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Overexpression of LAS21 in Cellulase-Displaying *Saccharomyces cerevisiae* for High-Yield Ethanol Production from Pretreated Sugarcane Bagasse

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Abstract: The valorization of lignocellulosic feedstocks into biofuels and biochemicals has received much attention due to its environmental friendliness and sustainability. However, engineering an ideal microorganism that can both produce sufficient cellulases and ferment ethanol is highly challenging. In this study, we have tested seven different genes that are involved in glycosylphosphatidylinositol (GPI) biosynthesis and remodeling for the improvement of cellulase activity tethered on the *S. cerevisiae* cell surface. It was found that the overexpression of LAS21 can improve β-glucosidase activity by 48.8% compared to the original strain. Then, the three cellulase genes (cellobiohydrolase, endoglucanase, and β-glucosidase) and the LAS21 gene were co-introduced into a diploid thermotolerant *S. cerevisiae* strain by a multiple-round transformation approach, resulting in the cellulolytic ECBLCCE5 strain. Further optimization of the bioprocess parameters was found to enhance the ethanol yield of the ECBLCCE5 strain. Scaling up the valorization of pretreated sugarcane bagasses in a 1 L bioreactor resulted in a maximum ethanol concentration of 28.0 g/L (86.5% of theoretical yield). Our study provides a promising way to improve the economic viability of second-generation ethanol production. Moreover, the engineering of genes involved in GPI biosynthesis and remodeling can be applied to other yeast cell surface display applications.

Keywords: *Saccharomyces cerevisiae*; cell surface display; cellulase; LAS21; ethanol production; lignocellulosic biomass

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1. Introduction

The rising concern about greenhouse gas emissions and global warming, which have affected the Earth in recent decades, has led to the discovery of more environmentally friendly substitutes for fossil fuels [1]. The utilization of renewable biofuels, especially second-generation biofuels derived from abundant lignocellulosic feedstocks, has great potential in reducing greenhouse gas emissions and preventing severe environmental impacts [2]. One of the most promising biofuels is bioethanol, which has been adopted in the transportation sector and can guarantee the security of the energy supply in several countries [3]. However, bioethanol produced from lignocellulosic biomasses requires a high quantity of cellulase cocktails for efficient biomass deconstruction, which limits the economic viability of industrial production [4]. The consolidated bioprocessing (CBP) concept, which integrates enzyme production, biomass hydrolysis, and fermentation into a single step, has been considered an ideal approach for improving the economic feasibility of cellulosic ethanol and other lignocellulose-valorized products [5]. However, constructing

efficient CBP microbes that can either produce sufficient cellulases or ferment ethanol is highly challenging [6].

Saccharomyces cerevisiae is one of the most attractive microorganisms to be employed in bioethanol production due to its high ethanol productivity, tolerance to industrial conditions, well-known physiochemical properties, and established genetic manipulation tools [7]. To obtain cellulolytic yeast strains, at least three kinds of cellulases—cellobiohydrolase (CBH), endoglucanase (EG), and β-glucosidase (BGL)—are needed [6]. Liu et al. (2017) [8] developed a cellulase-displaying strain of *S. cerevisiae* with the capability of producing 0.8 g/L ethanol from 25 g/L of pretreated rice straw. Furthermore, the CBP yeast containing five cellulase-displaying enzymes (BGL, XYN2, EG2, CBH1, and CBH2) was reported to produce up to 0.93 g/L ethanol from 5.0 g/L of ionic liquid-pretreated bagasse, which corresponds to 91.2% of the theoretical ethanol yield [9]. In this sense, the ethanol concentration from CBP is far lower than the practical yield obtained in the industrial process. Alternatively, a simultaneous saccharification process using the cellulolytic *S. cerevisiae* can remarkably reduce the amount of commercial cellulase additive needed while maintaining a reasonable bioethanol yield. The co-fermentation of different cellulase-secreting *S. cerevisiae* strains was able to produce approximately 14 g/L ethanol from pretreated rice straw with the addition of 10 FPU commercial cellulase/g glucan [10].

Yeast cell surface display (YSD) is a technique in which the target enzyme is immobilized onto the yeast cell surface by fusion with an anchoring protein motif [8]. YSD confers many advantages over secretion systems in terms of improved enzyme stability, easy enzyme separation, cell-enzyme recyclability, and proximity effects between displayed enzymes [11,12]. Most of the anchoring protein motifs were derived from the glycosylphosphatidylinositol (GPI)-protein family, such as AGα1 (SAG1), AGA1-AGA2, SED1, and 6_Kl [13,14]. These kinds of proteins undergo a posttranslational modification process by attaching a conserved glycolipid GPI anchor moiety, which plays an important role in targeting the proteins to the plasma membrane and, ultimately, to the cell wall [15]. The GPI moiety is synthesized in the endoplasmic reticulum (ER) and serves approximately 60 distinct GPI proteins during protein synthesis in yeast cells [16]. Although the availability of the GPI moiety might be a crucial factor that controls the quantity of displayed protein on the yeast cell surface, the effect of the overexpression of GPI biosynthesis and remodeling proteins on the YSD capacity is still unknown.

In this study, the effect of some genes encoding GPI biosynthesis and remodeling proteins was investigated for enhancing BGL activity on the yeast cell surface. To obtain the potent cellulase-displaying yeast strain, three cellulases genes (CBH, EG, BGL) and a GPI biosynthesis gene (LAS21) were introduced into a thermotolerant diploid strain of *S. cerevisiae*. To further improve the ethanol yield from pretreated sugarcane bagasse by the engineered *S. cerevisiae*, a number of bioprocess parameters, including initial pH, temperature, yeast inoculum size, and additives, were optimized. Finally, the upscaling of cellulosic ethanol production by the cellulase-displaying yeast was elucidated in a 1 L fermenter. This study reports, for the first time, the effect of overexpressing genes encoding GPI biosynthesis and remodeling proteins on the cell surface activity of BGL. The engineered cellulase-displaying yeast could efficiently produce bioethanol from pretreated sugarcane bagasse. Our combined strategy of bioengineering and bioprocess optimization provides a promising way to improve the economic viability of second-generation ethanol production and can be applied to other biochemicals from the valorization of lignocellulosic biomass.

2. Materials and Methods

2.1. Strains and Culture Conditions

S. cerevisiae INVSc1 (*MATa his3Δ1 leu2 trp1-289 ura3-52/MATα his3Δ1 leu2 trp1-289 ura3-52*; Invitrogen, Carlsbad, CA, USA) was used as a host for screening cellulase genes and GPI biosynthesis and remodeling proteins. The thermotolerant diploid strain *S. cerevisiae* TISTR5088 (Biodiversity Research Centre, BRC, Thailand) was used for the construction of cellulolytic yeast. Yeast transformants were% selected on synthetic complete (SC) medium

(0.67% *w*/*v* yeast nitrogen base without amino acids, 2% *w*/*v* glucose, and 1.5% *w*/*v* agar) with the required amino acid drop-out supplement (Clontech, Mountain View, CA, USA). Under the enriched conditions, all yeast strains were grown aerobically in YPD medium (1% *w*/*v* yeast extract, 2% *w*/*v* peptone, and 2% *w*/*v* glucose) at 30 °C.

The *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for DNA manipulation and plasmid propagation. A dam *E. coli* strain (New England Biolabs, Beverly, MA) was employed for pML104-derived plasmids. *E. coli* strains were cultivated in Luria–Bertani medium (1% *w*/*v* tryptone, 0.5% *w*/*v* yeast extract, and 1% *w*/*v* NaCl). The *E. coli* transformant was selected on LB medium with 100 μg/mL ampicillin.

2.2. Plasmid and Recombinant Strain Construction

To study the effect of GPI biosynthesis- and remodeling-related proteins on cellulase expression on the yeast cell surface, the co-expression vector pYES3-Ura (pY-U) was constructed from pYES3/CT (pY) by replacing the *TRP1* gene with the URA3 gene. Then, genes encoding GPI biosynthesis- and remodeling-related proteins, including BST1 (YFL025C), CWH43 (YCR017C), ERI1 (YPL096C-A), GPI1 (YGR216C), GUP1 (YGL084C), LAS21 (YJL062W), and PER1 (YCR044C), were amplified by PCR. These genes were cloned into pY-U at the *Bam*HI/*Sac*I and *Not*I restriction sites, resulting in intracellular expression of the genes under the control of the *GAL1* promoter. Then, the recombinant yeast containing the plasmid pSS_{SUC2}-*P*BGL1-6_Kl from our previous study [14] was transformed with pYES3-Ura harboring genes encoding GPI biosynthesis- and remodeling-related proteins. The plasmid pSS_{SUC2}-*P*BGL1-6_Kl consisted of the *Periconia* β-glucosidase 1 (*P*BGL1) gene that fused with 6_Kl at its C-terminal. A control yeast strain was constructed by introducing the empty plasmids pY and pY-U into *S. cerevisiae* INVSc1.

For the screening of different cellulolytic enzymes that are suitable for expression on the yeast cell surface, the plasmid pSS_{SUC2}-*P*BGL1-6_Kl was used as a template. Cellulase-encoding genes from *Aspergillus aculeatus* (*Aa*CBHB, *Aa*EG1, *Aa*EG2, and *Aa*BGL1), *Cochliobolus heterostrophus* (*Ch*CBH2, *Ch*EG2, and *Ch*BGL1), *Chrysosporium lucknowense* (*Cl*CBH2), *Humicola insolens* (*Hi*EG1), *Penicillium decumbens* (*Pd*BGL1), *Trichoderma reesei* (*Tr*CBH1, *Tr*CBH2, and *Tr*EG2), *Acremonium thermophilum* (*At*CBH1), and *Thielavia terrestris* (*Tt*EG1) were synthesized and cloned in place of the *P*BGL1 gene in the pSS_{SUC2}-*P*BGL1-6_Kl plasmid (Figure S1). The recombinant plasmids were then introduced into *S. cerevisiae* INVSc1 following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

To construct the cellulase-displaying yeast, *S. cerevisiae* TISTR5088 was used as the host strain, and the auxotrophic marker genes (*URA3*, *LEU2*, *TRP1*, *ADE2*, and *HIS3*) were disrupted by the CRISPR-Cas9 gene editing approach using the pML104 vector system, which was a gift from Dr. John Wyrick (Addgene plasmid #67638; http://n2t.net/addgene:67638 (accessed on 10 October 2022); RRID: Addgene_67638). Gene disruption by using the pML104 vector system was performed as described elsewhere [17]. To prepare the cellulase-displaying gene cassette, *Cl*CBH2 and *Tr*EG2 were fused with the 6_Kl anchoring protein motif, while *P*BGL1 was fused with the 21_Sc anchoring protein motif [14]. The plasmids pSS_{SUC2}-*P*BGL1-21_Sc, pSS_{SUC2}-*Cl*CBH2-6_Kl, pSS_{SUC2}-*Tr*EG2-6_Kl, and pY-U-LAS21 were digested by *Pvu*II and *Not*I to obtain the fragments SS_{suc2}-*P*BGL1-21_Sc, SS_{suc2}-*Cl*CBH2-6_Kl, SS_{suc2}-*Tr*EG2-6_Kl, and LAS21, respectively. Then, these fragments were subcloned into the pASB vector at the same restriction sites. The pASB vector contains a ribosomal integrating site for facilitating multicopy integration into the yeast genome and the *GPD1* promoter for the constitutive expression of each target gene. To construct the CBP strain, pASB derivative plasmids were digested with *Bsa*BI and transformed into an auxotrophic strain of *S. cerevisiae* TISTR5088 by electroporation, following an established protocol [18]. All primers and vectors used in this study are presented in Tables S1 and S2, respectively.

2.3. Enzyme Activity Assay on the Yeast Cell Surface

Intact yeast cells were used for the measurement of the enzyme activity on the yeast cell surface. For BGL, the activity was determined as described in Pheinluphon et al.

(2019) [14], using *p*-nitrophenyl-β-D glucopyranoside as a substrate. One unit of BGL activity refers to the amount of enzyme that produced 1 μmol *p*-nitrophenol per min under the assay conditions. The hydrolytic activity of CBH and EG on the yeast cell surface was examined by using phosphoric acid swollen cellulose (PASC) as a substrate. PASC was prepared from a modified protocol of Zhang et al. (2006) [19]. Five OD_{600} values of yeast cells were incubated with 1% PASC, 25 mM sodium acetate buffer (pH 5.0), and 75 mM methylglyoxal at 40 °C for 4 h. The yeast cells were then pelleted by centrifugation at 12,396× *g* for 1 min. The supernatant was collected, and the released reducing sugar concentration was determined by the 3,5-dinitrosalicylic acid (DNS) method [20].

2.4. Determination of Gene Copy Number by Quantitative Real-Time PCR

Quantitative real-time PCR was performed using SSOFast^{TM} EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All genomic DNA concentrations and purities were estimated using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The reaction contained 4 ng of genomic DNA and 0.2 μM gene-specific primers in 10 μL of supermix. The thermal cycling protocol was as follows: initial denaturation for 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature of each specific primer, and 30 s at 72 °C. The specificity of the PCR product was confirmed by melting curve analysis from 55 °C to 95 °C, with a continuous fluorescent reading at 0.5 °C increments. Tenfold serial dilutions of the pASB-*Cl*CBH2-Leu2, pASB-*Tr*EG2-Ura3, pASB-*P*BGL1-Ade2, pASB-LAS-Ade2, and pJET-Actin plasmids were used to generate standard curves for each gene. The gene copy number was calculated relative to that of the *actin* reference gene using the standard curve method [21]. The primers used for quantitative real-time PCR are listed in Table S3.

2.5. Pretreatment of Lignocellulosic Materials

Sugarcane bagasse was obtained locally in Thailand. It was physically processed using a cutting mill (an Ultra Centrifugal Mill SM2000) with a 0.5 cm mesh screen and sieved to particles that were 250–420 μm in size (Retsch, Haan, Germany). Lignocellulosic material was pretreated with 1% (*w*/*v*) sodium hydroxide at 90 °C for 90 min [22]. Next, alkali-pretreated bagasse was refined by wet milling. The pretreated sugarcane bagasse contained 57.01 ± 0.35% (*w*/*w*) cellulose, 24.46 ± 0.35% (*w*/*w*) hemicelluloses and 8.25 ± 0.87% (*w*/*w*) lignin, according to the standard TAPPI method [23].

2.6. Ethanol Production from Pretreated Sugarcane Bagasse

The yeast strains were precultivated in YPD medium at 30 °C and 200 rpm for 48 h. The cells were collected by centrifugation at 5000× *g* for 10 min at 4 °C and washed with sterile distilled water. The yeast cells were inoculated into YP medium (1% *w*/*v* yeast extract, 2% *w*/*v* peptone) containing 5% (*w*/*v*) pretreated sugarcane bagasse as the sole carbon source in a 50 mL screw cap tube at an initial yeast concentration of 100 g cell wet weight (CWW)/L. To study the effect of external cellulase loading, a commercial cellulase named Cellulase C (57 FPU/mL, Siam Victory Chemicals Company Limited, Thailand) was added at 2.5, 5, 7.5, 10, 15, and 20 FPU/g biomass into 50 mL of YP medium containing 5% (*w*/*v*) pretreated sugarcane bagasse. The medium was incubated at 50 °C with shaking at 200 rpm for 2 h for prehydrolysis. Then, 100 g CWW/L yeast cells were inoculated. Ethanol fermentation was carried out at 40 °C with shaking at 100 rpm for 72 h. The samples were centrifuged at 5000× *g* for 10 min at 4 °C, and the supernatant was collected.

For optimization of the ethanol fermentation by the CBP yeast strain, the effect of initial pH in the fermentation medium was evaluated by adjusting the pH to 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 before autoclaving. The recombinant yeast strain (100 g CWW/L) was inoculated in 10 mL of YP medium containing 5% (*w*/*v*) pretreated sugarcane bagasse and 7.5 FPU/g Cellulase C after prehydrolysis at 50 °C for 2 h. Ethanol fermentation was carried out at 40 °C with shaking at 100 rpm for 72 h. For the effect of temperature, YP medium containing 5% (*w*/*v*) pretreated sugarcane bagasse was prehydrolyzed with 7.5 FPU/g

Cellulase C for 2 h. Then, 100 g CWW/L yeast cells were inoculated, and fermentation was performed at 34, 37, 40, or 43 °C with shaking at 100 rpm for 72 h.

Thereafter, the fermentation reaction was set up at the optimum initial pH (pH 5.0) and optimum temperature (37 °C). To explore the effect of yeast concentration, different yeast inoculum sizes (50, 100, 150, 200, and 250 g CWW/L) were inoculated in 10 mL of YP medium containing 5% (*w*/*v*) pretreated sugarcane bagasse and 7.5 FPU/g Cellulase C. The 50 mL tube was shaken at 100 rpm for 72 h. For the effect of surfactants and divalent cations, 2% (*w*/*v*) Tween 20, 2% (*w*/*v*) Triton X-100, 5 mM $ZnSO_4$, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ were supplemented to the YP medium containing 5% (*w*/*v*) pretreated sugarcane bagasse, 7.5 FPU/g Cellulase C, and 200 g CWW/L yeast cells. The fermentation was performed at 100 rpm for 72 h. Ethanol concentrations were analyzed by high-performance liquid chromatography (HPLC; Shimadzu prominence LC-20 series, Shimadzu, Kyoto, Japan). All experiments were performed in triplicate.

2.7. Scale-Up of Ethanol Production in a 1 L Bioreactor

Ethanol fermentation was performed in a 1 L bioreactor (MDFT-N, B.E. Marubishi, Pathumthani, Thailand) with a working volume of 0.5 L. YP medium containing 5 or 10% (*w*/*v*) pretreated sugarcane bagasse was used as the fermentation medium. The initial pH of the medium was adjusted to 5.0 using 0.5 M H_2SO_4. After sterilization, the fermentation medium was supplemented with 7.5 FPU/g of Cellulase C or Cellic Ctec2 (161 FPU/mL, Novozymes A/S, Basgsværd, Denmark) and the additive solution containing 0.2% (*w*/*v*) Tween 20, 0.2% (*w*/*v*) Triton X-100, and 5 mM $ZnSO_4$. Prehydrolysis was performed at 50 °C and 350–500 rpm for 2 h. Then, ethanol fermentation was started by the inoculation of 200 or 400 g CWW/L yeast cells. The temperature was maintained at 37 °C with an agitation rate of 220 rpm for 96 h without any air supplement or pH control. Samples were taken every 12 h until 96 h. The samples were centrifuged at 5000× *g* for 10 min at 4 °C and stored until further analysis. The experiments were repeated twice.

2.8. Statistical Method

The statistical analysis of the displayed BGL activity was examined using one-way ANOVA followed by Dunnett's multiple comparison test. Data from 10 biological replicates were analyzed, and the control yeast strain without overexpression of enhancing genes was used for comparison. p values ≤ 0.05 (**) were considered statistically significant. For the study of initial pH, temperature, yeast inoculum, and additives on ethanol production, data from three biological replicates were applied for statistical analysis by using one-way ANOVA. Post hoc analysis by the Fisher's least significant test at $p \leq 0.05$ was conducted when the significance was observed by ANOVA.

3. Results and Discussion

3.1. Effect of GPI Biosynthesis and Remodeling Proteins on the Displayed BGL Activity

To improve the performance of cellulase-displaying activity in yeast cells, seven genes involved in GPI biosynthesis and remodeling (PER1, ERI1, CWH43, GUP1, BST1, GPI1, and LAS21) were selected and evaluated in the *P*BGL1-displaying yeast strain. The overexpression of CWH43, GUP1, BST1, GPI1, or LAS21 increased the BGL activity on the yeast cell surface compared to the control cells containing the empty vector (Figure 1A). The overexpression of GPI1 and LAS21 significantly improved the displayed BGL activity by 41.1% and 48.8%, respectively. The maximum BGL activity of 173.9 U/g CDW, corresponding to approximately 1.5 times that of the control strain (116.9 U/g CDW), was achieved with the yeast cells expressing LAS21. In contrast, the overexpression of PER1 or ERI1 led to a reduction in BGL activity on the yeast cell surface.

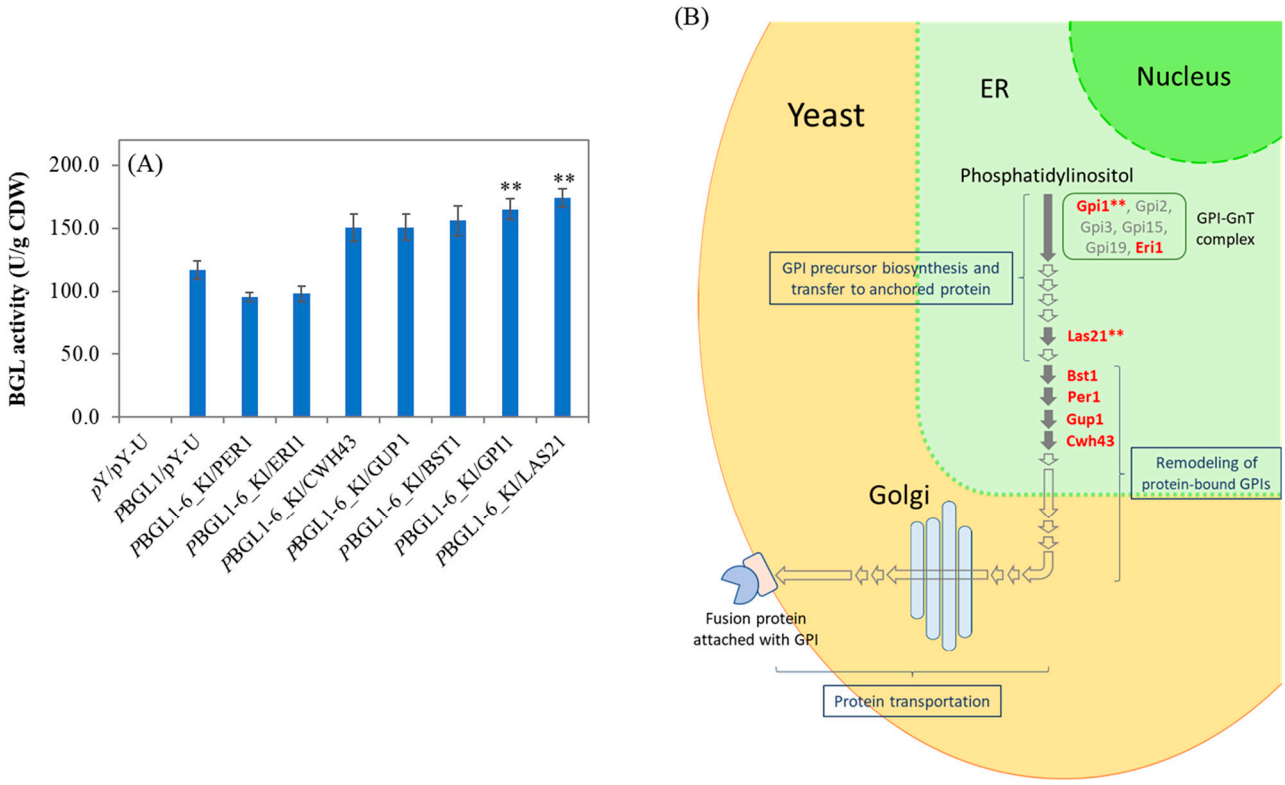

Figure 1. Effect of the overexpression of GPI biosynthesis- and remodeling-related proteins. (**A**) BGL activity on the cell surface. (**B**) Diagram illustrating the GPI biosynthesis and remodeling pathway. ** indicates statistical significance at $p \leq 0.05$.

As shown in Figure 1B, the GPI1 protein participates in the first step of GPI biosynthesis, while the LAS21 (GPI7) protein is a mannose-ethanolamine phosphotransferase catalyzing the transfer of phosphoethanolamine to the second mannose on the GPI moiety [16]. The absence of the LAS21 gene resulted in a significant reduction in GPI biosynthesis, a delay in the ER exit, and the trafficking of GPI-anchor proteins [24,25]. The posttranslational modification of the attachment of the GPI moiety onto the GPI-anchored protein precursor in the ER is essential for protein translocation to the cell surface. This GPI biosynthesis and remodeling process occurred in 14 sequential reactions involving 25 proteins [16]. We hypothesize that LAS21 and GPI1 proteins might be potential limiting factors for GPI-anchored protein synthesis. Increasing the intracellular expression of these proteins may contribute to higher levels of the GPI moiety, resulting in the enhanced expression level of BGL that was fused with GPI-anchored proteins (6_Kl). In this study, we demonstrated for the first time that the overexpression of proteins involved in GPI biosynthesis and remodeling processes could improve the displayed BGL activity. The combinatorial effect of GPI1 and LAS21 overexpression should be further investigated for its synergistic effect on the protein display capacity. In addition, the effect of GPI1 and LAS21 overexpression might vary among the cell surface displayed proteins; therefore, the investigation of their effects to different cellulases and other proteins should be explored in the future. As the overexpression of LAS21 showed the highest improvement in *P*BGL1 activity, it was chosen to be expressed along with the three cellulolytic enzymes on the cell surface of cellulolytic yeast.

3.2. Selection of Cellulolytic Enzymes for Yeast Cell Surface Display

The efficient degradation of cellulosic materials into glucose requires the synergistic action of CBH, EG, and BGL activities [26]. However, one of the major obstacles in the development of yeast as a CBP organism is an insufficient amount of cellulase expression [27].

In the present study, CBH-, EG-, and BGL-encoding genes from different microbial sources that are suitable for display on the yeast cell wall were explored. These cellulase genes were fused with 6_Kl as an anchoring protein, facilitating the incorporation of β-glucosidase into the yeast cell wall. Of the CBH enzymes, *Cl*CBH2 from *C. lucknowense* and *Tr*CBH2 from *T. reesei* yielded the highest reducing sugar products from the hydrolysis of PASC at 0.33 and 0.32 g/L, respectively (Figure 2A). The display of CBH2 exhibited a higher activity toward PASC than the display of CBH1 investigated in this study. For EG, endoglucanase 2 from *T. reesei* (*Tr*EG2) yielded the highest reducing sugar products (0.86 g/L) from PASC hydrolysis, corresponding to at least 1.5 times higher levels than other EGs (Figure 2B). The BGL activities of β-glucosidase from *Periconia* sp. (*P*BGL1) exhibited the highest BGL activity at 27.9 U/g cell dry weight (CDW) (Figure 2C). The high expression level of *Cl*CBH2 compared to other CBHs is consistent with previous findings on secreted enzyme forms in *S. cerevisiae* [10,28]. This can be explained by the same mRNA stability and through the sharing of some of the secretory pathway machinery between the extracellular secretory protein and the surface-display protein [29]. However, GPI proteins employ a specialized cargo protein, which is different from other secretory proteins [30]. Thus, *Cl*CBH2, *Tr*EG2, and *P*BGL1 were chosen for the construction of cellulolytic yeast.

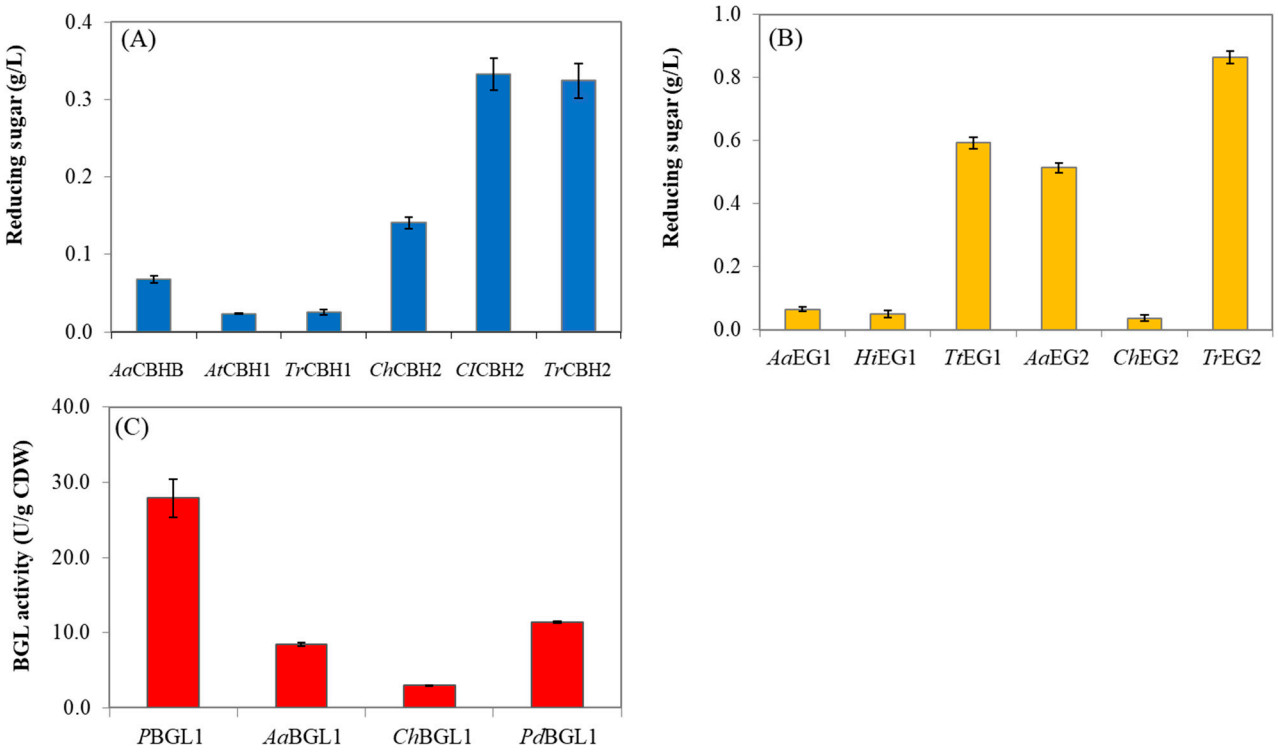

Figure 2. Cellulolytic activity of recombinant yeast cells displaying CBH, EG, or BGL. (**A**) Hydrolysis of PASC by CBH-displaying yeast strains, (**B**) hydrolysis of PASC by EG-displaying yeast strains, and (**C**) BGL activity on the cell surface of BGL-displaying yeast strains.

3.3. Construction of Thermotolerant Yeast Strains Displaying Cellulolytic Enzymes

The recombinant cellulase-displaying yeast strain was obtained from the multiple-round transformation of the pASB vector carrying *Tr*EG2-6_Kl, *Cl*CBH2-6_Kl, *P*BGL1-21_Sc, and LAS21 gene cassettes into an auxotrophic strain of a diploid thermotolerant *S. cerevisiae* TISTR5088 (Figure 3). The pASB vector system allows for the multicopy integration of the target genes into the ribosomal DNA (rDNA) site of the yeast genome. A strain, ECBLCCE5, which showed the highest ethanol-producing capability from pretreated sugarcane bagasse, was chosen. It exhibited a 2.2-fold higher reducing sugar production from PASC hydrolysis than its parental strain (Figure 4A). The corresponding PASC-degrading activity of the

ECBLCCE5 reached 27.2 mU/g CDW. Furthermore, the ethanol yield by the ECBLCCE5 strain was superior to that of its parental strain under CBP conditions (no external cellulase addition) using 5% (*w*/*v*) pretreated sugarcane bagasse as a substrate, especially after cultivation for 48 and 72 h at 40 °C (Figure 4B). The maximum ethanol concentration of 0.75 g/L was achieved at 72 h of fermentation, equivalent to approximately a 6-fold ethanol yield by the parental strain, and was in the same range as previous reports (0.80–0.93 g/L ethanol from ionic-liquid bagasse or pretreated rice straw) [8,9]. However, the cellulose composition and properties of the different biomasses greatly vary depending on the pretreatment methods and can affect the sugar products [31]. Figure 4C shows that the ECBLCCE5 strain grew slower than the parental strain on YPD medium. The previous study suggested that the cell-surface-displaying strain might encounter metabolic burden within the cell from various factors such as the heterologous gene source, gene dose, and trafficking of the enzyme to the yeast cell surface [32]. However, it did not seem to affect the ethanol yield obtained by the recombinant strain. The analysis of the gene copy number revealed that the genome of the ECBLCCE5 strain contained 16 *ClCBH2*, 9 *TrEG2*, 15 *PBGL1*, and 2 LAS21 genes (Table S4). In this study, the rDNA integration vector was used to construct the ECBLCCE5 strain. In the rDNA-mediated recombination system, the genetic stability of the heterologous gene could be maintained when the integrated plasmid size was not larger than the size of the chromosomal rDNA unit (9.1 kb) [33]. With the plasmid size control strategy, a recombinant *S. cerevisiae* strain containing a xylanase-encoding gene (xynHB) from *Bacillus* sp. integrated into the genome through the rDNA-mediated recombination showed genetic stability for more than 1000 generations of cultivation under non-selective conditions [34]. Therefore, all integrated fragments in this study were designed to not exceed 9.1 kb in size for the stable maintenance of the ECBLCCE5 strain.

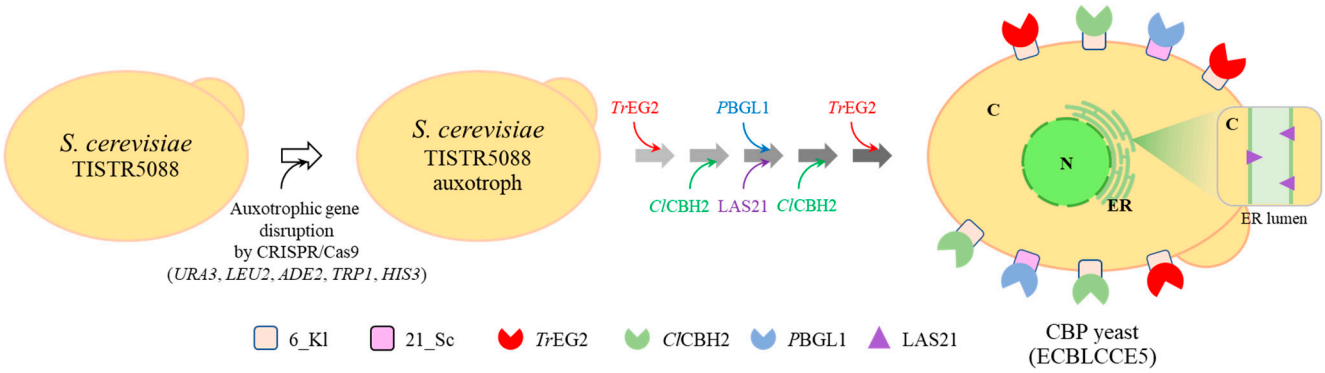

Figure 3. A scheme of the ECBLCCE5 construction.

3.4. Effect of Exogenous Cellulase on Ethanol Production from Sugarcane Bagasse

As the addition of exogenous cellulase is still necessary for efficient lignocellulose degradation, ethanol production from pretreated bagasse was conducted with the supplementation of varying commercial cellulase amounts. From the results, the ethanol production titer increased as the addition of external cellulase increased from 2.5 FPU/g to 20 FPU/g (Figure 4D). Compared to the wild-type strain, the ECBLCCE5 strain could produce 4.5–30.6% more ethanol depending on the quantity of commercial cellulase added in the fermentation reaction. The addition of 7.5 FPU/g Cellulase C into the fermentation of 5% pretreated sugarcane bagasse led to 7.91 g/L ethanol being produced by the ECBLCCE5 strain, which is equivalent to the ethanol concentration (7.94 g/L) obtained by the parental strain with the addition of 10 FPU/g Cellulase C. This indicates that the recombinant yeast enables up to a 25% reduction in the amount of external commercial cellulase needed, which is a major cost of cellulosic ethanol production [35]. These results suggest that the ECBLCCE5 strain exhibits a high performance in cellulolytic hydrolysis and alcoholic fermentation from the cellulosic substrate.

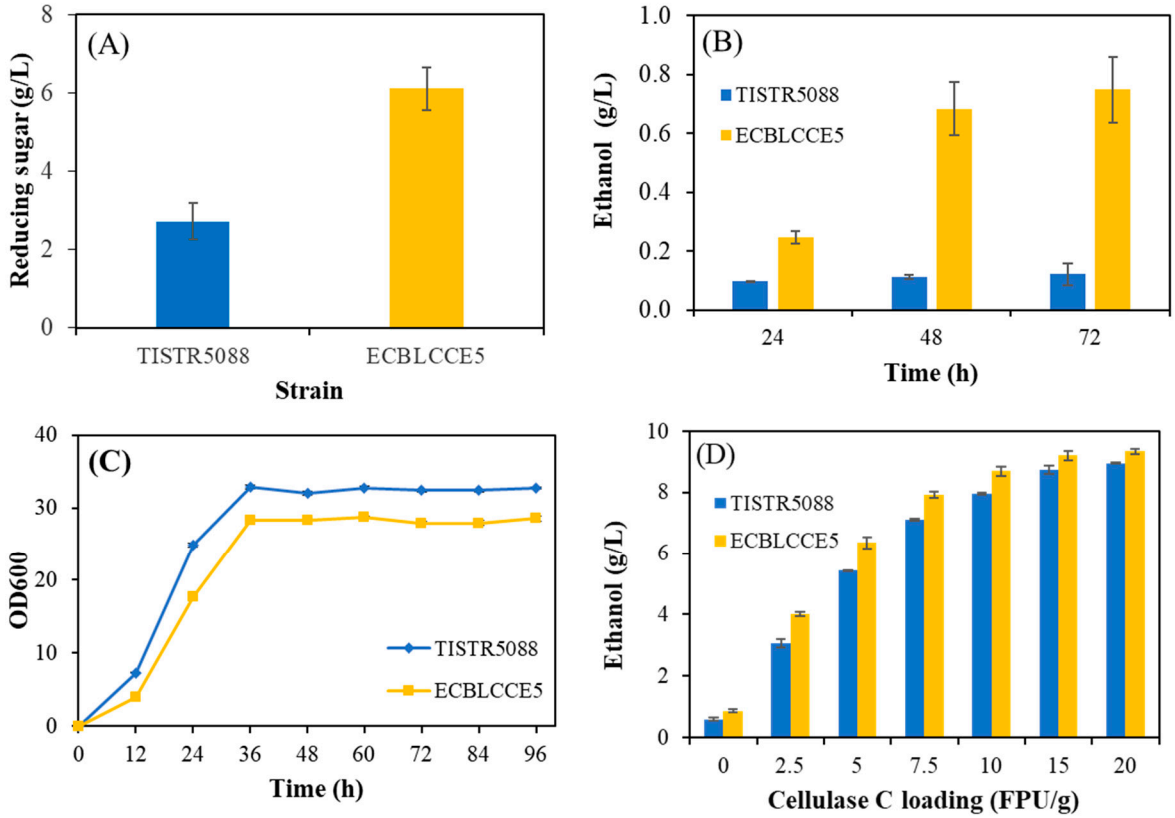

Figure 4. The characteristics of the ECBLCCE5 strain compared with those of its parental strain. (**A**) PASC hydrolysis at 40 °C for 4 h, (**B**) direct ethanol production from pretreated sugarcane bagasse, (**C**) growth rate on the YPD medium, and (**D**) effect of external cellulase supplementation on ethanol production from pretreated sugarcane bagasse.

3.5. Optimization of the Pretreated Sugarcane Bagasse Valorization Process with the ECBLCCE5 Strain

The pH and temperature of the culture have a significant effect on the optimal growth and ethanol production capacity of yeast cells, as well as the cellulolytic activity [36]. To investigate the effect of initial pH on ethanol production by the ECBLCCE5 strain, the fermentation of pretreated sugarcane bagasse was performed at different initial pH values (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5). As shown in Figure 5A, the increase in the initial pH in the fermentation medium from 5.0 to 7.5 caused a significant reduction in the ethanol product by the ECBLCCE5 strain. The maximum ethanol concentration of 7.96 g/L was obtained at pH 5.0, which is a 26.8% improvement from the non-pH adjustment condition. For the effect of temperature, the ECBLCCE5 yeast strains produced comparable amounts of ethanol from pretreated sugarcane bagasse under all tested temperatures and were not statistically significant at $p \leq 0.05$ (Figure 5B). The maximum ethanol concentration of 7.35 g/L was obtained from the recombinant yeast strain when grown at 37 °C. At 40 and 43 °C, ethanol production titers were decreased to 6.92 g/L and 6.89 g/L, respectively. Therefore, an initial pH of 5.0 and a temperature of 37 °C were selected for the bioethanol production process. Similarly, the optimized pH and temperature of ethanol production by the simultaneous saccharification and fermentation (SSF) of alkali-pretreated sugarcane bagasse using *S. cerevisiae* Y-2034 or *Kluyveromyces marxianus* NCYC-587 were pH 5.2 and 37 °C, respectively [37]. Then, the effect of the yeast inoculum size on ethanol production was explored, as presented in Figure 5C. After 72 h of cultivation, the inoculation with 200 g CWW/L yeast cells was able to produce the highest ethanol concentration of 8.89 g/L.

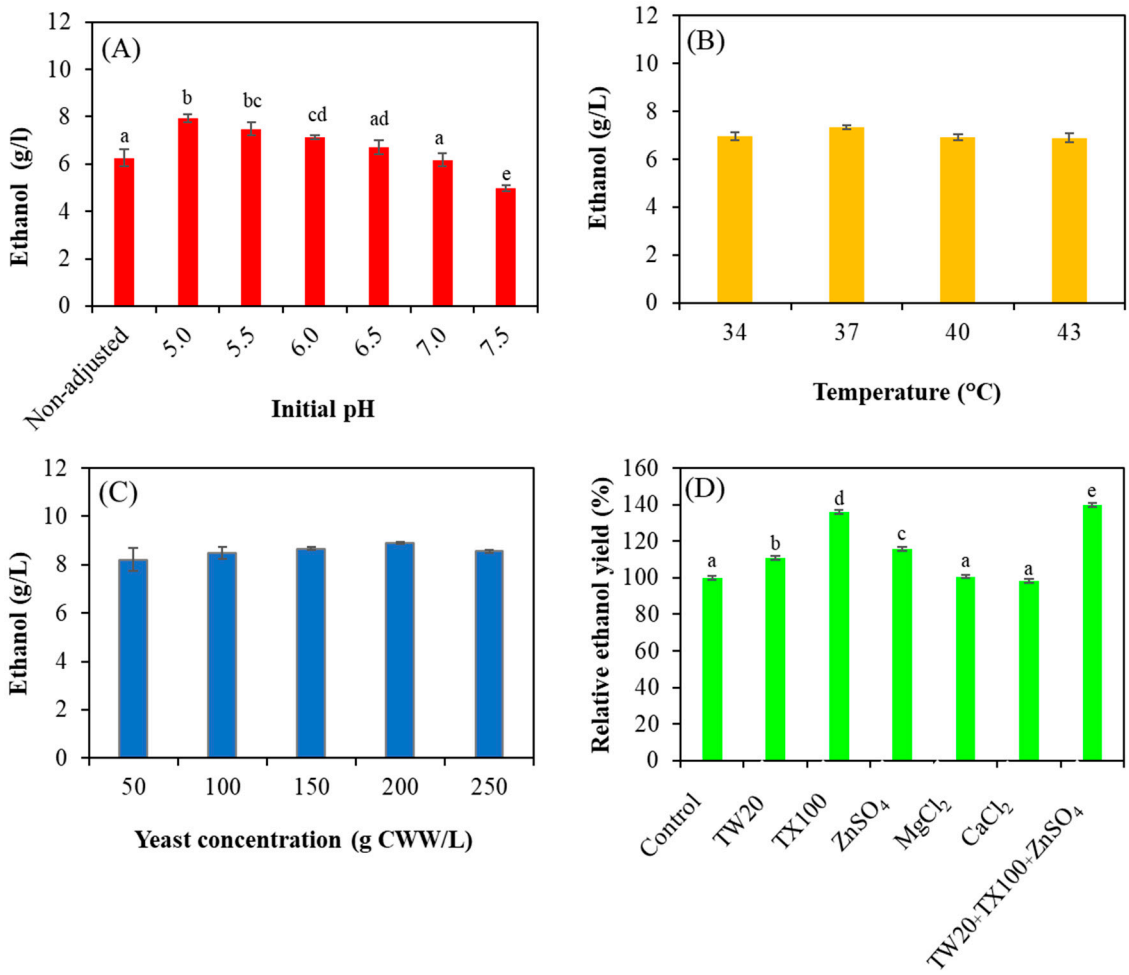

Figure 5. The influence of bioprocess parameters on direct ethanol production from pretreated sugarcane biomass. (**A**) Initial pH, (**B**) temperature, (**C**) yeast inoculum size, and (**D**) addition of surfactant and trace elements. Each datapoint with different letters marked above was significantly different ($p \leq 0.05$) according to the Fisher's least significant difference test.

To improve the ethanol production yield by the ECBLCCE5 strain, the supplementation of some nonionic surfactants and divalent cations into the fermentation medium was evaluated. As shown in Figure 5D, the addition of Tween 20, Triton X-100, and $ZnSO_4$ enhanced the ethanol production capabilities of the recombinant yeast by 10.7, 35.9, and 16.0%, respectively, compared to the control medium without any additives. The combination of 0.2% (*w*/*v*) Tween 20, 0.2% (*w*/*v*) Triton X-100, and 5 mM $ZnSO_4$ was found to significantly increase the ethanol concentration by 40% compared to the control medium. Surfactants such as Tween 20 and Triton X100 have been reported to enhance enzyme stability and yeast cell viability from thermal and shear deactivation during fermentation [38,39]. Divalent cations such as Zn^{2+} play an important role in the activity and stability of the enzyme [40]. In the case of the displayed enzyme, the Ca^{2+}, Mn^{2+}, and Zn^{2+} strongly activated the activity of *Thermomyces lanuginosus* lipase (TLL) immobilized onto the yeast cell surface [41]. It is interesting to note that these triple additives may have a positive effect on either yeast cells or displayed cellulolytic enzymes. From the results, the optimum conditions for ethanol production by the ECBLCCE5 strain were obtained at an initial pH of 5.0, a temperature of 37 °C, and 200 g CWW/L yeast inoculum, supplemented with Tween 20, Triton X-100, and $ZnSO_4$. However, the amount of yeast cells and additives might increase the cost of ethanol production or affect the downstream process. Further techno-economic assessment should be performed to obtain the feasibility of the developed process.

3.6. Scale-Up of Cellulosic Ethanol Production in a 1 L Bioreactor under Optimized Conditions

The bioethanol production from pretreated sugarcane bagasse by the ECBLCCE5 and TISTR5088 strains was carried out in a 1 L fermenter with 0.5 L of working volume. As shown in Figure 6A, at 5% (*w*/*v*) pretreated sugarcane bagasse and 200 g CWW/L yeast inoculum with 7.5 FPU/g Cellulase C, the highest ethanol concentration of 12.6 g/L (77.7% of theoretical yield) was achieved with the ECBLCCE5 after 96 h of cultivation. The ethanol yield from the cellulolytic strain was improved by up to 5% compared to its wild-type strain. Then, ethanol production was conducted with 10% (*w*/*v*) pretreated sugarcane bagasse and 400 g CWW/L yeast cell inoculum with 7.5 FPU/g Cellulase C or Ctec2 (Figure 6B). It was found that an increase in substrate loading and the yeast inoculum concentration led to an increase in the ethanol concentration. Ethanol concentrations of 17.9 g/L (55.2% of the theoretical yield) and 15.5 g/L (48.0% of the theoretical yield) were obtained with ECBLCCE5 and TISTR5088 at the 96 h fermentation time point, respectively. Interestingly, when Cellulase C was replaced with another commercial cellulase named Ctec2 at the same enzyme dose, a high level of ethanol product, up to 28.0 g/L (86.5% of the theoretical yield), was obtained with the ECBLCCE5 strain, while the wild-type strain produced up to 19.6 g/L ethanol (60.5% of the theoretical yield). By comparing cellulase activity per FPU, Ctec2 had 8.6 times higher BGL activity than Cellulase C (Table S5), which might help to reduce strong cellulase inhibition by cellobiose [42]. Ctec2 also contains a lytic polysaccharide monooxygenase that acts as a key auxiliary enzyme in lignocellulose degradation [43]. Overall, this led to the enhanced ethanol yield achieved by using this enzyme.

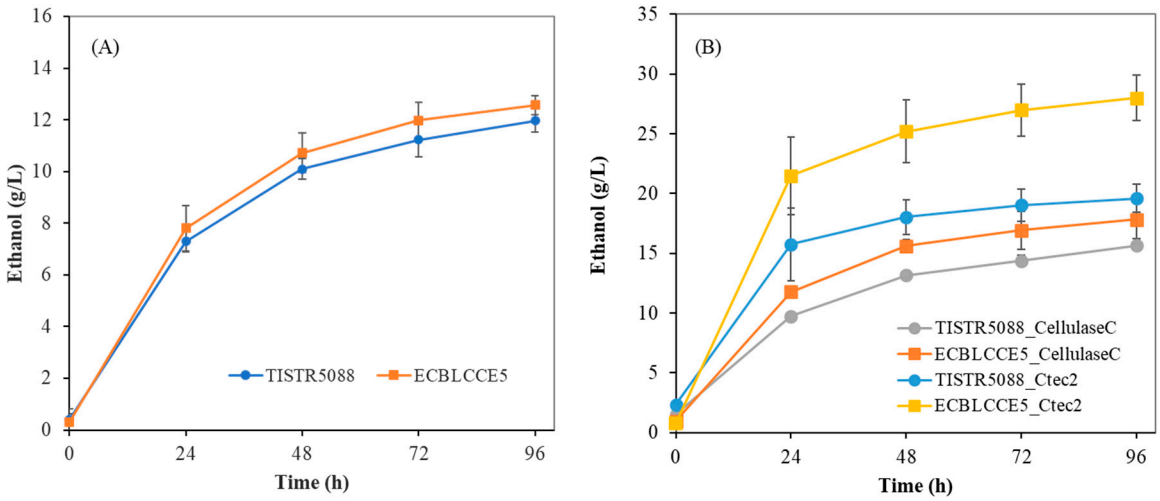

Figure 6. Scale-up of simultaneous saccharification and fermentation by the ECBLCCE5 strain in a 1 L bioreactor. (**A**) 5% (*w*/*v*) pretreated sugarcane bagasse with 7.5 FPU/g Cellulase C, (**B**) 10% (*w*/*v*) pretreated sugarcane bagasse with 7.5 FPU/g Cellulase C or Ctec2.

A comparison of previous reports for ethanol production from lignocellulosic biomass is presented in Table 1. Compared to the SSF process, the ethanol concentration obtained by CBP is quite low for industrial complementation. In contrast, the SSF processes using cellulolytic yeast strains apparently employed less exogenous cellulase loading to help destroy the cellulosic biomass structure compared to the conventional SSF process [44]. Lee et al. (2017) [10] constructed a yeast consortium with a secretion of cellulases and obtained 14 g/L or 79% theoretical yield of ethanol from 5% (*w*/*v*) pretreated rice straw. More recently, Inokuma et al. (2020) [45] optimized the anchorage position of cellulases on the yeast cell wall, which resulted in 8 g/L ethanol from 10% (*w*/*v*) pretreated rice straw with a small amount of commercial cellulases used (0.4 FPU/g biomass). In this study, the ethanol concentration of 28 g/L from 10% (*w*/*v*) pretreated sugarcane bagasse was higher than that in previous reports and promises the industrial application of second-generation ethanol from lignocellulosic biomasses. The utilization of the diploid thermotolerant

S. cerevisiae facilitates the ethanol production at high temperatures, which can save energy and reduce microbial contamination during the fermentation process [46]. The cellulolytic yeast cells could also be reutilized to reduce the yeast preparation cost [47]. Furthermore, the remaining xylose from the lignocellulose valorization can be transformed into more valuable biochemicals such as xylitol [48].

Table 1. Comparison of ethanol production from cellulosic biomasses by different processes and engineering strategies of *S. cerevisiae*.

Process	Properties of *S. cerevisiae*	Substrate	Substrate Loading (%*w*/*v*)	Commercial Cellulase Loading	Ethanol (g/L)	Ethanol Yield (g/g Biomass)	Ethanol Theoretical Yield (%)	References
SSF	Noncellulolytic	Sugarcane bagasse	10.0	100 U/g Celluclast 1.5 L	4.9	0.05	-	[44]
CBP	Cellulolytic (YSD)	Rice straw	2.5	No addition	0.80	0.03	-	[8]
CBP	Cellulolytic (YSD)	Sugarcane bagasse	0.5	No addition	0.93	0.19	91.2	[9]
SSF	Cellulolytic (Secretion)	Rice straw	5.0	10.0 FPU/g of Tec-mix	14.0	0.28	79.0	[10]
SSF	Cellulolytic (YSD)	Rice straw	10.0	0.4 FPU/g of CTec2	8.0	0.08	33.0	[45]
SSF	Cellulolytic (YSD)	Sugarcane bagasse	10.0	7.5 FPU/g of CTec2	28.0	0.28	86.5	This study

4. Conclusions

We report, for the first time, the enhancement of cell surface display technology by the overexpression of LAS21 and GPI1. By combining the engineering of cellulase-displaying yeast with the optimization of the bioprocess parameters, a 28.0 g/L (86.5% theoretical yield) ethanol concentration from pretreated sugarcane bagasse was achieved by the ECBLCCE5 strain using alkali-pretreated sugarcane bagasse as a substrate, which was a relatively high ethanol concentration compared to previous studies. The recombinant cellulolytic yeast strain and optimized bioprocessing conditions obtained in this study promise the efficient production of ethanol or other biobased chemicals from lignocellulosic biomass.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8110652/s1, Figure S1: Feature maps of the plasmids pSS$_{SUC2}$-*P*BGL1-6_Kl (A), pYES3-Ura (B), and pASB (C) series; Table S1: List of primers used in this study; Table S2: Recombinant vectors used in this study; Table S3: Primers for real-time PCR-based determination of the gene copy number; Table S4: Determination of the gene copy number in *S. cerevisiae* ECBLCCE5; Table S5: Enzyme activity profile of commercial cellulase.

Author Contributions: J.A., P.B., A.P. and P.D. performed the experimental work, the analysis of the data, and the writing of the initial draft paper. K.T., S.-n.P. and C.S. performed the text and figures formatting and the revision of the paper. C.T. and V.C. contributed to the revision of the paper. S.S. contributed to the revision of the paper, the supervision, and the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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References

1. Vasić, K.; Knez, Ž.; Leitgeb, M. Bioethanol production by enzymatic hydrolysis from different lignocellulosic sources. *Molecules* **2021**, *26*, 753. [CrossRef]
2. Hoang, T.-D.; Nghiem, N. Recent developments and current status of commercial production of fuel ethanol. *Fermentation* **2021**, *7*, 314. [CrossRef]
3. Rosales-Calderon, O.; Arantes, V. A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol. *Biotechnol. Biofuels* **2019**, *12*, 240. [CrossRef]
4. Cunha, J.T.; Gomes, D.G.; Romaní, A.; Inokuma, K.; Hasunuma, T.; Kondo, A.; Domingues, L. Cell surface engineering of *Saccharomyces cerevisiae* for simultaneous valorization of corn cob and cheese whey via ethanol production. *Energy Convers. Manag.* **2021**, *243*, 114359. [CrossRef]
5. Wang, N.; Yan, Z.; Liu, N.; Zhang, X.; Xu, C. Synergy of cellulase systems between *Acetivibrio thermocellus* and *Thermoclostridium stercorarium* in consolidated-bioprocessing for cellulosic ethanol. *Microorganisms* **2022**, *10*, 502. [CrossRef]
6. Chang, J.J.; Lin, Y.J.; Lay, C.H.; Thia, C.; Wu, Y.C.; Hou, Y.H.; Huang, C.C.; Li, W.H. Constructing a cellulosic yeast host with an efficient cellulase cocktail. *Biotechnol. Bioeng.* **2018**, *115*, 751–761. [CrossRef]
7. Cunha, J.T.; Romaní, A.; Inokuma, K.; Johansson, B.; Hasunuma, T.; Kondo, A.; Domingues, L. Consolidated bioprocessing of corn cob-derived hemicellulose: Engineered industrial *Saccharomyces cerevisiae* as efficient whole cell biocatalysts. *Biotechnol. Biofuels* **2020**, *13*, 138. [CrossRef]
8. Liu, Z.; Inokuma, K.; Ho, S.H.; den Haan, R.; van Zyl, W.H.; Hasunuma, T.; Kondo, A. Improvement of ethanol production from crystalline cellulose via optimizing cellulase ratios in cellulolytic *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2017**, *114*, 1201–1207. [CrossRef]
9. Amoah, J.; Ishizue, N.; Ishizaki, M.; Yasuda, M.; Takahashi, K.; Ninomiya, K.; Yamada, R.; Kondo, A.; Ogino, C. Development and evaluation of consolidated bioprocessing yeast for ethanol production from ionic liquid-pretreated bagasse. *Bioresour. Technol.* **2017**, *245*, 1413–1420. [CrossRef]
10. Lee, C.-R.; Sung, B.H.; Lim, K.-M.; Kim, M.-J.; Sohn, M.J.; Bae, J.-H.; Sohn, J.-H. Co-fermentation using recombinant *Saccharomyces cerevisiae* yeast strains hyper-secreting different cellulases for the production of cellulosic bioethanol. *Sci. Rep.* **2017**, *7*, 4428. [CrossRef]
11. Wu, Y.; Chen, Y.; Wei, N. Biocatalytic properties of cell surface display laccase for degradation of emerging contaminant acetaminophen in water reclamation. *Biotechnol. Bioeng.* **2020**, *117*, 342–353. [CrossRef]
12. Tabañag, I.D.F.; Chu, I.M.; Wei, Y.-H.; Tsai, S.-L. The role of yeast-surface-display techniques in creating biocatalysts for consolidated bioprocessing. *Catalysts* **2018**, *8*, 94. [CrossRef]
13. Lozančić, M.; Sk. Hossain, A.; Mrša, V.; Teparić, R. Surface display—An alternative to classic enzyme immobilization. *Catalysts* **2019**, *9*, 728. [CrossRef]
14. Phienluphon, A.; Mhuantong, W.; Boonyapakron, K.; Deenarn, P.; Champreda, V.; Wichadakul, D.; Suwannarangsee, S. Identification and evaluation of novel anchoring proteins for cell surface display on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 3085–3097. [CrossRef]
15. Muñiz, M.; Zurzolo, C. Sorting of GPI-anchored proteins from yeast to mammals-common pathways at different sites? *J. Cell Sci.* **2014**, *127*, 2793–2801. [CrossRef]
16. Pittet, M.; Conzelmann, A. Biosynthesis and function of GPI proteins in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **2007**, *1771*, 405–420. [CrossRef]
17. Laughery, M.F.; Hunter, T.; Brown, A.; Hoopes, J.; Ostbye, T.; Shumaker, T.; Wyrick, J.J. New vectors for simple and streamlined CRISPR-Cas9 genome editing in *Saccharomyces cerevisiae*. *Yeast* **2015**, *32*, 711–720. [CrossRef]
18. Helmuth, M.; Altrock, W.; Böckers, T.M.; Gundelfinger, E.D.; Kreutz, M.R. An electrotransfection protocol for yeast two-hybrid library screening. *Anal. Biochem.* **2001**, *293*, 149–152. [CrossRef]
19. Zhang, Y.H.; Cui, J.; Lynd, L.R.; Kuang, L.R. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: Evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules* **2006**, *7*, 644–648. [CrossRef]
20. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
21. Leelatanawit, R.; Klanchui, A.; Uawisetwathana, U.; Karoonuthaisiri, N. Validation of reference genes for real-time PCR of reproductive system in the black tiger shrimp. *PLoS ONE* **2012**, *7*, e52677. [CrossRef]
22. Suwannarangsee, S.; Arnthong, J.; Eurwilaichitr, L.; Champreda, V. Production and characterization of multi-polysaccharide degrading enzymes from *Aspergillus aculeatus* BCC199 for saccharification of agricultural residues. *J. Microbiol. Biotechnol.* **2014**, *24*, 1427–1437. [CrossRef]
23. Technical Association of the Pulp and Paper Industry. TAPPI Test Method T264 cm-97. Preparation of Wood for Chemical Analysis. Available online: https://www.scribd.com/document/340758480/Tappi-T264-Cm-97# (accessed on 10 October 2022).
24. Komath, S.S.; Singh, S.L.; Pratyusha, V.A.; Sah, S.K. Generating anchors only to lose them: The unusual story of glycosylphosphatidylinositol anchor biosynthesis and remodeling in yeast and fungi. *IUBMB Life* **2018**, *70*, 355–383. [CrossRef]
25. Benachour, A.; Sipos, G.; Flury, I.; Reggiori, F.; Canivenc-Gansel, E.; Vionnet, C.; Conzelmann, A.; Benghezal, M. Deletion of GPI7, a yeast gene required for addition of a side chain to the glycosylphosphatidylinositol (GPI) core structure, affects GPI protein transport, remodeling, and cell wall integrity. *J. Biol. Chem.* **1999**, *274*, 15251–15261. [CrossRef]

26. Dadwal, A.; Sharma, S.; Satyanarayana, T. Progress in ameliorating beneficial characteristics of microbial cellulases by genetic engineering approaches for cellulose saccharification. *Front. Microbiol.* **2020**, *11*, 1387. [CrossRef]
27. Oh, E.J.; Jin, Y.-S. Engineering of *Saccharomyces cerevisiae* for efficient fermentation of cellulose. *FEMS Yeast Res.* **2020**, *20*, foz089. [CrossRef]
28. 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.; et al. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* **2011**, *4*, 30. [CrossRef]
29. Tang, H.; Song, M.; He, Y.; Wang, J.; Wang, S.; Shen, Y.; Hou, J.; Bao, X. Engineering vesicle trafficking improves the extracellular activity and surface display efficiency of cellulases in *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* **2017**, *10*, 53. [CrossRef]
30. Lopez, S.; Rodriguez-Gallardo, S.; Sabido-Bozo, S.; Muñiz, M. Endoplasmic reticulum export of GPI-anchored proteins. *Int. J. Mol. Sci.* **2019**, *20*, 3506. [CrossRef]
31. Baruah, J.; Nath, B.K.; Sharma, R.; Kumar, S.; Deka, R.C.; Baruah, D.C.; Kalita, E. Recent trends in the pretreatment of lignocellulosic biomass for value-added products. *Front. Energy Res. Bioenergy Biofuels* **2018**, *6*, 141. [CrossRef]
32. Ding, J.; Liang, G.; Zhang, K.; Hong, J.; Zou, S.; Lu, H.; Ma, Y.; Zhang, M. Extra metabolic burden by displaying over secreting: Growth, fermentation and enzymatic activity in cellobiose of recombinant yeast expressing β-glucosidase. *Bioresour. Technol.* **2018**, *254*, 107–114. [CrossRef] [PubMed]
33. Lopes, T.S.; De Wijs, I.J.; Steenhauer, S.I.; Verbakel, J.; Planta, R.J. Factors affecting the mitotic stability of high-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*. *Yeast* **1996**, *12*, 467–477. [CrossRef]
34. Fang, C.; Wang, Q.; Selvaraj, J.N.; Zhou, Y.; Ma, L.; Zhang, G.; Ma, Y. High copy and stable expression of the xylanase XynHB in *Saccharomyces cerevisiae* by rDNA-mediated integration. *Sci. Rep.* **2017**, *7*, 8747. [CrossRef]
35. Zhang, Q.; Weng, C.; Huang, H.; Achal, V.; Wang, D. Optimization of bioethanol production using whole plant of water hyacinth as substrate in simultaneous saccharification and fermentation process. *Front. Microbiol.* **2016**, *6*, 1411. [CrossRef]
36. Mohapatra, S.; Jena, S.; Jena, P.K.; Badhai, J.; Acharya, A.N.; Thatoi, H. Partial consolidated bioprocessing of pretreated *Pennisetum* sp. by anaerobic thermophiles for enhanced bioethanol production. *Chemosphere* **2020**, *256*, 127126. [CrossRef]
37. Gao, Y.; Xu, J.; Yuan, Z.; Jiang, J.; Zhang, Z.; Li, C. Ethanol production from sugarcane bagasse by fed-batch simultaneous saccharification and fermentation at high solids loading. *Energy Sci. Eng.* **2018**, *6*, 810–818. [CrossRef]
38. Lou, H.; Zeng, M.; Hu, Q.; Cai, C.; Lin, X.; Qiu, X.; Yang, D.; Pang, Y. Nonionic surfactants enhanced enzymatic hydrolysis of cellulose by reducing cellulase deactivation caused by shear force and air-liquid interface. *Bioresour. Technol.* **2018**, *249*, 1–8. [CrossRef]
39. Lee, W.G.; Lee, J.S.; Lee, J.P.; Shin, C.S.; Kim, M.S.; Park, S.C. Effect of surfactants on ethanol fermentation using glucose and cellulosic hydrolyzates. *Biotechnol. Lett.* **1996**, *18*, 299–304. [CrossRef]
40. Pereira, J.D.C.; Giese, E.C.; SouzaMoretti, M.M.D.; Gomes, A.C.D.S.; Perrone, O.M.; Boscolo, M.; Silva, R.D.; Gomes, E.; Martins, D.A.B. Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. *Enzym. Inhib. Act.* **2017**, *29*, 139–164. [CrossRef]
41. Yang, J.; Huang, K.; Xu, X.; Miao, Y.; Lin, Y.; Han, S. Cell surface display of *Thermomyces lanuginosus* lipase in *Pichia pastoris*. *Front. Bioeng. Biotechnol.* **2020**, *8*, 544058. [CrossRef]
42. Gruno, M.; Väljamäe, P.; Pettersson, G.; Johansson, G. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. *Biotechnol. Bioeng.* **2004**, *86*, 503–511. [CrossRef] [PubMed]
43. Müller, G.; Chylenski, P.; Bissaro, B.; Eijsink, V.G.H.; Horn, S.J. The impact of hydrogen peroxide supply on LPMO activity and overall saccharification efficiency of a commercial cellulase cocktail. *Biotechnol. Biofuels* **2018**, *11*, 209. [CrossRef] [PubMed]
44. Jugwanth, Y.; Sewsynker-Sukai, Y.; Gueguim Kana, E.B. Valorization of sugarcane bagasse for bioethanol production through simultaneous saccharification and fermentation: Optimization and kinetic studies. *Fuel* **2020**, *262*, 116552. [CrossRef]
45. Inokuma, K.; Kurono, H.; den Haan, R.; van Zyl, W.H.; Hasunuma, T.; Kondo, A. Novel strategy for anchorage position control of GPI-attached proteins in the yeast cell wall using different GPI-anchoring domains. *Metab. Eng.* **2020**, *57*, 110–117. [CrossRef] [PubMed]
46. Nguyen, P.V.; Nguyen, K.H.; Nguyen, N.L.; Ho, X.T.; Truong, P.H.; Thi Nguyen, K.C. Lychee-Derived, Thermotolerant yeasts for second-generation bioethanol production. *Fermentation* **2022**, *8*, 515. [CrossRef]
47. Matano, Y.; Hasunuma, T.; Kondo, A. Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. *Bioresour. Technol.* **2013**, *135*, 403–409. [CrossRef]
48. Louie, T.M.; Louie, K.; DenHartog, S.; Gopishetty, S.; Subramanian, M.; Arnold, M.; Das, S. Production of bio-xylitol from D-xylose by an engineered *Pichia pastoris* expressing a recombinant xylose reductase did not require any auxiliary substrate as electron donor. *Microb. Cell Factories* **2021**, *20*, 50. [CrossRef]