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Increasing sodium lactate production by enhancement of Na^+ transmembrane transportation in *Pediococcus acidilactici*

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HIGHLIGHTS

- Na^+/H^+ antiporter overexpression led to the high Na^+ tolerance of *P. acidilactici*.
- Na^+/H^+ antiporter overexpression upregulated the sugar phosphorylation of *P. acidilactici*.
- A record high of 132.4 g/L of sodium lactate was produced from wheat straw by SSCF.

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Pediococcus acidilactici
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ABSTRACT

Fermentative production of sodium lactate generally is a low efficient process because of the high Na^+ osmatic stress on lactic acid bacterium cells. In this study, the homogeneous genes encoding Na^+/H^+ antiporters were screened and overexpressed in *Pediococcus acidilactici* for the enhancement of Na^+ transmembrane transportation. The function of the gene *RS02775* was identified and its overexpressing in *P. acidilactici* resulted in the significantly improved sodium lactate production. The recombinant not only accelerated the sugar consumption, but also achieved the record high titer of sodium lactate by 121.1 g/L using pure sugars and 132.4 g/L using wheat straw. The transcription analysis shows that the overexpression of Na^+/H^+ antiporter significantly upregulated the transcription of the sugar phosphorylation genes of *P. acidilactici* under high Na^+ stress. This study provides an effective method for high titer production of sodium lactate using both pure sugars and lignocellulose feedstocks.

1. Introduction

Sodium lactate is a commodity biobased product used as additive in food, pharmaceutical and cosmetics industries (Papadopoulos et al., 1991; Wiegers et al., 2019). Currently, the production of sodium lactate is by neutralizing lactic acid with sodium hydroxide during lactic acid fermentation. Certainly, the high titer of sodium lactate is crucially important for reduction of downstream purification cost. However, the higher sodium lactate titer inevitably leads to the higher concentration of aqueous sodium ion (Na^+) in fermentation broth and consequently leads to the higher inhibition on cell viability and fermentation efficiency (Maiorella et al., 1984; Vriesekoop et al., 2002; Wu et al., 2013). As the result of the Na^+ inhibition, the production of sodium lactate is in low fermentation efficency and the sodium lactate titer is unable to reach an economically favorable level.

To relieve the osmatic stress of high Na^+ level on fermenting microbes, enhancement of transportation of intracellular Na^+ into extracellular space mediated by Na^+/H^+ antiporter has been frequently used (Gao et al., 2018; Mager et al., 2011). In ethanologenic *Z. mobilis*, Yang et al. (2010) overexpressed the Na^+/H^+ antiporter gene *nhaA* (*ZMO0119*) and the sodium ion tolerance was effectively relieved; Liu et al. (2017) improved the sodium acetate tolerance of *Z. mobilis* by deleting a fragment of the promoter region of *ZMO0119*; Gao et al. (2018) overexpressed *ZMO0119* and the ethanol fermentability of *Z. mobilis* was improved in lignocellulose hydrolysate containing considerable Na^+. Na^+/H^+ antiporter genes in some plants were also used to improve salt tolerance by sodium ion efflux. Yang et al. (2009) improved the sodium ion tolerance of Transgenic *Arabidopsis* effectively by overexpressing *SOS1* or *SOS3* gene; Ma et al. (2014) found transgenic plants displayed superior growth and accumulated less Na^+ in roots in

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Table 1
Strains and plasmids.

Strains	Description	Sources
E. coli XLI-blue	Host for plasmid construction	Stratagene
A. resinae ZN1	Biodetoxification fungus	Zhang et al. (2010)
P. acidilactici DSM 20284	Wild strain	DSMZ (Scheiwg, Germany)
P. acidilactici ZY271	Engineered for L-lactic acid fermentation	Qiu et al. (2018)
P. acidilactici ZY271 (pZY36e)	*P. acidilactici* ZY271 harboring the empty plasmid pZY36e	This work
P. acidilactici ZY271 (*RS02775*)	*P. acidilactici* ZY271 harboring the plasmid pZY36e-*RS02775*	This work
P. acidilactici ZY271 (*RS02845*)	*P. acidilactici* ZY271 harboring the plasmid pZY36e-*RS02845*	This work
P. acidilactici ZY271 (*RS06435*)	*P. acidilactici* ZY271 harboring the plasmid pZY36e-*RS06435*	This work
P. acidilactici ZY271 (*RS08600*)	*P. acidilactici* ZY271 harboring the plasmid pZY36e-*RS08600*	This work
Plasmids	**Description**	**Sources**
pZY36e	Expression plasmid with *PldhD* replacing *P32* promoter of pMG36e	Qiu et al. (2020)
pZY36e-*RS02775*	*RS02775* was inserted into pZY36e at Xba I and Sal I sites	This work
pZY36e-*RS02845*	*RS02845* was inserted into pZY36e at Xba I and Sal I sites	This work
pZY36e-*RS06435*	*RS06435* was inserted into pZY36e at Xba I and Pst I sites	This work
pZY36e-*RS08600*	*RS08600* was inserted into pZY36e at Xba I and Sal I sites	This work

the high Na^+ pressure after co-expression of *Arabidopsis SOS* genes. Xu et al. (2008) proved that the expression of *TaSOS1* gene from wheat improved the salt tolerance of a yeast strain lacking the major Na^+-transporters by decreasing the cellular Na^+. These results suggest that the Na^+/H^+ antiporter effectively alleviates the Na^+ stress by reducing intracellular sodium ions. However, sodium ion stress on ethanol fermenting strains is still relatively mild because the addition of sodium ion is mainly for pH regulation in upstream processing (wet milling for corn oil extraction) or fermentation. Sodium lactate fermentation certainly leads to the much higher Na^+ concentration because Na^+ is one portion of the fermentation product (20% of sodium lactate by weight percentage). Therefore, the sodium lactate producing strain, lactic acid bacterium (LAB), certainly suffers a stronger Na^+ stress during sodium lactate fermentation. Till now, no report was found on applying Na^+/H^+ antiporter to lessen Na^+ stress in lactic acid bacterium and improve its sodium lactate fermentability.

In this study, we tested the concept of overexpressing Na^+/H^+ antiporter genes in the engineered lactic acid bacterium strain *Pediococcus acidilactici* ZY271 for chiral L-lactic acid production on increasing the sodium lactate fermentation performance. Since Na^+/H^+ antiporter is a membrane protein guided by a specific signal peptide for localization, heterogeneous Na^+/H^+ antiporter gene from *Z. mobilis* has rare chance to be correctly expressed in lactic acid bacterium. Therefore, we selected the homogenous Na^+/H^+ antiporter genes from *P. acidilactici* for enhancement of Na^+ transmembrane transportation. The selected gene *RS02775* was overexpressed in *P. acidilactici* ZY271 and the results showed that the Na^+ tolerance of *P. acidilactici* ZY271 was significantly improved. Correspondingly, the sugar consumption was accelerated and sodium lactate production was upgraded from both pure sugar and wheat straw feedstocks. This study provides an effective method for high fermentation performance of sodium lactate using different sugar feedstocks.

2. Materials and methods

2.1. Strains, media and culture conditions

The strains used are listed in Table 1. The L-lactic acid producing strain *Pediococcus acidilactici* ZY271 with xylose-assimilating ability was constructed in Qiu et al. (2018). The biodetoxification fungus *Amorphotheca resinae* ZN1 (CGMCC 7452) was isolated by Zhang et al. (2010). *Pediococcus acidilactici* DSM 20284 purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH was used for amplification of the Na^+/H^+ antiporter genes *RS02775*, *RS02845*, *RS06435* and *RS08600*.

P. acidilactici was grown at 42 °C in the simplified MRS medium described in Qiu et al. (2018). *A. resinae* ZN1 was cultured at 28 °C on a potato dextrose agar (PDA) slant containing 20 g/L glucose and 200 g/L potato extract juice. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. Erythromycin was added into the medium to screen positive *E. coli* (400 μg/mL) and *P. acidilactici* (5 μg/mL) recombinants.

2.2. Enzymes and reagents

Restriction endonucleases, DNA polymerase, T4 DNA ligase and RNAiso Plus were purchased from Takara (Otsu, Japan). Commercial cellulase Cellic CTec 2.0 was obtained from Novozymes China (Beijing, China) and its filter paper activity (203.2 FPU/mL), cellobiase activity (4,900 CBU/mL) and protein concentration (87.3 mg/mL) were measured in previous work (Qiu et al., 2018).

Genomic DNA Isolation Kit and Plasmid Isolation Kit were from Generay Biotech (Shanghai, China). Peptone and yeast extract were from Oxoid (Hampshire, UK). Glucose, xylose and other chemicals were from Sangon Biotech (Shanghai, China).

2.3. Construction of plasmids and recombinant strains

Primer sequences are listed in Supplementary Materials. The genomic DNA of *P. acidilactici* DSM 20284 was extracted using Genomic DNA Isolation Kit. The genes *RS02775*, *RS06435*, *RS02845 and RS08600* were PCR-amplified from the genome of *P.acidilactici* DSM 20284. The expression plasmid pZY36e was constructed by replacing the original promoter P32 in pMG36e (van de Guchte et al., 1989) with *PldhD* at *EcoR* I and *Xba* I to obtain the plasmid pZY36e (Qiu et al., 2020). The *RS06435* gene was then inserted into the expression plasmid pZY36e at *Xba* I and *Pst* I sites, other genes were inserted at *Xba* I and *Sal* I sites to obtain the recombinant plasmids pZY36e-*RS06435*, pZY36e-*RS02775*, pZY36e-*RS02845* and pZY36e-*RS08600*, respectively. These plasmids and empty plasmid pZY36e were introduced into *P. acidilactici* ZY271 by electroporation to generate the recombinant strains for fermentation.

2.4. Wheat straw feedstock and biorefinery operations

Wheat straw was collected from Nanyang City, Henan Province, China and was dry acid pretreated (Zhang et al., 2011; He et al., 2014). The pretreated feedstock was then biodetoxified for 48 h by inoculating *A. resinae* ZN1 spores to remove the inhibitors (He et al., 2016; Zhang et al., 2010). Furfural, HMF, acetic acid, vanillin and syringaldehyde were removed completely after biodetoxification. The contents of cellulose and hemicellulose in the pretreated and biodetoxified wheat

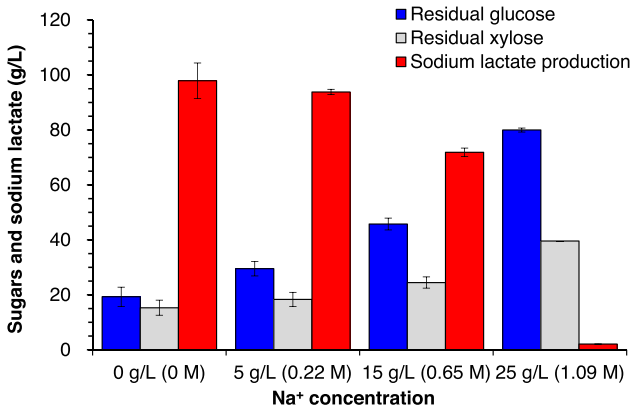


Fig. 1. Na^+ tolerance of *P. acidilactici* ZY271 under different sodium lactate concentrations. The simplified MRS medium containing 100 g/L glucose and 50 g/L xylose was added with NaCl to generate Na^+ gradients. Conditions: 42 °C, 150 rpm, pH adjusted to 5.5 in a one-liter fermentor under mild agitation.

straw were 36.2% (w/w) and 1.7% (w/w), respectively, using the two-step acid hydrolysis described in the protocols of NREL (Sluiter et al., 2008a, 2008b).

2.5. Sodium lactate fermentation

Sodium lactate fermentation using pure glucose (100 g/L) and xylose (50 g/L) sugars was carried out in a 3L bioreactor containing 600 mL simplified MRS medium. The fermentation was maintained for 72 h at 42 °C, 150 rpm. pH was automatically controlled at 5.5 by adding 13.5 M NaOH solution. 5 μg/mL erythromycin was added into the fermentation broth.

Simultaneous saccharification and sodium L-lactate co-fermentation (SSCF) from wheat straw feedstock was carried out in a 5 L bioreactor fitted with a helical ribbon agitator (Qiu et al., 2017). The pretreated and biodetoxified wheat straw at 25% (w/w) of solids loading was pre-hydrolyzed for 6 h by adding 4 mg cellulase protein per gram of dry wheat straw at 50 °C and mild stirring. *P. acidilactici* seeds were then added into the bioreactor at 10% (v/v) to initiate the SSCF at the same fermentation conditions.

2.6. HPLC analysis

Culture broth samples were periodically collected from the fermentation broth, then centrifuged at 13,000 × g for 5 min and filtered using 0.22 μm membrane filter. The concentration of glucose, xylose, and sodium L-lactate in the supernatant were quantified by HPLC described in Qiu et al. (2018). Arabinose, mannose and galactose in the supernatant were analyzed by HPLC according to the method described in Jin et al. (2019).

2.7. qRT-PCR assays

Cell mass of *P. acidilactici* was collected after culture in simplified MRS medium containing 100 g/L glucose and 50 g/L xylose for 12 h and then centrifuged at 12,000 rpm for 5 min at 4 °C for RNA extraction and qRT-PCR analysis. The reagents and instruments used for total RNA extraction, reverse transcription reactions and qRT-PCR reactions were referred to Jin et al. (2019). *P. acidilactici* ZY271 (pZY36e) was used as control strain. 16S rRNA gene was used as an endogenous control for data normalization. Primers used were listed in Supplementary materials.

3. Results and discussion

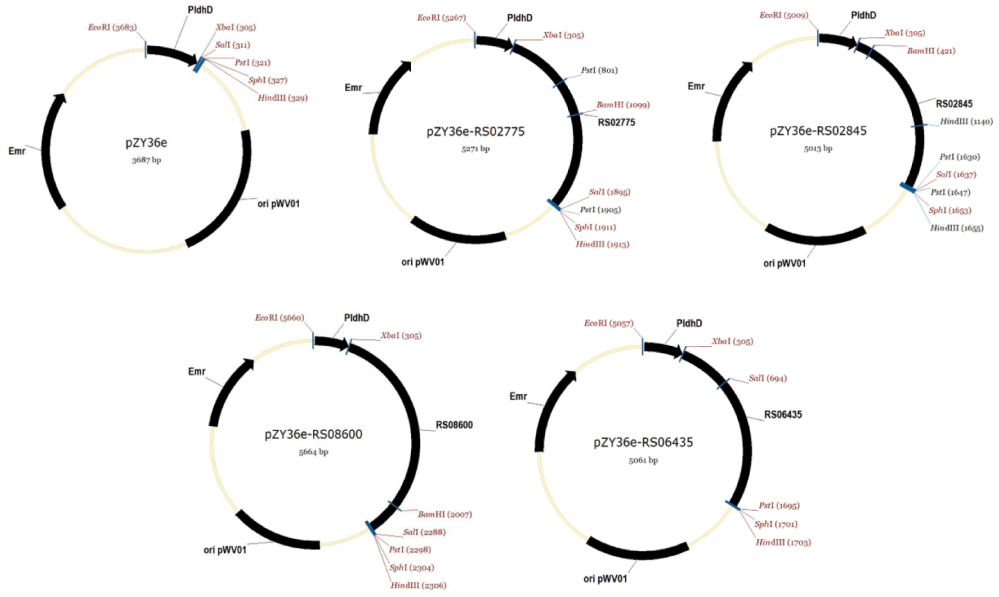
3.1. Evaluation of sodium ion tolerance of P. Acidilactici in L-lactic acid fermentation

The sodium ion accumulation and inhibition on the L-lactic acid bacterium *P. acidilactici* ZY271 was examined by adding extra sodium chloride to the culture medium. The Na^+ gradients were arranged to 0, 5, 15, and 25 g/L, corresponding to 0, 0.22, 0.65, 1.09 M of sodium ions (Fig. 1). The glucose and xylose utilization rates deceased with the increasing Na^+ concentration. The L-lactic acid generation also sharply declined and almost ceased when the Na^+ concentration reached 1.09 M. The Na^+ concentration of 1.09 M corresponded to 122 g/L of sodium lactate, a generally regarded as the minimum titer of lactic acid fermentation. The result clearly indicates that the *P. acidilactici* strain was highly sensitive to Na^+ concentration and the increase of sodium ion tolerance of lactic acid bacterium should endure the high Na^+ concentration for direct fermentative production of sodium lactate.

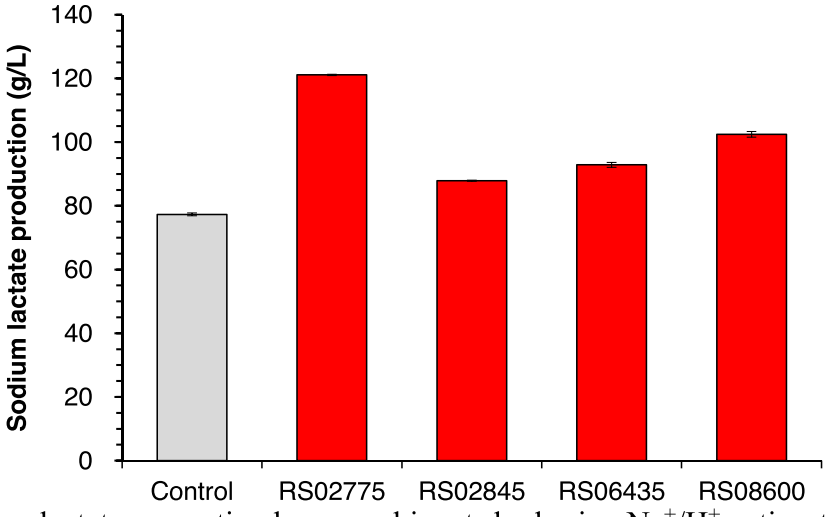
3.2. Constructing the Na^+ tolerant P. Acidilactici recombinant by overexpression of Na^+/H^+ antiporters

To improve the Na^+ tolerance of *P. acidilactici* and sodium lactate production level, we overexpressed four homogeneous genes encoding Na^+/H^+ antiporters responsible for the transmembrane transportation of sodium ion, including *RS02775*, *RS02845*, *RS06435* and *RS08600* according to the gene locus tag in the genome annotation of *P. acidilactic* DSM 20,284 (NCBI access: NZ_GL397067.1). These genes were separately inserted into the expression plasmid vector pZY36e (Fig. 2a) and the plasmids were introduced into *P. acidilactici* ZY271 to generate four recombinants. The sodium lactate fermentation performance of the recombinants was evaluated in the MRS medium containing high titer of Na^+ concentration (Fig. 2b). The results show that the overexpression of *RS02775* behaved the maximum sodium lactate production upgradation among the recombinants with expression of four Na^+/H^+ antiporter genes.

The *RS02775* gene was reasonably selected as the candidate for transmembrane transportation of Na^+. The recombinant *P. acidilactici* with the overexpression of *RS02775* was applied for sodium lactate fermentation using sodium hydroxide as neutralization agent (Fig. 3). When the pure glucose and xylose were used as feedstock (Fig. 3a), 121.1 g/L of sodium lactate was produced, approximately 24% more sodium lactate produced than that by the parental *P. acidilactici* (97.9 g/L). When wheat straw was used as feedstock (Fig. 3b), the simultaneous saccharification and co-fermentation (SSCF) of the dry acid pretreated and biodetoxified wheat straw produced 132.4 g/L of sodium lactate, approximately 31% more sodium lactate produced than that by the parental strain (101.3 g/L). The results confirm that the overexpression of the Na^+/H^+ antiporter *RS02775* gene in *P. acidilactici* ZY271 significantly relieved the sodium ion stress to the host cell, and achieved the higher sodium lactate production for both sugars and lignocellulose



(a) Construction of expression plasmids for Na^+/H^+ antiporter genes



(b) Sodium lactate generation by recombinants harboring Na^+/H^+ antiporter genes

Fig. 2. Evaluation of Na^+/H^+ antiporter genes expression on sodium lactate production in *P. acidilactici.* (a) Construction of expression plasmids for Na^+ antiporter genes. Four pZY36e plasmids harboring the genes *RS06435*, *RS02775*, *RS02845*, and *RS08600* were constructed and introduced into *P. acidilactici* ZY271, respectively. (b) Sodium lactate production by the *P. acidilactici* recombinants overexpressing different Na^+/H^+ antiporter genes. The fermentation was conducted at 42 °C at mild agitation, 10% (v/v) inoculum size in 3L bioreactor. The pH was adjusted to 5.5.

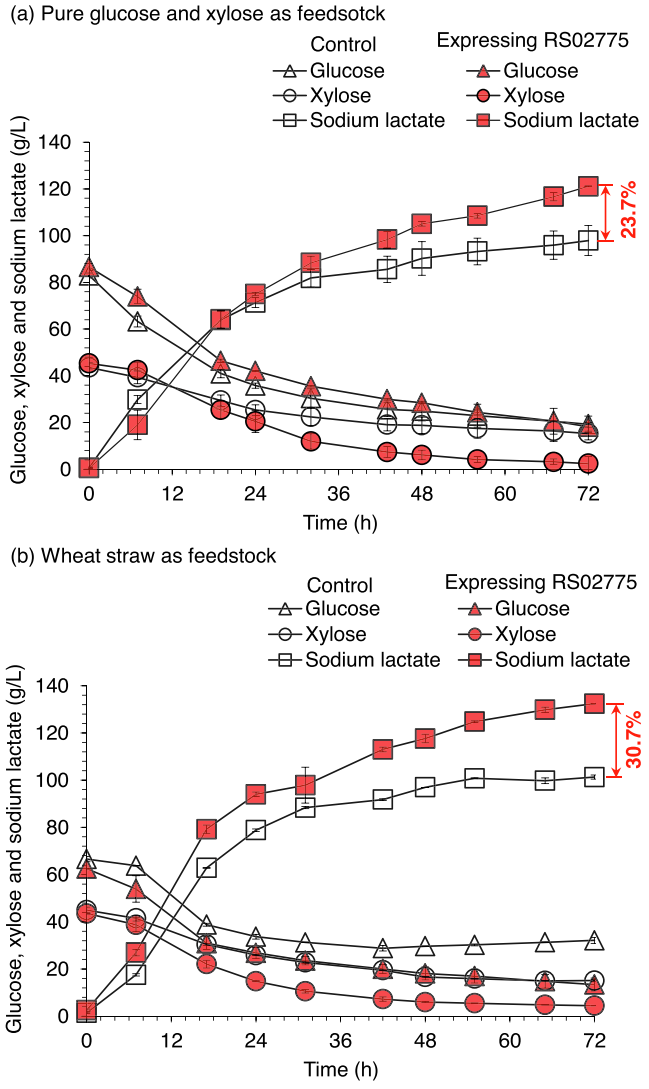


Fig. 3. Sodium lactate production from pure sugars (a) and wheat straw (b) by the parental and engineered *P. acidilactici* strains. Parental, *P. acidilactici* ZY271 harboring the empty plasmid pZY36e; the recombinants, *P. acidilactici* ZY271 (pZY36e-*RS02775*). Conditions: (a) 3L bioreactor with 600 mL simplified MRS medium. (b) Simultaneous saccharification and co-fermentation (SSCF) of the pretreated and biodetoxified wheat straw at 25% (w/w) solids loading in 5L bioreactor, 42 ℃, 150 rpm, pH adjusted to 5.5.

feedstocks.

3.3. Transcriptional analysis of P. Acidilactici recombinant under sodium ion stress

The function of the Na^+/H^+ antiporter *RS02775* gene on the alleviation of Na^+ stress and enhancement of sodium lactate production was investigated by examining its transcriptional changes. Quantitative real-time PCR (qRT-PCR) on the *P. acidilactici* recombinant with the overexpression of *RS02775* was performed with the parental *P. acidilactici* ZY271 as control (Fig. 4). The results show that the transcription of several important genes directly related to sugar metabolism and lactic acid synthesis were significantly upregulated for more than ten-folds than the control. The genes included (i) *manX_0095* (14.45), *manX_1353* (17.45), *manX_1749* (101.69), and *xylB* (19.42) responsible for the phosphorylation of glucose and xylose, (ii) the enolase gene *eno_1046* (20.05) for L-lactic acid synthesis, (iii) and the gene *pdhA* (11.88), *pdhD* (14.95) for pyruvate dehydrogenase and phosphate acetyltransferase gene *pta* (15.42) related to acetate synthesis.

The significant upregulation of the phosphorylation genes *manX_1749*, *manX_0095*, *manX_1353,* and *xylB* suggests that the reduced intracellular Na^+ stress significantly promoted the phosphorylation of the sugars, which was the first metabolic step after the sugars entered the cells. The EIIA/B of mannose-PTS catalyzed reaction is the most important sugar phosphorylase of lactic acid bacteria (Castro et al., 2009; Jamal et al., 2013). Xylulokinase gene (*xylB*) was also upregulated and xylose utilization was accelerated correspondingly, indicating that the suppression of sugar phosphorylation by high Na^+ osmotic pressure was reduced by the overexpression of Na^+/H^+ antiporter and the activity of the phosphorylation enzymes EIIA/B (*manX*) and xylulokinase (*xylB*) were recovered.

The upregulation of enolase gene (*eno*) re-activated the conversion from fructose 6-phosphate to L-lactic acid under high Na^+ stress and increased the synthesis flux of L-lactic acid. Pyruvate dehydrogenase genes *pdhA/D* and phosphate acetyltransferase gene *pta* from pyruvate to acetic acid synthesis were significantly up-regulated. However, the *ackA2* gene transforming acetyl phosphate to acetic acid has been completely knocked out while the activity of the residual acetate kinase encoded by *ackA1* gene is low (Qiu et al., 2018). Therefore, no obvious change in acetic acid generation was observed and the increased acetyl-CoA by the upregulated *pdh* step may enter the incomplete TCA cycle.

4. Conclusions

The endogenous gene *RS02775* responsible for encoding Na^+ /H^+ antiporter was found to play a key role in increasing the sodium ion tolerance of *P. acidilactici*. Overexpression of *RS02775* in *P. acidilactici* increased the intracellular Na^+ transmembrane transport capacity and alleviated the negative effects of sodium ions on sugar utilization and sodium lactate fermentation. The obtained *P. acidilactici* recombinant harboring *RS02775* gene produced 132.4 g/L of sodium lactate in the high solids loading SSCF of wheat straw and caused a great improvement (31%) in sodium lactate production.

CRediT authorship contribution statement

JB conceived and directed the research. NLH and CF conducted the experiment. NLH, ZYQ and JB wrote the 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.

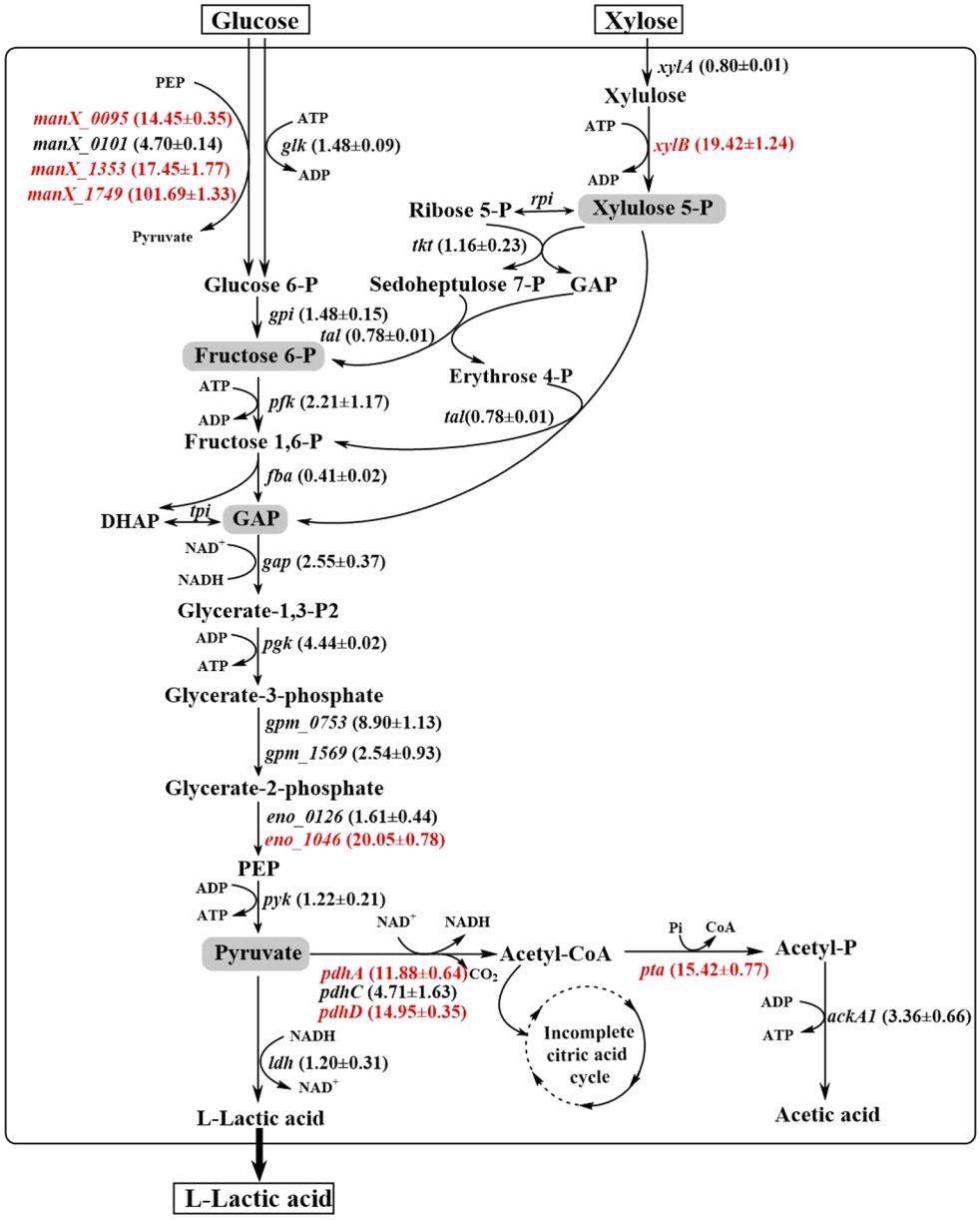


Fig. 4. Transcriptional analysis by qRT-PCR on *P. acidilactici* ZY271(pZY36e-*RS02775*) sugar metabolism genes and acetic acid synthesis genes under high Na^+ stress. The value in parentheses indicates the fold change of the gene expression for the recombinant strain , *P. acidilactici* ZY271(pZY36e-*RS02775*) , compared to the control strain, *P. acidilactici* ZY271 (pZY36e). Transcription levels of selected genes were quantifed using the $2^{-\Delta\Delta CT}$ method. Three biological and technical replicates were set up for the qRT-PCR analysis.

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