Improved bioconversion of lignocellulosic biomass by
Saccharomyces cerevisiae engineered for tolerance to acetic acid

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Abstract
Lignocellulosic biomass has considerable potential for the production of fuels and chemicals as a promising alternative to conventional fossil fuels. However, the bioconversion of lignocellulosic biomass to desired products must be improved to reach economic viability. One of the main technical hurdles is the presence of inhibitors in biomass hydrolysates, which hampers the bioconversion efficiency by biorefinery microbial platforms such as Saccharomyces cerevisiae in terms of both production yields and rates. In particular, acetic acid, a major inhibitor derived from lignocellulosic biomass, severely restrains the performance of engineered xylose-utilizing S. cerevisiae strains, resulting in decreased cell growth, xylose utilization rate, and product yield. In this study, the robustness of XUSE, one of the best xylose-utilizing strains, was improved for the efficient conversion of lignocellulosic biomass into bioethanol under the inhibitory condition of acetic acid stress. Through adaptive laboratory evolution, we successfully developed the evolved strain XUSAE57, which efficiently converted xylose to ethanol with high yields of 0.43–0.50 g ethanol/g xylose even under 2–5 g/L of acetic stress. XUSAE57 not only achieved twofold higher ethanol yields but also improved the xylose utilization rate by more than twofold compared to those of XUSE in the presence of 4 g/L of acetic acid. During fermentation of lignocellulosic hydrolysate, XUSAE57 simultaneously converted glucose and xylose with the highest ethanol yield reported to date (0.49 g ethanol/g sugars). This study demonstrates that the bioconversion of lignocellulosic biomass by an engineered strain could be significantly improved through adaptive laboratory evolution for acetate tolerance, which could help realize the development of an economically feasible lignocellulosic biorefinery to produce fuels and chemicals.

Keywords
acetic acid, bioethanol, lignocellulosic biomass, lignocellulosic hydrolysate, Saccharomyces cerevisiae, xylose
Lignocellulosic biomass has been considered a major source of biofuels production as a sustainable alternative to conventional fossil-derived fuels (Jonsson, Alriksson, & Nilvebrant, 2013). Owing to its abundance and potentially sustainable supply, there has been increased interest in lignocellulosic biomass as a promising renewable resource in a growing biorefinery (Nicolaus Dahmen, Zibek, & Weidtmann, 2019). Lignocellulosic biomass primarily consists of three polymers, cellulose, hemicellulose, and lignin, along with small amounts of substituents such as acetyl groups (Isikgor & Becer, 2015). The composition of biomass varies depending on the feedstock type including agricultural residues, wood wastes such as poplar, and dedicated energy crops, including miscanthus and switchgrass (Oliver & Khanna, 2017; Saini, Saini, & Tewari, 2015; Slade, Gross, & Bauen, 2011). Maize, wheat, rice straw, and sugarcane bagasse are responsible for generating the majority of agricultural residue feedstocks comprising 30%–50% cellulose, 25%–35% hemicellulose, 15%–25% lignin, and ~2% acetyl groups, whereas wood wastes contain higher amounts of lignin (15%–40%) with a more rigid structure (Ko et al., 2018; Ko, Kim, Ximenes, & Ladisch, 2015; Saini et al., 2015; Zhu & Pan, 2010). Given the varying compositions of lignocellulosic biomass, the development of integrated technologies for maximizing their bioconversion into target products is desired to achieve a cost-effective biorefinery at the commercial scale.

During the hydrolysis of lignocellulosic biomass, cellulose and hemicellulose are hydrolyzed into monomeric sugars (e.g., glucose and xylose), which are subsequently converted to biofuels via microbial fermentation. The economic feasibility of lignocellulosic biofuel production heavily relies on the microbial cell performance, requiring a strain that can utilize a broad range of carbon substrates along with robustness toward toxic compounds derived from the lignocellulosic biomass (Ko & Lee, 2018). The yeast *Saccharomyces cerevisiae* has been widely used as an industrial ethanol-producing microbial platform, which has made the goal of the commercial-scale production of lignocellulosic bioethanol feasible for the near future (Ko & Lee, 2018; Nielsen & Keasling, 2016). However, *S. cerevisiae* does not naturally utilize xylose and readily assimilate glucose. The development of engineered xylose-utilizing strains of *S. cerevisiae* harboring xylose reductase/xylitol dehydrogenase- or xylose isomerase-based pathways has significantly improved the prospects of establishing productive biorefineries by improving the bioconversion yields in lignocellulosic bioethanol production (Ko, Um, Woo, Kim, & Lee, 2016).

Such engineered strains can effectively convert the mixed sugars derived from biomass into biofuels; however, the presence of toxic inhibitors remains a critical hurdle for efficient biofuels production (Palmqvist & Hahn-Hägerdal, 2000). We previously developed a high-performance isomerase-based xylose-fermenting *S. cerevisiae* strain, XUSE, which could serve as a promising platform strain for biofuels and biochemical production in a lignocellulosic biorefinery. Through combinatorial rational and evolutionary engineering, XUSE exhibited the efficient conversion of xylose into ethanol, reaching a high yield of 0.43 g/L along with the simultaneous fermentation of glucose and xylose with negligible glucose inhibition (Tran Nguyen Hoang, Ko, Gong, Um, & Lee, 2018). However, the presence of acetic acid in lignocellulosic hydrolysates severely inhibited the xylose fermentation performance.

The bioconversion of lignocellulosic biomass via microbial fermentation is commonly preceded by physicochemical pretreatment processes that are designed to deconstruct the inherent recalcitrant structure. For example, hydrolysis processes such as hydrothermal pretreatments using dilute acid, steam explosion, or liquid hot water release by-products such as organic acids, sugar degradation products, and phenolic compounds that are toxic to microbial cells along with fermentable sugars (Dale & Ong, 2012; Gurram, Datta, Lin, Snyder, & Menkhaus, 2011). Since hemicellulose and lignin in the plant cell wall are usually substituted with 1%–6% acetyl groups, a substantial amount of acetic acid is inevitably produced at concentrations ranging from 1 to 15 g/L depending on the feedstock type and pretreatment condition (Klinke, Thomsen, & Ahring, 2004; Palmqvist & Hahn-Hägerdal, 2000). The normalized acetic acid concentrations in various lignocellulosic hydrolysates based on the xylose concentrations range from 1 to 9.5 g/L (per 20 g/L of xylose; Figure 1). As the most prevalent inhibitor in hydrolysates, acetic acid severely inhibits the cell growth and fermentation efficiency, especially in engineered xylose-utilizing *S. cerevisiae* strains, consequently reducing the bioconversion efficiency during lignocellulosic bioethanol production (Ko, Um, & Lee, 2016). Therefore, improving the robustness of yeast toward acetic acid is highly desired to achieve economically viable lignocellulosic biofuels production.

Despite numerous approaches developed to improve acid tolerance in yeast, few efforts have focused on engineered xylose-utilizing strains, which are more sensitive to acid stress, and these strategies have rarely been evaluated using lignocellulosic hydrolysates (Chen, Stabryla, & Wei, 2016; Oh, Wei, Kwak, Kim, & Jin, 2019; Wright et al., 2011). Although the inhibitor tolerance of XI-pathway-based strain was improved by mutagenesis followed by evolutionary engineering in lignocellulosic hydrolysates, the ethanol yield of the evolved strain was only 0.06 g ethanol/g xylose, which is 12% of the theoretical maximum yield (Smith, van Rensburg, & Görgens, 2014). Accordingly, the development of a lignocellulosic biorefinery using engineered strains with either xylose utilization or tolerance toward inhibitors has not
been realized to date. Given that engineered xylose-utilizing strains are more susceptible to acetic acid inhibition under industrially relevant conditions, it is important to combine these two strategies through the development of stress-tolerant xylose-utilizing strains so as to achieve a high overall biofuel yield from lignocellulosic biomass (Ko & Lee, 2018). Here, we developed a promising yeast strain platform as an efficient biorefinery of lignocellulosic biomass by improving the acetic acid tolerance of XUSE via an evolutionary engineering strategy which is a powerful approach to acquire the desired phenotype (Sandberg, Salazar, Weng, Palsson, & Feist, 2019). The newly developed strain (XUSAE57) showed a significantly improved bioconversion efficiency during lignocellulosic bioethanol production in the presence of acetic acid, demonstrating its potential for industrial scale use in a biorefinery.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

The previously developed engineered S. cerevisiae strain harboring the xylose-isomerase pathway XUSE (S. cerevisiae BY4741, xylA3*, TAL1, XKS1, ∆gre3, ∆pho13, evolved; Tran Nguyen Hoang et al., 2018) was used as the parental strain for the adaptive laboratory evolution experiments. S. cerevisiae cells were routinely cultured in yeast synthetic complete (YSC) medium containing 20 g/L of xylose, complete supplement mixture (CSM), and yeast nitrogen base. To examine the xylose fermentation performances of the parental engineered (XUSE) and adapted (XUSAE57) strains, seed culture was prepared in yeast extract–peptone–dextrose medium containing 20 g/L glucose by inoculation with a glycerol stock. The cells were then transferred to YSC medium containing 20 g/L xylose and incubated aerobically at 30°C for 1.5 days for pre-culture. The pre-cultured cells were collected and re-inoculated into fresh YSC medium containing 20 g/L xylose and 0–5 g/L acetic acid with an initial pH of 5. The fermentations were performed in 250 ml serum bottles with a working volume of 40 ml and an initial optical density at 600 nm (OD600) of 1 at 30°C and 200 rpm.

2.2 | Evolutionary engineering of xylose-utilizing strains under acetic acid stress

To improve the acetate tolerance of the engineered xylose-utilizing strain XUSE, we attempted serial subculturing in synthetic medium with xylose as the sole carbon source in the presence of increasing concentrations of acetic acid at pH 5. The cells were grown until reaching the mid-exponential phase (OD600 of 2–2.5) and serially transferred into fresh medium using 0.5% of the inoculum in biological triplicates. The selection pressure (0–5 g/L acetic acid at pH 5) was increased when the cell density of the evolving cultures increased. After 13 rounds of subcultures, the 58 largest colonies were isolated. The cell growth of these isolated variants was first evaluated using the TECAN Infinite Pro 200 system (Tecan Group Ltd.), followed by screening in 3 ml of CSM medium with 20 g/L xylose and 2 g/L acetic acid in 15 ml culture tubes. Finally, the best performing strain, XUSAE57, according to the highest xylose consumption rate and ethanol yield under acetic acid stress, was selected in microaerobic fermentation experiments using serum bottles (Figure S1).

2.3 | Lignocellulosic hydrolysate preparation

Sugarcane feedstock (obtained from the University of Florida) was processed for the preparation of lignocellulosic hydrolysates (Ko et al., 2018). For the pretreatment, 37 g of dry sugarcane bagasse was soaked in 250 ml of a 1% (w/v) dilute sulfuric acid solution in screw-capped bottles and autoclaved at 121°C for 30 min. The pH of the pretreatment slurries was adjusted to 5 with 1 M sodium hydroxide. The enzymatic hydrolysis was performed by adding 15 FPU/g glucan of Cellic CTec2 (Novozyme; North America Inc.) at 50°C for 96 hr. The liquid fraction was separated from the whole slurries and filter-sterilized before fermentation. The sugarcane hydrolysate was composed of 34 g/L glucose, 32 g/L xylose, 3.1 g/L acetic acid, and 0.7 g/L phenolics.
2.4 Analytical methods

The determination of glucose, xylose, and acetic acid concentrations was performed by high-performance liquid chromatography using a Hi-Plex H column (Agilent Technologies) maintained at 65°C. The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6 ml/min. For ethanol analysis, a gas chromatography system (Model 6890; Agilent Technologies) was used, which was equipped with a flame ionization detector and a 30 m × 0.25 μm × 0.25 μm HPINNOWax polyethylene glycol column.

For transcriptomic comparison of the XUSE and XUSAE57 strains, RNA-sequencing analyses were performed using tools from the commercial RNA-Seq service Ebiogen, Inc. After 16 hr of microaerobic fermentations with 20 g/L xylose under 4 g/L acetic acid stress reaching an OD₆₀₀ of 0.4, the yeast cells were harvested by centrifugation at 500 × g and 4°C for 5 min. Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The purity and integrity of each total RNA sample were assessed according to the 28S/18S ratio and RNA integrity number measured on the 2100 Bioanalyzer system (Agilent Technologies). The cDNA library was generated using the Clontech SMARTer Stranded RNA-Seq kit (Clontech). High-throughput sequencing was performed on an Illumina HiSeq 2500 system (Illumina, Inc.).

3 RESULTS

3.1 Xylose fermentation ability of the xylose isomerase-based strain under acetic acid stress

To improve the xylose fermentation and biofuel production of XUSE, we first examined the effect of acetic acid on its xylose fermentation performance in the presence of toxic levels of acetic acid with a low initial cell density (Figure 2). During 96 hr of microaerobic fermentation, XUSE utilized more than 83.5% of the initial xylose present in the medium buffered at pH 5; however, the extent of xylose utilization drastically dropped to 40% when the pH was not controlled. Moreover, the xylose utilization of XUSE was markedly inhibited with increasing acetic acid concentrations, suggesting that acetic acid stress should be relieved, even at low concentrations. Under 1 g/L acetic acid stress, the xylose utilization decreased from 83.5% (0 g/L, pH controlled) to 19.2% while the ethanol yield slightly decreased from 0.42 to 0.39 g/g. Ethanol yield decreased from 0.43 to 0.26 g/g with increasing acetic acid concentrations from 0 to 5 g/L. At the highest acetic acid concentration of 5 g/L, the yeast showed less than 5% xylose consumption with a poor ethanol yield of 0.26 g ethanol/g xylose, indicating the need for further attempts toward acetic acid tolerance improvement while maintaining efficient xylose utilization.

3.2 Adaptive laboratory evolution enhances the bioconversion efficiency of xylose under acetic acid stress

An adaptive laboratory evolution approach was employed to improve the acetic acid tolerance in the engineered xylose-fermenting yeast. Specifically, cells were transferred in fresh medium 13 times with increasing acetic acid concentrations, and 58 isolates were screened by evaluating their cell growth on xylose under acetic acid stress (Figure S1). The tolerance of XUSE toward acetic acid clearly improved by adaptive laboratory evolution, and strain XUSAE57 showed the best performance among the obtained evolved strains. Interestingly, XUSAE57 showed substantially (2.5-fold) higher xylose consumption efficiency than the parental strain XUSE even when the pH was not controlled (Figure 3). The reduction in the xylose consumption of XUSE in the absence of buffer might be caused by the pH drop due to the formation of acetic acid as a by-product during fermentation. After 96 hr fermentation, 0.5 g/L of acetic acid was produced with decreasing media pH from 5.0 to 3.4 in the absence of buffer (data not shown). When pH was controlled, the difference between the xylose consumption of XUSE and XUSAE57 was not significant suggesting the evolution toward more efficient xylose utilization seems to be minimal. XUSAE57 produced more ethanol than XUSE in the presence of acetic acid, resulting in higher ethanol yields (0.43–0.47 g/g vs. 0.22–0.29 g/g; Figure 4). Under 5 g/L of acetic acid stress, the xylose consumption of XUSAE57 was 2.5-fold higher than that of XUSE along with significant improvement in fermentation in terms of ethanol yield. In contrast to the decrease in the conversion of xylose into ethanol by XUSE from 0.43 to 0.22 g/g, the ethanol yield
Next, we evaluated the phenotypic differences between XUSE and XUSAE57 under 4 g/L acetic acid stress depending on the initial cell density (Figure 5), since fermentation under a high-cell-density fermentation is beneficial for achieving high productivity and yield at the industrial scale for growth-associated products (Kleman & Strohl, 1992). There was hardly any xylose consumed by XUSE at a low initial cell density (OD$_{600} = 1$) in the presence of 4 g/L acetic acid. However, under the same condition, the xylose consumption by XUSAE57 was increased by 5.1-fold when compared to that of XUSE, although the amount of xylose consumed was still extremely low (15.9% of the initial xylose), indicating inefficient fermentation performance (Figure 5a). However, with fermentation under a high cell density (OD$_{600} = 10$), XUSAE57 utilized 70% of the initial xylose within 96 hr, while XUSE still consumed less than 20%. Furthermore, the ethanol yield by XUSAE57 reached 0.5 g/g, which is equivalent to 98% of the maximum theoretical yield of 0.51 g/g, representing a five-time production increase compared to XUSE (Figure 5b,c). Specifically, the evolved strain XUSAE57 displayed a further increase in ethanol yield from 0.43 to 0.50 g/g under high-density fermentation compared to that produced under a low cell density, further supporting the benefit of high-cell-density fermentation under acetic acid stress conditions.

To dissociate the effects of decreased pH and acetic acid stress on the bioconversion of xylose into ethanol, we exposed the two strains to different levels of acetic acid-induced stress by changing the pH of the fermentation media. Specifically, fermentation experiments were performed in medium buffered at pH 6.5, in which the concentration of an undissociated form of acetic acid ($pK_a = 4.7$) at relatively high pH would have a relatively lower inhibitory effect on the cells. At pH 5, XUSAE57 showed a 5.8-fold higher xylose consumption rate (0.23 g L$^{-1}$ hr$^{-1}$; 0–48 hr) under 2 g/L acetic acid stress when compared to that of XUSE (0.04 g L$^{-1}$ hr$^{-1}$; 0–48 hr).
0–48 hr; Figure 6a,b). Of note, XUSAE57 converted xylose to ethanol with a yield of 0.46 g/g, which was 1.9-fold higher than that of XUSE (0.24 g/g) under 2 g/L acetic acid stress. At pH 6.5, almost all of the xylose was utilized by XUSAE57 within 48 hr at 0.41 g L⁻¹ hr⁻¹, which was two times higher than that of XUSAE57 at pH 5. Although the xylose consumption rate of XUSE was also enhanced at a higher pH, 65% of the initial xylose still remained after 120 hr of fermentation. Thus, for both strains, increasing the medium pH markedly enhanced the xylose consumption efficiency in a shorter time; however, the improvement was more evident with the evolved strain XUSAE57.

3.3 | Bioethanol production from lignocellulosic hydrolysates

The ability of the strains to convert lignocellulosic hydrolysates into ethanol was examined to compare their potential as practical hosts for the production of biofuels and chemicals in a lignocellulosic biorefinery. Microaerobic fermentation with non-detoxified sugarcane bagasse hydrolysates containing 2.5 g/L acetic acid was performed with a high cell density (OD₆₀₀ = 20) at pH 5 and 6.5. As shown in Figure 7, both strains rapidly utilized all of the glucose (about 25 g/L) within 10 hr with the simultaneous consumption of a significant amount of xylose (13–18 g/L), even in the presence of 3 g/L of acetic acid. Throughout fermentation, the acetic acid concentrations in the growth media reached up to 5–6 g/L as by-products of the reactions. In the early phase of fermentation (0–10 hr), xylose was consumed very quickly by both XUSE and XUSAE57 (1.3 and 1.8 g L⁻¹ hr⁻¹, respectively) at pH 5. Although the rate markedly dropped to 0.08 g L⁻¹ hr⁻¹ subsequently (10–96 hr) once glucose was depleted, XUSAE57 utilized more xylose (5.5 g/L) than XUSE.

The fermentation performances of XUSE and XUSAE57 at pH 6.5 were similar with those at pH 5. However, at higher pH, almost all of the glucose and xylose present in the sugarcane bagasse hydrolysates were utilized by XUSAE57 within 72 hr, with more evident improvement in xylose utilization than observed for XUSE. Thus, a higher pH is more favorable for the bioconversion of xylose during the fermentation of lignocellulosic hydrolysates by XUSZE57. Overall, XUSAE57 showed better fermentation performance of lignocellulosic hydrolysates with a higher specific xylose consumption rate (0.88 g L⁻¹ hr⁻¹; 0–24 hr) and ethanol yield (0.49 g/g) than XUSE (0.74 g L⁻¹ hr⁻¹; 0.46 g/g).

3.4 | Global transcriptome of the engineered and evolved strains under acetic acid stress

RNA-sequencing (Figure S2a) showed that the evolved strain XUSAE57 had reduced transcriptional levels of genes involved in the tricarboxylic acid cycle, electron transport chain, and oxidative phosphorylation, which produce massive amounts of energy in the form of ATP (Figure S2b), during fermentation under 4 g/L acetic acid. Meanwhile, genes involved in the plasma membrane H⁺-ATPase (PMA1), in coordination with those encoding proteins in the vacuolar membrane H⁺-ATPase complex (e.g., VMA1, VMA2, VMA4-8, and VMA22), were downregulated or not significantly changed (Figure S2a) compared to those of XUSE under acetic acid stress.
4 | DISCUSSION

A current challenge for the commercialization of lignocellulosic fuels and chemicals by the major microbial platform strain *S. cerevisiae* is to improve the low efficiency in the bioconversion of all available sugars derived from the biomass. This limitation is mainly due to the suboptimal xylose utilization capability and hindrance on cell performance by inhibitory compounds present in the biomass and produced as by-products during fermentation (Hasunuma & Kondo, 2012). In particular, the toxic effect of such inhibitors is more severe on engineered strains with xylose utilization capability, resulting in decreased cell growth, sugar utilization efficiency, and ethanol yield (Casey, Sedlak, Ho, & Mosier, 2010; Ko, Um, & Lee, 2016). Among the inhibitors present in lignocellulosic hydrolysates, acetic acid shows a preeminent effect with inevitable involvement during fermentation owing to the strong association with sugar polymers in lignocellulosic biomass. Here, we showed that upon exposure to low concentrations of acetic acid such as 1 g/L, the xylose utilization by XUSE, one of the best engineered xylose-utilizing strains developed to date, decreased by 77%. Moreover, the ethanol yield slightly decreased from 0.42 to 0.39 g/g with 1 g/L of acetate, and then markedly dropped to 0.22 g/g under more severe acetic acid stress. These significant decreases in the xylose utilization rate and ethanol yield of XUSE due to acetic acid are in accordance with a previous study showing a sensitive response to acetic acid in recombinant xylose-utilizing yeast strains (Ko, Um, & Lee, 2016).

To overcome this barrier, we developed the acetic acid-tolerant xylose-utilizing strain of *S. cerevisiae*, XUSAE57, through the adaptive laboratory evolution of XUSE. XUSAE57 showed significant improvement in ethanol yield and xylose utilization rates during fermentation under various acetic acid stress conditions, producing up to 8.9 g/L of ethanol from 20 g/L of xylose in the presence of 4 g/L of acetic acid under high-cell-density fermentation with an ethanol yield of 0.49 g/g. Both the xylose consumption rate and ethanol titer of the evolved strain XUSAE57 were sevenfold higher than those of XUSE. Despite this higher tolerance to acetic acid and consequent improved ethanol production, the cell biomass production of XUSAE57 was not increased, suggesting that utilization of the carbon source is directed more efficiently toward ethanol production, which would be a promising feature as a production host for lignocellulosic bioethanol production.

Such an evolutionary engineering approach can therefore promote the practical reality of applications of an engineered yeast strain utilizing lignocellulosic hydrolysates in biofuels production at the industrial scale. Given its enhanced acetic acid tolerance, XUSAE57 showed 1.3- to 1.4-fold improvements in both the maximal (0–10 hr) and overall (0–96 hr) xylose consumption rates compared to its parental strain XUSE. Ultimately, XUSAE57 produced bioethanol with the highest yield reported to date (0.49 g/g), which is close to the theoretical maximum, among previously engineered strains during lignocellulosic hydrolysate fermentation (Table 1). For example, the SXA-R2P-E strain is one of the best co-fermenting strains with the highest ethanol yield reported until now, with maximum and overall xylose consumption rates of 0.34 g L⁻¹ hr⁻¹.
(2–24 hr) and 0.19 g L⁻¹ hr⁻¹ (0–96 hr), respectively, during lignocellulosic hydrolysates containing 1.0 g/L of acetic acid (Ko, Um, Woo, et al., 2016). The XUSAE57 strain fermented hydrolysates containing a higher amount of acetic acid (2.5 g/L) with 5.3- and 1.4-fold higher maximum (1.8 g L⁻¹ hr⁻¹) and overall (0.27 g L⁻¹ hr⁻¹) xylose consumption rates and higher ethanol yield (0.49 g/g) than those of SXA-R2P-E. Moreover, XUSEA57, harboring a cofactor-neutral isomerase-based xylose utilization pathway, showed simultaneous glucose and xylose consumption during lignocellulosic hydrolysate fermentation, strongly indicating that xylose metabolism is not subjected to the catabolic repression of glucose, which is a significant barrier to the successful utilization of mixed sugars in lignocellulosic hydrolysates (Kim, Ha, Wei, Oh, & Jin, 2012). These results therefore highlight the potential of isomerase pathway-based yeast strains as ideal hosts for a lignocellulosic biorefinery.

Complex cellular functions are involved in the mechanisms of the yeast response against acetic acid to confer protection from acetic acid-induced inhibition. Thus, a variety of engineering strategies for improving acid tolerance in yeasts has been employed (Chen et al., 2016; Gonzalez-Ramos et al., 2016; Meijnen et al., 2016; Zhang et al., 2017; Zhang, Zhao, Cheng, & Bai, 2015). Studies on identifying mechanisms involved in yeast stress response to acetic acid have also provided insights into tolerance engineering and offered novel candidate genes (Meijnen et al., 2016; Mira, Palma, Guerreiro, & Sa-Correia, 2010). The previous rational engineering involved the direct manipulation of known genetic factors associated with acid transcriptional regulation (HAA1, MSN2), metal metabolism (AFT1), purine biosynthesis (ADE1, ADE13, ADE17), plasma membrane ergosterol synthesis (ERG-genes and PDR18), and others (Chen et al., 2016; Godinho et al., 2018; Oh et al., 2019; Tanaka, Ishii, Ogawa, & Shima, 2012; Zhang et al., 2019). However, due to the complex trait of the acetic acid stress response and the limited understanding of coordinated regulations of multiple genes (Ling, Teo, Chen, Leong, & Chang, 2014), it is still challenging to discover a set of effective gene targets for efficient sugar fermentation under acidic stress via knowledge-based rational engineering approaches. Moreover, the strategies to improve acetate tolerance in glucose-fermenting strains could not be effectively applied to engineered glucose/xylose co-fermenting strains since inhibitory effect of acetic acid on xylose utilization in engineered strains has shown to be more severe requiring integrated approaches of enhancing acetate tolerance and xylose utilization. Therefore, this study employed an evolutionary engineering strategy to improve acetic acid tolerance in the engineered S. cerevisiae with superior xylose utilization performance. Notably, using an evolutionary engineering approach, we successfully obtained an evolved yeast strain, XUSAE57, tolerant to acetic acid by enhancing bioconversion of lignocellulosic biomass into bioethanol with significantly improved bioconversion rates and the highest yield ever reported.

### TABLE 1  Comparison of ethanol yields obtained from the lignocellulosic hydrolysate fermentation using recombinant xylose-metabolizing *Saccharomyces cerevisiae*

| Strains          | Biomass          | Pretreatment   | Initial sugar level (g/L) | Acetic acid (g/L) | Other inhibitors (g/L) | Ethanol yield (g/g) | Reference                              |
|------------------|------------------|----------------|---------------------------|------------------|------------------------|--------------------|----------------------------------------|
| XUSAE57          | Sugarcane bagasse| Dilute acid    | 26.2                      | 27.7             | 2.5                    | Phenolics, 0.8      | 0.49 (This work)                       |
| XUSE              |                   |                |                           |                  |                        |                    | 0.46                                   |
| SXA-R2P-E        | Rice straw       | Dilute acid    | 27.7                      | 20.2             | 1.0                    | Phenolics, 0.8; Furfural, 0.2 | 0.46 (Ko, Um, Woo, et al., 2016)       |
|                  | Oak              | Dilute acid    | 26.8                      | 16               | 6.1                    | Phenolics, 1.3; Furfural, 0.6 | 0.43                                   |
| PE-XI            | Corn cob         | Autohydrolysis | 1.2                       | 28.3             | 4.4                    | HMF, 0.17; Furfural, 1.4 | 0.24 (Cunha, Soares, Romani, Thevelein, and Domingues, 2019) |
| PE-XR/XDH        |                   |                |                           |                  |                        |                    |                                        |
| CA11-XR/ XDH + XI|                   |                |                           |                  |                        |                    |                                        |
| GS1.11–26        | Spuce            | SO₂ steam-explosion | 48.9                  | 10.1             | 4.8                    | HMF, 1.1; Furfural, 1.6 | 0.43 (Demeke et al., 2013)               |
| 424A (LNH-ST)    | Poplar           | Dilute acid    | 41.4                      | 22.3             | 5.1                    | NA                 | 0.43 (Lu, Warner, Sedlak, Ho, and Mosier, 2009) |
|                  |                  |                |                           |                  |                        |                    | 0.46 (Lu et al., 2009)                  |
| Poplar           | SO₂ steam-explosion | 33.2              | 25.8                      | 6.2              | NA                    |                    |                                        |
| Corn fiber       | Dilute acid      | 41.7           | 39.9                      | 3.2              | NA                    |                    | 0.44 (Sedlak and Ho, 2004)              |
Based on the prior knowledge, the stressed cells employ an energy-intensive mechanism to maintain the intracellular pH homeostasis by pumping out the excess protons through membrane H⁺-ATPases at the expense of ATP, which ultimately compromises cell growth (Palma, Guerreiro, & Sa-Correia, 2018; Pamplula & Loureiro-Dias, 2000; Ullah, Chandrasekaran, Brul, & Smits, 2013). However, the RNA-sequencing results did not support the previously hypothesized molecular mechanisms of acetic acid detoxification in the acetic acid-stressed and evolved *S. cerevisiae* cells. Although the transcriptomic analysis showed limited differences in the overall gene expression profiles of XUSAE57 and XUSE, XUSAE57 seemed to switch the mode of metabolism from aerobic respiration to fermentation when growing under acetic acid stress, leading to a reduction in cell growth and increase in ethanol production.

Consequently, the evolved strain obtained in this study is compatible with industrial processes that involve acetic acid-containing hydrolysates along with other inhibitors such as phenolic compounds, and thus shows great potential as a microbial platform in a lignocellulosic biorefinery. The acquired novel stress tolerance of the evolved strains is not always expressed when the selective pressure is alleviated or changed (Mans, Daran, & Pronk, 2018; Wright et al., 2011). However, our evolved strain constitutively maintained its high performance of xylose fermentation in acetic acid-free medium, highlighting its robustness under various conditions. Moreover, the engineerability of XUSEA57, which was developed using a marker-free CRISPR-Cas system, opens up the possibility for this strain to serve as a platform host not only for bioethanol but also for other biofuels and the production of biochemicals from lignocellulosic biomass.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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