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Expressing an oxidative dehydrogenase gene in ethanologenic strain *Zymomonas mobilis* promotes the cellulosic ethanol fermentability

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ABSTRACT

Phenolic aldehydes from lignocellulose pretreatment harshly inhibit the viability and metabolism of ethanol fermenting strains. Direct conversion of phenolic aldehydes is usually incomplete due to their low water solubility and recalcitrance to bioconversion. Here we consolidated phenolic aldehydes bioconversion and ethanol fermentation in a typical ethanologenic bacterium *Zymomonas mobilis* by constructing an intracellular oxidative pathway. The gene PP_2680 encoding NAD^+-dependent aldehyde dehydrogenase from *Pseudomonas putida* KT2440 was expressed in *Z. mobilis* ZM4. The expression significantly improved both aldehyde inhibitor conversion and ethanol fermentability in corn stover hydrolysate. The purified PP_2680 aldehyde dehydrogenase showed strong *in vitro* oxidative capacity on phenolic aldehydes and its *in vivo* expression significantly up-regulated the key genes in the ED pathway and the oxidative phosphorylation. This study provided an important concept of simultaneous biodetoxification and fermentation in ethanologenic strains for the improvement of ethanol fermentability.

1. Introduction

Pretreatment is the prerequisite step to render lignocellulose biomass to enzymatic hydrolysis. In this harsh process, two groups of aldehyde compounds are produced including furan aldehydes (2-furylaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF)), as well as phenolic aldehydes (Klinke et al., 2004; Parawira and Tekere, 2011; Taylor et al., 2012; Chandel et al., 2013). Phenolic aldehydes include various derivatives of *p*-hydroxyphenyl (4-hydroxybenzaldehyde), guaiacyl (vanillin and ferulic acid), and syringyl (syringaldehyde) groups by their methoxylation degree (Klinke et al., 2004). These phenolic aldehydes significantly inhibit the cellulase enzyme activity and cell viability in consequent enzymatic hydrolysis and fermentation steps (Palmqvist and Hahn-Hägerdal, 2000; Zhang et al., 2010; Gu et al., 2015).

Biological conversion of phenolic aldehydes to less toxic derivatives (biodetoxification) effectively remove partial phenolic aldehydes, but the complete removal requires longer time and considerable fermentable sugars are consumed during this long term biodetoxification (Zhang et al., 2010; He et al., 2016). The residual phenolic aldehydes only slightly affect cell growth and metabolisms but obviously affect the ultimate ethanol fermentation efficiency. To achieve the complete removal of phenolic aldehydes, we propose a concept of combined biodetoxification and fermentation to fulfill simultaneously phenolic aldehyde conversion and ethanol fermentation. In the first step, furfural, HMF, acetic acid, and most of the phenolic aldehydes are removed from pretreated lignocellulose biomass in the biodetoxification step. When the “pre-detoxified” lignocellulose is sent to ethanol fermentation by ethanologenic fermenting microorganisms, the fermentable sugar (glucose and xylose) were fermented to ethanol and the residual phenolic aldehydes were simultaneously converted to non-toxic derivatives or even ultimately to CO_2 and H_2O through central carbon metabolism with high ethanol yield and productivity.

Here we constructed an oxidation pathway of phenolic aldehydes in the ethanologenic bacterium *Zymomonas mobilis* to realize the combined ethanol fermentation and phenolics removal. Regular conversion pathways of microorganisms reduce aldehydes to alcohols, but less likely to the corresponding acids (Franden et al., 2013; Yi et al., 2015; Almeida et al., 2008). On the other hand, only acid derivatives behave the potentials of complete assimilation into CO_2 and H_2O by the formation of protocatechuate before entering citric acid cycle (TCA). *Z. mobilis* is one of the most important strains for cellulosic ethanol fermentation for its high ethanol productivity, ethanol tolerance, and genetic manipulation feasibility (Zhang et al., 1995; Rogers et al., 2007). However, its viability and metabolism are relatively sensitive to the existence of phenolic aldehydes during fermentation (Franden et al.,

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Table 1
Strains and plasmids used in this study.

(a) Strains

	Genotype	Sources
A. resinae ZN1	Wild-type	Zhang et al. (2010)
E. coli BL21 (DE3)	F^-, *ompT*, *hsdS*$_B$ (rB^- mB^-), *gal dcm* (DE3)	Novagen
E. coli S17-1 λπ	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Purchased from ATCC
P. putida KT2440	Wild type	Purchased from ATCC
Z. mobilis ZM4	Wild type	Purchased from ATCC
S-ZMO0367	Glucose-6-phosphate 1-dehydrogenase gene ZMO0367 from *Z. mobilis* ZM4 in pHW20a	This study
S-ZMO1116	Oxidoreductase gene ZMO1116 from *Z. mobilis* ZM4 in pHW20a	This study
S-ZMO1696	Zn-binding alcohol dehydrogenase gene ZMO1696 from *Z. mobilis* ZM4 in pHW20a	This study
S-ZMO1885	NADH:flavin oxidoreductase/NADH oxidase gene ZMO1885 from *Z. mobilis* ZM4 in pHW20a	This study
S-Arz_2211_T1	Laccase gene Arz_2211_T1 from *A. resinae* ZN1 in pHW20a	This study
S-PP_1948	Benzaldehyde dehydrogenase gene PP_1948 from *P. putida* KT2440 in pHW20a	This study
S-PP_2680	Aldehyde dehydrogenase gene PP_2680 from *P. putida* KT2440 in pHW20a	This study
S-PP_3151	Aldehyde dehydrogenase gene PP_3151 from *P. putida* KT2440 in pHW20a	This study
S-PP_3357	Vanillin dehydrogenase gene PP_3357 from *P. putida* KT2440 in pHW20a	This study
S-Peno	Enolase (eno) promoter from *Z. mobilis* ZM4 in pHW20a	This study
S-Peno-ZMO1696	ZMO1696 in pHW20a-Peno	This study
S-Peno-PP_2680	PP_2680 in pHW20a-Peno	This study
S-Peno-ZMO1696-PP_2680	ZMO1696 and PP_2680 coexpressed in pHW20a-Peno	This study

(b) Plasmids

	Description	Sources
pHW20a	*Tc*^{r}, *mob* (RP4), *mob* (RSF1010), *lacZα*, MCS and *oriV*	Dong et al. (2011, 2013)
pHW20a-ZMO0367	Express ZMO0367 in *Z. mobilis* ZM4	This work
pHW20a-ZMO1116	Express ZMO1116 in *Z. mobilis* ZM4	Yi et al. (2015)
pHW20a-ZMO1696	Express ZMO1696 in *Z. mobilis* ZM4	Yi et al. (2015)
pHW20a-ZMO1885	Express ZMO1885 in *Z. mobilis* ZM4	Yi et al. (2015)
pHW20a-Arz_2211_T1	Express Arz_2211_T1 in *Z. mobilis* ZM4	This work
pHW20a-PP_1948	Express PP_1948 in *Z. mobilis* ZM4	This work
pHW20a-PP_2680	Express PP_2680 in *Z. mobilis* ZM4	This work
pHW20a-PP_3151	Express PP_3151 in *Z. mobilis* ZM4	This work
pHW20a-PP_3357	Express PP_3357 in *Z. mobilis* ZM4	This work
pET28a (+)	*Kan*^{r}, *lacI*, MCS, and *ori*	Stored in the lab

2013; Yang et al., 2014). The construction of oxidation pathway in *Z. mobilis* in this study improved aldehyde inhibitor conversion and ethanol fermentability by cofactor anaplerosis and the indirect expression strengthening of the growth-relevant genes. This study provided an important concept on elevating aldehyde inhibitor conversion and ethanol fermentability.

2. Materials and methods

2.1. Strains, plasmids, and medium

The details of the strains and plasmids used in this study are given in Table 1. *E. coli* stains were cultured in Luria-Bertani (LB) medium (pH 7.0) containing 10.0 g/L peptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl. *Amorphotheca resinae* ZN1 (CGMCC7452) was cultured on Potato dextrose agar (PDA) medium consisting of 200.0 g/L potatoes, 20.0 g/L glucose, and 20.0 g/L agar. *P. putida* KT2440 and *Z. mobilis* ZM4 were separately cultured in LB and Rich medium (RM) (pH 6.0) containing 2.0 g/L KH_2PO_4, 20.0 g/L glucose, and 10.0 g/L yeast extract.

2.2. Feedstock and reagents

Cellulase Youtell #7 was purchased from Hunan Youtell Biochem Co., Ltd., Yueyang, Hunan, China. The filter paper activity of cellulase was 63.0 FPU/g measured according to the NREL LAP-006 protocol (Adney and Baker, 1996), and the cellobiase activity was 102.0 IU/g determined according to Ghose (1987). The protein concentration was 49.5 mg/g according to Bradford (1976).

ABTS (2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was purchased from Sigma, Aldrich, St. Louis, MO. Furfural and 5-hydroxymethylfurfural (HMF) were purchased from J&K Scientific, Beijing, China. Both 4-Hydroxybenzaldehyde and syringaldehyde were purchased from Sangon Biotech, Shanghai, China, and vanillin was purchased from Aladdin Reagents, Shanghai, China.

2.3. Pretreatment and biodetoxification

Corn stover (CS) was harvested from Nanyang, Henan, China, in the fall of 2014. Corn stover (CS) was dry acid pretreated according to Zhang et al (2011) and He et al (2014). The pretreated corn stover contained 37.2% of cellulose and 8.2% of hemicellulose determined according to NREL LAP protocols (Sluiter et al., 2008a, 2008b). Hydrolysis of the pretreated corn stover was carried out in the bioreactor equipped with helical ribbon impeller for mixing (Zhang et al., 2010).

2.4. Recombinant construction

Z. mobilis ZM4 recombinants harboring the genes of ZMO0367, ZMO1116, ZMO1696, ZMO1885, Arz_2211_T1, PP_1948, PP_2680, PP_3151, and PP_3357 were constructed according to Dong et al. (2011; 2013) and Simon et al (1983) (Fig. 1; Table 1b). The genomic DNA of *Z. mobilis* ZM4 and *P. putida* KT2440 was extracted using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). The oligonucleotide primers used for DNA amplification in plasmid construction are listed in Table S1. *A. resinae* ZN1 was used to obtain the Arz_2211_T1 gene.

In order to harvest the purified PP_2680 protein, the PP-2680 gene was inserted into the plasmid pET28a (+) and overexpressed in *E. coli* BL21 and then cultured in LB medium supplemented with 25.0 μg/mL kanamycin at 37 °C shaking at 200 rpm. 0.1 mM IPTG was used at 6 h when $OD_{600\ nm}$ was 0.5. Elusion buffer (pH 6.0) containing 20 mM

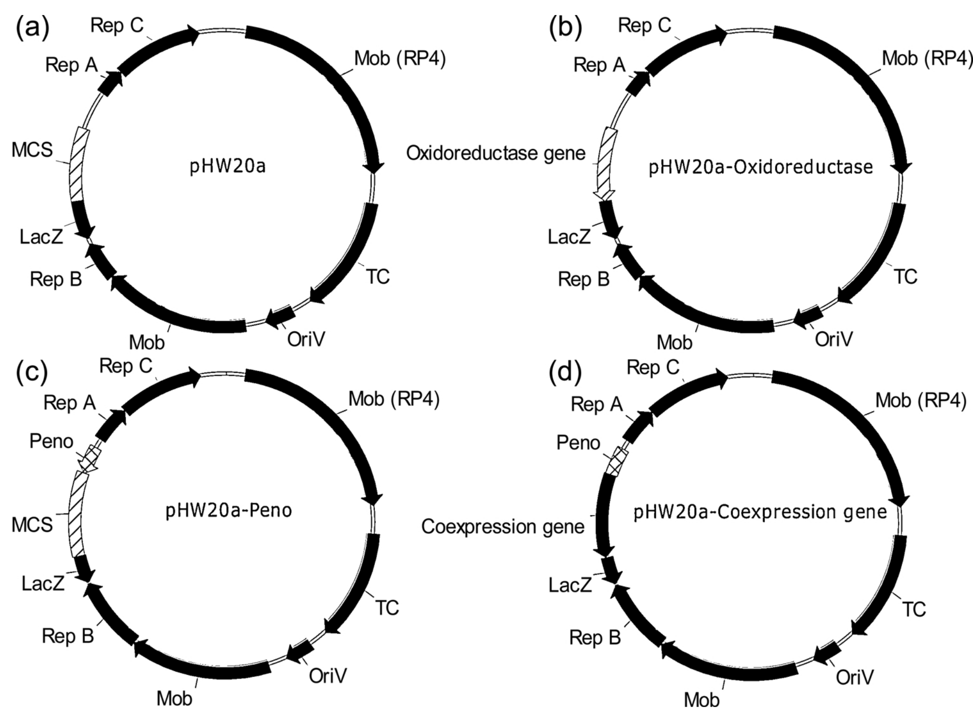

Fig. 1. Expression plasmid construction of the functional genes. (a) pHW20a; (b) pHW20a-oxidoreductase; (c) pHW20a-Peno; (d) pHW20a-Peno-coexpression gene.

Table 2
Crude enzyme activity of *Z. mobilis* ZM4 recombinants.

Genes	Encoding enzymes	Substrates	Enzyme activity (%)	References
(1) Reduction activity (gene from *Z. mobilis* ZM4)				
ZMO1116 (1473 bp)	Oxidoreductase	Vanillin	558.80	Abd E-Mawla and Beerhues (2002)
ZMO1696 (1017 bp)	Zinc-binding alcohol dehydrogenase		635.30	
ZMO1885 (1077 bp)	NADH: flavin oxidoreductase/NADH oxidase		35.30	
ZMO0367 (1458 bp)	Glucose-6-phosphate dehydrogenase	D-Glucose 6-phospate	176.30	Banerjee and Fraenkel (1972)
(2) Oxidation activity (gene from *A. resinae* ZN1 and *P. putida* KT2440, respectively)				
Arz_2211_T1 (1824 bp)	Laccase	ABTS	12.90	
PP_1948 (1479 bp)	Benzaldehyde dehydrogenase	Benzaldehyde	13.80	Nagai et al (2002)
PP_2680 (1521 bp)	NAD^+ Aldehyde dehydrogenase	4-Hydroxybenzaldehyde	29.40	
		Syringaldehyde	200.00	Abd E-Mawla and Beerhues (2002)
		Vanillin	8.30	
PP_3151 (1386 bp)	NAD^+ Aldehyde dehydrogenase	4-Hydroxybenzaldehyde	42.90	Liu et al (2008); Park et al (2011)
		Syringaldehyde	114.30	
		Vanillin	41.70	
PP_3357 (1449 bp)	Vanillin dehydrogenase	Vanillin	21.80	Ding et al (2015)

Na_3PO_4, 500 mM NaCl, and 10 mM imidazole, was used to re-suspend the collected cells after washed with 10.0 mL phosphate buffer saline (PBS) (pH 8.0) and then sonicate at 300 W for 5 s (at interval of 15 s, 98 cycles). For enzyme activity assay, the supernatant of the disrupted cell lysate filtered with a 0.44 μm filter after centrifuged at 4 °C, 15,000 × *g* for 30 min. 10 mM imidazole buffer (pH 7.8) was used to balance the Aogma Ni Aogarose 6 F F resin (Shanghai Chuzhi Biological Technology Co., Ltd., Shanghai, China).

The total RNA extracted from *A. resinae* ZN1 using Trizol (Invitrogen, Carlsbad, CA, USA). Arz_2211_T1 gene was amplified from the first strand of cDNA synthesized using ReverTra Ace qPCR RT Kit (Torobo Co., Osaka, Japan) using Arz_12286_T1 gene encoding actin as internal control.

Quantitative real time polymerase chain reaction (qRT-PCR) on CFX96™ Real-Time System with C10000TM Thermal Cycler (Bio-Rad Lab-oratories, Hercules, CA) was used to value the relative expression level of the genes in ED pathway and oxidative phosphorylation when PP_2680 heterogenously expressed in *Z. mobilis* ZM4. The primers of qRT-PCR were listed in Table S1. The first strand of cDNA was synthesized the same as the above. qRT-PCR was developed using an SYBR Green Real-time PCR Master Mix (Torobo Co., Osaka, Japan) according to the following procedure: 94 °C for 5 min, then 35 cycles at 94 °C for 2 min and 54 °C for 30 s, and 72 °C for 30 s. 16S rRNA gene (ZMOr003) was used as an internal control for data acquisition and normalization. Expression level of the candidate genes was analyzed according the method of Livak and Schmittgen (2001).

2.5. Enzyme activity assay

Z. mobilis recombinants were harvested and washed with 100 mM PBS (pH 7.0) twice, and then sonicated at 300 W for 1 s (at interval of 3 s, 8 cycles). The supernatant of the disrupted cell lysate was filtered by a 0.44 μm filter for the assay of crude enzyme activity after centrifuged at 12,000 × g for 5 min at 4 °C. The crude enzyme activity of glucose 6-phosphate dehydrogenase (ZMO0367) was detected according to Banerjee and Fraenkel (1972), and one unit crude enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1.0 μmol of $NADP^+$ per min at 25 °C.

The activity of oxidoreductases was performed according to Abd E-Mawla and Beerhues (2002). One unit crude enzyme activity was

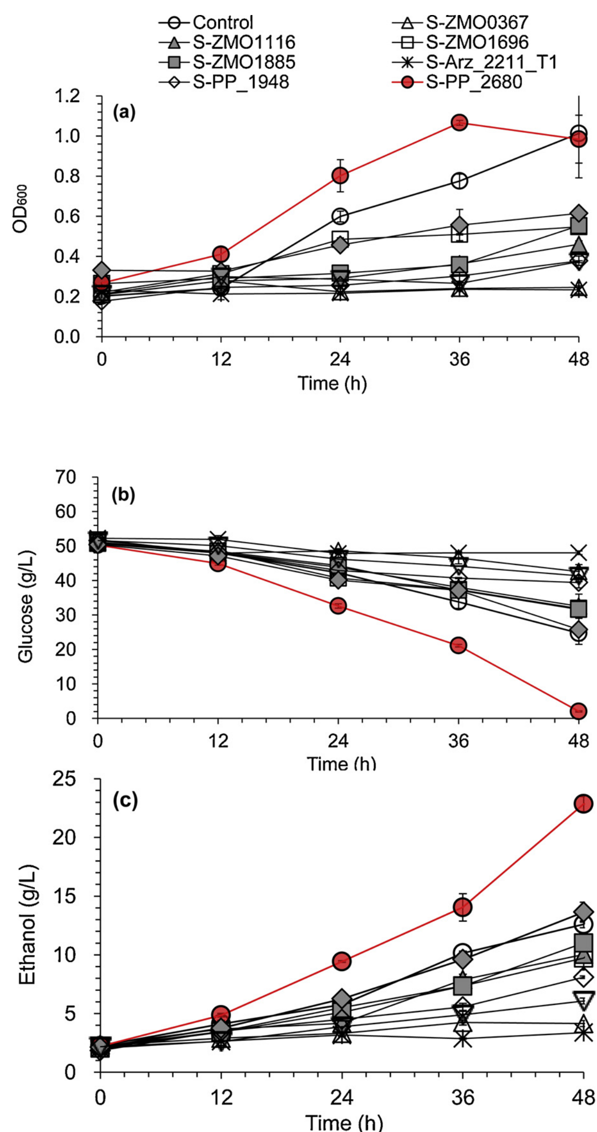

Fig. 2. Cellulosic ethanol fermentability of *Z. mobilis* recombinants in corn stover hydrolysate. (a) Cell growth; (b) Glucose consumption; (c) Ethanol titer. The fermentation was carried out at 30 °C without shaking and 10% inoculum in 15% (w/w) corn stover hydrolysates. Control indicated *Z. mobilis* ZM4 harboring empty pHW20a. S-ZMO0367, S-ZMO1116, S-ZMO1696, S-ZMO1885, S-Arz_2211_T1, S-PP_1948, S-PP_2680, S-PP_3151, and S-PP_3357, separately harbored pHW20a in *Z. mobilis* ZM4. All the experiments were conducted in triplicate. Corn sotver hydrolysate contained 56.1 g/L of glucose, 25.6 g/L of xylose, 0.75 g/L of furfural, 0.37 g/L of HMF, 3.44 g/L of acetic acid, 0.02 g/L 4-hydrobenzaldehyde, 0.20 g/L syringaldehyde, and 0.36 g/L vanillin.

Table 3
Enzyme activity of the purified PP_2680 protein in vitro.

Substrates	NAD^+ as cofactor (U/mg)	$NADP^+$ as cofactor (U/mg)
4-Hydroxybenzaldehyde	0.162 ± 0.038	0.086 ± 0.007
Syringaldehyde	0.216 ± 0.033	0.279 ± 0.012
Vanillin	0.127 ± 0.016	0.140 ± 0.003

defined as the amount of enzyme required to catalyze the reduction of 1.0 μmol of vanillin or benzaldehyde per min at 30 °C. The crude enzyme activity of laccase was developed according to Nagai et al (2002). One unit crude enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1.0 μmol of ABTS per min at 30 °C in 100 μL reaction system. The crude enzyme activity of NAD^+-dependent aldehyde dehydrogenase was detected according to Liu et al (2008) and Park et al (2011). One unit crude enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1.0 μmol of $NAD(P)^+$ per min at 30 °C. The crude enzyme activity of vanillin dehydrogenase (PP_3357) was carried out according to Ding et al (2015). One unit crude enzyme activity was defined as the amount of enzyme required to catalyze the reduction of 1.0 μmol of $NAD(P)^+$ per min at 30 °C. All the assays were performed in triplicate.

2.6. HPLC and GC–MS analysis

The supernatant was filtered through a 0.22 μm filter after centrifuged at 15,000 × *g* for 5 min at 4 °C for metabolites analysis by HPLC. Glucose, ethanol, and acetic acid were analyzed using HPLC according to Yi et al (2015) and Wang et al (2015). The aldehydes derivatives were identified by GC–MS according to Yi et al (2015).

3. Results and discussion

3.1. Construction of conversion pathways in Z. mobilis for aldehyde inhibitors

We expressed multiple oxidoreductase genes in *Z. mobilis* ZM4 to accelerate the conversion of furan and phenolic aldehyde inhibitors. For constructing reduction pathways, four genes from *Z. mobilis* ZM4 were selected based on their significant response to phenolic aldehydes reduction, including ZMO1116 encoding oxidoreductase, ZMO1696 encoding zinc-binding alcohol dehydrogenase, ZMO1885 encoding NADH: flavin oxidoreductase/NADH oxidase (Yi et al., 2015), and ZMO0367 encoding glucose 6-phosphate dehydrogenase (Gorsich et al., 2006; Nguyen et al., 2014). For reconstructing oxidative pathways, five genes were selected including four genes from *P. putida* KT2440, PP_1948 encoding benzaldehyde dehydrogenase, PP_2680 and PP_3151 encoding NAD^+ dependent aldehyde dehydrogenase, PP_3357 encoding vanillin dehydrogenase (Simon et al., 2014), and one gene from *A. resinae* ZN1, Arz_2211_T1 encoding laccase *lcc*2 (Cassland and

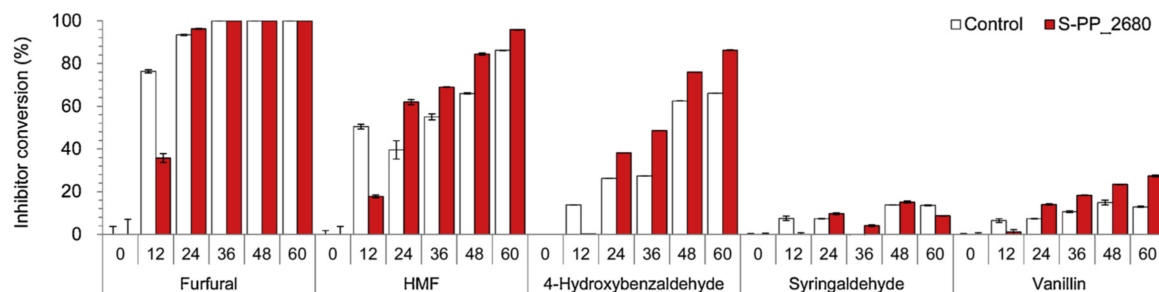

Fig. 3. Aldehyde inhibitors conversion of S-PP_2680 during ethanol fermentation in corn stover hydrolysate. The fermentation was carried out at 30 °C without shaking and 10% inoculum in 15% (w/w) corn stover hydrolysates containing 56.1 g/L of glucose, 25.6 g/L of xylose, 0.75 g/L of furfural, 0.37 g/L of HMF, 3.44 g/L of acetic acid, 0.02 g/L 4-hydrobenzaldehyde, 0.20 g/L syringaldehyde, and 0.36 g/L vanillin.

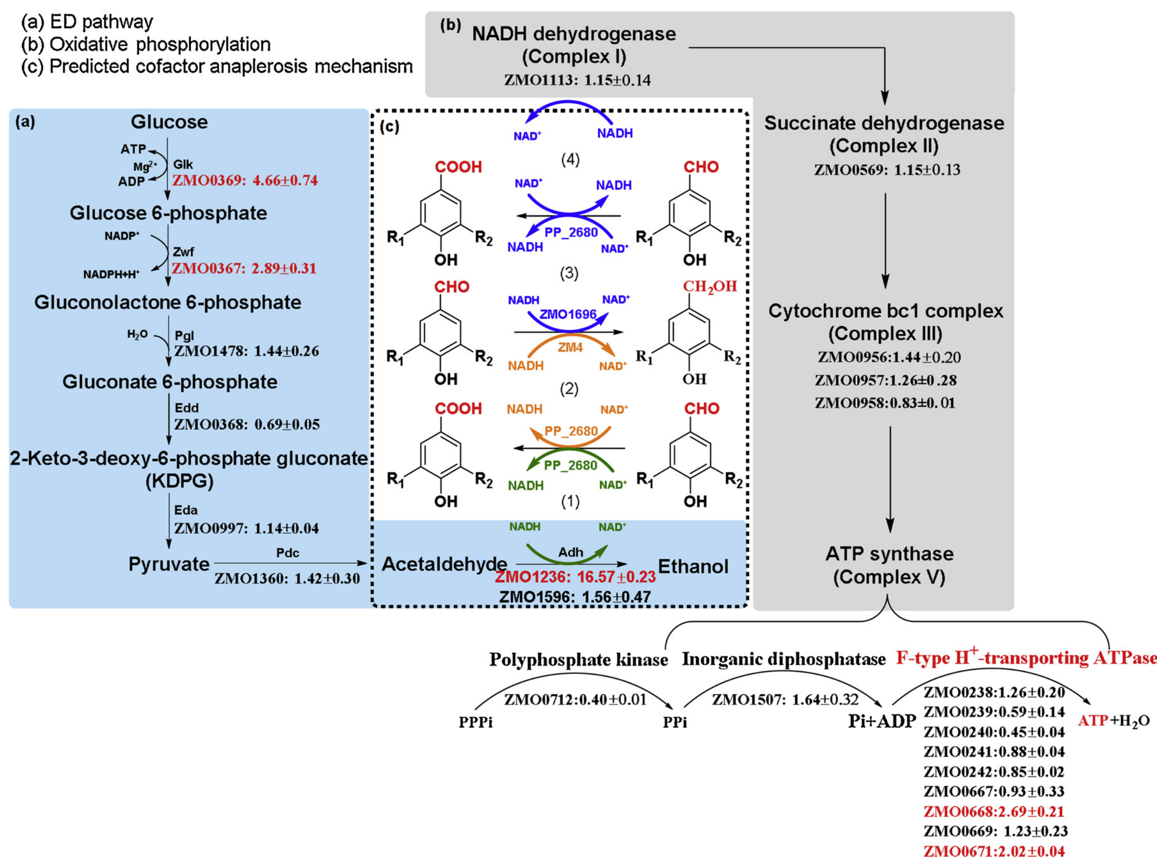

Fig. 4. Predicted cofactor anaplerotic reaction with PP_2680 expression in *Z. mobilis* ZM4. Light blue, ED pathway; Gray, oxidative phosphorylation. Fold change (increase or decrease) from the ratio of the recombinant and the control was used to indicate the relative expression level. It represented standard derivation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Jönsson, 1999). These genes were introduced and expressed in *Z. mobilis* ZM4 using a shuttle expression vector pHW20a according to the protocol by (Fig. 1).

The activity of the crude enzymes was assayed and the successful expression of the target genes were confirmed (Table 2). For the reductive genes, the expression of ZMO1116, ZMO1696, and ZMO1885 increased the vanillin conversion ratio by 558.8%, 635.3%, and 35.3%, while the over-expression of ZMO0367 enhanced the specific enzyme activity by 176.3% catalyzing D-glucose-6-phosphate substrate. For the oxidative genes, the specific activity of the expression of oxidative genes Arz_2211_T1, PP_1948, and PP_3357 increased the conversion of ABTS, benzaldehyde, and vanillin substrate by 12.9%, 13.8%, and 21.8%, respectively, while the specific activity of PP_2680 expression increased by 29.4%, 200.0%, and 8.3% on 4-hydroxybenzaldehyde, syringaldehyde, and vanillin, respectively; the specific enzyme activity of PP_3151 increased by 42.9%, 114.3%, and 41.7%, respectively.

The ethanol fermentability of the *Z. mobilis* recombinants expressing the reductive or oxidative genes was assayed in corn stover hydrolysate containing various inhibitors (Figs. 2 and 3). The ethanol fermentability was also increased by 95.5% in ethanol productivity, and 100.6% in ethanol yield at 48 h after inoculating (Fig. 2). However, no furan acids and phenolic acids were detected by GC–MS when furan aldehydes and phenolic aldehydes were converted, perhaps due to the fast assimilation of the acids in *Z. mobilis*. The PP_2680 expressing recombinant strain S-PP_2680 obviously increased the conversion rate of furfural, HMF, 4-hydroxybenzaldehyde, and vanillin (Fig. 3). No observable conversion of syringaldehyde was found, indicating that the relevant enzymes of *Z. mobilis* were not able to convert syringaldehyde less toxic for *Z. mobilis* ZM4 (Yi et al., 2015).

3.2. Catalytic activity evaluation of the PP_2680 aldehyde dehydrogenase

We overexpressed the PP_2680 gene in *E. coli* BL21 and the catalytic activity of the purified enzyme was measured. The high oxidative activity on phenolic aldehydes was identified using NAD^+ or $NADP^+$ as the cofactor, but no reductive activity was detected using NADH or NADPH as the cofactor (Table 3). We also identified the oxidative derivatives of 4-hydroxybenzoate (retention time 18.97 min) from 4-hydroxybenzaldehyde, vanillate (21.50 min) from vanillin by GC–MS, but no direct oxidative acid derivatives of phenolic aldehydes was detected (Table S2).

We further expressed the PP_2680 gene in *Z. mobilis* with ZMO1696 gene encoding NADH-dependent alcohol dehydrogenase co-expressed to enhance the regeneration of NADH from NAD^+. The expression of PP_2680 in *Z. mobilis* significantly improved the glucose consumption and ethanol fermentability (Fig. 5). There was significant difference in glucose consumption between recombinant strain S-Peno-PP_2680 (48 h) and S-Peno-ZMO1696 (48 h) ($p < 0.001$), S-Peno-PP_2680 (60 h) and S-Peno-ZMO1696 (60 h) ($p < 0.001$), and 4-hydroxybenzaldehyde between S-Peno-PP_2680 (48 h) and S-Peno-ZMO1696 (48 h) ($p < 0.001$), indicating the contribution of PP_2680 expression for the improvement of glucose consumption and 4-hydroxybenzaldehyde. The co-expression of PP_2680 and ZMO1696 led to the further improvement and the complete conversion of the most toxic aldehyde inhibitors including furfural and 4-hydroxybenzaldehyde (Fig. 5), indicating the cofactor anaplerosis contributed to the aldehyde inhibitors conversion and ethanol fermentability.

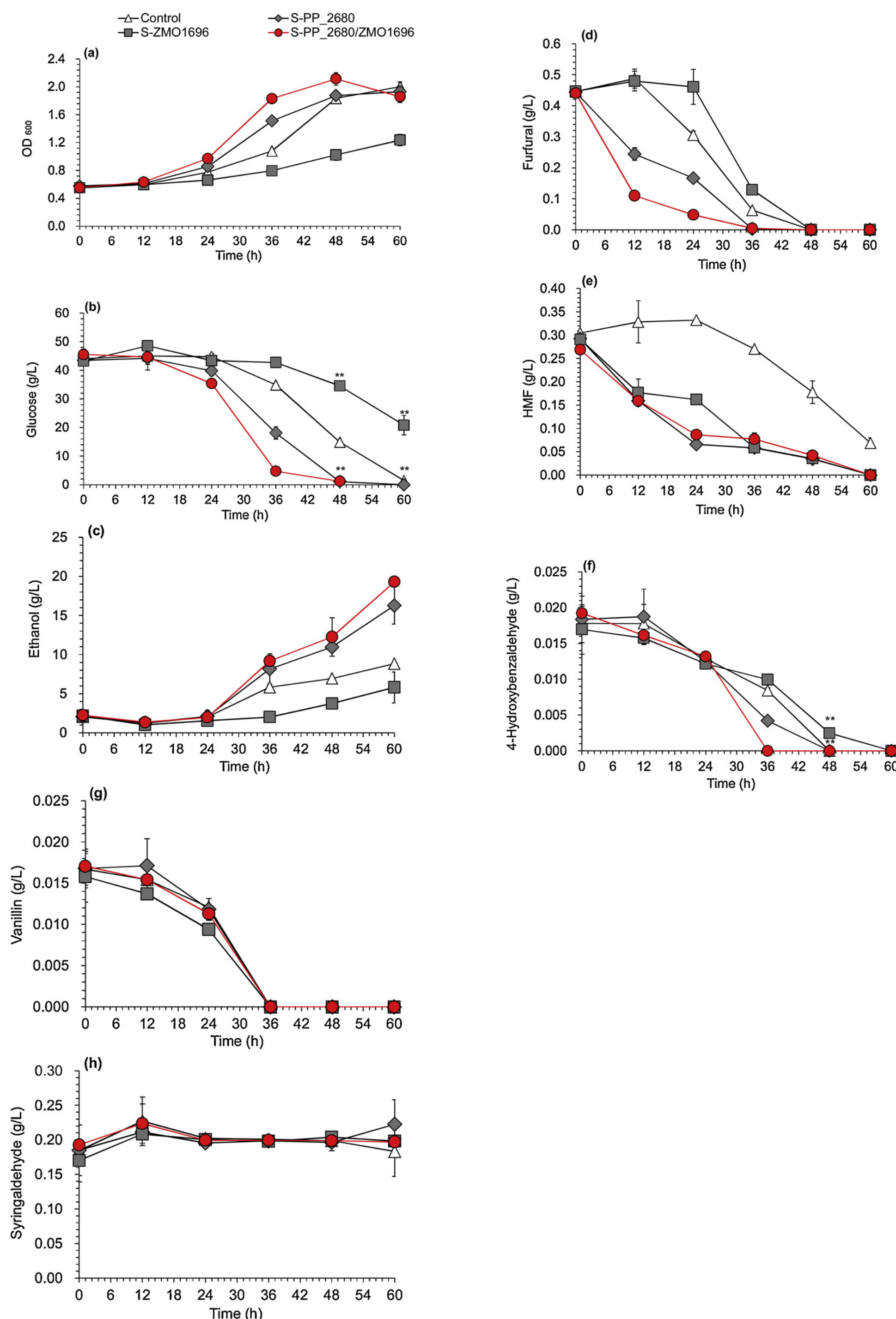

Fig. 5. Ethanol fermentability and inhibitors conversion in corn stover hydrolysate with PP_2680 and PP_1696 expressed in *Z. mobilis* strains. (a) Cell growth; (b) Glucose consumption; (c) Ethanol production; (d) Furfural conversion; (e) HMF conversion; (f) 4-Hydroxybenzaldehyde conversion; (g) Vanillin conversion; (h) Syringaldehyde conversion. The two asterisk (**) indicated the significant difference ($p < 0.001$) derived from Student's *t*-test (or the Mann-Whitney Rank Sum Test) wherever appropriate depending normality test by using the SigmaPlot software (version 12.5, SPSS, USA). A *p*-value of less than 0.05 was considered statistically significant with n = 3 for each group. Control indicated *Z. mobilis* ZM4 harboring empty pHW20a with Peno promoter in order to determine the most sensitive one. S-PP_2680, S-ZMO1696, and S-PP_2680/ZMO1696, were expressed in pHW20a-Peno-PP_2680/ZMO1696 in *Z. mobilis* ZM4, respectively. Corn sotver hydrolysate contained 51.9 g/L of glucose, 22.7 g/L of xylose, 0.46 g/L of furfural, 0.28 g/L of HMF, 4.02 g/L of acetic acid, 0.02 g/L 4-hydrobenzaldehyde, 0.15 g/L syringaldehyde, and 0.03 g/L vanillin.

3.3. Metabolic regulation of PP_2680 expression on ethanol synthesis in Z. mobilis

The mechanism of fermentability enhancement by PP_2680 expression in *Z. mobilis* ZM4 was further analyzed by examining the gene expression level of Entner-Doudoroff (ED) pathway and oxidative phosphorylation pathway by qRT-PCR (Fig. 4).

In ED pathway, the genes ZMO0369 encoding glucokinase (*glk*) and ZMO0367 encoding glucose 6-phosphate 1-dehydrogenase (*zwf*) were up-regulated by 4.66 and 2.89 folds, respectively, leading to the increase of NADPH regeneration and ATP production in ED pathway (Snoep et al., 1996). The genes ZMO1236 and ZMO1596 encoding alcohol dehydrogenases were also up-regulated by 16.57 and 1.56 folds, respectively, resulting in the improved cell growth and ethanol production. In oxidative phosphorylation pathway, the two genes ZMO0671 and ZMO0668 encoding H^+ transporting two-sector ATPases responsible for ATP production were up-regulated for 2.02 and 2.69 folds, respectively, by expression of PP_2680 in *Z. mobilis* preventing ATP exhaustion. The expression of PP_2680 promoted aldehyde inhibitor conversion and cellulosic ethanol fermentability was by the indirect upregulation instead of the expected direct oxidation of phenolic aldehydes to phenolic acids.

4. Conclusion

Construction of oxidation pathway by the heterologous expression

of PP_2680 in *Z. mobilis* increased aldehyde inhibitor conversion and cellulosic ethanol fermentability indirectly by improving cell growth depending on cofactor anaplerosis and gene expression enhancement including up-regulating the expression of glucokinase (*glk*), glucose 6-phosphate 1-dehydrogenase (*zwf*), alcohol dehydrogenase (*adh*) and H^+ transporting two-sector ATPase in ED pathway and oxidative phosphorylation. It's the first time to investigate the contribution of PP_2680 in the construction of tolerant strains. This discovery provided an important way to elevate aldehyde inhibitor conversion and ethanol fermentation between biodetoxification and fermentation of engineered fermenting strains.

Contributors

JB conceived the study. XY and JB prepared the manuscript. XY and LZ constructed recombinants, enzyme activity assays, conducted the qRT-PCR, and fermentation experiments. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.07.005.

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