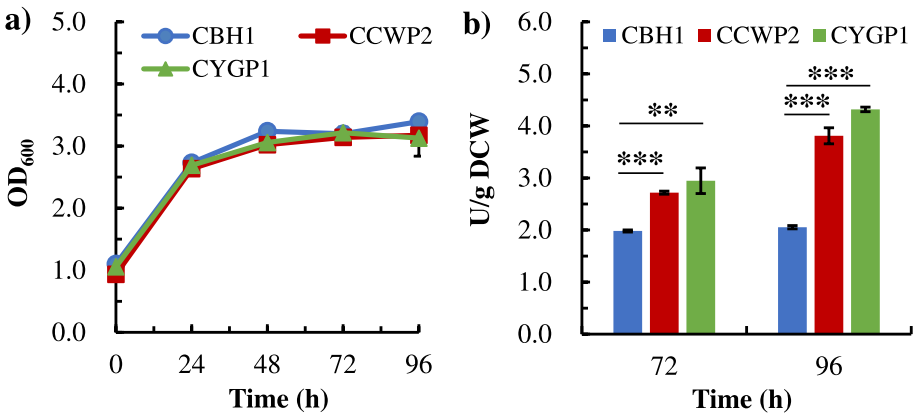

Fig. 3. Effect of *CWP2* and *YGP1* disruption on cell growth a) and CBH extracellular activity b). The cell growth and extracellular CBH activity of the previously developed yeast by *CWP2* discurption (Li et al. 2020) was used as a control.

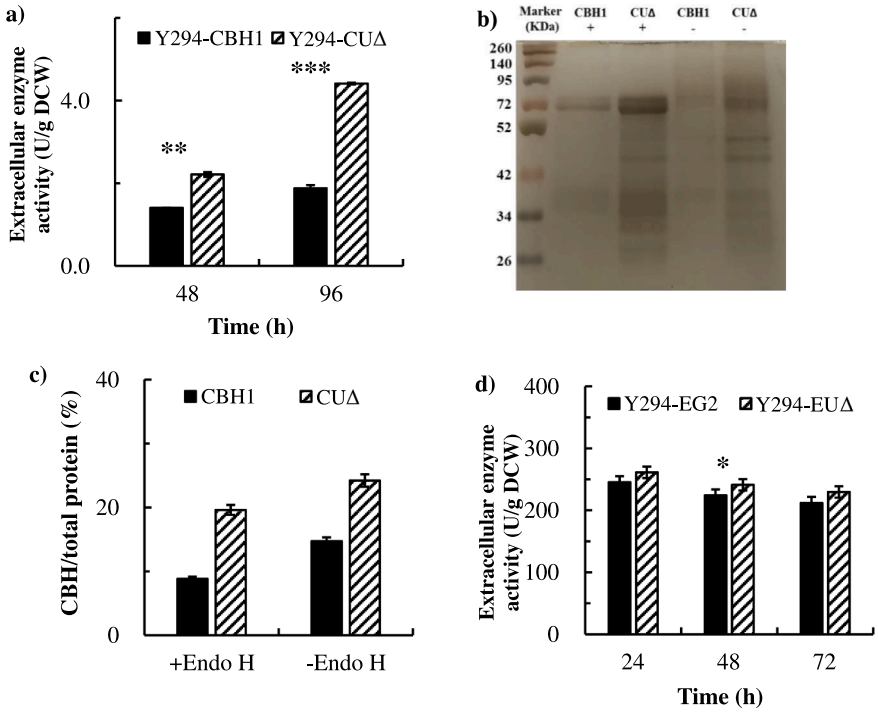

Fig. 4. Disruption of *UTH1* enhanced cellulase activity. a), extracellular CBH1 activity; b) and c), SDS-PAGE image of extracellular CBH secretion and quantification of the CBH amount with (+) or without (-) Endo H treatment, respectively; d), extracellular EG activity of the yeast strains.

similar with reported by disruption of *CWP2* (Li et al., 2020), it was also noticed *YGP1* disruption led to changes of genes related to protein synthesis, respiration and vesicle trafficking (see supplementary material). It is interesting whether the combined manipulation of *YGP1* with the target genes have synergistic effects on cellulase secretion, therefore, we selected five genes, which is *INO2*, *SYG1*, *SUR7*, *PDI1*, and *SED5* for the investigation. Among the constructed strains, *SED5* overexpression together with *YGP1* overexpression showed the most remarkable increase, followed by disruption of *INO2* together with *YGP1* overexpression. The extracellular CBH activity of the mutants was 194 %, 142 %, 134 %, 157 % and 228 %, respectively, comparing with that of the parent strain, whereas the cell growth was not apparently affected (Fig. 6). These results suggest that manipulating multiple genes are feasible methods to enhancing the extracellular cellulase production by the CBP yeast.

The yeast cell wall is an elastic structure which shapes cell morphology, maintains cell integrity, protects the cell from environmental stress and presents adhesive glycoproteins to other yeast cells (Levin 2011; Kahar et al., 2022; Ribeiro et al., 2022). Beneficial effects on lignocellulosic biorefinery by manipulating cell wall proteins has been reported, including cellulase secretion (Li et al., 2020) and cellulosic ethanol production (Kong et al., 2021). As a barrier to hinder the export of the heterologous protein to the medium, variation in wall composition and organization may impact heterologous protein secretion. Deletion of the key Golgi mannosyltransferases coding gene *MNN9* or *OCH1* could increase extracellular activities of BGL, EG and CBH by 135 %, 102 % and 144 % respectively (Tang et al. 2016). Sed5 is the SNARE (soluble-*N*-ethyl-maleimide-sensitive fusion protein attachment protein receptor) receptor subunit of SNARE complex, which is involved in endoplasmic reticulum to Golgi and intra-Golgi vesicle transport (Hardwick and Pelham, 1992). Sed5 also plays a role in yeast autophagy (Zou et al., 2017). In the previous study, overexpression of the single

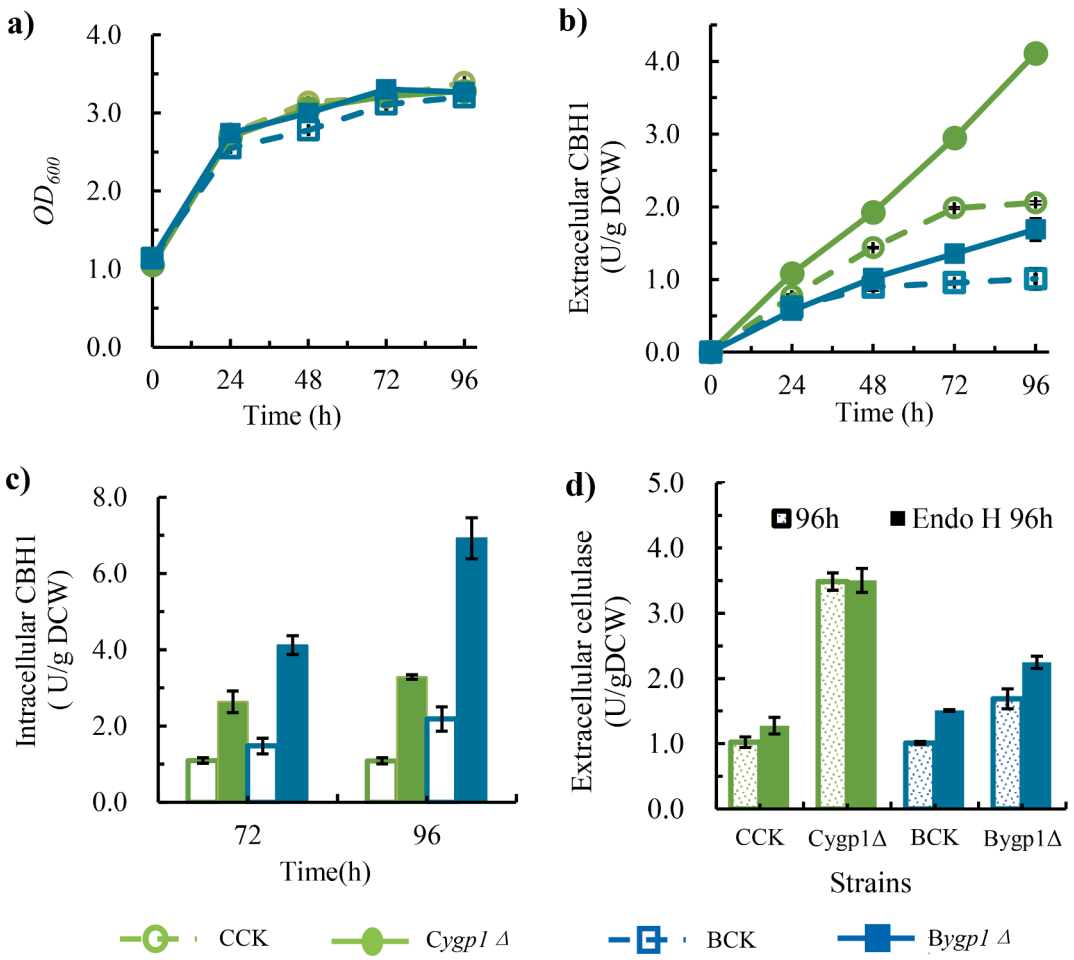

Fig. 5. Comparison of cellulase production by the *YGP1* disruptant and the control strain. a), Growth curve; b), Extracellular cellulase activity; c), Intracellular cellulase activity; d), Extracellular CBH1 activity after treated by glycosidase Endo H. CCK and BCK represent the parental strains Y294-CBH1 and Y294-BGL1 producing CBH1 and BGL1, respectively. C*ygp*1Δ and B*ygp*1Δ represent the *YGP1* disruption strains producing CBH1 and BGL1, respectively.

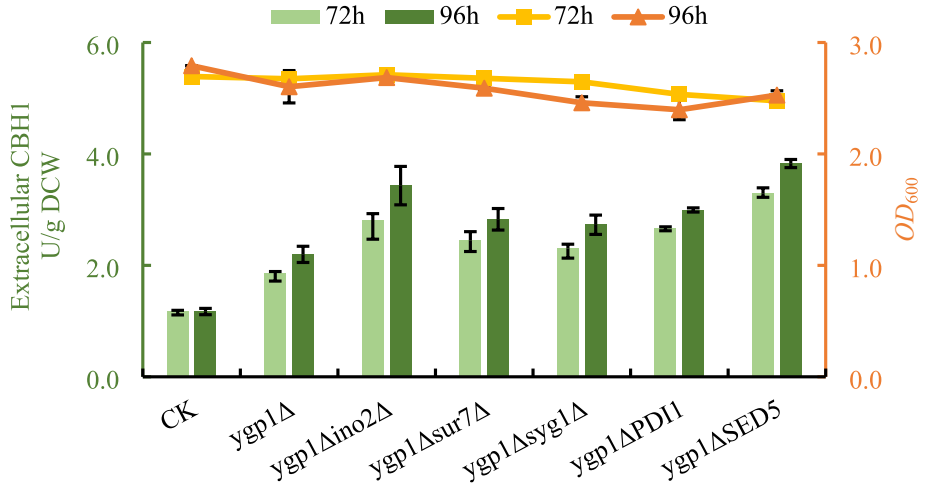

Fig. 6. Comparison of growth and the extracellular CBH activity in the double genes-editing strains and control strain. CK, control strain; *ypg*1Δ, the *YGP1* disruptant; *ypg*1Δ*ino*2Δ, *ypg*1Δ*sur*7Δ and *ypg*1Δ*syg*1Δ, recombinant strains with double deletion of *YGP1* and *INO2*, *SUR7*, *SYG1*, respectively; *ypg*1Δ*PDI1* and *ypg*1Δ*SED5*, recombinant strains with disruption of *YGP1* and overexpression of *PDI1* and *SED5*, respectively.

SED5 gene was found to improve CBH activity of the CBP yeast (Chetty et al., 2022). Here, simultaneous manipulation of *YGP1* and *SED5* showed considerably enhancement in extracellular CBH enzyme activity, 228 % of that of the parent strain. The results here suggest that simultaneous manipulation of genes in cell wall function and protein secretion would be a useful strategy to achieve efficient cellulase production.

Biorefinery of lignocellulosic biomass is the key solution for

sustainable production and carbon neutral economy. Increasing attention has been focused on production of cellulosic biofuels and biochemicals (Nawaz et al., 2022; Velvizhi et al., 2022). However, challenges remain for commercial production using cellulosic biomass, one of which is the high cost of the whole process (Chandel et al., 2018). Great efforts have been made to optimize cellulosic biorefinery, including developing efficient pretreatment technology (Saravanan et al., 2022), producing high grade cellulase using cheap substrates (Swathy et al., 2020), enhancing enzymatic hydrolysis (Kumar Saini et al., 2022; Sriariyanun et al., 2022), producing high-value products (Lu et al., 2022; Wang et al., 2021), improving stress tolerance and xylose utilization (Cunha et al., 2020, Zhang et al., 2019), as well as process integration (Qiao et al., 2022). Enzymatic hydrolysis of lignocellulosic biomass is environmentally friendly, and has been focused to obtain fermentable sugars for production of biofuels and biochemicals. CBP provides a promising strategy to integrate cellulase production with the subsequent microbial fermentation (den Haan et al., 2021; Liu et al., 2019). However, the efficiency of CBP is still not high. Various strategies have been developed to improve the CBP efficiency (Ilmén et al., 2011; Inokuma et al., 2021; Singhania et al., 2022). The findings in the current study are novel in that: 1) Mig1 derivative protein isolated from the industrial yeast endows the protein with regulatory function in cell wall. 2) The studies also documented that the functions of the $Mig1_{spsc01}$ is related to genes of *UTH1*, *CWP2* and *YGP1*, respectively, whose functions are related to cell wall biosynthesis and/or integrity. 3) This study proved that simultaneous manipulation of cell wall protein gene *YGP1* and the secretion related protein gene *SED5* have better effects on promoting cellulase secretion.

These results in this study encourage exploration of natural microbial strains to optimize the biosystems by multi-omics analysis and synthetic biology, and present novel targets to optimize yeast cell wall function. This study provides a feasible strategy to reduce cellulase production cost by engineering the CBP strains, including multi-omics analysis and simultaneous engineering of genes related to different cellular mechanisms. The limitation of the study includes: 1) authentic biomass feedstocks have not been tested, which will be explored in future studies to test the robust strains developed in this study for production of cellulosic biofuels or biochemicals. 2) On the other hand, we only tested secretion of single enzymes in this study. In the future, the CBP strains producing different cellulase component can be co-cultured to examine the effect on degradation of lignocellulosic biomass as previously reported (Lee et al., 2017). It can be expected that economic cellulosic biorefinery will be achieved by integrating robust strain development and enhanced process efficiency, which will contribute to carbon reduction, clean environment and sustainable development.

3. Conclusion

Overexpression of $MIG1_{spsc01}$ isolated from the industrial bioethanol yeast SPSC01 affected cell wall biogenesis and increased extracellular cellulase activity of the CBP yeast. Disruption of *CWP2, YGP1*, and *UTH1,* respectively, which are related to $MIG1_{spsc01}$, also enhanced the cellulase secretion. Further improved cellulase production was achieved by simultaneous disruption of *YGP1* and overexpression of *SED5*. As a result, the most significant increase in extracellular CBH activity of 2.2-fold over the control strain was achieved. These results provide a novel strategy to develop efficient CBP yeast for economic utilization of the carbon neutral lignocellulosic biomass by manipulating cell wall-related proteins.

CRediT authorship contribution statement

Jie Li: Investigation, Visualization, Formal analysis. **Yu Zeng:** Investigation, Validation, Formal analysis, Visualization. **Wei-Bin Wang:** Software, Writing – review & editing. **Qing-Qing Wan:** Investigation. **Chen-Guang Liu:** Writing – review & editing. **Riaan den Haan:** Methodology, Resources, Writing – review & editing. **Willem H. van Zyl:** Resources, Writing – review & editing. **Xin-Qing Zhao:** Supervision, Conceptualization, Resources, Data curation, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Amjith, L.R., Bavanish, B., 2022. A review on biomass and wind as renewable energy for sustainable environment. Chemosphere 293, 133579.
Arnthong, J., Ponjarat, J., Bussadee, P., Deenarn, P., Prommana, P., Phienluphon, A., Charoensri, S., Champreda, V., Zhao, X.Q., Suwannarangsee, S., 2022. Enhanced surface display efficiency of β-glucosidase in *Saccharomyces cerevisiae* by disruption of cell wall protein-encoding genes *YGP1* and *CWP2*. Biochem. Eng. J. 179, 108305.
Baral, P., Kumar, V., Agrawal, D., 2022. Emerging trends in high-solids enzymatic saccharification of lignocellulosic feedstocks for developing an efficient and industrially deployable sugar platform. Crit. Rev. Biotechnol. 42 (6), 873–891.
Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A.F., da Silva, S.S., 2018. The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. Bioresour. Technol. 264, 370–381.
Chetty, B.J., Inokuma, K., Hasunuma, T., van Zyl, W.H., den Haan, R., 2022. Improvement of cell-tethered cellulase activity in recombinant strains of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 106 (18), 6347–6361.
Cunha, J.T., Soares, P.O., Baptista, S.L., Costa, C.E., Domingues, L., 2020. Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. Bioengineered. 11 (1), 883–903.
De Bhowmick, G., Sarmah, A.K., Sen, R., 2018. Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products. Bioresour. Technol. 247, 1144–1154.
den Haan, R., Rose, S.H., Cripwell, R.A., Trollope, K.M., Myburgh, M.W., Viljoen-Bloom, M., van Zyl, W.H., 2021. Heterologous production of cellulose- and starch-degrading hydrolases to expand *Saccharomyces cerevisiae* substrate utilization: Lessons learnt. Biotechnol. Adv. 53, 107859.
Hardwick, K.G., Pelham, H.R., 1992. *SED5* encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. J. Cell Biol. 119 (3), 513–521.
He, L.Y., Zhao, X.Q., Ge, X.M., Bai, F.W., 2012. Identification and functional study of a new *FLO10*-derivative gene from the industrial flocculating yeast SPSC01. J. Ind. Microbiol. Biotechnol. 39 (8), 1135–1140.
Hou, J., Tyo, K.E., Liu, Z., Petranovic, D., Nielsen, J., 2012. Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. FEMS Yeast Res. 12 (5), 491–510.
Huang, M.T., Bao, J.C., Hallstrom, B.M., Petranovic, D., Nielsen, J., 2017. Efficient protein production by yeast requires global tuning of metabolism. Nat. Commun. 8 (1), 1131.
Ilmén, M., den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A., Koivula, A., Voutilainen, S.P., Siika-Aho, M., la Grange, D.C., Thorngren, N., Ahlgren, S., Mellon, M., Deleault, K., Rajgarhia, V., van Zyl, W.H., Penttilä, M., 2011. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. Biotechnol. Biofuels 4, 30.
Inokuma, K., Kitada, Y., Bamba, T., Kobayashi, Y., Yukawa, T., den Haan, R., van Zyl, W. H., Kondo, A., Hasunuma, T., 2021. Improving the functionality of surface-engineered yeast cells by altering the cell wall morphology of the host strain. Appl. Microbiol. Biot. 105 (14–15), 5895–5904.

Jensen, N.B., Strucko, T., Kildegaard, K.R., David, F., Maury, J., Mortensen, U.H., Forster, J., Nielsen, J., Borodina, I., 2014. EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. FEMS Yeast Res. 14 (2), 238–248.

Kahar, P., Itomi, A., Tsuboi, H., Ishizaki, M., Yasuda, M., Kihira, C., Otsuka, H., Azmi, N. B., Matsumoto, H., Ogino, C., Kondo, A., 2022. The flocculant *Saccharomyces cerevisiae* strain gains robustness via alteration of the cell wall hydrophobicity. Metab. Eng. 72, 82–96.

Kawahata, M., Masaki, K., Fujii, T., Iefuji, H., 2006. Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. FEMS Yeast Res. 6 (6), 924–936.

Kayikci, Ö., Nielsen, J., 2015. Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15 (6), fov068.

Kong, M., Li, X., Li, T., Zhao, X., Jin, M., Zhou, X., Gu, H., Mrša, V., Xiao, W., Cao, L., 2021. Overexpressing *CCW12* in *Saccharomyces cerevisiae* enables highly efficient ethanol production from lignocellulose hydrolysates. Bioresour. Technol. 337, 125487.

Kroukamp, H., den Haan, R., van Wyk, N., van Zyl, W.H., 2013. Overexpression of native *PSE1* and *SOD1* in *Saccharomyces cerevisiae* improved heterologous cellulase secretion. Appl. Energy 102, 150–156.

Kroukamp, H., den Haan, R., van Zyl, J.-H., van Zyl, W.H., 2018. Rational strain engineering interventions to enhance cellulase secretion by *Saccharomyces cerevisiae*. Biofuel. Bioprod. Bior. 12 (1), 108–124.

Kumar Saini, J., Himanshu, H., Kaur, A., Mathur, A., 2022. Strategies to enhance enzymatic hydrolysis of lignocellulosic biomass for biorefinery applications: A review. Bioresour. Technol. 127517.

Lee, C.R., Sung, B.H., Lim, K.M., Kim, M.J., Sohn, M.J., Bae, J.H., Sohn, J.H., 2017. Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol. Sci. Rep. 7 (1), 4428.

Levin, D.E., 2011. Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics 189 (4), 1145–1175.

Li, J., Zhang, M.M., Wan, C., Den Haan, R., Bai, F.W., Zhao, X.Q., 2020. Improved cellulase production in recombinant *Saccharomyces cerevisiae* by disrupting the cell wall protein-encoding gene *CWP2*. J. Biosci. Bioeng. 129 (2), 165–171.

Liu, H., Sun, J., Chang, J.S., Shukla, P., 2018. Engineering microbes for direct fermentation of cellulose to bioethanol. Crit. Rev. Biotechnol. 38 (7), 1089–1105.

Liu, C.G., Xiao, Y., Xia, X.X., Zhao, X.Q., Peng, L., Srinophakun, P., Bai, F.W., 2019. Cellulosic ethanol production: Progress, challenges and strategies for solutions. Biotechnol. Adv. 37 (3), 491–504.

Lu, H., Yadav, V., Bilal, M., Iqbal, H.M.N., 2022. Bioprospecting microbial hosts to valorize lignocellulose biomass – environmental perspectives and value-added bioproducts. Chemosphere 288 (Pt 2), 132574.

Moreno-García, J., Coi, A.L., Zara, G., García-Martínez, T., Mauricio, J.C., Budroni, M., 2018. Study of the role of the covalently linked cell wall protein (Ccw14p) and yeast glycoprotein (Ygp1p) within biofilm formation in a flor yeast strain. FEMS Yeast Res. 18 (2), foy005.

Nawaz, S., Ahmad, M., Asif, S., Klemeš, J.J., Mubashir, M., Munir, M., Zafar, M., Bokhari, A., Mukhtar, A., Saqib, S., Khoo, K.S., Show, P.L., 2022. Phyllosilicate derived catalysts for efficient conversion of lignocellulosic derived biomass to biodiesel: A review. Bioresour. Technol. 343, 126068.

Orlean, P., 2012. Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. Genetics 192 (3), 775–818.

Qiao, J., Cui, H., Wang, M., Fu, X., Wang, X., Li, X., Huang, H., 2022. Integrated biorefinery approaches for the industrialization of cellulosic ethanol fuel. Bioresour. Technol. 360, 127516.

Ribeiro, R.A., Bourbon-Melo, N., Sá-Correia, I., 2022. The cell wall and the response and tolerance to stresses of biotechnological relevance in yeasts. Front. Microbiol. 13, 953479.

Rippert, D., Backhaus, K., Rodicio, R., Heinisch, J.J., 2017. Cell wall synthesis and central carbohydrate metabolism are interconnected by the SNF1/Mig1 pathway in *Kluyveromyces lactis*. Eur. J. Cell Biol. 96 (1), 70–81.

Ritch, J.J., Davidson, S.M., Sheehan, J.J., Austriaco, N., 2010. The *Saccharomyces* SUN gene, *UTH1*, is involved in cell wall biogenesis. FEMS Yeast Res. 10 (2), 168–176.

Saravanan, A., Senthil Kumar, P., Jeevanantham, S., Karishma, S., Vo, D.N., 2022. Recent advances and sustainable development of biofuels production from lignocellulosic biomass. Bioresour. Technol. 344 (Pt B), 126203.

Sharma, J., Kumar, V., Prasad, R., Gaur, N.A., 2022. Engineering of *Saccharomyces cerevisiae* as a consolidated bioprocessing host to produce cellulosic ethanol: Recent advancements and current challenges. Biotechnol. Adv. 56, 107925.

Singhania, R.R., Patel, A.K., Singh, A., Haldar, D., Soam, S., Chen, C.W., Tsai, M.L., Dong, C.D., 2022. Consolidated bioprocessing of lignocellulosic biomass: Technological advances and challenges. Bioresour. Technol. 354, 127153.

Sriariyanun, M., Kitiborwornkul, N., Tantayotai, P., Rattanaporn, K., Show, P.L., 2022. One-pot ionic liquid-mediated bioprocess for pretreatment and enzymatic hydrolysis of rice straw with ionic liquid-tolerance bacterial cellulase. Bioengineering. 9 (1), 17.

Swathy, R., Rambabu, K., Banat, F., Ho, S.H., Chu, D.T., Show, P.L., 2020. Production and optimization of high grade cellulase from waste date seeds by *Cellulomonas uda* NCIM 2353 for biohydrogen production. Int. J. Hydrogen Energy 45 (42), 22260–22270.

Tang, H., Wang, S., Wang, J., Song, M., Xu, M., Zhang, M., Shen, Y., Hou, J., Bao, X., 2016. N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in *Saccharomyces cerevisiae*. Sci. Rep. 6, 25654.

Van Zyl, J.H., Den Haan, R., Van Zyl, W.H., 2016. Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes increased heterologous cellulase secretion. Appl. Microbiol. Biot. 100 (1), 505–518.

Velvizhi, G., Balakumar, K., Shetti, N.P., Ahmad, E., Kishore Pant, K., Aminabhavi, T.M., 2022. Integrated biorefinery processes for conversion of lignocellulosic biomass to value added materials: Paving a path towards circular economy. Bioresour. Technol. 343, 126151.

Wang, J., Chae, M., Beyene, D., Sauvageau, D., Bressler, D.C., 2021. Co-production of ethanol and cellulose nanocrystals through self-cycling fermentation of wood pulp hydrolysate. Bioresour. Technol. 330, 124969.

Welter, E., Montino, M., Reinhold, R., Schlotterhose, P., Krick, R., Dudek, J., Rehling, P., Thumm, M., 2013. Uth1 is a mitochondrial inner membrane protein dispensable for post-log-phase and rapamycin-induced mitophagy. FEBS J. 280 (20), 4970–4982.

Xu, J.R., Zhao, X.Q., Liu, C.G., Bai, F.W., 2018. Improving xylose utilization of *Saccharomyces cerevisiae* by expressing the *MIG1* mutant from the self-flocculating yeast SPSC01. Protein. Peptide. Lett. 25 (2), 202–207.

Zhang, M.M., Chen, H.Q., Ye, P.L., Wattanachaisaereekul, S., Bai, F.W., Zhao, X.Q., 2019. Development of robust yeast strains for lignocellulosic biorefineries based on genome-wide studies. Prog. Mol. Subcell. Biol. 58, 61–83.

Zhang, G.C., Kong, I.I., Kim, H., Liu, J.J., Cate, J.H.D., Jin, Y.S., 2014. Construction of a quadruple auxotrophic mutant of an industrial polyploid *Saccharomyces cerevisiae* strain by using RNA-guided Cas9 nuclease. Appl. Environ. Microb. 80 (24), 7694–7701.

Zou, S., Sun, D., Liang, Y., 2017. The roles of the SNARE protein Sed5 in autophagy in *Saccharomyces cerevisiae*. Mol. Cells 40 (9), 643–654.