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High-Titer Bioethanol Production from Steam-Exploded Corn Stover Using an Engineering *Saccharomyces cerevisiae* Strain with High Inhibitor Tolerance

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Abstract: Bioethanol is an important biofuel which can be produced from the abundant low-value lignocelluloses. However, the highly toxic inhibitory compounds formed in the hydrolysate and the ineffective utilization of xylose as a co-substrate are the primarily bottlenecks that hinder the commercialization of lignocellulosic bioethanol. In this study, aiming to properly solve the above obstacles, an engineered *Saccharomyces cerevisiae* strain was constructed by introducing the xylose reductase (XR)–xylitol dehydrogenase (XDH) pathway, overexpressing the non-oxidized pentose phosphate pathway, and deleting aldose reductase *GRE3* and alkaline phosphatase *PHO13* using a GTR-CRISPR system, followed by adaptive laboratory evolution (ALE). After screening, the isolated *S. cerevisiae* YL13-2 mutant was capable of robust xylose-utilizing, and exhibited high tolerance to the inhibitors in undetoxified steam-exploded corn stover hydrolysate (SECSH). An ethanol concentration of 22.96 g/L with a yield of 0.454 g/g can be obtained at the end of batch fermentation when using SECSH as substrate without nutrient supplementation. Moreover, aiming to simplify the downstream process and reduce the energy required in bioethanol production, fermentation using fed-batch hydrolyzed SECSH containing higher titer sugars with a YL13-2 strain was also investigated. As expect, a higher concentration of ethanol (51.12 g/L) was received, with an average productivity and yield of 0.71 g/L h and 0.436 g/g, respectively. The findings of this research provide an effective method for the production of bioethanol from lignocellulose, and could be used in large-scale applications in future works.

Keywords: *Saccharomyces cerevisiae*; bioethanol; steam explosion; metabolic engineering; evolution engineering

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Citation: Wu, Y.; Su, C.; Zhang, G.; Liao, Z.; Wen, J.; Wang, Y.; Jiang, Y.; Zhang, C.; Cai, D. High-Titer Bioethanol Production from Steam-Exploded Corn Stover Using an Engineering *Saccharomyces cerevisiae* Strain with High Inhibitor Tolerance. *Fermentation* **2023**, *9*, 906. https://doi.org/10.3390/fermentation9100906

Academic Editor: Kenji Sakai

Received: 14 September 2023
Revised: 7 October 2023
Accepted: 11 October 2023
Published: 13 October 2023

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1. Introduction

Second-generation (2G) bioethanol that is produced from lignocellulosic biomass materials has long been considered a primary renewable biofuel in responding to the severe environmental consequences caused by unsustainable fossil-based fuels [1–3]. However, at the current stage, the commercialization of 2G bioethanol is still challenging due to a series of technical barriers, including difficulties in monomeric sugar generation, the toxic effect of inhibitory compounds on the metabolism of microorganisms, and difficulties in pentose biotransformation [4–6].

For the generation of fermentable monomeric sugars from lignocelluloses, it is crucial to explore a pretreatment strategy to effectively wane the recalcitrant of the complex

lignocellulosic matrix so as to increase the accessibility of the cellulase to the cellulose fractions [7–9]. In recent decades, various pretreatment protocols, such as chemical, biological, physical protocols and their combinations, have been suggested for pretreating the lignocellulose matrix [10,11]. Among these, steam explosion (SE) is a relatively mature technique that is commonly used in 2 G bioethanol production, in which lignocelluloses are rapidly depressurized from high-pressure conditions into atmosphere pressure; thereby, the cellulosic fibers are expanded and the recalcitrant structure is depolymerized [12]. Compared with other pretreatment strategies, the SE process has obvious advantages in terms of energy saving, low waste water discharge, and proven effectiveness in demonstration plant [13,14]. Nonetheless, accompanied by depolymerization, by-products including organic acids, furans derivates and phenolic compounds are also released from lignocellulose matrices, leading to severe inhibition of sugar production and the following fermentation processes [15]. Generally, the inhibitory mechanisms of the toxic compounds in lignocellulose hydrolysate are ascribed to the intracellular production of reactive oxygen species (ROS), whereas the accumulation of intracellular ROS results in damage to DNA, proteins and organelles, leading to enzyme inactivation as well as decreasing protein translation and inducing apoptosis [16,17].

To this end, various detoxification strategies have been used in attempts to decrease the concentration of inhibitory compounds in SE hydrolysate, so as to decrease the toxic effect and improve the fermentation performance [18,19]. However, this complex detoxification process could negatively affect the overall monomeric sugars produced by lignocelluloses, and basically causes higher processing costs [20]. Another strategy to decrease the toxic effect of the inhibitors in lignocellulosic hydrolysate is to improve the robustness and the tolerance of the microorganism to the inhibitors in lignocellulosic hydrolysate via genetic engineering or an adaptive laboratory evolutionary (ALE) strategy [21–23]. For instance, to improve the inhibitor's tolerance of *S. cerevisiae*, a commonly used strain for bioethanol production in industry, the stress of lignocellulosic hydrolysate in an evolution process could receive phenotypically stable strains that are resistant to the synergistic toxicity of multiple inhibitors [24,25].

The ineffective utilization of pentose fractions (e.g., xylose and arabinose) in = lignocellulose hydrolysate is another technical obstacle in 2 G bioethanol production, because there are no natural pathways for pentose catabolism in wild *S. cerevisiae* [26,27]. Although other strains such as the *S. stipitis* and *P. tannophilus* have been also proposed to produce 2 G bioethanol in existing reports, the low ethanol yield and low resistance to inhibitors of these strains are still less comparable to those of *S. cerevisiae* [28,29]. Generally, current advances in the development of xylose-assimilating *S. cerevisiae* include three heterologous pathways: the xylose isomerase (XI) pathway, from bacteria; the xylose reductase (XR)–xylitol dehydrogenase (XDH) pathway, of fungal origin; and the oxidative and non-phosphorylative bacterial Weimberg pathway [24,30,31]. Among them, the introduction of the XI and XR-XDH pathways into wild *S. cerevisiae* is the primary strategy for accomplishing xylose-assimilating behaviors [27,32,33]. For instance, Chen et al. used systematic strategies including big data mining, rational modification, ancestral sequence reconstruction, and ALE to mine and optimize highly active xylose isomerase, and high-titer ethanol production can be achieved with lignocellulosic hydrolysate using the constructed *S. cerevisiae* CRD5HS [34,35]. In another research, Costa et al. constructed industrially engineered strains via heterologous introduction of xylose reductase gene *XYL1* (mutated for higher specificity for NADH) and xylitol dehydrogenase gene *XYL2*, and overexpression of xylulokinase gene *XKS1* and transketolase gene *TAL1*, followed by knockout of the aldose reductase gene *GRE3*. Ethanol yields of 0.40–0.46 g/g were obtained using xylose-enriched lignocellulosic hydrolysate [36]. Li et al. integrated two novel heterologous genes of the xylose-specific, glucose-insensitive transporter gene *MGT05196*^{N360F} and a xylose isomerase gene Ru-xylA; constructed with endogenous modifications and ALE, the engineered strain LF1 exhibited excellent xylose fermentation performance and enhanced

inhibitor resistance [24]. Nonetheless, works focusing on hyper bio-ethanol production directly from SE hydrolysate with high xylose utilization rates are still lacking.

In this study, aiming to produce high-titer 2 G bioethanol from undetoxified SE hydrolysate, a xylose-assimilating *S. cerevisiae* with tolerance of inhibitory compounds was constructed via metabolic engineering and ALE. Batch fermentation was conducted to verify the fermentation performance of the engineered strain. Aiming to produce higher-concentration bioethanol and therefore decrease the energy requirements of downstream processes, fed-batch SE hydrolysate containing a high concentration of monomeric sugars and inhibitors was also trialed as the substrate, directly. The evolved strain exhibited good fermentation performance, which provides a solid basis for future applications in biorefinery plants.

2. Materials and Methods

2.1. Chemicals and Raw Materials

The steam-exploded (SE) pulp was obtained from SDIC BIOTECH Co., Ltd. (Hailun, China). Generally, the CS was milled into 3–15 cm particles, followed by mixing with 1–3 wt% of sulfuric acid and exploding continuously at 170–190 °C. The SECS mainly consisted of 37.90 ± 1.12 wt% of cellulose, 12.31 ± 0.25 wt% of hemicellulose, 27.67 ± 2.03 wt% of lignin, and 22.12 ± 0.87 wt% of moisture. Cellulase (145 ± 5 FPU/mL) was purchased from Novozymes Co., Ltd. (Copenhagen, Denmark). Bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) = 99:1, D-xylose (>98 wt%), and D-glucose (>99 wt%) were purchased from Macklin Co., Ltd. (Shanghai, China). 2-Chlorobenzyl alcohol was purchased from Aladdin Co., Ltd. (Los Angeles, CA, USA). Other chemicals were purchased from Beijing Chemical Works, and were under AR grade.

2.2. Enzymatic Hydrolysis of the Steam Exploded Pulp

The enzymatic hydrolysis of SE pulp was carried out in a 2 L bioreactor with 1 L of working volume. Briefly, 0.05 M of H_3PO_4/KH_2PO_4 (pH 4.8) was adopted as the buffer. The hydrolysis process was performed at 50 °C and 180 rpm for 72 h, with 10 wt% of SE pulp dosage and 20 FPU/g of cellulase loading [37]. The SE corn stover hydrolysate (SECSH) was obtained after solid–liquid separation, and the pH of the SECSH was adjusted to 5.5 by ammonium hydroxide before using it as the fermentation medium for the screened strains. During the fed-batch hydrolysis of the SE pulp, after batch hydrolysis of the 10% (*w*/*v*) SE pulp and loading for an initial 12 h, an additional 20% (*w*/*v*) of SE pulp and cellulase were added into the bioreactor in the following 48 h intermittently, and the enzymatic saccharification was terminated after 96 h.

2.3. Strains Construction

E. coli strains constructed in this work were grown in Luria–Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl) at 37 °C and 200 rpm for 18 h. The *S. cerevisiae* M3013 was laboratory stored and used as a background strain for further metabolic engineering and ALE [38]. Wild-type *S. cerevisiae* M3013 was grown in yeast extract-peptone–dextrose (YPD) medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose). Xylose-assimilating strains (as summarized in Figure S1 and Table S1) were grown in YPX (20 g/L peptone, 10 g/L yeast extract, 20 g/L xylose). All the yeasts were grown at an optimal ethanol fermentation temperature of 30 °C and 200 rpm for 24 h before inoculation.

The plasmid, guide RNA, synthetic oligos, and primers used in this study are listed in Tables S2 and S3. The gene expression cassettes of the xylose reductase-xylitol dehydrogenase (XR-XDH) pathway were comparable with the previous work [30]. *RPE1*, *TAL1*, *RKI1*, and *TKL1* were isolated from *S. cerevisiae* S288C. All genes were amplified using PrimeSTAR GXL DNA from TaKaRa. The replicating plasmids for gene expression were constructed using a Gibson assembly Master Mix (New England BioLabs, Ipswich, UK) to ligate the pRS416 vector and the amplified gene fragments. The gRNA plasmids were constructed by

Golden Gate Cloning to ligate the pLacZ-SalI (Cas9), G418 (resistance screening markers) and guide RNA (gRNA). The gRNA sequences targeting a specific gene/genomic locus were found and compared at http://crispr.dbcls.jp (accessed on 23 September 2022). The plasmids' construction was confirmed via colony PCR using Taq RCR Master Mix from Biomed and sequencing.

GTR-CRISPR system was used for gene deletion and integration in *S. cerevisiae* M3013 [39]. Considering that the strain M3013 was non-nutrient-deficient, the plasmid pLacZ-SalI (Cas9) was modified by introducing G418 as a resistance-screening marker (Figure S2). In the integration process, the donors were PCR-amplified from the plasmid that constructed and co-transformed with the related vectors (pLacZ) into cells via electroporation [40].

2.4. Adaptive Laboratory Evolution of the Engineered Strains

An ALE of the xylose-assimilating strain was conducted in xylose and SECSH stress sequentially. Yeast seeds were incubated with a rate of 10% (*v*/*v*) at 30 °C and 200 rpm in oxygen-limited 100 mL non-baffled shake flasks containing 20 mL YPX20 (20 g/L xylose) or SECSH, respectively. Transfers were carried out when the strains reached the exponential growth phase and the initial OD_{600} reached 1. The cells' concentration was determined using a spectrophotometer detector (TU-1901) at 600 nm, and xylose was quantitatively detected by HPLC. Finally, cells were enriched on YPX plates and the best single colony was isolated and selected from the suspension of the evolved strain (Figure S1).

2.5. Batch Ethanol Fermentation

To guarantee good metabolic properties in the batch fermentation, strains were precultured for twice the amount of time in the YPX medium. Then, cells were transferred to a 250 mL shake flask containing 50 mL YPD medium, and were incubated at 30 °C and 200 rpm for 24 h. The seeds were finally inoculated for fermentation and maintained at an initial OD_{600} of 1. Batch fermentation of a synthetic medium with inhibitors (to match the concentration of inhibitors tested in SECSH: 8 g/L acetic acid, 5 g/L formic acid or lactic acid, 0.5 g/L furfural or 5-hydroxymethylfurfural (5-HMF), 0.1 g/L phenol, benzoic acid, or 4-hydroxymethylbenzoic acid, and 0.04 g/L of phenolic compounds (vanillin, vanillic acid, ferulic acid, coumaric acid, syringic acid, syringaldehyde, etc.) was added, respectively) was carried out, and realistic SECSH was used in a working volume of 20 mL. All fermentations had three parallel experimental groups.

2.6. Assay

Glucose, xylose and the organic acid by-products in the SECSH and the fermentation broth were analyzed via high-performance liquid chromatography (HPLC, Thermo Scientific™ UltiMate™ 3000, Waltham, MA, USA) with an HPX-87P (Bio-Rad Labs, Hercules, CA, USA) column at 65 °C and refractive index detector (RID) at 50 °C, as described in detail in a previous work [41]. Some 5 mM sulfuric acid was adopted as the mobile phase under a flow rate of 0.6 mL/min. Furan derivates including furfural and 5-hydroxymethylfurfural (5-HMF) were detected via HPLC using a 100-5-C18 column (Kromasil, Bohus, Sweden) at 35 °C and a UV detector at 270 nm. Some 50% (*v*/*v*) acetonitrile solution was used as the mobile phase.

Phenolic compounds including vanillin and vanillic acid were detected using GC/MS (Agilent 7890B GC and 5977B MS) [42]. For the derivatization, lignin in SECSH was extracted several times using ethyl acetate. Then, 100 µL of BSTFA:TMCS = 99:1 was added into the solid fraction after evaporation of the ethyl acetate, and the mixture was maintained at 60 °C for 30 min. During the process, 2-chlorobenzyl alcohol was used as an internal standard. The GC conditions were as follows: the oven temperature was maintained at 50 °C for 3 min, then increased to 150 °C at a speed of 10 °C/min, followed by ramping up to 220 °C at a speed of 5 °C/min, and finally increased to 300 °C at 10 °C/min and maintained for another 3 min. Helium was used as carrier gas at a flow rate

of 1.27 cm^3/min. The injector temperature was maintained at 280 °C in split mode with a 5:1 split ratio. The ion source temperature was 230 °C, and the quadrupole temperature was set at 150 °C in MS detector. All samples were replicated in triplicate.

2.7. Statistical Analysis

The software IBM SPSS Statistics 22 was used for statistical analysis to test significant differences between each treatment. All data are presented as means ± standard deviation of duplicate determination.

3. Results and Discussions

3.1. Composition of the SECSH and the Fermentation Performance of the Background Strain M3013

The SECSH was produced via batch saccharification containing 39.02 ± 0.27 g/L of glucose and 10.66 ± 0.91 g/L of xylose. In addition, by-products including organic acids, furan derivates, and phenolic compounds were also detected in the SECSH, and were likely they to the deacetylation of the hemicellulose fraction, the isomerization and dehydration; and were released from the polymerized lignin structure, respectively, during the SE pretreatment (Figure S1 and Table 1) [37,43–45]. These compounds are proven to cause severe inhibition of the growth of *S. cerevisiae* [46,47]. As shown in Table 1, furfural and 5-HMF are the primary furan inhibitors in SECSH, and their concentrations are 0.333 ± 0.001 g/L and 0.412 ± 0.004 g/L, respectively. The acetic acid concentration in SECSH was 2.110 ± 0.198 g/L, while 3.355 ± 0.136 g/L of lactic acid was also detected. The lactic acid was probably produced by the fermentation of lactic acid bacteria present in the CS during the silage process [48]. Meanwhile, the GC/MS spectra and the corresponding structural formulae of the phenolic compounds in Figure S1 demonstrated that the vanillin (0.064 ± 0.012 g/L) and vanillic acid (0.044 ± 0.001 g/L) were the main phenolic inhibitors in the SECSH, while low concentrations of 4-hydroxy-3-(4-hydroxyphenyl) propanoic acid (0.002 g/L), 4-hydroxy-benzenepropanoic acid (0.002 g/L), and 2-hydroxy-3-(4-hydroxy-3-methoxyphenyl) (0.001 g/L) propanoic acid were also detected.

Table 1. Inhibitors concentrations in SECSH.

Inhibitors	**Formate**	**Acetate**	**Lactate**	**Furfural**	**5-HMF**	**Phenol**	**4-Methylphenol**	**Benzoic Acid**	**4-Hydroxy-Benzaldehyde**
Concentration (g/L)	2.110 ± 0.198	6.153 ± 0.803	3.355 ± 0.136	0.333 ± 0.001	0.412 ± 0.004	0.021 ± 0.001	0.010 ± 0.000	0.123 ± 0.008	0.022 ± 0.004
Inhibitors	*p*-Hydroxyacetophenone	3-Hydroxy-3-(4-hydroxyphenyl)propanoic acid	4-Hydroxyphenethyl alcohol	4-Hydroxy benzonic acid	4-Hydroxy-benzenepropanoic acid	Methylhydroquinone	Protocatechuate	3-(4-Hydroxyphenyl)lactate	*p*-Coumaric acid
Concentration (g/L)	0.014 ± 0.002	0.002 ± 0.001	0.003 ± 0.001	0.015 ± 0.005	0.002 ± 0.001	0.002 ± 0.000	0.011 ± 0.004	0.032 ± 0.014	0.035 ± 0.006
Inhibitors	2-Methoxyphenol	Vanillin	4-Hydroxy-3-methoxyphenethylene glycol	Acetovanillone	Vanillic acid	4-Hydroxy-3-methoxybenzeneisopropanoic acid	4-Hydroxy-3-methoxyphenylacetic acid	Vanillylmandelic acid	2-hydroxy-3-(4-hydroxy-3-methoxyphenyl) propanoic acid
Concentration (g/L)	0.012 ± 0.004	0.064 ± 0.012	0.012 ± 0.005	0.010 ± 0.003	0.044 ± 0.001	0.007 ± 0.001	0.023 ± 0.007	0.008 ± 0.002	0.001 ± 0.000
Inhibitors	Ferulic acid	2,6-dimethoxyphenol	Syringaldehyde	Syringic acid					
Concentration (g/L)	0.034 ± 0.002	0.015 ± 0.006	0.010 ± 0.002	0.020 ± 0.007					

In order to investigate the degree to which the above inhibitors in SECSH inhibit the parent *S. cerevisiae* strain, batch fermentations were carried out in a synthesis medium with the addition of organic acids (refer to the results in Table 1, 5 g/L formic acid and lactic acid, 8 g/L acetic acid), furan derivates (0.5 g/L furfural and 5-HMF), and phenols (0.1 g/L phenol, benzoic acid and 4-hydroxy-benzaldehyde, 0.05 g/L *p*-coumaric acid, vanillin, vanillic acid, ferulic acid, syringaldehyde and syringic acid) were added). Figure 1 shows the tolerance of the *S. cerevisiae* M3013 to the various inhibitors (For details, please see Table S4). It is suggested that acetic acid exhibited the highest inhibition of the growth of the M3013 strain among the tested organic acids. Compared with the control group using a similar synthesis medium except for the addition of inhibitors (13.31 ± 0.56 of OD_{600max} and 0.472 ± 0.185 g/g of yield), levels of only 10.46 ± 0.16 of OD_{600max} and 0.447 ± 0.096 g/g of yield were accomplished in the acetic acid-containing group. In contrast to the inhibition of acetic acid, lactic acid had a slight negative influence on the M3013 strain. Similar to the phenomenon illustrated in the literature, the *S. cerevisiae* had a relatively higher tolerance to the furan derivatives than other inhibitors [49]. Despite the phenolic compound concentration in the medium being extremely low, severe inhibition of the strain growth and the ethanol yield was detected. Meanwhile, the phenolic compounds with carboxyl groups showed more severe inhibition of the M3013 strain than the aldehyde counterparts. This can be explained by the directly dissociated carboxyl groups containing phenols outside the cells, which lower the environmental pH. In contrast, the aldehyde groups containing phenols cause proteins to be denatured after they are transformed into cells [50,51].

3.2. Construction of Xylose-Fermenting *S. cerevisiae* Strains and Their Adaptive Evolution

The experimental results in Section 3.1 suggest that the strain *S. cerevisiae* M3013 possesses a good fermentation phenotype [38]. Hence, it was selected as a parent strain for the construction of the xylose-assimilating recombinant strains. As shown in Figure 2, the genome of the M3013 strain was modified by metabolic engineering through heterologous expression, knockout, and gene modification via the GTR-CRIPSR system. The XR-XDH pathway containing a mutant XR (R276H) gene *mXYL1*, XR gene *XYL1*, XDH gene *XYL2*, and XK gene *XKS1* was integrated into the specific locus in the genome of the *S. cerevisiae* chromosome (Table S3) [30]. To enhance xylose catabolism and reduce by-product production, the native aldose reductase gene *GRE3*, which inhibited XI activity and thereby reduced the production of xylitol, and the alkaline phosphatase gene *PHO13*, which promoted a reduction in ATP consumption, were deleted in the genome [52,53]. Then, the genes (including *TAL1*, *TKL1*, *RKI1* and *RPE1*) related to the non-oxidative pentose phosphate pathway were overexpressed.

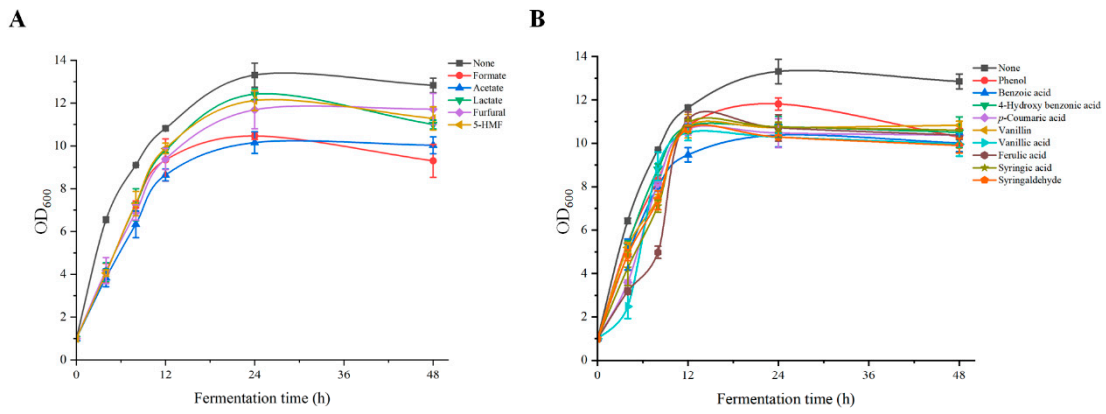

Figure 1. *Cont.*

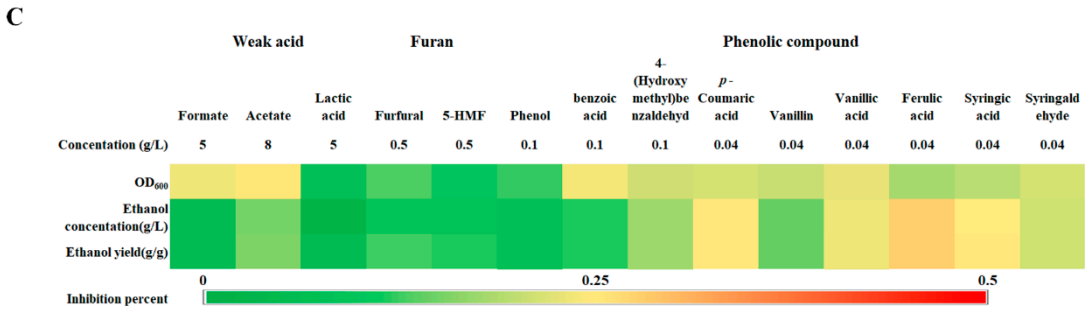

Figure 1. Inhibition of the metabolism of *S. cerevisiae* M3013 by inhibitors present in SECSH. Growth curves of *S. cerevisiae* M3013 in synthetic media containing (**A**) organic acids (5 g/L of formate and lactic acid, 8 g/L of acetate) and furan derivates (0.5 g/L of furfural and 5-HMF), and (**B**) phenolic compounds (0.1 g/L of phenol, benzoic acid and 4-(hydroxymethyl)benzaldehyde, 0.04 g/L of *p*-coumaric acid, vanillin, vanillic acid, ferulic acid, syringic acid and syringaldehyde). (**C**) Heat map for the inhibition degree of different inhibitors of *S. cerevisiae* M3013 in synthetic mediums. The inhibition degree of OD_{600} = 1 − (OD_{600max} − OD_{600min} of growth with inhibitors)/(OD_{600max} − OD_{600min} without inhibitors) × 100%. The inhibition degree of ethanol concentration = 1 − the ethanol concentration of the inhibitors/the ethanol concentration in the control without inhibitors × 100%. The inhibition degree of ethanol yield = 1 − the ethanol yield of the inhibitors/the ethanol yield in the control without inhibitors × 100%.

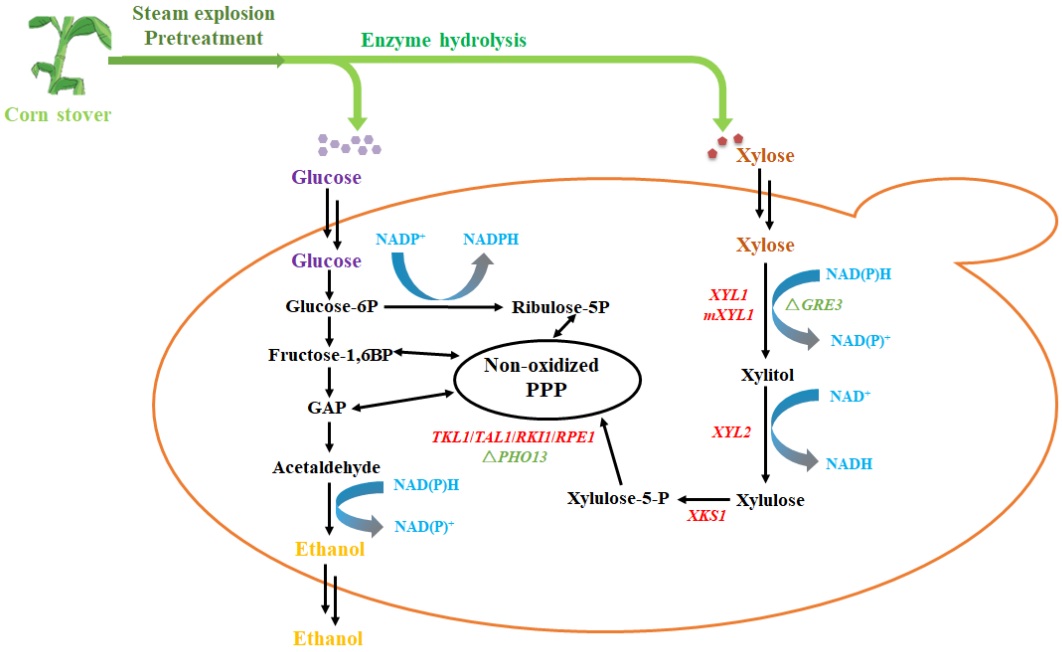

Figure 2. Schematics diagram of the construction of xylose-assimilating yeast for ethanol production. Heterologously expressed and overexpressed genes are shown in red, while knockout genes are shown in green. Glucose-6P: Glucose 6-phosphate; Ribulose-5P: Ribulose-5-phosphate; Fructose-1,6BP: Fructose-1,6-bisphosphate; GAP: Glyceraldehyde 3-phosphate; Xylulose-5-P: Xylulose-5-phosphate; and PPP: pentose phosphate pathway.

The resulting strain YL13 was isolated for ALE under the stress of xylose as a sole carbon source in synthetic medium. After 85 days of evolution, the mutant strain YL13-1 that significantly improved xylose assimilation was screened. To further improve the tolerance of inhibitors from SECSH, an ALE was further conducted using the YL13-1 strain with the stress of diluted SECSH. Several single colonies were used for evaluation, and the colony with the best phenotypes (xylose assimilation and inhibitor tolerance) was used for

subsequent fermentation. Finally, a single colony named by YL13-2 was isolated, which could tolerance the inhibitors and maintain stable hereditary traits in undetoxified SECSH when using xylose as a sole carbon source (Figure S4).

3.3. Fermentation Performance of the Evolved Strain YL13-2 Using SECSH

The fermentation performances of the evolved YL13-2 strain, with a high tolerance to the basic inhibitors present in the SECSH and high xylose-assimilating efficiency, was investigated. Before using the undetoxified SECSH as the substrate, the synthetic medium to which the key inhibitors were added was analyzed. Compared to strain YL13-1, *S. cerevisiae* YL13-2 showed better performance in biomass-growing and ethanol fermentation in various inhibitor-containing media (Figure 3 and Table S5), which reflected the significant effect of the ALE of the strain in the inhibitor-containing SECSH in enhancing inhibitor tolerance. In particular, yeast growth and ethanol yield after adding 5-HMF (13.98 ± 1.02 of OD_{600max} and 0.454 ± 0.004 g/g total sugar, respectively), syringic acid (13.84 ± 0.14 of OD_{600max} and 0.430 ± 0.005 g/g total sugar, respectively) and syringaldehyde (13.73 ± 0.66 of OD_{600max} and 0.439 ± 0.010 g/g total sugar, respectively) were very close to those of the control group with added inhibitors (14.02 ± 0.45 of OD_{600max} and 0.457 ± 0.014 g/g total sugar, respectively).

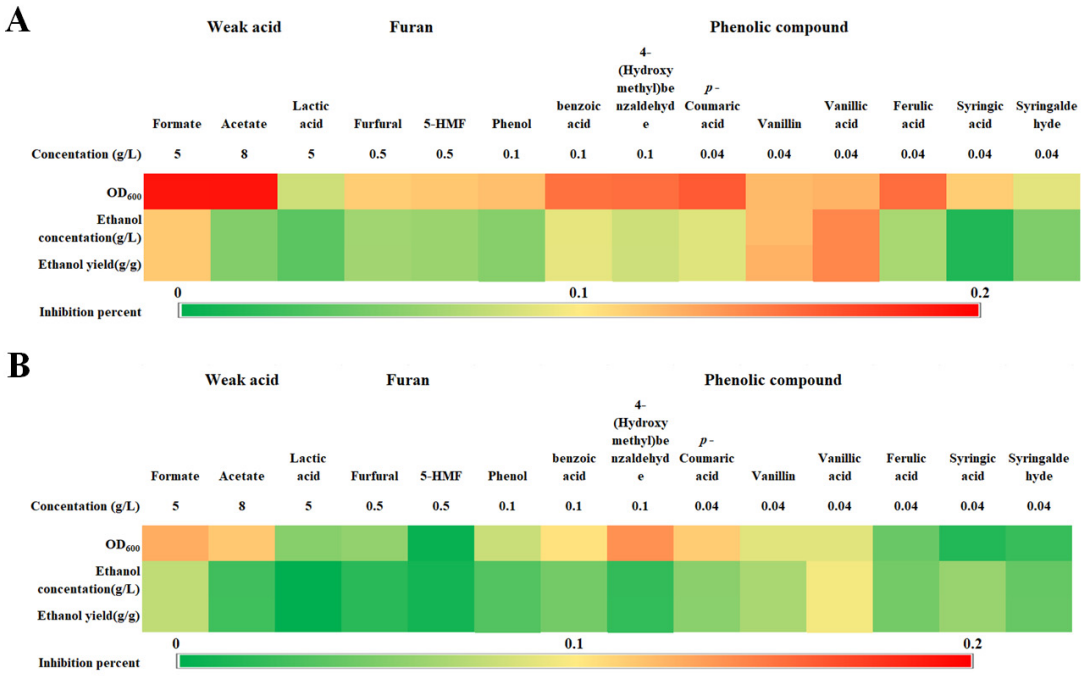

Figure 3. (**A**) Heat map for the degree of inhibition of *S. cerevisiae* YL13-1 in synthetic media containing toxic compounds. (**B**) Heat map for the degree of inhibition of *S. cerevisiae* YL13-2 in synthetic media.

The strong fermentation performance of the YL13-2 strain in the synthesis medium containing inhibitors justified further evaluation of fermentation performance when using undetoxified SECSH as a substrate. Herein, in order to better reflect the xylose utilization and the inhibitor tolerance of the engineered strain YL13-2, the fermentation kinetics of the background strain M3013 and the engineered strain YL13-1 were further investigated (Figure 4). In terms of glucose utilization in the SECSH, all the tested strains generally possessed similar kinetics in the batch fermentation process. As for the xylose assimilation using undetoxified SECSH, YL13-2 showed a significantly increased xylose utilization rate. After 96 h of inoculation, there was no xylose remaining in the fermentation broth of YL13-2, while 3.53 ± 0.59 g/L residual xylose was detected in the broth of YL13-1, and no xylose consumption was detected in the broth of M3013. This can be attributed to the ALE that promoted xylose assimilation in inhibitors in these conditions.

Meanwhile, the strain YL13-2 also showed a significant increase in ethanol production and yield (22.96 ± 1.73 g/L and 0.454 ± 0.034 g/g total sugar, respectively), compared to 22.43 ± 0.79 g/L and 0.442 ± 0.016 g/g total sugar in YL13-1. Our further work verified that the performance of the strain YL13-2 in ethanol fermentation with and without YP (10 g/L yeast extract and 20 g/L peptone) added to SECSH did not differ significantly (Table S5). Therefore, the strain YL13-2 exhibited better resistance to SECSH inhibitors, and did not require exogenously added nutrients to promote an intracellular oxidative stress response [54]. The above experimental results confirmed the excellent xylose consumption capability and inhibitor resistance property of strain YL13-2, which allowed hyper bioethanol production in undetoxified SECSH without any other nutrient supplementation.

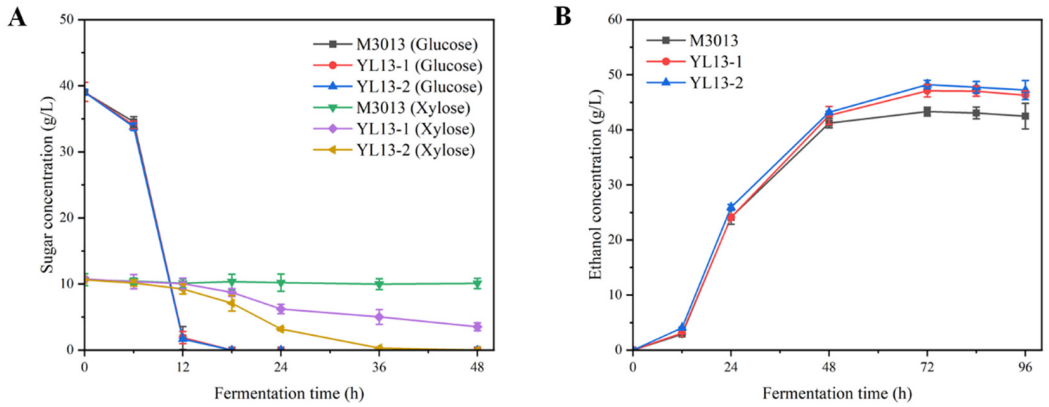

Figure 4. Fermentation performances of different *S. cerevisiae* for ethanol production using SECSH. (**A**) glucose, xylose, and (**B**) ethanol concentration.

3.4. Bioethanol Fermentation Using High-Concentration SECSH and Strain YL13-2

In order to reduce of the separation cost of downstream processes, high-concentration bioethanol production in broth is recommended [55]. It is estimated that the energy requirement for ethanol separation via distillation may be as low as one third of the heat of combustion (~30 MJ/kg) when feeding a broth containing ~3 wt% of ethanol [56]. Moreover, end-product inhibition can be achieved when the ethanol concentration in broth is above 5 wt% [57,58]. Therefore, a bioethanol concentration after fermentation maintained at >5 wt% may be more effective in concerns of the fermentation and separation efficiencies. Nonetheless, owing to poor mass transfer in the enzymatic hydrolysis stage, the initial sugar concentration in the substrate was far lower than the ideal titer in >5 wt% bioethanol production [59–61].

To realize high-concentration monomeric sugars in lignocellulose hydrolysate, fed-batch saccharification is suggested in the literature [62]. Based on intermittent feeding, the viscosity of the slurry during the enzymatic hydrolysis of the pulps can be maintained at a relatively lower level, and good homogenization and mass transfer can be realized [63,64]. As shown in Figure 5, in total, 30% (*w*/*v*) SECS loading was performed via fed-batch enzymatic hydrolysis. After 96 h of hydrolysis, 97.61 ± 0.59 g/L glucose and 22.56 ± 0.53 g/L xylose were achieved in the slurry. The slurry was directly used by the evolved strain YL13-2 for ethanol fermentation after pH neutralization, and there was no extra detoxification step and nutrient supplementation. It is worth noting here that the inhibitor concentration was also increased after fed-batch saccharification (for details, please see Figure S6) compared to the batch process. Therefore, more severe inhibition could be achieved when using fed-batch SECSH as substrate.

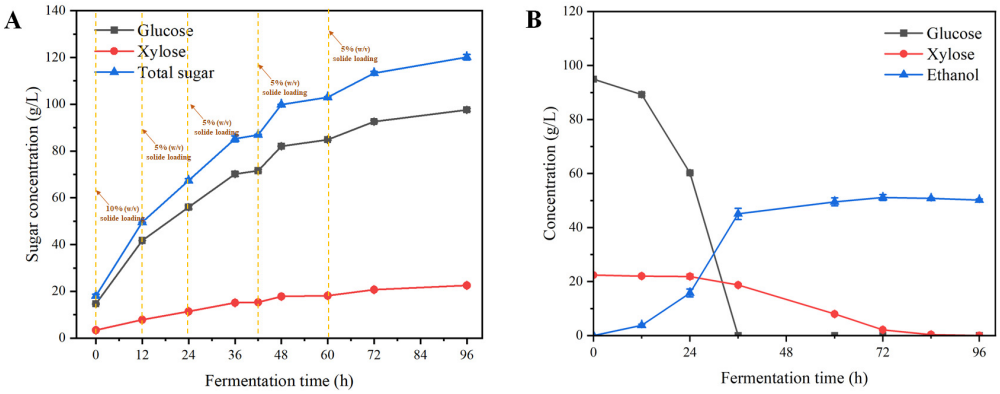

Figure 5. Ethanol production using SECSH containing high sugar levels. (**A**) Fed-batch hydrolysis of the SE pulp. (**B**) Batch fermentation of YL13-2 strain in a 5 L bioreactor.

As can be seen from Figure 5, attractive bioethanol fermentation results were realized using the evolved strain YL13-2. Despite being negatively affected by higher concentrations of inhibitors, YL13-2 depleted glucose and produced 45.06 ± 2.08 g/L ethanol in 36 h, and continued to produce up to 6.06 g/L of ethanol from xylose in the following 60 h. Even though the bioethanol yield was lower than in the process using batch SECSH (0.436 ± 0.009 g/g using fed-batch hydrolysate vs. 0.454 ± 0.034 in batch hydrolysate), a final ethanol titer of 51.12 ± 1.06 g/L with an average productivity of 0.71 ± 0.01 g/L h can be realized after 96 h of inoculation. The kinetics investigation suggested that obvious carbon catabolite repression occurs in the fermentation process, and consequently, glucose was almost completely consumed before the rapid utilization of xylose by YL13-2 [24,32,65]. Therefore, an important direction in future work may be the mitigation or elimination of carbon catabolite repression for more efficient bioethanol production.

3.5. Mass Balance

The mass balance of bioethanol production from CS by YL13-2 is shown in Figure 6, which shows that 778.8 g of SE pulp can be recovered from 1000 g of raw CS. After enzymatic hydrolysis of SE pulp, 325.4 g of glucose and 75.2 g of xylose can be obtained in the SECSH (based on the fed-batch process). Inhibitors including 0.212 g of phenolic compounds and 4.945 g of others were also co-generated after saccharification. However, due to the high tolerance of inhibitors and the boosting of xylose assimilation caused by the evolved strain YL13-2, 170.4 g of bioethanol can be obtained after fermentation. Table 2 summarizes the current advances in bioethanol production from different types of lignocelluloses. It shows that using strain YL13-2 in the current work produced a high ethanol yield from CS. More importantly, compared with other pieces of research, the avoiding of detoxification and nutrient supplementation simplified the overall production chain of bioethanol, and the relatively higher concentration of the bioethanol produced showed the potential of more energy saving in downstream purification processes. Overall, the evolved strain YL13-2 has great potential for application in scaled-up operations, and we will continue this research in future works.

Table 2. A summary of current advances in bioethanol production from different types of lignocelluloses using *S. cerevisiae*. * represents the ploidy of gene integration into the chromosome.

Strains	Substrate	Pretreatment Conditions	Description	Detoxification	Nutrient Supplements	Ethanol Concentration (g/L)	Ethanol Yield (g/g)	Refs.
S. cerevisiae YL13-2	Corn stover	SE	M3013, *mXYL1*, *XYL1*, *XYL2*, *XKS1*, *TAL1*, *TKL1*, *RKI1*, *RPE1*, Δ*GRE3*, Δ*PHO13*, ALE	No	Without additional nutrients	51.12	0.436	This study
S. cerevisiae XUSAE57	Sugarcane bagasse	Dilute acid pretreatment	BY4741, *xylA**3, *TAL1*, *XKS1*, *RPE1*, Δ*GRE3*, Δ*PHO13*, ALE	Yes	Without additional nutrients	23.2	0.49	[66]

Table 2. *Cont.*

Strains	Substrate	Pretreatment Conditions	Description	Detoxification	Nutrient Supplements	Ethanol Concentration (g/L)	Ethanol Yield (g/g)	Refs.
S. cerevisiae *XUSEA*	Micanthus sacchariflorus Goedae-Uksae	H_2SO_4 pretreatment	BY4741, *xylA**3, *TAL1*, *XKS1*, *RPE1*, Δ*GRE3*, Δ*PHO13*, Δ*ACS1*, ALE	Yes	Without additional nutrients	30.1	0.48	[67]
S. cerevisiae F12	Wheat straw	SE	TMB3001, *XYL1*, *XYL2*, *XKS1*, ALE	No	vitamin solution and ergosterol	23.7	0.43	[68]
S. cerevisiae PE-2ΔGRE3-X	Corn cob	Hydrothermal treatment	PE-2, *xylA*, Δ*GRE3*	Yes	Yeast extract 10 g/L, peptone 20 g/L	11.6	0.44	[69]
S. cerevisiae LF1	Corn stover	SE	BSIF (diploid), Ru-*xylA*, *TAL1*, *MGT05196*^{N360F}, *TKL1*, *RKI1*, *RPE1*, Δ*GRE3*, Δ*PHO13*, ALE	No	Yeast extract 10 g/L, peptone 20 g/L	50.81	0.413	[24]
S. cerevisiae XM20	Corn stover	Ethylenediamine (EDA) pretreatment	L2612, *XYL1*, *mXYL2*, *XKS1*, *TKL1*, *RKI1*, *SOD1*, *GSH1*, *GLR1*, *ZWF1*, *GND2*, *ACS1*, Δ*PHO13*	No	Without additional nutrients	90.7	/	[16]
S. cerevisiae CRD5HS	Corn stover	Alkaline/acidic pretreatment	CRD3, *hasxylA*, ALE	No	Yeast extract 5 g/L and tryptone 10 g/L	85.95	/	[34]
S. cerevisiae 6M-15	Corn straw	SE	LF1, ARTP mutagenesis	No	Yeast extract 10 g/L, peptone 20 g/L	30.95	0.43	[70]
S. cerevisiae Lg8-1	Wheat straw	Alkaline pre-extraction and acid catalyzed steam treatment	/	No	Without additional nutrients	54.5	0.46	[71]
S. cerevisiae MN8140X/TF-TF	Rice straw	Hydrothermally pretreated and mechanically milled	MT8-10, *XYL1*, *XYL2*, *XKS1*, *TAL1*, *FDH1*	No	Yeast extract 10 g/L, peptone 20 g/L	52.0	0.38	[72]

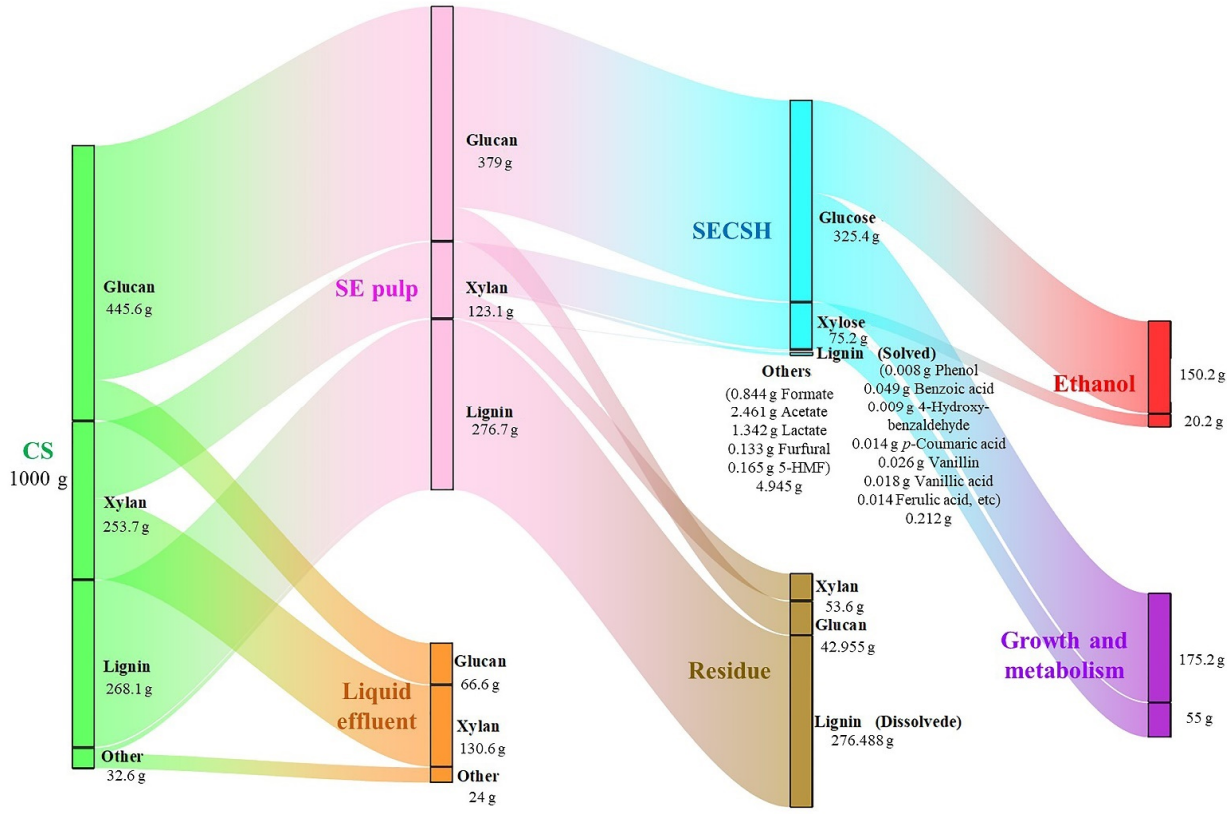

Figure 6. Mass balance of bioethanol production by *S. cerevisiae* YL13-2 using undetoxified SECSH.

4. Conclusions

The hydrolysis inhibitor tolerance of xylose-assimilating *S. cerevisiae* YL13-2 was engineered by introducing the heterologous mXR-XDH pathway and the ALE process under the stress of SECSH. Compared with the parent strain M3013, the evolved strain YL13-2 showed high robustness, and could effectively utilize the xylose fractions in undetoxified SECSH. A maximum of 51.12 g/L of bioethanol could be produced using the undetoxified SECSH, with a yield and productivity of 0.436 g/g and 0.71 g/L h, respectively. This work highlights the great potential of the production of bioethanol from undetoxified lignocellulose hydrolysate in practice. Future works will be concerned with the scaling up of fermentation using the evolved YL13-2 strain, and investigation of the specific molecular mechanisms of inhibitor tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9100906/s1, Figure S1: Lignin monomers presented in SECSH detected by GC/MS; Figure S2: Construction and the evaluation of the genetic strain. (A) Flowchart of the construction process of Saccharomyces cerevisiae YL13-2. (B) Xylose consumption rate and ethanol concentration after batch fermentation of YL13-2 strain using the synthetic medium. Figure S3: A gRNA-tRNA array for CRISPR-Cas9 (GTR-CRISPR). The screening marker replaced by *URA3* for *G418*. Figure S4: Schematic diagram of the xylose-assimilating enhancement and the inhibitors tolerance increases of *S. cerevisiae* YL 13 by the ALE strategy. Figure S5: (A) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium containing organic acids that presence in SE enzymatic hydrolysate. (B) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium that containing furan derivates (0.5 g/L of furfural and 5-HMF) presence in SE enzymatic hydrolysate. (C) (D) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium containing phenolic compounds (0.1 g/L of phenol, benzoic acid and 4-(hydroxymethyl)benzaldehyde, 0.04 g/L of p-coumaric acid, vanillin, vanillic acid, ferulic acid, syringic acid and syringaldehyde) presence in SE enzymatic hydrolysate. Figure S6: Inhibitors (organic acids, furan derivates and phenolic compounds) concentration in 10 % (*w*/*v*) and 30 % (*w*/*v*) of SECS loading. Table S1: Strains involved in this study. Table S2: Primers involved in this study. Table S3: Plasmid and gRNA involved in this study. Table S4: Key parameters of batch ethanol fermentation by different strains using the synthetic medium containing inhibitors. Table S5: Ethanol fermentation performances by strains YL13-2 using undetoxified SECSH without adding YP and with adding YP. Table S6: Ethanol fermentation performances by different strains using undetoxified SECSH.

Author Contributions: Conceptualization, Y.W. (Yilu Wu) and D.C.; methodology, Y.W. (Yilu Wu) and C.S.; validation, Y.W. (Yilu Wu), G.Z. and Z.L.; formal analysis, Y.W. (Yilu Wu) and Y.J.; investigation, Y.W. (Yankun Wang) and J.W.; resources, C.Z.; data curation, Y.W. (Yilu Wu) and D.C.; writing—original draft preparation, Y.W. (Yilu Wu); writing—review and editing, Y.W. (Yilu Wu) and D.C.; su-pervision, C.Z.; project administration, D.C.; funding acquisition, D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the National Natural Science Foundation of China (Grant No. 22078018).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Wenqiang Ren from Research Center for Eco-environmental Science, Chinese Academy of Science for kindly providing us with the help of testing.

Conflicts of Interest: The authors declare no conflict of interest.

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