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A short-chain dehydrogenase plays a key role in cellulosic D-lactic acid fermentability of *Pediococcus acidilactici*

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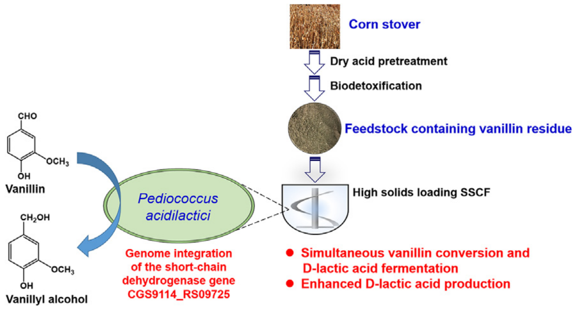
GRAPHICAL ABSTRACT



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ABSTRACT

Phenolic aldehydes from lignocellulose pretreatment are strong inhibitors of cell growth and metabolism of cellulosic lactic acid bacteria. Their low solubility and recalcitrance highly reduce the removal efficiency of various detoxification methods. This study shows a simultaneous conversion of phenolic aldehydes and fermentation of D-lactic acid by *Pediococcus acidilactici* using corn stover feedstock. Vanillin was found to be the strongest phenolic aldehyde inhibitor to *P. acidilactici*. The overexpression of a short-chain dehydrogenase encoded by the gene *CGS9114_RS09725* from *Corynebacterium glutamicum* was identified to play a key role in D-lactic acid fermentability of *P. acidilactici*. The engineered *P. acidilactici* with the genome integration of *CGS9114_RS09725* showed the accelerated vanillin reduction and improved cellulosic D-lactic acid production. This study reveals that vanillin conversion is crucial for D-lactic acid fermentation, and the direct expression of a specific vanillin reduction gene in lactic acid bacterium efficiently improves cellulosic D-lactic acid production.

1. Introduction

Pretreatment is the crucial step of the lignocellulose biorefinery chain (Chandel et al., 2018; Galbe and Zacchi, 2012; Wyman et al., 2005). One of the outcomes of the harsh pretreatment operation is the generation of various small molecular weight compounds such as furan aldehydes (furfural and 5-hydroxymethylfurfural (HMF)), weak acids (acetic acid and formic acid) and phenolic aldehydes (4-hydroxybenaldehyde, vanillin and syringaldehyde) (Jonsson et al., 2013; Klinke et al., 2004; Larsson et al., 1999; Parawira and Tekere, 2011). These compounds severely inhibit cellulase enzyme activity in the enzymatic hydrolysis step and reduce the viability of microbes in the

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fermentation step (Palmqvist and Hahn-Hägerdal, 2000; Zhang et al., 2010b). Therefore, the complete removal of inhibitors is essential for efficient production of biofuels and bio-based products from lignocellulose feedstock.

Phenolic aldehydes are lignin derivatives and the representatives include 4-hydroxybenaldehyde, vanillin and syringaldehyde by functional classification of the *p*-hydroxyphenyl group (H), guaiacyl group (G) and syringyl group (S), respectively. Complete removal of these phenolic aldehyde compounds by conventional detoxification methods such as water-washing and over-liming is difficult because of their low water solubility and hydrophobicity (Gu et al., 2015; Thomsen et al., 2009). Biological detoxification can completely degrade furfural, HMF, acetic acid and phenolic aldehydes but a long period is required ($>$ 72 h), resulting in the consumption of 30%–40% of the total xylose in pretreated lignocellulose biomass and a considerable loss of the final target product yield during the fermentation step (Gao et al., 2018; He et al., 2016; Wang et al., 2015; Yi et al., 2019; Zhang et al., 2010b). Ideally, a short biodetoxification period should be used to minimize xylose consumption and enable the complete degradation of furfural, HMF and acetic acid. However, a short biodetoxification period only partially degrades the phenolic aldehydes and the phenolic aldehyde residues in the lignocellulose feedstock negatively affect the fermentability of the fermentation strains (He et al., 2016; Yi et al., 2016). Thus, the complete removal of phenolic aldehydes leads to a considerable loss of xylose, whereas retaining the desired xylose is at the expense of incomplete removal of the toxic phenolic aldehydes. We propose to combine biodetoxification and fermentation into one simultaneous process to solve this issue. In the first step, the shortened biodetoxification is conducted to completely remove furfural, HMF, acetic acid, and partially remove phenolic aldehydes within a standard period (36–48 h), while xylose in the pretreated lignocellulose biomass is preserved. In the second step, the residual phenolic aldehydes and the sugars (glucose and xylose) in the biodetoxified lignocellulose are simultaneously converted into less toxic or non-toxic derivatives and the target products by microbial fermenting strains.

D-lactic acid is an important monomer chemical of the biodegradable poly-lactic acid (PLA) plastic (Abdel-Rahman et al., 2011; Farah et al., 2016; Nampoothiri et al., 2010). In our previous studies, a D-lactic acid producing strain *P. acidilactici* ZY15 was developed for efficient co-utilization of glucose and xylose from lignocellulose feedstock (corn stover and wheat straw) (Qiu et al., 2017; Yi et al., 2016). In this study, vanillin was identified to be the strongest phenolic aldehyde inhibitor of *P. acidilactici*. A heterologous short-chain dehydrogenase gene was discovered to be a key gene for increasing vanillin reduction conversion and accelerating D-lactic acid fermentability using corn stover feedstock. This study proved that directly expressing a heterologous vanillin reduction gene in the D-lactic acid bacterium strain improved the D-lactic acid fermentability from lignocellulose feedstock.

2. Materials and methods

2.1. Strains, media and growth conditions

The strains used in this study are shown in Supplementary materials. D-lactic acid producing strain *P. acidilactici* ZY15 with efficient xylose-assimilation ability was constructed previously (CGMCC 13612, Chinese General Microorganisms collection center, Beijing, China) (Qiu et al., 2017). *Escherichia coli* XLI-blue for construction of recombinant plasmids was stored in our laboratory. Biodetoxification strain *Amorphotheca resinae* ZN1 (CGMCC 7452) was isolated in previous work (Zhang et al., 2010b). *Zymomonas mobilis* ZM4 (ATCC 31821, American Type Culture Collection, Manassas, VA, USA), *Pseudomonas putida* KT2440 (ATCC 47054) and *Corynebacterium glutamicum* S9114 (SIIM B460, Shanghai Industrial Institute of Microorganism, Shanghai, China) were used for heterogenous oxidoreductase gene amplification.

P. acidilactici strains were grown at 42 °C in the simplified Man-Rogosa-Sharp (MRS) medium containing 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L ammonium citrate dibasic, 0.58 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L K_2HPO_4 and 0.25 g/L $MnSO_4 \cdot H_2O$ (Yi et al., 2016; Qiu et al., 2017). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C and 400 μg/mL of erythromycin was added to the medium for the construction of the recombinant plasmids. *A. resinae* ZN1 was maintained at 28 °C on a potato dextrose agar (PDA) slant (Zhang et al., 2010b).

2.2. Enzymes and reagents

Commercial cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes (Beijing, China). The filter paper activity, cellobiase activity and protein concentration of Cellic CTec 2.0 were 203.2 FPU/mL, 4,900 CBU/mL and 87.3 mg/mL, respectively, determined according to the methods described by Adney and Baker (1996), Ghose (1987) and Bradford (1976). DNA polymerase and the ligation kit were purchased from Takara (Otsu, Japan). The restriction endonucleases were from Thermo Scientific, Wilmington, DE, USA. The genomic DNA and plasmid extraction kits were from Generay Biotech, Shanghai, China. Erythromycin was from Biosharp Biotech, Beijing, China. Yeast extract and peptone were from Oxoid, Hampshire, UK. Phenolic aldehydes and their derivatives were purchased from local suppliers.

2.3. Construction of *P. acidilactici* recombinants

The plasmids and primers used are listed in Supplementary materials. The genomic DNA of *P. acidilactici* ZY15, *Z. mobilis* ZM4, *P. putida* KT2440 and *C. glutamicum* S9114 were extracted separately using the genome extraction kit. Promoter *PldhD* was amplified from the 300 bp upstream of the start codon of the D-lactate dehydrogenase gene *ldhD* of the *P. acidilactici* ZY15 genome. The *ZMO1885* gene was amplified from *Z. mobilis* ZM4. *PP_3151*, *PP_5120* and *PP_5258* were amplified from *P. putida* KT2440. *CGS9114_RS10340* and *CGS9114_RS09725* were amplified from *C. glutamicum* S9114.

The original promoter *P32* in pMG36e (van de Guchte et al., 1989) was replaced with *PldhD* at *EcoR* I and *Xba* I to obtain the plasmid pZY36e for gene expression in *P. acidilactici* ZY15. Genes *ZMO1885*, *PP_5120*, *CGS9114_RS10340* and *CGS9114_RS09725* were then inserted into pZY36e at *Xba* I and *Sal* I to obtain plasmids pZY36e-*ZMO1885,* pZY36e-*PP_5120*, pZY36e-*CGS9114_RS10340* and pZY36e-*CGS9114_RS09725*, respectively. Genes *PP_3151* and *PP_5258* were inserted into pZY36e at *Xba* I and *Pst* I to generate plasmids pZY36e-*PP_3152* and pZY36e-*PP_5258*, respectively. The above seven plasmids were transformed separately into *P. acidilactici* ZY15 to obtain the seven recombinants.

The pSET4E-Δ*ackA2* for deletion of the acetate kinase gene *ackA2* was constructed in Qiu et al. (2018). Expression cassette *PldhD_CGS9114_RS09725* was amplified from plasmid pZY36e-*CGS9114_RS09725*, and inserted into pSET4E-Δ*ackA2* at *Xba* I and *Pst* I to generate the integration plasmid pSET4E-Δ*ackA2*::*CGS9114_RS09725* for substitution of *ackA2* with the above expression cassette. The integration plasmid was transformed into *P. acidilactici* ZY15. The substitution of *ackA2* with *PldhD_CGS9114_RS09725* was conducted using the method described in Qiu et al. (2017) and Yi et al. (2015).

2.4. Dry acid pretreatment and biodetoxification

Corn stover was collected in the spring of 2018 from Nanyang, Henan, China. The cellulose and hemicellulose content in the raw corn stover were 34.4% (w/w) and 23.6% (w/w), respectively, which were determined using a cellulose analyzer (Cellulose Analyzer 220, Ankom Technology, Macedon, NY, USA).

Dry acid pretreatment of corn stover was conducted according to Zhang et al. (2011) and He et al. (2014). The pretreated corn stover contained 32.2 mg glucose and 144.3 mg xylose, as well as 5.8 mg furfural, 3.5 mg HMF, 23.0 mg acetic acid, 0.4 mg 4-hydroxybenzaldehyde, 3.3 mg vanillin and 2.2 mg syringaldehyde per gram of dry corn stover (dry matter, DM).

The pretreated corn stover was biodetoxified using *A. resinae* ZN1 to remove the inhibitors (He et al., 2016; Zhang et al., 2010b). Furfural, HMF and acetic acid were removed completely. The phenolic aldehydes were partially removed and the residual phenolic aldehydes in the pretreated and biodetoxified corn stover included 0.1 mg 4-hydroxybenzaldehyde, 0.6 mg vanillin and 1.5 mg syringaldehyde per gram DM. The cellulose and hemicellulose contents were essentially constant. The monosaccharides included 18.3 mg glucose and 124.3 mg xylose per gram DM.

2.5. Simultaneous saccharification and D-lactic acid co-fermentation (SSCF)

SSCF was carried out in a 5 L helical agitated bioreactor using the pretreated and biodetoxified corn stover at 30% (w/w) solids loading with addition of 10 mg cellulase protein/g cellulose (Qiu et al., 2017; Zhang et al., 2010a). After 6 h prehydrolysis at 50 °C and 150 rpm, SSCF was initiated by adding *P. acidilactici* seeds with 10% (v/v) inoculation at 42 °C, pH 5.5 and 150 rpm. The nutrients including 10 g/L peptone, 10 g/L yeast extract, 2 g/L ammonium citrate dibasic and 0.25 g/L $MgSO_4$ were added to the SSCF operation. The pH was maintained at 5.5 by adding a 25% (w/w) $Ca(OH)_2$ slurry.

The D-lactic acid yield in SSCF was defined according to Qiu et al. (2017).

2.6. Analytical methods

Cell growth was determined periodically by measuring the optical density at 600 nm (OD_{600}) using a Biomate 3S spectrophotometer (Thermo Scientific, Massachusetts, USA).

Glucose, xylose and D-lactic acid were determined using an HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with a Bio-Rad Aminex HPX-87H column. The mobile phase was a 5 mM sulfuric acid solution and operated at 65 °C with flow rate of 0.6 mL/min.

The phenolic compounds were determined by HPLC (UV/Vis detector SPD-20A, Shimadzu, Kyoto, Japan) with a YMC-Pack ODS-A column (YMC Co., Kyoto, Japan), according to the method described by Gu et al. (2015) and Khoddami et al. (2013).

3. Results and discussion

3.1. Tolerance evaluation of the D-lactic acid producing P. acidilactici to phenolic aldehydes

We tested the tolerance of the D-lactic acid producing strain *P. acidilactici* ZY15 to the three typical phenolic aldehydes (4-hydroxybenaldehyde, vanillin and syringaldehyde), as well as the corresponding alcohol and acid derivatives (Fig. 1). The concentrations of phenolic aldehydes were selected according to that found in the cellulosic lactic acid fermentation system (0.3 mM 4-hydroxybenzaldehyde, 1.5 mM vanillin and 3.1 mM syringaldehyde). Vanillin strongly inhibited *P. acidilactici* with an LC_{50} of 1.3 mM (LC_{50}, lethal concentration of 50% decrease in cell growth) (Fig. 1a), which is similar to the vanillin concentration found in cellulosic lactic acid fermentation (~1.5 mM). Syringaldehyde was also a strong inhibitor but its LC_{50} (4.4 mM) was about 3-fold greater than that of vanillin (~1.3 mM) and 1.4-fold greater than that found in cellulosic lactic acid fermentation (~3.1 mM). 4-Hydroxybenzaldehyde was a negligible inhibitor with 24.6 mM of 4-hydroxybenzaldehyde showing slight inhibition on *P. acidilactici*, which is approximately 19-fold greater than that of vanillin (~1.3 mM) and 80-fold greater than that found in cellulosic lactic acid fermentation (~0.3 mM). The corresponding alcohol derivatives (4-hydroxybenzyl alcohol, vanillyl alcohol and syringic alcohol) and acid derivatives (4-hydroxybenzoic acid, vanillic acid and syringic acid) showed only slight inhibition on *P. acidilactici* even at high concentrations (Fig. 1b and c). The conversion of phenolic aldehydes by *P. acidilactici* ZY15 was also evaluated. Fig. 2 shows that *P. acidilactici* was able to convert 4-hydroxybenzaldehyde into its alcohol and acid forms, vanillin was converted into its alcohol form (vanillyl alcohol) but not into its acid form, and syringaldehyde was stable and not converted into its alcohol or acid form.

The results showed that vanillin was the strongest phenolic aldehyde inhibitor of *P. acidilactici*, and *P. acidilactici* was able to convert vanillin into its less toxic alcohol form (vanillyl alcohol). Strengthening the conversion of vanillin into its less toxic alcohol or acid derivatives should potentially improve D-lactic acid fermentation by *P. acidilactici*.

3.2. Improving vanillin reduction conversion by integrating the CGS9114_RS09725 gene

Biodetoxification of the pretreated corn stover feedstock was conducted to completely remove the highly toxic and highly concentrated inhibitors such as furfural, HMF and acetic acid, but phenolic aldehydes were only partially removed. The residual phenolic aldehydes in the cellulosic fermentation broth still inhibited D-lactic acid production by *P. acidilactici* ZY15 from corn stover feedstock (Fig. 6). Acceleration of the conversion of toxic phenolic aldehydes into less toxic phenolic alcohol or acid derivatives is an effective strategy to improve the phenolic aldehyde tolerance in fermenting strains (Wang et al., 2016; Yi et al., 2015). Here, we overexpressed six oxidoreductase genes in *P. acidilactici* ZY15 for improving the conversion of phenolic aldehydes, especially vanillin. The genes were *ZMO1885* encoding NADH: flavin oxidoreductase/NADH oxidase from *Z. mobilis* ZM4 (Yi et al., 2015), *CGS9114_RS10340* encoding inositol 2-dehydrogenase, *CGS9114_RS09725* encoding short-chain dehydrogenase from *C. glutamicum* S9114 (Zhou et al., 2019), *PP_3151* encoding NAD^+-dependent succinate semialdehyde dehydrogenase, *PP_5120* encoding coniferyl aldehyde dehydrogenase and *PP_5258* encoding L-piperidine-6-carboxylate dehydrogenase from *P. putida* KT2440 (Simon et al., 2014). Among these genes, *ZMO1885*, *CGS9114_RS10340* and *CGS9114_RS09725* showed a strong response to phenolic aldehydes reduction, whereas *PP_3151*, *PP_5120* and *PP_5258* showed a significant response to vanillin oxidation.

The genes were inserted into the plasmid pZY36e under the control of promoter *PldhD* and then introduced into *P. acidilactici* to generate six recombinants. A modified MRS medium containing 1.3 mM vanillin, 3.3 mM syringaldehyde and 24.6 mM 4-hydroxybenaldehyde was designed to evaluate the D-lactic acid fermentability of the recombinants, which was based on their individual minimum inhibition concentrations of *P. acidilactici*. *CGS9114_RS09725* from *C. glutamicum* S9114 was the only gene that promoted D-lactic acid fermentation under phenolic aldehydes stress (Fig. 3).

To obtain a stable recombinant, the *CGS9114_RS09725* gene was integrated into the gene *ackA2* locus of the *P. acidilactici* ZY15 genome by thermo-sensitive homologous recombination with no antibiotic resistance markers remaining in the genome. The engineered strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* was examined in MRS medium containing gradient concentrations of vanillin (Fig. 4), and a significant improvement of D-lactic acid fermentation was observed when vanillin approached its LC_{50} concentration (1.3–1.6 mM). The time course of vanillin conversion at the LC_{50} of vanillin (i.e., 1.6 mM; Fig. 5) showed that about 30% of the original vanillin (0.46 mM) was converted into vanillyl alcohol (0.27 mM) within 24 h, which is more than double that of the parental strain (reduced 0.25 mM vanillin and produced 0.13 mM vanillyl alcohol). The imbalance between vanillin reduction and vanillyl alcohol production was observed in both strains. Several reasons may cause this imbalance: (1) conversion of vanillin to

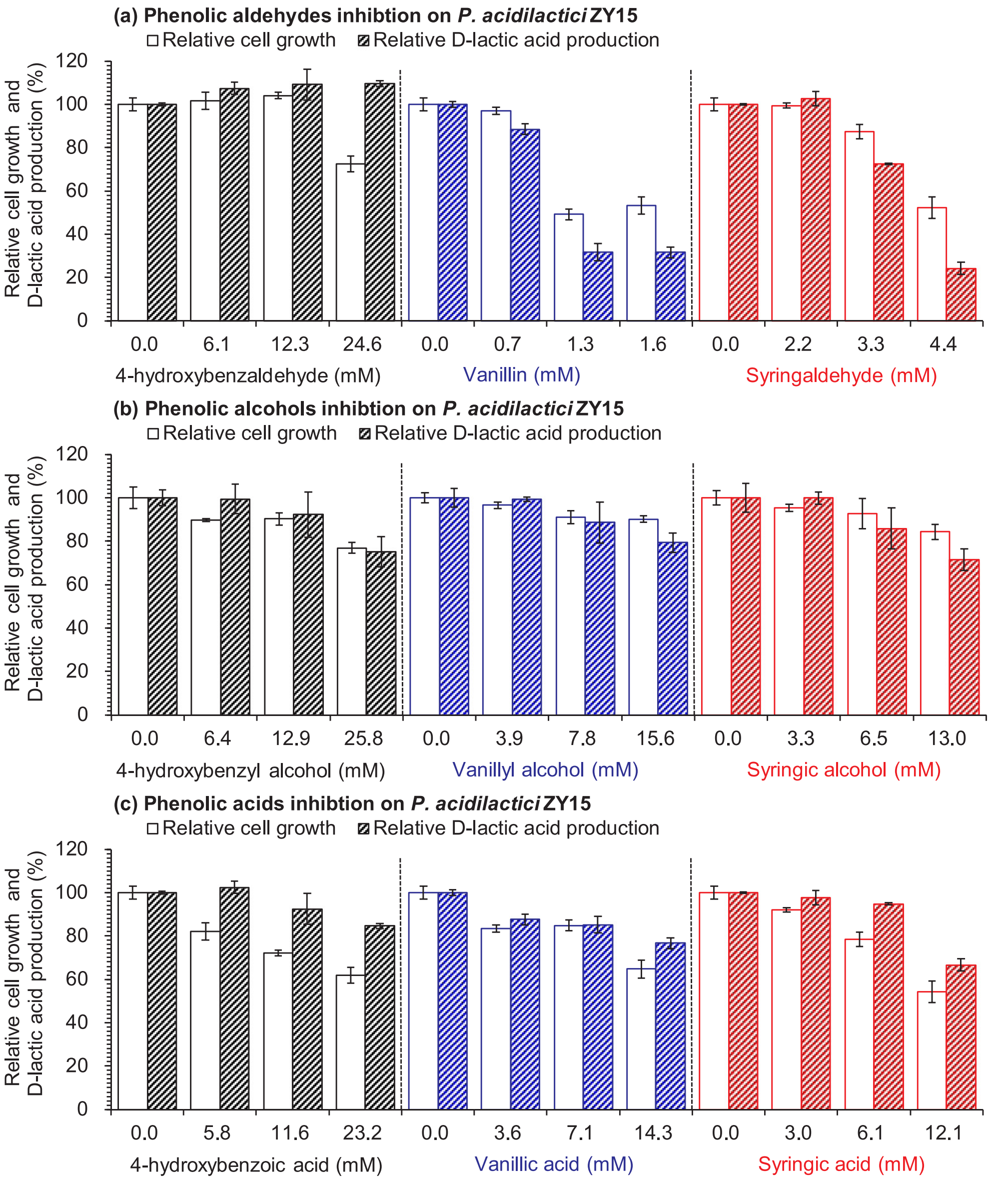


Fig. 1. Inhibition of phenolic compounds on the cell growth (OD_{600}) and D-lactic acid production of *P. acidilactici* ZY15. (a) Phenolic aldehydes inhibition on *P. acidilactici* ZY15; (b) Phenolic alcohols inhibition on *P. acidilactici* ZY15; (c) Phenolic acids inhibition on *P. acidilactici* ZY15. The relative cell growth (or relative D-lactic acid production) was defined as the percentage of the cell growth (or D-lactic acid production) under inhibitor stress to that without inhibitor stress in 12 h. The fermentation conditions: 50 mL of MRS medium with gradient concentration of phenolic compounds addition in 250 mL shaking flasks, 10% (v/v) inoculate size, 42 °C, 150 rpm. Cells were collected for measurement of optical density (OD) at 600 nm without pH control. D-lactic acid was measured with the pH value controlled by adding 0.6 g of $CaCO_3$ per g of glucose.

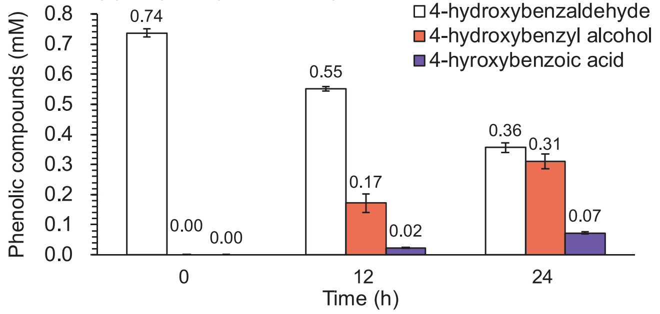
other vanillin derivatives other than vanillyl alcohol; (2) volatilization of vanillin at high culture temperature (42 °C for 24 h). Besides, no vanillic acid was detected, indicating that *CGS9114_RS09725* expression only improved the reduction of vanillin with no improvement in the oxidation of vanillin to vanillic acid.

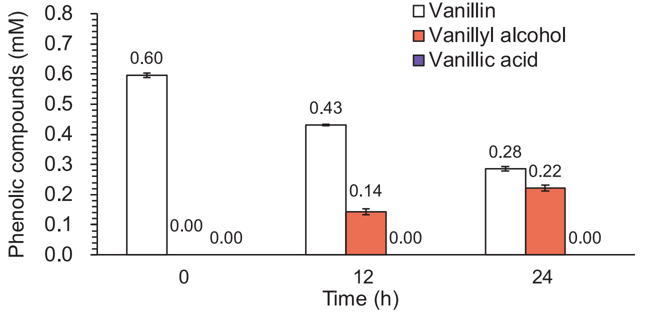
We failed to observe any improvements for the conversion of syringaldehyde (data not shown) and syringaldehyde tolerance (see Supplementary materials) by genome expression of *CGS9114_RS09725*, indicating that *CGS9114_RS09725* expression specifically accelerated the reduction of vanillin and consequently enhanced vanillin tolerance of *P. acidilactici*.

3.3. Cellulosic D-lactic acid fermentation by the engineered P. acidilactici strain

The engineered strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* was applied for D-lactic acid fermentation using corn stover feedstock for evaluating the improvement in fermentability. The corn stover feedstock was dry acid pretreated and then biodetoxified to completely remove furfural, HMF, acetic acid and partially remove the phenolic aldehydes with residuals of 0.1, 0.6 and 1.5 mg/g DM of 4-hydroxybenaldehyde, vanillin and syringaldehyde, respectively. The pretreated and biodetoxified corn stover was then simultaneously saccharified and fermented into D-lactic acid under 30% (w/w) solids loading (Fig. 6). The engineered *P. acidilactici* with improved vanillin tolerance significantly accelerated glucose consumption and increased D-lactic acid production by 13.9% in D-lactic acid titer (from 101 g/L to 115 g/L), 14.3% in productivity (from 1.4 g/L/h to 1.6 g/L/h) and 18.4% in overall yield (from 51.6% to 61.1%). We attempted to compare the vanillin reduction and vanillyl alcohol production between the engineered and parental strains, but it failed. This is probably because phenolic aldehydes usually have the low solubility or even insolubility (Palmqvist and Hahn-Hägerdal, 2000). Unlike the hydrolysate fermentation, high solid particles were contained in the SSCF. These slightly water-soluble phenolic aldehydes easily precipitated on solid particles and resulted in the inaccurate detection of vanillin and vanillyl alcohol (Thomsen et al., 2009; Gu et al., 2014).

Complete removal of phenolic aldehydes does not work by conventional water-washing and over-liming methods because of their low water solubility and hydrophobicity (Gu et al., 2015; Thomsen et al., 2009). Biodetoxification method is able to remove furfural, HMF and acetic acid completely, but the complete removal of phenolic aldehydes requires a very long period, which leads to the considerable loss of


(a) 4-hydroxybenzaldehyde conversion


(b) Vanillin conversion

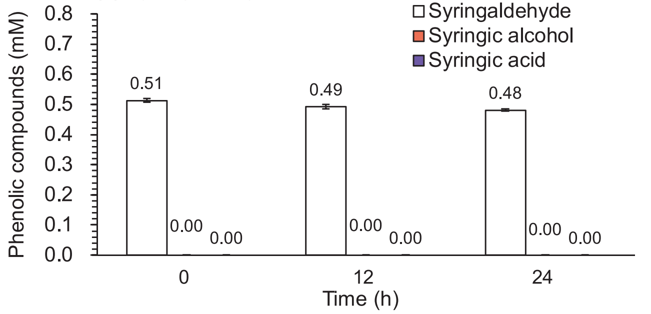
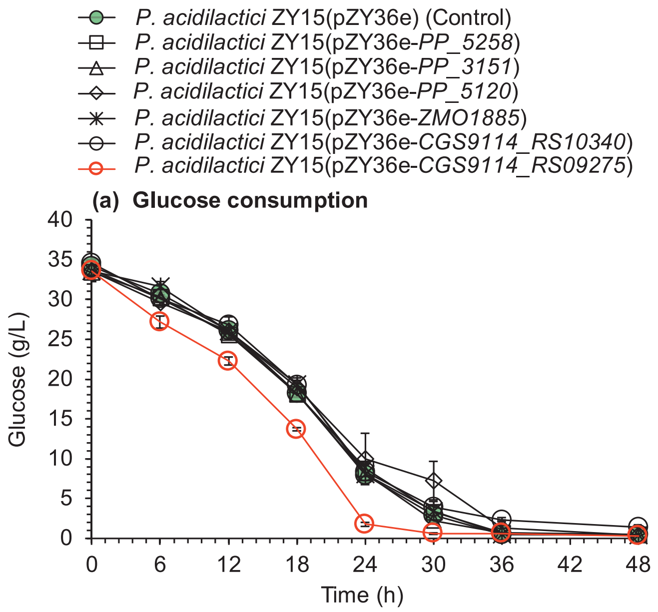

(c) Syringaldehyde conversion

Fig. 2. 4-hydroxybenzaldehyde, vanillin and syringaldehyde conversion evaluations of *P. acidilactici* ZY15. (a) 4-hydroxybenzaldehyde conversion; (b) vanillin conversion; (c) syringaldehyde conversion. The fermentation conditions: 50 mL of MRS medium with 4-hydroxybenzaldehyde, vanillin or syringaldehyde addition in the 250 mL shaking flasks, 10% (v/v) inoculate size, 42 °C, 150 rpm. The data were calculated by deducting the self-volatilization of phenolic aldehydes during the 24 h fermentation conducted without strains inoculation.



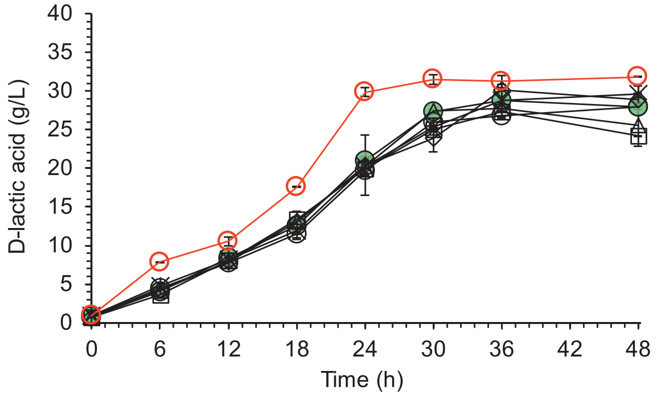

(b) D-lactic acid production

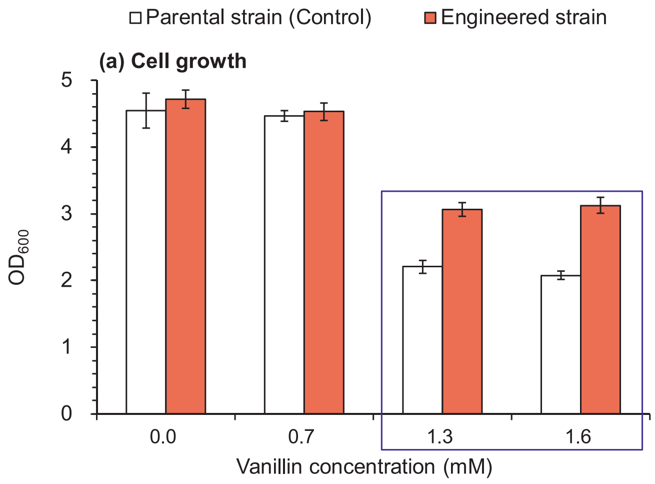
Fig. 3. Fermentation evaluation of *P. acidilactici* ZY15 recombinants expressing oxidoreductase genes under mixed phenolic aldehydes stress. (a) Glucose consumption; (b) D-lactic acid production. The fermentation conditions: 24.6 mM of 4-hydroxybenaldehyde, 1.3 mM of vanillin and 3.3 mM of syringaldehyde added; 50 mL of MRS medium in 250 mL shaking flasks with 5 μg/mL of erythromycin addition.

xylose (Gao et al., 2018; He et al., 2016;Wang et al., 2015; Yi et al., 2019; Zhang et al., 2010b). Thus, phenolic aldehydes are inevitably remained in the pretreated and biodetoxified lignocellulose feedstock to ensure that the fermentable xylose sugar is not lost and consequently the lactic acid fermentability was negatively affected. This study demonstrated a practical solution by applying the D-lactic acid fermenting strain to finalize the conversion of the residual phenolic aldehyde (vanillin) into its less toxic alcohol during the fermentation period. We accelerated the bioconversion of vanillin into vanillyl alcohol by the genome expression of a specific gene *CGS9114_RS09725* encoding a short-chain dehydrogenase from *C. glutamicum*, and the tolerance of *P. acidilactici* to vanillin was significantly improved. Consequently, the D-lactic acid fermentability from the dry acid pretreated and biodetoxified corn stover with vanillin contained was also improved.

Syringaldehyde is also a strong toxic phenolic aldehyde inhibitor, but the present *P. acidilactici* strain in this study was unable to convert syringaldehyde into its less toxic alcohol or acid derivative as in the case of vanillin (Fig. 2). This is probably because vanillin has one methoxyl group and syringaldehyde has two methoxyl groups, and the space shelling generated by two methoxyl groups may inhibit specific enzymes binding with syringaldehyde, which blocks the bioconversion of syringaldehyde (Klinke et al., 2004; Yi et al., 2015). This study failed to construct bioconversion pathways of syringaldehyde by overexpression of oxidoreductase genes, and failed to enhance the tolerance of *P. acidilactici* to syringaldehyde. Future efforts such as adaptive evolution should be made to enhance the tolerance of *P. acidilactici* to syringaldehyde for the construction of a robust cellulosic D-lactic acid bacterium.

4. Conclusion

Vanillin was found to be the strongest phenolic aldehyde inhibitor on *P. acidilactici* ZY15. Genome integration of an exogenous gene *CGS9114_RS09725* encoding short-chain dehydrogenase into *P. acidilactici* significantly enhanced vanillin reduction conversion, resulting in improved vanillin tolerance. The obtained strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* showed accelerated D-lactic acid fermentation from the dry acid pretreated and biodetoxified corn stover with phenolic aldehydes contained.



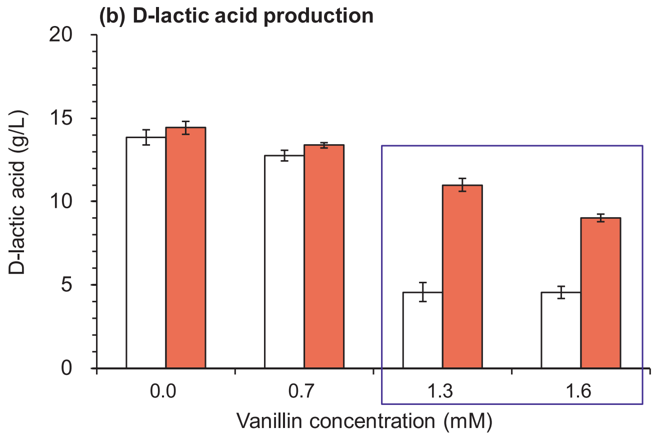


Fig. 4. D-lactic acid fermentability of the engineered strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* under stress of gradient concentrations of vanillin. (a) Cell growth (OD_{600}); (b) D-lactic acid production. The fermentation conditions: 50 mL of MRS medium in 250 mL shaking flasks, 10% (v/v) inoculate size, 42 °C, 150 rpm for 12 h. The parental strain *P. acidilactici* ZY15 was used as control.

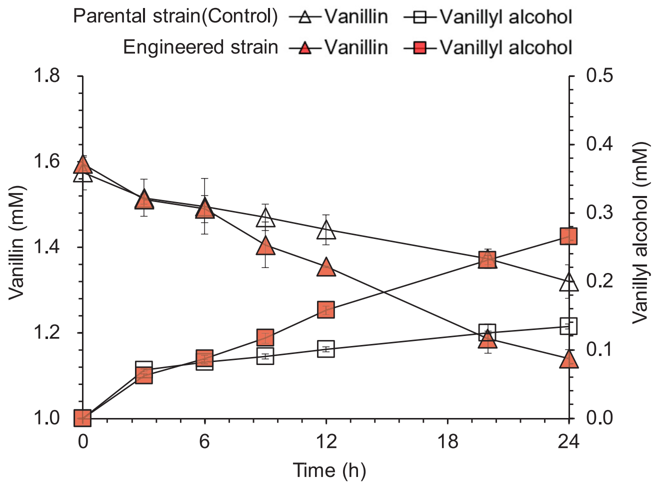


Fig. 5. Vanillin conversion evaluation of the engineered strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* in vanillin contained MRS medium. The fermentation conditions: 1.6 mM of vanillin added; 50 mL of MRS medium in 250 mL shaking flasks, 42 °C, 150 rpm, 10% (v/v) inoculate size. The parental strain *P. acidilactici* ZY15 was used as control. No vanillic acid was detected during the fermentation.

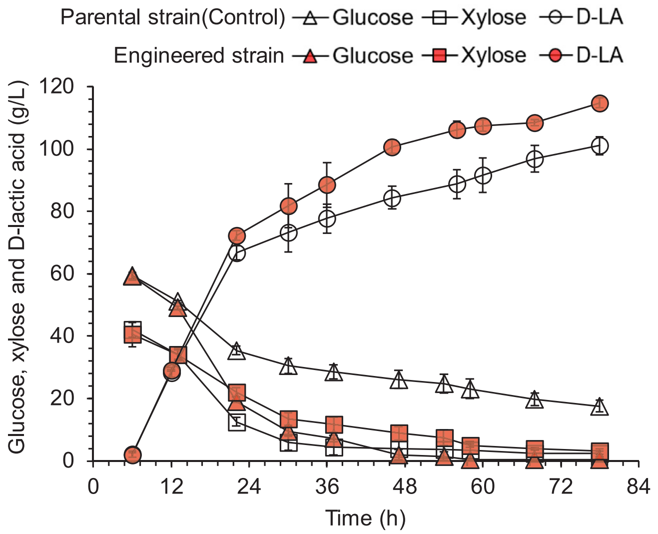


Fig. 6. Simultaneous saccharification and co-fermentation (SSCF) of D-lactic acid by the engineered strain *P. acidilactici* ZY15-Δ*ackA2*::*CGS9114_RS09725* using phenolic aldehydes contained corn stover feedstock. The SSCF was conducted using the 30% (w/w) solids loading of dry acid pretreated and biodetoxified corn stover, 10 mg cellulase protein per gram cellulose. The initial concentration of 4-hydroxybenaldehyde, vanillin and syringaldehyde in the SSCF system were 0.3, 1.5 and 3.1 mM, respectively. pH was maintained by automatic feeding of a 25% (w/w) $Ca(OH)_2$ slurry. The parental strain *P. acidilactici* ZY15 was used as control.

CRediT authorship contribution statement

Zhongyang Qiu: Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing - original draft, Writing - review & editing. **Chun Fang:** Data curation, Formal analysis, Investigation, Methodology, Resources. **Qiuqiang Gao:** Formal analysis, Investigation. **Jie Bao:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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

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