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BIOENERGY AND BIOFUELS

Improving furfural tolerance of *Zymomonas mobilis* by rewiring a sigma factor RpoD protein

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Abstract Furfural from lignocellulosic hydrolysates is the key inhibitor for bio-ethanol fermentation. In this study, we report a strategy of improving the furfural tolerance in *Zymomonas mobilis* on the transcriptional level by engineering its global transcription sigma factor (σ^{70}, RpoD) protein. Three furfural tolerance RpoD mutants (ZM4-MF1, ZM4-MF2, and ZM4-MF3) were identified from error-prone PCR libraries. The best furfural-tolerance strain ZM4-MF2 reached to the maximal cell density (OD_{600}) about 2.0 after approximately 30 h, while control strain ZM4-rpoD reached its highest cell density of about 1.3 under the same conditions. ZM4-MF2 also consumed glucose faster and yield higher ethanol; expression levels and key Entner-Doudoroff (ED) pathway enzymatic activities were also compared to control strain under furfural stress condition. Our results suggest that global transcription machinery engineering could potentially be used to improve stress tolerance and ethanol production in *Z. mobilis*.

Keywords Furfural tolerance · Ethanol production · Global transcription machinery engineering · Error-prone PCR · Sigma factor · RpoD protein

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Introduction

Lignocellulose contains up to 70 % carbohydrate by weight (35 to 45 % cellulose and 20 to 35 % hemicellulose) and represents an excellent potential source of sugars for microbial conversion into renewable fuels and chemicals (Katahira et al. 2006; Tokiwa and Calabia 2008). However, lignocellulose is designed by nature to resist deconstruction. Currently, dilute acid pretreatment of lignocellulose has been widely investigated to increase enzyme access to cellulose, and hydrolyze hemicellulose (Geddes et al. 2010b; Uppugundla et al. 2014). As a result, by-products such as furfural and 5-hydroxymethylfurfural (HMF), organic acids, vanillin, and lignin monomers will be produced which will retard microbial fermentation (Nichols et al. 2014; Parawira and Tekere 2011).

Furfural and HMF, formed by the dehydration of sugars (pentoses and hexoses, respectively) during pretreatment are the major inhibitors in lignocellulosic hydrolysates (Geddes et al. 2010a; Geddes et al. 2014; Wang et al. 2013), which have been previously proposed to inhibit growth by damaging DNA (Barciszewski et al. 1997; Khan et al. 1995; Miller et al. 2009), inhibiting glycolysis enzymes (Gorsich et al. 2006; Hristozova et al. 2008), and chemically reacting with cellular constituents (Barakat et al. 2012). One strategy to overcome the issue of inhibition is to remove the inhibitor after pretreatment from biomass physically or chemically, which requires extra equipment and time leading to increased costs. Another approach utilizes inhibitor tolerant microorganisms for efficient fermentation of lignocellulosic material to ethanol, and their utility is considered an industrial requirement (Dunlop 2011).

Zymomonas mobilis is a candidate microorganism for converting cellulosic biomass into ethanol or other

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valuable chemicals for its desirable industrial characteristics, such as high-specific productivity and ethanol yield, unique anaerobic use of the Entner-Doudoroff (ED) pathway that results in low cell mass formation, high ethanol tolerance, pH 3.5–7.5 range for ethanol production, and has a generally regarded as safe status (He et al. 2014; Rogers et al. 2007). However, ethanol fermentation of *Z. mobilis* is still affected by furfural and HMF, etc. To address these limitations, it is essential to obtain the mutant strains with improved stress tolerance using a variety of approaches (Zhang et al. 2010; Joachimsthal and Rogers 2000; Luo et al. 2009; Shi et al. 2009; Sridhar et al. 2002; Tao et al. 2005). It is well known that the stress tolerance of strain is results of multi-gene regulation involved in carbohydrate metabolism, cell membrane biogenesis, respiratory chain, DNA replication, DNA recombination, transcriptional regulation, some universal stress response, etc. (He et al. 2012a; He et al. 2012b; Yang et al. 2013). So it is still a difficult task to construct robust engineering strains to response various stresses. More recently, transcriptional engineering approach has started to captivate much interest in strain engineering. Several transcription factor, including zinc finger-containing artificial transcription factor (Lee et al. 2008; Park et al. 2003), sigma factor (Alper and Stephanopoulos 2007; Klein-Marcuschamer and Stephanopoulos 2008), Spt15 (Alper et al. 2006), H-NS (Hong et al. 2010b), Hha (Hong et al. 2010a), and cAMP (Chong et al. 2013) have been successfully engineered to improve strain tolerance or control biofilm formation. This strategy provides a route for identifying transcription factor mutants that tolerate various stresses. However, little information has been focused on the genetic improvement of furfural tolerance in *Z. mobilis* by this strategy.

In this work, the *rpoD* gene, which encodes the main sigma factor, σ^{70}, was subjected to random mutagenesis and introduced *Z. mobilis* to search for furfural tolerance strain. This sigma factor was chosen on the premise that the RNA polymerase sigma subunit (σ factor) is known to be essential to recognize promoters and initiate transcription at the correct site; mutations of σ factor will alter the promoter preferences of RNA polymerase affecting transcription levels and thus modulating the transcriptome at a global level (Gardella et al. 1989; Owens et al. 1998). Three error-prone PCR mutants with enhanced furfural resistance were identified in this study. All of the mutants showed better tolerance towards furfural as compared with control strain. The best mutant ZM4-MF2 was further subjected to evaluate its glucose utilization, ethanol production, and activities of key enzymes. Quantitative real-time PCR analysis was also performed on several metabolic pathways related genes in *Z. mobilis*.

Materials and methods

Materials

Z. mobilis ZM4 (ATCC31821) was used as the host organism and cultured in rich media (RM) at 30 °C without shaking. Plasmid pBBR1MCS-tet was the derivate of pBBR1MCS (Kovach et al. 1995). Restriction enzymes were purchased from Fermentas (Burlington, Canada). E.Z.N.A.® Gel Extraction Kit and E.Z.N.A. ® Plasmid Mini Kit I were obtained from Omega Bio-Tek (Norcross, GA, USA). T4 DNA ligase from Thermo Scientific (Ipswich, MA, USA) was used for ligation. GeneMorph® II Random Mutagenesis Kit was obtained from Stratagene (La Jolla, CA, USA). HotMaster Taq DNA polymerase was obtained from TIANGEN Biotech (Beijing, China).

Construction of variant library

The *rpoD* gene was subjected to error-prone PCR using Genemorph II Random Mutagenesis kit with primers 1623 *Xho*I F and 1623 *Xba*I R (Table 1). The PCR product was recovered from 1.0 % low melting agarose gel using E.Z.N.A.® Gel Extraction Kit, digested by restriction enzymes *Xho* I and *Xba* I, and inserted into the digested pBBR1MCS-tet backbone (containing the promoter and terminator of pyruvate decarboxylase) with T4 DNA ligase. The ligation mixture was transformed into *Z. mobilis* ZM4 competent cells by electroporation. The transformants were spread on RM-agar plates containing 5 μg/ml of tetracycline and scraped off to create a liquid library.

Mutant selection

Our primary experiment showed that *Z. mobilis* ZM4 was not able to grow on the medium containing the initial 1.5~2.0 g/l furfural. So, in this study, the initial 1~3 g/l furfural was added for mutant selection. Transformants were incubated in 5 ml RM medium at 30 °C. One percent (*v/v*) of the overnight cell culture was subcultured into 10 ml RM medium containing 1, 2, and 3 g/l furfural in sequence. Cells were subcultured twice in the same concentration furfural. In each period, mutants were incubated about 24~36 h in the presence of furfural. Then, colonies were spread on RM-agar plates containing 5 μg/ml of tetracycline and 3 g/l furfural to cultivate about 4~5 days. Individual colonies were picked up, and mutations were verified by DNA sequencing.

Cell growth profile

Cells were grown in RM medium at 30 °C to an OD_{600} of 1.0 and then serial dilutions of 10 times were made. Each dilution (5 μl) was spotted onto RM-agar plates containing different

Table 1 Primer sequences with restriction site underlined

Gene	Primer	Sequence
rpoD	1623 XhoI F	5′- CCG CTCGAG ATGGCAGAGA CGACTACGG-3′
	1623 XbaI R	5′-TGCTCTAGACTAGTGGTCG AGGAAGCTCC-3′
pdc	ZMO1360F	5′-TACAACCTCGTCCTTCTT-3′
	ZMO1360R	5′-CATAACCTTCTGCACTGA-3′
adh	ZMO1596F	5′-GGTATTAATTCTGCTGTT-3′
	ZMO1596R	5′-CGAAGTCTGAATTGTTAT-3′
edd	ZMO0368F	5′-AATTCTGGACCATTGAAG-3′
	ZMO0368R	5′-AACGAACAACAACGATAA-3′
glk	ZMO0369F	5′-TATTAAGACCAGCTACTC-3′
	ZMO0369R	5′-CAGATAAGAAGAATCCATAT-3′
me	ZMO1955F	5′-TAACCGATAATATGATGG-3′
	ZMO1955R	5′-GCTTACCTCTTCAATATC-3′
pyk	ZMO0152F	5′-ATTTACGAAGGTGCTGAT-3′
	ZMO0152R	5′-AACATAAGAAGCAATACGA-3′
pha	ZMO0057F	5′-GATAAAGCGACATTAAAGG-3′
	ZMO0057R	5′-TTCATCACCCAGTATTTC-3′
sulP	ZMO0546F	5′-TGTCCTGACTCATAATCT-3′
	ZMO0546R	5′-CGCTTATTCTCTTCATCA-3′
coQ7	ZMO1669F	5′-AGATGATTGCCGAATATC-3′
	ZMO1669R	5′-AACCCATATAAAACAGGAT-3′
dsbB	ZMO1668F	5′-GCTATCGGATTGATGGTCTT-3′
	ZMO1668R	5′-AGCCAGCAAGGTTAATATCT-3′
pspB	ZMO1064F	5′-TTAGTCTGCCTTATTCTG-3′
	ZMO1064R	5′-CTCATAAAGCTCTTCAATC-3′
xylR	ZMO0976F	5′-TTTACAGGCGATGATTTACG-3′
	ZMO0976R	5′-AAGCCAACACCGATTTATT-3′
Unknown1	ZMO1524F	5′-GCAATGGTTCTATCTGTTAA-3′
	ZMO1524R	5′-CAAAGCGATGGTATTAGC-3′
Unknown2	ZMO1936F	5′-CTCTACCCTGATGCCGAT-3′
	ZMO1936R	5′-CCGTCATGGATCAGATGT-3′
Unknown3	ZMO0055F	5′-AATTGTTGGTTCCGATAT-3′
	ZMO0055R	5′-CAATAAAGAAATCAGCATC-3′
rrsA	ZMOr009F	5′-TCAACTATAGACCAGTAAGT-3′
	ZMOr009R	5′-AGAACATAGAAGAGGTAAGT-3′

concentration furfural. Plates were incubated at 30 °C for 4~5 days. For measurement of growth curves of mutant strains and controls, cells were cultivated in a Bioscreen C system (Lab Systerms Helsinki, Finland). One-tenth of overnight seed (*v/v*) was inoculated into 1-ml fresh RM medium containing 2 and 3 g/l furfural with a similar initial OD_{600} value at 0.15~0.2, respectively. Then, cells were added into wells of Bioscreen plate with three replications. The working volume was 300 μl/well. Temperature was controlled at 30 °C and optical density at 600 nm. Absorbance values of the cell suspensions were automatically read at regular intervals of 1 h, over 48 h periods, and before each measurement, the cell cultures were automatically shaken for 60 s.

Table 2 Amino acid substitution in three mutant strains

Mutant	Amino acid substitution
ZM4-MF1	P195T V355M
ZM4-MF2	K219E V522G Q649L
ZM4-MF3	G14C L18P E145G L361F P511T L606.

Glucose utilization and ethanol analysis under furfural stress

Cells were grown in RM medium containing 20 g/l glucose at 30 °C to the mid-log phase. Ten milliliters of the culture was transferred into 100-ml fresh RM medium containing 3 g/l furfural with a initial OD_{600} value at ~0.2. Cells were grown at 30 °C for 2~3 days. One-milliliter sample was collected every 6 h within 54 h and was conserved at −20 °C for use. Cell growth was also determined by measuring the optical density at 600 nm with a UV765 spectrophotometer. Fermentation supernatant was prepared by passing through 0.2-μm membrane (Millipore) and used to determine the concentration of glucose and ethanol. High performance liquid chromatography (HPLC) (Agilent Hiplex 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.

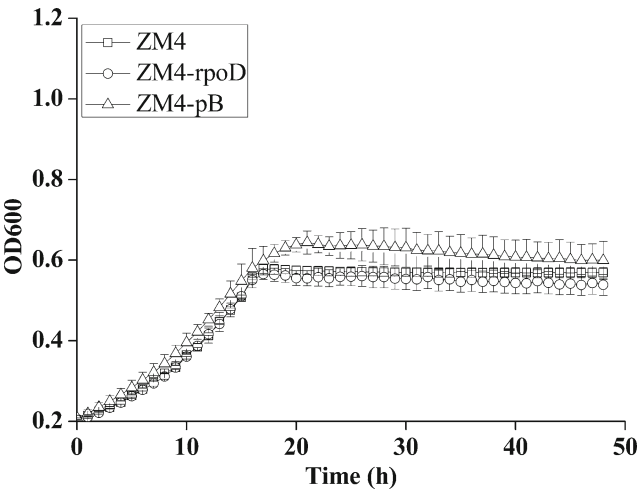

Fig. 1 Growth of the strains ZM4, ZM4-rpoD, and ZM4-pB. Cells were grown in RM medium containing 2 g/l furfural stress at 30 °C. ZM4-rpoD: the strain ZM4 harboring the unmutated version of *rpoD* gene, ZM4-pB: the strain ZM4 containing only a blank plasmid pBBR1MCS-tet

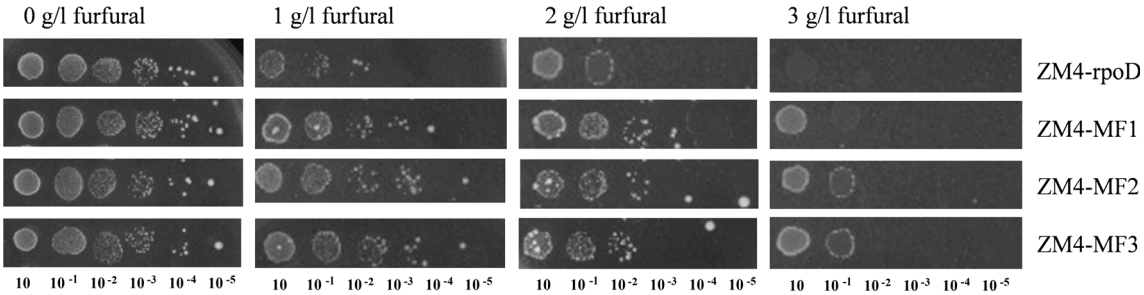

Fig. 2 Growths of the mutants and control strain in RM medium containing different concentration furfurals

RNA isolation and quantitative real-time reverse transcription PCR

Total cellular RNA was extracted from cells grown for 6 h with or without 3 g/L furfural using TRIzol reagent (Invitrogen). The quality and integrity of RNA were assessed using spectrophotometer and agarose gel electrophoresis. Reverse transcription step was carried out using QuantScript RT Kit (TIANGEN, Beijing, China) with random primer mix following the manufacturer's manual. Quantitative real-time reverse transcription PCR was performed in the Bio-Rad iQ5 real-time PCR detection system with SuperReal PreMix (SYBR Green) (TIANGEN, Beijing, China). The *rrsA* gene, encoding the 16S RNA was used as a reference gene for real-time PCR. The primers used in the PCR are listed in Table 1. The real-time PCR conditions were carried as follows: 15 min at 95 C, 40 cycles at 95 C for 10 s, followed by 50–55 °C for 20 s and 72 °C for 20 s, and final extension at 72 °C for 5 min. The ratios of the cycle threshold (Ct) values were determined from the Bio-Rad iQ5 Optical System Software provided. To analyze the gene expression level, the ΔΔCt method was chosen and standard curves of each primer were plotted to ensure similar amplification efficiency compared with the reference gene (Livak and Schmittgen, 2001).

Enzyme assays

PDC activity was determined by monitoring the pyruvic-acid-dependent oxidation of NADH with ADH as a coupling enzyme at pH 6.5, as previously described (Conway et al. 1987). The reaction was carried out at 25 °C in 50 mM sodium citrate buffer (pH 6.5) containing 0.15 mM NADH, 5 mM $MgCl_2$, 0.1 mM TPP, 5 mM pyruvate, and 10 μl (10 U) of ADH. The reaction was started by the addition of 10 μl of cell-free extract. The rate of NADH oxidation was measured at 340 nm.

ADHB activity was assayed by measuring the alcohol-dependent reduction of NAD^+ at pH 6.5. Cells were permeabilized for enzyme assays as previously described (Neale et al. 1986; Mackenzie et al. 1989), and the cell lysate (10~30 μl) was added to a final volume of 1 ml containing 333 mM ethanol and 8.3 mM NAD^+ in 50 mM sodium phosphate buffer, pH 6.5. The production of NADH was assayed from the change in absorbance at 340 nm. One unit of PDC/ADH activity was defined as the generation of 1 μmol NAD+/NADH per minute under the conditions specified. Enzyme activities were reported as international units per milligram of total cell protein. Protein was measured by the Lowry method with bovine serum albumin as a standard.

Results

Radom mutagenesis library construction and mutant selection

In order to select mutants with elevated tolerance towards furfural stress, error-prone PCR was performed to introduce mutations to RpoD and construct random mutagenesis libraries. With the enrichment selection of 1–3 g/l furfural, three mutants (ZM4-MF1, ZM4-MF2 and ZM4-MF3) that exhibited better tolerance towards furfural stress were selected from

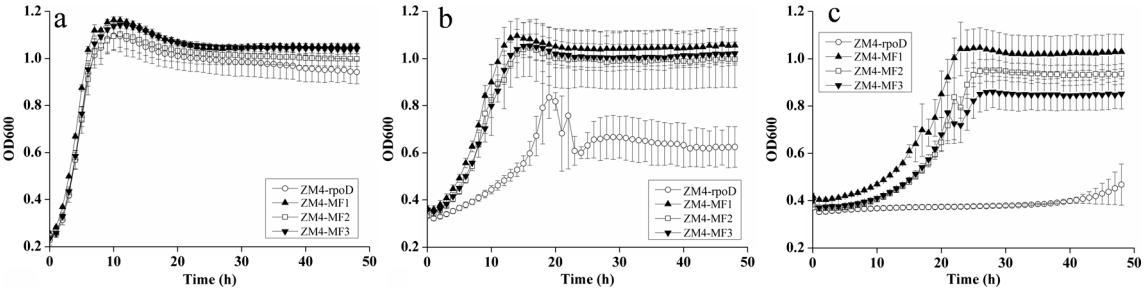

Fig. 3 Growth of RpoD mutants (ZM4-MF1, ZM4-MF2, and ZM4-MF3) and control strain in RM medium. Cells were grown in **a** 0 g/l furfural, **b** 2 g/l furfural, and **c** 3 g/l furfural stress at 30 °C

the library, and their amino acid substitutions are summarized in Table 2.

These mutants were compared in growth performance to those of control strains *Z. mobilis* ZM4, ZM4-rpoD (harboring the unmutated version of *rpoD* gene) and an alternative control (containing only a blank plasmid). Initial studies of growth characteristics of strains in the presence of 2 g/l furfural indicated that these control strains had similar growth rates (Fig. 1). As a result, we chose to use the strain ZM4-rpoD as the control strain for all further studies in this work.

The growth profile of mutants under furfural stress condition

The effect of furfural stress on the growth of RpoD mutants and control stain were firstly investigated. As shown in Fig. 2, growth of mutants and control strain was no significant difference when growing on RM medium without furfural addition. When the concentration of furfural in the medium was increased, a drastically decreased growth of control strain ZM4-rpoD was observed as compared to those of mutants. Almost no growth of control stain was detected when it was cultured on RM medium containing 3 g/l furfural.

The growth curves of mutant strains and control strain on the medium containing different concentration furfural (0, 2 and 3 g/l) were also determined. As can be seen from Fig. 3, with increasing furfural concentration in the culture medium, ZM4-MF1, ZM4-MF2, and ZM4-MF3 also demonstrated better growth than that of control strain. All mutants and control strain presented similar cell growth trend under normal condition. In the presence of 2 g/l furfural stress, all mutant strains reached their cell density of about 1.0 (OD_{600}), while that of control strain was about 0.8. When the furfural concentration increased to 3 g/l, the highest cell densities of three mutants were about 0.8~1.0 (OD_{600}), but the control strain stopped growing and the cell density remained unchanged. These results indicated that three mutants were significantly improved under furfural stress condition.

Effect of furfural stress on glucose utilization and ethanol yield

During ethanol fermentation, furfural stress in the medium may adversely affect the glucose consumption and ethanol production (Fig. 4). In the RM medium containing 3 g/l furfural stress, ZM4-MF2 reached to the maximal cell density (OD_{600}) about 2.0 after approximately 30 h, while ZM4-rpoD reached its highest cell density of about 1.3 (OD_{600}) under the same conditions. ZM4-MF2 also consumed glucose faster under furfural stress conditions, nearly 22 % of the initial glucose remaining after 24 h

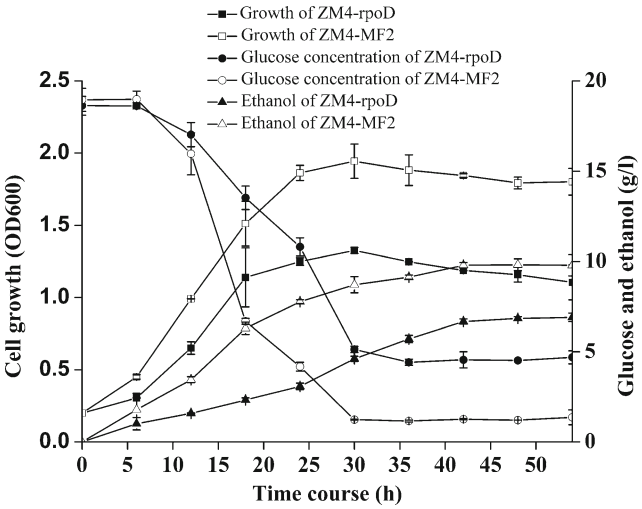

Fig. 4 Effects of furfural stress on growth, glucose utilization, and ethanol yield of ZM4-MF2 and control strain. The data come from mean values of triplicate experiments. Cells were grown in 3 g/l furfural stress at 30 °C

incubation, versus about 58 % for ZM4-rpoD. When fermentation for 54 h in the presence of 3 g/l furfural, the initial glucose remained in the cultures of ZM4-rpoD and ZM4-MF2 was about 25 and 7.2 %, respectively. As a result, the ethanol yield of ZM4-MF2 was about 9.8 g/l after fermentation for 54 h, which was significantly greater than that (6.9 g/l) of ZM4-rpoD.

Assay of key enzyme activity

Furfural stress in the medium may have negative effect on the key enzymes in Entner-Doudoroff (ED) pathway. So PDC and ADH needed to be monitored to describe the changes of activity between mutants and control strain. As shown in Fig. 5, when compared to control strain, the activity of PDC and ADH in ZM4-MF2 showed dramatically higher at 24 and 48 h under 3 g/l furfural stress (except for activity of ADH

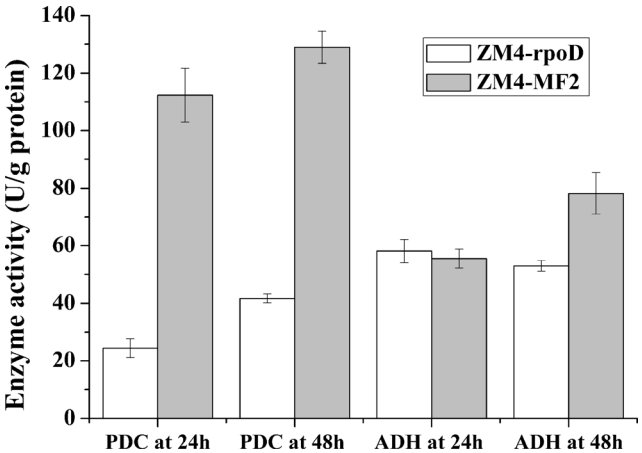

Fig. 5 Enzyme activity of PDC and ADH in ZM4-MF2 and control strain under 3 g/l furfural stress

at 24 h). Especially for PDC, the activity of PDC in ZM4-MF2 was about 4.6 and 3.1 times as that of control strain cultured for 24 and 48 h, respectively. These results indicated that the mutant strain ZM4-MF2 may be response to furfural stress via up-regulated *pdc* and *adh* gene expression.

Analysis of gene expression by qRT-PCR

Genes for qRT-PCR analysis were chosen based on Entner-Doudoroff (ED) pathway (*glk*, *edd*, *pdc*, *adh*, etc.) and their relevance to furfural stress (He et al. 2012a), including genes related to metabolism (*coQ7*, *dsbB*), cell process (*pha*), information transfer (*pspB*), transporter (*sulP*), and some other unknown. As shown in Table 2, among ED pathway related genes, *pdc* (pyruvate decarboxylase) and *adh* (alcohol dehydrogenase) had a 11.8- and 1.86-fold enhanced expression, respectively, in ZM4-MF2 at 3 g/l furfural stress compared to the control, which was consistent with their higher PDC and ADH enzyme activity under furfural stress. At the same time, other genes of ED pathway revealed down-regulation to some extent. For example, *glk* (glucokinase) and *edd* (6-phosphogluconate dehydratase) had about 1.731- and 1.439-fold down-regulation, respectively, in ZM4-MF2 after furfural treatment compared to the control. Apart from ED pathway related genes, other genes were also showed up-regulation or down-regulation to some extent under the furfural stress condition. For example, information transfer associated gene *pspB* (phage shock protein B) was transcriptionally activated in ZM4-MF2 at furfural stress, while metabolism related gene *coQ7* (ubiquinone biosynthesis protein COQ7) and *dsdB* (disulfide bond formation protein) was down-regulated about 2.147- and 3.03-fold, respectively. Especially for *xylR* (aldo/keto reductase) gene, it revealed significant difference in ZM4-MF2 and control, which up-regulation was about 15.83-fold at furfural stress compared to control strain.

Discussion

Z. mobilis has received considerable scientific and commercial interests as a potential microbe for converting lignocelluloses to ethanol (He et al. 2014). However, furfural and furan derivatives generated from the pretreatment of lignocelluloses have negative effects on the growth and ethanol production in *Z. mobilis*. Our previous study on genome-wide scale firstly demonstrated its mechanisms on molecular level under furfural stress (He et al. 2012a). However, developing an engineering strain tolerance to furfural is still limited by its multi-gene regulation and transcriptional responses. Although many efforts have also been performed by gene knock or over-expression 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 aimed to improve *Z. mobilis* furfural tolerance via engineering its global transcription factor RpoD. The

Table 3 qRT-PCR results on selected expression (Avg±std, $p<0.05$)

Gene	Control expression		ZM4-MF2 expression		Fold change[a] (+F)ZM4-MF2/control
	−F	+F	−F	+F	
glk	1.299±0.230	0.585±0.127	0.963±0.206	0.338±0.114	−1.731
edd	0.834±0.199	0.482±0.149	1.000±0.262	0.335±0.130	−1.439
pdc	1.869±2.88	0.057±0.020	1.381±0.247	0.675±0.167	11.84
adh	0.908±0.258	0.375±0.161	1.000±0.164	0.698±0.119	1.860
me	1.000±0.286	0.610±0.195	0.684±0.149	0.343±0.061	−1.778
pyk	1.000±0.307	0.137±0.045	0.157±0.091	0.091±0.026	−1.505
xylR	1.565±0.268	0.065±0.019	0.158±0.064	1.029±0.253	15.83
pha	2.287±0.495	0.251±0.048	1.246±0.305	0.313±0.045	1.250
sulP	1.121±0.337	2.415±0.290	0.636±0.214	2.877±0.359	1.191
coQ7	0.210±0.056	1.922±0.372	0.322±0.096	0.895±0.241	−2.147
dsbB	1.491±0.377	3.118±0.305	0.253±0.098	1.029±0.311	−3.030
pspB	1.017±0.233	0.602±0.233	0.909±0.145	1.725±0.516	2.87
ZMO1524	1.830±0.492	0.799±0.133	1.666±0.315	0.709±0.153	−1.127
ZMO1936	1.000±0.439	0.609±0.141	0.850±0.252	1.174±0.162	1.928
ZMO0055	0.885±0.213	0.584±0.268	0.781±0.271	1.029±0.331	1.762

−F cultured without furfural stress, *+F* cultured with 3 g/l furfural stress

[a] Positive and negative signs refer to gene up-regulation and down-regulation, respectively

random mutagenesis libraries were first constructed via error-prone PCR method and the libraries were then challenged with furfural stress. Three mutants (ZM4-MF1, ZM4-MF2, and ZM4-MF3) were identified with enhanced performance to furfural. To the best of our knowledge, this is the first report on using transcriptional engineering approach to enhance *Z. mobilis* phenotype under high furfural conditions. We believe that RpoD engineering can be implemented as a useful technology for *Z. mobilis* strain development.

Bacterial sigma factors focus the promoter selectivity of RNA polymerase suggests that mutations in key residues can alter this preference (Burgess and Anthony 2001). RpoD is one of the RNA polymerase sigma subunit composed of N-terminal domain, non-essential region, and C-terminal domain (NCBI conserved domain 2015) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?SEQUENCE=56544093&FULL). Analysis of mutations in ZM4-MF1, ZM4-MF2, and ZM4-MF3 mutants reveals a few meaningful features. First, we found that simple modification to sigma factor RpoD could result in enhanced strain tolerance towards furfural stress. Second, there are 11 point mutations in total are adopted by these RpoD mutants to cope with the furfural stress (Table 2). Among them, point mutation E145G and V522G is located at conserved region 1.2 and region 3, respectively (NCBI conserved domain 2015) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?SEQUENCE=56544093&FULL). Two point mutations (Q649L and L606.) fall onto the conserved region 4, which is known to be responsible for binding to the promoter region and to anti-sigma factors. Interestingly, the mutation of L606 led to the formation of a truncated factor, which apparently contributes to increasing overall furfural tolerance. This result concurred with previous findings that a truncated mutant resulted in increasing ethanol tolerance in *E. coli* (Alper and Stephanopoulos 2007), and the reason is that a truncated form may have an increased binding affinity to anti-sigma factors relative to that of the full protein (Sharma et al. 1999). However, the structure-function relationship between these mutations and the observed tolerances toward furfural remains to be investigated. Further studies are required to identify its direct target genes or its interacting partners to elucidate the molecular mechanisms of RpoD mutation in conferring improved furfural stress tolerance in *Z. mobilis*. It will be interesting to ascertain the global transcriptional differences in strains harboring the mutation to identify the gene expression changes resulting in the enhanced furfural tolerance.

Our results showed that simple changes in RpoD lead to tolerance alteration to furfural. Why do these changes appear? As we all know, complicated phenotypes of strains are caused from complex interactions of thousands of genes and gene products. Transcription factors can regulate the expression of hundreds of genes. Modification to these transcription factors will change their DNA binding specificity, their partner proteins, and their influence on transcription. As revealed in our qRT-PCR data, the simple mutations to sigma factor could reprogram the transcription profile and alter cell phenotype accordingly. As shown in Table 3, *pdc* and *adh*, the key enzyme genes of ED pathway, were up-regulated when subjected to furfural stress in ZM4-MF2, which complies with our enzymatic assay results-PDC and ADH activities were enhanced by more than several fold in ZM4-MF2 with respect to the control. In addition, the gene ZMO0976, annotated as a putative aldose reductase, was activated about 15.83-fold at furfural stress compared to the control (Table 3). Agrawal et al. illustrated that the gene ZMO0976 not only plays an important role in converting xylose to xylitol, but also alleviates the toxicity of furfural and other inhibitors (Agrawal and Chen 2011). Taken together, we can assume that the gene ZMO0976 may also play a key role in the metabolism of furfural in *Z. mobilis*. On the other hand, the *glk* and *edd* gene have showed minor down-regulation in our real-time PCR, as also showed in our previous global profiling studies (He et al. 2012a). The mutation of RpoD may hinder the combination of DNA-binding site of RpoD and the promoter region of these down-regulation genes, which resulted in the down-regulation of gene expression. We speculated that the changes of gene expression may minimize furfural-induced damage, so as to improve cell growth in the presence of furfural. Further work may be performed by overexpression and knockout of these genes to demonstrate the relationship between the down-regulation of the gene expression and improvement of cell growth under furfural stress.

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