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High Tolerance and Physiological Mechanism of *Zymomonas Mobilis* to Phenolic Inhibitors in Ethanol Fermentation of Corncob Residue

Hanqi Gu, Jian Zhang, Jie Bao

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China; telephone: +86-21-64251799; e-mail: jbao@ecust.edu.cn

ABSTRACT: Corncob residue as the lignocellulosic biomass accumulated phenolic compounds generated from xylitol production industry. For utilization of this biomass, *Zymomonas mobilis* ZM4 was tested as the ethanol fermenting strain and presented a better performance of cell growth (2.8×10^8 CFU/mL) and ethanol fermentability (54.42 g/L) in the simultaneous saccharification and fermentation (SSF) than the typical robust strain *Saccharomyces cerevisiae* DQ1 (cell growth of 2.9×10^7 CFU/mL, ethanol titer of 48.6 g/L). The physiological response of *Z. mobilis* ZM4 to the twelve typical phenolic compounds derived from lignocellulose was assayed and compared with that of *S. cerevisiae* DQ1. *Z. mobilis* ZM4 showed nearly the same tolerance to the phenolic aldehydes with *S. cerevisiae* DQ1, but the stronger tolerance to the phenolic acids existing in corncob residue (2-furoic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, ferulic acid, and syringic acid). The tolerance mechanism of *Z. mobilis* was investigated in terms of inhibitor degradation, cell morphology and membrane permeability under the stress of phenolics using GC-MS, scanning and transmission electron microscopies (SEM and TEM), as well as fluorescent probes. The results reveal that *Z. mobilis* ZM4 has the capability for in situ detoxification of phenolic aldehydes, and the lipopolysaccharide aggregation on the cell outer membrane of *Z. mobilis* ZM4 provided the permeable barrier to the attack of phenolic acids.

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KEYWORDS: *Zymomonas mobilis*; tolerance; phenolic inhibitors; lignocellulose; corncob residue; ethanol fermentation

Pretreatment of lignocellulosic biomass generates at least three groups of toxic compounds that inhibit the fermenting microbes, including furans (furfural, 5-hydromethylfurfural (HMF)), weak organic acids (acetic acid, formic acid, levulinic acid), and phenolic compounds (Jonsson et al., 2013). Among the three groups, furan derivatives and weak organic acids are considered as the major inhibitors because of their relatively high contents in the pretreated biomass. Phenolic compounds from lignin degradation are composed by various phenolic aldehydes, alcohols, and acids such as *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, coniferyl aldehyde, guaiacol, ferulic acid, cinnamic acid, etc. (Klinke et al., 2004), and exert the stronger inhibition on the fermenting microbes than furans and weak organic acids (Mills et al., 2009). Generally, the phenolics are not the first priority when the inhibition phenomenon is considered because the less apparent inhibition was caused by each single phenolic compound at relatively low concentration.

However, phenolics accumulation may create special cases with stronger phenolic stress than furans and organic acids in lignocellulose biorefinery practices as demonstrated in the xylitol production industry. In this process, corncob is used as feedstock for xylose production by dilute acid hydrolysis and hydrolysate extraction, then the solid corncob residue is left over and considered as a promising lignocellulosic material for cellulosic ethanol production due to its high cellulose content (Lei et al., 2014; Liu et al., 2012; Tang et al., 2011). The major inhibitory compounds generated from the dilute acid hydrolysis such as furans, organic acids, and some phenolic compounds with high water-solubility are removed from corncob residue during the multiple washing steps. Whereas, the slightly water-soluble or water-insoluble phenolic compounds accumulate on the solid corncob residue and reach a considerable high concentration, leading to a strong inhibition on the yeast cells growth and fermentability in the consequent ethanol fermentation (Gu et al., 2014).

For alleviation of the phenolic inhibition on ethanol fermenting strains, washing or over-liming methods do not work effectively because of the low water solubility and the increased hydrophobicity of phenolics after pretreatment (Gu et al., 2014; Thomsen et al., 2009). Another new method, biodetoxification, is also in low efficiency on degrading these insoluble phenolics in the thoroughly washed corncob residue (Zhang et al., 2010b). Although the evolutionary adaptation improves the tolerance of yeast strains to

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phenolic inhibitors, the tolerance is temporary unless a stable mutant is obtained (Fan et al., 2013; Gu et al., 2014). In this context, the screening of a specific ethanologenic strain with inherent tolerance to phenolics would be the best option for the phenolics enriched lignocellulosic biomass.

Zymomonas mobilis is becoming one of the important ethanol fermenting strains for lignocellulosic biomass feedstock (Lau and Dale, 2009; Lau et al., 2010). Generally, *S. cerevisiae* is considered as the dominant ethanol fermenting strain in the ethanol industry and more tolerance to lignocellulose derived inhibitors than *Z. mobilis* (Klinke et al., 2004) due to its capacity of the in situ biodegradation of the most toxic furan aldehydes (Liu, 2011). Different species of *S. cerevisiae* behave different tolerance. For the purpose of a fair comparison with *Z. mobilis*, a high thermotolerant and inhibitor tolerant robust strain, *S. cerevisiae* DQ1, was selected as the control strain. *S. cerevisiae* DQ1 was obtained by UV radiated mutation and screened by the long term adaptation in the pretreated corn stover hydrolysate medium (Chu et al., 2012). It demonstrated the high ethanol fermentability (ethanol yield above 75% and productivity above 1.1 g/L/h) and strong tolerance to high temperature (42°C), high lignocellulosic solids loading (35%), and inhibitors (acetic acid, furfural, and HMF) during the simultaneous saccharification and fermentation of the pretreated corn stover, wheat straw, rice straw and other agricultural biomass (Chu et al., 2012; Zhang et al., 2010a,2011). Although *Z. mobilis* demonstrates advantages in ethanol fermentation (Yang et al., 2013), the low tolerance to inhibitory compounds makes it is less frequently used when the inhibitor is at a high level (Su et al., 2013; Zhang and Lynd, 2010). The tolerance of *Z. mobilis* to furans and weak acids had been investigated extensively (Dong et al., 2013; Skerker et al., 2013; Yang et al., 2010, 2014), but few studies focus on the toxic effect of phenolic inhibitors on *Z. mobilis*. Delgenes (Delgenes et al., 1996) investigated the tolerance and degradation of *Z. mobilis* ATCC 10988 to three phenolic aldehydes and found that it was sensitive to the phenolic aldehydes but unable to degrade the aldehydes. Franden (Franden et al., 2013a) found that *Z. mobilis* 8b was capable of converting *p*-hydroxybenzaldedyde, vanillin and syringaldehyde to their alcohol forms, but the tolerance mechanism to the phenolics was not clear.

Z. mobilis was found to behave a better cell growth and ethanol fermentability than *S. cerevisiae* when the phenolics enriched corncob residue was used as feedstock at high solids content. To confirm this surprising phenomenon, 12 typical phenolic compounds produced from lignocellulose pretreatment were selected and used for testing the tolerance of *Z. mobilis* and *S. cerevisiae*. The results revealed that *Z. mobilis* did behave high tolerance to the phenolic acids, which was abundant in the corncob residue (Gu et al., 2014). The tolerance mechanism was investigated by examining the responses of *Z. mobilis* on biodegradation and the cell membrane permeability to the phenolic compounds, and the lipopolysaccharide aggregation on the cell outer membrane of *Z. mobilis* might be responsible for the strong tolerance of phenolic acids. This study discovered that *Z. mobilis* is a robust ethanologenic strain to phenolic inhibitors and provided a practical choice for ethanol fermentation using the phenolics enriched lignocellulosic biomass such as corncob residue from xylitol industry.

Materials and Methods

Raw Materials and Enzyme

Corncob residue was supplied by Longlive Biotechnology Co., Yucheng, Shandong, China. The water content of the original corncob residue was 64.0% and the composition of the dry corncob residue was determined according to the NREL protocol (Sluiter et al., 2011), including $51.8 \pm 0.1\%$ of cellulose, $5.4 \pm 0.1\%$ of xylan, $14.9 \pm 0.3\%$ of lignin, and $7.6 \pm 0.1\%$ ash. The total phenolic content of corncob residue was 16.8 mg/g dry mater and the major phenolics included guaiacol, 2-furoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and ferulic acid (Gu et al., 2014). The materials were disk milled to 100–200 μm in diameter before use.

The cellulase enzyme Youtell 6# was supplied by Hunan Youtell Biotechnology Co., Yueyang, Hunan, China. The protein content of 9% (w/w) was determined by Bradford method (Bradford, 1976). The filter paper activity of 135 FPU and the cellobiase activity of 344 IU per gram enzyme reagent were determined according to the protocol of NREL LAP-006 (Adney and Baker, 2008) and Ghose (Ghose, 1987), respectively.

The phenolic compounds including *p*-hydroxybenzyl alcohol, vanillyl alcohol, syringyl alcohol, coniferyl alcohol, guaiacol, *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, coniferyl aldehyde, 2-furoic acid, cinnamic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, ferulic acid, and syringic acid were reagent grade and purchased from Sigma–Aldrich (St Louis, MO). Glucose and other chemicals used in this study were analytical grade and purchased from Lingfeng Chemical Reagent Co., Shanghai, China.

Strains and Culture Medium

Zymomonas mobilis ZM4 (ATCC 31821) was purchased from American Type Culture Collection (Manassas, VA). *Saccharomyces cerevisiae* DQ1 (CGMCC 2528) was a mutant strain used for cellulosic ethanol fermentation at high solids content and deposited in Chinese General Microorganisms Collection Center, Beijing, China. The strains were stored and maintained at −80°C in 2.0 mL aliquot vials containing 30% (w/w) glycerol.

The culture media: *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 got the better performance in the rich medium and synthetic medium, respectively (date not shown). (1) Rich medium used for inoculum preparation of *Z. mobilis* ZM4 containing 20 g/L of glucose, 10 g/L of yeast extract, and 2 g/L of KH_2PO_4 (Dong et al., 2011, 2013); (2) Synthetic medium for inoculum preparation of *S. cerevisiae* DQ1 containing 20 g/L of glucose, 2 g/L of KH_2PO_4, 1 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4 \cdot 7H_2O$, and 1 g/L of yeast extract (Chu et al., 2012; Zhang et al., 2010a, 2011); (3) Fermentation medium for *Z. mobilis* ZM4 contained 2 g/L of KH_2PO_4 and 10 g/L of yeast extract; (4) Fermentation medium for *S. cerevisiae* DQ1 contained 2 g/L of KH_2PO_4, 1 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4 \cdot 7H_2O$, and 10 g/L of yeast extract.

Simultaneous Saccharification and Ethanol Fermentation (SSF)

The SSF operation was carried out in a 5L bioreactor equipped with a helical impeller as described in our previous study (Zhang et al.,

2010a). Corncob residue feedstock was prehydrolyzed at 50°C and pH 4.8 for 12 h, and then the seed culture of *S. cerevisiae* DQ1 or *Z. mobilis* ZM4 with the initial OD_{600} of 8.8–9.1 and 2.1–2.4, respectively, was inoculated and lasted for the 60 h. The pH was maintained by addition of 5M NaOH solution. Samples were withdrawn at regular intervals and centrifuged at 11167 × g for 5 min to obtain the supernatant for HPLC analysis. All the data are the mean values with standard deviations of the duplicate experiments.

The cell growth in the SSF process was measured by counting colony-forming units (CFU). The cell growth of *Z. mobilis* ZM4 or *S. cerevisiae* DQ1 was assayed by CFU per mL of the fermentation slurry because the solids containing slurry blocked the direct observation of optical density. 100 μL of the 10^5–10^6 diluted fermentation slurry samples was spread onto the Petri dishes with rich medium or synthetic medium and the single colony number growing on the dishes at 30°C for 48 h was counted as the CFU number. An average value of triplicate samples represented for the cell growth performance.

Analysis of Tolerance to Phenolic Compounds

The tolerance of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 to the phenolic compounds was investigated by culturing the strains in the pure mediums containing single phenolic compound. Because the phenolic compounds were insoluble or slightly soluble in water, DMSO as a commonly used non-toxic and powerful solvent was used for increasing the solubility of phenolics (Franden et al., 2013b; Gu et al., 2014; Xiros et al., 2011). Each phenolic compound was dissolved into DMSO for stock solution preparation and added to the sterilized culture media (the rich medium used for *Z. mobilis* ZM4 and the synthetic medium for *S. cerevisiae* DQ1). The final DMSO content in all medium was kept below 0.7% (v/v), and no concentration change of the phenolic compounds was detected in the inhibitor medium without cell inoculation for 48h. The addition of DMSO at 0.7% (v/v) in the culture medium was assayed and no inhibition on the growth and fermentation of both strains was observed. 250 mL flask containing 40 mL of the rich medium or the synthetic medium with the individual phenolic compound was incubated at desired concentration, 10% (v/v) of inoculation ratio, 30°C for 24 h. *S. cerevisiae* DQ1 was shake at 150 rpm, while *Z. mobilis* ZM4 was cultured statically.

During the fermentation in the pure medium added with phenolic inhibitors, the cell growth of both tow strains was evaluated by specific growth rate, which was determined as the slope of logarithm (ln) of the dry cell mass plotted against time (Ask et al., 2013) and calculated by Equation (1):

$$\mu = \frac{\ln(x_t/x_c)}{t} \tag{1}$$

where μ is the maximum specific growth rate during the log phase (h^{-1}), x_t is the dry cell mass at the time t (g/L), x_c is the initial dry cell mass concentration (g/L), and t is the time interval between x_c and x_t (h). The dry cell mass were calculated from the optical density at 600 nm based on the previous studies: OD_{600} at 1.0 equals to 0.5 g/L of dry cell mass of *S. cerevisiae* DQ1 (Chu et al., 2012) and to 0.31 g/L dry cell weight of *Z. mobilis* ZM4 (Dong et al., 2011).

The biodegradation reaction of the phenolic compounds is represented by the first order reaction with its linear form in Equation (2):

$$\text{in}[C] = -kt + \text{in}[C]_0 \tag{2}$$

where $[C]$ is the concentration of phenolic compound (mM), $[C]_0$ is the initial concentration of phenolic compound (mM), k is the first order rate constant (1/s), t is the reaction time (s).

GC-MS Analysis

GC-MS analysis was used for identification of the intermediate compounds from biodegradation of phenolic compounds by *Z. mobilis* ZM4. Samples were taken at 0 h and 24 h after inoculation and concentrated by rotary evaporator with vacuum system, then dissolved in ethyl acetate and acetonitrile solution (2:1, v/v) and silylated with NO-bis-trimethylsilyl trifluoro-acetamide according to (Klinke et al., 2002; Varga et al., 2004). The treated samples were analyzed using Agilent 6890 GC-MS fitted with a HP-5 MS column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA) under the temperature from 80°C (held for 4 min) to 280°C at 8°C/min. One micro liter sample was injected and detected under splitless condition.

HPLC Analysis

Glucose and ethanol were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with a Bio-Rad Aminex HPX-87H column at 65°C. Five millimolar H_2SO_4 was used as a mobile phase at flow rate of 0.6 mL/min. The phenolic compounds were analyzed using HPLC (UV/Vis detector SPD-20A, at 270 nm, Shimadzu, Kyoto, Japan) fitted with YMC-Pack ODS-A column (YMC Co., Kyoto, Japan) at 35°C. The mobile phase are the eluent A (0.1% formic acid in water), and the eluent B (acetonitrile) at flow rate of 1.0 mL/min. Elution started at 10% of eluent B and raised to 35% in 4 min and held at 35% for 11 min. Then, it was decreased from 35% to 10% in 5 min, and held at 10% of eluent B for 10 min (Khoddami et al., 2013). All samples were filtered through a 0.22 μm filter before analysis.

Electron Microscopy Characterization

The cell morphology of *Z. mobilis* ZM4 or *S. cerevisiae* DQ1 was examined using the scanning electron microscopy (SEM) and the transmission electron microscopy (TEM). The cell culture was collected at 8 h after the incubation in the phenolic inhibitor containing medium and centrifuged at 5000 × g for 10 min. The supernatant was re-suspended into 0.05 M sodium phosphate buffer (pH 7.0) and fixed with 2% glutaraldehyde for 30 min. Then the cells were dehydrated by a graded ethanol series beginning with 10% and changing to solutions of 20%, 50%, 75%, 95%, and 100%, in each grade for 30 min, air-dried, and sputtered coat with 1–2 nm gold. The treated cells were observed on Hitachi S3400 N SEM (Hitachi, Kyoto, Japan) with the accelerating voltage of 15 kV.

The cell preparation used for TEM analysis was similar to that of SEM analysis. The cells were post-fixed with 2% osmium tetraoxide (OsO_4) for 1 h, the fixed cells were dehydrated by subsequent rinse with a graded ethanol series as above mentioned. Cells were embedded, sectioned, and mounted on the copper grids, then stored in a desiccator before microscopic examination. Subsequently, the ultrathin sections were imaged at 80 kV using a Hitachi H-7650 TEM (Hitachi, Kyoto, Japan).

Outer Membrane Permeability Analysis

Outer membrane permeability measurement based on the uptake of N-Phenyl-α-naphthylamine (NPN) (Loh et al., 1984). *Z. mobilis* ZM4 cells were cultured in the rich medium with or without ferulic acid for 8 h, centrifuged at 7000 × g for 5 min, and re-suspended in 0.05 M sodium phosphate buffer (pH 7.0) to make sure an OD_{600} of 0.5. NPN was dissolved in acetone and added into 1 mL of cell suspension, in which the NPN concentration was 10 mM. 100 μL samples were added to the 96-well plate, then fluorescence intensity was measured using a Synergy H1 (BioTek Instruments, Winooski, VT) with excitation and emission wavelengths set to 350 and 420 nm, respectively. All the membrane permeability data were triplicated in this experiment.

Calculation of Ethanol Yield

The ethanol yield is calculated using the method in Equation (3) by (Zhang and Bao, 2012):

$$\text{Ethanolyield}(\%) = \frac{[C] \times W}{976.9 - 0.804 \times [C]} \times \frac{1}{0.511 \times f \times [CRR] \times m \times 1.111} \times 100\% \quad (3)$$

where $[C]$ (g/L) is the ethanol concentration in SSF fermentation broth, $[CCR]$ (g/g) is the dry corncob residue concentration at the beginning of SSF, f is the cellulose content of the dry corncob residue (g/g), W is the total water in SSF (g), m is the initial total weight of the SSF system, 1.111 is the conversion factor for cellulose to equivalent glucose, 0.511 is the conversion factor for ethanol from glucose based on the yeast biochemical reaction stoichiometry.

Results and Discussion

Ethanol Fermentation of Phenolics Enriched Corncob Residue by *Z. mobilis* and *S. cerevisiae*

Corncob residue contains considerable amount of phenolic compounds (16.8 mg/g dry mass) and the major components of slightly water-soluble or water-insoluble phenolic compounds included guaiacol, 2-furoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid and ferulic acid (Gu et al., 2014). When this cellulose riched corncob residue is used as the feedstock for ethanol fermentation, the ethanol productivity of the typical ethanologenic strain *Saccharomyces cerevisiae* was inhibited significantly to 0.61 g/L/h, which was approximately 1/3 when the pretreated corn stover feedstock was used (Gu et al., 2014). The growth and fermentability of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 at the corncob residue content of 30% (w/w) were measured as shown in Figure 1. The highest ethanol titers of 54.4 g/L and 48.6 g/L were obtained by *Z. mobilis* ZM4 at 30°C (Fig. 1a) and by *S. cerevisiae* DQ1 at 37°C (Fig. 1b), respectively. During the initial 12 h, the ethanol productivity of *Z. mobilis* ZM4 was as high as 2.43 and 2.92 g/L/h at 30 and 37°C, respectively, whereas *S. cerevisiae* DQ1 was only 0.46 and 0.31 g/L/h. The cell growth of *Z. mobilis* ZM4 indicated by the colony-forming units (CFU) increased to $\sim 3 \times 10^8$ shortly after inoculation, approximately one order of magnitude greater than that of *S. cerevisiae* DQ1 at $\sim 3 \times 10^7$ after inoculation. The cell viability of *Z. mobilis* ZM4 was $2.5\text{–}3.0 \times 10^8$ CFU during the SSF at 30°C, and quickly decreased to less than 1.0×10^8 in the later stage of SSF at 37°C (Fig. 1b), partially due to the relatively weak tolerance of *Z. mobilis* ZM4 to 37°C, similar to *Z. mobilis* 8b observed by Zhang and Lynd (2010). On the other hand, the cell viability of *S. cerevisiae* DQ1 was also reduced from 3.0×10^7 to 1.5×10^7 CFU after SSF for 36 h at both 30 and 37°C. The cell viability decrease of both *Z. mobilis* and *S. cerevisiae* might be the effect of the sugar (glucose) disappearance in the late stage of SSF on the cell growth.

The ethanol fermentability of *Z. mobilis* ZM4 using the phenolics enriched corncob residue feedstock was carefully investigated as shown in Figure 2. The ethanol concentration increased with the increasing corncob residue content from 20%, 25%, to 30% (w/w) (Fig. 2a). When the cellulase dosage increased from 7.5 to 15 FPU/g DM, the ethanol yield increased significantly, but the further increase to 30 FPU/g DM only gave a slight increase (Fig. 2b). The ethanol yield at 30°C was only slightly higher than that at 37°C and this difference might be attributed to the higher viable cell number of *Z. mobilis* at 30°C (Fig. 2c). No significant increase in the ethanol yield was observed when pH was changed in the range of 5.0–5.5 for both enzymatic hydrolysis and ethanol fermentation (Fig. 2d). When corncob residue was pre-hydrolyzed for 3, 12, and 24 h, the initial glucose concentration increased from 47.8, 69.8, to 87.4 g/L, respectively, whereas the final ethanol titers were almost same (48.2, 54.4, and 47.7 g/L) (Fig. 2e). Although the initial glucose increased with the prehydrolysis time prolonging, the left glucose hydrolyzed during the SSF was reduced. The amount of corncob residue was determined, the final glucose converted from the corncob residue was a fixed value. Therefore, the final ethanol concentration of these three group was almost same during the SSF. This phenomenon coincided with the results observed from the SSF of pretreated corn stover by *S. cerevisiae* (Öhgren et al., 2007; Zhang et al., 2010a). The results provided an excellent option of *Z. mobilis* ZM4 for efficiently fermenting the phenolics enriched lignocellulosic biomass such as corncob residue into ethanol.

Tolerance of *Z. mobilis* and *S. cerevisiae* to the Phenolic Inhibitors

The fermentation results proved that *Z. mobilis* ZM4 is a better ethanol fermenting strain than *S. cerevisiae* DQ1 for the phenolics enriched corncob residue feedstock. To confirm the phenomenon, the physiological response of *Z. mobilis* to the phenolic inhibitor

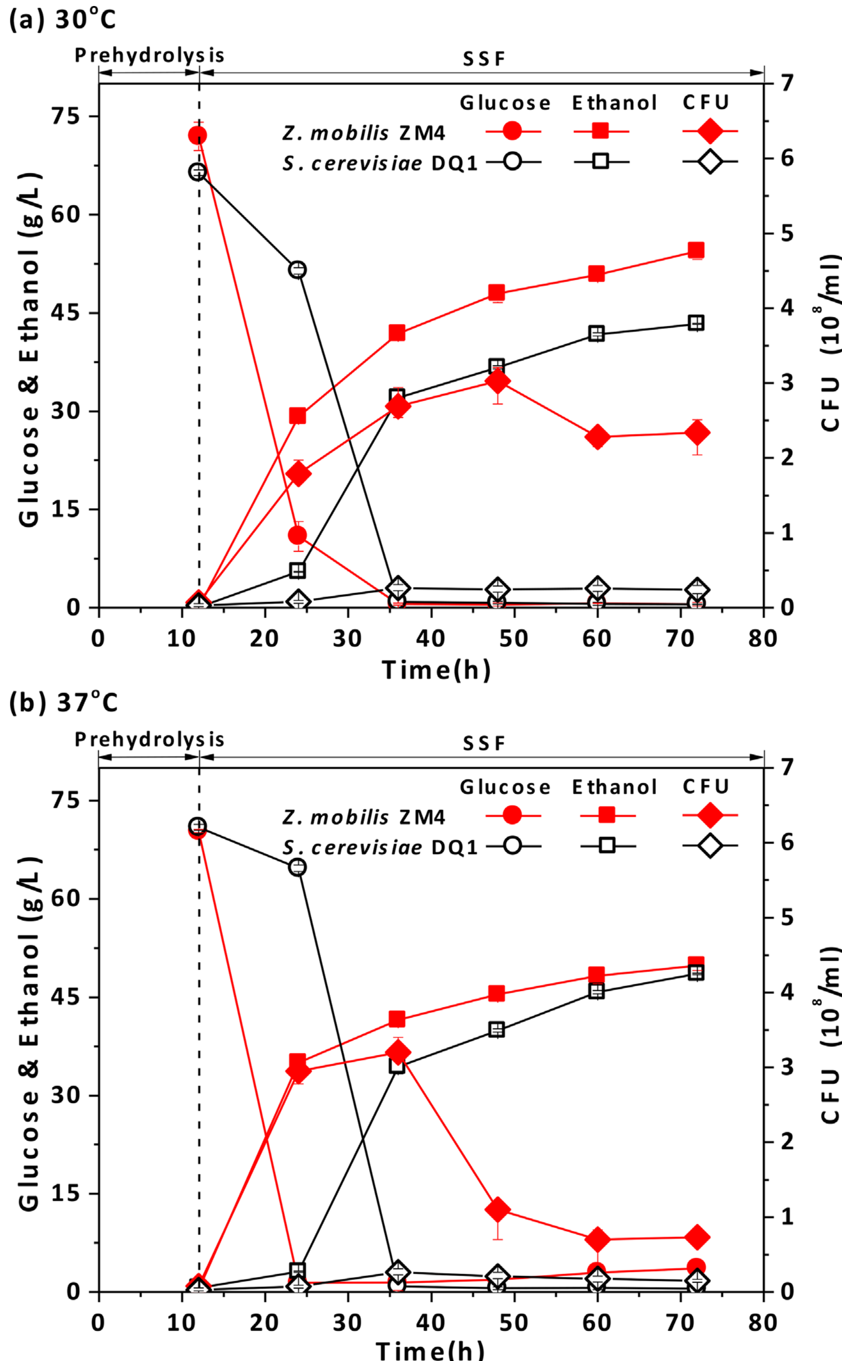

Figure 1. SSF of corncob residue by *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 at high solids loading. (a) 30°C; (b) 37°C. The solids loading was 30% (w/w), the enzyme dosage was 15 FPU/g DM. The prehydrolysis was performed at 50°C and pH 4.8 for 12 h, then SSF was initiated at 30°C or 37°C at pH 5.5. The cell growth was represented by the colony-forming units (CFU) of the strains on petri dishes.

stress was examined by culturing *Z. mobilis* ZM4 in the medium containing the phenolic compounds, with *S. cerevisiae* DQ1 as the control strain in the same culture conditions. Totally 12 typical phenolic compounds derived from lignin degradation in pretreatment operations were selected as the model compounds, including 5 phenolic aldehydes (*p*-hydroxybenaldehyde, vanillin, syringaldehyde, coniferyl aldehyde, and guaiacol), and 7 phenolic carboxylates (*p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, and 2-furoic acid) (Alvira et al., 2013; Jonsson et al., 2013; Klinke et al., 2002, 2004).

Figures 3a and 3b show the inhibition of these phenolic compounds on the specific growth rate of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 in the tested concentration range. For phenolic aldehydes, the specific growth rate of *Z. mobilis* ZM4 was

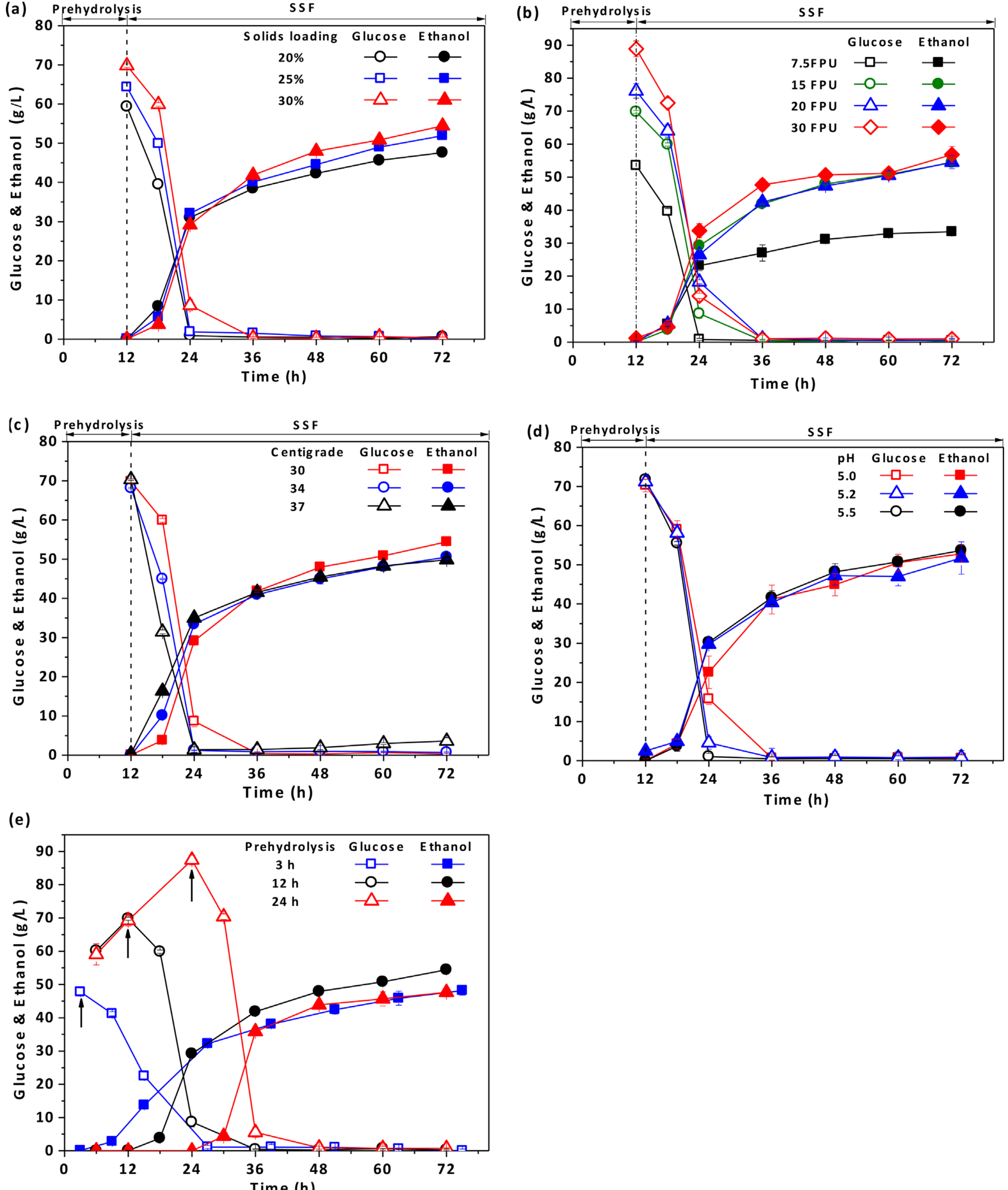

Figure 2. SSF of corncob residue by *Z. mobilis* ZM4 at different fermentation parameters. The prehydrolysis was at 50°C and pH 4.8 for 12 h unless mentioned elsewhere. (a) Effect of solids loading. Solids loading of 20%, 25%, and 30% (w/w), cellulase dosage of 15 FPU/g DM, 30°C, pH 5.5. (b) Effect of cellulase dosage. Cellulase dosage of 7.5, 15, 20, and 30 FPU/g DM. Solids loading of 30% (w/w), 30°C, pH 5.5. (c) Effect of SSF temperature. SSF at 30, 34, and 37°C. Solids loading of 30% (w/w), cellulase dosage of 15 FPU/g DM, pH 5.5. (d) Effect of pH. pH at 5.0, 5.2, and 5.5. Solids loading of 30% (w/w), cellulase dosage of 15 FPU/g DM, 30°C. (e) Effect of prehydrolysis time. Prehydrolysis for 3, 12, and 24 h. Solids loading of 30% (w/w), cellulase dosage of 15 FPU/g DM, 30°C, pH 5.5.

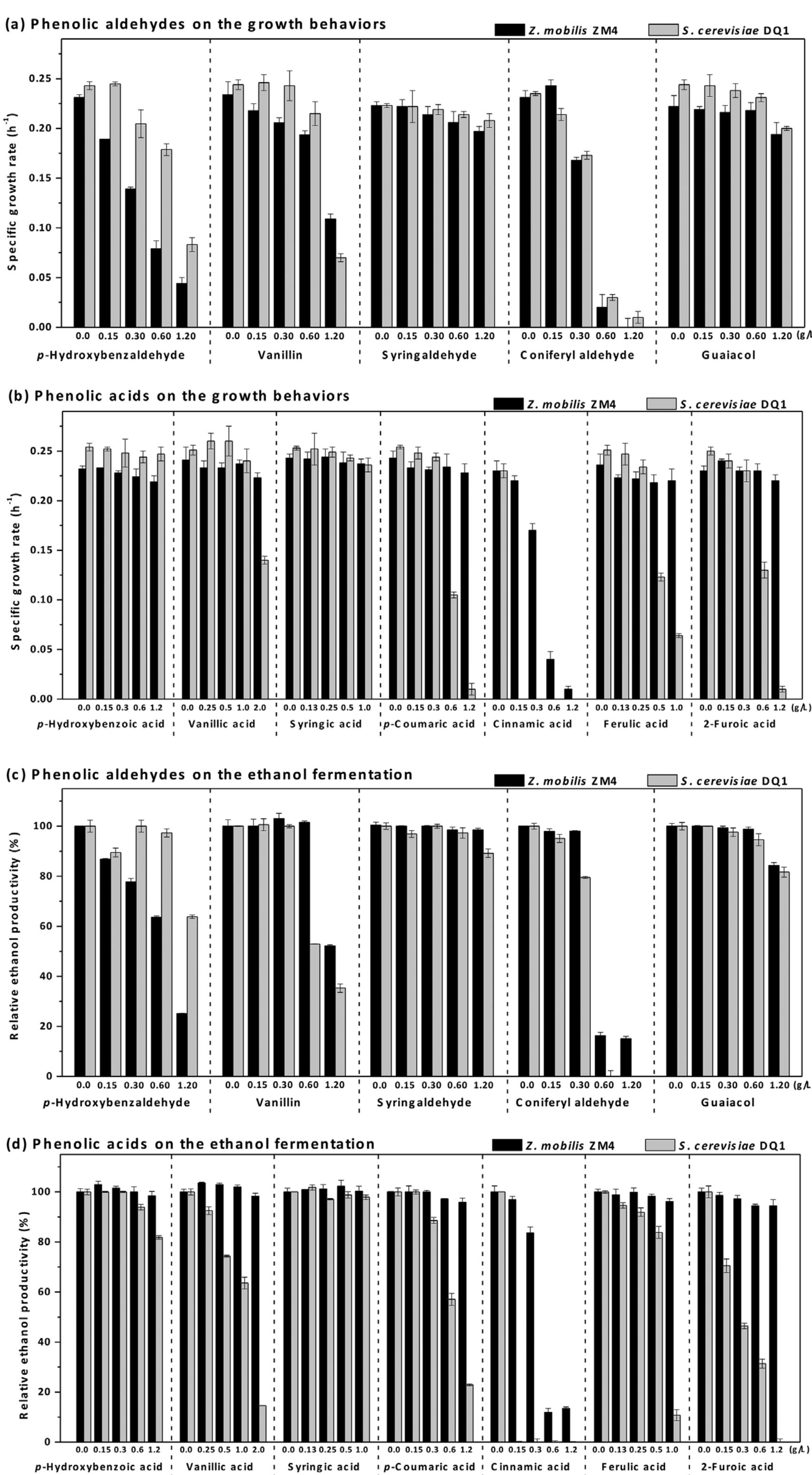

Figure 3. Inhibition of phenolics on the growth behaviors and the ethanol fermentation of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1. (a) Phenolic aldehydes on the growth behaviors. (b) Phenolic acids on the growth behaviors. (c) Phenolic aldehydes on the ethanol fermentation. (d) Phenolic acids on the ethanol fermentation. Conditions: 250 mL flask containing 40 mL of the rich medium or synthetic medium containing the individual phenolic compound at desired concentration, 10% (v/v) of inoculation ratio, 30°C for 24 h; *S. cerevisiae* DQ1 cultured at 150 rpm, *Z. mobilis* ZM4 cultured at static condition. The specific growth rate was defined as the maximum specific growth rate in the exponential growth phase. The relative ethanol productivity was defined as the percentage of the maximum ethanol productivity under inhibitor stress to that without inhibitor stress in the 24 h. The data were the mean and standard deviation of the twice independent experiments.

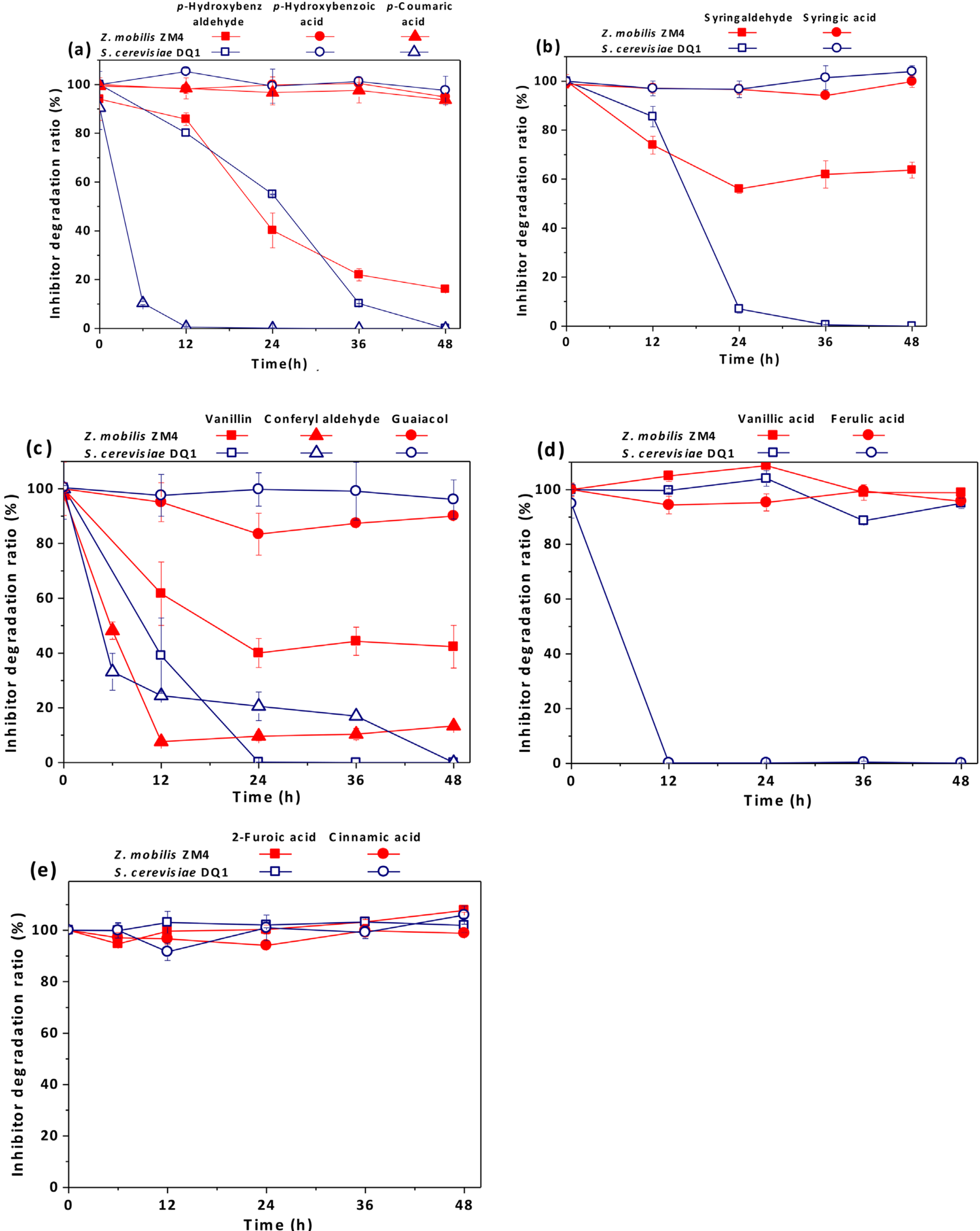

Figure 4. Biodegradation profiles of phenolic compounds by *Z. mobilis* ZM4 and *S. cerevisiae* DQ1. 250 mL flask containing 40 mL of the rich medium or synthetic medium with the individual phenolic compounds concentration at the 50% growth inhibition, 10% (v/v) inoculation ratio, 30°C for 48 h; *S. cerevisiae* DQ1 cultured at 150 rpm, *Z. mobilis* ZM4 cultured at static condition. *p*-Hydroxyphenyl compounds (H group): (a) *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and *p*-coumaric acid; Syringyl compounds (S group): (b) syringaldehyde and syringic acid; Guaiacyl compounds (G group): (c) vanillin, coniferyl aldehyde, guaiacol, and (d) vanillic acid, ferulic acid; Other aromatic acids: (e) cinnamic acid and 2-furoic acid.

Table I. GC-MS analysis of composition and structure of phenolic compounds from biodegradation by *Z. mobilis* ZM4.

		Trimethylsilylated phenolic compounds		
Phenolic compounds	Structures	RT (min)	MW	Quantification ions
p-Hydroxybenzyl aldehyde		12.44	194	89,121,151,179,194
p-Hydroxybenzyl alcohol		14.64	268	73,147,179,208,268
Vanillin		15.14	224	73,91,151,194,224
Vanillyl alcohol		16.72	298	147,179,209,268,298
Syringaldehyde		17.59	254	96,153,181,224,254
Syringyl alcohol		18.64	328	147,209,239,298,328
Coniferyl aldehyde		19.48	250	102,166,192,220,250
Coniferyl alcohol		20.69	324	131,204,235,294,324

RT, retention time; MW, molecular weight.

significantly inhibited from 0.23 h^{-1} (control) to 0.19, 0.21, 0.19, 0.21, and 0.17 h^{-1} when the concentrations of *p*-hydroxybenaldehyde, vanillin, guaiacol, syringaldehyde and coniferyl aldehyde were above 0, 0, 0.6, 1.2, and 0.15 g/L, respectively. On the other hand, the specific growth rate of *S. cerevisiae* DQ1 was reduced from 0.25 h^{-1} (control) to 0.20, 0.21, 0.20, 0.21, and 0.20 h^{-1} when these phenolics were above 0.15, 0.3, 0.15, 1.2, and t 0.15 g/L, respectively (Fig. 3a). The results show that *Z. mobilis* ZM4 was significantly less tolerant to *p*-hydroxybenaldehyde and vanillin but more tolerant to guaiacol than *S. cerevisiae* DQ1, and the similar tolerance to syringaldehyde and coniferyl aldehyde with *S. cerevisiae* DQ1 (Fig. 3a). For phenolic acids, *Z. mobilis* ZM4 was more tolerant to the most phenolic acids than *S. cerevisiae* DQ1. The specific growth rate of *Z. mobilis* ZM4 was not influenced when the concentration of *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and 2-furoic acid was at the highest level. Only when

Table II. Biodegradation of phenolic compounds by *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 in inhibitor medium.

Phenolic compounds	Concentration (mM)	Rate constant *k* of phenolic biodegradation reactions (1/s)	
		Z .mobilis ZM4	*S. cerevisiae* DQ1
Phenolic aldehydes			
p-hydroxybenzyl aldehyde	4.62	1.17×10^{-5}	2.65×10^{-5}
Vanillin	3.96	1.26×10^{-5}	7.79×10^{-5}
Coniferyl aldehyde	1.64	5.95×10^{-5}	5.85×10^{-5}
Guaiacol	12.10	—	—
Syringaldehyde	4.91	0.63×10^{-5}	5.54×10^{-5}
Phenolic acids			
p-hydroxybenzoic acid	9.35	—	—
p-coumaric acid	2.94	—	1.17×10^{-4}
Vanillic acid	5.95	—	—
Ferulic acid	2.49		2.91×10^{-4}
Syringic acid	2.99	—	—
Cinnamic acid	1.01	—	—
2-furoic acid	5.54	—	—

—: Not detected.

cinnamic acid was above 0.3 g/L, the specific growth rate of *Z. mobilis* ZM4 was significantly inhibited from 0.23 to 0.17 h^{-1}. While the specific growth rate of *S. cerevisiae* DQ1 was significantly inhibited from 0.25 h^{-1} (control) to 0.13, 0.10, 0.0, 0.13, and 0.12 h^{-1} when the concentration of vanillic acid, *p*-coumaric acid, cinnamic acid, ferulic acid and 2-furoic acid was were above 1.0, 0.3, 0.15, 0.25, and t 0.3 g/L, respectively (Fig. 3b).

Figures 3c and 3d show the inhibition of these phenolics on the ethanol productivity of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 in the tested concentration range. The ethanol productivity of *Z. mobilis* ZM4 was slightly greater than that of *S. cerevisiae* DQ1 in the presence of phenolic aldehydes. When the concentrations of vanillin, syringaldehyde, coniferyl aldehyde, and guaiacol were at 0.6, 1.2, 0.3, and 0.6 g/L, the inhibition on the ethanol productivity of *Z. mobilis* ZM4 was not observed, while the relative ethanol productivity of *S. cerevisiae* DQ1 was reduced to 53.6%, 88.7%, 79.0%, and 94.3%, respectively. Only when *p*-hydroxybenzaldehyde was above 0.6 g/L, the relative ethanol

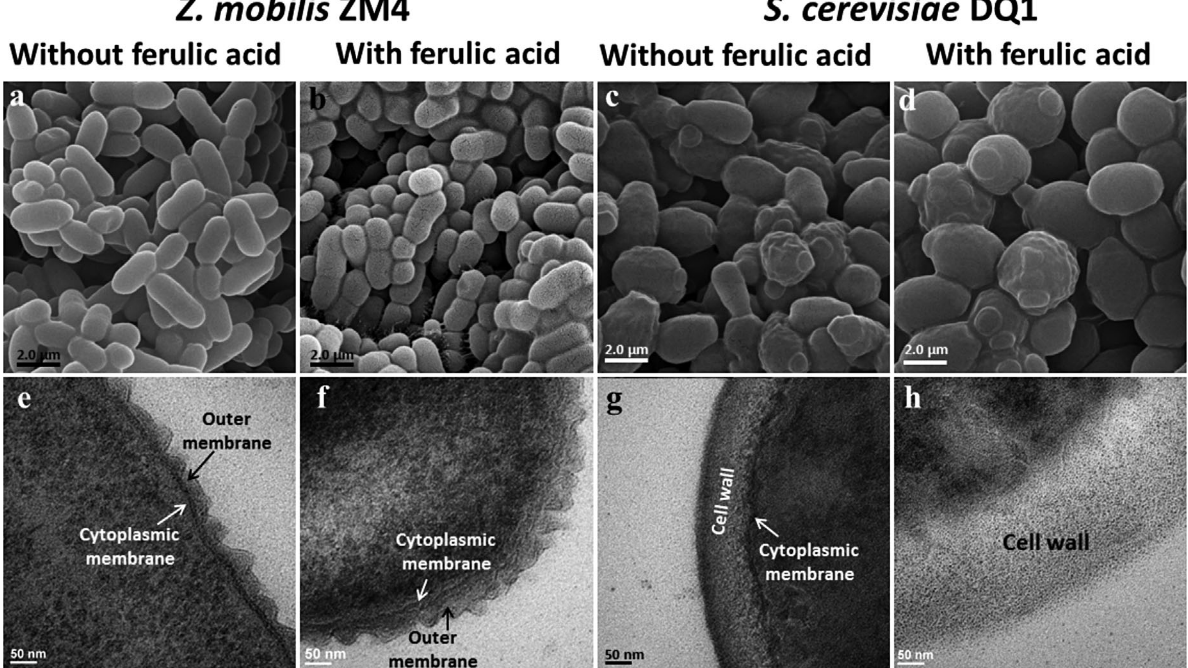

Figure 5. SEM and TEM images of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1. (a–d) SEM images of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 with and without ferulic acid, respectively. (e–h) TEM images of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 with and without ferulic acid.

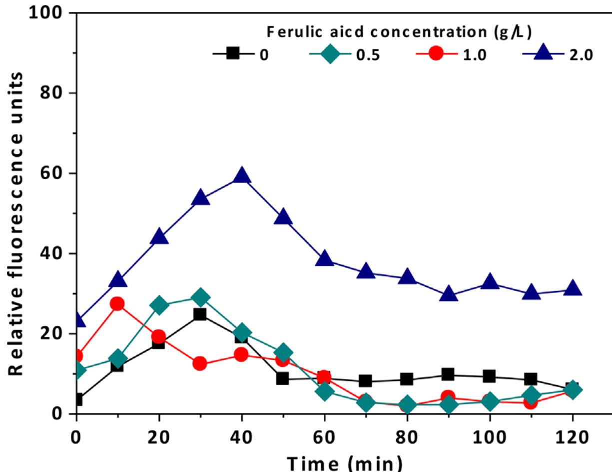

Figure 6. Effect of ferulic acid on the outer membrane permeability of *Z. mobilis* ZM4. Condition: 250 mL flask containing 40 mL of the rich medium with 0.5, 1.0, and 2.0 g/L of ferulic acid, 10% (v/v) inoculation ratio, at 30°C for 8 h. The cells were harvested, rinsed, and resuspended in 0.05 M sodium phosphate buffer (pH 7.0) to an OD_{600} of 0.5. NPN was added to the cell suspension, and then the fluorescence of sample was monitored at 420 nm for 120 min. Each value represented the mean of triplicate measurements, and the deviation from the mean was below 5%.

productivity of *Z. mobilis* ZM4 was reduced to 63.2%, while *S. cerevisiae* DQ1 was not inhibited (Fig. 3c). For phenolic acids, it is worth noting that the ethanol productivity of *Z. mobilis* ZM4 was much less affected by the phenolic acids in the tested range than that of *S. cerevisiae* DQ1 (Fig. 3d). When the concentrations of *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, cinnamic acid, ferulic acid, and 2-furoic acid were at 0.6, 0.25, 0.3, 0.15, 0.13, and 0.15 g/L, the ethanol productivity of *Z. mobilis* ZM4 was not inhibited, while *S. cerevisiae* DQ1 was significantly reduced to 94.3%, 92.1%, 88.4%, 0, 93.9%, and 69.7%, respectively. A typical example was for cinnamic acid: the inhibition on *Z. mobilis* ZM4 occurred only at the high concentration of 0.6 g/L, while *S. cerevisiae* DQ1 was completely inhibited to produce ethanol at the lowest concentration in the tested range of 0.15 g/L. In addition, syringic acid did not inhibit the ethanol productivity of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 at the range of 0–1.0 g/L.

The results reveal that *Z. mobilis* was significantly tolerant to phenolic acids than *S. cerevisiae*, although the tolerance to phenolic aldehydes was almost the same. Considering the high level of the phenolic acids, such as guaiacol, 2-furoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid and ferulic acid, accumulated on corncob residue (Gu et al., 2014), the results may explain why *Z. mobilis* behaved the better ethanol performance than *S. cerevisiae* when corncob residue was used as the feedstock.

Biodegradation of Phenolic Compounds by *Z. mobilis* and *S. cerevisiae*

Inhibitor tolerance of microorganisms is generally understood as their capability of converting the strong toxic inhibitors into the less toxic metabolites (Liu et al., 2009; Liu and Moon, 2009). Therefore, the degradation capacity of *Z. mobilis* on the 12 model phenolic compounds was examined and compared to that of *S. cerevisiae*.

Figure 4 shows that both *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 were capable to degrade four of the five selected phenolic aldehydes, with the only exception of guaiacol. For phenolic acids, *Z. mobilis* ZM4 was unable to degrade all the seven selected phenolic acids, while *S. cerevisiae* DQ1 degraded two of them: *p*-coumaric acid and ferulic acid. The degraded products of the phenolic aldehydes by *Z. mobilis* ZM4 were identified on GC-MS and quantitatively measured on HPLC. The results show that the phenolic aldehydes were converted into the corresponding alcohols within the initial 12–24 h (Table I and Figure S1). The degradation kinetic data (Table II) indicate that *Z. mobilis* ZM4 was slower in degrading vanillin and syringaldehyde, and similar in degrading *p*-hydroxybenzaldehyde and coniferyl aldehyde, compared with that of *S. cerevisiae* DQ1.

These results indicate that *Z. mobilis* ZM4 was capable of the in situ detoxification phenolic aldehydes to the less toxic alcohols, perhaps due to its reduction enzyme of NAD(P)H-dependent alcohol dehydrogenases (He et al., 2012; Larroy et al., 2002), but unable to degrade the phenolic acids that enriched in corncob residue feedstock. Therefore, the biodegradation performance of *Z. mobilis* proved its tolerance to phenolic aldehydes, but could not explain its higher tolerance to phenolic acids and the better fermentation performance to corncob residue.

Analysis of Phenolic Acid Tolerance by Cell Morphology Characterization

The outer membrane structure of Gram-negative bacteria plays a role in the selective barrier under the inhibitors stress (Nikaido, 2003). To search the possible mechanism of the high tolerance of *Z. mobilis* to phenolic acids, the cell morphology and membrane structure of *Z. mobilis* ZM4 and *S. cerevisiae* DQ1 were observed by the scanning electron microscope (SEM) and transmission electron microscope (TEM) under the stress of ferulic acid, a typical phenolic acid, as shown in Figure 5.

The SEM images show that *Z. mobilis* ZM4 cells without ferulic acid stress exhibited a regular cell size and structural morphology with the smooth cell surface (Fig. 5a). When furulic acid stress was added, the *Z. mobilis* cells exhibited the obvious adherence with each other and the cell surfaces appeared to rough with lots of protrusions (Fig. 5b). On the other hand, *S. cerevisiae* DQ1 cells with and without ferulic acid stress did not show significant changes in the surface morphology (Figs. 5c and 5d).

The TEM images of *Z. mobilis* ZM4 under the ferulic acid stress show the rough surface and thicker outer membrane with little protrusions, comparing to that without ferulic acid stress (Figs. 5e and 5f). On the other hand, *S. cerevisiae* DQ1 shows the looser cell walls and disappearance of cytoplasmic membrane in the cells cultured under the ferulic acid compared with the cells cultured without stress (Figs. 5g and 5h).

The changes of the cell surface and the outer membrane structure of *Z. mobilis* ZM4 under the ferulic acid stress might provide an effective permeability barrier to hydrophobic molecules and kept the outer membrane structure stable (Nikaido, 2003). The increased protrusions on the cell surface may attribute to the

lipopolysaccharide aggregation on the cell outer membrane (Barrow et al., 1984; Kotra et al., 1999). Lipopolysaccharide is the major composition of outer membrane of Gram-negative bacteria and the protrusions of lipopolysaccharide become tighter with the neighbor protrusion under chemical stress (Amro et al., 2000). For *S. cerevisiae*, the changes in inner microstructure of the loosed cell wall and the disappeared cytoplasmic membrane might be caused by the phenolic acids permeability to the cell wall, and damage of the membrane integrity, and disruption of the proton gradient (Mills et al., 2009; Zaldivar and Ingram, 1999). The poor cell growth and ethanol productivity of *S. cerevisiae* DQ1 could be attributed to these morphological and structural changes.

In order to investigate the outer membrane barrier function of *Z. mobilis* ZM4 cells in the presence of ferulic acid, the fluorescence titration was carried out using a hydrophobic fluorescent probe N-Phenyl-α-naphthylamine (NPN). NPN has a low fluorescence quantum yield in aqueous solution but a high fluorescence in hydrophobic environment of the biological membrane. Thus, the fluorescence value and its increased rate were used to indicate the outer membrane permeability. Figure 6 shows the outer membranes of *Z. mobilis* ZM4 cells cultured with 0.5 and 1.0 g/L of ferulic acid for 8 h had very low permeability, similar to the performance of control, whereas their outer membranes showed a higher permeability when the ferulic acid concentration was increased to 2.0 g/L. The results suggest that the lower ferulic acid (below 1.0 g/L) did not have significant effect on the outer membranes permeability of *Z. mobilis* ZM4, while the high ferulic acid may damage the outer membrane integrity. Therefore, the alteration of cell outer membrane structure for maintaining its function of permeable barrier to phenolic acid could explain the higher tolerance of *Z. mobilis* to phenolic acids, and provided a robust ethanologenic strain for ethanol fermentation from the phenolics enriched lignocellulosic material.

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