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Co-Fermentation of Glucose–Xylose–Cellobiose–XOS Mixtures Using a Synthetic Consortium of Recombinant *Saccharomyces cerevisiae* Strains

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Abstract: The efficient conversion of cellulosic sugars is vital for the economically viable production of biofuels/biochemicals from lignocellulosic biomass hydrolysates. Based on comprehensive screening, *Saccharomyces cerevisiae* RC212 was chosen as the chassis strain for multiple integrations of heterologous β-glucosidase and β-xylosidase genes in the present study. The resulting recombinant BLN26 and LF1 form a binary synthetic consortium, and this co-culture system achieved partial fermentation of four sugars (glucose, xylose, cellobiose, and xylo-oligosaccharides). Then, we developed a ternary *S. cerevisiae* consortium consisting of LF1, BSGIBX, and 102SB. Almost all four sugars were efficiently fermented to ethanol within 24 h, and the ethanol yield is 0.482 g g^{-1} based on the consumed sugar. To our knowledge, this study represents the first exploration of the conversion of mixtures of glucose, xylose, cellobiose, and xylo-oligosaccharides by a synthetic consortium of recombinant *S. cerevisiae* strains. This synthetic consortium and subsequent improved ones have the potential to be used as microbial platforms to produce a wide array of biochemicals from lignocellulosic hydrolysates.

Keywords: synthetic consortium; bioethanol; yeast; cellobiose; xylo-oligosaccharides; co-culture system

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Citation: Yan, N.; Luan, T.; Yin, M.; Niu, Y.; Wu, L.; Yang, S.; Li, Z.; Li, H.; Zhao, J.; Bao, X. Co-Fermentation of Glucose–Xylose–Cellobiose–XOS Mixtures Using a Synthetic Consortium of Recombinant *Saccharomyces cerevisiae* Strains. *Fermentation* **2023**, *9*, 775. https://doi.org/10.3390/fermentation9080775

Academic Editor: Ronnie G. Willaert

Received: 17 July 2023
Revised: 12 August 2023
Accepted: 16 August 2023
Published: 19 August 2023

1. Introduction

Continued oil prices rise due to factors such as the Ukraine conflict have seriously disrupted the world economy. In the United States, the Federal Reserve System estimates that every USD 10 per barrel rise in oil prices cuts GDP (gross domestic product) growth by 0.1 percentage points and increases inflation by 0.2 percentage points. In an attempt to gain some control over spiking energy prices in the U.S., President Biden has set to order the release of as much as 1 million barrels of oil a day from the nation's strategic oil reserves. In the eurozone, as a rule of thumb, every 10% rise in the oil price in euro terms increases eurozone inflation by 0.1 to 0.2 points. Asia, the region with the world's most enormous demand for oil and the fastest growth in demand, is also severely hit. Furthermore, legitimate concerns regarding the petrochemical industry's negative environmental impact and unsustainability have resulted in extensive exploration of microbial production of fuels and chemicals, which are sustainable and environmentally friendly based on renewable resources [1,2]. Currently, most industrialized biochemical processes utilize crop sugar as substrate, which is non-ideal for many reasons, including minimal reductions in greenhouse gas emissions, food vs. fuel controversy, and uncompetitive margins compared to petrochemical counterparts [3].

For its abundance, sustainability, and low price, lignocellulosic biomass (LB) continues to attract global interest as a sustainable alternative to produce second-generation biofuels and other biobased chemicals without compromising global food security [4,5]. These include agricultural wastes, forest residues, dedicated crops, and short rotation coppices [4]. LB is mainly composed of cellulose, hemicellulose, and lignin, with proportions of each component depending on the sources of plant biomass, making a complex assembly of polymers naturally recalcitrant to enzymatic conversion, and thus, pretreatment is a necessary procedure before enzyme hydrolysis of LB [6].

Although LB-based products offer multiple environmental and socioeconomic advantages, their current economic state renders the biorenewables industry incapable of competing with the petrochemical industry [7]. Multiple technical hurdles must be overcome to efficiently and economically convert LB to biofuels/biochemicals. One of the most important is efficiently utilizing all types of sugars from LB hydrolysates [8]. However, there are many critical challenges to achieving this goal [9]. After pretreatment and saccharification processing, the resulting LB hydrolysates are mixtures of various hexoses, pentoses, and oligosaccharides. Cellulose hydrolysis requires the synergistic use of cellulase, primarily including endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BGL) [10]. However, cellulase from different filamentous fungi such as *Trichoderma reesei*, which currently dominates the industrial applications of cellulase, is short of the β-glucosidase activity [11]. Therefore, a certain amount of cellobiose, one of the main end products of *T. reesei* cellulase, was usually accumulated. Moreover, cellobiose also acts as a potent inhibitor, especially for CBH, which plays a key role in cellulase [12]. To maximize the yield of xylose and minimize the production of inhibitors, milder pretreatment methods have been commonly adopted; however, a certain amount of xylan and xylo-oligosaccharides (XOS, 2–7 xylose units) will remain [13]. Recent work has shown that XOS vastly reduces the efficiency of cellulose hydrolysis by cellulase, and this inhibition cannot be effectively relieved by increasing the loading of the cellulose substrate or cellulase [14]. However, in most cellulase secreted by filamentous fungi (such as *T. reesei*) and in commercially available enzymes, the β-xylosidase activity is also deficient. Worse still, the vast majority of non-genetically engineered microorganisms, including *Saccharomyces cerevisiae* and *Escherichia coli*, can efficiently utilize glucose as the optimal fermentation substrate, whereas non-glucose sugars such as pentose (particularly D-xylose, the second most abundant sugar in LB materials) and oligosaccharides (cellobiose and XOS for example) are utilized at much lower efficiencies or are not metabolized [15] due to the lack of upstream metabolic modules and the corresponding glycoside hydrolases. Therefore, measures must be taken to consume xylose, cellobiose, XOS, and other monosaccharides in the LB-derived fermentation materials.

The budding yeast *S. cerevisiae* is a prominent microorganism that has traditionally been used in industrial bioethanol production because of its numerous inherent advantages, and it is also the microorganism of choice for the production of advanced fuels and chemicals based on LB feedstocks [16]. Extensive efforts have been made to improve the capacity of xylose metabolism, including XR/XDH (xylose reductase and xylitol dehydrogenase) or XI (xylose isomerase) pathway engineering, cofactor engineering, transporter engineering, evolutionary engineering, etc. [4,5,17–20].

In terms of cellobiose and XOS metabolism, instead of the expensive measure, such as increment of activities in the enzymatic hydrolysis system by supplementing of exogenous commercial β-glucosidase and β-xylosidase [12], another alternative is the heterologous expression of these enzymes in fermentation microbes [21]. Various origins of β-glucosidase genes have been isolated and heterologously expressed in *S. cerevisiae* to enable the growth and fermentation of cellobiose [12,22,23]. The β-xylosidase coding genes were also expressed in *S. cerevisiae* single or together with the xylanase/β-glucosidase gene to utilize XOS or xylan as a carbohydrate source [13,21,24] or to co-ferment cellulose and xylan [25].

So far, research on the fermentation of LB hydrolysates by *S. cerevisiae* has focused on the development of 'generalist' yeast strains capable of fermenting mixtures of two or

three kinds of LB-derived sugars [26,27]. The current lack of simultaneous mixed-sugar utilization, caused by negative factors such as metabolic burden, limits achievable titers, yields, and productivities. As an additional note, laboratory evolution experiments with engineered pentose-fermenting generalist yeast strains have shown progressive degeneration of their pentose fermentation kinetics during prolonged growth in repeated batch cultures, according to multiple laboratory reports [26,28,29]. Therefore, the development of microbial platforms capable of fermenting mixed sugars simultaneously from LB hydrolysates is essential for economic industry-scale production, particularly for compounds with marginal profits [4].

Microbial consortia are ubiquitous in nature, and synergistic interactions among community species are established after long-term evolution in specific environments. In recent years, engineering microbial communities have garnered increasing attention and represent a new frontier of synthetic biology [30]. Unlike monocultures of 'generalist' strain, the consortium in the microbial co-culture system enables the partitioning of multi-step metabolic pathways to each member. It thus can alleviate the excessive metabolic burden on a single member and perform complex tasks via labor division [9]. In addition, the co-culture system can provide a competitive advantage of modularity compared to the single-culture system and can be tuned to deal with fluctuations in feedstock composition to achieve robust and cost-effective biofuel/biochemical production from LB hydrolysate. Both natural and synthetic consortia have been applied to enhance mixed-sugar consumption and improve biochemical production [9,26,31–33].

In summary, a microbial consortium in a co-culture system possesses an excellent potential for efficiently utilizing all types of LB-derived sugars, which can improve the competitiveness of the biorenewables industry. In addition, the fermentation performance of microorganisms was significantly reduced by various inhibitors formed during the pretreatment and saccharification of LB feedstocks [34]. One of the effective measures is the selection of parent strains with inherent robustness to the construct synthetic microbial consortium. Based on comprehensive screening, *S. cerevisiae* RC212 was chosen as the chassis strain for multiple integrations of heterologous β-glucosidase and β-xylosidase genes in the present study. The resulting recombinant BLN26 and LF1 (obtained in our previous work and could co-ferments glucose and xylose efficiently and synchronously) were selected as the synthetic microbial consortium members. In addition, three *S. cerevisiae* strains (LF1, 102SB, and BSGIBX) were also selected to form another consortium. By adjusting the inoculum ratios and initial inoculum amount of these consortia, we explored the possibility of achieving co-utilization of the four LB-derived sugars (glucose–xylose–cellobiose–XOS). In this work, bioethanol was simply used as a reporter molecule for assessing mixed-sugar assimilation competence, and it is the hope that these synthetic *S. cerevisiae* consortia can serve as microbial platforms to produce a wide array of LB-based biochemicals.

2. Materials and Methods

2.1. Microbial Strains and Media

The genetic properties of the microbial strains used and constructed in the present study are summarized in Table 1. *E. coli* Trans5α was used as the host strain for recombinant plasmid amplification in Luria–Bertani (LB) medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl; pH 7.0), and 100 mg L^{-1} of ampicillin was added as necessary.

The wild-type diploid *S. cerevisiae* strain RC212 with robustness and higher intracellular trehalose content was used as a chassis for the heterologous overexpression of genes coding β-glucosidase and β-xylosidase. All the strains were precultured at 30 °C in YEPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose), and an appropriate concentration of G418 was added as necessary.

Table 1. Microbial strains and plasmids used in the present study.

Strains and Plasmids	Description	Source/Reference
Strains		
NAN27	Used in starch-based ethanol production	Laboratory reserved
RC212	Isolated from grape or wine production regions	Laboratory reserved
LSF	Diploid *S. cerevisiae* strain separated from commercially available products (Lesaffre Yeast Corporation)	Laboratory reserved
RedStar	Diploid *S. cerevisiae* strain separated from commercially available products	Laboratory reserved
BSIF	Diploid *S. cerevisiae* strain isolated from a tropical fruit in Thailand	Laboratory reserved [35]
6508	Used in starch-based ethanol production	Laboratory reserved [35]
SQ	Diploid *S. cerevisiae* strain separated from commercially available products	Laboratory reserved
CEN.PK102-5B	Diploid *S. cerevisiae* strain MATa; *URA*3-52, *HIS*3Δ1, *LEU*2-3,112	Laboratory reserved [36,37]
LF1	Recombinant glucose/xylose cofermenting strain derived from BSIF (*pho13*::XI, *3δ*::XI, *gre3*::PPP, XK, AE-PCS, *N360F*, AE)	Laboratory preserved [16]
RBK	RC212 derivative; (*δ*::BGL& KanMX)	This work
RB	RBK derivative; (*δ*::BGL)	This work
BLN26	RB derivative; (*δ*::BGL& KanMX, *ADH2*::IBX, *δ*::IBX)	This work
BSGIBX	BSPXO42 derivative; pJXIHIBX	Laboratory preserved [21]
102SB	102-Δ*TPI* derivative; CPOTSB	Laboratory preserved [12]
Plasmids		
YEp-CH	YEp24 derivative; *GAL1p-Cre-CYC1t*, *TEF1p-hygB-TEF1t*	Laboratory preserved [16]
CPOTSSB	CPOT with β-glucosidase gene *BGL1* from *Saccharomycopsis fibuligera*; *SUC2* signal peptide	Laboratory preserved [12]
pUC-N360F	pUC19-based yeast integration plasmid containing *GRE3*-targeting recombinant arms, an overexpression cassette for $MGT05196^{N360F}$, the upstream activating sequence (UAS elements) UAS_{CLB}, and the selectable marker loxP-KanMX4-loxP	Laboratory preserved [16]
pUG6	*E. coli* plasmid with segment LoxP-KanMX4-LoxP	[38]
pJXIHIBX	pJXIH-PC with β-xylosidase gene *xyl3A* from *Penicillium oxalicum*; signal peptide of *Kluyveromyces INU*	Laboratory preserved [21]
pXIδ	pUC19-based yeast integration plasmid containing the δ-sequence-targeting recombinant arms, three tandem expression cassettes of Ru-*xylA*, and the selectable marker loxP-KanMX4-loxP	Laboratory preserved [16]
pUCδBK	pUC19-based yeast integration plasmid containing the δ-sequence-targeting recombinant arms, an expression cassette of BGL, and the selectable marker loxP-KanMX4-loxP	This work
pIBXδ	pUC19-based yeast integration plasmid containing the δ-sequence-targeting recombinant arms, β-xylosidase gene *xyl3A* from *P. oxalicum* with signal peptide of *Kluyveromyces INU*, and the selectable marker loxP-KanMX4-loxP	This work

YP medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) supplemented with different sugars as the carbon source was used for oxygen-limited growth and fermentation. When the glucose–cellobiose or glucose–xylose–cellobiose mixture was used, the medium was abbreviated as YPGC or YPGXC. If a glucose–XOS (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) mixture or glucose–xylose–cellobiose-XOS was used, the medium was abbreviated as YPGO or YPGXCO. When the carbon source was a mixture of glucose–xylose–cellobiose and XOS pretreated with xylanase, the medium was abbreviated as $YPGXCO_{pre}$.

2.2. Stress Tolerance Assays

The growth performance of selected *S. cerevisiae* strains under the typical stresses of furfural, acetic acid, vanillin, and ethanol, as well as hyper-osmotic stress [39], oxidative stress [40], and high temperature [41], were characterized through a spot dilution growth assay. Optical density measurement in a spectrophotometer at 600 nm (OD_{600}) was used to determine cell concentration. The dry cell weight (DCW) was determined by a correlation with OD_{600} using a calibration curve [21]. Cells were harvested from an overnight culture in a YEPD liquid medium and washed twice with sterile water. The density of the resuspended cells was normalized to an OD_{600} of 1.0. A 10-fold serial dilution of this suspension (10^0, 10^{-1}, 10^{-2}, and 10^{-3}) was prepared, and 4 µL of each dilute suspension was spotted onto the appropriate solid medium [42].

To determine the oxidative stress resistance of each strain, *S. cerevisiae* cells were washed with 100 µL sterile water and resuspended. The density was adjusted to OD_{600} = 2, and the cells were mixed with 20 mL YEPD solid medium (cooled to approximately 50 °C) and immediately poured into a plate. Then, a sterile filter paper (0.5 mm diameter) with 6 µL 30% H_2O_2 was placed in the center of each plate. The oxidative stress resistance of each strain was demonstrated by the diameter of the zone of growth inhibition (mm) after cultivation for 2 days at 30 °C. A smaller inhibition zone was interpreted as a higher resistance to oxidative stress [35].

2.3. Construction of Plasmids and Integrating Fragments

The PCR primers used in this study are listed in Table S1. The characteristics of the plasmids used and constructed in the present study are summarized in Table 1. The linear fragment L-pUC was derived from plasmid pUC-N360F via linearized with the restriction endonucleases NheI/BamHI. The fragment BGL and the fragment loxP-KanMX-loxP with homologous sequences of the fragment L-pUC were amplified from the plasmid CPOTSSB [12], and the pUG6 with primers BGL-up/BGL-R and KAN-F/KAN-R, respectively. Two homologous sequences, δ1 and δ2, were amplified from the genomic DNA of RC212 using primers Up-arm-F/Up-arm-R and Down-arm-F/Down-arm-R, respectively. Then, the fragments δ1/BGL and δ2/loxP-KanMX-loxP were fused by overlapping PCR to obtain fragments δ1-BGL and loxP-KanMX-loxP-δ2, respectively. Then, the plasmid vector pUCδBK was obtained by one-step ISO assembly [43] of three fragments (L-pUC, δ1-BGL, and loxP-KanMX-loxP-δ2). The construction of recombinant plasmid pUCδBK is demonstrated in Figure S1.

The fragment adh/in-adh and loxP-KanMX-loxP were amplified from the genomic DNA of RC212 and plasmid pUG6 with primers adh2-F&adh2-R/in-adh2-F&in-adh2-R and KA-XY-F/KA-R. The fragment upADH and IBXBOX (β-xylosidase expression cassette with signal peptide of *Kluyveromyces INU*) were amplified from the genomic DNA of RC212 and plasmid pXIHIBX with primers upADH-XY-F&upADH-R and XYL-F&XYL-R. Then, fragment adh/in-adh and loxP-KanMX-loxP were fused by PCR to obtain fragment loxP-KanMX-loxP-adh/in-adh; fragment upADH and IBXBOX were fused by PCR to obtain fragment upADH-IBXBOX. Finally, the two fragments were further fused by PCR to obtain the fragments upADH-IBXBOX-loxP-KanMX-loxP-adh and upADH-IBXBOX-loxP-KanMX-loxP-in-adh.

The linear fragment L-pδ was derived from plasmid pXIδ via linearization with the restriction endonucleases SalI/BamHI. The fragment BamHI-IBX-SalI was amplified from the plasmid pXIHIBX with primers BamHI-IBX-F/SalI-IBX-R and then digested with SalI/BamHI. The above two fragments were ligated by DNA ligase to obtain plasmid pIBXδ. The construction of recombinant plasmid pIBXδ is demonstrated in Figure S2.

2.4. Determination of Intracellular Trehalose Content

The *S. cerevisiae* cells, after preculture, were transferred into 50 mL YEPD medium at 30 °C, 200 rpm, with an initial OD_{600} of 0.5. After culturing for 12 h, the culture temperature was increased by 1 °C every 21 min until being stopped at 37 °C, and then the samples were

taken to determine intracellular trehalose. The quantification of trehalose was performed using the anthrone–sulfuric acid method, and trehalose was used for the standard curve [44]. Each experimental group was performed in triplicate.

2.5. Determination of the Ratio of Reduced GSH (Glutathione) to Oxidized GSH (GSSG)

The determination of the GSH/GSSG ratio adopts the 'GSH and GSSG assay kit' (S0053, Beyotime Biotechnology, Shanghai, China), and the specific operation and precautions were carried out according to the instructions provided in the kit. The cells were cultured in YEPD medium at the initial OD_{600} of 0.2 and collected when the OD_{600} reached 4.0 and then washed with PBS buffer. The mixture of cells was added with three times the volume of deproteinization buffer M from the GSH and GSSG Assay Kit and then was alternately subjected to multi-gelation twice in liquid nitrogen and 37 °C water. After centrifugation (4 °C, 13,000 rpm, 15 min), the supernatant was collected for GSH and GSSG determination. Each experimental group was performed in triplicate.

2.6. Assays of β-Glucosidase and β-Xylosidase Activity

The activity of β-glucosidase or β-xylosidase was measured using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) or *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) as a substrate, respectively, as previously reported in the literature [12,21,45]. Briefly, 50 µL of 1.0 mg mL^{-1} *p*NPG or *p*NPX (dissolved in 50 mM sodium phosphate buffer, pH 4.8) and 100 µL of appropriately diluted crude enzyme were thoroughly mixed and incubated at 50 °C for 30 min. Then, the reaction system was stopped by adding 150 µL of 1 M Na_2CO_3, and the absorbance at 405 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol *p*-nitrophenol per minute under assay conditions.

Overnight cultures were transferred into fresh YEPD medium with an initial OD_{600} of 0.2 at 30 °C. To assay the extracellular activity, the culture broth was withdrawn and centrifuged at regular intervals, and the resulting supernatant was used to determine the enzyme activity. The protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China).

2.7. Oxygen-Limited Growth and Fermentation

The *S. cerevisiae* strains were aerobically precultured in a proper volume of YEPD medium at 30 °C overnight and then transferred into fresh YEPD medium at initial OD_{600} 0.2 for culture overnight. The resulting strains were withdrawn and inoculated into a fermentation medium in 120 mL serum bottles with a rubber stopper plug in a syringe needle (the oxygen-limited condition). The preparation of strain BSGIBX was carried out according to the previous operation [21]. The initial cell density was OD_{600} = 1 or OD_{600} = 10 according to specific experimental requirements. Samples were taken at regular intervals. All shake flask experiments were performed on an orbital shaker at 200 rpm.

2.8. Analysis of Hydrolysis and Fermentation Products

The glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined using high-performance liquid chromatography (HPLC) with Alliance Separations module e2695 (Waters, Milford, MA, USA). The compounds were separated on an Aminex HPX-87H ion exclusion column (300 × 7.8 mm, Bio-Rad, Richmond, CA, USA) at a column temperature of 45 °C as previously described [21]. The mobile phase was 5 mmol L^{-1} H_2SO_4 with a flow rate of 0.6 mL min^{-1} and subsequently detected with a Waters 2414 refractive index detector.

The concentrations of cellobiose, xylobiose, xylotriose, and xylotetraose were determined with the Dionex ICS-3000 ion chromatography system [46,47]. The compounds were separated on a Dionex™ CarboPac™ PA100 IC column with gradient elution. An operating temperature of 30 °C was used to ensure reproducible resolution and retention. The mobile phase was 100 mmol L^{-1} NaOH and 500 mmol L^{-1} CH_3COONa with a flow rate of 0.3 mL min^{-1} and subsequently detected with a Thermo Scientific ICS-3000 ED electrochemical detector.

3. Results

3.1. Evaluation of Industrial S. cerevisiae Strains as the Chassis Cell for the Synthetic Consortium

3.1.1. Collection of *S. cerevisiae* Strains

In this section, seven wild-type *S. cerevisiae* strains were collected and used from several strain preservation institutions, ethanol production companies, and commercial active dry yeasts. Laboratory strain CEN.PK102-5B and engineered diploid strain LF1 were used as controls. The detailed strain sources are listed in Table 1.

3.1.2. Intracellular Trehalose Content of *S. cerevisiae* Strains

Trehalose is a nonreducing disaccharide that accumulates in bacteria, algae, fungi, yeast, invertebrates, and some resurrection plants in response to drought, salinity, temperature variations, and heavy metal stress [48]. Trehalose contains two glucose units linked by α, α-1, and 1-glycosidic bonds [49]. It is vital for maintaining cell longevity, avoiding mitochondrial mutation, and improving ethanol production [50]. Moreover, trehalose synthesis and transport are crucial for adapting yeast cells to stress conditions [51]. In the yeast *S. cerevisiae*, trehalose is essential for survival after long-term desiccation; the elevation of intracellular trehalose in dividing yeast by its import from the media converts yeast from extreme desiccation sensitivity to a high level of desiccation tolerance [52].

The *S. cerevisiae* strain LF1 co-ferments glucose and xylose efficiently and synchronously [16]. Furthermore, it is considered one of the potential engineering yeast strains that can be used to produce second-generation bioethanol. Although the current recombinant *S. cerevisiae* strains have excellent fermentation capacities for xylose in a synthetic medium, they showed lower conversion efficiencies of lignocellulosic hydrolysates because of the effects of multiple inhibitors generated during the pretreatment process [5]. In addition, when LF1 was used to prepare active dry yeast, its survival rate was low (data not shown). The insufficient accumulation of trehalose in LF1 cells is the possible reason for the above phenomenon. Therefore, the intracellular trehalose content is the first indicator in our strain screening.

The intracellular trehalose contents of yeast strains are shown in Figure 1. LSF, NAN27, RC212, and SQ have relatively high trehalose content. Moreover, as we speculated, the trehalose content of strain LF1 is much lower than other strains.

3.1.3. Determination of the Ratio of the Two Forms of Glutathione (GSH:GSSG)

Glutathione (GSH, γ-glutamyl-cysteinyl-glycine), the primary intracellular thiol compound, is a ubiquitous tripeptide practically synthesized in all cells of living organisms, and it is the primary mechanism of antioxidant defense against reactive oxygen species (ROS) and electrophiles [53]. Intracellular glutathione (GSH) is essential in the adaptive response to heat-shock stress [54] and ethanol-induced oxidative stress [55].

GSH is a sensitive compound that can be oxidized to its disulfide analog (GSSG). The ratio of the two forms of glutathione (GSH:GSSG) determines the cell's redox state and has proven to be of particular importance for living organisms [53]. Thus, the ratio of GSH:GSSG may be used as a marker of oxidative stress [56] and could reflect the resistance or tolerance of yeast cells to a certain extent.

According to the results of intracellular trehalose content, the strains LSF, NAN27, RC212, and SQ were selected to determine the GSH/GSSG ratio and the laboratory strain CEN.PK102-5B was used as a control. The results are shown in Figure 2, strains SQ and RC212 have higher GSH/GSSG ratios than other strains.

3.1.4. Evaluation of Tolerance of *S. cerevisiae* Strains to Individual Stress Factors

In the production of second-generation fuel ethanol using LB as raw material, inhibitors toxic to microorganisms are formed with the release of sugars during the pretreatment and hydrolysis processes. Therefore, the yeast strain used in lignocellulosic bioethanol production requires high ethanol yields from all of the fermentable sugars and robustness in its harsh working environment [35].

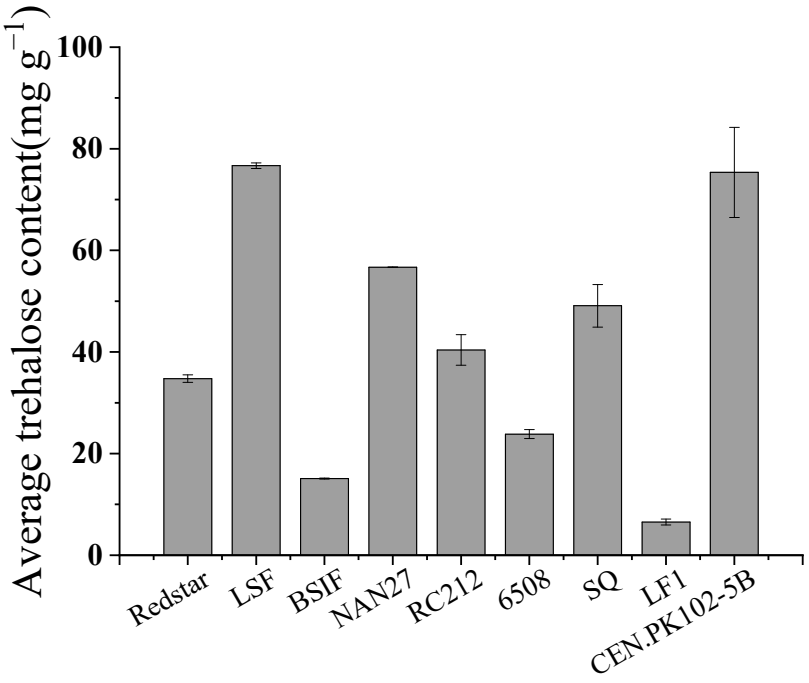

Figure 1. Intracellular trehalose content of *S. cerevisiae* strains. The quantification of trehalose was performed using the anthrone–sulfuric acid method, and each experimental group was performed in triplicate.

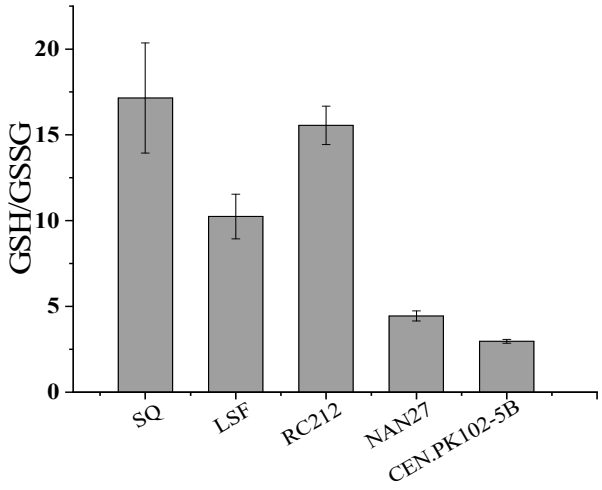

Figure 2. Glutathione content of *S. cerevisiae* strains (the ratio of cellular GSH to GSSG). The GSH and GSSG Assay Kit (Beyotime S0053, Haimen, China) was used for GSH and GSSG determination. Each experimental group was performed in triplicate.

Considering the stress conditions that the fermentation strain may encounter in the second-generation fuel ethanol production process, the growth performances of the five selected strains were tested under aerobic conditions with several stress factors [35], including high temperature, high osmotic pressure, and some typical inhibitors such as acetic acid, ethanol, furfural, and vanillin, etc.

As shown in Figure S3, the performances of the five strains were different. Strain RC212 grew better than other strains at a higher temperature, 41 °C. The hypertonic stress tolerance of SQ and RC212 was slightly higher than the tolerance of other strains on plates with 0.7 mol L^{-1} KCl. NAN27 and LSF were more sensitive to oxidative stress by 30% H_2O_2 than other strains. RC212 showed more furfural and vanillin tolerance but less acetic acid. Strain LSF and NAN27 showed better tolerance to acetic acid; On the plate

of ethanol stress, compared with other strains, strains SQ and RC212 had a better growth tendency. The tested strains showed distinct resistance to various inhibitory factors that may be encountered in the production of second-generation fuel ethanol, but no single strain showed the highest tolerance to all of the different inhibitors.

The evaluation of the five strains is summarized in Table 2. Based on the comprehensive screening results, we chose the strain RC212, which exhibited better performance in multiple detection items, as the chassis cell for subsequent experiments.

Table 2. Summary of the performance evaluation of different *S. cerevisiae* strains.

		LSF	SQ	RC212	NAN27	CEN.PK102-5B
1	Trehalose content	+++	++	++	++	+++
2	GSH/GSSG	++	+++	+++	+	+
3	High temperature	/	/	+++	++	+
4	Hyper-osmotic stress	+	++	++	+	+++
5	Oxidative stress	+	++	++	+	+++
6	High concentration of ethanol	+	++	+++	+++	++
7	Furfural stress	++	++	++	+++	+

The greater the number of '+' symbols, the better the performance or resistance of the strain.

3.2. Construction of BLN26 with β-Glucosidase and β-Xylosidase Expression Activity

Integrating the gene of interest into the *S. cerevisiae* genome allows for its stable presence. The δ-sequence is the long terminal repeat sequence in the yeast retrotransposon *Ty1*. About 400 copies of δ-sequences are thought to be scattered throughout the chromosomes of *S. cerevisiae*, with slightly different DNA sequences [57]. Given that the multiple repeats of the δ-sequence in the chromosome could lead to multiple integrated copies of the β-glucosidase (BGL) gene in the genome [57], a pair of δ-targeted recombinant arms, δ1/δ2, and β-glucosidase expression cassette were introduced into pUC-N360F, resulting in pUCδBK (Figure S1). Additionally, the β-xylosidase expression cassette was introduced to pXIδ containing δ-targeted recombinant arms, δ1′/δ2′, resulting in pIBXδ (Figure S2).

The linearized fragment δ1-BGL-loxP-KanMX-loxP-δ2, derived from plasmid vector pUCδBK (linearized with SwaI), was verified and transformed into RC212 using the standard lithium acetate method [21] on the δ-sequence site. The resulting recombinant *S. cerevisiae strain* was named RBK, as shown in Table 1. The G418 resistance marker of recombinant strain RBK was removed before integrating the allogenic β-xylosidase expression cassette IBXBOX, and the resulting strain was RB. Two fragments, upADH-IBXBOX-loxP-KanMX-loxP-adh and upADH-IBXBOX-loxP-KanMX-loxP-in-adh, were verified and transformed into RB using the standard lithium acetate method on the *ADH2* site. Relative positions of homologous arms adh, upADH, and in-adh on the *ADH2* loci on the two chromosomes are shown in Figure S4. The resulting recombinant *S. cerevisiae strain* was named RBKX2, and then RBX2 was obtained by removing the G418 resistance marker. Then, the linearized fragment δ1′-KanMX-IBX-δ2′ was obtained from pIBXδ via linearization with the restriction endonucleases EcoRI/SphI and was then transformed into RBX2 to get BLN26.

The extracellular activities of β-glucosidase and β-xylosidase used *p*NPG and *p*NPX as a substrate, respectively. As shown in Figure 3, during the cultivation of the engineering strain BLN26, the extracellular activity of the two enzymes demonstrated an increasing trend. By 48 h of cultivation, the extracellular specific activities of β-glucosidase and β-xylosidase were 0.255 U mg^{-1} protein (2.18 U mL^{-1}) and 0.068 U mg^{-1} protein (0.56 U mL^{-1}), respectively. The activities were lower than those of engineered *S. cerevisiae* we previously reported based on laboratory strains. Tang et al. reported a *S. cerevisiae* haploid strain 102SB with high β-glucosidase secretion activity of 5.2 U mL^{-1}, 1.39 times higher than that of BLN26 [12]. Niu et al. reported *S. cerevisiae* haploid strain BSGIBX that showed β-xylosidase specific activity of 6 U mg^{-1} protein at 30 h and was 87.2 times higher

than BLN26 [21]. Claes et al. successfully expressed seven secreted lignocellulolytic enzymes in a single second-generation industrial *S. cerevisiae* strain MD4 using CRISPR/Cas9 methodology. The β-glucosidase activity of the recombinant strain AC1 was 19.1 U g^{-1} CDW (approximately 0.525 U mg^{-1} protein), and the β-xylosidase activity of AC7 was approximately 0.341 U mg^{-1} protein [58].

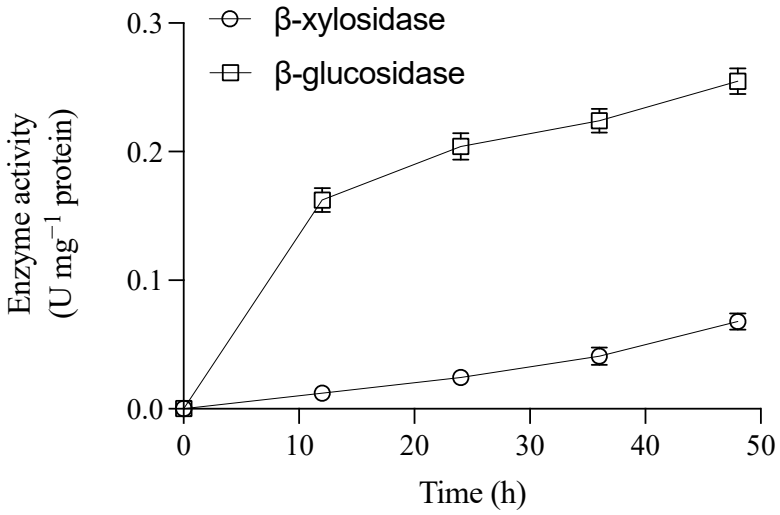

Figure 3. Specific activities of β-glucosidase and β-xylosidase of recombinant *S. cerevisiae* strain BLN26. Overnight cultures were transferred into fresh YEPD medium with an initial OD_{600} of 0.2 at 30 °C. Then, the culture broth was withdrawn and centrifuged at regular intervals, and the resulting supernatant was used to determine the enzyme activity. The protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Each experimental group was performed in triplicate. The symbols: □, β-glucosidase; ○, β-xylosidase.

3.3. Oxygen-Limited Fermentation of BLN26 and a Binary Synthetic Consortium

3.3.1. Fermentation of BLN26 with the Mixture of Glucose–Cellobiose or Glucose–XOS

The recombinant strain BLN26 was cultured with an initial cell density of OD_{600} = 1 in a YP medium with a mixture of glucose–cellobiose (YPGC, ~20 g L^{-1} glucose, and ~22 g L^{-1} cellobiose) or glucose–XOS (YPGO, ~20 g L^{-1} glucose and ~20 g L^{-1} XOS) as the carbon source. As shown in Figure 4, BLN26 can consume all the glucose in about 8 h. Almost all the cellobiose was fermented to ethanol in 48 h; the ethanol yield was 0.442 g g^{-1} based on the consumed sugar (glucose and cellobiose), as shown in Figure 4a. These results indicated that the engineered strain BLN26 maintained its excellent glucose metabolism ability and was endowed with better cellobiose fermentation ability. In fermentation with YPGO, as shown in Figure 4b, the initial concentrations of xylobiose, xylotriose, and xylotetraose are approximately 5.84 g L^{-1}, 4.91 g L^{-1}, and 2.58 g L^{-1}. Under the catalysis of β-xylosidase secreted by BLN26, a small amount of XOS was degraded, corresponding to about 0.61 g L^{-1} of xylobiose, 1.20 g L^{-1} of xylotriose and 0.51 g L^{-1} of xylotetraose. Since the natural *S. cerevisiae* RC212 and its successor BLN26 could not metabolize xylose, the above-mentioned degraded XOS eventually led to about 3 g L^{-1} xylose accumulation in the culture medium.

3.3.2. Fermentation of Binary Synthetic Consortium with the Mixture of Glucose–Xylose–Cellobiose

Since BLN26 cannot metabolize xylose, we used a binary synthetic consortium consisting of two kinds of *S. cerevisiae* (LF1 + BLN26) to explore the ability of this co-culture system to metabolize three sugars, that is, glucose, xylose, and cellulose, which are commonly found in LB hydrolysate. As shown in Figure 5a, when the inoculation ratio of LF1 and BLN26 was 5:5, and the total initial inoculum amount was the same as above (OD_{600} = 1), the metabolic capacity of cellobiose was significantly reduced, after 48 h of fermentation,

67.9% of cellobiose was consumed and 6.8 g L^{-1} of which remained. Due to the excellent co-fermentation ability of glucose and xylose in LF1, the glucose in the co-culture system was still exhausted within 8 h. The entire ~22 g L^{-1} of xylose was consumed in about 16 h.

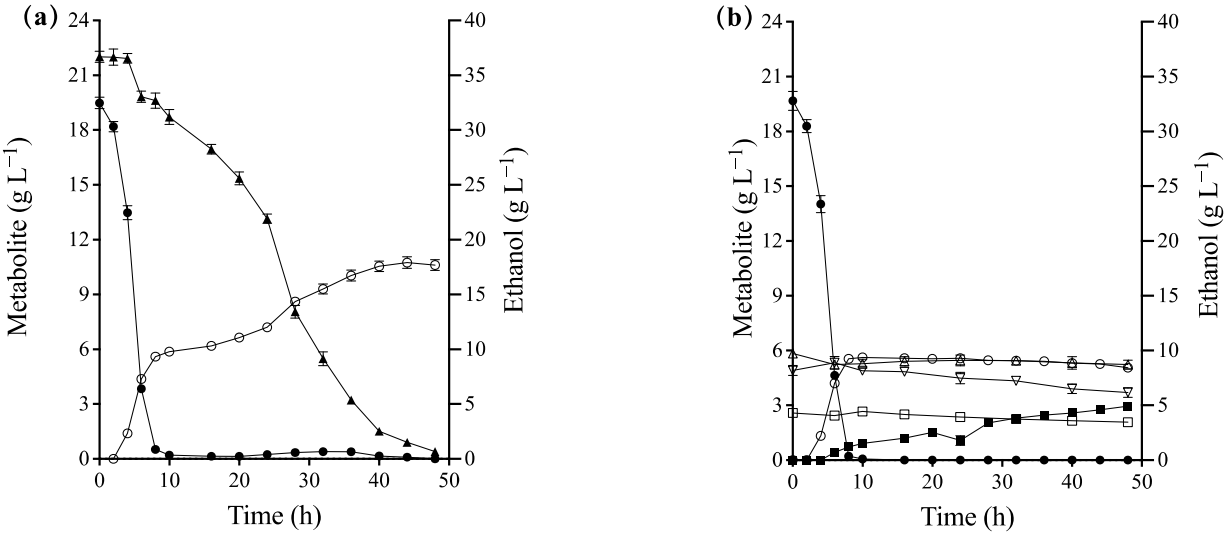

Figure 4. Fermentation characteristics of BLN26 with the mixture of glucose–cellobiose (YPGC, **a**) or glucose–XOS (YPGO, **b**). The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The initial cell density was OD_{600} = 1, and the oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: ●, glucose; ■, xylose; ▲, cellobiose; △, xylobiose; ▽, xylotriose; □, xylotetraose; ○, ethanol.

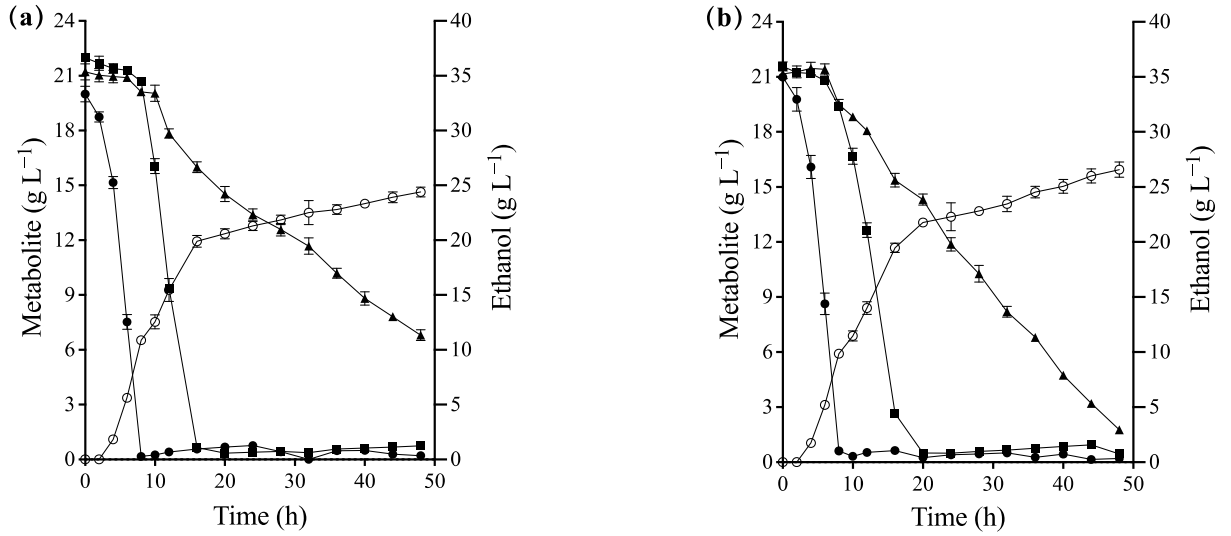

Figure 5. Co-culture of two *S. cerevisiae* strains in a ratio of 5:5 or 3:7 in the media of YPGXC. (**a**) LF1:BLN26 = 5:5 (**b**) LF1:BLN26 = 3:7. The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The total initial cell density was OD_{600} = 1, and the oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: ●, glucose; ■, xylose; ▲, cellobiose; ○, ethanol.

As the initial inoculum of strain BLN26 was reduced compared with that of Figure 4a, the cellobiose fermentation ability dropped sharply, so we adjusted the inoculum ratio of LF1 and BLN26 to 3:7; the results are shown in Figure 5b, as expected, after 48 h of fermentation, 91.7% of cellobiose was metabolized (increased by 34.9% compared with that

of Figure 5a). At the same time, due to the reduction of LF1 inoculum, the utilization of xylose was slightly slowed down, and all xylose was consumed within 20 h of fermentation.

3.3.3. Fermentation of Binary Synthetic Consortium with the Mixture of Glucose–Xylose–Cellobiose–XOS

As previously mentioned, the engineered strain BLN26 could metabolize cellobiose to produce ethanol, and its secreted β-xylosidase can also degrade a certain amount of XOS to produce xylose. Furthermore, the co-culture system of LF1 and BLN26 was used to achieve the co-fermentation of three sugars in hydrolysates (glucose, xylose, and cellobiose; Figure 5). Therefore, we conducted further experiments to investigate whether this binary system composed of LF1 and BLN26 could achieve the co-fermentation of four sugars. For this purpose, YPGXCO (glucose, xylose, cellobiose, and XOS) was selected as the fermentation medium. As shown in Figure 6a, cellobiose utilization was further reduced compared with Figure 5a. After 48 h of fermentation, only 44.8% of cellobiose was utilized (34.0% lower than that in Figure 5a), and 11.65 g L^{-1} of cellobiose remained in the medium. This result indicates that the presence of XOS harms cellobiose degradation. Although increasing the initial inoculation amount of BLN26, as shown in Figure 6b, the utilization rate of cellobiose increased to 67.1%, close to the level of Figure 5a, there was still about 7 g L^{-1} of cellobiose remaining. In the binary system of LF1 and BLN26, a small amount of XOS was still utilized. In Figure 6a, 6.30% of xylotriose, 20.86% of xylotriose, and 14.20% of xylotetraose were consumed after 48 h of fermentation, respectively, decreasing by 39.73%, 14.97%, and 27.51% compared with Figure 4b. With the increase in the initial inoculation amount of BLN26 in Figure 6b, the utilization ability of XOS recovered to the level shown in Figure 4b.

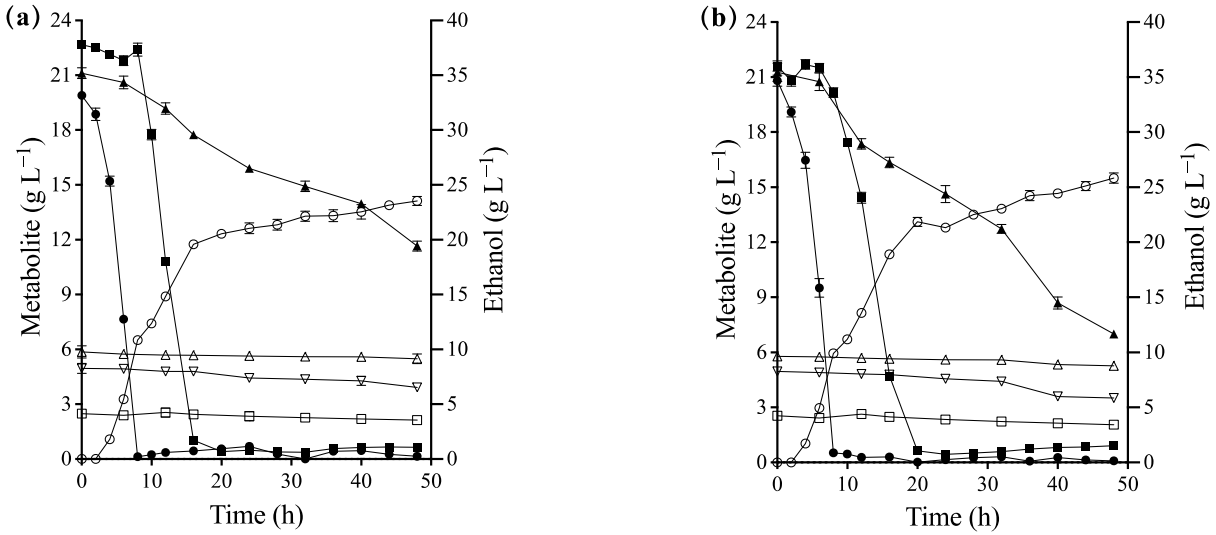

Figure 6. Co-culture of two *S. cerevisiae* strains in a ratio of 5:5 or 3:7 in the media of YPGXCO. (**a**) LF1:BLN26 = 5:5 (**b**) LF1:BLN26 = 3:7. The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The total initial cell density was OD_{600} = 1, and the oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: ●, glucose; ■, xylose; ▲, cellobiose; △, xylobiose; ▽, xylotriose; □, xylotetraose; ○, ethanol.

3.3.4. Fermentation of Binary Synthetic Consortium with the Mixture of Glucose–Xylose-Cellobiose–XOS_{pre}

To take full advantage of the components in XOS, xylanase (with the activity of approximately 170 U mg^{-1} protein, kindly provided by Qingdao Vland Biotech Inc., Qingdao, China) was added to pretreat XOS for 18 h at 50 °C with an enzyme dosage of 3 mg g^{-1} XOS [21]. In the 20 g L^{-1} XOS system, after pretreatment, the concentrations of xylobiose and xylose increased from ~5.8 and 0.07 g L^{-1} to approximately 15.7 and 2.3 g L^{-1}, respectively.

The concentrations of xylotriose and xylotetraose decreased from ~5.0 and ~2.5 g L^{-1} to 1.1 and 1.0 g L^{-1}, respectively. After pretreatment, almost all of the XOS is presented as xylose and xylobiose.

As shown in Figure 7a, the fermentation medium contained glucose, xylose, cellobiose, and pre-hydrolyzed XOS. Under the co-culture of LF1 and BLN26, the metabolic curves of glucose and xylose were similar to those in Figure 6b, except that the initial concentration of xylose increased by about 2.3 g L^{-1}. At the end of fermentation, 4.18 g L^{-1} of xylobiose (accounting for 27.0% of the total xylobiose) was consumed, 8.1 times that of Figure 6b. In addition, 16.32 g L^{-1} of cellobiose was metabolized, an increase of 2.06 g L^{-1} compared to Figure 6b.

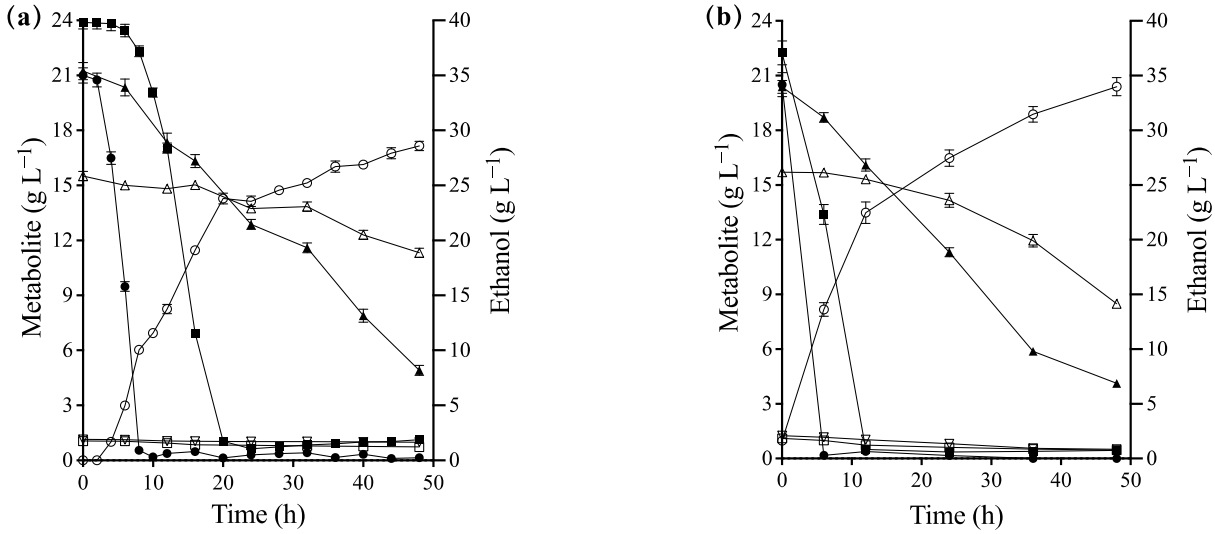

Figure 7. Co-culture of two *S. cerevisiae* strains LF1 and BLN26 in a ratio of 3:7 in the media of $YPGXCO_{pre}$. The total initial cell density was OD_{600} = 1 (**a**) and OD_{600} = 10 (**b**). XOS was pretreated with xylanase. The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: ●, glucose; ■, xylose; ▲, cellobiose; △, xylobiose; ▽, xylotriose; □, xylotetraose; ○, ethanol.

Although some improvement was achieved in the fermentation results after the XOS pretreatment was applied, we still need to meet our expectation: to achieve the co-metabolism of the four sugars by co-culturing different yeasts. As shown in Figure 7a, after 48 h of fermentation, there were still about 11.32 g L^{-1} of xylobiose and 4.91 g L^{-1} of cellobiose remaining. Further, we tried to solve this problem by increasing the inoculation amount. As shown in Figure 7b, the initial total inoculation amount of the binary co-culture system of LF1 and BLN26 was increased to OD_{600} = 10, which was ten times that of before. The fermentation rates of glucose and xylose were significantly increased (glucose and xylose were entirely consumed after 6 and 12 h of fermentation, respectively). However, disappointingly, the fermentation rates of xylobiose and cellobiose did not increase accordingly, and there were still 8.49 g L^{-1} of xylobiose and 4.12 g L^{-1} of cellobiose that needed to be utilized.

3.4. Oxygen-Limited Fermentation of a Ternary Synthetic Consortium with the Mixture of Glucose–Xylose–Cellobiose–XOS_{pre}

Although the strain BLN26 could consume all the cellobiose and produce corresponding ethanol within 48 h when fermented alone (Figure 4a), cellobiose cannot be completely decomposed within 48 h when xylose is present in the culture medium (Figure 5), especially when XOS is present (Figure 6). Even if XOS was pretreated using enzymatic hydrolysis (Figure 7a) and the initial inoculation amount of the binary consortium (composed of LF1 and BLN26) is increased (Figure 7b), cellobiose cannot be completely metabolized. In

addition, the fermentation of xylobiose also has similar problems, and its metabolic performance is even worse than that of cellobiose. We speculate that the possible reason is that BLN26 expresses and secretes β-glucosidase and β-xylosidase simultaneously, resulting in its metabolic burden. Therefore, to achieve the co-fermentation of four sugars in LB hydrolysate, we consider choosing two other engineering strains of *S. cerevisiae*.

S. cerevisiae strain BSGIBX, constructed in our previous work [21], in which the xylose isomerase and β-xylosidase from *P. oxalicum* with selected signal peptides were successfully co-expressed. BSGIBX possesses better xylose metabolism, and the β-xylosidase secreted could rapidly convert xylobiose and xylotriose to xylose, which would then be metabolized to ethanol. *S. cerevisiae* strain 102SB [12] has an effect secretion of β-glucosidase and can improve the efficiency of cellulase hydrolysis and ethanol production in simultaneous saccharification and fermentation. The ternary system composed of LF1, BSGIBX, and 102SB was used in the following experiments.

As shown in Figure 8a, when the initial inoculation ratio of LF1: BSGIBX:102SB was 1:1:1 and the total inoculation amount was OD_{600} = 1, most glucose and xylose were consumed after 16 and 32 h of fermentation, respectively. After 48 h of fermentation, 12.53 and 10.1 g L^{-1} of xylobiose and cellobiose were consumed, 80.57% and 48.77% of the initial concentration, respectively. When the inoculation ratio of the three yeasts was adjusted to 1:2:2, as shown in Figure 8b, the metabolic rate of glucose and xylose decreased slightly. However, the fermentation performance of xylobiose and cellobiose improved significantly. After 48 h of fermentation, 15.36 and 17.35 g L^{-1} of xylobiose and cellobiose were metabolized, respectively, 98.23% and 83.51% of the initial concentration.

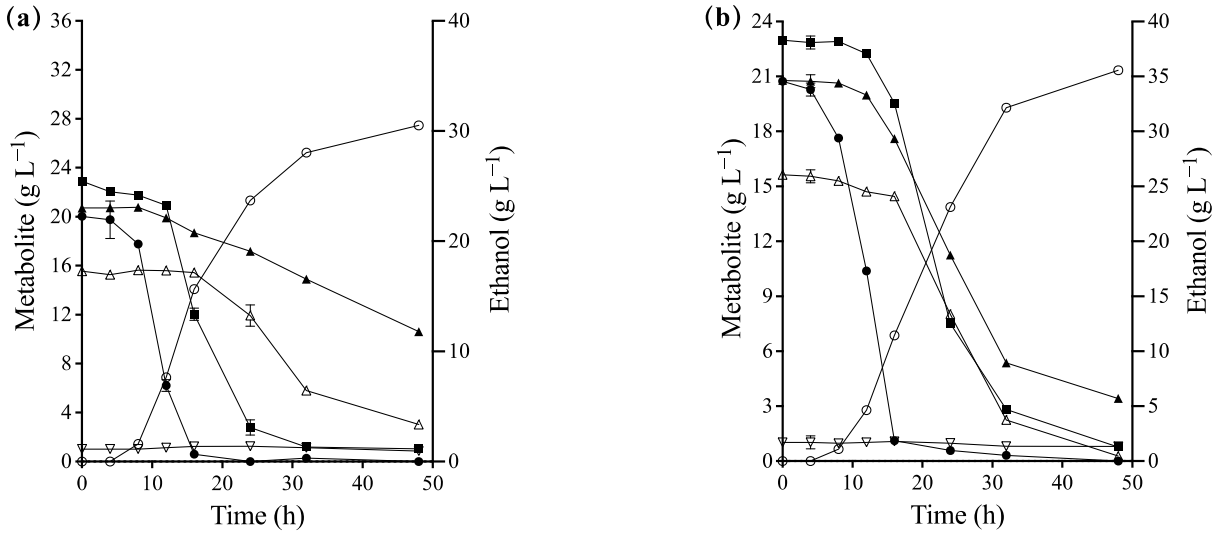

Figure 8. Co-culture of three *S. cerevisiae* strains in a ratio of 1:1:1 or 1:2:2 in the media of $YPGXCO_{pre}$. (**a**) LF1:BSGIBX:102SB = 1:1:1 (**b**) LF1:BSGIBX:102SB = 1:2:2. The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The total initial cell density was OD_{600} = 1, and the oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: •, glucose; ■, xylose; ▲, cellobiose; △, xylobiose; ▽, xylotriose; □, xylotetraose; ○, ethanol.

We then increased the total initial inoculum to OD_{600} = 3.5 (approximately 0.65 g DCW L^{-1}) as shown in Figure 9, all four sugars (glucose, xylose, xylobiose, and cellobiose) can be consumed in 24 h, the concentration of ethanol reached to 39.85 g L^{-1}, and the ethanol yield based on the consumed sugar was 0.482 g g^{-1} based on the consumed sugar. We also noticed that the ternary system metabolized 92.43% of glucose, 90.77% of xylose, and 91.92% of xylobiose at 6, 11, and 18 h of fermentation, respectively. In addition, 75.34% of cellobiose was consumed in 18 h. In the ternary co-culture system, the utilization rate of xylobiose and cellobiose is lower than that of glucose and xylose. Claes et al. reported a recombinant

S. cerevisiae strain AC14 using industrial Ethanol Red^{TM} as chassis, capable of secreting seven different lignocellulosic enzymes and fermenting xylose and glucose [58]. Then, the consolidated bioprocessing profile of the AC14 strain was investigated in synthetic media composed of approximately 16 g L^{-1} xylose, 11 g L^{-1} glucose, 12 g L^{-1} cellobiose, 6 g L^{-1} corncob xylan, 20 g L^{-1} peptone, and 10 g L^{-1} yeast extract [27]. Glucose, xylose, and cellobiose were consumed within 6 h, while xylan hydrolysis was slightly slower; all potential sugars were consumed after 30 h. Considering a process duration of 6 h, where the majority of the potential sugars had been released and fermented (92%), ethanol titer, productivity, and yield obtained were 26.8 g L^{-1}, 4.46 g L^{-1} h^{-1}, and 0.5 g g^{-1}, respectively. However, for yeast cells used in the fermentation run under high cell load, equivalent to 80 g DCW L^{-1} (OD_{600} = 100), which was 122 times higher than that of Figure 9. Therefore, when considering the sugar composition in the actual LB hydrolysate and moderately increasing the cell load, we believe that our ternary synthetic consortium has the potential to realize the simultaneous fermentation of multiple sugars. In conclusion, within a short fermentation time of 24 h, the ternary synthetic consortium we developed substantially achieved the complete fermentation of the four sugars and produced ethanol with high efficiency. To our knowledge, this study represents the first exploration of the conversion of mixtures of glucose, xylose, cellobiose, and xylo-oligosaccharides using a synthetic consortium of recombinant *S. cerevisiae* strains. This synthetic consortium and subsequent improved ones have the potential to be used as microbial platforms to produce a wide array of biochemicals from LB-hydrolysate.

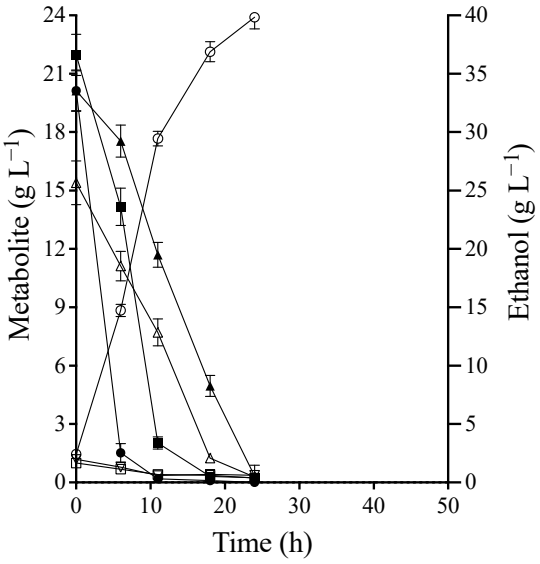

Figure 9. Co-culture of three *S. cerevisiae* strains in a ratio of LF1:BSGIBX:102SB = 1:2:2 in the media of $YPGXCO_{pre}$. The strains were cultured at 30 °C with agitation at 200 rpm in 120 mL serum bottles with a working volume of 40 mL. The total initial cell density was OD_{600} = 3.5, and the oxygen-limited condition was provided by a rubber stopper plug in a syringe needle. Each experimental group was performed in triplicate. Symbols: ●, glucose; ■, xylose; ▲, cellobiose; △, xylobiose; ▽, xylotriose; □, xylotetraose; ○, ethanol.

4. Discussion

Active dried yeast (ADY) preparation is usually applied in first- and second-generation bioethanol production [59]. Traditionally, yeast cells are cultured and propagated until the cell number desired for ethanol fermentation is achieved. This process begins with slant cultivation followed by gradual expansion of the cultivation scale in a liquid medium to increase the volume. As an alternative, many factories use commercial ADY to initiate ethanol fermentation, which can cut down the initial lag time for fermentation and reduce yeast culture plant numbers and the risk of bacterial contamination [60]. Increased accumulation of intracellular trehalose contributes to the higher cell viability of ADY after dehydration

and rehydration [60]. As is shown in Figure 1, LF1 has a minor intracellular trehalose content, which explains its low ADY viability. To make up for the above deficiencies, the intracellular trehalose content was used as the primary screening index in the present work. It should be noted that LF1 is still a member of the binary or ternary microbial consortium used in this work, which means that we still need to take measures to increase the trehalose content of LF1 to improve its ADY viability. The protoplast fusion method or rational metabolic engineering of trehalose synthesis pathways is currently conducted to address this issue.

As mentioned above, LB materials must be pretreated before enzymic hydrolysis for their recalcitrant nature. However, compounds inhibitory for the enzymes or fermenting organism are released during this process. Through HPLC and mass-spectroscopy analyses, Kont et al. reported that the strong cellulase inhibitors existing in the hydrolysate mainly consisted of gluco-oligosaccharides (GOS) and xylo-oligosaccharides (XOS) [61]. In addition, some GOS and XOS are also produced during enzymatic hydrolysis of pretreated lignocelluloses because the cellulose and hemicellulose can be considerably reserved in the pretreated lignocelluloses material after pretreatments under neutral and alkali conditions [14]. Cellobiose (the predominant hydrolysis product of GOS) is primarily responsible for the inhibition of GOS on cellulase, and this inhibitory effect could be reduced by the supplementation of β-glucosidase (BGL) in vitro [62]. In addition, certain hemicellulose-derived sugars, including sparingly soluble xylan and soluble XOS, were widely reported to be strong inhibitors of cellulase and negatively influenced enzyme hydrolysis [61,63]. Qing et al. reported that XOS had a greater impact on enzymatic hydrolysis than xylose or xylan [64], and the supplementation of xylanase and β-xylosidase could reduce the inhibition of xylo-oligomer and xylan to enzymatic hydrolysis of cellulose and pretreated corn stover [65]. As shown in Figure 6a, the presence of XOS has a negative effect on cellobiose degradation. This result is consistent with the above conclusion that XOS inhibits cellulase activity. Accompanied by the hydrolysis of XOS by supplementation of β-xylosidase (Figure 7a vs. Figure 6b) and its faster consumption rate by β-xylosidase secreted by BSGIBX (Figure 8b vs. Figure 7a), its inhibition of cellulase is further released, resulting in a gradual increase in the rate of cellobiose utilization.

Although high expression of the target gene can be obtained using the 2 μ plasmids as an expression vector, maintaining the selection pressure obviously increases the cost of culturing, limiting its application in industrial production [57]. Integrating a target gene into the genome allows for the stable existence of the gene in *S. cerevisiae*. In the present work, the expression cassettes of β-glucosidase and β-xylosidase were introduced into the δ-sequence site of the RC212 genome through fragments derived from plasmid pUCδBK and pIBXδ, respectively. The subsequent degradation of cellobiose and xylobiose demonstrated that the heterologous β-glucosidase and β-xylosidase were successfully expressed and secreted in the resulting strain BLN26. However, the degradative ability of BLN26 to cellobiose, especially XOS, was much lower than expected. Low copy numbers of integrated heterologous genes should take the main responsibility. There were eight copies of the β-glucosidase gene and five copies of the β-xylosidase gene in the genome of AC1 and AC7, respectively [58]. Additionally, this could be used to explain that the corresponding enzyme activity was higher than that of BLN26. Zheng et al. developed a simple and high-efficiency strategy for *rDNA*-mediated multicopy gene integration based on the dynamic balance of *rDNA* in *S. cerevisiae* and integrated 18.0 copies of the xylose isomerase gene into the *S. cerevisiae* genome in a single-step [57]. Shi et al. developed a Di-CRISPR platform, which uses CRISPR-Cas to generate double-strand breaks (DSBs) in the delta sites of *S. cerevisiae* chromosome, to integrate up to 18 copies of 24 kb combined genes in the δ sites in a single-step [66]. Wang et al. developed a CRISPR–Cas9-assisted multiplex genome editing (CMGE) approach; ~10 copies of the GFP gene were integrated into the *rDNA* region [67]. This novel multicopy genome integration strategy provides a convenient and efficient tool for further metabolic engineering of *S. cerevisiae* and should be used in our future work to improve the comprehensive capacity of the synthetic *S. cerevisiae* consortium.

S. cerevisiae is a robust cell factory to secrete or surface-display cellulase and amylase to convert agricultural residues into valuable chemicals. Engineering the secretory pathway is a well-known strategy for overproducing these enzymes. Chen et al. systematically studied the effect of engineering cell wall biosynthesis on the activity of cellulolytic enzyme β-glucosidase (BGL1) by comparing seventy-nine gene knockout *S. cerevisiae* strains and newly identified that inactivation of *DFG5*, *YPK1*, *FYV5*, *CCW12*, and *KRE1* improved BGL1 secretion and surface-display. Combinatorial modifications of these genes, particularly double deletion of *FVY5* and *CCW12*, along with the use of a rich medium, increased the activity of secreted and surface-displayed BGL1 by 6.13-fold and 7.99-fold, respectively [68]. This strategy could be applied to improve the activities of β-glucosidase and β-xylosidase in BLN26 for efficient degradation of cellobiose and XOS.

Haploid *S. cerevisiae* strains are unattractive for industrial applications for their lower genetic stability and robustness [69]. The diploid or polyploid industrial *S. cerevisiae* strains are usually more robust than the haploid strains, and the whole-genome duplication in yeast was proposed to lead to an efficient fermentation system [35,70]. Xie et al. demonstrated a CRISPR/Cas9-mediated method to enable rapid and highly efficient mating-type switching in *S. cerevisiae* [71]. This method is generally helpful in building polyploids of a defined genotype and expedites strain construction, for example, in constructing fully a/a/α/α isogenic tetraploids. In addition, Peris et al. developed an iterative method of Hybrid Production (iHyPr) to combine the genomes of multiple budding yeast species, generating *Saccharomyces* allopolyploids of at least six species, which have potential applications for the study of polyploidy, genome stability, chromosome segregation, and bioenergy [72]. Since the synthetic *S. cerevisiae* consortium used in this work contained two haploid yeast strains, it is essential to obtain the corresponding diploid or polyploid progeny to efficiently consume all the LB-derived sugars in the presence of various inhibitory factors. To this end, traditional protoplast fusion [73], iHyPr, or the CRISPR/Cas9-mediated method could be used in our future work.

The cost-effective bioconversion of lignocellulose via monoculture remains a challenge. The application of artificial microbial consortia in biotechnological production processes offers great potential for the improvement of established as well as the development of novel processes [74]. Several review articles are available on microbial consortia for LB bioconversion [75]. In which the application of these systems in the detoxification of LB hydrolysate, cellulase production, co-utilization of lignocellulosic sugars, valorization of lignin, and production of value-added products were presented in detail [33,74–76]. The uncontrollability of natural consortia limits their wide application in producing specific biochemicals at high titers. To this end, synthetic microbial consortia have made significant progress in their relatively simple interplays [77]. The simplest design is to build a synthetic community with strains from the same species, which avoids growth incompatibility issues. In the present work, two kinds of synthetic *S. cerevisiae* consortium (a binary consortium consisting of BLN26 and LF1; a ternary consortium consisting of LF1, 102SB, and BSGIBX) were built, and their fermentation performances on the mixture of four LB-derived sugars (glucose–xylose–cellobiose–XOS) were analyzed.

It is still challenging to achieve effective co-utilization of mixed sugars in LB hydrolysate, and carbon catabolite repression (CCR) is considered a bottleneck problem in the field of lignocellulosic biorefineries [75]. By constructing parallel utilization pathways of pentose and hexose in different microorganisms of consortia, the coculture systems can simultaneously utilize LB-derived mixed sugars based on a dedicated metabolic pathway. Both *E. coli* and *S. cerevisiae* have been engineered to be this kind of 'specialist' strain [26,78–80]. However, the *S. cerevisiae* strains used in this work were not 'specialist' strains, glucose metabolism was preserved in all the strains. For example, LF1 and BSGIBX can utilize glucose and xylose efficiently and synchronously for their multiple modifications in xylose metabolism [16,21], and this largely alleviated the severe inhibition of xylose metabolism by glucose. As shown in Figure 9, all four LB-derived sugars were consumed in 24 h by our ternary consortium. To the

best of our knowledge, this is the first work aiming at co-fermenting four LB-derived sugars by synthetic *S. cerevisiae* consortia.

Meanwhile, the construction of 'specialist' strains is also on our agenda for convenient, dynamic modeling and to enable faster and more accurate responses to environmental fluctuations, such as the variation in the type and concentration of sugars derived from different LB materials [30]. In addition, LB-hydrolysates also contain substantial amounts of L-arabinose (usually 2–3%, although some hydrolysates contain up to 20% L-arabinose) [26], and based on this, the construction of yeast strains that rapidly metabolize L-arabinose is in progress. Together with detoxified microorganisms (such as *F. striatum* UdL-TA-3.335 and *Amorphotheca resinae* ZN1 [81]), we hope to obtain a five-component microbial consortium, which is capable of rapidly fermenting all sugars from non-detoxified LB hydrolysate, to serve as a microbial platform to produce a wide array of biochemicals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9080775/s1, Table S1: Primers used in this work; Figure S1: The construction schematic diagram of recombinant plasmid pUCδBK; Figure S2: The construction schematic diagram of recombinant plasmid pIBXδ; Figure S3: Growth of *S. cerevisiae* strains in the solid medium under different stress conditions; Figure S4: Relative positions of homologous arms adh, upADH and in-adh on the *ADH2* loci on the two chromosomes.

Author Contributions: Conceptualization, H.L. and X.B.; methodology, H.L. and J.Z.; validation, N.Y., T.L., M.Y. and L.W.; formal analysis, N.Y., T.L. and Y.N.; investigation, N.Y., T.L., Y.N., L.W. and S.Y.; resources, Z.L.; data curation, N.Y., T.L., Y.N. and H.L.; writing, original draft preparation, N.Y., T.L. and H.L.; writing, review and editing, H.L., J.Z. and X.B.; visualization, H.L. and X.B.; supervision, H.L.; project administration, H.L.; funding acquisition, H.L. and X.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Project of China (2018YFB1501401), Key innovation Project of Qilu University of Technology (Shandong Academy of Sciences) (2022JBZ01-06), the Shandong Provincial Technical Innovation Boot Program (02055183), the Shandong Provincial Natural Science Foundation (ZR2020MC016), the Foundation (ZZ20190303, ZZ20190313) of State Key Laboratory of Biobased Material and Green Papermaking of Qilu University of Technology (Shandong Academy of Sciences), the Foundation (No. 202005) of Qilu University of Technology of Cultivating Subject for Biology and Biochemistry, the National Natural Science Foundation of China (31870063), the Key R&D Project of Shandong Province (2019JZZY010906).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest with the publication of this paper.

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