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

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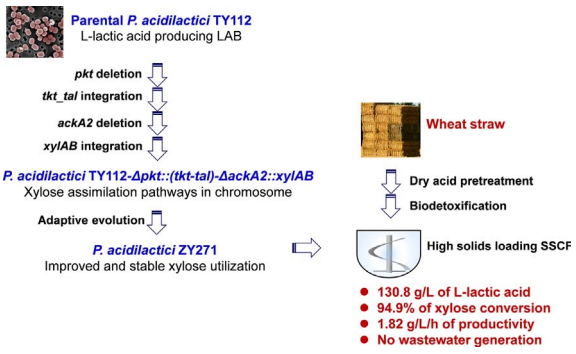
GRAPHICAL ABSTRACT



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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