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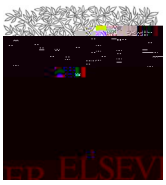

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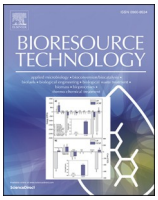

Increasing extracellular cellulase activity of the recombinant *Saccharomyces cerevisiae* by engineering cell wall-related proteins for improved consolidated processing of carbon neutral lignocellulosic biomass

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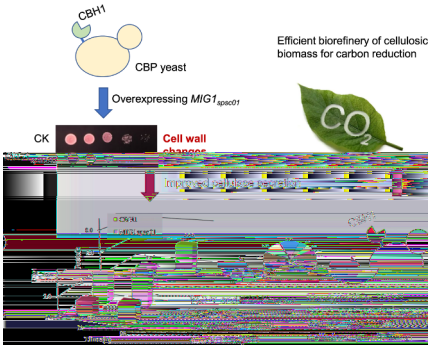
HIGHLIGHTS

- 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

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

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 *Saccharomyces cerevisiae* strains. In a 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 [(CWP2 or YGP1 Mig1 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).

2.3. Disruption of *UTH1*

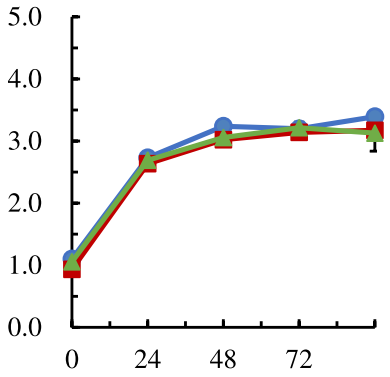

Fig. 3. Effect of *CWP2* and *YGP1* disruption on cell growth a) and CBH extracellular activity b). The cell growth and extracellular CBH activity of the previously developed yeast by *CWP2* discurption (Li et al. 2020) was used as a control.

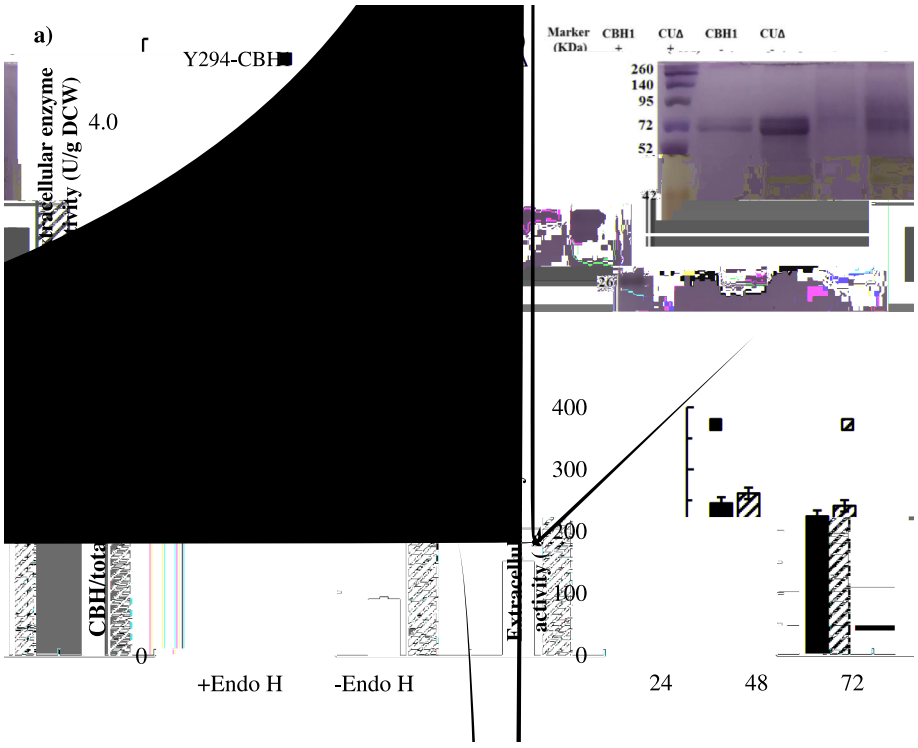

Fig. 4. Disruption of *UTH1* enhanced cellulase activity. a), extracellular CBH1 activity; b) and c), SDS-PAGE image of extracellular CBH secretion and quantification of the CBH amount with (+) or without (-) Endo H treatment, respectively; d), extracellular EG activity of the yeast strains.

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

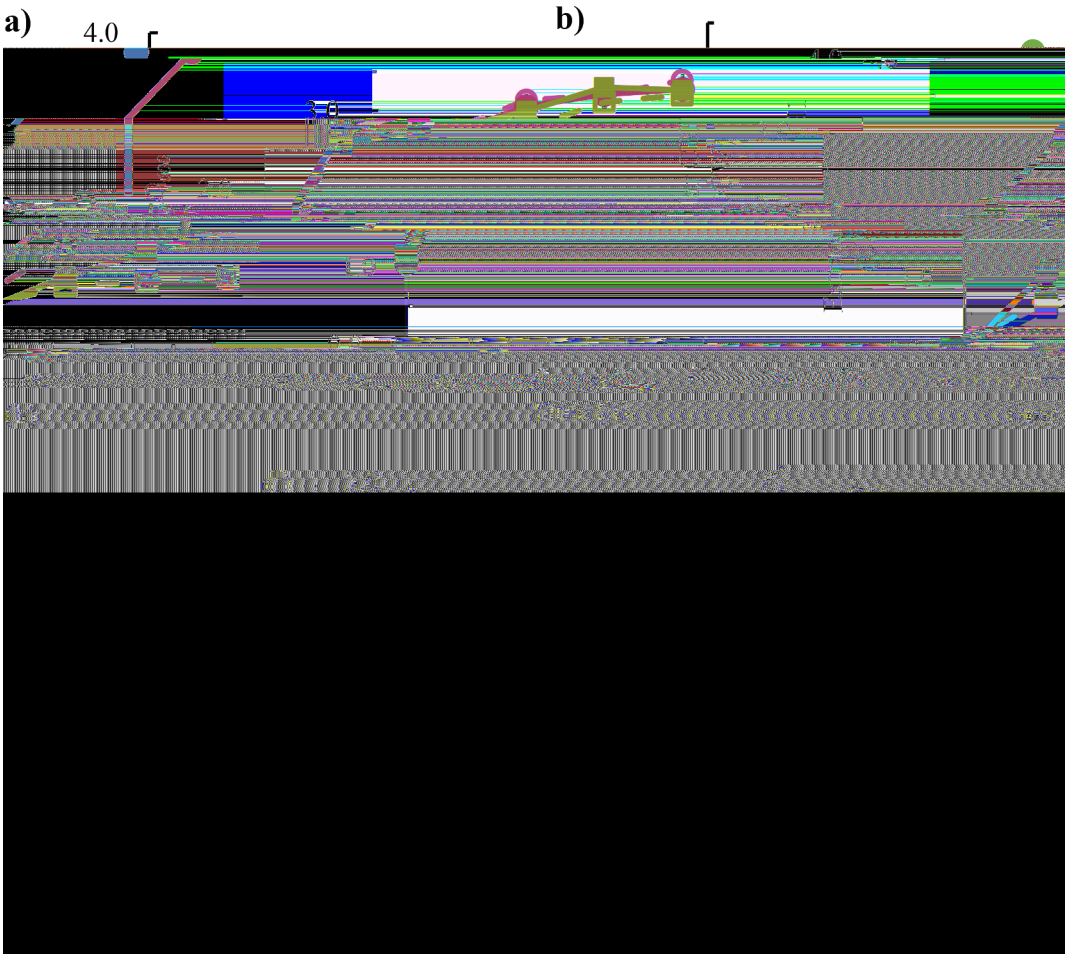

Fig. 5. Comparison of cellulase production by the *YGP1* disruptant and the control strain. a), Growth curve; b), Extracellular cellulase activity; c), Intracellular cellulase activity; d), Extracellular CBH1 activity after treated by glycosidase Endo H. CCK and BCK represent the parental strains Y294-CBH1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

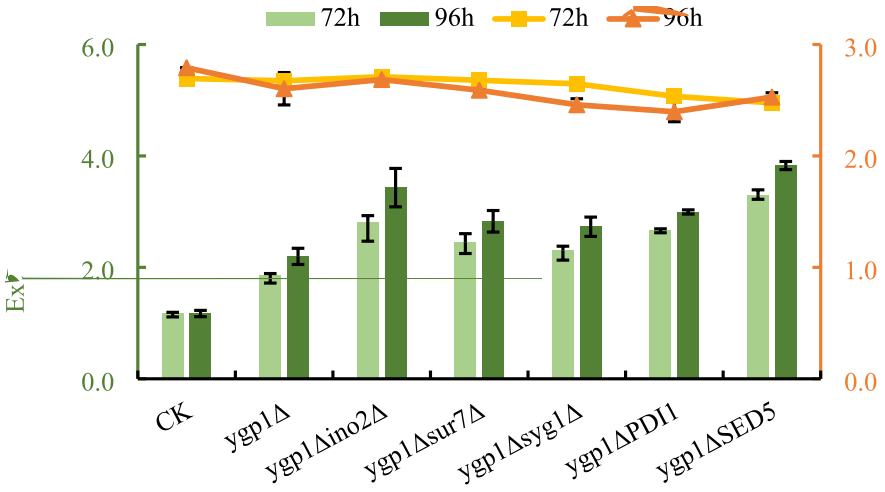

Fig. 6. Comparison of growth and the extracellular CBH activity in the double genes-editing strains and control strain. CK, control strain; *ygp*1Δ, the *YGP1* disruptant; *ygp*1Δ*ino*2Δ, *ygp*1Δ*sur*7Δ and *ygp*1Δ*syg*1Δ, recombinant strains with double deletion of *YGP1* and *INO2*, *SUR7*, *SYG1*,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

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