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Improving cellulosic ethanol fermentability of *Zymomonas mobilis* by overexpression of sodium ion tolerance gene *ZMO0119*

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ABSTRACT

Inhibition of sodium ion (Na^+) on *Zymomonas mobilis* represents an important obstacle for efficient cellulosic ethanol production. This study screened and overexpressed the genes responsible for transporting metal ions in *Z. mobilis* for increasing its Na^+ tolerance. The *ZMO0119* gene encoding Na^+/H^+ antiporter was identified to be highly effective for reducing intracellular Na^+ concentration of *Z. mobilis* by improving the Na^+ transport capacity. Overexpression of *ZMO0119* gene in *Z. mobilis* significantly accelerated the cell growth, glucose consumption, and cellulosic ethanol production from the dry acid pretreated and biodetoxified corn stover feedstock. This study provided an important gene responsible for increasing the cellulosic ethanol fermentability by *Z. mobilis*.

1. Introduction

Metal ions in lignocellulose hydrolysates such as sodium ion (Na^+), potassium ion (K^+) and ammonia ion (NH_4^+) come from ash in the feedstock, chemical and alkaline reagents addition during pretreatment and pH neutralization, or corrosion products of pretreatment reactor (Lynd et al., 2001; Ranatunga et al., 2000). These metal ions inhibit the fermenting cell viability when the ion concentrations reach to the threshold levels (Klinke et al., 2004; Lynd et al., 2001; Maiorella et al., 1984; Park and Baratti, 1993).

Zymomonas mobilis is a promising strain for cellulosic ethanol production (He et al., 2014; Panesar et al., 2006), but it is sensitive to inhibitors including metal ions in cellulosic hydrolysates (Bajpai and Margaritis, 1984; Doelle et al., 1990; Franden et al., 2013; Stevnsborg and Lawford, 1986). Especially, the cell growth and ethanol production of *Z. mobilis* are inhibited even by a low Na^+ concentration (0.175 M) (Vriesekoop and Rasmusson, 2002). Na^+ also has an inhibitory synergy with other inhibitors such as acetate and formate (Franden et al., 2013). The tolerance of *Z. mobilis* against Na^+ is important for achieving high cellulosic ethanol. Yang et al. (2010) identified the sodium-proton antiporter gene *nhaA* (*ZMO0119*) and its elevation on sodium acetate tolerance for *Z. mobilis*. Recently, Liu et al. (2017) deleted a short sequence of the promoter region of *ZMO0119* and the tolerance of *Z. mobilis* to sodium acetate was significantly improved. These studies focused on the mechanism of the gene but the function was not yet evaluated and confirmed in the practical Na^+ containing fermentations using lignocellulose feedstock.

In this study, five endogenous genes of *Z. mobilis* ZM4 associated with ion transport were evaluated for increasing both Na^+ tolerance and cellulosic ethanol fermentation using a dry dilute acid pretreated corn stover hydrolysate. The overexpression of *ZMO0119* was found to be effective in alleviating the inhibition of Na^+ on *Z. mobilis* with higher ethanol yields. The study provided an insight for improving ethanol fermentability of *Z. mobilis* in lignocellulose biorefining processes.

2. Materials and methods

2.1. Strains, plasmids, media and culture conditions

The strains and plasmids used in this study are shown in Table 1.

Amorphotheca resinae ZN1 used for biodetoxification of the pretreated corn stover feedstock was isolated in our previous study and stored in China General Microbiological Culture Collection Center (CGMCC, Beijing, China) with the registration number 7452 (Zhang et al., 2010b). *A. resinae ZN1* was grown at 28 °C on the potato dextrose agar (PDA) slant containing 200 g/L potato extract juice, 20 g/L glucose, and 15 g/L agar.

Zymomonas mobilis ZM4 (ATCC) used for ethanol fermentation and the parental strain for recombinant construction was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). *Z. mobilis* strain and the derived recombinants were inoculated in rich medium (RM) containing 2 g/L KH_2PO_4, 20 g/L glucose and 10 g/L yeast extract, and incubated at 30 °C without shaking. 20 μg/mL of

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Table 1
Microbial strains, plasmids and primers used in this study.

Strains	Genotype	Sources/ references
Zymomonas mobilis ZM4	Wild-type strain, ATCC31821	ATCC
Escherichia coli S 17-1 λπ	*Pro*, *res*, *mod*$^+$; chromosomal integrated RP4, 2-*Tc*::Mu-*Km*::*Tn7*; *Tp*, *sm*	Simon et al. (1983)
Amorphotheca resinae ZN1	Wild-type strain	Zhang et al., (2010b)

Plasmids	Description	Sources/ references
pHW20a	*Tc*^{r}, *mob* (RP4), *mob* (RSF1010), *lacZα*, MCS, and *oriV*	Dong et al. (2011)
pHW20a-*ZMO0119*	Na^+/H^+ antiporter gene *ZMO0119* from ZM4 in pHW20a	This study
pHW20a-*ZMO0204*	Cation efflux protein gene *ZMO0204* from ZM4 in pHW20a	This study
pHW20a-*ZMO0578*	Sodium/dicarboxylate symporter gene *ZMO0578* from ZM4 in pHW20a	This study
pHW20a-*ZMO0846*	Sodium/hydrogen exchanger gene *ZMO0846* from ZM4 in pHW20a	This study
pHW20a-*ZMO1209*	Potassium transporter Kup gene *ZMO1209* from ZM4 in pHW20a	This study

Primers	Sequence (5'-3')
ZMO0119-F	CCCAAGCTTATGCGGTTTTCTATTCGTCG
ZMO0119-R	CGGAATTCTCAAACGCCTTTTGCCG
ZMO0204-F	CCCAAGCTTATGATGGGTAGGAAATGTTG
ZMO0204-R	GCTCTAGATTAGCAAACAGATAAAGATCG
ZMO0578-F	CCCAAGCTTGTGGCAAAAAGGCTCAC
ZMO0578-R	GCTCTAGATTATTCAGCCCCTATTTTTG
ZMO0846-F	CCCAAGCTTATGCATATGAATTCTTCTCTT
ZMO0846-R	GCTCTAGATTATGCTTCAGGCATAATG
ZMO1209-F	CCCAAGCTTATGAGTAACGATACTTCCC
ZMO1209-R	GCTCTAGACTATTTTCCGTTACTGCC

Note: The underline indicates the digestion sites.

tetracycline and 30 μg/mL of nalidixic acid were added for the culture of *Z. mobilis* recombinants.

Escherichia coli S17-1 λπ used for conjugation with *Z. mobilis* ZM4 was cultured at 37 °C, 220 rpm in the Luria-Bertani (LB) medium containing 10 g/L NaCl, 5 g/L yeast extract and 10 g/L tryptone. 20 μg/mL of tetracycline was added to screen positive *E. coli* recombinants.

2.2. Reagents and enzymes

Tryptone and yeast extract were purchased from Oxoid, Hampshire, UK. Tetracycline and nalidixic acid were from Sigma-Aldrich, St. Louis, MO, USA. Acetic acid, glucose and other chemical reagents were of analytical reagent grade and purchased from Sinopharm Chemical Reagent Co., Shanghai, China.

The PrimeSTAR HS DNA Polymerase was purchased from Takara, Otsu, Japan. TIANamp Bacterial DNA Kit was purchased from Tiangen, Beijing, China. Restriction endonuclease and T4 DNA ligase were purchased from Thermo Scientific, Wilmington, DE, USA.

Cellulase enzyme Youtell #6 was purchased from Hunan Youtell Biochemical Co., Yueyang, Hunan, China. The filter paper activity was 135 FPU/g determined using the NREL protocol LAP-006 (Adney and Baker, 1996), the cellobiase activity was 344 CBU/g determined using the method of Ghose (1987), and the protein content was 90 mg/g cellulase enzyme determined by Bradford method using bovine serum albumin (BSA) as protein standard (Bradford, 1976).

2.3. Corn stover hydrolysate and the biorefinery processing

Corn stover was harvested in fall 2014 from Nanyang, Henan, China. The corn stover was coarsely chopped, water washed to remove field dirt, stones and metals, and air dried before milling to pass through the mesh with 10 mm in diameter. The composition of corn stover after pre-handling treatment was determined using cellulose analyzer (Cellulose Analyzer 220, Ankom Technology, Macedon, NY, USA) to be 35.8% of cellulose, 19.4% of hemicellulose, 28.4% of lignin, and 3.6% of ash on dry weight base (w/w).

Corn stover was pretreated using the dry acid pretreatment according to Zhang et al. (2011), He et al. (2016) and Liu et al. (2018). Briefly, g of corn stover feedstock (dry base) and 600 g of 5% (w/w) dilute sulfuric acid solution was co-currently fed into a 20 L pretreatment reactor under the mild helical agitation and remained at 175 °C for 5 min. The solids loading of the pretreated corn stover was around 50% (w/w) and no free liquid fraction was generated. The pretreated corn stover was neutralized to pH 5.0 using 5 M NaOH, then biodetoxified by *A. resinae* ZN1 at 28 °C for two days to remove furfural and HMF, but with acetic acid residue (He et al., 2016).

Corn stover hydrolysate was prepared by hydrolyzing the biodetoxified or non-detoxified corn stover feedstock in a 5 L bioreactor with a cellulase dosage of 15 FPU per gram of dry solid matter (DM) at 50 °C, pH 4.8 and mild agitation for 48 h. The hydrolysate slurries were centrifuged at ×g for 10 min, autoclaved at 115 °C for 20 min, and then filtered to yield the hydrolysates. Four hydrolysates were prepared by hydrolyzing 15% (w/w) of non-detoxified corn stover solids to yield the multiple inhibitors containing hydrolysate, and by hydrolyzing 15%, 20% and 25% (w/w) of biodetoxified corn stover solids to yield three hydrolysates containing different levels of Na^+. The non-detoxified hydrolysate contained 54.3/L of glucose, 24.4/L of xylose, 3.6/L of acetic acid, 0.35/L of furfural, 0.23/L of 5-hydroxymethylfurfural (HMF). The detoxified hydrolysates contained: (1) 15% (w/w) solids, 56.6/L of glucose, 24.9/L of xylose, 3.1/L of acetic acid, 0.06/L of furfural, 0.03/L of HMF; (2) 20% (w/w) solids, 69.4/L of glucose, 31.8/L of xylose, 4.2/L of acetic acid, 0.10/L of furfural, 0.07/L of HMF; (3) 25% (w/w) solids, 80.1/L of glucose, 38.0/L of xylose, 5.9/L of acetic acid, 0.13/L of furfural, 0.10/L of HMF. The Na^+ content in the 15% (for both detoxified and non-detoxified hydrolysates), 20% and 25% (w/w) solids loading hydrolysates was 90, 120 and 150 mM respectively, while the K^+ content was less than 15 mM (Han and Bao, 2018).

The simultaneous saccharification and fermentation (SSF) was conducted in a 5 L bioreactor equipped with helical ribbon impeller (Zhang et al., 2010a). The feedstock used was 25% (w/w) solids loading of dry acid pretreated and biodetoxified corn stover. After prehydrolysis at 50 °C, 150 rpm and pH 4.8 for 12 h using cellulase Youtell #6 at the cellulase dosage of 15 FPU/g DM, seed cultures were inoculated at 10% (v/v) inoculum size. The SSF was lasted for 84 h at 30 °C, 100 rpm and pH 5.0.

2.4. Construction of Z. mobilis recombinants and fermentation evaluation

The genomic DNA of *Z. mobilis* ZM4 was extracted using TIANamp Bacterial DNA Kit (Tiangen Biotech, Beijing, China). The primers used for amplification of *ZMO0119*, *ZMO0204*, *ZMO0578*, *ZMO0846*, and *ZMO1209* genes and the plasmids harboring the genes are shown in Table 1. The plasmid transformation followed the method of Dong et al. (2011).

A single colony of the recombinant strain was inoculated into 5 mL rich medium (RM), pre-cultured at 30 °C for 20 h, and then the whole culture broth was transferred to 50 mL of the fresh RM and incubated overnight as the seed culture. Ethanol fermentation evaluations in synthetic medium and non-detoxified corn stover hydrolysate were conducted in 250 mL Erlenmeyer flasks containing 50 mL of the medium at 30 °C, pH 5.0 without shaking. Ethanol fermentation evaluations in the three detoxified hydrolysates were conducted in a 3 L fermentor at 30 °C, pH 5.0, and mild agitation. Simultaneous saccharification and fermentation (SSF) evaluation using 25% (w/w) solids loading of pretreated and biodetoxified corn stover was conducted in a

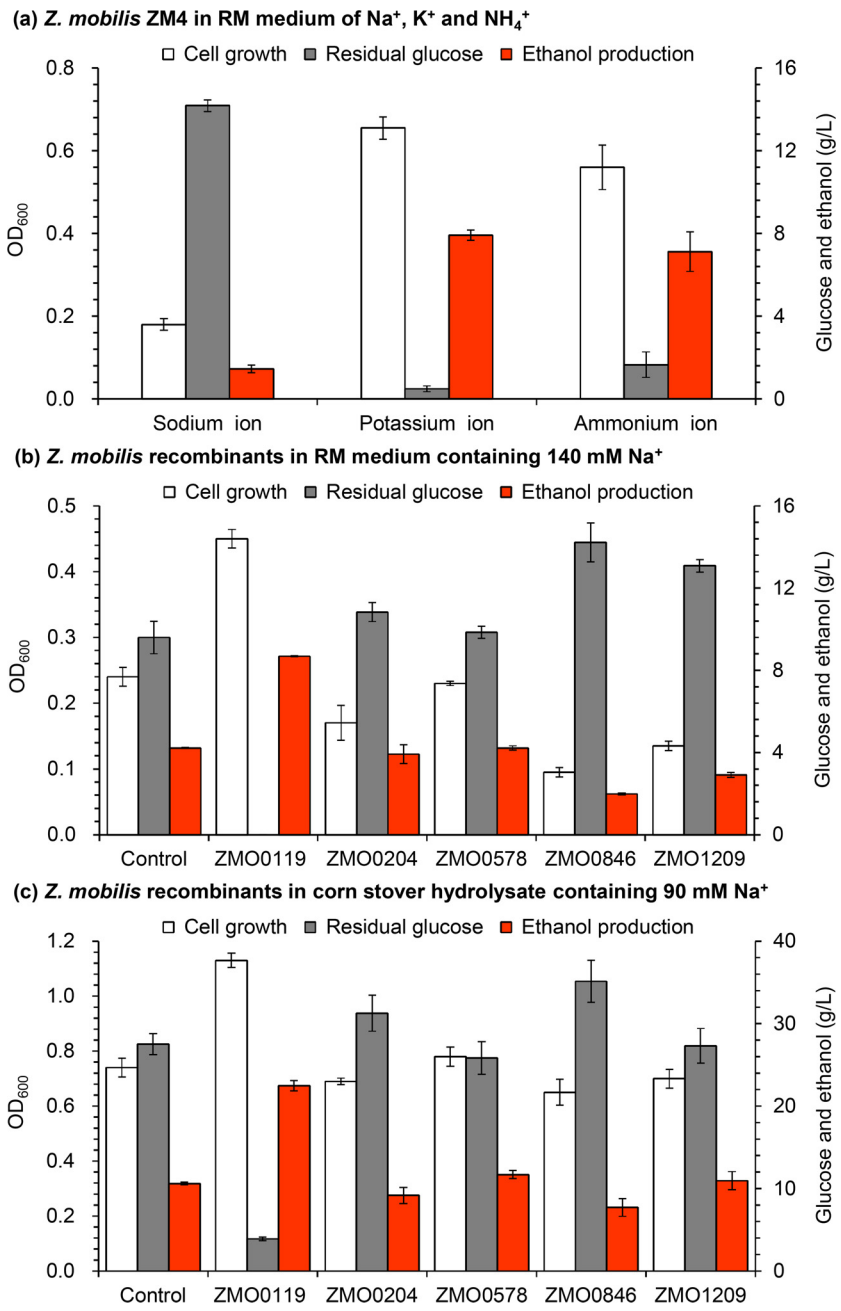

Fig. 1. Inhibition of sodium, potassium and ammonium ions on cell growth and ethanol fermentation of *Z. mobilis* strains. (a) *Z. mobilis* ZM4 in RM medium containing Na^+, K^+, and NH_4^+; (b) *Z. mobilis* recombinants in RM medium containing 140 mM Na^+; and (c) *Z. mobilis* recombinants in corn stover hydrolysate containing 90 mM Na^+. 5 M NaOH, KOH or NH_4OH was used to titrate the RM medium containing 10 g/L of acetic acid to pH 5.0 to generate fermentation broth containing 140 mM of Na^+, 160 mM of K^+, or 140 mM of NH_4^+, respectively. 5 M NaOH was used to neutralize the hydrolyate to pH 5.0 to prepare hydrolyate containing 90 mM of Na^+. The cell growth, glucose consumption and ethanol production were measured at 12 h for inhibition tests, and 36 h for fermentation performance evaluation tests. Conditions: 10% (v/v) inoculum size, 30 °C, in static state culture. Mean values are presented with error bars representing two standard deviations.

5 L bioreactor equipped with helical ribbon impeller (Zhang et al., 2010a).

2.5. Intracellular Na^+ and K^+ content analysis

Intracellular Na^+ and K^+ of *Z. mobilis* cells were extracted following Kolacna et al. (2005). Briefly, the cells were collected after growth for 24 h in RM medium containing 140 mM Na^+, then rapidly washed with 20 mM $MgCl_2$ solution and acid extracted with 0.1 M HCl solution for 24 h. The Na^+ and K^+ contents were analyzed on ICP-AES Agilent 725ES equipped with a simultaneous CCD detector at 1.2 kW of power, 1.5 L/min of plasma gas flow, 1.5 L/min of auxiliary gas flow, 0.75 L/min of nebuliser flow, 15 rpm of pump speed, 35 s of sample delay time, and 10 s of stabilization.

2.6. Sugar, ethanol and inhibitor analysis

Glucose, xylose, ethanol, acetic acid, furfural and HMF were analyzed on HPLC (LC-, Shimazu, Kyoto, Japan) equipped with a refractive index detector RID-10 A (Shimadzu, Kyoto, Japan) and Bio-rad Aminex HPX-87H column (Bio-rad, Hercules, CA, USA). The column temperature was controlled at 65 °C and 5 mM H_2SO_4 solution was used as the mobile phase with a flow rate of 0.6 mL/min.

3. Results and Discussion

3.1. Evaluation of ion transport genes on fermentation improvement for Z. mobilis ZM4

The inhibition of Na^+, K^+ and NH_4^+ on *Z. mobilis* ZM4 was examined in the RM medium by titrating 10 g/L of acetic acid to pH 5.0 using 5 M NaOH, KOH, or NH_4OH, respectively, to generate 140 mM of

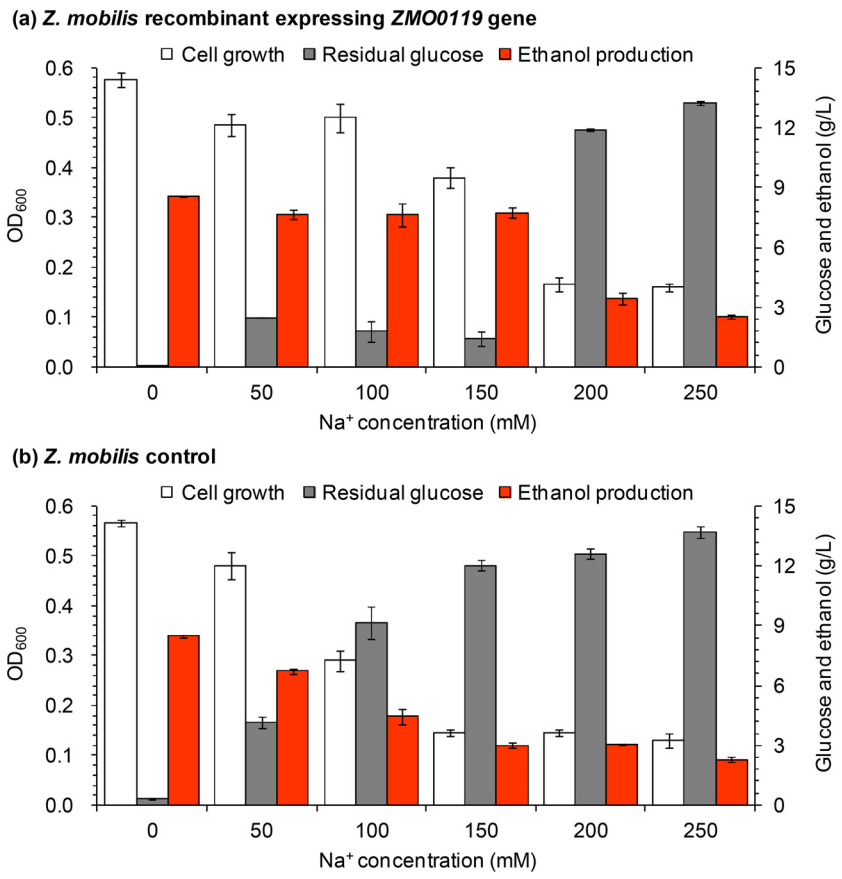

Fig. 2. Cell growth and ethanol fermentation of *Z. mobilis* strains in RM medium containing different Na^+ contents ranging from 0 to 250 mM. (a) *Z. mobilis* recombinant overexpressing *ZMO0119* gene; (b) *Z. mobilis* control. The cell growth, glucose consumption and ethanol production were measured at 12 h for 0 and 50 mM Na^+; 24 h for 100 mM Na^+; 36 h for 150 mM Na^+; and 48 h for 200 and 250 mM Na^+. Conditions: 10% (v/v) inoculum size, 30 °C, in static state culture. Mean values are presented with error bars representing two standard deviations.

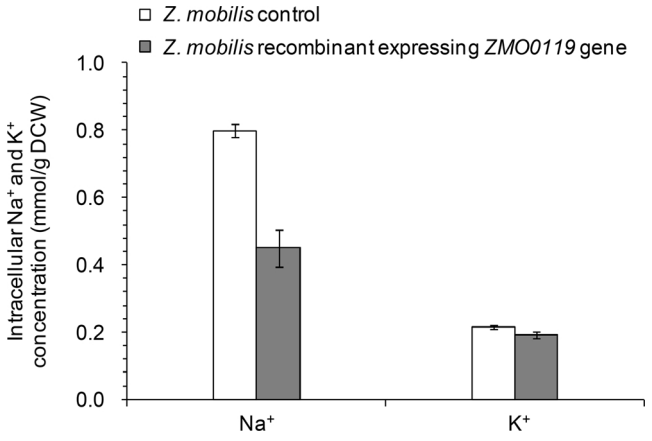

Fig. 3. Intracellular Na^+ and K^+ contents of *Z. mobilis* recombinants overexpressing Na^+/H^+ antiporter gene *ZMO0119*. *Z. mobilis* cells were cultured in RM medium containing 140 mM Na^+ by addition of NaOH to neutralize the RM medium containing 10 g/L of acetic acid to pH 5.0. After growth for 24 h, cells were collected and the ionic content was acid extracted for determination. The Na^+ and K^+ concentration was calculated on the dry cell weight (DCW) basis. Mean values are presented with error bars representing two standard deviations.

Na^+, 160 mM of K^+, or 140 mM of NH_4^+. The results show that cell growth, glucose consumption and ethanol production by *Z. mobilis* were significantly inhibited by Na^+, but the existence of K^+ and NH_4^+ did not give the strong inhibition on *Z. mobilis* (Fig. 1a). The different toxicity between Na^+, K^+ and NH_4^+ is probably due to the relative higher synergistic inhibition of Na^+ with acetic acid than the other two ions of K^+ and NH_4^+ on *Z. mobilis* ZM4, which has been proved to be highly sensitive to sodium acetate compared to potassium acetate and ammonium acetate (Franden et al., 2013; Yang et al., 2010). To improve the tolerance of Na^+ of *Z. mobilis*, five genes responsible for the transmembrane uptake and/or efflux of metal ions were screened from the genome of *Z. mobilis* ZM4 (GenBank: AE008692.2) as candidates, including ZMO0119 encoding Na^+/H^+ antiporter, ZMO0846 encoding sodium/hydrogen exchanger, ZMO0578 encoding sodium/dicarboxylate symporter, ZMO1209 encoding potassium transporter, and ZMO0204 encoding cation efflux protein (Table 1). These genes were inserted into the expression plasmid vector pHW20a and transformed into *Z. mobilis* ZM4 cells to generate five recombinants. The fermentation performances of the obtained recombinants were evaluated in RM medium containing 140 mM Na^+ (Fig. 1b). The results show that the overexpression of ZMO0119 gene in *Z. mobilis* ZM4 significantly improved the cell growth, glucose consumption and ethanol production, while the overexpression of the other four genes ZMO0204, ZMO0578, ZMO0846 and ZMO1209 did not show any observable improvements (Fig. 1b).

The cell growth and fermentation performances of the obtained recombinants were further examined in a corn stover hydrolysate containing 90 mM Na^+ (Fig. 1c). Similarly, the cell growth, glucose consumption and ethanol production in the hydrolysate were obviously improved by the overexpression of *ZMO0119* gene, but no observable improvement was obtained for the other four genes. These results revealed the unique function of *ZMO0119* gene on relieving the Na^+ stress on *Z. mobilis* cells, which is consistent with the previous reports that Na^+/H^+ antiporter (homogeneous) genes in various yeasts and bacteria strains contribute to Na^+ tolerance (Hahnenberger et al., 1996; Jia et al., 1992; Prior et al., 1996; Yang et al., 2010).

The ethanol production capacity of the *Z. mobilis* recombinant overexpressing *ZMO0119* gene was further examined in RM medium containing different Na^+ concentrations ranging from 0 to 250 mM (Fig. 2). The obvious improvement was observed when the concentration of Na^+ reached to a high level of 150 mM (Fig. 2). The result

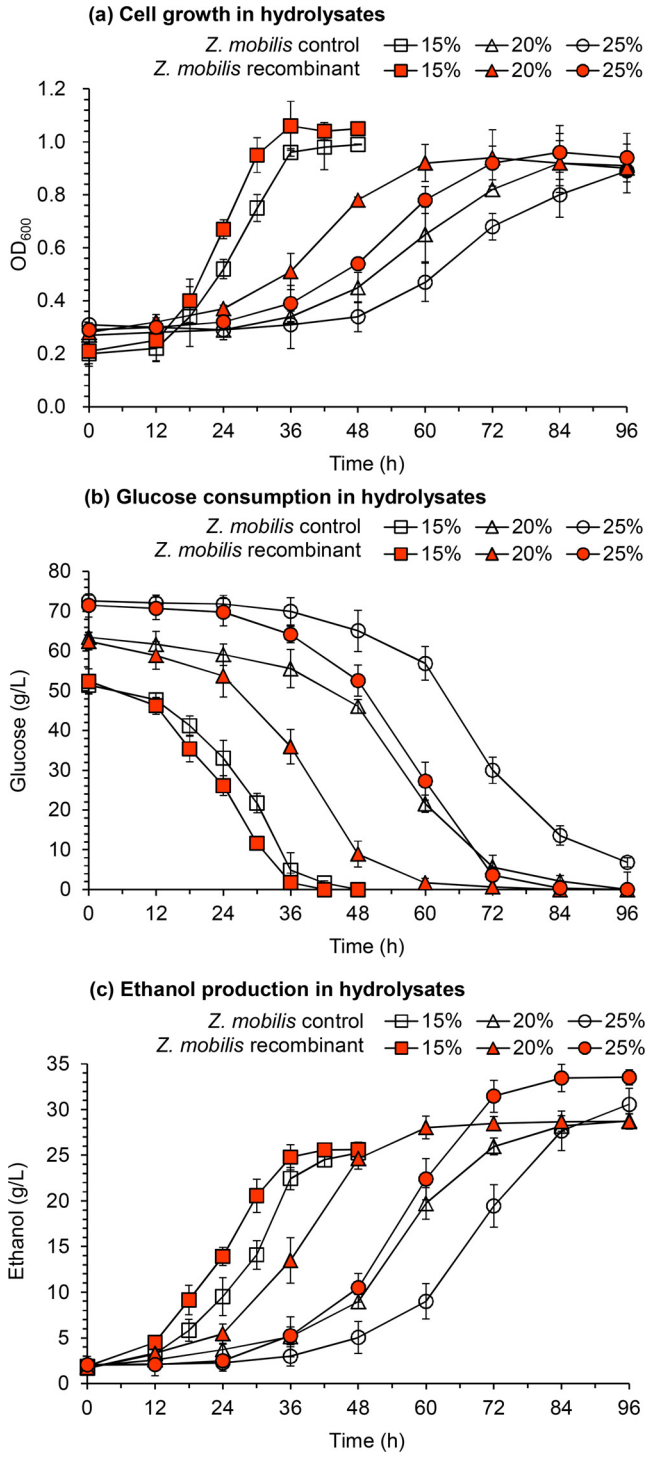

Fig. 4. Ethanol fermentation in corn stover hydrolysates by *Z. mobilis* recombinant overexpressing *ZMO0119* gene. (a) Cell growth; (b) Glucose consumption; (c) Ethanol production. The hydrolysates used were prepared at 15%, 20% and 25% (w/w) of corn stover solids containing 90, 120, and 150 mM of Na^+, respectively. Conditions: 10% (v/v) inoculum size, 30 °C, 100 rpm, pH 5.0 adjusted by 5 M NaOH. Mean values are presented with error bars representing two standard deviations.

indicates that function of *ZMO0119* is greatly related to the induction of higher Na^+ concentration. The intracellular Na^+ content of the *Z. mobilis* recombinant harboring *ZMO0119* gene cultured in the RM medium containing 140 mM Na^+ was measured (Fig. 3). Compared to

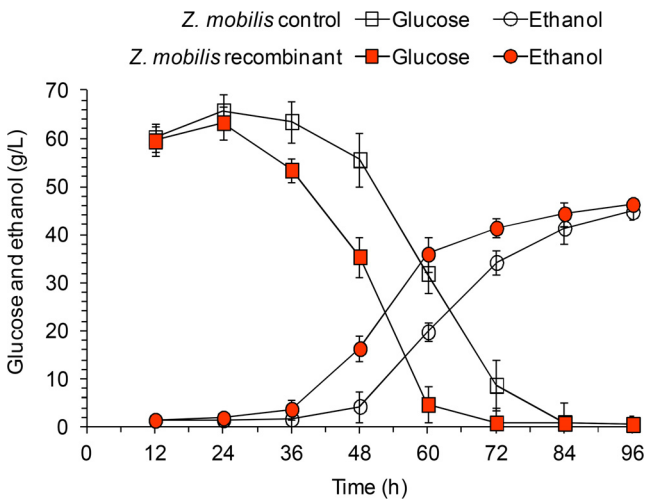

Fig. 5. Simultaneous saccharification and fermentation (SSF) of cellulosic ethanol by *Z. mobilis* recombinant overexpressing *ZMO0119* gene. The feedstock used was 25% (w/w) solids loading of dry acid pretreated and biodetoxified corn stover. Corn stover feedstock was pre-hydrolyzed for 12 h at the cellulase dosage of 15 FPU/g DM, 50 °C, 150 rpm and pH 4.8, then the seed broth was inoculated at 10% of inoculation ratio to start the SSF at 30 °C, 100 rpm, and pH 5.0 adjusted by 5 M NaOH. Mean values are presented with error bars representing two standard deviations.

the control strain, the overexpression of *ZMO0119* gene reduced the intracellular Na^+ content by 44%. On the other hand, no obvious change of the intracellular K^+ content was found for overexpressing *ZMO0119* gene (Fig. 3). The result suggests that the overexpression of *ZMO0119* specifically expelled the intracellular Na^+ out of cells, thus the intracellular Na^+ content was reduced and the tolerance to Na^+ was improved. The function of the Na^+/H^+ antiporter was identified in yeasts and bacteria by importing Na^+ into the cytoplasm while exporting H^+ in *Methanosarcina barkeri* (Thauer et al., 2008), or reversely exporting Na^+/Li^+ or Na^+/K^+ from the cytoplasm while importing H^+ in *E. coli* and *Saccharomyces cerevisiae* (Arkin et al., 2007; Banuelos et al., 1998). Based on the finding of the similar structure of *Z. mobilis* to *E. coli* antiporter (Yang et al., 2010), this study found that the function of Na^+/H^+ antiporter in *Z. mobilis* was also similar to that in *E. coli*. The expulsion of Na^+ by Na^+/H^+ antiporter alleviates the inhibition of Na^+ on the cell metabolism.

3.2. Improved cellulosic ethanol production at high Na^+ content

Ethanol fermentation using the pretreated corn stover hydrolysate containing high Na^+ content was conducted for verification of the function of *ZMO0119* expression on relieving Na^+ stress (Fig. 4). Three corn stover hydrolysates were prepared by hydrolyzing 15%, 20% and 25% (w/w) of the pretreated and biodetoxified corn stover. The obtained hydrolysates contained of 90, 120 and 150 mM of Na^+ content, respectively. The *Z. mobilis* recombinant harboring *ZMO0119* gene showed obviously accelerated cell growth, glucose consumption and ethanol production (Fig. 4). For ethanol fermentation of both 20% and 25% (w/w) solids content hydrolysates, the time required for achieving maximum cell growth and ethanol production was shortened by about 24 h by overexpressing the *ZMO0119* gene (Fig. 4).

The high Na^+ tolerance of the *Z. mobilis* recombinant was further tested in high corn stover feedstock loading (25%, w/w) simultaneous saccharification and fermentation (SSF) (Fig. 5). Again the *Z. mobilis* recombinant overexpressing *ZMO0119* gene showed significantly accelerated glucose consumption and ethanol production. The required fermentation period was shortened by approximate 12 h for achieving the similar ethanol titer by overexpressing this gene (Fig. 5). All these

results clearly showed the important role of *ZMO0119* gene on fermentation performance improvement for *Z. mobilis* ZM4 during ethanol production from the pretreated lignocellulose feedstock.

4. Conclusions

The endogenous *ZMO0119* gene encoding Na^+/H^+ antiporter was identified to be highly responsible for improving Na^+ tolerance of *Z. mobilis*. Overexpression of this gene in *Z. mobilis* increased the Na^+ transport capacity and reduced the intracellular Na^+ concentration. The obtained *Z. mobilis* recombinant harboring *ZMO0119* gene showed significantly improved cell growth and ethanol fermentation performance from the pretreated and biodetoxified corn stover feedstock.

Contributors

XCG and JB designed the experiment; JB and QQG conceived the study; XCG conducted the experiment; QQG analyzed the methods; XCG, QQG and JB wrote the manuscript. All authors read and approved the manuscript.

Ethical and to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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