Effect of a novel alpha/beta hydrolase domain protein on tolerance of *K. marxianus* to lignocellulosic biomass derived inhibitors

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Research

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Abstract

Background: Lignocellulosic biomass is a promising renewable and sustainable resource for biofuels and chemicals. However, the inhibitors generated during lignocellulosic biomass pretreatment hinder microbial growth and interfere with fermentation. Studies on the tolerance to inhibitors, especially multiple inhibitors, are important in the construction of robust strains that can utilize lignocellulosic biomass.

Results: The expression of an uncharted α/β hydrolase (ABHD) from *Kluyveromyces marxianus* was found to be up-regulated in the presence of single or multiple inhibitors; this enzyme was termed KmYME in this study. Disruption of KmYME made the yeast more susceptible to inhibitors, particularly to acetic acid, furfural and 5-hydroxymethylfurfural. KmYME was located in the mitoplast of mitochondria with targeting signals in the N-terminal 1-40 amino acid residues. Under inhibitor stress, the intracellular concentration of ATP, NAD(P) in the KmYME disrupted strain decreased. In addition, the KmYME disrupted strain lost more mitochondrial membrane potential, showed increased plasma membrane permeability, a more severe redox imbalance, and increased ROS accumulation, compared with those of the non-disrupted strain in the presence of the same inhibitors. The recombinant KmYME possessed esterase/thioesterase activity and preferred short-chain pNP aliphatic esters, long-chain acyl-CoA, and succinyl-CoA as substrates. The retro-complementation results indicated that the enzymatic activity of KmYME was necessary for the resistance to inhibitors. In addition, KmYME was also required for the resistance to other stresses including ethanol, temperature, and osmotic pressure. Disruption of two possible homologous genes in *S. cerevisiae* also reduced its tolerance to inhibitors.

Conclusion: As an uncharted α/β hydrolase, KmYME which had esterase/thioesterase activity and located in mitochondrial matrix was identified for the first time. The esterase/thioesterase activity was necessary for the tolerance to lignocellulosic biomass derived inhibitors in *K. marxianus*. Disruption of KmYME reduced the tolerance to inhibitors by interfering with ATP, NAD and NADP synthesis. KmYME also correlated with tolerance to other stresses such as osmotic, ethanol, and temperature. This study may provide useful information for better understanding the resistance mechanism of multiple inhibitors, which is important in the creation of a robust strain for industrial fermentation that can use cellulosic biomass as substrate.

Background

Lignocellulosic biomass is deemed as a promising resource for renewable biofuels and other chemicals due to its low cost, large-scale availability, and non-competition with food production [1–3]. However, during lignocellulosic biomass pretreatment, inhibitors are generated as by-products of sugar and lignin degradation, including weak acids, furan derivatives and phenolic compounds. These compounds have different toxic mechanisms and synergistic effects that inhibit microbial cell metabolism and fermentation [4, 5]. Hence, determining the mechanism for tolerance to these inhibitors is important in the construction of a robust strain for industrial fermentation.

Kluyveromyces marxianus is a 'generally regarded as safe' (GRAS) microorganism that has attracted increasing attention in bioethanol fermentation of lignocellulosic biomass due to its thermo-tolerance, high growth rate, and broad substrate spectrum [6]. *K. marxianus* strains can grow with a growth rate of $0.86\text{--}0.99\text{ h}^{-1}$ at $40\text{ }^{\circ}\text{C}$, and some strains even grow at temperatures as high as $52\text{ }^{\circ}\text{C}$ [7]. Elevated temperature is suitable for cellulolytic enzymes used in lignocellulose saccharification (optimal temperature $45\text{--}50\text{ }^{\circ}\text{C}$) and provides advantages for the simultaneous saccharification and fermentation (SSF), as well as simultaneous saccharification and co-fermentation (SSCF) [8, 9]. Moreover, industrial fermentation at elevated temperature reduces cooling cost and risk of contamination [7]. Xylose is one of the main hydrolysis products of lignocellulosic biomass, aside from glucose, and *K. marxianus* can natively utilize xylose [10]. However, the tolerance of *K. marxianus* to multiple inhibitors is not very strong and the knowledge of *K. marxianus* tolerance to multiple inhibitors was scarce in previous studies [11, 12].

In our previous study, the expression of an uncharacterized α/β -hydrolase domain (ABHD)-containing gene (GenBank BAP69980, locus tag KMAR_10772) of *K. marxianus* was significantly up-regulated in the presence of inhibitors derived from the pretreatment of lignocellulosic biomass [13]. In this study, after the up-regulation of its expression was confirmed through real time PCR, this gene was disrupted or overexpressed to evaluate its effect on tolerance to inhibitors. The intracellular location of this protein was identified and the effect of the gene disruption on the mitochondria, such as the intracellular ATP concentration, intracellular NAD and NADP concentration, mitochondria membrane potential (MMP), plasma membrane integrity, mitochondrial morphology and intracellular ROS level was determined. Next, the enzymatic activity of the recombinant protein of this gene and its mutants was characterized. The tolerance to inhibitors of the strains which were retro-complemented with mutants was also evaluated. Subsequently, the effect of the gene disruption on other stresses, including osmotic pressure (sugar and salt), ethanol and temperature was evaluated. Finally, the homologous genes in *Saccharomyces cerevisiae* were disrupted and the effect on the tolerance to inhibitors of *S. cerevisiae* was also evaluated.

Results

KmYME was significantly up-regulated in the presence of lignocellulosic-derived inhibitors

In our previous study, the gene KMAR_10772, encoding an uncharacterized ABHD-containing protein was significantly up-regulated when the cells were cultivated with multiple inhibitors (acetate acid, furfural, HMF, and phenols) [13]. In this study, this gene was named KmYME and its expression in YHJ010 cells (Table 1) in the presence of each single kind of inhibitor (2.5 g/L acetic acid, 1.5 g/L furfural + 1.5 g/L 5-HMF, or 1.0 g/L phenols (4-hydroxybenzaldehyde, syringaldehyde, catechol and vanillin), respectively) at $42\text{ }^{\circ}\text{C}$ was also determined by quantitative real-time PCR (qPCR) (Fig. 1). The change of expression was shown as the $\log_2$ fold change (FC) ($\log_2$ FC). As shown in Fig. 1, KmYME was up-regulated with a $\log_2$ FC value of 5.95 ± 0.29 , 7.17 ± 0.27 , and 6.73 ± 0.26 , corresponding to acetic acid, furfural + 5-HMF, and phenols, respectively, compared with the $\log_2$ FC value of 0.12 ± 0.18 with no inhibitor. Therefore,

regardless of the presence of the inhibitor mixture or a single inhibitor, the expression of KmYME was enhanced.

Disruption of KmYME reduced the tolerance to inhibitors in *K. marxianus*

After the significantly up-regulated expression of KmYME was confirmed, its effect on tolerance to inhibitors was evaluated through gene disruption and retro-complementation. As shown in Fig. 2a, when cultivated at 42 °C in YPD medium without inhibitors, the growth of YWD003 (KmYME disrupted strain) was similar to those of YWD004 (KmYME retro-complemented strain) and YWD010 (YHJ010 complemented with URA3 and TRP1, as a control) (Table 1 and Fig. 3). With the inhibitor mixture or each single inhibitor, however, the growth of all strains was repressed with a longer lag phase, slower growth rate, and less final biomass yield (OD_{600}) (Fig. 2b, c, d, e). The YWD003 showed worse performance, particularly to acetic acid, furfural and 5-hydroxymethylfurfural.

Table 1
Yeast strains used in this study

Strains	Relevant genotype	Reference
YHJ010	<i>K. marxianus</i> , Δ KmURA3::KAN ^R , Δ KmLEU2::HISG, Δ KmTRP1::HISG	[14]
W303 1A	<i>S. cerevisiae</i> ATCC 208352, MATa, ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100	ATCC 208352
YWD001	<i>K. marxianus</i> , YHJ010, Δ KmYME::ScURA3	This study
YWD002	<i>K. marxianus</i> , YHJ010, pWD003, KmYME	This study
YWD003	<i>K. marxianus</i> , YWD001, ScTRP1	This study
YWD004	<i>K. marxianus</i> , YWD001, pWD003, KmYME	This study
YWD005	<i>K. marxianus</i> , YHJ010, ScURA3	This study
YWD009	<i>K. marxianus</i> , YHJ010, ScTRP1	This study
YWD010	<i>K. marxianus</i> , YWD005, ScTRP1	This study
YWD021	<i>K. marxianus</i> , YHJ010, pWD004, EGFP	This study
YWD022	<i>K. marxianus</i> , YHJ010, pWD005, KmYME-EGFP	This study
YWD024	<i>K. marxianus</i> , YWD021, pWD006, KmCox -RFP	This study
YWD026	<i>K. marxianus</i> , YWD022, pWD006, KmCox -RFP	This study
YWD028	<i>K. marxianus</i> , YHJ010, pWD007, KmYME (1–20 aa)-EGFP	This study
YWD030	<i>K. marxianus</i> , YHJ010, pWD008, KmYME (1–40 aa)-EGFP	This study
YWD032	<i>K. marxianus</i> , YHJ010, pWD009, KmYME (41–360 aa)-EGFP	This study
YWD034	<i>S. cerevisiae</i> W303 1A, Δ NP_011545::ScURA3	This study
YWD036	<i>S. cerevisiae</i> W303 1A, Δ NP_011529::ScURA3	This study
YWD037	<i>S. cerevisiae</i> W303 1A, YWD034, Δ ScURA3	This study
YWD038	<i>S. cerevisiae</i> W303 1A, YWD037, Δ NP_011529::ScURA3	This study
YWD040	<i>S. cerevisiae</i> W303 1A, ScURA3	This study
YWD046	<i>K. marxianus</i> , YWD003, Δ ScURA3, pWD026, KmCox -RFP	This study
YWD047	<i>K. marxianus</i> , YWD004, Δ ScURA3, pWD026, KmCox -RFP	This study
YWD048	<i>K. marxianus</i> , YWD010, Δ ScURA3, pWD026, KmCox -RFP	This study
YWD051	<i>S. cerevisiae</i> W303 1A, YWD038, ScTRP1	This study
YWD052	<i>S. cerevisiae</i> W303 1A, YWD038, pWD003, KmYME	This study

Strains	Relevant genotype	Reference
YWD053	<i>S. cerevisiae</i> W303 1A, YWD040, ScTRP1	This study
YWD074	<i>K. marxianus</i> , YWD001, pWD032, KmYME (GYSLG→GHSMG)	This study
YWD076	<i>K. marxianus</i> , YWD001, pWD033, KmYME (GYSLG→AYALA)	This study

Specifically, when the cells were cultured in YPD medium with the inhibitor mixture (3.0 g/L acetate acid, 0.7 g/L furfural, 0.7 g/L HMF, and 0.28 g/L phenols) (Fig. 2b), 2.5 g/L acetic acid (pH 4) (Fig. 2c) or 1.5 g/L furfural + 1.5 g/L HMF (Fig. 2d), respectively, the lag phase of YWD003 was longer than that of YWD004 and YWD010; and the exponential phase maximum growth rate (h^{-1}) of YWD003 (0.20 ± 0.01 , 0.27 ± 0.02 , 0.13 ± 0.07) was slower than that of YWD004 (0.31 ± 0.01 , 0.41 ± 0.02 , 0.33 ± 0.02) and YWD010 (0.28 ± 0.01 , 0.41 ± 0.01 , 0.33 ± 0.00). The biomass yield (OD_{600} maximum) of YWD003 was also less than that of YWD004 and YWD010. However, the difference of the growth in the presence of 1.0 g/L phenols was not obvious among those three strains except that the biomass yield (OD_{600} maximum) of YWD003 was a little lower than that of YWD004, and YWD010 (Fig. 2e).

Afterwards, tolerance to inhibitors of KmYME overexpressed strains (YWD002) (Table 1 and Fig. 3) was also evaluated by cultivating the cells with or without inhibitors. The results indicated that overexpression KmYME did not improve *K. marxianus* tolerance to inhibitors (Additional file 1: Fig. S1).

Moreover, the growth of YWD003, YWD004 and YWD010 in synthetic dropout medium (SD) was also determined. The tolerance of these strains to inhibitors all decreased and the strains only grew under lower concentrations of inhibitors. YWD003 again showed a worse performance than YWD004 and YWD010 in the presence of the inhibitor mixture (Additional file 1: Fig. S2).

Because acetic acid in the medium reduces the pH and acetic acid is often produced during yeast fermentation [15], a synergistic effect of acetate and pH was determined. As shown in Additional file 1: Fig. S3, the growth of YWD003 showed a worse performance than YWD004 and YWD010 in YPD with acetate (pH 6). Also, the acetate and pH showed a synergistic effect on the inhibition of *K. marxianus* growth. Disruption of KmYME led the strain (YWD003) to be more sensitive to acetate and a low pH.

The KmYME protein was located in the mitochondrial mitoplast

To determine the roles of KmYME in the tolerance to inhibitors, the intracellular localization of KmYME was investigated. KmYME with an EGFP fused at its C-terminus (KmYME-EGFP) was expressed in strain YWD022 (Table 1 and Fig. 3) to determine its intracellular location. These data suggested that the fusion protein was located in the mitochondria. Therefore, KmCox (GenBank: XP_022674814), a subunit of cytochrome oxidase on the inner mitochondrial membrane [16], was used to co-localize the position of KmYME. KmCox-RFP, as a mitochondria marker, was co-expressed with KmYME-EGFP in strain YWD026 (Table 1 and Fig. 3). KmCox-RFP was expressed in the EGFP-expressing strain YWD024 as a control (Table 1 and Fig. 3). As shown in Fig. 4a, KmYME was expressed in mitochondria and co-localized with

KmCox-RFP. Subsequently, a series of truncated (1–20 aa, 1–40 aa or 41–360 aa) or full length (1–360 aa) KmYME constructs were expressed with EGFP to determine the mitochondrial signal sequence of KmYME. The results indicated that the mitochondrial targeting sequence of KmYME was within 1–40 aa of the N- terminus, but longer than the first 20 aa (Additional file 1: Fig. S4).

To clarify the function of KmYME, a more accurate position in the mitochondria was determined using western blotting. The mitochondria of YWD026, which co-expressed KmCox-RFP and KmYME-EGFP was extracted for analysis. As shown in Fig. 4b, most of the KmYME-EGFP and KmCox-RFP was present in the fraction containing the mitochondrial mitoplast (inner-membrane and matrix). Since there was no transmembrane domain found in KmYME using informatics analysis, KmYME was possibly a matrix protein.

Disruption of KmYME led to decreased intracellular ATP concentrations in the presence of inhibitors

Mitochondria are "the powerhouse of the cell" and supply most of the intracellular ATP [17]. Because KmYME was located in the mitochondria, intracellular ATP levels were determined to evaluate if the disruption of KmYME affected ATP production. As shown in Table 2, without inhibitors, the intracellular ATP concentration of YWD003 ($3.20 \pm 0.17 \mu\text{mol/g DCW}$ (dry cell weight)), YWD004 ($3.47 \pm 0.13 \mu\text{mol/g DCW}$), YWD010 ($3.32 \pm 0.01 \mu\text{mol/g DCW}$) were similar. With the addition of inhibitors however, the ATP concentration in all strains obviously decreased. Specifically, the ATP concentration of YWD003 in the presence of the inhibitor mixture, acetic acid, and furfural + 5-HMF was $0.43 \pm 0.07 \mu\text{mol/g DCW}$, $0.96 \pm 0.10 \mu\text{mol/g DCW}$, and $0.74 \pm 0.10 \mu\text{mol/g DCW}$, respectively, which was obviously less than those of YWD004 ($0.89 \pm 0.14 \mu\text{mol/g DCW}$, $1.66 \pm 0.12 \mu\text{mol/g DCW}$, and $1.41 \pm 0.04 \mu\text{mol/g DCW}$, respectively) and YWD010 ($1.03 \pm 0.10 \mu\text{mol/g DCW}$, $1.60 \pm 0.06 \mu\text{mol/g DCW}$, and $1.34 \pm 0.22 \mu\text{mol/g DCW}$, respectively). These results indicated that disruption of KmYME led to a significant decrease of intracellular ATP concentration. However, the difference in intracellular ATP concentration was not so obvious among YWD003 ($1.11 \pm 0.04 \mu\text{mol/g DCW}$), YWD004 ($1.39 \pm 0.19 \mu\text{mol/g DCW}$) and YWD010 ($1.17 \pm 0.09 \mu\text{mol/g DCW}$) in the presence of phenols (Table 2), which was consistent with the growth analysis (Fig. 2).

Table 2
Intracellular ATP concentration ($\mu\text{mol/g DCW}$)

Inhibitor	None	Mixture	Acetic acid	Furfural + 5-HMF	Phenols
YWD003	3.20 ± 0.17	0.43 ± 0.07	0.96 ± 0.10	0.74 ± 0.10	1.11 ± 0.04
YWD004	3.47 ± 0.13	0.89 ± 0.14	1.66 ± 0.12	1.41 ± 0.04	1.39 ± 0.19
YWD010	3.32 ± 0.01	1.03 ± 0.10	1.60 ± 0.06	1.34 ± 0.22	1.17 ± 0.09
All values are the means of three biological replicates $\pm$ standard deviation.					

Disruption of KmYME led to decreased intracellular NAD and NADP concentrations in the presence of inhibitors

Mitochondrial matrix proteins usually participate in various biochemical reactions such as the citric acid cycle, oxidative phosphorylation, oxidation of pyruvate and the beta oxidation of fatty acids to produce NAD(P)H and ATP [17]. Therefore, the intracellular concentrations of NAD ($\text{NAD}^+ + \text{NADH}$) and NADP ($\text{NADP}^+ + \text{NADPH}$) were determined. As shown in Fig. 5a, without inhibitors (N), the intracellular NAD ($\text{NAD}^+ + \text{NADH}$) concentrations in YWD003, YWD004, and YWD010 were similar. However, with the addition of inhibitors, the intracellular NAD concentration of all strains decreased. The NAD concentration of YWD003 decreased the most, with only $24.9 \pm 3.1\%$ remaining in the presence of the inhibitor mixture (M) and $21.0 \pm 1.6\%$ remaining in the presence of acetic acid (A) compared to the concentrations without inhibitors (N). The next was YWD010, the NAD concentration was $55.0 \pm 2.6\%$ in the presence of the inhibitor mixture (M) and $50.0 \pm 3.0\%$ in the presence of acetic acid (A) compared to the concentrations without inhibitors (N). The NAD concentration of YWD004 remained at $87.3 \pm 12.0\%$ in the presence of the inhibitor mixture (M) and $72.2 \pm 1.7\%$ in the presence of acetic acid (A) compared to the concentrations without inhibitors (N) (Fig. 5a). However, in the presence of phenols (P), the intracellular NAD concentration of YWD003 was only slightly lower than that of YWD004 and YWD010, while in the presence of furfural + 5-HMF (F), the NAD concentration of the three strains decreased to a similar degree (Fig. 5a).

The ratio of NADH/NAD^+ was also determined. As shown in Fig. 5b, the ratios of NADH/NAD^+ in YWD003, YWD004 and YWD010 were similar without inhibitors (N). In the presence of the inhibitor mixture (M) or acetic acid (A), the ratio of NADH/NAD^+ in YWD003 decreased, whereas the ratios in YWD004 and YWD010 increased. However, in the presence of phenols (P) or furfural + 5-HMF (F), the ratio of NADH/NAD^+ in all three strains obviously decreased.

The intracellular concentration of NADP ($\text{NADP}^+ + \text{NADPH}$) and the ratio of $\text{NADPH}/\text{NADP}^+$ were also determined. As shown in Fig. 5c, without inhibitors, the intracellular NADP concentration of YWD003 was lower than the other two strains. Though the intracellular concentration of NADP was 20-fold lower than that of NAD, the pattern of the change in the presence of the various inhibitors was similar to that of NAD for all three strains; the change in the ratios of $\text{NADPH}/\text{NADP}^+$ were also similar to the ratios of NADH/NAD^+ (Fig. 5d).

Disruption of KmYME injured the integrity of the plasma membrane, reduced the MMP and increased intracellular ROS accumulation

Mitochondrial function, a key indicator of cell health, can be assessed by monitoring changes in the mitochondrial membrane potential (MMP) [18] using Rhodamine 123 (Rh123), a positively charged molecule that can accumulate in energized mitochondria. Decline of the MMP will cause leakage of Rh123 from the mitochondria, resulting in the decline of green fluorescence intensity (Rh123^-) [19]. Propidium iodide (PI) was used to measure cell plasma membrane integrity. This dye can enter the

damaged membranes of dead cells to bind to DNA and produces red fluorescence [20]. Therefore, the permeabilized plasma membrane of a dead cell will result in higher red fluorescence intensity (PI⁺). In our study, the MMP and plasma membrane integrity of cells in response to various inhibitors was determined with Rh123 and PI double staining using flow cytometry to evaluate mitochondrial function and cell death.

As shown in Fig. 6 and Additional file 1: Fig. S5, compared with those without inhibitors, the ratio of viable cells (Rh123⁺/PI⁻) (Fig. 6a) decreased while the percentage of dead cells (PI⁺) (Fig. 6b) or cells with reduced MMP (Rh123⁻) (Fig. 6c) of all strains increased in the presence of various inhibitors. Notably, in the presence of the inhibitor mixture (M), acetic acid (A) or furfural + 5-HMF (F), the ratio of viable cells of YWD003 ($8.8 \pm 0.6\%$; $34.8 \pm 2.3\%$; $55.4 \pm 1.8\%$, respectively) was much lower than that of YWD004 ($23.5 \pm 1.3\%$; $58.6 \pm 3.4\%$; $69.4 \pm 0.9\%$, respectively) and YWD010 ($22.8 \pm 1.5\%$; $57.8 \pm 0.8\%$; $65.0 \pm 0.3\%$, respectively); a higher ratio of dead cells or cells with reduced MMP was detected in strain YWD003 compared to that of strains YWD004 and YWD010. In the presence of phenols (P), the ratio of viable cells, dead cells (PI⁺) or cells with reduced MMP in YWD003 was not significantly different from those in YWD004 and YWD010. These results indicated that disruption of KmYME reduced the MMP and increased the permeability of the plasma membrane in the presence of acetic acid and furfural + 5-HMF, which then caused loss of mitochondrial function and cell death.

In addition, in the presence of inhibitors, the morphology of the mitochondria in *K. marxianus* obviously changed. As shown in Additional file 1: Fig. S6, mitochondria in all three strains were fissured into small, short and round shapes and some mitochondria appeared swollen in the presence of various inhibitors, compared with those reticula form of mitochondria in the cells without inhibitors, suggesting that the mitochondria were impaired by the inhibitors. However, there was no obvious difference among strains YWD046 (KmYME disrupted strain), YWD047 (KmYME retro-complemented strain) and YWD048 (YHJ010 complemented with URA3 and TRP1, as a control). Even the intracellular concentration of NAD and ATP were different under the same conditions. It is possible that the inhibitors had a strong ability to disrupt the mitochondrial morphology, but the difference was not obvious enough to be detected by microscopy.

Next, levels of intracellular ROS were determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining to determine if disruption of KmYME could influence ROS accumulation in the presence of multiple inhibitors. As shown in Fig. 6d, ROS accumulation obviously increased in the presence of various inhibitors. The levels of ROS in YWD003 were the highest in the presence of all inhibitors, compared with the levels in the other two strains, indicating that disruption of KmYME improved the accumulation of intracellular ROS in response to the presence of the inhibitors. It is noteworthy that the pattern of the amount of dead cells was not consistent with that of ROS accumulation (Fig. 6b and d). Though the percent of dead cells was the highest in the presence of the mixture of inhibitors and the second highest was in the presence of acetic acid, the highest levels of ROS accumulation was induced by the presence of phenols, and the second highest levels by the presence of furfural + 5-HMF, indicating that phenols and furfural + 5-HMF may play a leading role among the inhibitors in ROS accumulation.

KmYME had esterase and thioesterase activity

KmYME is described as an uncharacterized ABHD-containing protein YGR015C in GenBank. The ABHD superfamily includes proteases, lipases, esterases, dehalogenases, peroxidases, and epoxide hydrolases. Most of the homologous proteins of KmYME from other organisms are uncharacterized proteins. In this study, the KmYME gene was expressed in *Escherichia coli* BL21 (DE3) cells and the recombinant enzyme was purified. The enzyme was tested for the following activities: peroxidase, acetylcholinesterase (AChE), esterase or thioesterase using H_2O_2 , 2-mercaptoethyl-trimethylammonium iodide acetate, butyryl-CoA (C4-CoA) and p-nitrophenyl butyrate (pNPC4) as substrates, respectively. As a result, KmYME could hydrolyze C4-CoA and pNPC4 but could not hydrolyze H_2O_2 and 2-mercaptoethyl-trimethylammonium iodide acetate. Thus, the KmYME protein was provisionally identified as an esterase or a thioesterase.

Enzymatic properties of KmYME and its mutants

The consensus pentapeptide GX SXG is found in virtually all lipases/esterases and generally contains the active site serine [21] (Additional file 1: Fig. S7). In KmYME and ABHD11, a mammalian homolog, the conserved amino acid residues were GYSLG and GHSMG, respectively. Therefore, the amino acid residues GYSLG in the KmYME protein were substituted with GHSMG or AYALA. Then, the KmYME and its mutants were recombinantly expressed in *E. coli* BL21 (DE3) cells and purified (Additional file 1: Fig. S8). The enzymes were characterized using various substrates with different length carbon chains (Table 3). For pNP aliphatic ester substrates, the activity of KmYME was 2303.61 ± 154.69 nmol/min/mg and 600.59 ± 5.90 nmol/min/mg for p-nitrophenyl acetate (pNPC2) and pNPC4, respectively. There was no activity detected with p-nitrophenyl decanoate (pNPC10). Moreover, the K_m value of pNPC2 (189.73 ± 12.20 μM) was lower than that of pNPC4 (258.41 ± 5.97 μM). These results suggested that the KmYME esterase preferred short-chain pNP aliphatic ester substrates. For acyl-CoA substrates, KmYME showed a higher enzyme activity and lower K_m value with decanoyl-CoA (C10-CoA) (321.77 ± 34.20 nmol/min/mg and 329.20 ± 12.75 μM , respectively), compared with those of C4-CoA (48.32 ± 1.68 nmol/min/mg and 468.44 ± 20.62 μM , respectively) and succinic-CoA (44.22 ± 2.28 nmol/min/mg and 387.58 ± 3.42 μM , respectively). There was no enzyme activity detected with acetyl-CoA (C2-CoA), indicating that KmYME preferred long-chain acyl-CoA substrates.

Table 3
Comparison of the enzymatic properties of KmYME and its mutants

Substrates	KmYME		KmYME (GYSLG→GHSMG)		KmYME (GYSLG→AYALA)	
	Enzyme activity (nmol/min/mg)	Km (μM)	Enzyme activity (nmol/min/mg)	Km (μM)	Enzyme activity (nmol/min/mg)	Km (μM)
pNPC2	2303.61 ± 154.69	189.73 ± 12.20	157.80 ± 9.18	193.89 ± 10.00	N.D.	N.D.
pNPC4	600.59 ± 5.90	258.41 ± 5.97	45.67 ± 9.07	685.66 ± 34.83	N.D.	N.D.
pNPC10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C2-CoA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
C4-CoA	48.32 ± 1.68	468.44 ± 20.62	18.63 ± 0.81	1138.20 ± 185.83	N.D.	N.D.
C10-CoA	321.77 ± 34.20	329.20 ± 12.75	122.98 ± 17.86	449.88 ± 87.78	N.D.	N.D.
Succinyl-CoA	44.22 ± 2.28	387.58 ± 3.42	22.46 ± 3.22	1022.84 ± 145.83	N.D.	N.D.
Note: N.D. not detected.						

Interestingly, the enzyme activities and substrate affinity of KmYME (GYSLG→GHSMG) notably declined (Table 3). However, the preference characteristics of this mutant with the pNP aliphatic esters or acyl-CoA substrates was the same as those of KmYME (Table 3). KmYME (GYSLG→AYALA) had no enzyme activities with any of the pNP aliphatic ester or acyl-CoA substrates. These results indicated that the consensus pentapeptide GX SXG was essential for the esterase and thioesterase activity of KmYME.

Enzymatic activity was required for KmYME resistance to inhibitors

After analysis of the KmYME esterase and thioesterase activity, their effect on the tolerance to inhibitors was evaluated by expressing KmYME or its mutants in KmYME deficient strains. The strains expressing KmYME, KmYME (GYSLG→GHSMG), or (GYSLG→AYALA) were YWD004, YWD074, and YWD076, respectively (Table 1 and Fig. 3). Then, the growth of YWD003, YWD004, YWD010, YWD074, and YWD076 was measured with or without inhibitors. As shown in Fig. 7, although there was no obvious difference in the growth among the strains without inhibitors, in the presence of the inhibitor mixture (3.0 g/l acetic acid, 0.75 g/l furfural, 0.75 g/l 5-HMF, 0.3 g/l phenols), the growth of YWD076 was obviously repressed and similar to that of YWD003, and the growth of YWD074 was a little slower than that of YWD004 and YWD010. The growth performance was consistent with the enzymatic activity of KmYME and its mutants. These results suggested that the enzymatic activity (esterase or thioesterase) was necessary for the function of KmYME in the tolerance to inhibitors.

Transcriptomic analysis of the KmYME disrupted strain in the presence of multiple inhibitors

The transcriptomic analysis of *K. marxianus* YWD001 (KmYME disrupted) and YWD005 (no disruption of KmYME) (Table 1 and Fig. 3) with or without the inhibitor mixture was conducted using RNA-sequencing (RNA-seq). The RNA-seq results were analyzed in the following two relevant pairwise comparisons of gene expression levels: YWD001-I vs YWD001-C (*K. marxianus* YWD001 with vs without inhibitors), and YWD005-I vs YWD005-C (*K. marxianus* YWD005 with vs without inhibitors).

Unexpectedly, compared to those under the no stress conditions, most of the differentially expressed genes (DEGs) (about 87.30%, data not shown) in YWD001 in the presence of multiple inhibitors (YWD001-I vs YWD001-C) were also found in YWD005 with the same inhibitors stress conditions (YWD005-I vs YWD005-C). Additionally, most of them showed the same up- or down-regulation trends, although the relative expression levels (the fold changes, shown as $\log_2$ FC) were different between the pairwise comparisons. The 215 unique DEGs (about 12.70%) (not crossed with YWD005-I vs YWD005-C) in YWD001-I vs YWD001-C pairwise comparison were too decentralized by KEGG or GO enrichment analysis, so we focused on the total DEGs in the YWD001-I vs YWD001-C group, regardless of the comparison or not with those in the YWD005-I vs YWD005-C group, especially those DEGs related to the mitochondrial respiratory chain, coenzyme-dependent proteins, NAD⁺ biosynthesis, ROS reduction, and fatty acid biosynthesis and degradation.

As shown in Additional file 1: Table S1, in the presence of multiple inhibitors, quite a few DEGs related to NAD(P)⁺ dependence were differentially regulated. Among those DEGs related to central carbon metabolism, ADH3/4, ALD2/5, GUT2 etc., were down-regulated and led to less NAD(P)H production. Meanwhile, all DEGs related to the tricarboxylic acid cycle (TCA cycle) such as SDHs, MDH2 etc., and those related to glutamate metabolism such as GDH1 and UGA2, were up-regulated. Also, TDH2 and ADH6 were up-regulated, suggesting an increase of NAD(P)H production. In addition, some NAD(P)H-dependent DEGs coding for dehydrogenases and oxidoreductases such as GRE2, LYS1 etc., were also up-regulated in response to the resistance to the oxidative stress induced by inhibitors (Additional file 1: Table S1). From these results it was difficult to conclude that the NAD(P)H production was enhanced in response to the stress of the inhibitors.

For those DEGs related to NAD⁺ biosynthetic enzymes and related proteins, such as BNA3, FUN26, NMNAT, and URH1, all of them were up-regulated except PNC1, coding for nicotinamidase (Additional file 1: Table S1). Another gene NUDT12, coding for NADH pyrophosphatase, which was related to nicotinate and nicotinamide metabolism, was also up-regulated. These results suggested an enhancement of NAD⁺ production in response to the stress of multiple inhibitors.

The respiratory chain of the inner mitochondrial membrane is a unique assembly of protein complexes that transfers the electrons of reducing equivalents to molecular oxygen to generate a proton-motive force as the primary energy source for cellular ATP-synthesis [22]. For those DEGs related to the complexes within the mitochondrial respiratory chain, in both YWD005-I vs YWD005-C and YWD001-I vs

YWD001-C pairwise comparisons, under the stress of multiple inhibitors, NDI1 coding for rotenone-insensitive NADH-ubiquinone oxidoreductase, NDH1 coding for the external NADH-ubiquinone oxidoreductase 1, and the SDHs coding for succinate dehydrogenase, and COX2 coding for cytochrome c oxidase subunit 2 were up-regulated (Additional file 1: Table S1). There were no significant changes in the genes encoding the F_1F_0 ATP synthase subunits (data not shown). These results suggested that when exposed to inhibitors, cells regulated their energy metabolism towards increased generation of ATP.

As expected, when exposed to the stress of multiple inhibitors, most of the DEGs related to ROS detoxification were up-regulated. These genes encode proteins including superoxide dismutases (SOD1, SOD2), glutathione peroxidase 2 (GPX2), the thioredoxin system (TRR1, PRX1, DOT5, HYR1) and the glutathione/glutaredoxin system (GSH1) etc. The only two exceptions were CTT1 and a gene coding for glutaredoxin-like protein YLR364W, which were down-regulated (Additional file 1: Table S1). These results indicated that the defense systems were activated to detoxify ROS and to repair the damage caused by ROS.

We also noticed that most of the DEGs related to fatty acid biosynthesis, elongation and fatty acid degradation were down-regulated except MECR, PECL and ACADM, coding for a probable trans-2-enoyl-CoA reductase, 3,2-trans-enoyl-CoA isomerase and acyl-CoA dehydrogenase family member 11, respectively, in both pairwise comparisons (Additional file 1: Table S1). These data indicated that the fatty acid metabolism process was depressed by the stress of multiple inhibitors, regardless of whether KmYME was disrupted or not.

Disruption of KmYME reduced the tolerance to other stresses in *K. marxianus*

Because the disruption of KmYME reduced ATP, NAD(P) production, reduced MMP, and increased ROS accumulation, and thereafter affected the tolerance to inhibitors, it is possible that the disruption of KmYME reduced the tolerance to other stresses. In industrial production, osmotic pressure, ethanol and temperature can affect microorganism growth and fermentation. Therefore, the cell growth of YWD003, YWD004, and YWD010 cultivated at 42 °C in YPD with 180 g/L glucose, 0.5 M NaCl, or 20 g/L ethanol were conducted to evaluate the effect of the stress of sugar, salt, and ethanol, respectively. Furthermore, growth at 45 °C was also conducted to evaluate the effect of temperature stress.

As shown in Fig. 8, with the relatively high concentrations of glucose and salt (Fig. 8b, c), the growth of YWD003 (KmYME disrupted strain) was weaker than that of strains YWD004 and YWD010, though there was no obvious difference among them without stress (Fig. 8a). The YWD003 strain was also more sensitive to ethanol and weaker growth was detected with 20 g/L ethanol (Fig. 8d). When the temperature was increased to 45 °C, the growth of all strains decreased with a longer lag phase, lower specific growth rate, and less biomass (OD_{600}) (Fig. 8e); the growth of strain YWD003 showed the weakest growth among these strains. These results indicated that disruption of KmYME led to not only decreased tolerance to lignocellulosic inhibitors, but also decreased tolerance to osmotic pressure, ethanol, and temperature stresses.

Double disruption of two homologous proteins reduced the tolerance to inhibitors in *S. cerevisiae*

After confirming the decreased tolerance to the inhibitors by the disruption of KmYME in *K. marxianus*, we tested homologous genes in other yeast. Two ABHD-containing proteins (GenBank: NP_011545, NP_011529) homologous to KmYME were found in *S. cerevisiae*. The alignment of the amino acid sequences between KmYME and these two homologs is shown in Additional file 1: Fig. S7. Subsequently, these two genes in *S. cerevisiae* W303 1A were disrupted one by one. Two single-disruption strains (YWD034 and YWD036) and one double-disruption strain (YWD038) were obtained (Table 1 and Fig. 3). The strain YWD040 (*S. cerevisiae* W303 1A complemented with ScURA3) was used as the non-disrupted control. As shown in Fig. 9, in the presence of the multiple inhibitor mixture, the growth of all strains decreased with a longer lag phase and YWD038 showed the worst performance (Fig. 9b), though there was no obvious difference among the growth of these strains without the inhibitors (Fig. 9a). These results indicated that the single disruption of NP_011545 or NP_011529 did not reduce the tolerance to the inhibitors, while the double disruption led *S. cerevisiae* to be more sensitive to the inhibitors.

Subsequently, the KmYME gene was expressed in *S. cerevisiae* YWD038 to determine if the reduced tolerance to the inhibitors could be rescued. YWD051 (YWD038 complemented with TRP1 as non-overexpressing control), YWD052 (KmYME gene expressed in YWD038), YWD053 (YWD040 complemented with the TRP1 as non-disrupted control) were obtained (Table 1 and Fig. 3) and then cultivated in YPD medium with or without inhibitors. As shown in Fig. 9c and 9d, under the inhibitor mixture treatment, the growth of YWD051 and YWD052 was slower than that of YWD053, though there was no obvious difference among these three strains without inhibitors. This suggested that the expression of KmYME in YWD038 did not rescue the tolerance to inhibitors of the double-disrupted strain.

Discussion

Exploring the toxicity of lignocellulose-derived inhibitors to yeast and developing strains with enhanced tolerance is becoming more critical in producing chemical products from lignocellulosic materials. Considering the synergistic effects of these inhibitors, the construction of a strain with tolerance to multiple inhibitors has an increased practical application value. In our previous study, the significant up-regulation of KmYME was detected in the presence of multiple inhibitors [13]. Here, its upregulation was confirmed in the presence of multiple inhibitors or each single kind of inhibitor (Fig. 1), and this uncharacterized ABHD protein aroused great interest.

KmYME was shown to be essential in the tolerance to inhibitors by gene disruption and retro-complementation (Fig. 2). However, overexpression of KmYME did not enhance the tolerance (Additional file 1: Fig. S1). It is possible that the amount of KmYME required for tolerance to inhibitors was relatively small and overexpression of KmYME saturated that requirement. The retro-complementation with KmYME mutants with no or weak activity also suggested this possibility. Through site-directed mutagenesis of the consensus pentapeptide GX SXG, which is found in lipases/esterases and generally contains the active site serine, the low activity and no activity mutants were obtained (Table 3). The strain

expressing the KmYME mutant with no activity (YWD076) did not rescue the tolerance to the inhibitors of the KmYME disrupted strain, while the strain expressing the low-activity mutant (YWD074) partly rescued the tolerance to the inhibitors (Fig. 7). The tolerance to the inhibitors of the strains expressing the mutants was consistent with the enzymatic activity (Fig. 7). However, the native substrate of KmYME in yeast cells is still unknown. ABHD11, the mammalian homolog to KmYME (Addition file 1: Fig. S7 and S9), has high expression in metabolically active tissues such as brown adipose tissue, heart and skeletal muscle, and is related to many diseases such as Williams-Beuren syndrome [23] and lung adenocarcinoma [24], though the causal connection of ABHD11 to these diseases is not clear. Human ABHD11 is reported to hydrolyze pNPC2 and pNPC4, but cannot hydrolyze pNPC8 and pNPC10 and the long-chain acyl-CoA in vitro. In addition, the native substrate of human ABHD11 in cells is also not clear [25]. Clearly, the identification of the function of the novel KmYME protein in the mitochondrial matrix is critical for achieving a better understanding of the ABHD family proteins. It is noteworthy that KmYME could hydrolyze succinyl-CoA, which is a part of the TCA cycle. Succinyl-CoA synthetase can couple the hydrolysis of succinyl-CoA to ATP synthesis in yeast [26]. KmYME disruption led to decreased intracellular ATP concentration compared to that in the non-disrupted strain in the presence of inhibitors. Hence, it is possible that KmYME takes part in succinyl-CoA metabolism in vivo to regulate energy metabolism, though this needs to be confirmed in a future study.

Disruption of KmYME interfered with NAD^+ production. KmYME was shown to be localized to the mitochondria (Fig. 4), which is one of the places for NAD^+ production [27]. As shown in Fig. 5, the presence of inhibitors led to a relatively low intracellular NAD and NADP co-enzyme concentration, and disruption of KmYME caused a further decrease in intracellular NAD and NADP co-enzyme concentrations. However, regardless of whether KmYME was disrupted or not, the transcriptome results indicated that genes coding for NAD^+ biosynthetic enzymes such as BNA3, FUN26, NMNAT, URH1, and NUDT12 etc. were up-regulated in the presence of inhibitors (Addition File 1: Table S1). Nevertheless, the intracellular concentration of ATP, which is the adenylyl-backbone donor for NAD^+ [28] decreased in the presence of inhibitors (Table 2), and this may have led to the decreased concentration of NAD^+ . NADP^+ is generated from phosphorylation of NAD^+ by NAD^+ kinase [29], so the intracellular NADP^+ concentration also decreased. In addition, it is well known that the mitochondria is a place to produce NAD(P)H through the TCA cycle and NAD(P)H is required for the conversion of furfural, HMF and phenol inhibitors into low toxicity compounds [30, 31]. The removal of excess ROS induced by inhibitors also requires increased NAD(P)H [32]. Thus, the $\text{NAD(P)H}/\text{NAD(P)}^+$ ratio was very low and showed no obvious difference among the three strains in the presence of furfural, HMF and phenols (Fig. 5). Regarding the resistance to acetic acid, acetic acid is pumped out with the consumption of ATP and the procedure does not use reduced NAD(P)H co-enzyme directly. Furthermore, acetic acid can be converted into acetyl-CoA, which is a precursor substrate in the TCA, by acetyl-CoA synthetase (ACS). The reduced NAD(P)H coenzyme production may be enhanced in this situation due to the increased acetyl-CoA or just in response to stress, whereas the degradation of acetic acid requires less reduced NAD(P)H coenzyme. Therefore, the $\text{NAD(P)H}/\text{NAD(P)}^+$ ratio in the non-disrupted strains was elevated in the presence of acetic acid. For the KmYME disruption strain, because the expression pattern of the NAD(P)H dependent genes in the

KmYME disrupted strain and non-disrupted strains were similar, the decreased NAD(P)H/NAD(P)⁺ ratio was not due to the change in gene expression, and may be due to the interference of the KmYME disruption in NAD (NAD⁺, NADH) and NADP (NADP⁺, NADPH) synthesis, especially reductive NAD(P)H synthesis.

The disruption of KmYME interfered with ATP production. In eukaryotes, mitochondria supply ATP through oxidative phosphorylation and the TCA cycle. Though the transcriptomic analysis indicated that the expression of genes related to ATP synthesis were up-regulated and the expression of genes related ATP consumption were down-regulated in the presence of inhibitors (Addition File 1: Table S1), the intracellular ATP concentration decreased in all strains, and the KmYME disrupted strain (YWD003) had the lowest concentration among the strains (Table 2). A significant decrease in the ATP concentration due to the disruption of KmYME indicated that KmYME affected ATP production. There may be several reasons. 1) The inhibitors lead to the disruption of the proton gradient by uncoupling the respiratory chain and the oxidative phosphorylation of ADP, so that ATP regeneration is inhibited [30]; 2) The nonspecific hydrolysis of ATP increases in the presence of furfural [33]; 3) Yeast cells have been reported to re-direct the energy to fix the damage by reduced intracellular ATP and NAD(P)H levels either by enzymatic inhibition or consumption/regeneration of cofactors [5, 34]; 4) In addition, ATP is consumed to pump out the toxic inhibitors (weak acid) from the cell [33, 35]; 5) Finally, the low concentration of NADH could lead to decreased ATP concentration. Therefore, it is not unexpected that the intracellular concentration of ATP decreased in the presence of inhibitors (Table 2). The reduced intracellular concentration of NAD(P)H and the more significant decline of the MMP in KmYME disrupted strains compared to non-disrupted strains (Fig. 5, Fig. 6) led to a more severe decrease in ATP production (Table 2).

Disruption of KmYME led to a higher accumulation of ROS. Acetic acid, furfural and phenols have been reported to induce ROS accumulation [36–38] in yeast cells and excessive ROS can damage many cellular components, including DNA, proteins and lipids [39] and ultimately lead to apoptosis of the cell [40]. In our study, intracellular ROS level increased in the presence of inhibitors. Furthermore, the KmYME disrupted strain (YWD003) showed more ROS accumulation (Fig. 6d). The RNA-seq results showed that regardless of whether KmYME was disrupted or not, genes coding for proteins responsible for ROS removal and maintaining the redox balance such as superoxide dismutases, peroxidase, thioredoxin reductase, glutathione/glutaredoxin systems were up-regulated in response to the stress of multiple inhibitors (Addition File 1: Table S1). In addition, KmYME was shown to have no peroxidase activity. These results hinted that the increased accumulation of ROS in the KmYME disrupted strain was not due to the change in expression levels of ROS removal enzymes. Therefore, disruption of KmYME led to increased ROS accumulation due to the decreased reducing power of NAD(P)H, which is used by thioredoxin peroxidase and glutathione oxoreductase [32]. Interestingly, the amount of cell death was not similar to ROS levels (Fig. 6d); this may be explained by other lethal effects of the inhibitors, in addition to ROS accumulation.

The disruption of ATP and NAD(P)H synthesis led to a decreased tolerance to other stresses in the KmYME disrupted strain. In previous studies, ATP and NAD(P)H are necessary for the detoxification of

weak acids, furan derivatives and phenolic inhibitors [34, 41, 42]. NADPH donates electrons for glutathione and thioredoxin reduction and maintains the glutathione peroxidase or peroxiredoxin antioxidant systems to eliminate ROS generation [32]. In addition, NAD(P)H is reported to play an important role in aldehyde inhibitor detoxification [43]. NADH can be used to produce ATP via the electron transport chain [44] and ATP is required for pumping inhibitors out of cells. Moreover, ATP and NAD(P)H are necessary for biomolecule synthesis and cell division. Even though the genes coding for ATP and NAD(P)H synthesis were up-regulated (Addition File 1: Table S1), the detoxification of inhibitors consumed a large amount of ATP and NAD(P)H. As a result, the intracellular ATP and NAD(P)H concentrations decreased in the presence of inhibitors (Table 2 and Fig. 5). The disruption of KmYME further reduced the ability to synthesize ATP and NAD(P)H; therefore, the shortage of ATP and NAD(P)H became more severe and eventually led the cell to be sensitive to the inhibitors. This may also be the reason why disruption of KmYME reduced the tolerance of cells to other stresses including ethanol, temperature, and osmotic pressure (Fig. 8).

The KmYME disrupted strain had no obvious changes in death rate and growth compared to non-disrupted strains in the presence of the phenolic mixture. Because phenolic compounds cause the loss of membrane integrity, including the cell membrane and mitochondrial membranes, they inhibit microbial growth and fermentation [4, 5]. Because of the complexity of the compound structure with different functional groups and functional side groups, the mechanism of yeast tolerance to each phenolic compound is quite different [45]. In this study, KmYME may not contribute to membrane repair. Also, the contribution of KmYME to the tolerance of phenolic inhibitors may be eclipsed due to the different tolerance mechanisms to the phenolic compounds in the mixture.

Two homologous genes were found in the genome of *S. cerevisiae* (Additional file 1: Fig. S7, S9), with no report on the determination of function. In our study, the disruption of either NP_011545 (YGR031W) or NP_011529 in *S. cerevisiae* did not change the tolerance to the inhibitors (Fig. 9). However, the double disruption led to a decreased tolerance to the inhibitors (Fig. 9). We speculated that the function of NP_011545 or NP_011529 may be similar or overlapping. However, overexpression of KmYME could not remedy the decreased tolerance to the inhibitors of the double disruption strain of *S. cerevisiae*, suggesting that the mechanisms of KmYME in tolerance to inhibitors in *K. marxianus* may be different than the homologous proteins in *S. cerevisiae*. However, this novel ABHD family protein located in the mitochondrial matrix was applicable to other stresses and other organisms, which suggests a wide variety of promising applications.

Conclusion

An uncharacterized ABHD protein KmYME was required for tolerance to multiple inhibitors. Tolerance to the inhibitors decreased in the KmYME disrupted strain while overexpression of KmYME did not improve the tolerance to inhibitors in *K. marxianus*. The enzymatic activity of esterase/thioesterase of KmYME was necessary for the tolerance to the inhibitors. Disruption of KmYME did not result in a significant change of gene expression at the transcriptional level; thus, it was possible that KmYME affected the

tolerance to the inhibitors through interfering with NAD(P)^+ , NAD(P)H and/or ATP synthesis. In other words, K_{MYE} disruption led to a down-regulated energy metabolism. Disruption of two possible homologous genes in *S. cerevisiae* also reduced tolerance to inhibitors. The results of this study will enhance the understanding of the tolerance mechanisms in yeast to lignocellulosic hydrolysate inhibitors.

Methods

Culture and microorganisms

Yeast extract/peptone dextrose (YPD) medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose was used to culture yeast. Synthetic dropout (SD) medium containing 6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose supplemented with appropriate supplements were used to screen yeast transformants. Luria–Bertani (LB) medium with 100 µg/mL ampicillin was used to culture *Escherichia coli* Top10 for gene cloning and BL21 (DE3) for protein expression. *K. marxianus* NBRC1777 was obtained from the NITE Biological Resource Center (Tokyo, Japan). *K. marxianus* YHJ010 is the TRP1, LEU2, URA3 auxotrophic strain of NBRC1777 [14]. *S. cerevisiae* W303 1A is the ADE2, TRP1, LEU2, URA3 and HIS3 auxotrophic strain (ATCC 208352).

Plasmid construction

All of the plasmids used in this study are listed in Table 4. Plasmid construction is described in Additional file 2 in detail. Primer pairs are listed in Additional file 2: Table S1.

Table 4
Plasmids used in this study

Plasmids	Selection marker and description	Reference
YEGAP	ScTRP1, P _{ScGAPDH} , T _{ScGAPDH}	[46]
YEUKmPGK	ScURA3, P _{KmPGK} , T _{ScGAPDH}	[47]
pMD18T-ΔScURA3	Amp ^R , no function ScURA3	[9]
pCF.1676	Amp ^R , pJK148- ase1P*-klp3(1-335)-TagRFP-tom22	[48]
pCG	Amp ^R , CBM-EGFP- pTWIN1	[49]
pWD001	Amp ^R , KmYME-T vector	This study
pWD002	Amp ^R , KmYME inserted with ScURA3	This study
pWD003	Amp ^R , ScTRP1, P _{ScGAPDH} - KmYME -T _{ScGAPDH}	This study
pWD004	Amp ^R , ScURA3, P _{KmPGK} -EGFP- T _{ScGAPDH}	This study
pWD005	Amp ^R , ScURA3, P _{KmPGK} - KmYME -EGFP-T _{ScGAPDH}	This study
pWD006	Amp ^R , ScTRP1, P _{ScGAPDH} -KmCox -RFP-T _{ScGAPDH}	This study
pWD007	Amp ^R , ScURA3, P _{KmPGK} - KmYME (1–20 aa)-EGFP -T _{ScGAPDH}	This study
pWD008	Amp ^R , ScURA3, P _{KmPGK} - KmYME (1–40 aa)-EGFP -T _{ScGAPDH}	This study
pWD009	Amp ^R , ScURA3, P _{KmPGK} - KmYME (41–360 aa)-EGFP -T _{ScGAPDH}	This study
pWD010	Amp ^R , KmYME expressed under P _{T7}	This study
pWD024	Amp ^R , NP_011545 inserted with ScURA3	This study
pWD025	Amp ^R , NP_011529 inserted with ScURA3	This study
pWD026	Amp ^R , ScURA3, P _{KmPGK} -KmCox -RFP-T _{ScGAPDH}	This study
pWD032	Amp ^R , ScTRP1, P _{ScGAPDH} - KmYME (GYSLG→GHSMG) -T _{ScGAPDH}	This study
pWD033	Amp ^R , ScTRP1, P _{ScGAPDH} - KmYME (GYSLG→AYALA) -T _{ScGAPDH}	This study
pWD034	Amp ^R , KmYME (GYSLG→GHSMG) expressed under P _{T7}	This study

Plasmids	Selection marker and description	Reference
pWD035	Amp ^R , KmYME (GYSLG→AYALA) expressed under P _{T7}	This study

Briefly, pWD002, pWD024 and pWD025 were constructed for the disruption cassette of KmYME, NP_011545 and NP_011529, respectively. pWD004 was constructed for the expression of EGFP; pWD005 and pWD006 were constructed for the expression of KmYME-EGFP and KmCox-RFP in the KmYME localization assay. pWD007, pWD008 and pWD009 were constructed for the expression of the truncated KmYME (1–20 aa)-EGFP, KmYME (1–40 aa)-EGFP and KmYME (41–360 aa)-EGFP to identify the mitochondrial signal peptide. pWD026 was constructed for the expression of KmCox-RFP with a URA3 label to observe the mitochondrial morphology of the KmYME disrupted and non-disrupted strains. pWD010, pWD034 (GYSLG→GHSMG from pWD010) and pWD035 (GYSLG→AYALA from pWD010) were constructed for the expression of KmYME and KmYME mutants under the T₇ promoter in E.coli BL21 (DE3) cells. pWD003, pWD032 (GYSLG→GHSMG from pWD003) and pWD033 (GYSLG→AYALA from pWD003) were used to overexpress KmYME and the KmYME mutants under the ScGAPDH promoter in *K. marxianus*.

Strain construction

All of the yeast strains used in this study are listed in Table 1 and illustrated in Fig. 3. Transformation was conducted using the lithium acetate method [50]. The transformants were screened on SD medium with appropriate supplements and confirmed by PCR using genomic DNA as a template. The detailed strain construction is described in Additional File 2.

Samples preparation and transcriptome analysis

Cultivation conditions

K. marxianus YWD001 and YWD005 were pre-cultivated in 5 mL of YPD medium at 42 °C overnight. Then, the cells were transferred into 500-mL Erlenmeyer flasks containing 100 mL of YPD medium with an initial OD₆₀₀ of 0.5 and cultivated at 42 °C with shaking at 250 rpm in an orbital shaker until the OD₆₀₀ = 6 (the early-exponential phase of growth). The cells were then incubated without or in the presence of the inhibitor mixture containing 5.3 g/L acetate acid, 1.3 g/L furfural, 1.3 g/L HMF, and 0.5 g/L phenols (4-hydroxybenzaldehyde, syringaldehyde, catechol and vanillin with 0.13 g/L of each compound) for 1 h. Yeast cells were then recovered and stored at -80 °C until RNA isolation.

RNA-Seq analysis

Total RNA of each sample was extracted and cDNA libraries were prepared as previously described [13]. The resulting cDNA library products were then shotgun sequenced (101 bp paired-end read) with the Illumina HiSeq 4000 instrument (Illumina, San Diego, CA, USA) using a customer sequencing service (Majorbio Co., Ltd, Shanghai, China).

Annotation and bioinformatics analysis were performed as previously reported [13]. Clean reads were mapped to the reference genomic sequence of *K. marxianus* NBRC1777 from GenBank with accession No. AP014599-AP014607 [51] using TopHat (<http://tophat.cbcb.umd.edu>). In addition, information from the DEGs was subjected to GO and KEGG significant enrichment analyses to identify biological functions and metabolic pathways in which these genes participated. For differential gene expression analysis, reads or fragments per kilobase of exon model per million mapped reads (FPKM) was used as a value of normalized gene expression. Genes were considered differentially expressed in a given library when p -value < 0.05 and a greater than two-fold change in expression across libraries was observed.

Extraction of RNA and qPCR analysis

Total RNA was isolated using a yeast total RNA extraction kit (Sangon Biotech Co. Shanghai, China). Isolated RNA was treated with RNase-free DNase I (Toyobo, Osaka, Japan) and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan) as described [13]. Real-time PCR was conducted on a Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA, USA) using the THUNDERBIRD SYBR qPCR mix kit (Toyobo, Osaka, Japan). The primers for KmYME and the ACT1 internal control are shown in Additional file 2: Table. S1. The cycle threshold values (C_T) were determined and the relative fold differences were calculated using the $2^{-\Delta\Delta C_T}$ method [52] with ACT1 as the endogenous reference gene. The fold change was shown as the sign of the $\log_2$ transformed fold change (FC) values ($\log_2$ FC).

Fluorescence microscopy

Localization of KmYME in *K. marxianus* was conducted as described previously [53]. Imaging was conducted using a Perkin Elmer spinning-disk confocal microscope (PerkinElmer, Inc., Norwalk, CT, USA) equipped with a Zeiss PlanApo 100X/1.4 NA objective and a Photometrics EMCCD camera Evolve 51234. Images were captured by the DeltaVision softWoRx software (Applied Precision) and processed by deconvolution and z-stack projection. All images were analyzed using ImageJ.

Mitochondrial protein separation and extraction

Purification of mitochondria was performed as previously described [54]. Briefly, cells were harvested at $3000 \times g$ for 5 min, washed twice with ddH₂O, resuspended in DTT buffer (100 mM Tris/H₂SO₄ (pH 9.4), 10 mM dithiothreitol) and shaken for 20 min at 37 °C. The cells were recovered and resuspended in snailase solution (20 mM potassium phosphate (pH 6.0), 1.2 M sorbitol and 40 mg/g (wet weight cells) snailase). After 1 h incubation at 37 °C, the cells were harvested and resuspended in ice-cold homogenization buffer (10 mM Tris/HCl (pH 7.4), 0.6 M sorbitol, 1 mM EDTA, 0.2% (w/v) BSA). The cells were disrupted by sonication in an ice bath until half of the cell membranes were broken. Subsequently, mitochondria were isolated by differential centrifugation.

Second, the mitochondrial outer-membrane proteins and mitoplast (inner-membrane plus matrix) proteins were isolated by adding 0.15 mg/mL digitonin (Sangon Biotech Co. Shanghai, China) and vigorously agitated for 15 min using a vortex mixer. Then, the mixture was centrifuged at $10,000 \times g$ for 30 min at

4 °C. The resulting supernatant was the outer-membrane protein fraction and the pellet was the mitoplast protein (inner-membrane plus matrix) fraction [55].

Western blotting

Western blotting was performed as described previously [55]. Anti-GFP antibody and anti-RFP antibody were purchased from YEASEN Biotech Co., Shanghai, China.

Preparation of the samples for the measurement of intracellular ATP, NAD(P)⁺, NAD(P)H, MMP, plasma membrane permeability, mitochondria morphology and ROS detection

Cells were cultivated in YPD medium until the OD₆₀₀ = 6, then they were recovered and incubated for another 2 h without inhibitors, or in the presence of the inhibitor mixture (5.3 g/L acetate acid, 1.3 g/L furfural, 1.3 g/L HMF, and 0.5 g/L phenols), 3 g/L acetic acid, 6 g/L furfural + 6 g/L 5-HMF, or 2 g/L phenols, respectively at 42 °C. The cells were collected by centrifugation for 5 min at 3000 × g for the detection of intracellular ATP and NAD(P)⁺, NAD(P)H, MMP, plasma membrane permeability, mitochondria morphology and intracellular ROS level.

Intracellular ATP extraction and quantification

Half milliliter of cells were vortexed for 3 min in 0.5 mL 5% cold trichloroacetic acid to extract ATP. Then the samples were centrifuged at 10,000 × g for 10 min at 4 °C. The resulting supernatant was diluted 1000 times with 0.05 mol/L Tris-acetate (pH 7.8). The amount of ATP was determined using the ENLITEN® ATP Assay System Bioluminescence kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instruction. At OD₆₀₀ = 1, the concentration of the cells was equivalent to 0.411 g/L dry cell weight (DCW) [56].

Quantification of the intracellular NAD(P)⁺ and NAD(P)H

A 5-mL sample of yeast culture was withdrawn and sprayed into quenching solution (60% methanol and 70 mM HEPES). Then, the intracellular coenzymes NAD(P)H and NAD(P)⁺ were extracted and quantified using an EnzyChrom™ NAD(P)⁺/NAD(P)H assay kit (BioAssay Systems, Hayward, California, USA) following the manufacturer's instruction. Briefly, the quenched pellets were resuspended in acid extraction buffer or base extraction buffer and incubated at 65 °C for 5 min to extract oxidized pyridine nucleotides or reduced pyridine nucleotides, respectively. The relative amounts of NAD(P)⁺ and NAD(P)H in each extract were then quantified by enzymatic methods using an NADP⁺-specific glucose-6-phosphate dehydrogenase and an NAD⁺-specific yeast alcohol dehydrogenase (BioAssay Systems) [13].

Rhodamine 123 (Rh123) and propidium iodide (PI) double fluorescent staining

After washing twice with phosphate buffered saline (PBS), 100 µL yeast cells were collected and suspended in 10 mM 2-morpholinoethanesulfonic acid buffer (MES) containing 0.1 mM MgCl₂ and 2%

(w/v) glucose. Then, Rh123 (YEASEN Biotech Co., Shanghai, China) was added to a final concentration of 10 μ M and the sample was incubated for 20 min at 37 °C in the dark. After the sample was washed twice with PBS, PI (YEASEN Biotech Co., Shanghai, China) was added to a final concentration of 100 μ g/mL and the sample was incubated for 1 min at 37 °C in the dark. At last, the cells were resuspended in 1 mL PBS and immediately examined by CytoFLEX flow cytometry (Beckman Coulter, Inc., USA). The excitation wavelength was set to 480 nm, the application of side scatter (SSC) and forward scatter (FSC) were linearly amplified, and a logarithmic amplification was performed for fluorescence channel FL1 (FITC) and FL3 (PC5.5). The results were analyzed using the FlowJo software.

ROS assay

Cells were washed twice with PBS, then resuspended in PBS at a final concentration of 10^7 cells/mL with the addition of 10 μ g 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma, USA) [57]. After incubation at 37 °C for 60 min, the cells were washed twice with PBS. The fluorescence (excitation, 488 nm; emission, 525 nm) was detected using a CytoFLEX flow cytometer. The results were analyzed using the FlowJo software.

Recombinant expression and purification of KmYME and its mutants

To recombinantly express KmYME and its mutants, *E. coli* BL21 (DE3) was transformed with the plasmids pWD010, pWD034 or pWD035 (Table 4) and the resulting strains were cultivated in a 1 L shaking flask containing 400 mL LB medium with 100 μ g/mL ampicillin at 37 °C until $OD_{600} = 0.6-0.8$. Then, expression was induced with 0.5 mM IPTG at 37 °C for 4 h, the cells were recovered and lysed in lysate buffer (20 mM Tris-HCl pH 7.8, 0.3 M NaCl) by sonication (Sonics Vibra cell Model VCX130, SONICS & MATERIALS INC. Newtown, CT, USA). After centrifugation, the lysate supernatant was used for purification using a nickel-sepharose resin column (TransGen Biotech Co., Beijing, China) following the manufacturer's instruction. The obtained protein was dialyzed to remove imidazole and analyzed by SDS-PAGE. Protein concentration was determined using a Modified Bradford Protein Assay Kit (Sangon Biotech Co. Shanghai, China).

Enzymatic activity assays

One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μ mol of substrate in 1 min. The enzymes were assayed as follows. The thioesterase activity was assayed as described by McMahon et al. [58]. In brief, the enzyme was incubated with 100 μ M acyl-CoA, 1 mM DTNB (5,5'-Dithiobis-(2-nitrobenzoic acid)) (Sangon Biotech Co. Shanghai, China) in 100 mM HEPES, pH 8.0 at 37 °C for 5 min. The progress of the reaction was monitored through the change in absorbance at 412 nm using the molar extinction coefficient of 5-thio-2-nitrobenzoate ($14,150 \text{ M}^{-1} \text{ cm}^{-1}$), which is formed when DTNB reacts with free CoA. Acetyl-CoA(C2-CoA), butyryl-CoA(C4-CoA, succinyl-CoA) and decanoyl-CoA (C10-CoA) (Sigma, USA) were used as substrates.

Esterase activity was assayed as described by Kademi et al. [59]. In brief, the enzyme was incubated with 100 μ M pNP aliphatic esters in 50 mM Tris-HCl, pH 8.0 at 37 °C for 5 min. The amount of produced pNP was calculated in absorbance at 410 nm. pNPC2), p-nitrophenyl butyrate (pNPC4) or p-nitrophenyl decanoate (pNPC10) (Sigma, USA) were used as substrates.

Acetylcholinesterase (AChE) activity was assayed as described by Ellman et al. [60]. The enzyme was incubated with 100 μ M 2-mercaptoethyl-trimethylammonium iodide acetate (Sangon Biotech Co. Shanghai, China), 1 mM DTNB in 50 mM sodium phosphate buffer, pH 7.5 at 37 °C for 5 min. The measurement method was the same as that of the thioesterase activity assay described above.

Peroxidase activity was assayed as described by Yaaser et al [61]. The enzyme was incubated with 8 mM H₂O₂, 40 mM guaiacol in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C for 10 min. The brown red product tetramethoxyphenol was detected at OD₄₇₀ using a spectrophotometer.

Abbreviations

DEGs: differentially expressed genes; SD: synthetic dropout medium; DCW: dry cell weight; MMP: mitochondrial membrane potential; TCA cycle: tricarboxylic acid cycle; pNPC2: p-nitrophenyl acetate; pNPC4: p-nitrophenyl butyrate; pNPC10: p-nitrophenyl decanoate; C2-CoA: acetyl-CoA; C4-CoA: butyryl-CoA; C10-CoA: decanoyl-CoA; Log₂ FC: log₂ transformed fold change values; DTNB: 5,5'-Dithiobis-(2-nitrobenzoic acid); Rh123: rhodamine 123; PI: propidium iodide. ROS: reactive oxygen species; PBS: phosphate buffer saline.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consented on the publication of this work.

Authors' contributions

DW performed most of the experiments and collected data. DMW carried out RT-PCR experiments and conducted the transcriptomic analysis. JH and DMW conceived the concept and 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

The authors declare that they have no competing interests.

Availability of data and materials

The dataset supporting the conclusions of this article are included within the article and its additional files.

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Figures

Figure 1

qPCR results of KmYME expression in YHJ010 cells with or without lignocellulose-derived inhibitors. All values are the means of three biological replicates $\pm$ standard deviation.

Figure 2

The growth of YWD003, YWD004 and YWD010 in YPD containing a) no inhibitor, b) inhibitor mixture, c) acetic acid, d) furfural + 5-HMF, or e) phenols. All values are the means of three biological replicates $\pm$ standard deviation at each of the time points.

Figure 3

Schematic diagram of the strains construction. A key for the colored boxes with strains is shown in the lower right corner.

Figure 4

Analysis of the location of KmYME. a) Subcellular localization. YWD024: expressing EGFP and KmCox-RFP. YWD026: expressing KmYME-EGFP and KmCox-RFP. KmCox-RFP was used as a mitochondria marker. b) Intra-mitochondrial localization by western blotting. IM+Matrix: inner-membrane and matrix fraction of mitochondria, OM: outer-membrane fraction of mitochondria.

Figure 5

The intracellular concentration of the NAD and NADP coenzymes without inhibitors (N), and in the presence of the inhibitor mixture (M), acetic acid (A), furfural+5-HMF (F), or phenols (P). a) NAD (NAD⁺⁺+NADH) concentration, b) Ratio of NADH/NAD⁺, c) NADP (NADP⁺⁺+NADPH) concentration, d) Ratio of NADPH/NADP⁺. All values are the means of three biological replicates $\pm$ standard deviation.

Figure 6

The flow cytometry results of a) viable cells (Rh123+/PI-), b) dead cells (PI+), c) cells with reduced MMP (Rh123-), and d) ROS levels in YWD003, YWD004, YWD010 without inhibitors (N), or in the presence of the inhibitor mixture (M), acetic acid (A), furfural and 5-HMF (F), or phenols (P).

Figure 7

The growth of strains YWD003, YWD004, YWD010, YWD074 and YWD076 in YPD a) without inhibitors, b) in the presence of the inhibitor mixture. All values are the means of three biological replicates $\pm$ standard deviation.

Figure 8

The growth of strains YWD003, YWD004 and YWD010 in YPD medium with various stresses. a) No stress (42 °C); b) 180 g/L glucose at 42 °C; c) 0.5 M NaCl at 42 °C; d) 20 g/L ethanol at 42 °C; e) 45 °C. All values are the means of three biological replicates $\pm$ standard deviation.

Figure 9

The growth of *S. cerevisiae* strains with the KmYME homologous genes disrupted at 30 °C. a), c), without inhibitors; b), d), in the presence of the inhibitor mixture. All values are the means of three biological replicates $\pm$ standard deviation at each of the time points.

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