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BIOENERGY AND BIOFUELS

Adaptive laboratory evolution of ethanologenic *Zymomonas mobilis* strain tolerant to furfural and acetic acid inhibitors

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Abstract Furfural and acetic acid from lignocellulosic hydrolysates are the prevalent inhibitors to *Zymomonas mobilis* during cellulosic ethanol production. Developing a strain tolerant to furfural or acetic acid inhibitors is difficul by using rational engineering strategies due to poor understanding of their underlying molecular mechanisms. In this study, strategy of adaptive laboratory evolution (ALE) was used for development of a furfural and acetic acid-tolerant strain. After three round evolution, four evolved mutants (ZMA7-2, ZMA7-3, ZMF3-2, and ZMF3-3) that showed higher growth capacity were successfully obtained via ALE method. Based on the results of profiling of cell growth, glucose utilization, ethanol yield, and activity of key enzymes, two desired strains, ZMA7-2 and ZMF3-3, were achieved, which showed higher tolerance under 7 g/l acetic acid and 3 g/l furfural stress condition. Especially, it is the first report of *Z. mobilis* strain that could tolerate higher furfural. The best strain, *Z. mobilis* ZMF3-3, has showed 94.84 % theoretical ethanol yield under 3-g/l furfural stress condition, and the theoretical ethanol yield of ZM4 is only 9.89 %. Our study also demonstrated that ALE method might also be used as a powerful metabolic engineering tool for metabolic engineering in *Z. mobilis*. Furthermore, the two best strains could be used as novel host for further metabolic engineering in cellulosic ethanol or future biorefinery. Importantly, the two strains may also be used as novel-tolerant model organisms for the genetic mechanism on the "omics" level, which will provide some useful information for inverse metabolic engineering.

Keywords Adaptive laboratory evolution (ALE) · Lignocellulosic hydrolysates · Furfural · Acetic acid · Bioethanol · *Zymomonas mobilis*

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Introduction

Furfural and acetic acid from lignocellulosic hydrolysates are the prevalent inhibitors to *Zymomonas mobilis* during cellulosic ethanol production or biorefinery, especially inhibit cell growth, and lower its ethanol productivity (Franden et al. 2009, 2013; Gutierrez-Padilla and Karim 2005; He et al. 2012; Mills et al. 2009; Ranatunga et al. 1997). Although removal of inhibitors is technically feasible, i.e., ion exchange resins and ion exchange membranes (Han et al. 2006), the economics of ethanol production calls for an alternative approach. For example, the yield of ethanol was decreased by 20.5 % under furfural stress condition in *Z. mobilis* (He et al. 2012). Although two studies have focused on the response of *Z. mobilis* to furfural stress (He et al. 2012; Yang et al. 2010b), the genetic mechanisms of furfural and acetic acid stress in *Z. mobilis* are also poorly understood. In our previous study, genome-wide transcriptional response to furfural was first investigated in *Z. mobilis* using microarray analysis, and 433 genes were differentially expressed in the response to

furfural (He et al. 2012). Although these results revealed that furfural had effects on multiple aspects of cellular metabolism at the transcriptional level and membrane might play important roles in response to furfural, developing a strain tolerant to furfural and acetic acid inhibitors is also difficult by using rational engineering strategies due to our poor understanding of their underlying molecular mechanisms. On the other hand, different sodium acetate-tolerant *Z. mobilis* strains such as ZM4/AcR (Joachimsthal et al. 1998), *Z. mobilis* ZM4/Ac^R (pZB5) (Jeon et al. 2002), and ZM4/Ac^R (pZB5, pJX1) (Jeon et al. 2005) have been obtained from previous studies. Although genome changes associated with *Z. mobilis* sodium acetate-tolerant mutant ZM4/Ac^R were characterized by using microarray comparative genome sequencing (CGS) and 454-pyrosequencing (Yang et al. 2010a), developing an acetic acid-tolerant strain will also be a difficult task for *Z. mobilis*.

So far, different strategies, i.e., conventional mutagenesis, transposon mutagenesis, traditional genetic engineering, have also been used for strain improvement in *Z. mobilis*, and different strains were also achieved (He et al. 2014). Development of industrial strains with high tolerance toward the inhibitors released during biomass pretreatment is critical for bioethanol production. However, little study was focused on development of a furfural-tolerant strain in *Z. mobilis*. Recently, adaptive laboratory evolution (ALE) emerged as a valuable method in metabolic engineering for strain development and optimization (Chatterjee and Yuan 2006; Conrad et al. 2011; Dragosits and Mattanovich 2013; Pál et al. 2005; Portnoy et al. 2011), which has been successfully used in model organisms, such as *Escherichia coli* (Hua et al. 2007; Lee and Palsson 2010) and *Saccharomyces cerevisiae* (Cakar et al. 2012; Demeke et al. 2013; Dhar et al. 2011; Wallace-Salinas and Gorwa-Grauslund 2013). ALE is a powerful method to improve certain features of common industrial strains (e.g., inhibitor tolerance, substrate utilization, growth temperature) without requiring knowledge of any underlying genetic mechanisms, as long as the desired trait can be coupled with growth. In contrast to rational engineering strategies and directed modification of specific enzymes, ALE has the advantage of letting nonintuitive beneficial mutations occur in many different genes and regulatory regions in parallel (Dragosits and Mattanovich 2013; Portnoy et al. 2011). Currently, ALE strategy was also employed for *Z. mobilis* strain improvement, highly efficient xylose-fermenting, and acetic acid-tolerant strains obtained, respectively (Agrawal et al. 2011; Ali Mohagheghi et al. 2014; Wang 2008). These results demonstrated that ALE method might also be used as a powerful metabolic engineering tool for metabolic engineering in *Z. mobilis*.

In this study, ALE strategy was used for development of inhibitor-tolerant strains. The goal of this research is to develop furfural and acetic acid-tolerant strains for future cellulosic ethanol fermentation. An adaptive mutation under stress pressure will be developed, and inhibitor-tolerant strains may be selected. Finally, full characterization of its cell growth behavior, ethanol fermentation characteristics, and activity of key enzymes will also be performed.

Material and methods

Bacterial strains and culture conditions

Z. mobilis ZM4 (ATCC31821) was used as a starting strain for the laboratory evolution, which was maintained on glucose agar (20.0 g/l glucose, 10.0 g/l yeast extract, and 15.0 g/l agar). *Z. mobilis* ZM4 was cultured in Rich media (RM) (Goodman et al. 1982) at 30 °C without shaking. Evolved mutants were developed from the wild-type strain by ALE experiments as followed. Selected tolerant strains were cultured with RM medium containing 115 g/l glucose for investigation on its profiling of cell growth, glucose utilization, and ethanol yield under normal or stress conditions. For long-term storage, all strains were kept at −80 °C by mixing 500-μl steriled RM medium with overnight culture with 500-μl 60 % (*w*/*v*) glycerol solution.

Adaptive laboratory evolution experiments

The concentration of furfural and acetic acid in lignocellulosic hydrolysates was measured in the range from 0.5 to 11 g/l (Almeida et al. 2007) and 1 to 10 g/l (Mills et al. 2009). Our primary experiment showed that *Z. mobilis* ZM4 was not able to grow on the medium added with 1.5~2.0 g/l of furfural (initially added). So, in this study, furfural (1 to 3 g/l) and acetic acid (4 to 7 g/l) were added when necessary. In this study, the initial furfural concentration was 1 g/l for all populations and was gradually increased to 2 g/l, 3 g/l during the evolution to maintain an approximately constant selective pressure. And, the other adaptation experiment was also carried out with acetic acid concentration, 4, 6, and 7 g/l, respectively.

The procedure of adaptive mutation is illustrated in Fig. 1. In the first round adaptation, *Z. mobilis* ZM4 strain grew in RM medium in the absence of furfural (or acetic acid) at pH 7.0 and 30 °C without shaking. After overnight growth, the *Z. mobilis* was inoculated into a new 10-ml Falcon tubes containing 5 ml of RM medium supplemented with 1 g/l furfural (or 4 g/l acetic-acid), and then cultured into the same medium by the method of serial batch transfer (i.e., performed by a tube-to-tube transfer of the inoculums into the new media), repeated ten times. After this process, overnight culture was sprayed onto solid RM medium supplemented with 1 g/l furfural (or 4 g/l acetic-acid) at 30 °C. Twenty bigger clones were selected for assessment by cell growth under stress condition, and the best strain (named F1-X or A4-X) was selected

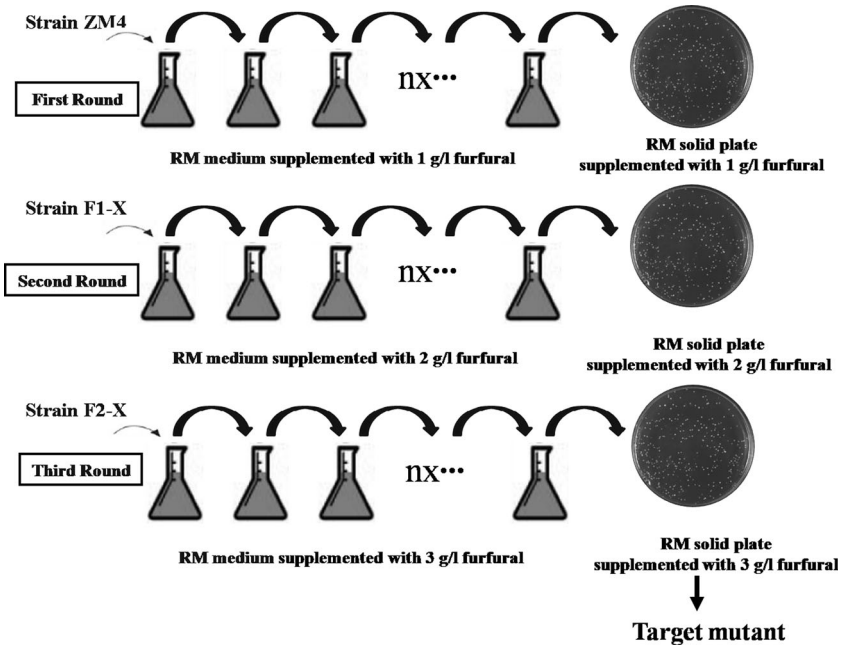

Fig. 1 The process of adaptive laboratory evolution (ALE) used in this study

for the second round adaptation experiment (2 g/l furfural for F2-X and 6 g/l acetic acid for A6-X, respectively). Repeat this process for the second and third round adaptation experiments under higher furfural concentration (3 g/l furfural and 7 g/l acetic-acid). After three round adaptation experiments, another ten bigger clones were selected for assessment by cell growth under stress condition, and the best strain (named F3-X or A7-X) was selected for further analysis.

Cell growth, glucose, and ethanol analysis

Mutant library of ALE was first screened by BioScreen C analyzer (Lab Systerms Helsinki, Finland). Incubations were performed at 30 °C, and absorbance readings were taken every 30 s. Operation of the Bioscreen C and collection of turbidity measurements ($OD_{420–580}$) were computer automated with EZ experiment (Franden et al. 2013). Cell growth was also determined by monitoring the optical density at 600 nm by using multi-scanner spectrometer (Thermo Inc.) at 6-h intervals. Fermentation supernatant was prepared by passing through 0.2-μm membrane (Millipore) and used to determine the concentrations of glucose and ethanol. High-performance liquid chromatography (HPLC) (Agilent Hi-plex H, 300 mm × 7.7 mm) was applied to measure the concentration of glucose and ethanol with sulfuric acid (0.05 M) as mobile phase at a flow rate of 0.6 ml/min and a column temperature of 35 °C. Glucose and ethanol were quantified by comparing their peak areas with standard sugar and ethanol solutions of known concentrations.

Identification of tolerant strain by 16 s rRNA and random amplified polymorphic DNA (RAPD)

The expected tolerant evolved mutants and *Z. mobilis* ZM4 cells were cultured in RM medium containing relevant inhibitors when necessary, respectively. Cells were harvested when the OD_{600} was 1.0, and then used for obtaining genomic DNA by genomic DNA extraction kit (Omega) according to the manufacturer's instructions. A DNA fragment was amplified with genomic DNA as template by using the following primers: (ZM16sRDNA-493F: 5′-ACGAAAGCGTGGGT AGCAAACA-3′) and (ZM16sRDNA493-R: 5′-TCTGTC ACCGCCATTGTAGCAC-3′). Subsequently, PCR products were examined by 1 % agarose gel electrophoresis. Purified PCR products were cloned into pGEM-T vector and sequenced by GENEWIZ Company (Suzhou, China). Sequence similarity analysis was performed by NCBI/BLAST/blastn.

On the other hand, random amplified polymorphic DNA (RAPD) was also performed by using OPERON random primers (Operon Technologies, USA), as listed in Table 1. The amplification was conducted with predenaturation at 94 °C for 5 min, followed by 45 cycles of thermal denaturation at 94 °C (45 s), primer annealing at 36 °C (45 s), and extension at 72 °C (2 min). After that, a 10-min final extension at 72 °C was conducted to stabilize the amplified DNA products. Such amplified products were examined by 1 % agarose gel electrophoresis.

Assay of key enzyme activity

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are the key enzymes in Entner-Doudoroff (ED)

Table 1 Primers used for random amplified polymorphic DNA in this study

Primer name	10 m in length—5′ to 3′
RAPD-1 (OPERON A01)	CAGGCCCTTC
RAPD-2 (OPERON A-04)	AATCGGGCTG
RAPD-3 (OPERON A-07	GAAACGGGTG
RAPD-4 (OPERON A-11)	CAATCGCCGT
RAPD-5 (OPERON A-15)	TTCCGAACCC
RAPD-6 (OPERON B-01)	GTTTCGCTCC
RAPD-7 (OPERON B-04)	GGACTGGAGT
RAPD-8 (OPERON B-07)	GGTGACGCAG
RAPD-9 (OPERON B-11)	GTAGACCCGT
RAPD-10 (OPERON B-15)	GGAGGGTGTT
RAPD-11 (OPERON C-01)	TTCGAGCCAG
RAPD-12 (OPERON C-04)	CCGCATCTAC
RAPD-13 (OPERON C-07	GTCCCGACGA
RAPD-14 (OPERON C-11)	AAAGCTGCGG
RAPD-15 (OPERON C-15)	GACGGATCAG
RAPD-16 (OPERON D-01)	ACCGCGAAGG
RAPD-17 (OPERON D-07)	TTGGCACGGG
RAPD-18 (OPERON D-11)	AGCGCCATTG
RAPD-19 (OPERON D-15)	CATCCGTGCT

pathway, which needed to be monitored to describe the changes of activity between mutants and parent strains. The evolved cells and *Z. mobilis* ZM4 cells were cultured in RM medium and RM medium containing furfural or acetic acid, respectively, which were harvested when the OD_{600} was 1.0 to preparation of cell extract by centrifugation at 3000*g*, 4 °C for 5 min. Cells were dissolved by Yeast Protein Extraction Kit (CW0890A) for 20 min in an ice bath and slightly shaked every several minutes. Cell debris was removed by centrifugation at 12,000 rpm, 4 °C for 10 min. The supernatant was collected for subsequent protein determination and enzyme assays according to previous studies (Shin and Rogers 1995; Zhang et al. 2010).

The activity of PDC was assayed by coupling the decarboxylation reaction with the ADH-mediated reaction and monitoring the oxidation of NADH to NAD^+ at 340 nm. The reaction mixture consisted of (μl) 200 mM sodium citrate buffer (pH 6.0) 945, 10 mg/ml NADH (sodium salt, Sigma Chem. Co., Product no. N8129) 10, 100 mg/ml sodium pyruvate 32, 10 mg/ml alcohol dehydrogenase (Solarbio Chem. Co., Product no. A-8400) 3, enzyme sample 10. One unit of enzyme activity is defined as that activity which converts 1.0 μM of pyruvate to acetaldehyde/min at pH 6.0 and 25 °C. The activity of the enzyme was monitored as NAD^+ formation by changes in absorbance at 340 nm.

The reaction for determination of ADH activity is the oxidation of ethanol to acetaldehyde with monitoring of the reduction of NAD^+ to NADH. The reaction mixture consisted of (μl) 35 mM Tris–HCl buffer (pH 8.5) 930, 20 mg/ml NAD^+ (Sigma Chem. Co., Product no. N7004) 0, absolute ethanol 30, enzyme sample 10. One unit of enzyme activity was defined as that activity which converts 1.0 μM ethanol to acetaldehyde/min at pH 8.5 and 37 °C. The activity of the enzyme was monitored as NADH formation by changes in absorbance at 340 nm. Protein was measured by the Bradford method with bovine serum albumin as a standard.

Results

Adaptive evolution and isolation of higher tolerant mutants

Adaptive mutation by gradually increasing the acetic acid concentration was carried out with different acetic acid concentration, 4, 6, and 7 g/l, respectively. Meanwhile, adaptive mutation under furfural stress was also carried out with 1, 2, and 3 g/l furfural concentration. After previous round selection, 20 colonies were investigated by BioScreening C analyzer, and the best clone was used for next round adaptive evolution (data not shown). After third round evolution, the culture with the highest acetic acid and furfural concentration (in this case, named A7 and F3) was plated on RM agar plates containing relevant inhibitors. Nearly 50 and 30 colonies were obtained under 7 g/l acetic acid and 3 g/l furfural stress conditions, respectively. Six evolved mutants (named ZMA7/F3-1, 2, 3, 4, 5, 6) were used for further screening. The interrelations of the evolved mutants obtained from this work are shown in Fig. 2.

The selected six colonies were used for further screening by spraying onto RM agar plate containing different concentration of acetic acid and furfural in manner of gradient dilution. As shown in Fig. 3, evolved mutants ZMA7-1, 2, 3, 4, 5, 6 grown better than ZM4 under 6 g/l acetic acid stress condition. However, ZM4 could not grow under 7 g/l acetic acid stress conditions. Similarly, evolved mutants ZMF3-1, 2, 3, 4, 5, 6 grew better than ZM4 under 2 g/l furfural stress condition. However, ZM4 could not hardly grow under 3 g/l furfural stress conditions.

On the other hand, cell growth of all evolved mutants (ZMA7/F3-1, 2, 3, 4, 5, 6) were also investigated by monitoring its optical density at 600 nm under normal and stress conditions (data not shown), and all evolved mutants showed higher growth than ZM4, especially, strains ZMA7-2, 3 and ZMF3-2, 3 showed better growth among all evolved mutants. So, four mutants were obtained by third round adaptive evolution, which were used for further analysis.

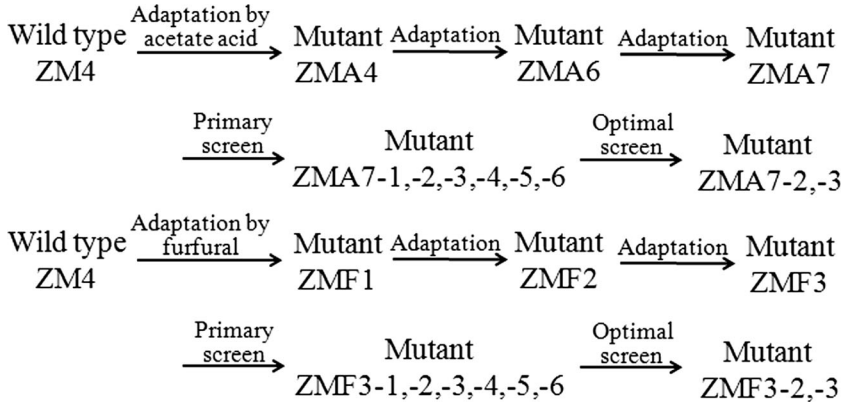

Fig. 2 The interrelations of the evolved mutants obtained from this work. These evolved mutants obtained were named using a combination of letters and numbers. The letters "ZM" are taken from the name of the microorganism "*Z. mobilis.*" Alphabet "A" and alphabet "F" following "ZM" denote acetate acid and furfural separately used in the adaptation, and the following number indicates the acetic acid or furfural concentration (g/l). The number after hyphen represents one of different single colonies. For example, ZMA7-1 is one of plate colonies obtained using 7 g/l acetic acid concentration

Profiling of cell growth, glucose utilization, and ethanol yield

Second, profiling of cell growth of these tolerant strains was also investigated. As shown in Fig. 4a, b, all evolved mutants showed the same cell growth trend as wild-type ZM4 under normal condition. ZM4 reached its maximum cell density (OD_{600}) to 3.247 after 72-h postinoculation under normal condition, while the highest cell density was 1.841 (after 66-h inoculation) and 0.584 (after 72-h inoculation) under 7 g/l acetic acid and 3 g/l furfural stress conditions, respectively. These results indicated that furfural and acetic acid have a negative effect on cell growth. However, evolved mutants ZMA7-2, ZMA7-3, ZMF3-2, and ZMF3-3 showed higher growth than ZM4 under stress conditions. Actually, all evolved mutants starting growth after 6-h initial inoculation, however, ZM4 starting growth after 12 h. After cultured with 18 h, the cell density of ZMA7-2, ZMA7-3, and ZM4 was 0.67, 0.249, and 0.184 under 7 g/l acetic acid, which showed increasing of 0.35–2.64 times than wild-type strain. After 54 h, all strains reached the highest cell density, which also showed an increasing with 29–33 % than ZM4 (Fig. 4a). These results also indicated that ZMA7-2 had higher growth than ZMA7-3. Similarly, after cultured with 18 h, the cell density of ZMF3-2, ZMF3-3, and ZM4 was 0.505, 0.532, and 0.164 under 3 g/l furfural, which showed increasing of 2.08–2.24

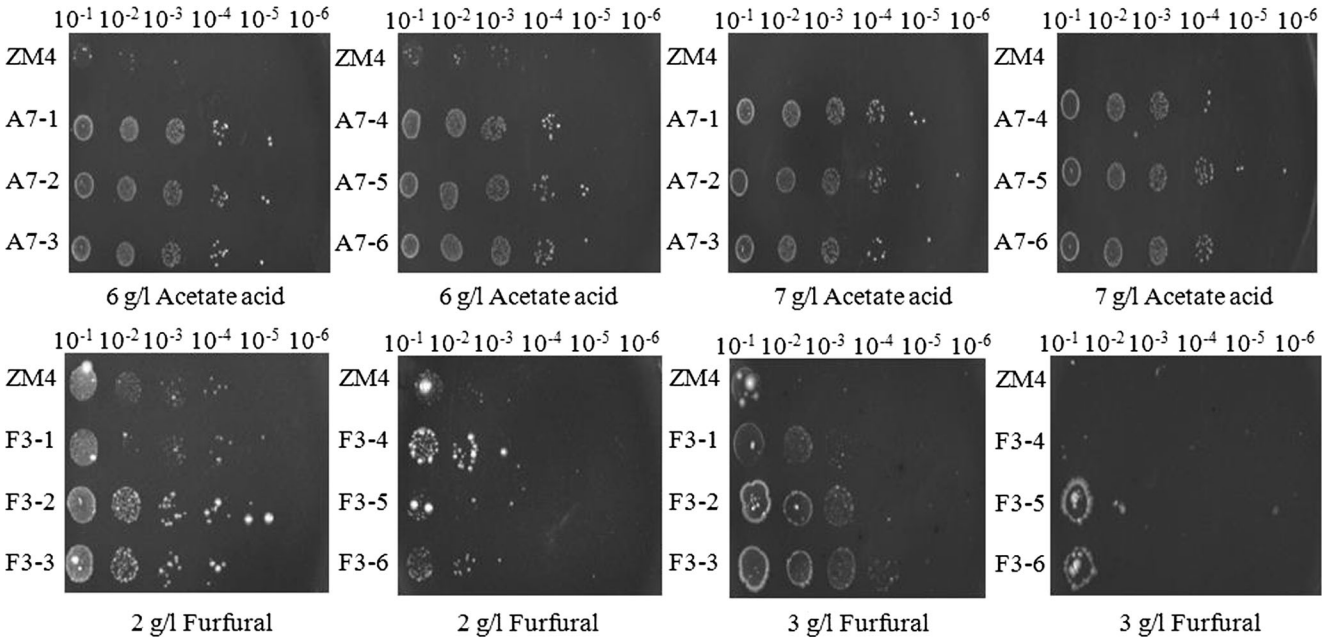

Fig. 3 Growth of the parent strain and the evolved mutants in RM medium containing of furfural and acetic acid

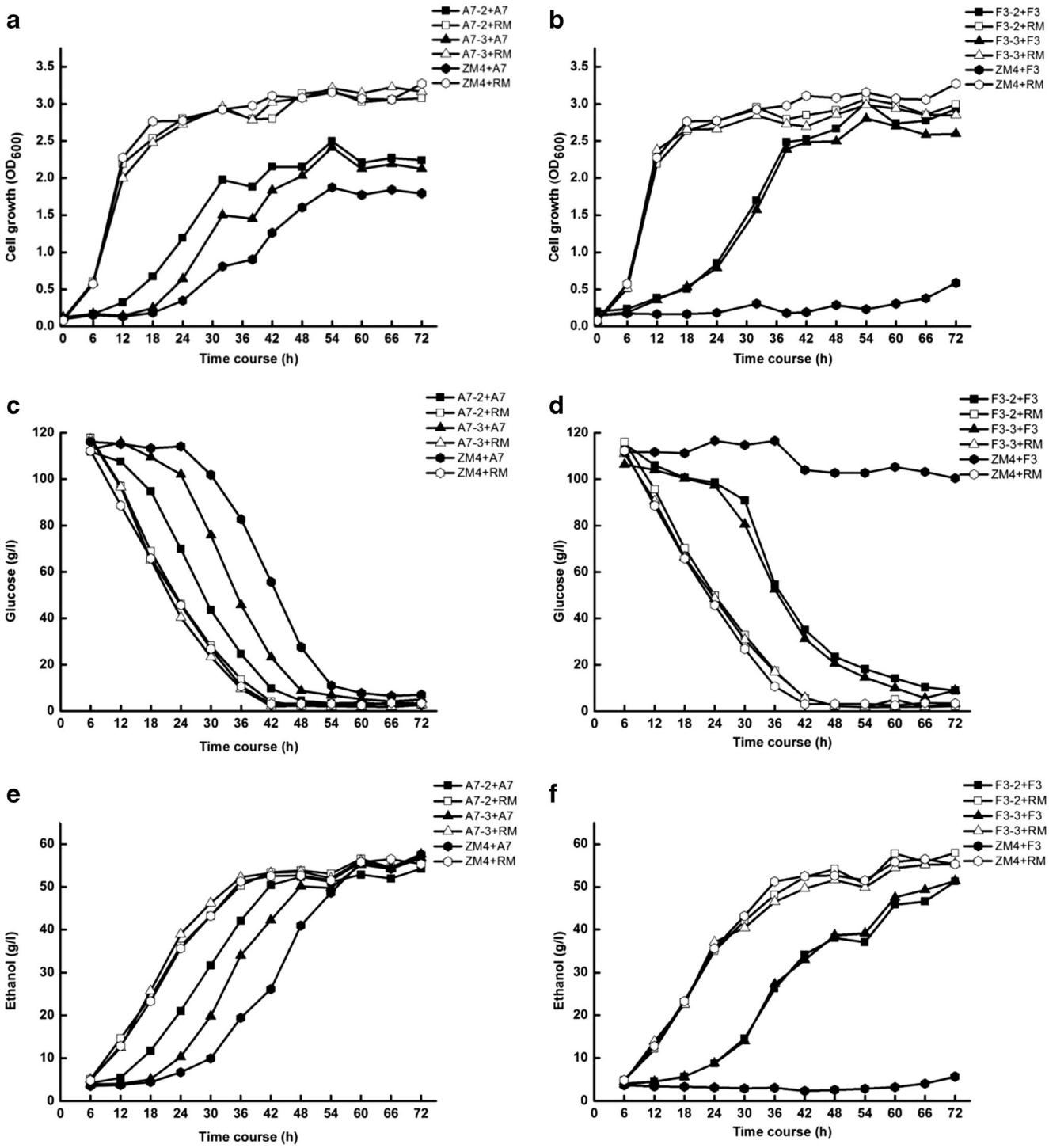

Fig. 4 Profiling of cell growth, glucose utilization, and ethanol yield of tolerant mutant. The data come from mean values of triplicate experiments

times than wild-type strain. Furfural tolerant strains reached the highest cell density of (OD_{600}) 3.023 and 2.803 after 54 h, while the time of *Z. mobilis* needed to reach its highest cell density of 0.584 delayed until 72 h after initial inoculation under 3 g/l furfural stress conditions (Fig. 4b). These results indicated that cell growth of ZMF3-2 and ZMF3-3 was significantly improved by ALE strategies.

Third, glucose utilization and ethanol yield were also performed by HPLC analysis. As shown in Fig 4c, d, *Z. mobilis* ZM4 consumed glucose more slowly under acetic acid stress condition, with more than 24 % of the initial glucose concentration (27.57 g/l) remaining after 48-h incubation. In opposite, 92.3–96 % of the glucose has been utilized at this time point under 7 g/l acetic acid stress condition. Furthermore,

ZMA7-2 showed higher rate of glucose utilization under acetic acid stress condition (Fig. 4c). Similarly, ZM4 consumed glucose also more slowly under furfural stress condition, with more than 91.9 % of the initial glucose concentration remaining after 48-h incubation. In opposite, nearly 80 % of the glucose has been utilized at this time point under 3 g/l furfural stress condition. Furthermore, only 10 % of the glucose has been consumed by ZM4 after 72-h fermentation under 3 g/l furfural stress condition (Fig. 4d). Although ZMA7-2 and ZMA7-3 have showed higher growth than ZM4, there was no significant difference in the yield of ethanol under acetic acid stress condition (Fig. 4e). However, the ethanol yield of ZM4 was significantly decreased by 89.8 % under furfural stress condition when compared to normal condition. On the other hand, ZMF3-2 and ZMF3-3 have higher ethanol yield than ZM4 under 3 g/l furfural stress condition, which showed 87.27 and 94.84 % theoretical ethanol yield. And, the theoretical ethanol yield of ZM4 is only 9.89 %. These results indicated that ZMF3-3 has higher ethanol yield than ZMF3-2 under furfural stress condition (Fig. 4f).

Taken together, two best strains, ZMA7-2 (stored at China General Microbiological Culture Collection Center, CGMCC No.9986) and ZMF3-3 (China General Microbiological Culture Collection Center, CGMCC No.9987), were obtained in this study, which could be used as novel host for further metabolic engineering in cellulosic ethanol or future biorefinery.

Characterization of selected evolved mutants

First, to verify those phenotype changes in adaptively evolving populations were not due to contamination, samples were also identificated by 16 s rRNA. An expected 493-bp fragment of 16 s rRNA gene sequence (ZMOr009, *Z. mobilis* ZM4 complete genome Accession No. AE008692.2) was obtained from all evolved mutants. Sequence comparison showed that all strains belong to *Z. mobilis*, which indicated that these evolved mutants are not due to contamination.

Second, to obtain molecular evidence of the occurrence of evolution events during ALE, we also compared the amplification profiles of parental strains and the evolved mutants by random amplified polymorphic DNA analysis (RAPD). As expected, some primers amplified DNA fragments that failed to show significant polymorphism among all tested strains, which showed no single nucleotide polymorphism (SNPs) in some specific genome locus. However, a large number of DNA bands were also obtained from the templates of the genomes, and significant differences were clearly observed between the RAPD profiles of the parents and evolved mutants by using primer RAPD-5, 6, and 13, respectively (as shown in Fig. 5). Especially, RAPD profiles of evolved mutant ZMF3-3 and ZMA7-2 also showed difference, which indicated that different SNPs are responsible for its tolerant phenotypes.

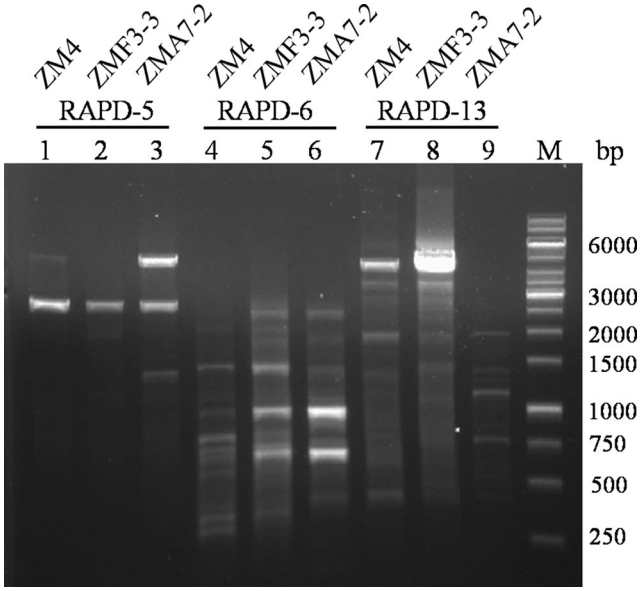

Fig. 5 Genetic variation of the evolved mutants by random amplified polymorphic DNA (RAPD) analysis. M: 1 kb DNA ladder (Fermentas); 1, 2, 3: RAPD profiles of ZM4, ZMF3-3 and ZMA7-2 by using primer RAPD-5; 4, 5, 6: RAPD profiles of ZM4, ZMF3-3, and ZMA7-2 by using primer RAPD-6; 7, 8, 9: RAPD profiles of ZM4, ZMF3-3, and ZMA7-2 by using primer RAPD-13

Third, enzyme activity of PDC and ADH was also compared under different conditions. As summarized in Table 2, significant differences were shown for enzyme activity of PDC and ADH between ZM4 wild-type and the evolved strains when cultured with RM or RM containing relevant inhibitor, respectively. Expectedly, both furfural and acetic acid have negative effect on key enzymes of ED pathway. For example, activity of PDC and ADH in ZM4 was decreased to 72.6 and 84.3 % when cultured with 3 g/l furfural stress condition. At the same time, its activity was also decreased to 80.1 and 6.2 % during acetic acid stress condition, respectively. On the other hand, although the activity of PDC

Table 2 Enzyme activity of PDC and ADH in evolved and parent strains after 24-h culture

Strains	Normal condition[a]		Stress conditions[b,c]	
	PDC (U/g)	ADH (U/g)	PDC (U/g)	ADH (U/g)
ZM4	156.5	62.4	42.9[b]	9.8[b]
			31.2[c]	58.52[c]
ZMF3-3	167.6	25.08	62.8[b]	54.2[b]
ZMA7-2	84.2	13.9	25.3[c]	103.9[c]

[a] Cultured with RM medium

[b,c] Cultured with RM medium with 3 g/l furfural and 7 g/l acetic acid for ZMF3-3 and ZMA7-2, respectively

All data come from two independent experiments

and ADH in evolved mutants was lower than wild type under normal condition (except for activity of PDC in ZMF3-3), and its activity showed dramatically increasing under stress conditions when compared to ZM4 (except for activity of PDC in ZMA7-2). For example, ADH activity of ZMA7-2 and ZMF3-3 was 103.9 and 54.2 U/g under relevant stress conditions, which showed increasing of 43.7 % and 5.54 times higher than parent strain, respectively. PDC activity of ZMF3-3 has also increased to 46.4 % during furfural stress condition. However, there is no significant difference in PDC activity between ZMA7-2 and ZM4 under acetic acid stress condition, which was consistent with its no significant difference in the yield of ethanol under acetic acid stress condition (Fig. 4e). These results indicated that furfural have more negative effect on activity of PDC and ADH than acetic acid, especially for ADH. Taken together, the difference profiling of PDC and ADH activities between furfural and acetic acid-tolerant strain, which may be due to different stress response mechanisms. Importantly, the evolved mutants may respond to stress via upregulated *pdc* and *adh* gene expression.

Discussion

Z. mobilis has a number of positive attributes as an ethanologen for converting cellulosic biomass into ethanol or other valuable chemicals. However, furfural and acetic acid from lignocellulosic hydrolysates are key inhibitors to *Z. mobilis* during cellulosic ethanol production or biorefinery, which will affect its cell growth, glucose, and ethanol yield. In the present study, we also confirmed the fact that the presence of furfural and acetic acid will negatively affect those phenotypes, especially for cell growth, ethanol yield, and key enzymes of ED pathway.

Our previous study on genome-wide scale first demonstrated its mechanisms on molecular level under furfural stress condition (He et al. 2012). However, developing a furfural-tolerant strain also be limited by its multiple affect on gene transcriptional responses. Although many efforts have also been performed by gene knockout or overexpression of some furfural tolerant related gene in *Z. mobilis* in our lab, no desired strain showed higher tolerance obtained in our previous studies by conventional genetic engineering. In this study, we applied an adaptive evolution approach using acetic acid and furfural as the selective pressure in order to generate nongenetically modified ZM4 strains with enhanced tolerance. Four mutants that showed higher furfural and acetic acid-tolerant strain were successfully screened by ALE strategy, and growth behavior and ethanol fermentation characteristics were also performed. Finally, two novel acetic acid and furfural tolerant strains (named ZMA7-2 and ZMF3-3, respectively) were obtained for further use. Currently, ALE strategy has been successfully used in *S. cerevisiae* (Cakar et al. 2012; Demeke et al. 2013; Dhar et al. 2011; Wallace-Salinas and Gorwa-Grauslund 2013) and *E. coli* (Hua et al. 2007; Lee and Palsson 2010), which will lead to the activation of latent metabolic pathways, phenotype optimization, increasing environmental factor tolerance, and improving bacterial fitness (Dragosits and Mattanovich 2013; Portnoy et al. 2011). However, the potential of ALE for other microorganisms is now being recognized. Our study also demonstrated that ALE could also work well in *Z. mobilis*, especially for improving its stress tolerance, which may be used as a powerful tool for *Z. mobilis* strain improvement. To our knowledge, we describe here the first *Z. mobilis* strain that could tolerate higher furfural.

Actually, Joachimsthal et al. first screened an acetate-tolerant *Z. mobilis* strain (ZM4/Ac^R) by chemical mutagenesis and selection in the presence of acetate (Joachimsthal et al. 1998), and also used as a host for constructing of engineered tolerant *Z. mobilis* strain, i.e., ZM4/Ac^R (pZB5) and ZM4/Ac^R (pZB5, pJX1) (Jeon et al. 2002, 2005; Mohagheghi et al. 2004; Yamada et al. 2002). However, all studies are focusing on sodium acetate inhibition, not acetic acid. As we all know, acetic acid is a compound commonly found in hemicellulosic hydrolysates and strongly influenced the bioconversion of sugar containing hydrolysates, which showed higher toxicity than sodium acetate. Although, a few differential expression genes were also found by DNA microarray under 4 g/l acetic acid stress condition in our lab (unpublished data), so far, no mechanism has been proposed for *Z. mobilis* to explain how this organism responds to the acetic acid stress. Until now, little study has demonstrated that some key genes are responsible for acetic acid tolerance, i.e., *him*A (ZMO1122) (Viitanen et al. 2009, 2012), *hfq* (ZMO0347) (Yang et al. 2010b), *nha*A (ZMO0117) (Yang et al. 2010a), which provide some target genes for metabolic engineering. Another recombinant *Z. mobilis* CP4 (pJB99-2) was also constructed to enhance its acid tolerance by introducing a proton-buffering peptide (Pbp) from *E. coli* K-12 (Baumler et al. 2006). An adaptive mutation procedure combination of NTG mutagenesis was first developed for screening of acetic acid-tolerant *Z. mobilis*, and a desired evolved mutant (ZM6014) obtained (Wang 2008). In this study, we also successfully obtained a novel acetic acid-tolerant *Z. mobilis* A7-2 without any previous mutagenesis.

Although two novel furfural and acetic acid-tolerant *Z. mobilis* have been screened by ALE strategy, both *Z. mobilis* A7-2 and F3-3 could not use xylose as sole carbon source. For purpose of increasing in the substrate utilization range of *Z. mobilis*, many efforts will be necessary. First, the two strains should be used as a novel host for further metabolic engineering, i.e., introducing xylose utilization metabolic pathway to make it directly convert pentose into ethanol. Second, this ALE strategy could also be used for improvement of other recombinant *Z. mobilis* strains, especially phenotype

of tolerance or substrate optimization. Third, the two strains may be used as novel model organisms for searching of its genetic mechanism on furfural and acetic acid stress response by using comparative genome sequencing (CGS), 454-pyrosequencing, transcriptomics, proteomics, metabolomics, etc, which will provide some useful information for inverse metabolic engineering.

In this study, 16 primers amplified DNA fragments that failed to show significant polymorphism, whereas 3 primers showed expected DNA bands. Although the differences between activity of key enzymes and profiling of RAPD in all strains were compared by biochemistry and molecular techniques, without using genome analysis techniques in our current study, the mutations responsible for furfural and acetic acid-tolerant *Z. mobilis* strains are still unknown. With the cost of genome sequencing, further work is also necessary to determine the underlying mechanisms involved in stress responses. Importantly, ALE can be used to address fundamental mechanism about adaptation to stress or the process of evolution by genome resequencing or other "omic" technology. The integration of ALE into metabolic engineering of *Z. mobilis* will offer tuning possibilities at multiple levels of metabolic engineering, especially for inverse metabolic engineering.

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