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ORIGINAL RESEARCH

A C6/C5 co-fermenting *Saccharomyces cerevisiae* strain with the alleviation of antagonism between xylose utilization and robustness

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Abstract

During second-generation bioethanol production from lignocellulosic biomass, the desired traits for fermenting microorganisms, such as *Saccharomyces cerevisiae*, are high xylose utilization and high robustness to inhibitors in lignocellulosic hydrolysates. However, as observed previously, these two traits easily showed the antagonism, one rising and the other falling, in the C6/C5 co-fermenting *S. cerevisiae* strain. In this study, LF1 obtained in our previous study is an engineered budding yeast strain with a superior co-fermentation capacity of glucose and xylose, and was then mutated by atmospheric and room temperature plasma (ARTP) mutagenesis to improve its robustness. The ARTP-treated cells were grown in 50% (v/v) leachate from lignocellulose pretreatment with high inhibitors content for adaptive evolution. After 30 days, the generated mutant LF1-6 showed significantly enhanced tolerance, with a six-fold increase in cell density in the above leachate. Unfortunately, its xylose utilization dropped markedly, indicating the recurrence of the negative correlation between xylose utilization and robustness. To alleviate this antagonism, LF1-6 cells were iteratively mutated with ARTP mutagenesis and then anaerobically grown using xylose as the sole carbon source, and xylose utilization was restored in the resulting strain 6M-15. 6M-15 also exhibited increased co-fermentation performance of xylose and glucose with the highest ethanol productivity reported to date (0.525 g g^{-1} h^{-1}) in high-level mixed sugars (80 g L^{-1} glucose and 40 g L^{-1} xylose) with no inhibitors. Meanwhile, its fermentation time was shortened by 8 h compared to that of LF1. During the fermentation of non-detoxified lignocellulosic hydrolysate with high inhibitor concentrations at pH ~3.5, 6M-15 can efficiently convert glucose and xylose with an ethanol yield of 0.43 g g^{-1}. 6M-15 is also regarded as a potential chassis cell for further design of a customized strain suitable for production of second-generation bioethanol or other high value-added products from lignocellulosic biomass.

Fangqing Wei and Menglei Li contributed equally to this work.

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KEYWORDS

anaerobically breeding, antagonism, ARTP, inhibitor tolerance, lignocellulose bioconversion, *Saccharomyces cerevisiae*, xylose utilization

1 | INTRODUCTION

Lignocellulosic biomass is the most abundant renewable energy resource on earth with an annual production of more than a trillion tons (Sun, & Chen, 2007). With the gradual depletion of fossil fuels, the conversion of lignocellulosic biomass into biofuels and chemicals via microbial fermentation is considered promising as a sustainable alternative to already established petroleum-based processes (de Paula et al., 2019; Liu et al., 2019). Generally, lignocellulosic biomass is difficult to be utilized directly through microbial processes because of its stubborn resistance to degradation. Therefore, to release the available sugars for microbial utilization, pretreatment and hydrolysis of lignocellulose are generally required (Abo et al., 2019; Hasunuma et al., 2013). *Saccharomyces cerevisiae* is regarded as one of the most promising industrial agents for converting lignocellulosic hydrolysates into biofuels, such as bioethanol, because of its powerful fermentation capacity, robustness, and well-developed genetic tools (Borodina & Nielsen, 2014; Ko & Lee, 2018; Lian et al., 2018). In second-generation bioethanol production using lignocellulosic biomass, the desired traits of high xylose utilization and robustness to inhibitors present in lignocellulosic hydrolysates are essential for any promising *S. cerevisiae* strains for biorefining. The economic feasibility of lignocellulose biorefining is mainly challenged by the difficulty of achieving complete bioconversion of all degraded sugars, especially pentoses such as xylose. Indeed, *S. cerevisiae* does not naturally utilize xylose (the second most abundant sugar in lignocellulosic hydrolysates) because of the lack of an upstream module of the xylose metabolic pathway. Extensive efforts have been made to improve the capacity of xylose metabolism, including xylose reductase and xylitol dehydrogenase or xylose isomerase (XI) pathway engineering, cofactor engineering, transporter engineering, evolutionary engineering, and other related metabolic engineering (Farwick et al., 2014; Ho et al., 1998; Hou et al., 2014; Karhumaa et al., 2007; Matsushika et al., 2010; Watanabe et al., 2007). In our previous study, recombinant *S. cerevisiae* LF1 was constructed with efficient xylose catabolism by combining rational metabolic engineering and evolutionary engineering based on higher activity XI from rumen metagenome and a specific xylose transporter MGT05196N360F from *Meyerozyma guilliermondii* (Hou, Shen, et al., 2016; Li et al., 2016; Wang et al., 2015). This strain was able to completely consume high-level mixed sugars (80 g L^{-1} glucose and 40 g L^{-1} xylose) within 20 h to achieve an ethanol yield of 0.48 g g^{-1} total sugars.

Although the current recombinant *S. cerevisiae* strains have excellent fermentation capacities for xylose in synthetic medium, they showed lower conversion efficiencies of lignocellulosic hydrolysates because of the effects of multiple inhibitors generated during the pretreatment process (Jönsson et al., 2013). Among these inhibitors, acetic acid, a major inhibitor of lignocellulosic hydrolysates, severely inhibits cell growth and reduces sugar metabolism by destroying membrane integrity and intracellular redox homeostasis, especially inhibiting xylose utilization (Bellissimi et al., 2009; Ko et al., 2016). Similar to acetic acid, other inhibitors such as hydroxymethylfurfural (HMF), furfural, and phenols also reduce microbial fermentation efficiency through different mechanisms (Jönsson et al., 2013). In the past decade, numerous rational strategies, including the genetic manipulation of related oxidoreductase genes, transcription factors, and modulation of the expression of the purine biosynthesis pathway, have been effectively applied to improve individual inhibitor tolerance (Chen et al., 2016; Liu et al., 2008; Moon & Liu, 2012; Oh et al., 2019; Tanaka et al., 2012; Zhang et al., 2019). However, these rational strategies were frequently insufficient in providing the yeast phenotypes with simultaneous tolerance of various inhibitors attributed to different inhibitory mechanisms. In particular, a mixture of several inhibitors can impose a higher toxicity on cells, even at low concentrations because of synergistic inhibitory effects (Chen et al., 2019; Jönsson & Martín, 2016; Palmqvist et al., 1999). Therefore, irrational strategies, including random mutagenesis, adaptive evolutionary engineering and genome shuffling, were considered as efficient approaches to improve cellular tolerance to multiple inhibitors (Mans et al., 2018; Smith et al., 2014). However, to our knowledge, most of the current irrational strategies still focus on the improvement of individual inhibitor tolerance of yeast strains in lignocellulosic hydrolysates (Giannattasio et al., 2013; Palma et al., 2018).

In contrast to adaptive evolution over a long time, physical and chemical mutagenesis provide enormous scope for genetic mutation over a short period. Atmospheric and room temperature plasma (ARTP) is currently emerging as a powerful mutagenesis approach for microbial breeding because it is safe, fast, and has high operational flexibility compared with conventional random mutagenesis methods such as ultraviolet (UV) and chemical mutagenesis (Zhang et al., 2014). ARTP has been widely used for the phenotypic improvement

of production efficiency, growth rate, and toxicity tolerance in bacteria, fungi, and plants (Zhang et al., 2015). However, despite the power of ARTP, no single approach has emerged to achieve broad desired phenotypes. Therefore, the desired mutant may be obtained by combining several strategies matched with effective screening methods for special phenotypes, or an efficient strategy may be repeatedly carried out to accumulate positive mutations.

Previously, to reduce the effects of inhibitors on microbial fermentation efficiency, detoxification and/or pH adjustment are generally performed on lignocellulosic hydrolysates prior to fermentation, which will increase production costs. Therefore, directly performing microbial fermentation of lignocellulosic hydrolysates without any prior treatment is an aspirational goal for researchers. It is essential to simultaneously improve xylose utilization and tolerance to multiple inhibitors of *S. cerevisiae* strains. However, the antagonism between high xylose utilization and robustness was observed in the reported xylose-utilizing strains (Demeke et al., 2013; Koppram et al., 2012; Li et al., 2016). In this study, by combining iterative ARTP mutagenesis and anaerobic screening, the resulting strain 6M-15 with significantly enhanced tolerance to multiple inhibitors was achieved based on our previous strain LF1, one of the best glucose/xylose co-fermentation strains (Hoang Nguyen Tran et al., 2020; Li et al., 2016). Simultaneously, xylose fermentation performance was further improved in the inhibitor-tolerant strain with alleviated antagonism of high xylose utilization and robustness. The 6M-15 strain exhibited excellent fermentation performance in non-detoxified lignocellulosic hydrolysates. Consequently, the strategies applied in this work provide a promising route to develop a robust *S. cerevisiae* strain for economically feasible production of biofuels under harsh fermentation conditions. This approach helps match microbial phenotypes with process demands in industrial production.

2 | MATERIALS AND METHODS

2.1 | Strains, medium, and cultivation

The *S. cerevisiae* strain LF1, one of the best industrial strains with a superior co-fermentation capacity of glucose and xylose, was constructed in our previous study, and served as the original strain in this study (Li et al., 2016). Yeast extract peptone (YP) medium composed of 10 g L^{-1} yeast extract and 20 g L^{-1} peptone was used. The yeast extract peptone xylose (YPX) and yeast extract peptone dextrose xylose (YPDX) media were produced from YP medium supplemented with 40 g L^{-1} xylose and a mixture of 80 g L^{-1} glucose and 40 g L^{-1} xylose, respectively. The leachate media were produced from YP medium by adding different proportions of leachate and were used for the screening and fermentation tests of the inhibitor-tolerant strains. The corresponding solid medium was prepared by adding 20 g L^{-1} agar. Generally, except for special instructions, the strains were cultivated at 30°C and 200 rpm in 120-ml serum bottles with working volume of 40 ml. Oxygen-limited conditions were maintained using a rubber stopper with a syringe needle to allow carbon dioxide release.

2.2 | Composition analysis and toxicity test of the leachates from three pretreatment samples of corn straw

Three pretreatment samples of corn straw were provided by three companies (named A, B, and C). The corresponding leachate was obtained according to the following process. Deionized water was added to 150 g of dry weight equivalent of the pretreated corn straw sample to a volume of 500 ml. The mixture was warmed at 80°C in a water bath for 1 h to dissolve the degradation products including various sugars and inhibitors generated during the pretreatment process (Sun & Chen, 2007). The mixture was filtered through four layers of gauze to remove solids. The resulting liquor was corn straw pretreatment leachate as used in this study. The leachate was centrifuged at 13,800 *g* for 5 min to further remove residual solids. Subsequently, the components, including the main sugars and inhibitors, were quantified using high-performance liquid chromatography (HPLC). To test the toxicity of the three leachates, LF1 was cultivated in 1 ml of the YPX medium containing different proportions of leachate (10–50%, v/v) at an initial OD_{600} (optical density at 600 nm) of 0.1. The cell growth was monitored using a microplate reader (Biotek Instruments Inc.), and the variable OD_{600} value reflected the degree of inhibition imposed by the leachate.

2.3 | ARTP mutagenesis and adaptive evolution

Saccharomyces cerevisiae cells were grown through the log phase, collected, and washed twice with sterile water. The cells were suspended in sterile water to obtain an OD_{600} value of 0.6–0.8. The cell suspension (10 μl) was spread on a coated metal slide and irradiated (0–60 s) in a Type M ARTP Mutagenesis Bio-breeding Machine (Wuxi Tmaxtree Biotechnology Co., Ltd.) to evaluate the lethal rate. The radio-frequency power input was set at 120 W, and the jet temperature was controlled at 22°C. High-purity helium gas served as the working gas at a flow rate of 10 standard liters per minute (SLM). Based on the lethal-rate curve, the treatment time was set at 15 s for subsequent experiments. ARTP-treated cells were spread on YP medium plates

containing 60% (v/v) leachate to screen the inhibitor-tolerant mutant strains. Colonies with relatively large diameters were selected to verify the tolerant phenotype in 50% (v/v) leachate medium using the BioScreen system (Oy Growth Curves Ab Ltd), and the verified strains were repeatedly tested in shake flasks. Adaptive evolution was alternately performed for the strain with the highest inhibitor tolerance, through a round of ARTP mutagenesis, in 100-ml serum bottles containing 20 ml of YPX medium (40 g L^{-1} xylose) or YP medium with 50% (v/v) leachate at 30°C with stirring at 200 rpm. The culture was sampled every 2 h according to the growth state of strain, and cell growth was monitored by measuring OD_{600}. Once the stationary growth phase was reached, the culture was transferred into a fresh medium with an initial OD_{600} of 0.05. The resulting colony with the highest OD_{600} value was applied in the next round of ARTP mutagenesis. ARTP-treated cells with inhibitor-tolerant phenotype were cultivated on YPX medium plates placed in a simple anaerobic bag to maintain anaerobic conditions. The colonies with relatively large diameters were selected for further investigation of xylose utilization capacity under oxygen-limited conditions.

2.4 | Stress tolerance assays

The stress tolerance of yeast was evaluated by a spot assay. The strains were cultivated at 30°C in the YPX medium overnight, and the broth was adjusted to an OD_{600} reading of 0.5 with sterile water. According to the tolerance pretest of different concentrations inhibitors, tenfold diluted suspensions (4 μl) were spotted on YPX plates containing 6 g L^{-1} acetic acid, 1.5 g L^{-1} furfural, 1.5 g L^{-1} HMF, 1.0 g L^{-1} vanillin, and mixed inhibitors (0.6 g L^{-1} acetic acid, 0.63 g L^{-1} HMF, 0.48 g L^{-1} Furfural, and 0.76 g L^{-1} vanillin; Wimalasena et al., 2014).

2.5 | Batch fermentation

The seed culture was prepared by activating cells twice in the YPX medium for all batch fermentations. YPX medium containing 40 g L^{-1} xylose, YPDX medium containing 80 g L^{-1} glucose and 40 g L^{-1} xylose, and YP medium supplemented with 50% (v/v) leachate were used to investigate the fermentation performance of the final strain. Specifically, the 50% (v/v) leachate medium contained 6.8 g L^{-1} glucose and 21.5 g L^{-1} xylose, and an inhibitor mixture of 1.8 g L^{-1} acetic acid, 1.3 g L^{-1} HMF, 1.7 g L^{-1} phenol compounds, and 0.15 g L^{-1} furfural at pH ~3.0. The corn straw hydrolysate provided by company B contained 50 g L^{-1} glucose, 25 g L^{-1} xylose, and a mixture of inhibitors (4.3 g L^{-1} acetic acid, 1.0 g L^{-1} HMF, 4.78 g L^{-1} phenol compounds, and 0.35 g L^{-1} furfural) at pH 3.5. The oxygen-limited fermentation of leachate and hydrolysate was carried out in a working volume of 40 ml at a low cell density with an initial OD_{600} value of 0.2 or at a high cell density with an initial OD_{600} value of 10.

2.6 | Analytical methods

The concentrations of glucose, xylose, acetic acid, and ethanol were determined using an HPLC (Waters e2695) with an Aminex HPX-87H ion exchange column (300 × 7.8 mm; Bio-Rad) at 35°C, and a Waters 2414 refractive index detector. Sulfuric acid (5 mM) was used as the mobile phase at a flow rate of 0.6 ml min^{-1}. The concentrations of the inhibitors, including HMF and furfural, were determined using HPLC with a WondaSil C18 column (4.6 × 250 mm, 5 μm; Shimadzu) at 40°C and a PDA-2998 UV detector (Waters) and 40% methanol as the mobile phase with a flow rate of 0.6 ml min^{-1}. Total phenolics were determined using the Folin phenol method as described in previous reports, and vanillin was used as a standard (Li et al., 2016).

The dry cell weight (DCW) was measured using a previously described method based on the relationship between OD_{600} and DCW (DCW g L^{-1} = 0.19 × OD_{600} − 0.0065). The linear regression coefficient of the plot of ln(OD_{600}) versus time during the exponential growth phase was taken as the maximum growth rate (μ_{max}). The specific consumption or production rates of glucose, xylose, and ethanol were calculated as previously described (Li et al., 2016; Peng et al., 2012). The ethanol yield was calculated based on the sugars consumed.

3 | RESULTS

3.1 | The growth of xylose-utilizing *S. cerevisiae* LF1 in the leachates of pretreated corn straw pretreated by different pretreatment methods

In our previous study, the engineered *S. cerevisiae* LF1, reported as one of the best xylose-utilizing strains, was constructed using rational metabolic and adaptive evolution engineering methods. It was able to consume 80 g L^{-1} glucose and 40 g L^{-1} xylose in a synthetic medium within 20 h to produce an ethanol yield of 0.48 g g^{-1} total sugars (Li et al., 2016). Therefore, LF1 is considered a great potential host for second-generation bioethanol production. In this study, we first investigated the growth of LF1 in the leachates of three corn straw pretreatments provided by different companies in China. The three leachates were named A, B, and C, and their chemical compositions are listed in Table 1. Xylose accounted for the highest proportion of total sugars, which

TABLE 1 Concentrations of main sugars and inhibitors in different leachates of pretreated corn straws from different sources

	A	B	C
Main sugars (g L^{-1})			
Glucose	0.66 ± 0.09	13.56 ± 0.47	1.28 ± 0.09
Xylose	1.26 ± 0.17	42.91 ± 0.45	11.8 ± 0.43
Cellobiose	0.38 ± 0.08	0.51 ± 0.14	0.38 ± 0.04
Galactose	0.32 ± 0.02	0.83 ± 0.11	1.50 ± 0.22
Arabinose	0.40 ± 0.13	2.09 ± 0.33	2.30 ± 0.31
Main inhibitors (g L^{-1})			
Acetic acid	4.60 ± 0.05	3.46 ± 0.18	0.21 ± 0.01
HMF	1.86 ± 0.03	2.70 ± 0.18	0.92 ± 0.13
Furfural	0.28 ± 0.01	0.31 ± 0.06	0.14 ± 0.02
Total phenol	4.0 ± 0.09	3.28 ± 0.12	2.04 ± 0.11
pH	1.98	1.52	4.5

Abbreviation: HMF, hydroxymethylfurfural.

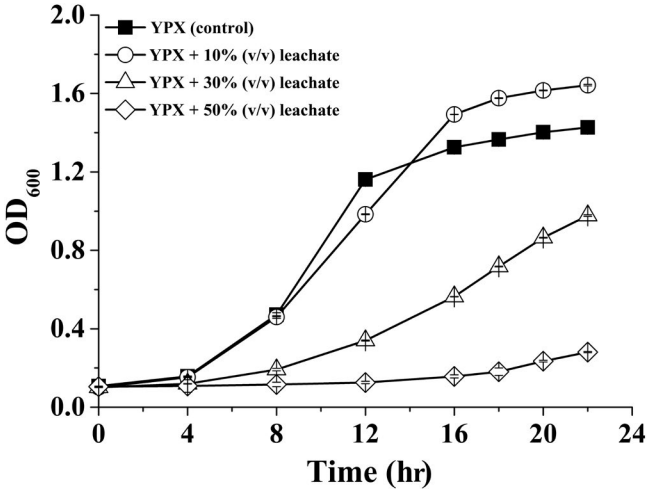

FIGURE 1 Inhibition performance on strain LF1 in YPX medium with different proportions of leachates of pretreated corn straw from company B, China. The strains were cultivated in a BioTek microplate reader containing 1 ml medium with an initial OD_{600} of 0.1 for 24 h at 200 rpm, 30°C. The error bars represent the standard deviation of biological triplicates

indicates that the hemicellulose component of corn straw was preferentially depolymerized through the three pretreatment methods. Leachates A and B contained high levels of inhibitors at low pH, while leachate C contained a low level of inhibitors and low levels of sugars. Among the inhibitors, acetic acid was the most abundant, followed by phenolic compounds and HMF in leachates A and B. The chemically defined medium, supplemented with the different proportions (v/v) of leachate, was designed to test the toxicity of the three leachates on LF1. The results showed that the inhibition of cell growth was more serious as the proportion of leachate in the medium increased. As shown in Figure 1 and Figure S1, cell growth was completely inhibited in the presence of 30% (v/v) leachate A and 50% (v/v) leachate B, respectively, but only had a minor effect in the presence of 50% (v/v) leachate C. Thus, the toxicity of the three leachates on cells was ranked in the order of A > B > C. Inhibitors that are mainly generated during lignocellulose pretreatment processes seriously inhibit cell growth and microbial fermentation efficiency, resulting in the decreased economic feasibility of second-generation bioethanol production. Hence, there is an urgent need to develop strains that are robust against this inhibition. Given that leachate B contained high levels of sugars and moderate levels of inhibitors, it was selected as the optimum feedstock for improving the tolerance of LF1 to inhibitors in this study.

3.2 | Improving inhibitor tolerance of xylose-utilizing *S. cerevisiae* via ARTP mutagenesis but reducing xylose consumption

To improve the inhibitor tolerance of LF1, we aimed to generate a mutant collection of LF1 via ARTP mutagenesis. Prior to mutagenesis, the optimal lethal rate of ARTP was determined. A high lethal rate was achieved using a radio-frequency power input of 120 W and a flow rate of 10 SLM, resulting in a cellular lethal rate of 97.2% at 15 s and no cell survival over 45 s (Figure S2). Generally, a lethal rate of 90%–99% is considered appropriate (Wu et al., 2019). Therefore, LF1 cells were treated with ARTP at 120 W and 10 SLM for 15 s at room temperature.

To enrich positive mutations, the ARTP-treated cells were adaptively evolved in 50% (v/v) leachate, and then spread on YP plates containing 60% (v/v) leachate as a selective pressure. Then, a set of larger colonies were cultivated in 50% (v/v) leachate medium to screen mutants with improved inhibitor tolerance in the BioScreen system (Figure S3). To further verify the tolerance dependability of the mutants, three mutants with high growth rates were repeatedly cultivated under the same leachate stress. Finally, mutant LF1-6, which had the highest growth rate and tolerance stability, was selected. Subsequently, to investigate whether ARTP mutagenesis influenced xylose metabolism capacity, LF1-6 was cultivated with pure xylose as the sole carbon source under oxygen-limited conditions. As shown in Figure 2b–d, xylose utilization of LF1-6 was decreased significantly, along with cell growth and ethanol production. The results showed an apparent antagonism between inhibitor tolerance and xylose utilization. It is noteworthy that this phenomenon was observed in xylose-utilizing strains before LF1 in our previous study. In addition, the antagonism between glucose utilization and xylose utilization appeared in our mixed sugar co-fermentation strains (Li et al., 2016). Therefore, we speculate that antagonism between different phenotypes frequently occurs during microbial breeding.

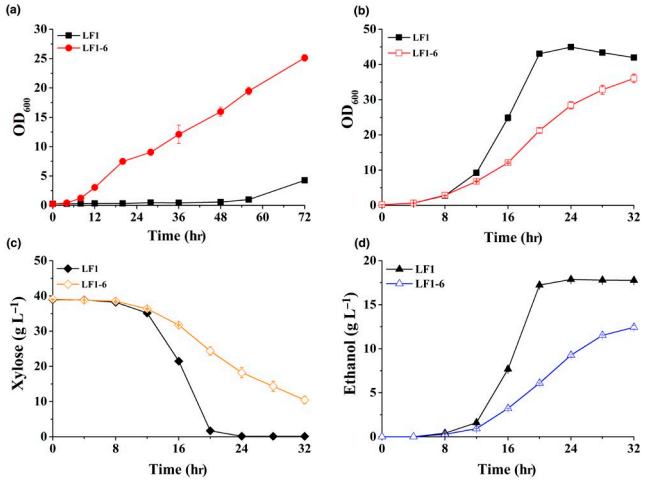

FIGURE 2 Oxygen-limited fermentation performance of the control strain LF1 and the mutant strain LF1-6 in YPX medium with/without 50% (v/v) leachate. (a) Growth of LF1 and LF1-6 in YPX medium with 50% (v/v) leachate. (b) Growth, (c) xylose consumption, and (d) ethanol production by LF1 and LF1-6 in YPX medium (40 g L^{-1} xylose). The strains were cultivated in serum bottles with an initial OD_{600} of 0.2 for 32 h at 200 rpm, 30°C. The error bars represent the standard deviation of biological triplicates

3.3 | Significantly alleviating the antagonism between robustness to inhibitors and xylose utilization using iterative ARTP mutagenesis combined with anaerobic screening

To rescue xylose utilization in the inhibitor-tolerant strain, we performed adaptive evolution on LF1-6. LF1-6 was alternately evolved in YPX medium (40 g L^{-1} xylose) and YP medium with 50% (v/v) leachate for 1 month. The final culture was spread on the YPX plate, and the 20 largest colonies were picked up for the evaluation of xylose fermentation capacity. However, the resulting mutant LF1-6M showed only slightly improved xylose utilization, which was still significantly lower than the initial strain LF1 (Figure 3b; Table 2), indicating that the antagonism between inhibitor tolerance and xylose utilization was still present. In addition, under aerobic conditions, the growth and xylose utilization of LF1-6M were equivalent to that of LF1 (Figure S4), indicating that the oxygen level may be a potential factor regulating this antagonism. Given that the oxygen level is limited in industrial ethanol production to avoid the reoxidation of ethanol, resulting in ethanol loss. Therefore, eliminating this antagonism is essential for improving ethanol production under oxygen-limited conditions.

To further alleviate this antagonism, anaerobic conditions were used as the screening pressure for mutant strains in subsequent work. The LF1-6M cells were treated by the second round of ARTP mutagenesis and then cultivated on xylose plates placed in an anaerobic environment. Through the evaluation of xylose

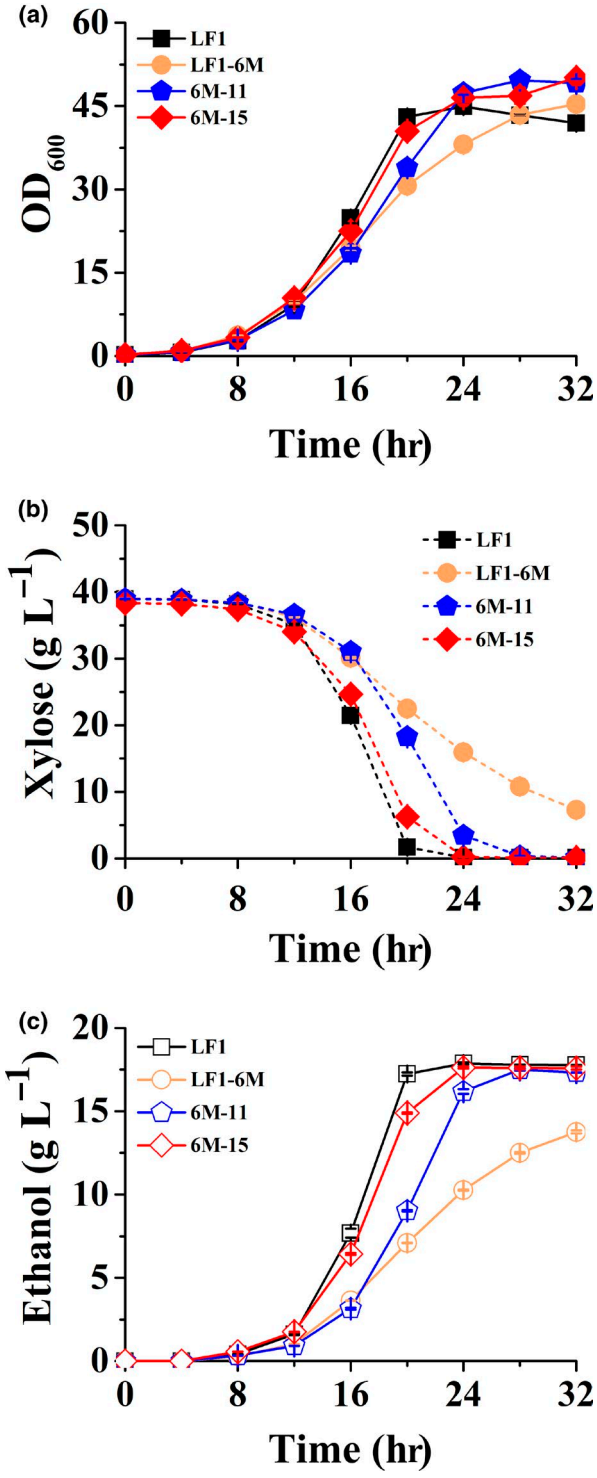

FIGURE 3 Oxygen-limited fermentation performance of the control strain LF1 and the mutant strains LF1-6M, 6M-11, and 6M-15 in YPX medium (40 g L^{-1} xylose). Growth (a), xylose consumption rate (b), and ethanol production (c) by the mutant strains increased gradually compared with the control strain. The strains were cultivated in serum bottles with an initial OD_{600} of 0.2 for 32 h at 200 rpm, 30°C. The error bars represent the standard deviation of biological triplicates

fermentation, the resulting 6M-11 showed an enhanced xylose consumption rate. As shown in Figure 3 and Table 2, 6M-11 completely consumed 40 g L^{-1} xylose

TABLE 2 Metabolic characteristics of the initial strain and inhibitor-tolerant *Saccharomyces cerevisiae* strains in different mediums

Strains	Medium[a]	Initial glucose (g L^{-1})	Initial xylose (g L^{-1})	Xylose conversion (%)	μ_{max}	Specific consumption or production rate (g g^{-1} DCW h^{-1})[b]		Ethanol yield (g g^{-1} sugar)[c]
						Xylose	Ethanol	
LF1	YPX	—	38.95	99.68	0.265	0.557	0.245	0.461
6M	YPX	—	39.02	81.30	0.177	0.304	0.122	0.434
6M-11	YPX	—	38.97	99.68	0.206	0.407	0.169	0.453
6M-15	YPX	—	38.33	99.66	0.268	0.533	0.236	0.463
LF1	YPDX	76.26	39.46	94.98	0.386	0.183	0.458	0.457
6M-15	YPDX	76.32	39.61	99.14	0.403	0.210	0.525	0.464
LF1	YP + 50% (v/v) leachate	5.75	21.72	6.68	—	0.025	0.016	0.094
6M-15	YP + 50% (v/v) leachate	5.50	21.07	96.54	0.025	0.129	0.079	0.341
LF1	Hydrolysate	49.77	26.22	15.95	—	0.023	0.035	0.236
6M-15	Hydrolysate	46.87	25.11	94.56	0.028	0.061	0.085	0.426

[a]Fermentation in YPX (with 40 g L^{-1} as carbon source), YPDX (with 80 g L^{-1} glucose and 40 g L^{-1} xylose as carbon sources), leachate, and hydrolysate medium in 32, 36, 76, and 96 h, respectively.

[b]The specific consumption rate for xylose was calculated based on xylose consumption phase, and the specific production for ethanol was calculated based on all consumed sugars.

[c]Ethanol yields were calculated based on all consumed sugars.

within 28 h to produce 18 g L^{-1} ethanol with a yield of 0.45 g g^{-1} at a low initial OD_{600} value of 0.2. By comparison, strain LF1-6M did not utilize 28% of the available xylose. Moreover, the specific xylose consumption rate of 6M-11 was 34% higher than that of LF1-6M, while the specific maximum growth rate (μ_{max}) and the specific ethanol production rate were 16% and 39% higher, respectively. To further increase xylose utilization, the third round of ARTP mutagenesis was performed based on 6M-11 according to the above screening procedure. The xylose fermentation performance of the resulting strain 6M-15 was close to that of LF1 when using xylose as the sole carbon source. Specifically, both 6M-15 and LF1 gave total exhaustion of xylose within 24 h at an approximate xylose consumption rate. These results demonstrate that the strategy of combining iterative ARTP mutagenesis and anaerobic screening was efficient to rescue xylose utilization in inhibitor-tolerant strains within a short time, thereby alleviating the antagonism between xylose utilization and inhibitor tolerance.

3.4 | Evaluation of tolerance of mutant strains to individual inhibitor and mixed inhibitors

To further confirm the enhanced inhibitor tolerance of mutant strains together with increased xylose utilization capacity, we evaluated cell growth on various representative inhibitors with different concentrations, including acetic acid, HMF, furfural, vanillin, and their mixtures, using spot assays. As shown in Figure 4, all the strains showed almost identical growth under non-inhibitor stress. Strain 6M-15 clearly showed the best growth in the presence of 6 g L^{-1} acetic acid, while LF1-6M and 6M-15 grew better than the other strains in 1.5 g L^{-1} HMF, 1.5 g L^{-1} furfural, 1.0 g L^{-1} vanillin, and in mixed inhibitors. It is noteworthy that the mixture of inhibitors (0.6 g L^{-1} acetic acid, 0.63 g L^{-1} HMF, 0.48 g L^{-1} furfural, and 0.74 g L^{-1} vanillin) severely inhibited cell growth, indicating synergistic inhibitory effects at a low mixture concentration. In addition, the inhibitor tolerance of mutant strains generated from the three rounds of ARTP mutagenesis were monitored throughout the screening process, and 6M-15 still showed excellent tolerance to 50% (v/v) leachate (data not shown). These results indicate that final strain 6M-15 retained good xylose utilization capacity and showed multiple inhibitor tolerance compared with parent strain LF1.

3.5 | Evaluation of the co-fermentation capacity of 6M-15 in high-level mixed sugars

In this study, we sought to obtain an industrial strain for the direct fermentation of harsh lignocellulosic hydrolysates to produce ethanol. Prior to the fermentation of lignocellulosic hydrolysates, we investigated

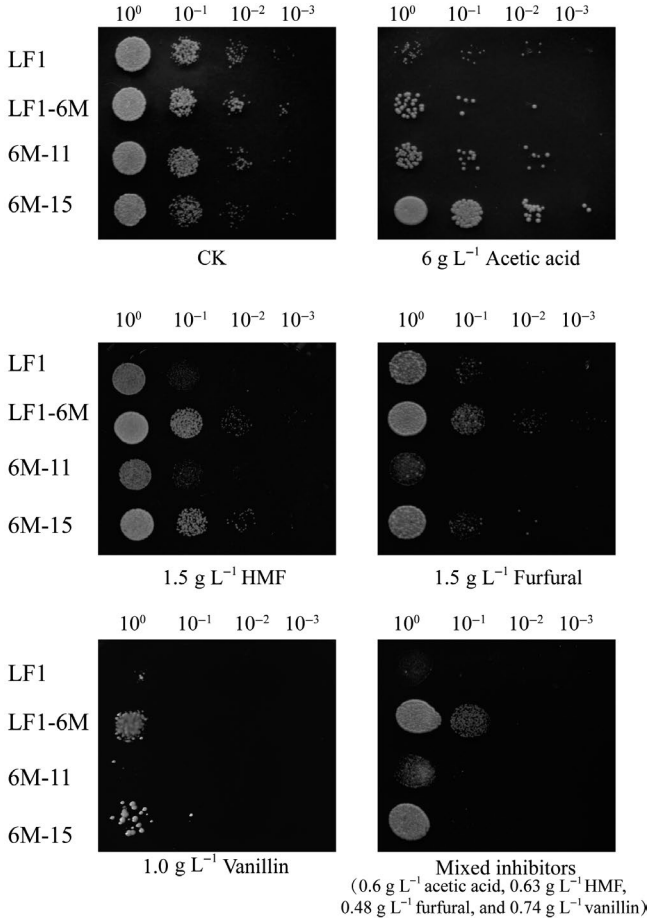

FIGURE 4 Plate growth of the mutant strains under stress conditions. Aliquots (4 μl) of each cell suspension from a 10-fold serial dilution with an initial OD_{600} of 0.5 were spotted onto plates containing acetic acid, hydroxymethylfurfural (HMF), furfural, vanillin, and mixed inhibitors

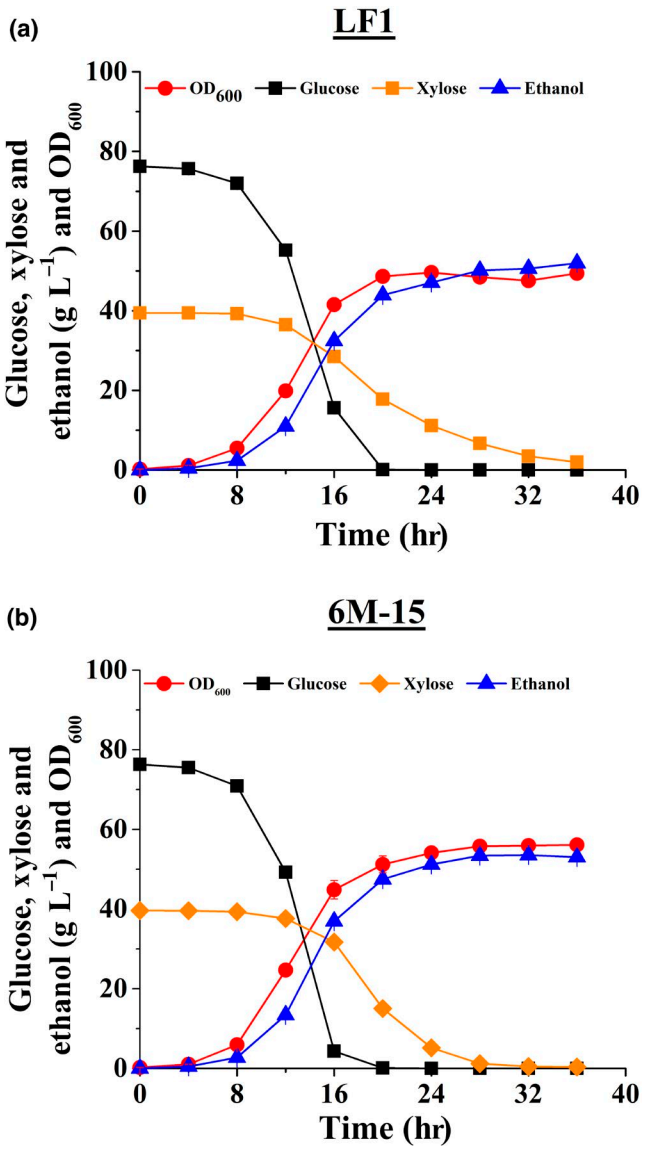

FIGURE 5 Oxygen-limited fermentation performance of the control strain LF1 (a) and the final mutant strain, 6M-15 (b) in a high level of mixed sugars (80 g L^{-1} glucose and 40 g L^{-1} xylose). The strains were cultivated in serum bottles with an initial OD_{600} of 0.2 for 32 h at 200 rpm, 30°C. The error bars represent the standard deviation of biological triplicates

the fermentation performance of 6M-15 in high-level mixed sugar substrates composed of 80 g L^{-1} glucose and 40 g L^{-1} xylose, which are considered the target concentrations of sugars in lignocellulosic hydrolysates for economical production of bioethanol on an industrial scale. As shown in Figure 5 and Table 2, at a low initial OD_{600} of 0.2, 6M-15 consumed 80 g L^{-1} glucose within 16 h, whereas the control strain LF1 consumed 61 g L^{-1} glucose, indicating that 6M-15 showed a higher glucose consumption rate than LF1. During the glucose consumption phase, 6M-15 and LF1 showed low xylose consumption rates because of the glucose repressive effect (metabolism of other sugars is repressed when glucose is present). Along with the depletion of glucose, 6M-15 showed a xylose consumption rate of 15% higher than that of LF1. The overall ethanol productivity was 0.525 g g^{-1} DCW h^{-1}, which was 15% higher than that of LF1. All sugars were completely consumed by 6M-15 within 28 h with an ethanol yield of 0.46 g g^{-1} total sugars, reducing the fermentation time by 8 h when compared with LF1. These results demonstrate that 6M-15 not only showed high inhibitor tolerance but also enhanced the co-fermentation performance of high-concentration mixed sugars (glucose and xylose), which indicates that 6M-15 has great potential for converting lignocellulosic hydrolysates into biofuels.

3.6 | Fermentation performance of the strains with multiple inhibitors tolerance in leachate and hydrolysate of lignocellulose

The direct microbial conversion of the non-detoxified lignocellulosic hydrolysates remains a challenge because

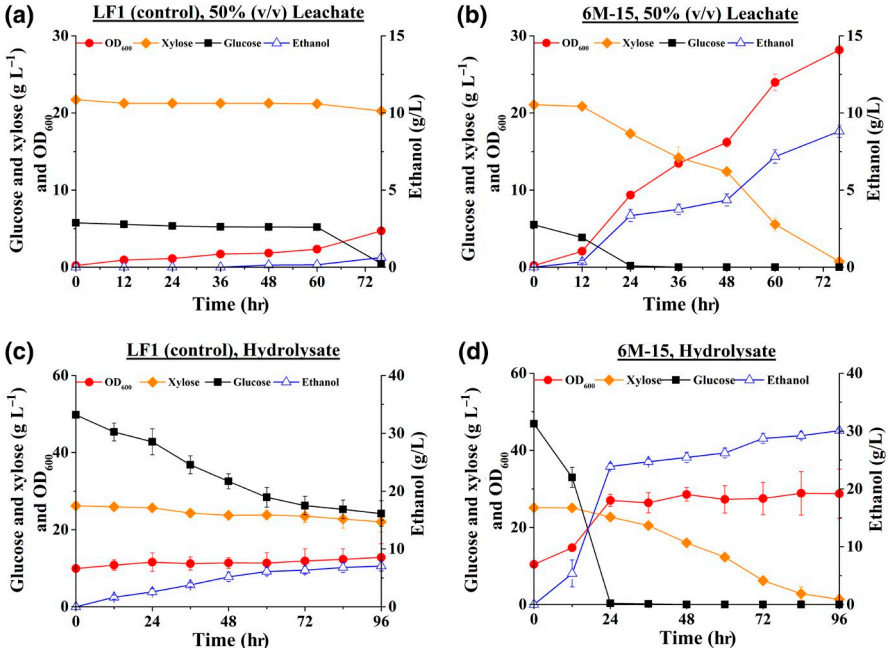

FIGURE 6 Oxygen-limited fermentation performance of the control strain LF1 (a, c) and the resulting strain 6M-15 (b, d) in YP medium with 50% (v/v) leachate and lignocellulosic hydrolysate, respectively. The strains were cultivated in serum bottles with an initial OD_{600} of 0.2 (in leachate) or 10 (in lignocellulosic hydrolysate) at 200 rpm, 30°C. The error bars represent the standard deviation of biological triplicates

yeast cell growth and ethanol yield are seriously affected by the inhibitors generated during the pretreatment of lignocellulose. To overcome this challenge, we developed a superior robust yeast strain 6M-15 against harsh lignocellulosic hydrolysates in the present study. The fermentation performance of 6M-15 was first examined in YP medium containing 50% (v/v) leachate at low pH (~3.0), in which the mixture of inhibitors (1.8 g L^{-1} acetic acid, 1.3 g L^{-1} HMF, 1.7 g L^{-1} phenol compounds, and 0.15 g L^{-1} furfural) was present. As shown in Figure 6 and Table 2, the growth of the control strain LF1 was mostly inhibited together with sugar utilization and ethanol production. In contrast, 6M-15 showed good growth without a long lag phase at a low initial OD_{600} of 0.2. Xylose was slowly consumed during the glucose consumption phase, and the consumption then speed up after glucose exhaustion. 6M-15 completely consumed all the glucose and xylose within 76 h with an ethanol yield of 0.34 g g^{-1}. Then the fermentation performance of 6M-15 was evaluated in corn straw hydrolysate with a high concentration of mixed inhibitors (4.3 g L^{-1} acetic acid, 1.0 g L^{-1} HMF, 4.78 g L^{-1} phenol compounds, and 0.35 g L^{-1} furfural). During fermentation with a high cell density (OD_{600} = 10), 6M-15 rapidly consumed 46.8 g L^{-1} glucose within 24 h to produce 23.9 g L^{-1} ethanol with a yield of 0.50 g g^{-1}, whereas LF1 consumed a small amount of glucose. This indicated that glucose utilization was not inhibited in the presence of a high concentration of mixed inhibitors at low pH. Although xylose utilization was inhibited to some extent, the conversion reached 94.6%, achieving an overall ethanol yield of 0.43 g g^{-1}. These results demonstrate that 6M-15 exhibited excellent co-fermentation capacity of glucose and xylose in lignocellulosic hydrolysate with high inhibitory compounds contents at low pH.

4 | DISCUSSION

To achieve the economic feasibility of bioethanol production by a microbial cell factory from lignocellulosic biomass, it is essential that the ethanol-producing *S. cerevisiae* strain is equipped with high xylose utilization capacity and high tolerance of inhibitors. In our previous study, the engineered strain LF1 was constructed through rational metabolic engineering and adaptive evolution engineering, and showed efficient co-fermentation of glucose and xylose. It rapidly consumed 120 g L^{-1} of a 2:1 glucose/xylose mixture to give an ethanol yield of 0.48 g g^{-1} (Li et al., 2016). Subsequently, two *S. cerevisiae* strains, STXQ and XUSEA, with comparable fermentation capacities have been reported (Table 3; Hoang Nguyen Tran et al., 2020; Liu et al., 2018). Although the xylose fermentation capacity of the engineered *S. cerevisiae* strain has been improved significantly, and even equal to the glucose fermentation capacity through numerous efforts contributed by researchers around the world, the inhibitor tolerance remains low, hampering the microbial conversion efficiency of lignocellulosic hydrolysates. Most reported strains show preferred tolerance to individual inhibitors, such as acetic acid, and give better fermentation performance in detoxified lignocellulosic hydrolysates with low concentrations of mixed inhibitors (Table 3; Chen et al., 2019; González-Ramos et al., 2016; Ko et al., 2019; Tian et al., 2011). Moreover, the synergistic inhibitory effects of multiple inhibitors present in lignocellulosic hydrolysates have been observed (Jönsson & Martín, 2016), and it is, therefore, of great importance to achieve simultaneous tolerance of *S. cerevisiae* to multiple inhibitors for efficient lignocellulosic biorefining (Cunha, Romaní, et al., 2019). In contrast, the detoxification processes increase freshwater

TABLE 3 Comparison of co-fermentation performance of 6M-15 with those of previously reported recombinant *Saccharomyces cerevisiae* strains in lignocellulosic hydrolysate medium

Strain	Culture medium	Initial glucose ($g\ L^{-1}$)	Initial xylose ($g\ L^{-1}$)	Inhibitor concentration ($g\ L^{-1}$)	Medium pH	Detoxified	Overall total sugar consumption rate ($g\ g^{-1}\ h^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Reference
6M-15	YP + Corn straw hydrolysate	46.87	25.11	4.3 $g\ L^{-1}$ acetic acid 1.0 $g\ L^{-1}$ HMF 0.35 $g\ L^{-1}$ furfural 4.78 $g\ L^{-1}$ total phenol	3.5	No	0.20	0.43	This study
LF1	YP + Corn straw hydrolysate	49.77	26.22	4.3 $g\ L^{-1}$ acetic acid 1.0 $g\ L^{-1}$ HMF 0.35 $g\ L^{-1}$ furfural 4.78 $g\ L^{-1}$ total phenol	3.5	No	0.15	0.24	
LF1	Urea + Corn straw hydrolysate	86.60	39.10	4.52 $g\ L^{-1}$ acetic acid 0.66 $g\ L^{-1}$ HMF 0.47 $g\ L^{-1}$ furfural 4.05 $g\ L^{-1}$ total phenol	6.0	No	0.61	0.41	Li et al. (2016)
	YP + Papermaking residue	55.00	23.80	—	6.0	No	0.97	0.42	
XUSEA	SC + Saccharomate hydrolysate	39.60	23.10	—	5.0	Yes	0.66	0.48	Hoang Nguyen Tran et al. (2020)
STXQ	YP + Oil palm empty fruit bunch (OPEFB) hydrolysate was	41.8	30	—	4.5	NO	—	0.42	Liu et al. (2018)
GLBRCY 128	YP + Corn stover hydrolysate	60.00	30.00	—	5.0	Yes	0.57	0.39	Parreiras et al. (2014)
SXA-R2P-E	YSC + Hardwood hydrolysate	26.80	16.00	6.1 $g\ L^{-1}$acetic acid 0.6 $g\ L^{-1}$ furfural 1.3 $g\ L^{-1}$ total phenol	5.0	No	0.11	0.43	Ko et al. (2016)
	YSC + Rice straw hydrolysate	27.70	20.20	1.0 $g\ L^{-1}$ acetic acid 0.2 $g\ L^{-1}$ furfural 0.8 $g\ L^{-1}$ total phenol	5.0	No	0.13	0.46	
36aS1.10.4	YP + Wheat straw hydrolysate	82.66	43.96	7.42 $g\ L^{-1}$ acetate 0.088 $g\ L^{-1}$ furfural	5.5	No	0.55	0.44	Huang et al. (2019)
PE-2ΔGRE3-XI	SC + Corn cob hemicellulosic hydrolysate	1.15 1.15	28.3 28.3	4.36 $g\ L^{-1}$ acetic acid 0.17 $g\ L^{-1}$ HMF 1.36 $g\ L^{-1}$ furfural	5.0	Yes No	— —	0.44 0.38	Cunha, Soares, et al. (2019)

(Continues)

TABLE 1 (Continued)

Strain	Culture medium	Initial glucose ($g\ L^{-1}$)	Initial xylose ($g\ L^{-1}$)	Inhibitor concentration ($g\ L^{-1}$)	Medium pH	Detoxified	Overall total sugar consumption rate ($g\ g^{-1}\ h^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Reference
MN8140X/TF-TF	YP + Rice straw hydrolysate	8.30	12.10	0.98 $g\ L^{-1}$ acetic acid 0.13 $g\ L^{-1}$ HMF 0.25 $g\ L^{-1}$ furfural 0.73 $g\ L^{-1}$ formic acid	5.0	No	—	0.38	Sasaki et al. (2015)

Abbreviation: HMF, hydroxymethylfurfural.

usage, wastewater discharge, cause sugar loss and raise the cost of production.

In this study, we sought to develop a robust yeast strain with high tolerance to the complex toxicity of multiple inhibitors and with high xylose utilization for the direct fermentation of non-detoxified lignocellulosic hydrolysates. Given the complexity of the tolerance mechanism of multiple inhibitors in yeast cells, random mutagenesis may be presented a more practical method than rational metabolic engineering to improve tolerance to inhibitors. ARTP mutagenesis, a relatively novel powerful random mutagenesis technique was applied to improve the inhibitor tolerance of our previous strain, LF1. To screen the mutant strain for simultaneously enhanced inhibitor tolerance and xylose metabolism, a leachate of corn straw pretreatment with high xylose and multiple inhibitors at low pH was obtained from industrial treatment in China. Under such harsh pressure, the mutant strain, LF1-6, which had a high growth rate in 50% (v/v) leachate, was generated through one round of ARTP mutagenesis. However, we found that the xylose utilization rate of LF1-6 dropped dramatically compared with that of the parent strain, LF1. Although the xylose consumption rate showed slight improvement through adaptive evolution, it was significantly lower than that of LF1 (0.304 $g\ g^{-1}\ h^{-1}$ vs. 0.557 $g\ g^{-1}\ h^{-1}$). We speculated that the antagonism occurred between xylose utilization and inhibitor tolerance, similar to the phenomenon between glucose metabolism and xylose utilization that occurred in the construction of LF1 (Demeke et al., 2013; Li et al., 2016). Interestingly, xylose utilization of inhibitor-tolerant strain did not decrease under aerobic conditions, where oxygen may have served as an electron acceptor for the regeneration of NAD^+ to facilitate xylose consumption (Wasylenko & Stephanopoulos, 2015). It is reasonable that both LF1-6 and LF1-6M preferred aerobic growth on xylose because of the initial aerobic screening condition. Given that industrial ethanol production requires an anaerobic or oxygen-limited environment to minimize ethanol reoxidation, it is essential to rescue xylose metabolism in the inhibitor-tolerant strain under oxygen-limited conditions. To this end, the subsequent ARTP-treated cells were cultivated under anaerobic conditions to screen strains with enhanced xylose utilization. The resulting strain 6M-15 exhibited a xylose consumption rate and ethanol yield that were similar to those of LF1. This indicated that the strategy of iterative ARTP mutagenesis combined with anaerobic screening was able to alleviate the above-mentioned antagonism by gradually increasing xylose consumption rate in the inhibitor-tolerant strain. Surprisingly, 6M-15 showed a moderate improvement on co-fermentation performance of high-level mixed sugars (80 $g\ L^{-1}$ glucose and 40 $g\ L^{-1}$ xylose), thereby reducing the fermentation time required for complete consumption of total sugars from 36 h for LF1 to 28 h. To our knowledge, 6M-15 showed the shortest

fermentation time and the highest ethanol production rate, 28 h and 0.525 g g^{-1} h^{-1}, respectively, of any previously reported strains under similar fermentation conditions (Li et al., 2016; Liu et al., 2018).

In lignocellulosic biorefining, efficient pretreatment methods, such as diluted acid pretreatment, can enhance hydrolysis efficiency, but can simultaneously result in the release of multiple inhibitors and low pH conditions. It is not reasonable to accept a poor sugar yield and a consequent poor target product titer because of the use of insufficient pretreatment conditions. Although a variety of detoxification treatments or pH adjustments can reduce the toxicity of lignocellulosic hydrolysates, these processes would increase the cost of production. In this study, we developed a robust yeast strain, 6M-15, to withstand multiple inhibitors and low pH in lignocellulosic hydrolysates. We evaluated the fermentation performance of 6M-15 in the non-detoxification leachate (pH 3.0) and hydrolysate (pH 3.5) of pretreated corn straw with high levels of multiple inhibitors. Strain 6M-15 showed great inhibitor tolerance and co-fermentation performance of glucose and xylose with a short lag phase compared with the control strain. During the fermentation of the leachate, the xylose consumption rate was low in the first 48 h because of glucose repression and multiple inhibitor pressure, but the xylose consumption rate increased after 48 h. During the fermentation of corn straw hydrolysate, 6M-15 rapidly consumed all glucose within 24 h with an ethanol yield of 0.50 g g^{-1}. Although xylose utilization was inhibited to some extent under harsh conditions, 6M-15 still exhibited a high overall ethanol yield of 0.43 g g^{-1} (based on total glucose and xylose). Cunha, Soares, et al. (2019) reported a set of inhibitor-tolerant *S. cerevisiae* strains with ethanol yields in the range of 0.24–0.38 g g^{-1} in non-detoxified corn cob hydrolysate at pH 5.0. It is possible that the decreased ethanol yield in fermentation of lignocellulosic hydrolysates is caused by the expense of additional energy, NADPH/NADH, and carbon source against multiple inhibitors and low pH pressures because of activating or enhancing complex cellular response pathways (Cunha, Romaní, et al., 2019). For example, to maintain intracellular pH homeostasis, cells need to pump out protons via membrane H+-ATPases at the expense of ATP (Ullah et al., 2013). In this study, the low pH (3.0 or 3.5) would have restricted acetic acid (p*K*a = 4.7) to its protonated form, which has a more severe inhibitory effect on cell growth and metabolism, especially on xylose metabolism (Bellissimi et al., 2009; Casey et al., 2010; Ko et al., 2019). A set of previously reported genes participating in the yeast response against acetic acid and other inhibitors were investigated at the expression level in this study (Figure S5). Among them, the expression levels of three *ADE* genes (*ADE1*, *ADE13*, and *ADE17*) involved in purine metabolism, two *FLO* genes (*FLO1* and *FLO9*) involved in flocculation, and *FRM2* encoding NADH-dependent type II nitroreductase were significantly upregulated, which could improve the cell tolerance to multiple inhibitors (de Oliveira et al., 2010; Westman et al., 2014; Zhang et al., 2019). The upregulation of *TFC3* encoding a transcription factor and downregulation of *ASK10* encoding an activator of *SKn7* may contribute to the recovery of xylose utilization in the improved tolerance strain (Hou et al., 2016; Wei et al., 2019). Additionally, the ATP content of 6M-15 increased by 28% compared to that of the starting strain LF1 (Figure S5), which can compensate for the expense of ATP in cellular responses against inhibitors. However, owing to the complex mechanism of multiple inhibitors together with xylose metabolism, it is still challenging to completely understand the coordinated regulation of multiple related genes.

In conclusion, this study has developed a multiple inhibitor-tolerant *S. cerevisiae* strain for the direct fermentation of lignocellulosic hydrolysates by alleviating the antagonism between inhibitor tolerance and xylose metabolism. To date, apart from 6M-15, no other strain has been reported to exhibit excellent fermentation performance in harsh lignocellulosic hydrolysates with higher multiple inhibitors content at a pH below 4.0 (Table 3). We also demonstrated that the strategy applied in this study is efficient for breeding customized second-generation ethanol-producing strains suitable for lignocellulose pretreatment processes. In future studies, we hope to discover a group of effective gene parts that regulate sugar metabolism and inhibitor tolerance through genomic, transcriptomic, and metabolomic methods based on the inhibitor-tolerant strains obtained in this study. Such studies will further improve the fermentation capacity and inhibitor tolerance of current chassis cells by rational metabolic engineering.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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SUPPORTING INFORMATION

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

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