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Enhanced fermentative performance under stresses of multiple lignocellulose-derived inhibitors by overexpression of a typical 2-Cys peroxiredoxin from *Kluyveromyces marxianus*

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Abstract

Background: Bioethanol from lignocellulosic materials is of great significance to the production of renewable fuels due to its wide sources. However, multiple inhibitors generated from pretreatments represent great challenges for its industrial-scale fermentation. Despite the complex toxicity mechanisms, lignocellulose-derived inhibitors have been reported to be related to the levels of intracellular reactive oxygen species (ROS), which makes oxidoreductase a potential target for the enhancement of the tolerance of yeasts to these inhibitors.

Results: A typical 2-Cys peroxiredoxin from *Kluyveromyces marxianus* Y179 (*KmTPX1*) was identified, and its overexpression was achieved in *Saccharomyces cerevisiae* 280. Strain TPX1 with overexpressed *KmTPX1* gene showed an enhanced tolerance to oxidative stresses. Serial dilution assay indicated that *KmTPX1* gene contributed to a better cellular growth behavior, when the cells were exposed to multiple lignocellulose-derived inhibitors, such as formic acid, acetic acid, furfural, ethanol, and salt. In particular, *KmTPX1* expression also possessed enhanced tolerance to a mixture of formic acid, acetic acid, and furfural (FAF) with a shorter lag period. The maximum glucose consumption rate and ethanol generation rate in *KmTPX1*-expressing strain were significantly improved, compared with the control. The mechanism of improved tolerance to FAF depends on the lower level of intracellular ROS for cell survival under stress.

Conclusion: A new functional gene *KmTPX1* from *K. marxianus* is firstly associated with the enhanced tolerance to multiple lignocellulose-derived inhibitors in *S. cerevisiae*. We provided a possible detoxification mechanism of the *KmTPX1* for further theoretical research; meanwhile, we provided a powerful potential for application of the *KmTPX1* overexpressing strain in ethanol production from lignocellulosic materials.

Keywords: Lignocellulose, Inhibitors, Tolerance, Peroxiredoxin, Ethanol fermentation, *Kluyveromyces marxianus*

Background

Fuel ethanol has been the earliest and most mature bio-fuel product so far and widely considered as one of the most promising biomass energies. Bioethanol from lignocellulosic materials represents the most promising renewable fuel due to its wide range of sources. However,

the complex structure of lignocellulose requires a pretreatment step to produce monosaccharides for *Saccharomyces cerevisiae*. Current efficient pretreatment of lignocellulose by steam explosion, acids, or alkali may generate inhibitors to restrain the growth of yeasts in the fermentation, which represents a great challenge for the scale-up of ethanol production from lignocellulosic materials [1].

Lignocellulose-derived inhibitors in hydrolysates include weak acids (formic acid and acetic acid), furan

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derivatives (furfural and 5-hydroxymethyl furfural), and phenolic compounds (phenol and *O*-methoxyphenol) [1]. Toxic mechanisms of inhibitors in lignocellulosic hydrolysates are supposed to be extremely complicated. The inhibitors may be removed by detoxification of lignocellulosic hydrolysates [2], which should not be regarded as the preferred choice considering loss of sugars and increased costs. Therefore, exploring the toxicity of distinctive inhibitors to cells and its mechanism, and developing excellent strains with enhanced tolerance are becoming a more critical component of ethanol production from lignocellulosic materials. However, mechanisms of toxicities of these inhibitors in yeasts are very complex and greatly variable depending on strains [3].

Inhibitors like acetic acid, furfural, and phenol have been reported to be related to the redox state inside cells, inducing reactive oxygen species (ROS) generation [4–6]. Acetic acid generally affects cell metabolism and stabilities of proteins by a drop in intracellular pH and potential, leading to a net negative effect on yeasts' cell growth and proliferation [7]. Acetic acid diffusing across plasma membrane damages cells by accumulating ROS [4], and therefore the expression of some oxidoreductases like mitochondrial cytochrome C oxidase chaperone gene (encoded by *COX20*) may improve tolerance to weak acids, especially acetic acid in *S. cerevisiae* [8]. Moreover, an enhancement of intracellular proline concentration by the addition of proline or overexpression of a proline synthesis-related gene (*PRO1*) led to an obvious increase in tolerance to both acetic acid and furfural [9]; unlike acetic acid, the toxicity of furfural results from the inhibition of glycolytic and fermentative enzymes essential to central metabolic pathways, which reduces cell growth rates eventually [6]. Interestingly, furfural also induces the accumulation of ROS inside cells by lowering activities of intracellular oxidoreductases [10]. Consequently, some oxidoreductases, such as alcohol dehydrogenase *ADH1* [11] and *ADH6* [12], 3-methylglyoxal reductase *GRE2* [13], aldehyde reductase *ARI1* [14], and xylose reductase *XYL1* [15], help yeast cells convert furfural into the less toxic alcohols and then increase tolerance to furfural; phenolic compounds alter the permeability of biological membranes and caused irreversible damages to the cells. Phenolic compounds are supposed to be even more toxic than furfural by generating ROS like peroxides and superoxides inside the cells [6]. It has been indicated that oxidoreductases could be quite effective in enhancing the tolerance of yeast cells to phenolic compounds in lignocellulosic hydrolysates [16]. But beyond that, ionic liquids and hydrogen peroxide have become a new kind of inhibitors as the pretreatment technologies evolve. Hence, oxidoreductases have shown a perfect application in the enhancement of tolerance to multiple inhibitors from pretreatment of lignocellulose.

High-throughput sequencing is a powerful tool to gain insight into new genes and their new functions [16–19]. Our previous study on the transcriptional analysis of the non-conventional yeast *Kluyveromyces marxianus*, which possesses the advantages of high-temperature resistance, rapid growth rate, and diversity of substrates, identified lots of differentially expressed genes (DEGs) [20]. A greatly up-regulated gene under aerobic conditions (ORF controlled at -130 mV) from *K. marxianus* Y179 has attracted our attention. According to the sequence alignment, this gene (*KmTPX1*) belongs to a large and highly conserved peroxiredoxin family. *KmTPX1* is homologous to one of the five peroxiredoxins (Prxs) in *S. cerevisiae* (*TSA1/TPX1*) [21], which is the most abundant Prxs in cells [22]. *Tsa1p* in *S. cerevisiae* has been reported to participate in oxidation–reduction reactions to remove excess ROS like peroxides [23], and the antioxidant role of *Tsa1p* in regulating the concentrations of intracellular peroxides protects cells from DNA damage and cell death [24, 25]. However, so far, no reports have combined *Tsa1p* in *S. cerevisiae* with tolerance to lignocellulose-derived inhibitors, and the homologous protein in *K. marxianus* (encoded by *KmTPX1*) has never been studied.

Therefore, in this study, *KmTPX1* gene was cloned and then overexpressed in *S. cerevisiae* to analyze its potential functions in yeast cells. Afterwards, tolerance of the recombinant *S. cerevisiae* to multiple inhibitors or stressors in lignocellulosic hydrolysates was evaluated. The enhanced tolerance to formic acid, acetic acid, furfural, and salt makes great difference to ethanol production from lignocellulosic materials and provides more theoretical references for its industrial scale-up in the future.

Results and discussion

Identification of a hypothetical typical 2-Cys peroxiredoxin from *K. marxianus*

Sequence analysis of *KmTPX1* gene from *K. marxianus* Y179 was conducted in this study. *KmTPX1* can be classified into the typical 2-Cys Prx family, and the two conserved cysteine residues in *KmTPX1*, serving as the site of oxidation by peroxides, are named as “Peroxidatic” Cys (C_p) and “Resolving” Cys (C_R). To perform its functions in oxidation–reduction reactions, this typical 2-Cys protein needs to exist in dimer model, which indicates an inter-subunit disulfide formation between the C_p -SOH and the C_R -SH in the other subunit [23]. A specific catalytic cycle of *TPX1* protein is illustrated in Fig. 1a.

Furthermore, the construction of evolutionary tree by several typical 2-Cys Prxs from *S. cerevisiae*, *Kluyveromyces lactis*, *Scheffersomyces stipites*, and *Candida tropicalis* indicates that the evolutionary distance between

KmTPX1 and Prxs from *K. lactis* is the shortest, followed by Tsa1p from *S. cerevisiae*. The other four Prxs from *S. cerevisiae* have the lowest homologies with *KmTPX1*, which makes us even more certain that *KmTPX1* should be identified as a hypothetical typical 2-Cys Prx (Fig. 1b).

Simultaneously, a simple function annotation was conducted using amino acid sequence from *K. marxianus*, Y179. Firstly, we determined the accurate positions of two cysteine residues in *KmTPX1*, which were found at the sites of 47 and 171, respectively. Besides, an active domain, PXXXTXXC_p, has been reported to exist in all Prxs [26], and this domain lies in amino acids between the 40th and 47th sites in *KmTPX1*. Meanwhile, the 146th Arg, another active site, is supposed to be close to the above active domain in 3D structure while it is far away from C_p in 2D sequence [26, 27]. Finally, Tairum et al. [27] constructed the three-dimensional structure model of Tsa1 protein, which showed that the 50th Glu and the 46th Arg are two essential sites for disulfide formation. These two functional sites in *KmTPX1* were also found to be highly conserved from the alignment results (Fig. 1c).

Enhanced tolerance of TPX1 strain to oxidative stresses

To validate the possible functions of *KmTPX1*, we constructed the overexpression vector containing the gene *KmTPX1*, and the overexpression was achieved in *S. cerevisiae* 280 after verification by restriction enzyme digestion. Meanwhile, real-time quantitative PCR was performed to test the levels of expression. Expression of *KmTPX1* gene in strain TPX1 can be detected, compared with the control strain 423 containing only empty vector (an additional file shows this in more detail [see Additional file 1]).

Prxs have been reported to play an important role in regulating intracellular redox state by the participation of oxidation–reduction reactions [23, 28]. Therefore, *KmTPX1* is supposed to be related to the removal of excess peroxides inside the cells for a much better chance of survival at high concentrations of ROS. On the one hand, serial dilution assay was conducted on SC-His agar plates with or without H₂O₂. As shown in Fig. 2a, no significant differences in growth were detected between TPX1 and 423 without H₂O₂. However, when 3 mM of H₂O₂ was added to the plates, TPX1 achieved a much better growth status than the strain 423, which indicated that the gene *KmTPX1* might increase the tolerance of *S. cerevisiae* to H₂O₂. Similarly, a kind of 2-Cys Prxs from *Oryza sativa* [29] and the thioredoxin from *Endocarpon pusillum* [30] both have been proved to enhance the tolerance of *S. cerevisiae* to peroxides, including H₂O₂ and 2-methyl-1,4-naphthoquinone (MD).

On the other hand, we carried out liquid fermentation experiments at different concentrations of H₂O₂ to further confirm our findings (Fig. 2b). There were no obvious differences in growth between two strains under low concentration (0.2 mM). An elevated concentration of 3 mM could inhibit the growth of the control strain 423 significantly, while strain TPX1 had been less affected, which was in line with the results reported by Kim et al. [29]. Consequently, an enhanced tolerance of *S. cerevisiae* 280 with a normally functioning *KmTPX1* gene provided a good foundation for further analysis and validation of other possible functions.

Responses to multiple lignocellulose-derived inhibitors in *KmTPX1*-overexpressing yeast

Lignocellulose-derived inhibitors, such as acetic acid, furfural, and phenol, induce the generation of intracellular ROS that is closely related to the redox state in cells. Hence, we further explored the potential applications of gene *KmTPX1* in an enhanced tolerance of yeasts to the mixed inhibitors in the lignocellulosic hydrolysates. As a result, weak acids (formic acid and acetic acid), phenolic compounds (phenol and *O*-methoxyphenol), and furan derivative (furfural and 5-HMF), as the representative inhibitors in hydrolysates, were selected to test their effects on *S. cerevisiae* overexpressing *KmTPX1*.

As shown in Fig. 3, gene *KmTPX1* played a positive role in the enhanced tolerance to weak acids and furfural. Overexpression of *KmTPX1* would not affect the cell growth (Fig. 2a), while the growth of control strain 423 had been greatly repressed on plates with 0.3 g/L of formic acid, 1.5 g/L of acetic acid, or 1.0 g/L of furfural, which was 1–2 gradients less than the strain TPX1 in the serial dilution assay. These results have been supported by a variety of similar oxidoreductases that might be contributed to the increased tolerance of *S. cerevisiae* to the inhibitors [8–15, 29]. However, despite sharing a similar mechanism of toxicity with furfural, the tolerance of TPX1 strain to 5-HMF was only slightly improved compared with the control strain.

Considering the possibilities of applications in industrial-scale ethanol production from lignocellulosic materials, the enhanced tolerance to a single inhibitor makes little sense. So we further evaluated the abilities of *KmTPX1* to tolerate a mixture of lignocellulose-derived inhibitors. An improved tolerance of strain TPX1 to mixed FAF inhibitors was observed. Interestingly, a much more obvious difference in cell growth was detected when the two strains were exposed to mixed FAFH inhibitors, which might be attributed to the comprehensive stress compared with the FAF inhibitors. A similar publication reported that the addition of proline or overexpression of

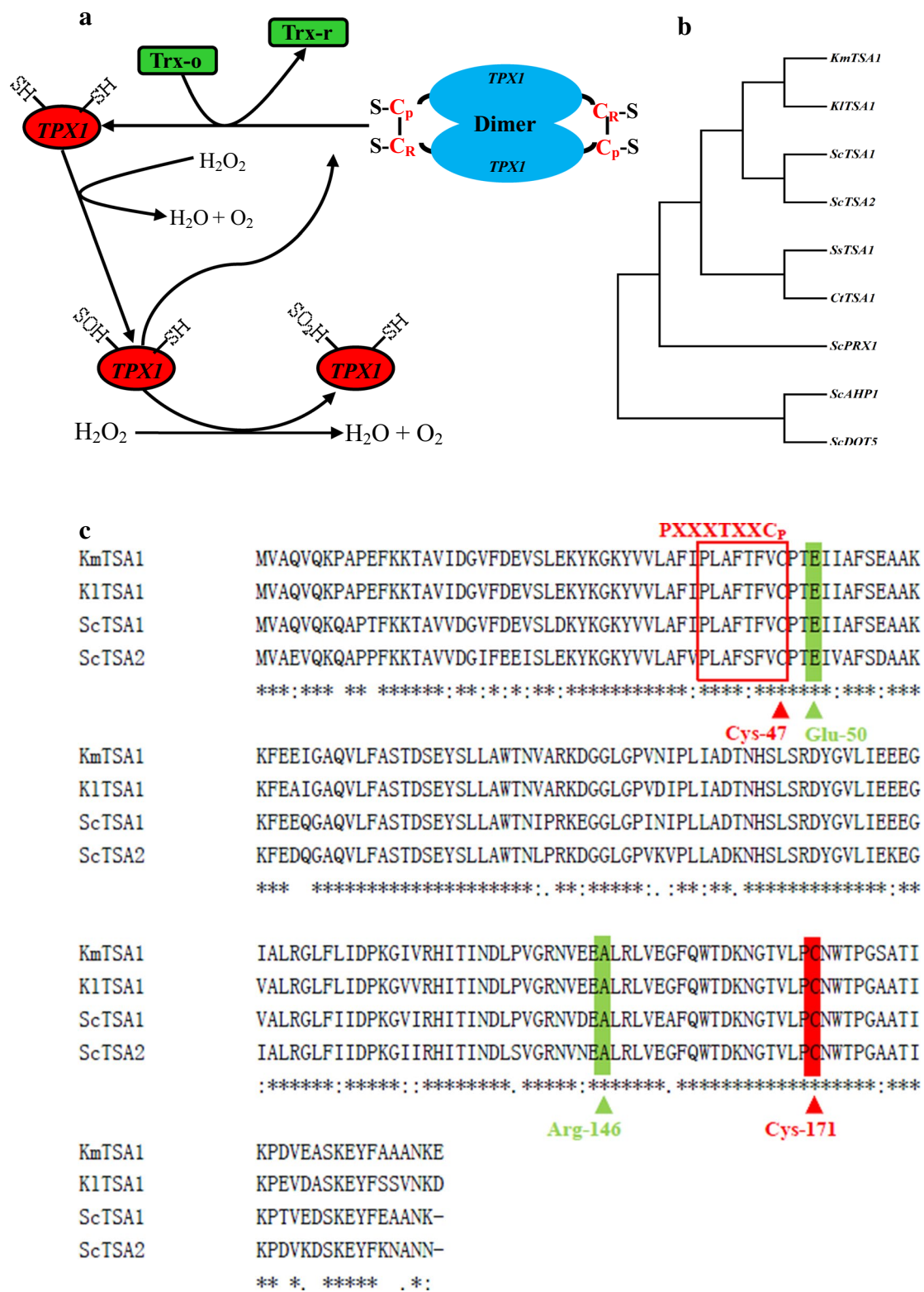
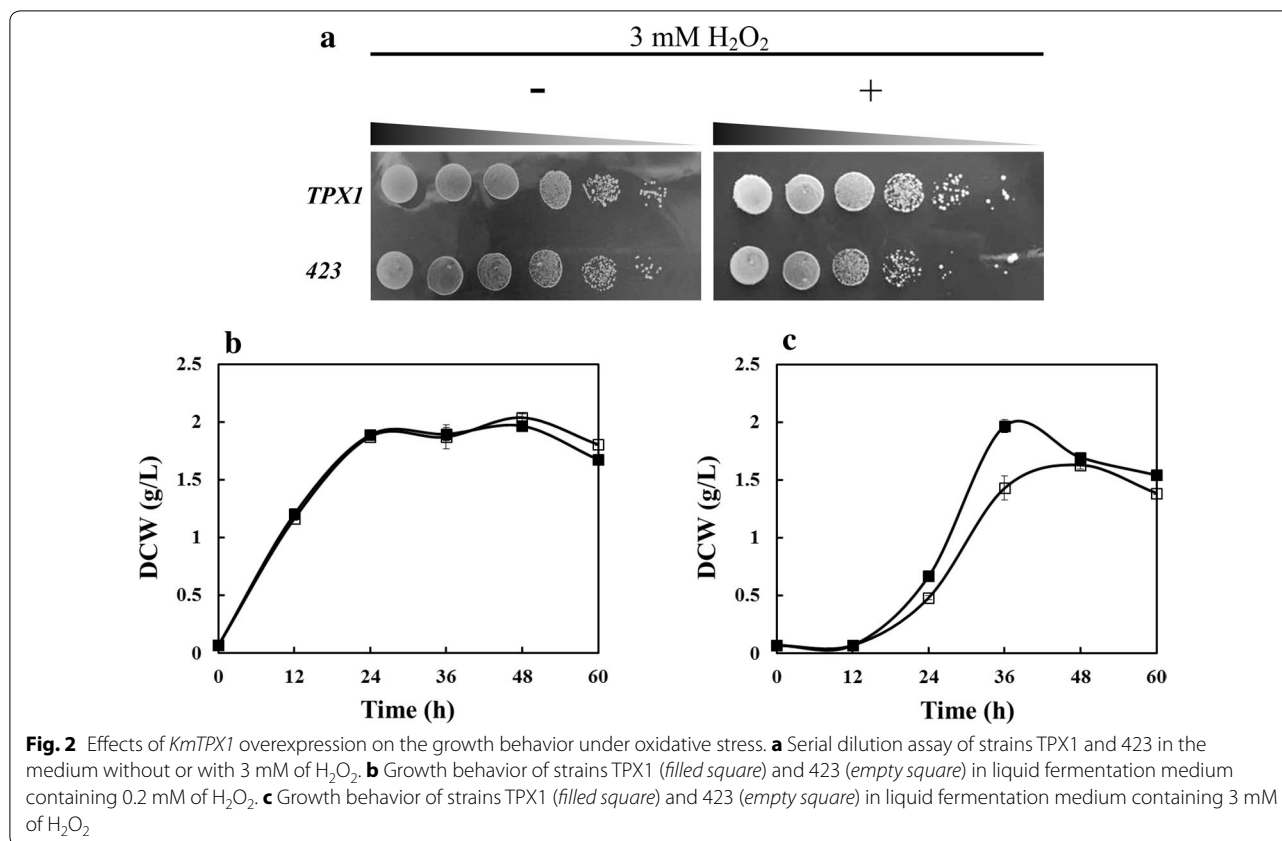


Fig. 1 Typical 2-Cys peroxiredoxin in common yeasts. **a** A typical catalytic cycle of *TPXI* protein as a dimer structure. **b** Evolutionary tree of some typical 2-Cys Prxs from Y179 and their allied species using *MEGA 4* software [41]. **c** Alignment of amino acid sequences of typical 2-Cys Prxs from Y179, *K. lactis*, and *S. cerevisiae* indicates some active domains and sites



a proline synthesis-related gene (encoded by *PRO1*) might also achieve enhanced tolerance to a mixture of furfural, acetic acid, and phenol [9]. In this case, a generally elevated tolerance of yeasts to the mixed lignocellulose-derived inhibitors due to some key genes makes a major breakthrough in the biofuel industry.

Besides, our tests on phenol and *O*-methoxyphenol indicated that *KmTPX1* did not work for these two inhibitors despite marginally increased tolerance to *O*-methoxyphenol. Except for lignocellulose-derived inhibitors, phenol and *O*-methoxyphenol are also two typical environmental pollutants, which might make a great difference if we achieved both enhanced tolerance and degradation. The elevated effect of *KmTPX1* on these two inhibitors was not obvious, which can be attributed to two main reasons: on the one hand, test conditions were not quite suitable; on the other hand, some doubts remain on whether these two inhibitors will generate ROS.

In particular, we also evaluated the tolerance of strain TPX1 to salt, ethanol, and high temperature from a global perspective of ethanol production. An enhanced tolerance of strain TPX1 to high concentration of salt was just as expected, which showed similar results with some related Prxs [29, 30]. This finding is of great significance to reducing the costs for scale-up of ethanol production.

Surprisingly, *KmTPX1* played a positive role in growth at high concentrations of ethanol. No previous results showed that ethanol had possible relations with intracellular redox states, so oxidoreductases might also be applied in increasing tolerance to ethanol. Therefore, this special result makes great sense in the field of ethanol fermentation. Finally, no significant effect of *KmTPX1* in cell growth at high temperature was observed in spite of the relations between high temperature and intracellular ROS [31]. In contrast, another Prx family protein, thioredoxin from *Endocarpon pusillum*, showed a positive role in increasing yeast tolerance to high temperature [30].

The principal advantage of *KmTPX1* gene tends to increase the tolerance to mixed lignocellulose-derived inhibitors. Although some other oxidoreductases have been proved to promote cell growth when exposed to a single inhibitor, a comprehensively enhanced tolerance to multiple stressors may provide more references and potential applications for ethanol production from lignocellulosic materials.

Effect of initial pH on acetic acid tolerance in transgenic yeast cells

KmTPX1 gene has shown a distinct effect on increasing tolerance of *S. cerevisiae* to multiple

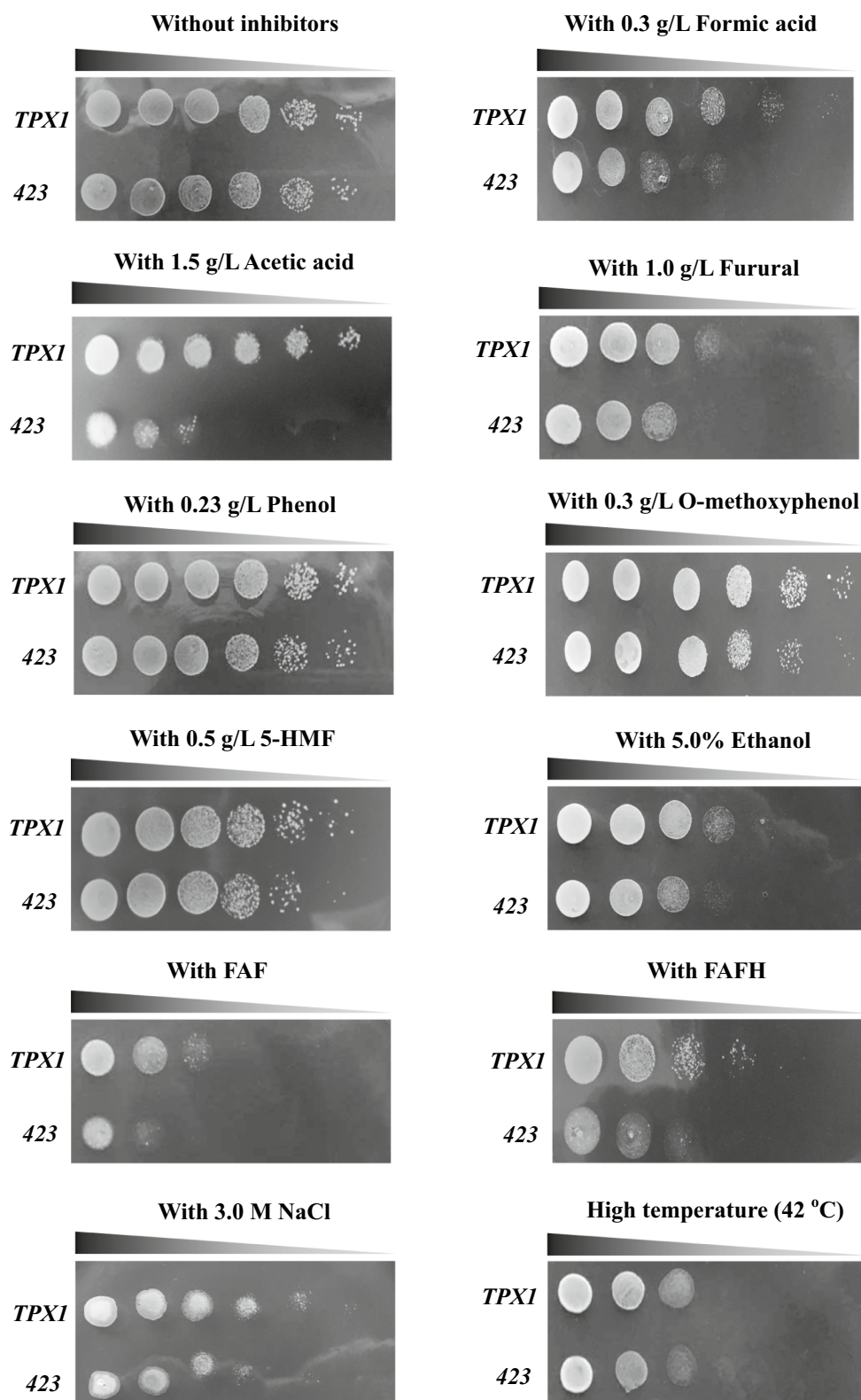


Fig. 3 Stress response of *KmTPX1* overexpression to the presence of multiple lignocellulose-derived inhibitors by serial dilution assay. Cells with 10 g/L of DCW in log phase were serially diluted to 10^{-5} and then spotted onto SC-His plates containing various inhibitors. Cells were cultivated at 30 or 42 °C for 3 days and then photographed

lignocellulose-derived inhibitors. In our experiments, a lack of adequate nutrition in SC medium led to an obvious reduction in tolerance to acetic acid. Concentrations of acetic acid in lignocellulosic hydrolysates have been reported to achieve up to 5 g/L [32], so the relatively low concentration of acetic acid in FAF affects its possibility of application in industrial-scale production. Ionization of some inhibitors under certain pH values may change the degree of toxicity of the compounds. Therefore, the initial pH of the medium was adjusted to improve cell growth as much as possible under the current conditions [33].

Without adjusting the initial pH after the addition of inhibitors, overexpressing strain TPX1 could grow well in the medium with 1.5 g/L of acetic acid compared with the serious repression of control strain. Both strains stopped growth as the acetic acid amount was increased to 5 g/L; when the initial pH was adjusted to around 4.5 after the addition of inhibitors, an enhanced tolerance to acetic acid was detected. TPX1 was tolerant to 5 g/L of acetic acid. Under these conditions, both strains were not still able to

survive in more than 10 g/L of acetic acid (Fig. 4). Even so, strain TPX1 that could tolerate 5 g/L of acetic acid in SC medium, which is supposed to be much higher in normal media, has already satisfied the requirement of industrial-scale ethanol production from lignocellulosic materials. Moreover, a mitochondrial cytochrome C oxidase chaperone gene (encoded by *COX20*) has also been reported to contribute to a perfect survival with the acetic acid concentration up to 75 mM (about 3.5 g/L) [4]. Thus, it can be seen that oxidoreductases have broad applications in increasing tolerance of *S. cerevisiae* to acetic acid, which may be further strengthened by adjusting the initial pH values.

Batch fermentation by *KmTPX1*-overexpressing yeast with FAF

To test the potential performance of ethanol fermentation using inhibitors in *S. cerevisiae* with *KmTPX1* gene, we conducted batch ethanol fermentation in both flasks (an additional file shows this in more detail [see Additional file 2, Additional file 3]) and bioreactors (Fig. 5; Table 1) containing 50 g/L of glucose and FAF inhibitors.

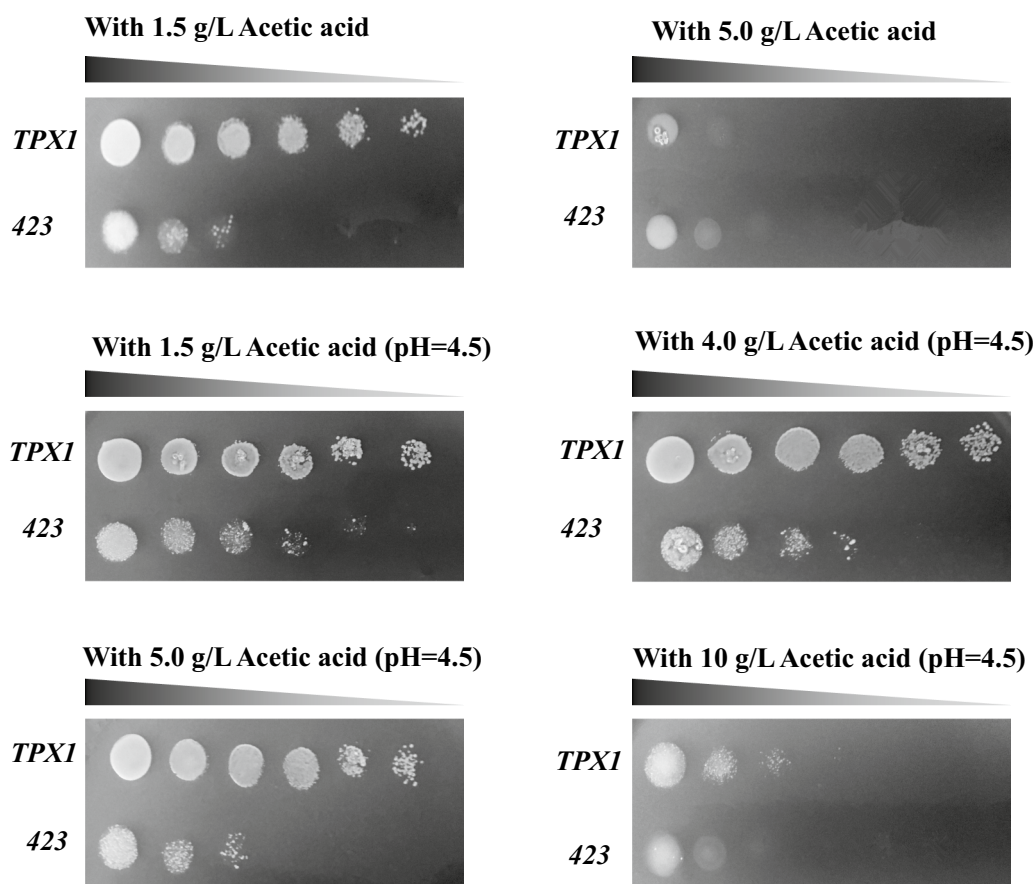


Fig. 4 Effects of initial pH on the tolerance of yeast cells to acetic acid. Serial dilution assay was performed on SC-His plates containing different concentrations of acetic acid. To investigate the effects of pH, the initial pH in plates was adjusted to 4.5 with 3 N NaOH when required

As shown in Fig. 5 and Additional file 2: Figure S2, the overexpression of *KmTPX1* gene in cells helped accelerate the process of ethanol fermentation under inhibitors and greatly reduce the lag phase in both flasks and bioreactors, which showed a similar trend. Considering the experiments in the 3-L bioreactors as an example (Fig. 5; Table 1), fermentative performance was greatly enhanced whether controlling the pH or not. Without adjusting the initial pH after the addition of inhibitors (Fig. 5a, b), the lag phase was slashed by almost 24 h compared with that in the control strain. Glucose consumption rate and ethanol productivity of TPX1 were achieved up to 0.86 and 0.36 g/L/h, respectively, both of which were double those of the control strain. Particularly, a lower residual glucose concentration for strain TPX1 was observed because of an accelerated fermentative process (Table 1). This may make great differences to industrial-scale ethanol production.

In addition, controlling pH at 4.5 greatly improved both the fermentative performance and the tolerance to acetic acid in TPX1 strain. As mentioned above, the poor nutrition in SC media led to the decrease of acetic acid concentration in FAF inhibitors. Fortunately, adjusting the initial pH in serial dilution assay helped solve this problem. Therefore, fermentations were conducted in bioreactors to control the pH at around 4.5 in media throughout the process. As shown in Fig. 5c, d and Table 1, TPX1 strain could tolerate the FAF inhibitors containing an elevated concentration of acetic acid without an obvious lag phase. The glucose consumption and ethanol production achieved a reasonable result within 48 h; in contrast, the control strain hardly grew under the same conditions, which was mainly manifested in the extremely low consumption rate of glucose (less than 10 g/L within 48 h).

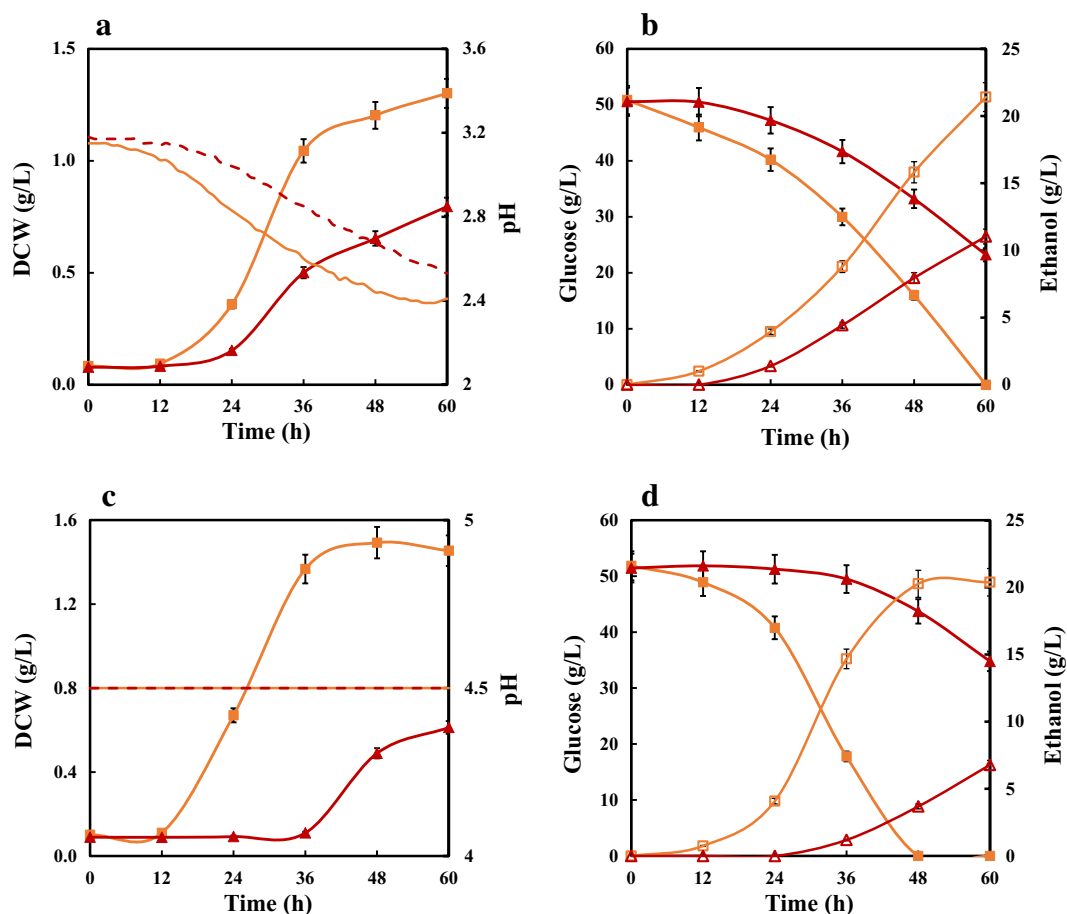


Fig. 5 Batch ethanol fermentation by *KmTPX1*-expressing *S. cerevisiae* under FAF stress. **a, b** Fermentative profiles of two strains within 60 h without controlling pH, or controlling pH at 4.5, respectively. **c, d** Fermentative profiles of two strains within 60 h when controlling pH at 4.5. Cells were pre-cultured in SC-His medium containing 1 mM H_2O_2 for 16–18 h. 5% of seed culture was inoculated into a 3-L bioreactor with a 1 L working volume. Data are given as mean \pm SD, $n = 2$

Table 1 Fermentative performance under FAF inhibitors in 3-L bioreactors

	TPX1	423	TPX1-pH ^a	423-pH ^a
Lag phase, h	0	24	0	36
Biomass, g/L	1.30 ± 0.06	0.80 ± 0.05	1.49 ± 0.12	0.61 ± 0.01
Initial glucose, g/L	51.84 ± 2.60	51.45 ± 1.89	50.86 ± 1.01	50.56 ± 2.27
Residual glucose, g/L	0	23.22 ± 1.24	0	34.78 ± 1.91
Ethanol, g/L	21.41 ± 0.83	11.03 ± 0.51	20.38 ± 1.02	8.50 ± 1.33
Glucose consumption rate, g/L/h	0.86 ± 0.04	0.47 ± 0.08	1.06 ± 0.02	0.33 ± 0.02
Productivity, g/L	0.36 ± 0.01	0.18 ± 0.01	0.42 ± 0.02	0.18 ± 0.03

^a pH in the media with an elevated concentration of acetic acid was controlled at 4.5

Consequently, *KmTPX1* gene overexpressed in *S. cerevisiae* increased the tolerance to lignocellulose-derived inhibitors, thus shortening the lag period and accelerating the fermentation process. Besides, a shorter lag period is supposed to be the general characterization for strains with an enhanced tolerance [9, 29]. We would see a better fermentative performance in strain TPX1, as was described previously by Kim et al. [29], if higher concentrations of initial glucose and final ethanol were adopted.

Increasing stress tolerance related to intracellular ROS levels

Despite the complicated mechanisms that lignocellulose-derived inhibitors are toxic to cells, they may induce cells to generate intracellular ROS, either FAF or phenol [4–6]. Intracellular ROS damages cells by altering the growth and metabolism characteristics. Therefore, oxidoreductases help remove the excess ROS, maintaining it at a normal level, to achieve an elevated tolerance and reduce damages to cells. Intracellular ROS in cells with an increased tolerance has been reported to decrease under inhibitors, no matter what strategies were adopted [9, 29, 34].

According to our results and existing theories, the possible mechanism of *KmTPX1* gene is inferred to be related to the levels of intracellular ROS. When the cells were exposed to FAF inhibitors, ROS like ·OH, H₂O₂, and O₂^{·-} might be generated. Hence, on the one hand, these ROS caused molecular damages and cellular effects to the normal cells, which eventually led to cell death [16]. However, on the other hand, overexpressed cells with *KmTPX1* gene achieved functional dimer proteins after transcription and translation under peroxides [23]. The activated dimers removed excess ROS inside the cells to maintain normal cell metabolism and to ensure a high rate of cell viabilities (Fig. 6a).

Fortunately, the measurement of intracellular ROS provided strong supports for our inference. Without FAF inhibitors, the level of intracellular ROS in TPX1 was

slightly lower than that in 423, which was proved to have no differences. Rather, with inhibitors of FAF, the level of intracellular ROS in control strain increased dramatically, achieving up to 3 times more than that in strain TPX1, and a lower amount of ROS was an important guarantee of growth, metabolism, and multiplication of yeasts (Fig. 6b). Above all, our results and some previous reports all confirmed that oxidoreductases maintained a normal level of oxidoreductases, which eventually manifested as an apparently enhanced tolerance of *S. cerevisiae* to multiple lignocellulose-derived inhibitors [9, 29, 34].

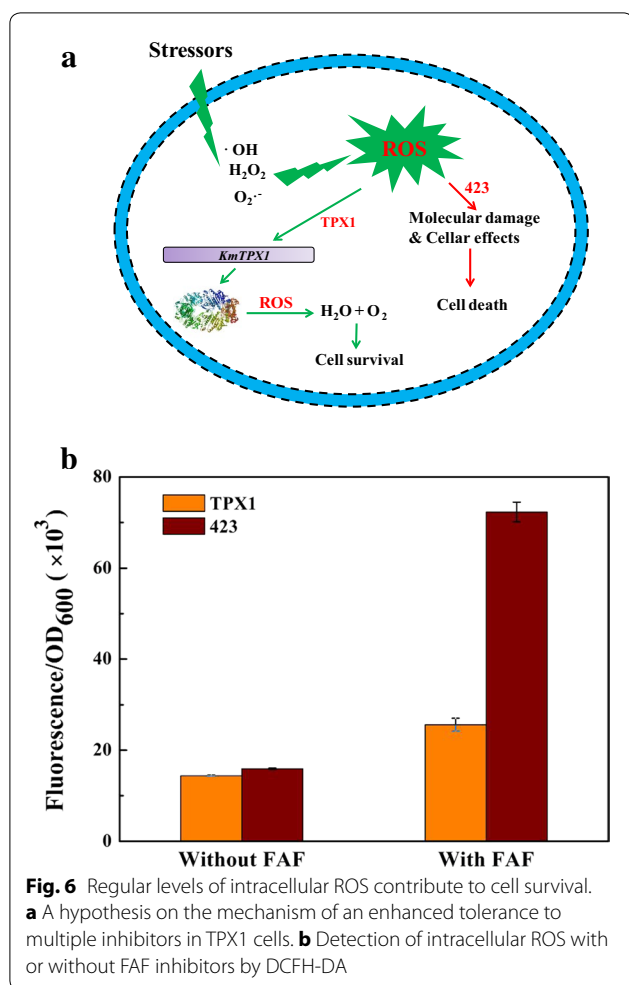
Conclusion

Ethanol production from lignocellulosic materials has been considered as one of the most promising substitutions for fossil fuels. In this study, through a comprehensive transcriptional analysis, we identified a typical 2-Cys peroxiredoxin from *K. marxianus* Y179 (*KmTPX1*). Meanwhile, the overexpression of *KmTPX1* gene has been proved to regulate the levels of intracellular ROS, which correspondingly increased the tolerance of *S. cerevisiae* to both oxidative stress and multiple lignocellulose-derived inhibitors. Particularly, a reasonable fermentation profile of the overexpressing strain TPX1 was achieved in the presence of FAF inhibitors. These findings lay a good foundation for further research on industrial-scale ethanol production from lignocellulosic materials.

Methods

Strains, media, and growth conditions

All strains used in this study are listed in Table 2. Yeast cells were grown at 30 °C in YPD medium (2% glucose, 1% yeast extract, and 2% peptone) or in synthetic complete medium (SC) containing 2% glucose and 0.67% yeast nitrogen base, supplemented with the appropriate amino acids. *Escherichia Coli* DH5α was cultivated at 37 °C in LB medium (0.5% yeast extract, 1% peptone, and 1% sodium chloride).



Plasmid construction and transformation

To construct an overexpression vector with the *KmTPX1* gene, plasmid pRS423 [35] was used for DNA manipulation and cloning. DNA manipulation was performed by standard procedures [36]. *KmTPX1* gene fragment was amplified from genome of *K. marxianus* Y179 with primers TPX1-F and TPX1-R. The obtained fragment with a coding region and a native promoter of *KmTPX1* gene was inserted into pRS423 to generate pRS423-TPX1 after digestion with *SacI* and *SacII* (Table 2; Additional file 1: Figure S1a). The constructs were verified by both digestion of restriction enzyme and sequencing (Additional file 1: Figure S1b).

Transformation of *E. coli* cells was performed using the CaCl₂ method [37]. For screening the transformants, ampicillin (100 µg/mL) was added into LB selective plates. Yeast transformation was conducted using the LiAc/PEG method described by Gietz et al. [38], and the transformants were screened by SC medium without histidine (SC-His). Transformants with the recombinant plasmid of pRS423-TPX1 were marked as TPX1, and strains with the plasmid pRS423 were set as control (423) in this study. All transformants were verified by PCR.

Stress tolerance assay

To test the functions of *KmTPX1* gene, serial dilution assay was performed using strain TPX1, and strain 423 was used as the control. Yeast cells were cultivated in SC-His medium with 1 mM H₂O₂ for 16–18 h at 30 °C and 150 rpm and then collected by centrifugation at 3000×g

Table 2 Strains, plasmids, and primers used in this study

Strains	Genotype	Source
Y179	<i>K. marxianus</i> , wild-type	Lab stock
280	<i>S. cerevisiae</i> , MATa, his3-1, leu2-0, met 15-0, ura3	Lab stock
DH5a	<i>E. coli</i> , for genetic manipulation	Lab stock
TPX1	<i>S. cerevisiae</i> overexpressing <i>KmTPX1</i> gene, transformed from 280	This study
423	<i>S. cerevisiae</i> with pRS423 plasmid, transformed from 280	This study
Plasmids	Characteristic	Source and reference
pRS423	Yeast episomal vector with <i>HIS3</i> marker	[37]
p423TPX	<i>KmTPX1</i> in pRS423	This study
Primers		
KmTPX1-F	5'-CTTgagctcAATGTCTCGTCTCGTCTCGT-3'	
KmTPX1-R	5'-TCCccgcggGGCTAAGCCAATAACTTATT-3'	
RT-TPX1-F	5'-CTCAAGTTTTGTTTCGTTCCAC-3'	
RT-TPX2-R	5'-AAGTCGTTGATGGTGATGTGTCT-3'	
ACT1-F	5'-ACGTTGTTCCAATCTACGCC-3'	
ACT1-R	5'-CTTGTTGGAAGTCCAAAGCG-3'	

for 5 min. The collected cells (final cell density 10 g/L of DCW) were serially diluted with distilled water, after which 10 μ L of the diluted cells was loaded onto SC-His agar plates containing a single or mixed lignocellulose-derived inhibitors. The inhibitors applied in this section included 3 mM H₂O₂, 0.3 g/L formic acid, 1.5 g/L acetic acid, 1.0 g/L furfural, 0.23 g/L phenol, 0.3 g/L *O*-methoxyphenol, 0.5 g/L 5-HMF, 5% (v/v) ethanol, 3 M NaCl, FAF mixture (0.3 g/L formic acid, 1.5 g/L acetic acid and 0.6 g/L furfural), and FAFH mixture (0.2 g/L formic acid, 0.8 g/L acetic acid, 0.3 g/L furfural, and 0.3 g/L 5-HMF). All plates were incubated for 2–3 days at 30 °C and then photographed. For high-temperature test, plates were incubated at 42 °C.

Fermentation experiments with H₂O₂ were also conducted to further verify the potential function of *KmTPX1*. Overnight cultures of two strains were inoculated into 100 mL SC-His medium supplemented with 0.2 mM or 3 mM H₂O₂, adjusting the initial cell density to around 0.05 g/L of DCW. The fermentation was performed at 30 °C and 150 rpm, and the samples were taken every 12 h to test cell growth.

The potential of an enhanced acetic acid tolerance was tested by serial dilution assay as was described above with the concentration of acetic acid increasing from 1.5 to 5 g/L. Effects of pH on acetic acid tolerance were evaluated by adjusting the initial pH to 4.5 with 3 N NaOH. The concentrations of acetic acid in SD-His plates were 1.5, 4, 5, and 10 g/L, respectively (Fig. 4).

Laboratory-scale batch fermentation

To evaluate the fermentative performance of strain TPX1, batch fermentation was conducted in bioreactors for 60 h. Pre-cultures of yeast cells were carried out in 100 mL of SC-His medium in 250-mL flasks for 16–18 h, and then 5% of cells were inoculated in a 3-L fermenter with 1 L of SC-His medium containing 50 g/L of glucose and FAF mixture (0.3 g/L formic acid, 1.2 g/L acetic acid, and 0.5 g/L furfural) at 30 °C and 150 rpm. The initial cell density was adjusted to 0.08–0.09 g/L of DCW, and the aeration rate was kept at 0.05 vvm.

To test the effect of initial pH on the fermentation and tolerance of TPX1 strain, batch fermentation was performed at an elevated concentration of acetic acid in FAF inhibitors (0.3 g/L formic acid, 5 g/L acetic acid, and 0.5 g/L furfural). Before inoculation, the initial pH of the medium with inhibitors was adjusted to around 4.5, and other environmental parameters were kept the same as above.

Samples were taken every 12 h to test cell growth, sugar and ethanol.

Bioinformatics analysis

Expression pattern analysis of differentially expressed genes related to oxidative stress from transcriptome of *K. marxianus* Y179 [20] was clustered using Cluster software [39] and Java TreeView software [40]. The hierarchical clustering of the chosen experimental conditions and genes was carried out using Euclidean Distance as the formula of the distance matrix. Evolutionary tree of some typical 2-Cys Prxs from Y179 and its allied species using *MEGA 4* software [41], and amino acid sequences applied are listed as follows: *KmTSA1/KmTPX1*, Tsa1 protein from *K. marxianus* Y179 in this study; *KlTSA1*, Tsa1 protein from *K. lactis* (Accession No. XP_451603.1); *ScTSA1*, Tsa1 protein from *S. cerevisiae* (Accession No. NP_013684.1); *ScTSA2*, Tsa2 protein from *S. cerevisiae* (Accession No. NP_010741.1); *SsTSA1*, Tsa1 protein from *S. stipitis* (Accession No. XP_001382622.1); *CtTSA1*, Tsa1 protein from *C. tropicalis* (Accession No. XP_002547929.1); *ScPRX1*, Pxr1 protein from *S. cerevisiae* (Accession No. NP_009489.1); *ScAHP1*, Ahp1 protein from *S. cerevisiae* (Accession No. NP_013210.1); and *ScDOT5*, Dot5 protein from *S. cerevisiae* (Accession No. NP_012255.3). Alignment was conducted using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) software (<http://clustalw.ddbj.nig.ac.jp/>).

ROS level analysis

Levels of ROS inside cells were tested by a common method using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-35845, dissolved in absolute ethanol) as an indicator. Cells after pre-culture were cultivated for 16–18 h in SD-His medium with FAF (0.3 g/L formic acid, 1.2 g/L acetic acid, and 0.5 g/L furfural) at 30 °C and 150 rpm. Cell pellets were washed twice with distilled water and then re-suspended in 0.5 mL of 10 mM PBS (pH 7.0) containing 10 μ M DCFH-DA. After incubation at 37 °C for 60 min, fluorescence was measured by a Multiskan spectrum microplate spectrophotometer (PerkinElmer, USA).

Analytical methods

The cell concentration was measured in dry cell weight (DCW) following the previous protocol [42]. Concentrations of glucose, ethanol, and glycerol were analyzed by Aminex HPX-87H column (300 \times 7.8 mm; Bio-Rad, Hercules) in HPLC system, which used 0.01 mol/L H₂SO₄ as the mobile phase and was eluted at 50 °C with a flow rate of 0.5 mL/min. All analyses in this study were done in duplicate except indicated, and the mean values are shown in the Results and discussion section.

Additional files

Additional file 1: Figure S1. Construction of overexpressing vector and subsequent verification. a) The schematic of overexpressing vector containing KmTPX1 gene and its own promoter. b) PCR and restriction enzyme digestion verification with a band of 1042 bp. c) Relative abundance of KmTPX1 overexpression in SC-His medium by real-time quantitative PCR technology.

Additional file 2: Figure S2. Fermentation profile in *KmTPX1*-expressing *S. cerevisiae* cells during batch ethanol production process under FAF stress in flasks. a) Growth behavior of two strains at first 60 h. b) Glucose consumption and ethanol production under FAF stress. Cells were pre-cultured in SC-His medium containing 1 mM H₂O₂ for 16–18 h. 1% of seed culture was inoculated into a 250 mL flask with a 100 mL working volume. Data are given as means ± SD, n = 2.

Additional file 3: Table S1. Fermentative performance under FAF inhibitors within 72 h in flasks.

Abbreviations

ROS: reactive oxygen species; DEGs: differentially expressed genes; Prxs: peroxiredoxins; ORP: oxidoreduction potential (mV); FAF: a mixture of formic acid, acetic acid and furfural inhibitors; FAFH: a mixture of formic acid, acetic acid, furfural, and 5-HMF inhibitors; SC: synthetic complete medium; SC-His: SC medium without histidine; DCW: dry cell weight (g/L); DCFH-DA: 2',7'-dichlorofluorescein diacetate.

Authors' contributions

JQG designed the research, performed most of the experiments, collected data, and prepared the manuscript. WJY conceived the concept and design of the experiment. HLF, SBH, and YML carried out microbiological sampling study and conducted part of the analyses. SJZ and FWB participated in the design of the experiment. The manuscript was written with the contributions of all the authors. All authors read and approved the final manuscript.

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Competing interests

All authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in the published article.

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