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Engineering *Pediococcus acidilactici* with xylose assimilation pathway for high titer cellulosic L-lactic acid fermentation

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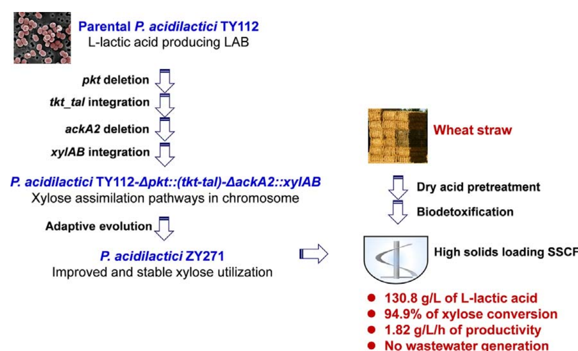
GRAPHICAL ABSTRACT



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ABSTRACT

Xylose-assimilating pathways were constructed in the parental *Pediococcus acidilactici* strain and evolutionarily adapted to yield a highly stable co-fermentation strain for L-lactic acid production. The phosphoketolase pathway (PK) was blocked for reduction of acetic acid generation by disrupting phosphoketolase (*pkt*) gene. The pentose phosphate pathway (PPP) was reconstructed for xylose assimilation by integrating four heterologous genes encoding transketolase (*tkt*), transaldolase (*tal*), xylose isomerase (*xylA*) and xylulokinase (*xylB*) into the *P. acidilactici* chromosome. The xylose-assimilating ability of the constructed strain was significantly improved by long term adaptive evolution. The engineered strain was applied to the simultaneous saccharification and co-fermentation (SSCF) under high solids loading of wheat straw. The L-lactic acid titer, productivity and xylose conversion reached the record high at 130.8 ± 1.6 g/L, 1.82 ± 0.0 g/L/h, and 94.9 ± 0.0%, respectively. This study provided an important strain and process prototype for production of high titer cellulosic L-lactic acid.

1. Introduction

L-Lactic acid is the monomer chemical of biodegradable poly-lactic acid (PLA) plastic (Nampoothiri et al., 2010; Farah et al., 2016). Currently, approximately 90% of the commercially available L-lactic acid is produced from corn and the corn feedstock accounts for 70% of the overall production cost (Abdel-Rahman et al., 2011). The future perspective of PLA as the alternative of petroleum derived polyethylene (PE), polypropylene (PP), and polystyrene (PS) certainly requires a more sustainable feedstock supply, other than food crop starch. Lignocellulose biomass provides the best and only practical feedstock option for its availability, great abundance and the post-harvest pressure of agriculture (Taha et al., 2016).

Xylose utilization is crucially important in lignocellulose biorefinery

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because it accounts for 30% of the total carbohydrates in lignocellulose biomass. Recently, several wild type *Bacillus* strains were found to behave the xylose assimilation capacity for L-lactic acid fermentation (Maas et al., 2008; Hu et al., 2015; Zhang et al., 2016). However, *Bacillus* species are not lactic acid bacterium (LAB) strains and the optical purity of L-lactic acid is relatively low (around 98%). For polymerization application, the extra high optical purity is preferred, and in case of L-lactic acid, the optical purity should be greater than 99% (Litchfield, 2009). In this aspect, lactic acid bacterium (LAB) has unique advantages in high optical purity of lactic acid and less byproduct generation. In our previous work, we engineered a wild *Pediococcus acidilactici* strain for L-lactic acid and applied to cellulosic L-lactic acid production (Yi et al., 2016; Liu et al., 2015). Both the L-lactic acid yield (104.5 g/L) and the optical purity (99.8%) reached the satisfactory levels when corn stover was used as the feedstock after the dry acid pretreatment and biodetoxification. Besides, no wastewater was generated in pretreatment and detoxification steps. However, the engineered *P. acidilactici* TY112 strain was not able to utilize xylose.

There are two potential pathways for xylose assimilation in LAB strains: pentose phosphate pathway (PPP) produces only lactic acid and phosphoketolase pathway (PK) produces both lactic acid and acetic acid (Tanaka et al., 2002). Few wild LABs such as *Lactococcus lactis* IO-1, *Leuconostoc lactis* SHO-47 and *Lactobacillus pentosus* FL0421 are known to metabolize xylose via the PK pathway, but the lactic acid yield from xylose are very low (Tanaka et al., 2002; Ohara et al., 2006; Hu et al., 2016). Several LAB strains were engineered to utilize xylose via the PP pathway. Shinkawa et al. (2011) engineered *Lactococcus lactis* IL 1403 strain for L-lactic acid by disruption of *pkt* gene, integration of *tkt* gene and expression of *xylRAB* genes in a plasmid, but the co-fermentation ability of this recombinant was not assayed in practical lignocellulose fermentations. Okano et al. (2009), Yoshida et al. (2011), and Hama et al. (2015) engineered *Lactobacillus plantarum*, Qiu et al. (2017) engineered *P. acidilactici* for D-lactic acid production from lignocellulose feedstock, but not for L-lactic acid. To our knowledge, no efficient xylose-assimilating LAB strains for L-lactic acid production had been developed and practically tested using lignocellulose as fermentation feedstock up to date.

In this study, the xylose-assimilating pathways were constructed into the chromosome of *P. acidilactici* by introducing four heterologous genes *xylA*, *xylB*, *tkt* and *tal*, and disrupting the endogenous genes *pkt* and acetate kinase (*ackA2*). The xylose-assimilating ability of the newly constructed strain was significantly improved by long term adaptive evolution and a highly stable co-fermentation strain *P. acidilactici* ZY271 for L-lactic acid production was obtained. The engineered strain was applied to the high solids loading SSCF of wheat straw. The L-lactic acid titer, productivity and xylose conversion reached the record high at 130.8 ± 1.6 g/L, 1.82 ± 0.0 g/L/h, and 94.9 ± 0.0%, respectively. This study provided an important strain and process prototype for production of high titer cellulosic L-lactic acid.

2. Materials and methods

2.1. Strains, media and culture conditions

The strains used in this study are listed in Table 1. The L-lactic acid producing strain *P. acidilactici* TY112 was an engineered strain by knocking-out the *ldhD* gene encoding d-lactate dehydrogenase from the wild D/L-lactic acid producing strain *P. acidilactici* DQ2 (Yi et al., 2016), and stored at Chinese General Microorganisms collection center (CGMCC), Beijing, China, with the registration number of 8664. The xylose-assimilating strain *P. acidilactici* ZY271 constructed in this study was stored at CGMCC with the registration number of 13611. *P. acidilactici* DSM 20284 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Biodetoxification fungus *Amorphotheca resinae* ZN1 was isolated in our lab and stored in CGMCC with the registration number of 7452 (Zhang et al., 2010a).

Escherichia coli XLI-blue was cultured at 37 °C in Luria-Bertani (LB) medium. The fungus *A. resinae* ZN1 was maintained on a potato dextrose agar medium (PDA) slant containing 200 g of potato extract juice and 20 g of glucose in 1 L deionized water. *P. acidilactici* strains were grown at 42 °C in two Man-Rogosa-Sharp (MRS) media: (1) the regular MRS medium containing 20.0 g/L of glucose, 10.0 g/L of peptone, 4.0 g/L of yeast extract, 8.0 g/L of beef extract, 3.0 g/L of sodium acetate, 2.0 g/L of ammonium citrate dibasic, 2.0 g/L of dipotassium phosphate, 0.2 g/L of magnesium sulfate heptahydrate, 0.05 g/L of manganese sulfate monohydrate, and 1.0 mL of Tween 80; (2) the simplified MRS medium containing 20.0 g/L of glucose, 10.0 g/L of peptone, 10.0 g/L of yeast extract, 5.0 g/L of sodium acetate, 2.0 g/L of ammonium citrate dibasic, 0.58 g/L of magnesium sulfate heptahydrate, 2.0 g/L of dipotassium phosphate, 0.25 g/L of manganese sulfate monohydrate. 150 μg/mL of erythromycin and 5 μg/mL of erythromycin were added into the medium to screen positive recombinants for *E. coli* and *P. acidilactici*, respectively.

2.2. Enzyme

Commercial cellulase enzyme Cellic CTec 2.0 was kindly provided by Novozymes (China), Beijing, China. The filter paper activity of 203.2 FPU and the cellobiase activity of 4900 CBU per mL enzyme solution were determined according to the NREL protocol LAP-006 (Adney and Baker, 1996) and Ghose (Ghose, 1987). The protein concentration was 87.3 mg total proteins per mL of enzyme solution determined by Bradford method using bovine serum albumin (BSA) as protein standard (Bradford, 1976). DNA polymerase was purchased from Takara, Otsu, Japan. Restriction endonuclease was purchased from Thermo Scientific, Wilmington, DE, USA.

2.3. Plasmid construction

All plasmids used in this study are listed in Table 1. The plasmids pSET4E-Δ*pkt* for *pkt* deletion and pSET4E-Δ*pkt*::(*tkt_tal*) for integration of expression cassette P*ldhD_tkt_tal* into *pkt* locus were constructed by Qiu et al. (2017). A 700 bp upstream fragment (*ackA2-up*) of acetate kinase (*ackA2*) gene was amplified with the primer pair *ackA2-up-F* (CCCAAGCTTTTCGCGAATTTGTTAACGCT) and *ackA2-up-R* (CGCGGATCCGCGCCGCTCGAGCCAAATGGCAGGTGATTAATT), a 700 bp downstream fragment (*ackA2-down*) of gene *ackA2* was amplified with the primer pair *ackA2-down-F* (CGCGGATCCTTAGCGTAGAAGAAGTCGTTGAC) and *ackA2-down-R* (CCGGAATTCAGACAAAACCAAAAGAGCGTG), from the genomic DNA of *P. acidilactici* TY112, respectively; expression cassette P*ldhD-xylAB_2911* was amplified from pMG36e-P*ldhD_xylAB_2911* using the primer pair P*ldhD-xylAB_2911-F* (CCGCTCGAGTGCTCTGGTGTGCAGACCAGAC) and P*ldhD-xylAB_2911-R* (CGCGGATCCTTACAACATTTCACGCCGGTAATTC). The *ackA2-up* fragment was inserted into the thermo-sensitive plasmid pSET4E at *Hind* III and *Bam*H I, then *ackA2-down* fragment was inserted at *Bam*H I and *Eco*R I, P*ldhD_xylAB_2911* was finally inserted at *Xho* I and *Bam*H I to obtain plasmid pSET4E-Δ*ackA2*::*xylAB* for integration of *xylAB* into the *ackA2* locus of the *P. acidilactici* genome.

The genes disruption and integration were based on a thermo-sensitive homologous recombination system and no antibiotic resistance marker gene remained in the genome. This system was described clearly in our previous works (Yi et al., 2016; Qiu et al., 2017).

2.4. Feedstock and biorefining operations

Wheat straw was harvested from Jinan, Shandong, China in fall 2016. The collected wheat straw was washed to remove field dirt, stones and metals. The dried wheat straw material was milled coarsely using a beater pulverizer and screened through a mesh with the circle diameter of 10 mm. The virgin wheat straw contained 36.6 ± 0.2% of

Table 1
Strains and plasmids used.

Strains	Characteristics	Sources
Escherichia coli XLI-blue	Host for plasmid construction	Stratagene
Amorphotheca resinae ZN1 (CGMCC 7542)	Biodetoxification fungus isolated in our lab	Zhang et al. (2010a)
Pediococcus acidilactici DSM 20284	Xylose assimilation genes *xylAB_2911*, *tkt* and *tal* contained	Stratagene
P. acidilactici TY112	L-lactic acid producing strain with D-lactate dehydrogenase encoding gene (*ldhD*) deficiency	Yi et al. (2016)
P. acidilactici TY112 (pMG36e)	*P. acidilactici* TY112 harboring empty plasmid pMG36e	This work
P. acidilactici TY112 (pMG36e-P*ldhD_xylAB_2911*)	*P. acidilactici* TY112 harboring *xylAB* expression plasmid pMG36e-P*ldhD_xylAB_2911*	This work
P. acidilactici TY112-Δ*pkt*	*pkt* deficient of *P. acidilactici* TY112	This work
P. acidilactici TY112-Δ*pkt* (pMG36e-P*ldhD_xylAB_2911*)	*P. acidilactici* TY112-Δ*pkt* harboring *xylAB* expression plasmid pMG36e-P*ldhD_xylAB_2911*	This work
P. acidilactici TY112-Δ*pkt*::(*tkt_tal*)	Integration of the expression cassette P*ldhD_tkt_tal* into *pkt* locus of *P. acidilactici* TY112-Δ*pkt*	This work
P. acidilactici TY112-Δ*pkt*::(*tkt_tal*) (pMG36e-P*ldhD_xylAB_2911*)	*P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*) harboring *xylAB* expression plasmid pMG36e-P*ldhD_xylAB_2911*	This work
P. acidilactici TY112-Δ*pkt*::(*tkt_tal*)-Δ*ackA2*::*xylAB*	Integration of the expression cassette P*ldhD_xylAB_2911* into *ackA2* locus of *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*)	This work
P. acidilactici ZY271	The adapted strain of *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*)-Δ*ackA2*::*xylAB*	This work

Plasmids	Characteristics	Sources
pMG36e	Expression vector for *P. acidilactici*, P32 promoter	van de Guchte et al. (1989)
pMG36e-P*ldhD_xylAB_2911*	Expression vector containing *xylAB_2911* expression cassette	Qiu et al. (2017)
pSET4E	Em^r replacing Spc^r marker of pSET4S, temperature sensitive vector	Yi et al. (2016)
pSET4E-Δ*pkt*	Vector for deletion of the *pkt* gene	Qiu et al. (2017)
pSET4E-Δ*pkt*::(*tkt_tal*)	Vector for integration of expression cassette P*ldhD_tkt_tal* into *pkt* locus	Qiu et al. (2017)
pSET4E-Δ*ackA2*	Vector for deletion of the *ackA2* gene	This work
pSET4E-Δ*ackA2*::*xylAB*	Vector for integration of expression cassette P*ldhD_xylAB_2911* into *ackA2* locus	This work

cellulose and 27.2 ± 0.3% of hemicellulose determined by cellulose analyzer (Cellulose Analyzer 220, Ankom Technology, Macedon, NY, USA).

Wheat straw was pretreated using the dry acid pretreatment method according to Zhang et al. (2011) and He et al. (2014). Briefly, 1200 g of dry wheat straw and approximately 600 g of 4.5% (w/w) dilute sulfuric acid solution was co-currently fed into the 20 L pretreatment reactor with the solids/liquid ratio of 2:1 (w/w), equivalent to 2.25 g sulfuric acid per 100 g of the dried wheat straw. The pretreatment was conducted at 175 °C, 50 rpm for 5 min. The pretreated wheat straw contained 38.1 ± 0.3% of cellulose and 3.3 ± 0.0% of hemicellulose determined using the two-step acid hydrolysis according to NREL LAP protocols (Sluiter et al., 2008a,b). The dissolved sugars in the dry pretreated wheat straw included 20.4 ± 0.2 mg/g DM of glucose and 155.9 ± 1.0 mg/g DM of xylose. The major inhibitors content were 1.7 ± 0.0 mg/g DM of furfural, 0.9 ± 0.1 mg/g DM of 5-hydroxymethylfurfural (HMF), 11.5 ± 0.3 mg/g DM of acetic acid, 2.0 ± 0.0 mg/g DM of vanillin, 0.9 ± 0.1 mg/g DM of syringaldehyde and 0.05 ± 0.0 mg/g DM of 4-hydroxybenzaldehyde.

The pretreated wheat straw was biodetoxified to remove the inhibitors according to He et al. (2016). Briefly, the pretreated wheat straw was adjusted to pH 5.5 using 20% (w/w) $Ca(OH)_2$ slurry and then disk milled to remove the long fibers in the pretreated wheat straw. *A. resinae* ZN1 seeds were cultured on the pretreated wheat straw materials at 28 °C for 5 days by inoculating spores from PDA slant. Then the seed solids were inoculated onto the freshly pretreated wheat straw at 10% (w/w) inoculation ratio in a 15 L bioreactor. Biodetoxification was operated at 28 °C and 0.8 vvm of aeration for 72 h. After biodetoxification, the cellulose and hemicellulose content maintained stable, the dissolved sugars in the dry wheat straw were 10.7 ± 0.4 mg/g DM of glucose and 140.2 ± 0.6 mg/g DM of xylose, equivalent to 0.9 ± 0.0% of cellulose and 12.3 ± 0.1% of hemicellulose, respectively. The major inhibitors including furfural, acetic acid, HMF, 4-hydroxybenzaldehyde, vanillin, and syringaldehyde were removed completely.

2.5. Adaptive evolution and SSCF

The adaptive evolution was conducted by the continuous transfer of culture broth into the fresh simplified MRS containing xylose as the sole carbon source. Briefly, the *P. acidilactici* strain was cultured in the simplified MRS containing 40 g/L of xylose as the sole carbon source, then followed by a series transfer of 10% (v/v) culture broth into the fresh simplified MRS every 24 h. The culture condition was 42 °C, 150 rpm, liquid volume ratio 20% in 250 mL shaking flasks. 60 g of $CaCO_3$ per 100 g of xylose was added to control pH in flasks to measure the concentration of xylose, L-lactic acid and acetic acid. Cell growth was measured by detecting the optical density (OD) at 600 nm without $CaCO_3$ addition. The successive transfer was repeated for 66 days until xylose, L-lactic acid concentration and cell growth tended to be stable.

The xylose-assimilating performance comparison of strains before and after adaptive evolution were carried out in the simplified MRS medium containing 35 g/L of xylose as the sole carbon source, or 40 g/L of glucose and 40 g/L of xylose as the mixed carbon source. One vial of the adapted strain was inoculated into a 250 mL flask containing 50 mL of simplified MRS medium with 20 g/L of glucose and cultured at 42 °C, 150 rpm for 12 h. Then the cells were transferred into a 250 mL flask containing 50 mL of the simplified MRS medium containing only xylose or mixed carbon source at 10% (v/v) of inoculation, and cultured at the same condition. Cells were collected every 2 h for measurement of optical density (OD) at 600 nm without pH control. Xylose, glucose, L-lactic acid and acetic acid were measured every 12 h with adding 60 g of $CaCO_3$ per 100 g of sugars to control pH.

The SSCF was conducted in the 5 L bioreactor equipped with a helical ribbon stirrer (Zhang et al., 2010b). For seeds cultivation, one vial of the adapted *P. acidilactici* ZY271 strain was inoculated into a 100 mL flask containing 20 mL of the simplified MRS medium and cultured at 42 °C, 150 rpm for 12 h. Then all the cells were transferred into a 500 mL flask containing 200 mL of the fresh simplified MRS medium cultured at 42 °C, 150 rpm for 5 h; 1% (v/v) glucoamylase solution was added into the seeds culture broth to prevent cells flocculation (Liu et al., 2015). Distilled water and cellulase enzyme Cellic CTec 2.0 solution was first added into the bioreactor, the pretreated and biodetoxified wheat straw was gradually fed into the bioreactor until the

solids loading at 30% (w/w) and the prehydrolysis was performed at 50 °C for 6 h. The SSCF was started by inoculating the seeds broth at 10% (v/v) of inoculation and adding the nutrient solution including 10.0 g/L of peptone, 15.0 g/L of yeast extract, 2.0 g/L of ammonium citrate dibasic and 0.25 g/L of manganese sulfate monohydrate. The SSCF was performed at 42 °C, 150 rpm and pH 5.5 for 72 h. The cellulase dosage used was 10 mg total protein per gram of cellulose in the pretreated wheat straw. The pH was adjusted by automatic regulation with 25% (w/w) $Ca(OH)_2$ slurry during the SSCF stage.

2.6. Analysis of glucose, xylose, L-lactic acid and acetic acid

Samples were withdrawn periodically from the culture broth and centrifuged at 13,000*g* for 5 min and filtered through 0.22 μm filters. Glucose, xylose, L-lactic acid and acetic acid were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with a Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65 °C and 0.6 mL/min using 5 mM H_2SO_4 as the mobile phase.

The D/L chiral purity of lactic acid was assayed using the Megazyme D-/L-Lactic Acid Kit (Megazyme International Ireland, Bray, Wicklow, Ireland) according to the manufacturer's protocol.

2.7. L-lactic acid yield and xylose conversion ratio calculation

The L-lactic acid yield in SSCF was calculated based on the method by Qiu et al. (2017). L-Lactic acid yield was defined as the ratio of the actually obtained L-lactic acid to the theoretical L-lactic acid calculated based on the chemical stoichiometry from cellulose and xylose in the SSCF:

$$\text{L-lactic acid yield} = \frac{[LA] \times V \text{-} [LA]_0 \times V0}{[Biomass] \times ([Cellulose] \times 1.111 + [Xylose]) \times 1} \times 100\%$$

where [*Biomass*] is the dry feedstock content at the beginning of SSCF (g/L), [*Cellulose*] is the cellulose fraction of dry feedstock (g/g), 1.111 is the conversion factor for cellulose to equivalent glucose, [*Xylose*] is the xylose content of dry feedstock (g/g), 1 is the conversion factor for glucose and xylose to L-lactic acid, [*LA*] is the L-lactic acid concentration at the end (g/L) and $[LA]_0$ is the starting concentration (g/L) (comes from the seed inoculation) in the liquid fraction (not the whole slurry), *V* is the volume of the SSCF system including $Ca(OH)_2$ solution added, V_0 is the volume of the SSCF system at the starting point. The liquid volume was calculated based on the water mass balance of SSCF operation.

Xylose conversion ratio is calculated by the decreased xylose concentration in the hydrolysate at the starting and the end of the SSCF operation based on the xylose concentration in the starting of SSCF.

3. Results and discussion

3.1. Construction of xylose-assimilating pathways in L-lactic acid producing P. acidilactici

The L-lactic acid producing strain *P. acidilactici* TY112 was used as the parental strain for constructing xylose-assimilating pathway. The expression plasmid pMG36e-P*ldhD_xylAB_2911* harboring xylose isomerase (*xylA*) and xylulokinase (*xylB*) was transformed into *P. acidilactici* TY112, and 5.6 ± 0.2 g/L of L-lactic acid was produced from 12.6 ± 0.5 g/L of xylose within 48 h but 6.7 ± 0.4 g/L of the by-product acetic acid was also produced (Table 2). This result indicates that the PK pathway is active in the present *P. acidilactici*. Then the PK pathway was blocked by knockout of phosphoketolase gene (*pkt*) and acetic acid was reduced to 0.2 ± 0.0 g/L. However, xylose conversion and L-lactic acid production were dramatically decreased in the resulting recombinant *P. acidilactici* TY112-Δ*pkt* (pMG36e-

Table 2
Xylose fermentation performance comparison of the recombinant *P. acidilactici* strains.

P. acidilactici strains	Properties	Cell growth (OD_{600})	Xylose consumed (g/L)	L-lactic acid produced (g/L)	Acetic acid produced (g/L)
P. acidilactici TY112 (pMG36e)	Parental L-lactic acid producing strain harboring empty plasmid	1.0 ± 0.0	–	–	–
P. acidilactici TY112 (pMG36e-*PldhD_xylAB_2911*)	*xylAB* expression plasmid was induced to the parental strain to initiate xylose utilization but acetic acid was produced	1.6 ± 0.0	12.6 ± 0.5	5.6 ± 0.2	6.7 ± 0.4
P. acidilactici TY112-Δ*pkt* (pMG36e-*PldhD_xylAB_2911*)	*pkt* gene was knocked out and both L-lactic acid and acetic acid production were significantly decreased	0.4 ± 0.0	4.9 ± 0.5	0.2 ± 0.0	0.2 ± 0.0
P. acidilactici TY112-Δ*pkt::(tkt_tal)* (pMG36e-*PldhD_xylAB_2911*)	L-lactic acid production was restored by integrating the *tkt* and *tal* genes, while maintained acetic acid yield at low level	1.1 ± 0.1	9.6 ± 0.0	5.9 ± 0.2	1.7 ± 0.0
P. acidilactici TY112-Δ*pkt::(tkt_tal)*-Δ*ackA2::xylAB*	Integration of *tkt*, *tal* and *xylAB* genes into the chromosome and knockout of *pkt* and *ackA2* genes in the parental strain	1.7 ± 0.1	14.0 ± 0.3	9.8 ± 0.2	2.3 ± 0.1
P. acidilactici ZY271	Final stable strain after long term adaptive evolution of *P. acidilactici* TY112-Δ*pkt::(tkt_tal)*-Δ*ackA2::xylAB*	4.5 ± 0.1	34.2 ± 0.3	28.3 ± 0.3	0.9 ± 0.0

Note: The *P. acidilactici* strains were cultured in the simplified MRS medium containing 35 g/L xylose as sole carbon source. The cells were collected at 12 h for detecting the optical density (OD) at 600 nm. The fermentation broth was collected at 48 h for measuring xylose, L-lactic acid and acetic acid with pH value controlled by adding 60 g of $CaCO_3$ per 100 g of xylose. The fermentation conditions: 42 °C, 150 rpm, the inoculate size 10% and liquid volume ratio 20% in 250 mL shaking flasks.

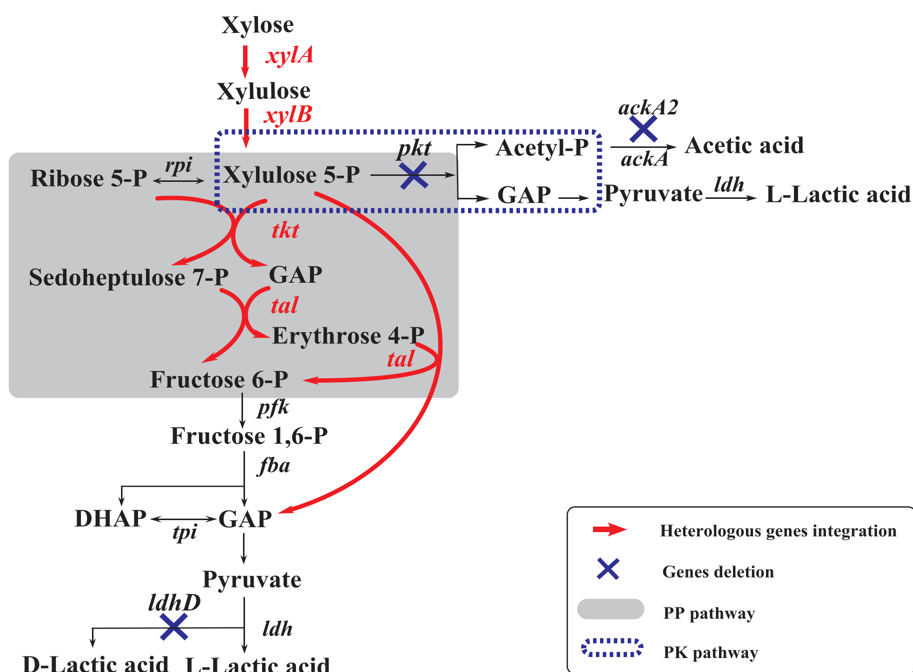

Fig. 1. Construction of xylose-assimilating pathways in *P. acidilactici* for L-lactic acid synthesis. Red arrows represent the heterologous genes integrated into the chromosome of the parental strain *P. acidilactici* TY112. Blue crosses show the deleted genes of *P. acidilactici* TY112. Gray filled box represents the PP pathway. Blue dashed outline indicates the PK pathway. *xylA*, xylose isomerase; *xylB*, xylulokinase; *tkt*, transketolase; *tal*, transaldolase; *rpi*, ribulose-phosphate 3-epimerase; *pfk*, 6-phosphofructokinase; *fba*, fructose-bisphosphate aldolase; *tpi*, triosephosphate isomerase; *pkt*, phosphoketolase; *ackA* and *ackA2*, acetate kinase; *ldhD*, D-lactate dehydrogenase; *ldh*, L-lactate dehydrogenase; GAP, glyceraldehyde-3-phosphate; DHAP, 1,3-dihydroxyacetone phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P*ldhD_xylAB_2911*) (Table 2), indicating that the PK pathway might be the primary way to convert xylose into L-lactic acid.

To boost the L-lactic acid production from xylose in the *P. acidilactici* recombinant, the PP pathway was reconstructed by introducing two heterologous genes transketolase (*tkt*) and transaldolase (*tal*) from *P. acidilactici* DSM 20284 under the control of P*ldhD* (Fig. 1). The two genes were integrated at the disrupted *pkt* locus to yield an engineered strain *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*). The L-lactic acid generation by *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*) harboring xylose assimilating *xylAB* plasmid (pMG36e-P*ldhD_xylAB_2911*) was restored (5.9 ± 0.2 g/L of L-lactic acid produced from 9.6 ± 0.0 g/L of xylose), and acetic acid was reduced from 6.7 ± 0.4 g/L to 1.7 ± 0.0 g/L (Table 2). The result indicates that the reconstructed PP pathway successfully worked in *P. acidilactici* with significant reduction of acetic acid. As the final step, the expression cassette P*ldhD_xylAB_2911* was integrated into the gene *ackA2* locus of *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*) to yield the stable xylose-assimilating strain *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*)-Δ*ackA2*::*xylAB* with all heterologous genes in the chromosome (Fig. 1).

3.2. Adaptive evolution and application in simultaneous saccharification and co-fermentation (SSCF)

The newly constructed xylose-assimilating strain *P. acidilactici* TY112-Δ*pkt*::(*tkt_tal*)-Δ*ackA2*::*xylAB* was cultured in the xylose containing MRS medium and considerable xylose was converted into L-lactic acid with less acetic acid generation (9.8 ± 0.2 g/L of L-lactic acid and 2.3 ± 0.1 g/L of acetic acid produced from 14.0 ± 0.3 g/L of xylose) (Table 2). To accelerate the xylose assimilation rate and elevate the L-lactic acid yield, adaptive evolution was conducted by successive transfer of the engineered strain every 24 h in the simplified MRS medium containing 40 g/L of xylose. After 66 successive transfers (66 days), the xylose-assimilating ability of the resulting strain *P. acidilactici* ZY271 was significantly stabilized and improved (Fig. 2). The cell growth (OD_{600}) increased from 1.0 ± 0.0 to 5.1 ± 0.1, and L-lactic acid production increased from 4.8 ± 0.1 g/L to 30.2 ± 0.2 g/L, and the residue xylose decreased from 33.1 ± 0.4 g/L to 3.3 ± 0.2 g/L.

The adapted strain *P. acidilactici* ZY271 was compared with the starting strain in the two simplified MRS media containing xylose as the sole carbon source, and containing mixed glucose and xylose,

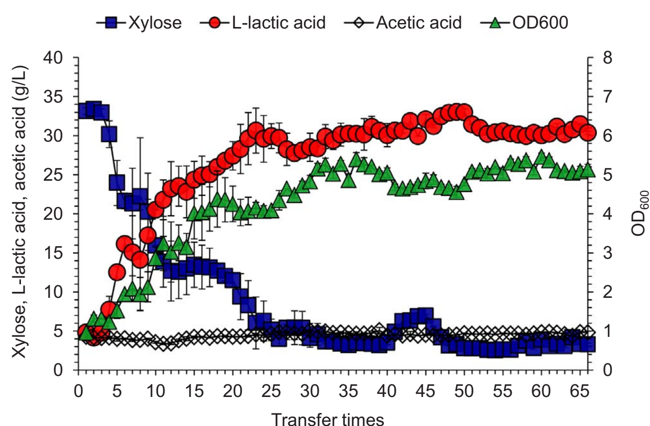

Fig. 2. Adaptive evolution of the newly constructed *P. acidilactici* strain TY112-Δ*pkt*::(*tkt_tal*)-Δ*ackA2*::*xylAB*. A series of transfers to the fresh simplified MRS containing xylose as the sole carbon source was done every 24 h at 10% (v/v) inoculation ratio. The initial xylose and acetic acid concentration in the medium were 40 g/L and 5 g/L, respectively. Cell growth was measured by detecting the optical density (OD) at 600 nm without pH control. Xylose, L-lactic acid and acetic acid were measured with pH controlled by adding 60 g of $CaCO_3$ per 100 g of xylose. The fermentation was conducted at 42 °C, 150 rpm, liquid volume ratio 20% in 250 mL shaking flasks.

respectively (Fig. 3). In the xylose only medium, the cell growth, xylose conversion, L-lactic acid productivity of the adapted *P. acidilactici* ZY271 were significantly improved (Fig. 3a and b). In the mixed glucose and xylose medium, the cell growth rate and glucose consumption rate of the adapted and the starting strains were almost same, but the adapted strain consumed 91.2 ± 0.2% of xylose within 48 h, while only 56.9 ± 0.5% of xylose was consumed by the starting strain (Fig. 3c and d). Interestingly, the glucose and xylose utilization of the adapted strain *P. acidilactici* ZY271 worked simultaneously without carbon catabolite repression. The generation of byproduct acetic acid was negligible (less than 1.0 g/L).

The pretreated and biodetoxified wheat straw contained 38.1 ± 0.3% of cellulose and 3.3 ± 0.0% of hemicellulose, 10.7 ± 0.4 mg/g DM of glucose and 140.2 ± 0.6 mg/g DM of xylose. High content of xylose makes this feedstock suitable for testing the xylose utilization performance and the higher L-lactic acid production

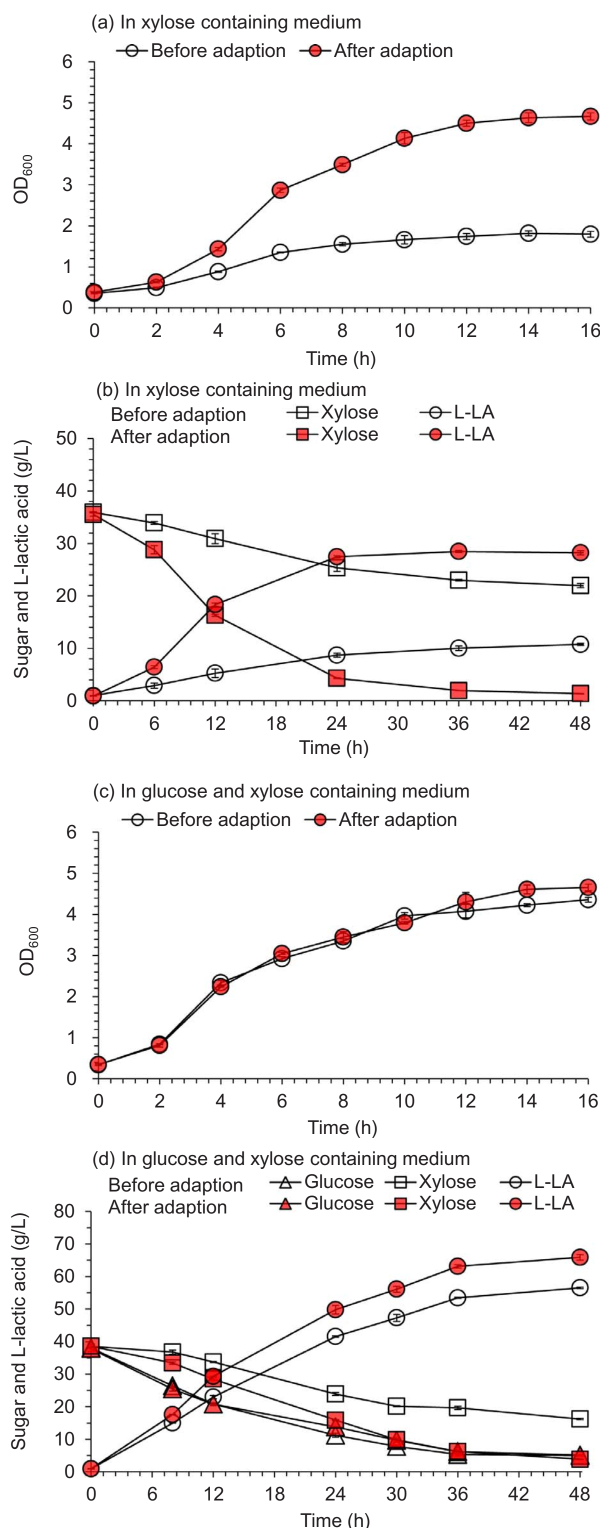

Fig. 3. L-lactic acid fermentation of the xylose-assimilating *P. acidilactici* strains before and after adaptive evolution. (a) (b) Cell growth and L-lactic acid fermentation in the simplified MRS medium containing 35 g/L of xylose; (c) (d) Cell growth and L-lactic acid fermentation in the simplified MRS medium containing 40 g/L of glucose and 40 g/L of xylose. The fermentation conditions: 42 °C, 150 rpm, the inoculate size 10%, and liquid volume ratio 20% in 250 mL shaking flasks.

of the newly constructed xylose-assimilating strain *P. acidilactici* ZY271. The SSCF was conducted using the high solids loading (30%, w/w) pretreated and biodetoxified wheat straw feedstock (Fig. 4). Comparing

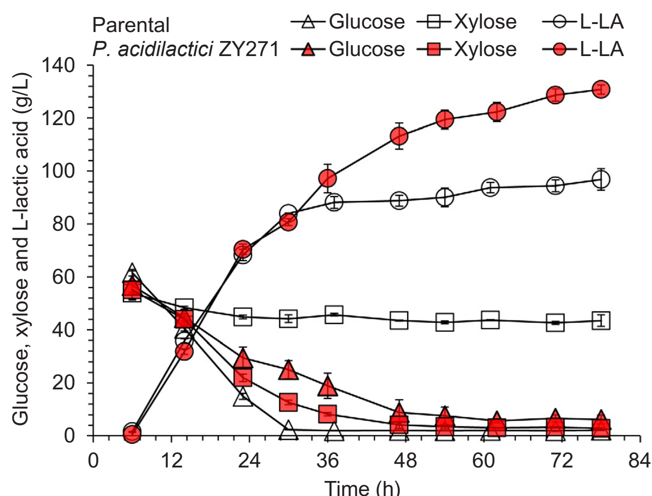

Fig. 4. Simultaneous saccharification and co-fermentation (SSCF) of cellulosic L-lactic acid by *P. acidilactici* ZY271. The feedstock used was 30% (w/w) solids loading of dry acid pretreated and biodetoxified wheat straw. Wheat straw feedstock was pre-hydrolyzed for 6 h at 50 °C and pH 4.8, then the seed broth was inoculated at 10% of inoculation ratio to start the SSCF at 42 °C and pH 5.5. pH was maintained by automatic feeding of 25% (w/w) $Ca(OH)_2$ solution. The cellulase dosage was 10 mg total protein per gram of cellulose in the pretreated wheat straw feedstock.

with the parental *P. acidilactici* TY112 (without xylose utilization), *P. acidilactici* ZY271 consumed glucose and xylose simultaneously and 94.9 ± 0.0% of xylose (55.1 ± 3.5 g/L of initial xylose concentration in SSCF) was converted within 72 h, while no observable xylose consumption of *P. acidilactici* TY112. *P. acidilactici* ZY271 produced 130.8 ± 1.6 g/L of L-lactic acid with the yield of 67.7 ± 0.3% from both glucose and xylose in wheat straw, which was 35% more L-lactic acid produced than that of the parental *P. acidilactici* TY112 (96.8 ± 4.0 g/L). No observable acetic acid was produced during the SSCF of *P. acidilactici* ZY271.

3.3. Analysis on L-lactic acid fermenting strains and cellulosic L-lactic acid fermentation

Xylose is generally released from hemicellulose during the pretreatment step, but the inhibitors such as furfural, 5-hydromethylfurfural (HMF), acetic acid are also generated from pretreatment. A difficulty always exists in lignocellulose biorefining process: inhibitor removal generally leads to the loss of xylose in the regular detoxification (conditioning) methods such as water washing or overliming, besides its disadvantage of massive wastewater generation. The situation makes the co-fermentation of xylose less meaningful on increasing L-lactic acid production. Studies on cellulosic L-lactic acid production by SSCF and xylose-utilizing strains were reported recently. Hu et al. (2015, 2016) and Zhang et al. (2016) water-washed the pretreated corn stover or corncob to obtain the "pure" feedstock, but huge fresh water was consumed and huge wastewater was generated (5 tons of fresh water input in pretreatment and 8 tons of fresh water input in inhibitors washing, totally 13 tons of fresh water were used per ton of dry corn stover or corncob). The SSCF of the "pure" feedstock produced 92.3 g/L of L-lactic acid from corn stover by *Lactobacillus pentosus* FL0421 with the generation of 34.3 g/L of acetic acid (Hu et al., 2016); 97.6 g/L of L-lactic acid by *B. coagulans* LA204 from corn stover (Hu et al., 2015); 123.0 g/L and 118.6 g/L of L-lactic acid were produced from corncob (different pretreatment methods) by *B. coagulans* LA204 (Zhang et al., 2016). In other cases, the pretreated feedstock was directly used without water washing to remove the inhibitors. Zhang et al. (2016) also tested the unwashed corncob as feedstock but L-lactic acid titer dramatically decreased to 79.5 g/L from 118.6 g/L by the same *B. coagulans* LA204 strain. Maas et al. (2008) used the lime

pretreated wheat straw as feedstock and no water washing was done although fresh water usage was still high in the pretreatment step only (10 tons of fresh water usage per ton of wheat straw), and 40.7 g/L of L-lactic acid was produced by *B. coagulans* DSM 2314 with very low productivity (0.68 g/L/h).

This study applied a unique biodetoxification method by a filamentous fungus *A. resinae* ZN1 to remove the inhibitors from the pretreated wheat straw solids quickly, selectively, completely and without any wastewater generation (Zhang et al., 2010a; He et al., 2016). *A. resinae* ZN1 also has the unique substrate priority of inhibitors to xylose and glucose, thus xylose was well conserved in the biodetoxification step (Ran et al., 2014; Wang et al., 2015). The maximum L-lactic acid of 130.8 ± 1.6 g/L was obtained from dry acid pretreated and biodetoxified wheat straw. The xylose conversion was 94.9 ± 0.0% from 55.1 ± 3.5 g/L of the initial xylose in SSCF. The optical purity of the obtained L-lactic acid was as high as 99.6 ± 0.1%. The cellulase enzyme usage was reduced to 10 mg cellulase protein per gram cellulose in the pretreated wheat straw feedstock, only accounted less than half of the above mentioned cases (23 mg/g cellulase protein per gram cellulose for Hu et al., 2015; 22 mg/g for Hu et al., 2016; 21 mg/g for Zhang et al., 2016; 32 mg/g for Maas et al., 2008). No wastewater stream was generated starting from the pretreatment till the end of fermentation. The fresh water usage was orders of magnitude smaller than that of the other cases. The combination of dry acid pretreatment, biodetoxification and high solids SSCF maximized the advantage of the xylose assimilating strain *P. acidilactici* ZY271 for cellulosic L-lactic acid production.

4. Conclusion

The xylose-assimilating pathways were constructed into the chromosome of *P. acidilactici* by introducing four heterologous genes *xylA*, *xylB*, *tkt* and *tal*, and disrupting the endogenous genes *pkt* and *ackA2*. The xylose-assimilating ability of the newly constructed strain was significantly improved by adaptive evolution and an efficient xylose-assimilating strain *P. acidilactici* ZY271 for L-lactic acid production was obtained. The engineered strain was applied to the high solids loading SSCF of wheat straw. The L-lactic acid titer, productivity and xylose conversion reached the record high at 130.8 ± 1.6 g/L, 1.82 ± 0.0 g/L/h, and 94.9 ± 0.0%, respectively.

References

Abdel-Rahman, M., Tashiro, Y., Sonomoto, K., 2011. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. J. Biotechnol. 156, 286–301.

Adney, B., Baker, J., 1996. Measurement of cellulase activities. Laboratory analytical procedure. Technical Report NREL/TP510-42628. National Renewable Energy Laboratory (NREL), Golden CO.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Farah, S., Anderson, D.G., Langer, R., 2016. Physical and mechanical properties of PLA, and their functions in widespread applications – a comprehensive review. Adv. Drug. Deliv. Rev. 107, 367–392.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

Hama, S., Mizuno, S., Kihara, M., Tanaka, T., Ogino, C., Noda, H., Kondo, A., 2015. Production of D-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. Bioresour. Technol. 187, 167–172.

He, Y.Q., Zhang, L.P., Zhang, J., Bao, J., 2014. Helically agitated mixing in dry dilute acid pretreatment enhances the bioconversion of corn stover into ethanol. Biotechnol. Biofuels 7, 1.

He, Y.Q., Zhang, J., Bao, J., 2016. Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation. Biotechnol. Biofuels 9, 19.

Hu, J.L., Zhang, Z.T., Lin, Y.X., Zhao, S.M., Mei, Y.X., Liang, Y.X., Peng, N., 2015. High titer lactic acid production from NaOH-pretreated corn stover by Bacilluscoagulans LA204 using fed-batch simultaneous saccharification and fermentation under non-sterile condition. Bioresour. Technol. 182, 251–257.

Hu, J.L.Y.X., Lin, Z.T., Zhang, T., Xiang, Y.X., Mei, S.M., Zhao, Y.X., Liang, N., 2016. High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. Bioresour. Technol. 214, 74–80.

Litchfield, J.H., 2009. Lactic acid, microbially produced. In: Schaechter, M.O. (Ed.), Encyclopedia of Microbiology. Academic Press, Oxford, pp. 362–372.

Liu, G., Sun, J., Zhang, J., Tu, Y., Bao, J., 2015. High titer l-lactic acid production from corn stover with minimum wastewater generation and techno-economic evaluation based on Aspen plus modeling. Bioresour. Technol. 198, 803–810.

Maas, R.H.W., Bakker, R.R., Jansen, M.L.A., Ed de Jong, D.V., Eggink, G., Weusthuis, R.A., 2008. Lactic acid production from lime-treated wheat straw by *Bacillus coagulans*: neutralization of acid by fed-batch addition of alkaline substrate. Appl. Microbiol. Biotechnol. 78, 751–758.

Nampoothiri, N.K., Nair, N.R., John, R.P., 2010. An overview of the recent developments in polylactide (PLA) research. Bioresour. Technol. 101 (22), 8493–8501.

Ohara, H., Owaki, M., Sonomoto, K., 2006. Xylooligosaccharide fermentation with *Leuconostoc lactis*. J. Biosci. Bioeng. 101 (5), 415–420.

Okano, K., Yoshida, S., Yamada, R., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2009. Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. Appl. Environ. Microbiol. 75 (24), 7858–7861.

Qiu, Z.Y., Gao, Q.Q., Bao, J., 2017. Constructing xylose-assimilating pathways in *Pediococcus acidilactici* for high titer d-lactic acid fermentation from corn stover feedstock. Bioresour. Technol in press.

Ran, H., Zhang, J., Gao, Q.Q., Lin, Z.L., Bao, J., 2014. Analysis of biodegradation performance of furfural and 5-hydroxymethylfurfural by *Amorphotheca resinae* ZN1. Biotechnol. Biofuels 7, 51.

Shinkawa, S., Okano, K., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2011. Improved homo L-lactic acid fermentation from xylose by abolishment of the phosphoketolase pathway and enhancement of the pentose phosphate pathway in genetically modified xylose-assimilating *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 91 (6), 1537–1544.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Technical Report NREL/TP-510-42623. National Renewable Energy Laboratory (NREL), Golden CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlat, C., Sluiter, J., Templeton, D., Crocker, D., 2008a. Determination of Structural carbohydrates and lignin in biomass: laboratory analytical procedure. Technical Report NREL/TP-510-42618. National Renewable Energy Laboratory (NREL), Golden CO.

Taha, M., Foda, M., Shahsavari, E., Aburto-Medina, A., Adetutu, E., Ball, A., 2016. Commercial feasibility of lignocellulose biodegradation: possibilities and challenges. Curr. Opin. Biotechnol. 38, 190–197.

Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S.J., Stanbury, P.F., 2002. Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. Appl. Microbiol. Biotechnol. 60, 160–167.

van de Guchte, M., van der Vossen, J.M., Kok, J., Venema, G., 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. lactis. Appl. Environ. Microbiol. 55 (1), 224–228.

Wang, X., Gao, Q.Q., Bao, J., 2015. Transcriptional analysis of *Amorphotheca resinae* ZN1 on biological degradation of furfural and 5-hydroxymethylfurfural derived from lignocellulose pretreatment. Biotechnol. Biofuels 8, 136.

Yi, X., Zhang, P., Sun, J., Tu, Y., Gao, Q.Q., Zhang, J., Bao, J., 2016. Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock. J. Biotechnol. 217, 112–121.

Yoshida, S., Okano, K., Tanaka, T., Ogino, C., Kondo, A., 2011. Homo-D-lactic acid production from mixed sugars using xylose-assimilating operon-integrated *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 92, 67–76.

Zhang, J., Chu, D.Q., Huang, J., Yu, Z.C., Dai, G.C., Bao, J., 2010b. Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor. Biotechnol. Bioeng. 105 (4), 718–728.

Zhang, Z.T., Xie, Y.J., He, X.L., Li, X.L., Hu, J.L., Ruan, Z.Y., Zhao, S.M., Peng, N., Liang, Y.X., 2016. Comparison of high-titer lactic acid fermentation from NaOH and NH_3-H_2O_2-pretreated corncob by *Bacillus coagulans* using simultaneous saccharification and fermentation. Sci. Rep. 6, 37245.

Zhang, J., Zhu, Z.N., Wang, X.F., Wang, N., Wang, W., Bao, J., 2010a. Biodetoxification of toxins generated from lignocellulose pretreatment using a newly isolated fungus *Amorphotheca resinae* ZN1 and the consequent ethanol fermentation. Biotechnol. Biofuels 3, 26.

Zhang, J., Wang, X.S., Chu, D.Q., He, Y.Q., Bao, J., 2011. Dry pretreatment of lignocellulose with extremely low steam and water usage for bioethanol production. Bioresour. Technol. 102, 4480–4488.