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Increasing the chiral purity of cellulosic L-lactic acid by D-Lactate oxidase-catalyzed oxidation in engineered lactic acid bacterium *Pediococcus acidilactici*

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ABSTRACT

Trace amounts of D-lactic acid derived from lignocellulose feedstock and nitrogen additives frequently reduce the chiral purity of L-lactic acid below the polymerization standard during biorefinery fermentations. The spontaneous D-lactic acid degradation catalyzed by the highly substrate specific D-lactate oxidase in lactic acid bacterium provides an ideal solution. This study used a D-lactate oxidase GOX2071 with relatively high activity for construction of D-lactic acid oxidation pathway in the L-lactic acid producing strain *Pediococcus acidilactici* ZY271. The engineered *P. acidilactici* LJ2071 strain demonstrated an active D-lactic acid oxidation performance while the high L-lactic acid production property was well maintained. A L-lactic acid chiral purity of 99.63 % was obtained using the D-lactic acid containing wheat straw as feedstock and 40 % of corn steep liquor (CSL) as nitrogen additive to alternate expensive yeast extract. This study provided a practical method for upgrading the chirality of cellulosic L-lactic acid using lignocellulose feedstock and cheap nitrogen additives.

1. Introduction

High chiral purity of L-lactic acid is essential for chiral lactide synthesis and subsequent ring-opening polymerization for production of polylactic acid (PLA) (Abdel-Rahman and Sonomoto, 2016). The chirality of cellulosic L-lactic acid using lignocellulose as carbohydrate feedstock is highly affected by the trace amount of D-lactic acid contained in lignocellulosic feedstock (Guo et al., 2024). Furthermore, lactic acid fermentation by lactic acid bacterium (LAB) generally requires high nitrogen additives as amino acid supplementation. The average prices of nitrogen additives such as industrial grade yeast extract, corn steep liquor, and soybean meals were 3938.1, 98.5, and 395.2 USD/ton respectively according to local suppliers (Zhang et al., 2024). The use of expensive yeast extract (YE) additives significantly increases the production cost of L-lactic acid (De la Torre et al., 2018), and the use of alternative nitrogen additives such as soybean or cottonseed meals contain less amino acid nutrients than the required amount by lactic acid bacterium (Zhang et al., 2024). However, the most commonly used cheap nitrogen additives such as corn steep liquor (CSL) is unable to be used because of high racemic lactic acid contents (14 %-20 %) (Zhang et al., 2022; Okano et al., 2022). For these two reasons, endowing L-lactic acid-producing strains with spontaneous D-lactic acid degradation capacity is an effective strategy to cut down the production cost of L-lactic acid and the final PLA products by reducing the D-lactic acid content in L-lactic acid to meet the required L-lactic acid chirality of 99.5 % or greater (Chen et al., 2001; Litchfield., 2009).

Lactate oxidase (LOX) is a soluble flavin protein with strict chiral substrate-specific property that catalyzes the oxidation of either L-lactic acid or D-lactic acid with molecular oxygen as a direct electron acceptor without the need of exogenous cofactors (Desguin et al., 2014; Maeda-Yorita et al., 1995). Okano et al. (2022) expressed L-lactate oxidase in *Lactobacillus plantarum* KOLP7 and 0.52 g/L of L-lactic acid derived from CSL additive was removed, resulting in the D-lactic acid product with over 99.9 % chiral purity. However, unlike L-lactate oxidase with relative rich natural existences, D-lactate oxidase for D-lactic acid oxidation is a rarely identified protein. To our knowledge, GOX2071 derived from *Gluconobacter oxydans* 621 H is the only confirmed D-lactate oxidase by in vitro assessment for catalysis of D-lactic acid oxidation (Sheng et al., 2015). Different from L-lactate oxidase with flavin mononucleotide (FMN) as cofactor, D-lactate oxidase uses flavin adenine dinucleotide (FAD) as cofactor to catalyze the oxidation of D-lactic acid and produce pyruvate and H_2O_2, although it is considered to have a similar ping-pong reaction mechanism to L-lactate oxidase (Sheng et al., 2015; Sheng et al., 2016). No previous study was

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Table 1
Strains, plasmids and primers used in this study.

(a) Strains

	Characteristics	Sources
Escherichia coli BL21	Host for plasmid construction and inducible expression	Lab storage
E. coli XLI-blue	Host for plasmid construction	Lab storage
Gluconobacter oxydans 621 H	Putative D-lactate oxidase gene *gox2071* and catalase gene *gox1138* contained	Lab storage
Lactobacillus plantarum ATCC8014	Putative D-lactate oxidase gene *lploxd* contained	Lab storage
Pediococcus acidilactici ZY271	L-lactic acid producing strain with xylose assimilation pathway	Lab storage
P. acidilactici LJ2071	Integration of D-lactate oxidase *gox2071* into *ackA3* locus of *P. acidilactici* ZY271	This work

(b) Plasmids

	Characteristics	Sources
pET28a	Inducible expression vector for *E. coli* BL21, T7 promotor	Lab storage
pET28a-*gox2071*	Inducible expression plasmid for GOX2071 from *G. oxydans* 621 H	This work
pET28a-*lploxd*	Inducible expression plasmid for LpLoxD from *L. plantarum* ATCC8014	This work
pET28a-*fadpro*	Inducible expression plasmid for FAD binding protein from *L. paracasei* ATCC 334	Synthesized
pET28a-*glcD*	Inducible expression plasmid for GlcD from *S. inulinus* NBRC 13595	Synthesized
pZY36e	Expression vector for *P. acidilactici*	Lab storage
pZY36e-*gox2071*	Expression plasmid for GOX2071 from *G. oxydans* 621 H in *P. acidilactici* ZY271	This work
pZY36e-*lploxd*	Expression plasmid for LpLoxD from *L. plantarum* ATCC8014 in *P. acidilactici* ZY271	This work
pZY36e-*gox1138*	Expression plasmid for *gox1138* in *P. acidilactici* LJ2071	This work
pSET4E	Em^r replacing Spc^r marker of pSET4E, temperature sensitive vector	Lab storage
pSET4E-Δ*ackA3*::*gox2071*	Plasmid for integration cassette *PldhD_gox2071* into *ackA3* locus of *P. acidilactici* ZY271	This work

(c) Primers

Forward	Sequences (5'→3')	Reverse	Sequences (5'→3')
gox2071-F-28a	GAATTCGAGCTCATGCCGGAACCAGTCA	*gox2071*-R-28a	GCTGTTTACACGGGCTGACTCGAGCACC
lploxd -F-28a	CGAGCTCTTGACAGTCTTTCGCGCA	*lploxd* -R-28a	CTCGAGCGGTTAATTGATTTTACGTTTCGGGTTCAAAATATTC
Series28a-F	ATACGACTCACTATAGGGGAATT	Series28a-R	CCGGATATAGTTCCTCCTTTCA
gox2071-F-36e	TAATATTACATCTAGAATGCCGGAACCAGTCATGAC	*gox2071*-R-36e	ATGCCTGCAGGTCGACTCAGCCCGTGTAAACAGCACCGG
lploxd -F-36e	TAATATTACATCTAGATTGACAGTCTTTCGCGCATTTTCT	*lploxd* -R-36e	ATGCCTGCAGGTCGACTTAATTGATTTTACGTTTCGGGTTCAAAATATTCATCG
gox1138-F-36e	AAAAAAGAAAGGGGTAATATTACATCTAGAATGAGCACGGACAAACGCAAAACCGTCACC	*gox1138*-R-36e	CTTTGCAAGCTTGCATGCCTGCAGGTCGACTCAGACAGCGTGGGTTCTGGCTTCCCGGTC
Series36e-F	CAAGGCGCTAAATATACCCG	Series36e-R	ATCTCAACAATGTGAAGTCAGC
F-up-*ackA3*	AGTGAATTCGAGCTCGGTACCCGGGGATCAATTCGCTTCTTTGGTGAAAAACTTTCGGGA	R-up-*ackA3*	TGCAATCCCAATTCTCGAGCGGCGGGATCCCCTAATTACCTTCCTGAAAATCTTTATTTG
F-down-*ackA3*	GAAGGTAATTAGGGGATCCCGCCGCTCGAGAATTGGGATTGCAAATAGTACGTTTGATTA	R-down-*ackA3*	CAAGCTTGCATGCCTGCAGGTCGACTCTAGAAATCTGCAGCATACGCGCCAATGTAATGTT
F-*PldhD-gox2071*	AAGATTTTCAGGAAGGTAATTAGGGGATCCTGCTCTGGTGTGCAGACCAGACGTTGTACA	R-*PldhD-gox2071*	AACGTACTATTTGCAATCCCAATTCTCGAGTCAGCCCGTGTAAACAGCACCGGGCGGAAG
Series4e-F	AAAACCTCTGACACATGCAG	Series4e-R	ATCCCCTGATTCTGTGGATAA
Homo-*ackA3*-F	TGCGTAAATTACAATCCCAAATCA	Homo-*ackA3*-R	CGACTCTACTTCCGAGACTT

Note: The underline indicates the digestion site.

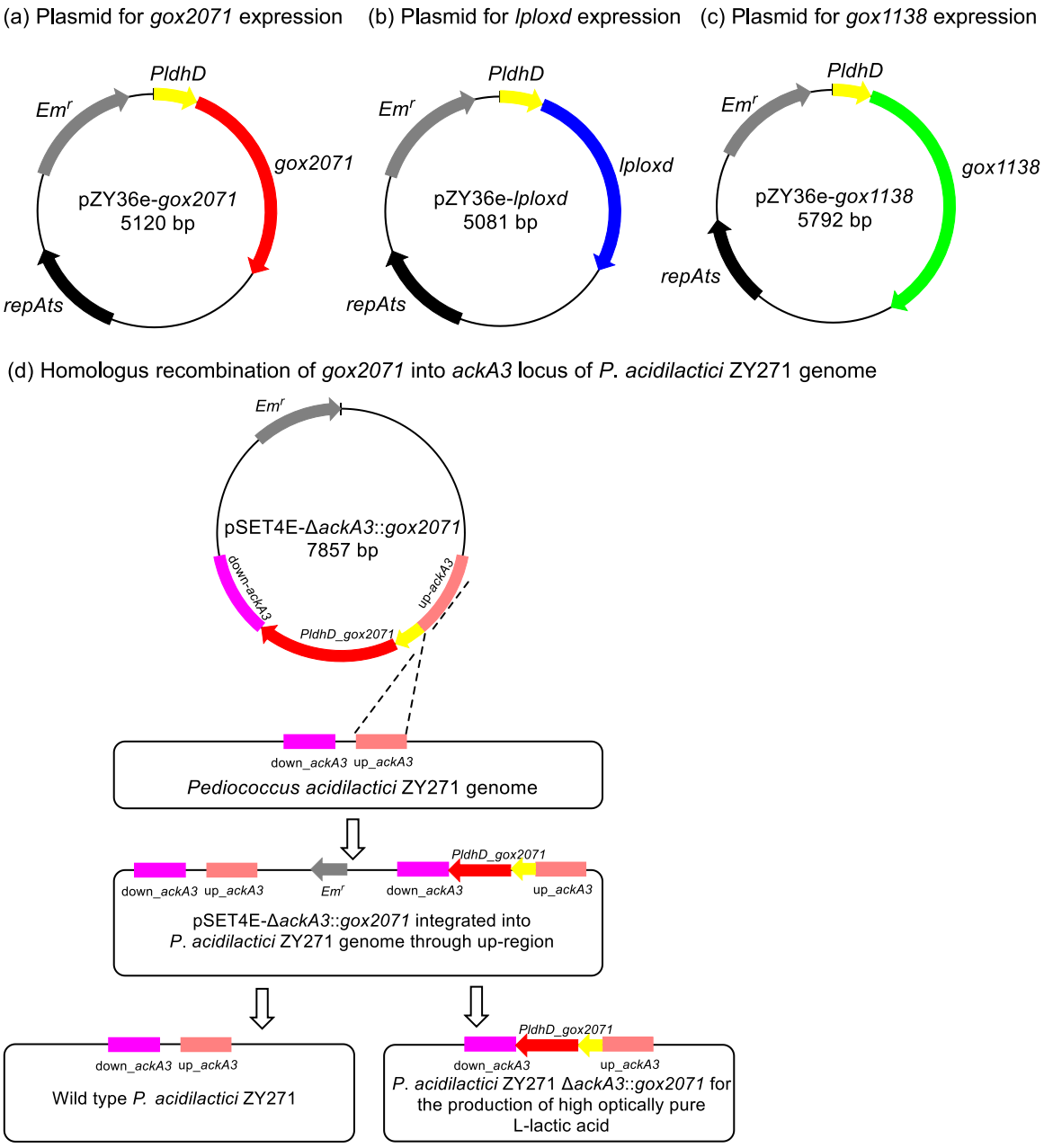

Fig. 1. Detailed maps of plasmid construction and genome integration. (a) Plasmid for *gox2071* expression in pZY36e vector; (b) plasmid for *lplox* expression in pZY36e vector; (c) Plasmid for *gox1138* expression in pZY36e vector; (d) homologus recombination of *gox2071* into *ackA3* locus of *P. acidilactici* ZY271 genome.

Table 2
Putative D-lactate oxidase genes and the D-lactic acid oxidation assay.

Proteins	Strain sources	D-lactate activity of crude enzyme (U/mg)	D-lactic acid conversion by engineered *P. acidilactici* (%)	Residual D-lactic acid by engineered *P. acidilactici* (g/L)
GOX2071 (WP_024717105.1)	*Gluconobacter oxydans* 621 H	0.12	28.2 ± 3.5	0.539 ± 0.026
LploxD (WP_015825086.1)	*Lactobacillus plantarum* ATCC8014	Inclusion body	10.7 ± 5.4	0.670 ± 0.041
FADpro (WP_011674855.1)	*Lacticaseibacillus paracasei* ATCC 334	Not detected	/	/
GlcD (WP_047035604.1)	*Sporolactobacillus inulinus* NBRC 13595	Not detected	/	/

reported on D-lactic acid oxidation in L-lactic acid bacterium strains to elevate the chiral purity production of L-lactic acid.

This study mined the potential candidates of D-lactate oxidase genes based on the available amino acid sequence of the only identified GOX2071 protein and then overexpressed the most favorable protein in a high L-lactic acid-producing strain *Pediococcus acidilactici* ZY271. The

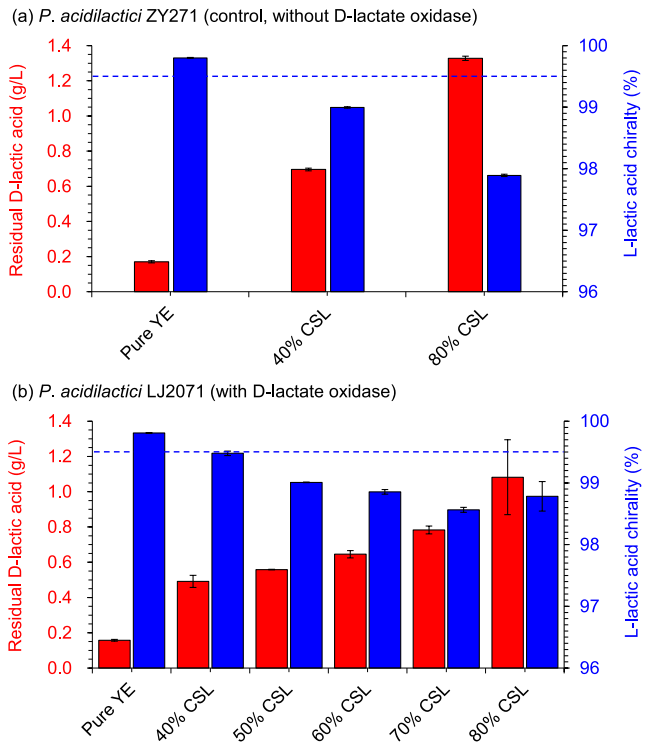

Fig. 2. Assay of D-lactic acid conversion by *P. acidilactici* LJ2071 using corn steep liquor with high D-lactic acid content. Fermentation was carried out in 250 mL shaking flasks containing 50 mL of simplified sugar-free MRS medium with initial sugar concentration of 80 g/L glucose and 40 g/L xylose under the condition of 42 ℃ and 150 rpm for 48 h. pH was adjusted to 5.5 with 0.6 g $CaCO_3$ per gram of sugars.

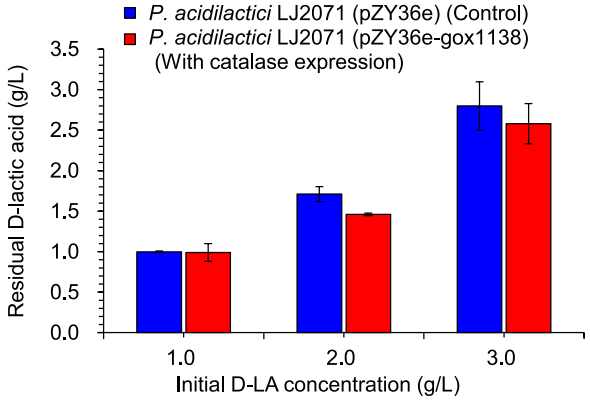

Fig. 3. Impact of H_2O_2 on D-lactic acid conversion by *P. acidilactici* LJ2071. Conditions: D-lactic acid conversion was performed in 250 mL shaking flasks containing 50 mL of simplified sugar-free MRS medium with sugar concentration of 40 g/L glucose and 20 g/L xylose under the condition of 42 ℃ and 150 rpm. The pH was adjusted to 5.5 with 0.6 g $CaCO_3$/g sugar for 48 h. The initial D-lactic acid concentration was set at 1.0, 2.0, and 3.0 g/L respectively.

engineered strain was able to oxidize minor D-lactic acid in wheat straw feedstock or in cheap nitrogen additives, leading to an elevated L-lactic acid chirality to 99.63 %, which met the need for L-lactide synthesis. The result provides a practical method for upgrading the chirality of cellulosic L-lactic acid using lignocellulose feedstock and cheap nitrogen additives.

2. Materials and methods

2.1. Strains, media and culture conditions

The microorganisms used in this study are shown in Table 1. The genes encoding for putative D-lactate oxidases WP_024717105.1 and WP_015825086.1 were derived from *Gluconobacter oxydans* 621 H (GenBank Accession Number: CP000009.1) and *Lactobacillus plantarum* ATCC8014 (GenBank Accession Number: CP024413.1), respectively. *P. acidilactici* ZY271 (CGMCC 13611) is an L-lactic acid-producing strain constructed in the previous work (Qiu et al., 2018).

E. coli BL21 and *E. coli* XLI-blue were cultivated in LB medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl at 37 ℃, 200 rpm. The solid medium used in petri dishes was added by an extra 20 g/L agar. *E. coli* BL21 carrying the inducible expression plasmid pET28a(+) required the addition of kanamycin (~50 μg/mL). *E. coli* XLI-blue carrying the expression plasmid pZY36e and the temperature-sensitive shuttle plasmid pSET4E required the addition of erythromycin (~200 μg/mL in liquid medium and ~400 μg/mL on solid medium). *P. acidilactici* ZY271 and *L. plantarum* ATCC8014 were cultured in the simplified MRS medium containing 20 g/L glucose, 10 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium acetate, 2 g/L diammonium hydrogen citrate, 2 g/L K_2HPO_4, 0.58 g/L $MgSO_4{\cdot}7\ H_2O$, and 0.25 g/L $MnSO_4{\cdot}H_2O$. *P. acidilactici* ZY271 was cultured at 42 ℃ and 150 rpm. *L. plantarum* ATCC8014 was cultured at 37 ℃ and 180 rpm. *G. oxydans* 621 H was cultured in the YS medium containing 80 g/L sorbitol, 20 g/L yeast extract, 0.5 g/L $MgSO_4{\cdot}7\ H_2O$, 1.5 g/L $KH_2PO_4{\cdot}3\ H_2O$, and 1.5 g/L $(NH4)_2SO_4$ at 30 ℃, 200 rpm.

2.2. Enzymes and reagents

Commercial cellulase Cellic CTec 3HS (CTec3) was purchased from Novozymes China, Beijing, China. The filter paper activity (256.1 FPU/mL) were determined by NREL protocols LAP-006 (Adney and Baker, 1996) and the method of Ghose (Ghose, 1987). The protein concentration (90.1 mg/ mL) was determined by Bradford method (Bradford, 1976).

Yeast extract and tryptone were purchased from Oxoid, Hampshire, UK. Industrial grade yeast extract was purchased from Angelyeast, Yichang, Hubei, China. Liquid corn steep liquor (CSL) was provided by Cathay Industrial Biotech Inc, Shanghai, China, with 37.53 % (w/w) of dry matter content, 2.59 % of the total nitrogen and 0.37 % of the ammonia nitrogen based on the dry CSL weight.

2.3. Plasmid construction and genome integration

All plasmids and primers used in this study are listed in Table 1. The expression plasmid pET28a(+) was used in *E. coli* BL21 (DE3); pZY36e for gene overexpression (Qiu et al., 2020) and pSET4E for gene integration (Yi et al., 2016) were used in *P. acidilactici*. The inducible expression plasmids with pET28a(+) backbone adopted SacI and XhoI as digestion sites. The putative D-lactate oxidase genes were amplified from the genomes of *G. oxydans* 621 H and *L. plantarum* ATCC8014 using primers *gox2071*-F-28a, *gox2071*-R-28a and *lploxd*-F-28a, *lploxd*-R-28a, respectively. The DNA fragments were ligated with linearized vector pET28a(+). The plasmids pET28a-*fadpro* and pET28a-*glcD* were synthesized directly by Tsingke, Shanghai, China. The plasmids used were transformed to *E. coli* by $CaCl_2$ method.

The overexpression plasmids were constructed in pZY36e with SalI and XbaI as the digestion sites. The putative D-lactate oxidase genes were amplified from the genomes of *G. oxydans* 621 H and *L. plantarum* ATCC8014 using the primers *gox2071*-F-36e, *gox2071*-R-36e and *lploxd*-F-36e, *lploxd*-R-36e, respectively. The catalase gene was amplified from the genome of *G. oxydans* 621 H using the primers *gox1138*-F-36e, *gox1138*-R-36e. The DNA fragments were ligated with the linearized vector pZY36e (Figs. 1a-1c). The plasmids were transformed to *P.*

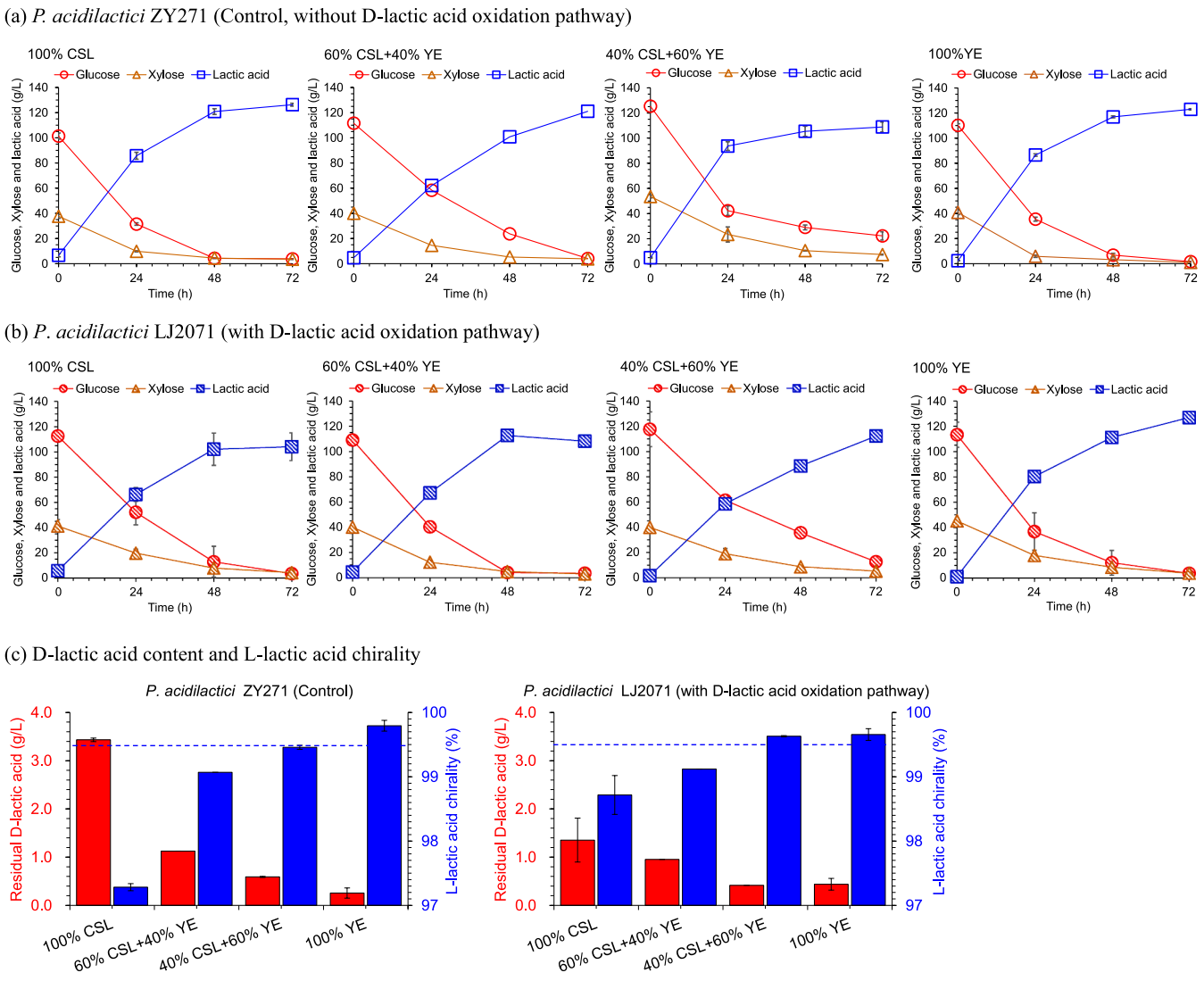

Fig. 4. D-lactic acid conversion in L-lactic acid fermentation using wheat straw feedstock and corn steep liquor. (a) Cellulosic L-lactic acid fermentation by *P. acidilactici* ZY271 (control) without D-lactic acid oxidation pathway. (b) Cellulosic L-lactic acid fermentation by *P. acidilactici* LJ2071 with D-lactic acid oxidation pathway. (c) Residual D-lactic acid and chirality of L-lactic acid products of the control *P. acidilactici* ZY271 and *P. acidilactici* LJ2071. Conditions: fermentation was conducted in 3 L bioreactor with 30 % (w/w) solids loading of the pretreated and detoxified wheat straw at 42 ℃ and 300 rpm for 72 h. Totally 25 g/L of corn steep liquor and (or) yeast extract (DM) was used as organic nitrogen source. 10 g/L ammonium sulfate and 0.25 g/L manganese sulfate monohydrate were used as nutrition. pH was adjusted to 5.5 with 25 % (w/v) $Ca(OH)_2$ slurry.

acidilactici by electrotransformation. The temperature-sensitive shuttle plasmid pSET4E was used for the integration of *gox2071* gene into the *ackA3* locus of the genome of *P. acidilactici* ZY271 (Fig. 1d). Single colonies carrying the expression plasmids were inoculated into 5 mL of the fresh LB medium containing kanamycin and cultured overnight at 37 ℃ and 200 rpm. Then the broth was transferred to the fresh liquid medium at 1 % (v/v) inoculum and cultured for ~3 h till the OD_{600} of 0.4–0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.1 mM to induce the expression of putative D-lactate oxidases after 8 h at 30 ℃ and 180 rpm. The cells were collected and washed twice with PBS buffer (pH 7.5) and then re-collected by centrifugation at 12,000 rpm for 10 min at 4 ℃. The cells were re-suspended and for ultrasonically treated to release the proteins into the supernatant. The supernatants and cell debris were separated before the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. D-lactic acid oxidation assay

Putative D-Lactate oxidases were expressed using the plasmid pET-28a(+) in *E. coli* BL21 (DE3). The activity of the putative D-lactate oxidases was assayed in 50 mM Tris-HCl buffer (pH 7.5) with 0.80 g/L of D-lactic acid and 200 μL of the putative D-lactate oxidase crude enzyme (cell-free extract). The total crude protein concentration of the cell free extract was determined as 0.45 g/L by Bradford method (Bradford, 1976). The D-lactic acid oxidation reaction was carried out at 30 ℃, 200 rpm for 1 h, then the reaction system was boiled for 10 min. One unit (U) of D-lactate oxidase was defined as the amount of enzymes to catalyze the oxidation of 1 μmol D-lactic acid in one minute.

Putative D-Lactate oxidases were expressed using the plasmid pZY36e in *P. acidilactici* ZY271. The D-lactic acid conversion was assayed in 50 mL simplified MRS medium containing 20 g/L of glucose and 0.75 g/L of D-lactic acid in a 250 mL shaking flask at 42 ℃, 150 rpm for 48 h with an inoculum of 10 % (v/v). pH was adjusted to 5.5 with the addition of 0.6 g $CaCO_3$ per gram of sugars.

2.5. Wheat straw feedstock and biorefinery operations

Wheat straw was harvested from Yangqu county, Shanxi province, China in fall of 2024 provided by Cathay Biotech Inc in Bioindustry Park, Taiyuan, Shanxi Province, China. Wheat straw was mechanically milled into the pieces with the average length of 10–30 mm before pretreatment operation. The soil and ash in the wheat straw was vibrationally removed during the mechanic milling. The composition of the raw wheat straw included 31.2 % cellulose, 24.3 % xylan, 19.4 % lignin and 9.6 % ash as measured by two-step acid digestion method according to NREL (Sluiter et al., 2008; Sluiter et al., 2012).

The wheat straw was pretreated in a 10 m^3 industrial reactor in the Bioindustry Park, Taiyuan, Shanxi Province. Briefly, dry wheat straw and dilute sulfuric acid solution at a solid-liquid ratio of 2:1 (w/w) were co-currently fed into the pretreatment reactor equipped with a single helical impeller under the condition of 175 °C and 10 rpm for 5 min. The sulfuric acid dosage was 40 mg per gram dry wheat straw. Pre-hydrolysis was carried out in a 5 L bioreactor with helical impeller stirring at 50 ℃ and 200 rpm for 12 h at a 30 % (w/w) high solid loading.The cellulase dosage was 4 mg total cellulase proteins per gram dry wheat straw. The slurry was immediately transferred to 3 L bioreactor for submerged liquid detoxification by adding the bio-detoxification strain *Paecilomyces variotii* FN89 at an inoculum of 10 % (v/v) with 750 rpm stirring and 1 vvm aeration. Then the L-lactic acid fermentation was initiated by inoculating 10 % (v/v) of *P. acidilactici* seed broth for simultaneous saccharification and co-fermentation (SSCF) at 42 ℃ and 300 rpm for 72 h. The nutrients addition included 25 g/L of corn steep liquor (dry base) and industrial grade yeast extract with varying ratio, 10 g/L of ammonium sulfate, and 0.25 g/L of manganese sulfate monohydrate. The pH value was maintained at 5.5 by adding 25 % (w/w) $Ca(OH)_2$ slurry.

2.6. Analytical methods

D-lactic acid content was determined using HPLC with UV detector SPD-20A (Shimadzu, Kyoto, Japan) and chiral column MCI GEL CRS10W (Mitsubishi, Kyoto, Japan) at 254 nm and 25 ℃. The mobile phase was 2 mM $CuSO_4$ with a flow rate of 0.5 mL/min.

Glucose, xylose and total lactic acids (both L- and D-lactic acid) were measured using HPLC with refractive index detector RID-10A (Shimadzu, Kyoto, Japan) and Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65 ℃. The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min.

2.7. Chiral purity calculation

The chirality of L-lactic acid is defined as the ratio of L-lactic acid content to the total lactic acid contents (the sum of L-lactic acid and D-lactic acid). L-lactic acid content is calculated as the total lactic acid content minus D-lactic acid content.

3. Results and discussion

3.1. Mining the putative D-lactate oxidases (D-LOX) for D-lactic acid oxidation

The putative D-lactate oxidase genes in lactic acid bacterium strains used for L-lactic acid fermentation were mined to search the homogenous genes for D-lactic acid removal. D-lactate oxidase GOX2071 from *G. oxydans* 621 H was found to contain two conserved domains, the FAD_binding_4 domain and the FAD-oxidase_C domain using online prediction tool SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/). Based on the amino acid sequences of the two conserved domains, the putative D-lactate oxidases were mined among lactic acid bacteria including *P. acidilactici*, *L. plantarum*, *Lacticaseibacillus paracasei*, *Sporolactobacillus inulinus,* and *Bacillus cogulans*.

The four putative D-lactate oxidase genes were identified as listed in Table 2. The enzyme activities of the crude proteins by overexpression the genes in *E. coli* and the D-lactic acid conversion by *P. acidilatici* were assayed. The *E. coli* overexpression showed that GOX2071 from *G. oxydans* 621 H, FADpro from *L. paracasei* ATCC 334, and GlcD from *S. inulinus* NBRC 13595 were soluble proteins, while LpLoxD from *L. plantarum* ATCC 8014 was mainly present as inclusion body. The enzyme activity was assayed under the initial D-lactic acid concentration of 10 mM (~0.9 g/L) according to Sheng et al. (2015) and Guo et al. (2024). The enzyme activity of GOX2071 was determined as 0.12 U/mg, while the activity of FADpro and GlcD were not detected, indicating that amino acid sequentce alignment of the two conserved domains were only structurally similar and the substrate specifity might be different (Tsvik et al., 2022).

GOX2071 and LpLoxD (inclusion body for *E. coli*, but still potentially active in lactic acid bacteria) were further overexpressed in *P. acidilactici* ZY271 using the plasmid pZY36e. The D-lactic acid conversions of the two recombinants *P. acidilactici* pZY36e-*gox2071* and *P. acidilactici* pZY36e-*lploxd* were assayed in shaking flasks with the initial D-lactic acid concentration of 0.75 g/L. *P. acidilactici* pZY36e-*gox2071* reduced the initial D-lactic acid from 0.75 g/L to 0.54 g/L after 48 h with a D-lactic acid conversion of 28.2 ± 3.5 %. For *P. acidilactici* pZY36e-*lploxd*, the D-lactic acid was only reduced from 0.75 g/L to 0.67 g/L at 48 h with a D-lactic acid conversion of 10.7 ± 5.4 % (Table 2).

The *gox2071* gene encoding GOX2071 was interated into the genome of *P. acidiactici* ZY271 at *ackA3* locus, resulting in an engineered strain *P. acidilactici* LJ2071. Firstly, the D-lactic acid removal were conducted in MRS medium using the D-lactic acid-containing corn steep liquor as nitrogen additive to replace the expensive yeast extract with the ratio (dry basis) of 0 %, 40 %, 50 %, 60 %, 70 %, and 80 % (Fig. 2). The residual D-lactic acid increased and the L-lactic acid chirality decreased with the increasing dosage of corn steep liquor. When yeast extract was used as the sole nitrogen source, the residual D-lactic acid was 0.157 g/L and the L-lactic acid chirality was 99.81 % by the newly constructed *P. acidilactici* LJ2071 strain, similar to that of 0.170 g/L of the residual D-lactic acid and 99.80 % of L-lactic acid chirality by the control strain *P. acidilactici* ZY271. When 40 % yeast extract was replaced by corn steep liquor (the total nitrogen additive content was maintained at 25 g/L), *P. acidilactici* LJ2071 converted 38.0 % of D-lactic acid (0.492 g/L of D-lactic acid residue) and achieved 99.48 % of the L-lactic acid chirality, while the control strain converted 12.7 % of D-lactic acid (0.696 g/L of D-lactic acid residue) and achieved 99.00 % of the L-lactic acid chirality. When 80 % of yeast extract was replaced by corn steep liquor, *P. acidilactici* LJ2071 converted D-lactic acid to the residual concentration of 1.082 g/L, resulting in a L-lactic acid chirality of 98.78 %, which was below the required L-lactic acid purity of 99.5 %for synthesis of L-lactide. The control strain *P. acidilactici* ZY271 maintained ~1.328 g/L of D-lactic acid during fermentation and even lower chirality of 97.89 %. These results indicated that engineered *P. acidilactici* LJ2071 was capable of degrading partial D-lactic acid derived from lignocellulose feedstock and corn steep liquor (up to 40 % of the total nitrogen additives) to the required L-lactic acid chirality (99.5 %), but the complete replacement of corn steep liquor to yeast extract was still not achieved (Fig. 2b).

3.2. Impact of hydrogen peroxide (H_2O_2) generation and decomposition on D-lactic acid oxidation by D-lactic acid oxidase

Hydrogen peroxide (H_2O_2) is the inevitable product of D-lactic acid oxidation by D-lactate oxidase and frequently inhibits the metabolic activity of microbial cells (Li et al., 2020; Sheng et al., 2015; Sheng et al., 2016). To alleviate toxic effect of H_2O_2 on lactic acid bacterium strain, a catalase gene *gox1138* from *G. oxydans* 621 H was overexpressed by the plasmid pZY36e in *P. acidilactici* LJ2071. Fig. 3 indicates that the *gox1138* expression in *P. acidilactici* LJ2071 (pZY36e-*gox1138*) exhibited 14.65 % and 7.84 % higher D-lactic acid conversion when D-lactic

acid content was at 2.0 or 3.0 g/L, respectively, compared to that of the control *P. acidilactici* LJ2071. However, when the D-lactic acid concentration was at 1.0 g/L, no significant change of D-lactic acid conversion was observed, probably due to the H_2O_2 at low concentration was degraded spontaneously without the participation of catalase.

These results indicated that the expression of catalase was able to further enhance relative conversion rate of D-lactic acid at high D-lactic acid concentrations (above 2.0–3.0 g/L). However, the typical concentration of D-lactic acid in cellulosic L-lactic acid fermentation broth during cellulose L-lactic acid fermentations was usually not high (usually less 1 g/L) (Guo et al., 2024), therefore the expression of catalase might not make observable changes on the enhancement of minor D-lactic acid oxidation.

3.3. Corn steep liquor as nitrogen source for L-lactic acid fermentation

The D-lactic acid oxidation property of *P. acidilactici* LJ2071 was applied to improve the chirality of L-lactic acid fermentation using wheat straw as feedstock and corn steep liquor as nitrogen additive (Fig. 4). The L- and D-lactic acid contents in the liquid corn steep liquor were determined as 38.8 ± 1.6 g/L and 58.8 ± 0.7 g/L, respectively. In the simultaneous saccharification and co-fermentation (SSCF) of L-lactic acid using the pretreated and biodetoxified wheat straw at 30 % solid loading (w/w), the L-lactic acid generation using corn steep liquor (CSL) as the nitrogen additive was similar to that of yeast extract (YE) as the additive in the range of 100 % CSL, 40 % CSL + 60 % YE, 60 % CSL + 40 % YE, and 100 % YE, by either the control strain *P. acidilactici* ZY271 (Fig. 4a) or the engineered strain *P. acidilactici* LJ2071 (Fig. 4b). The content of D-lactic acid decreased rapidly during the first 24 h by *P. acidilactici* LJ2071 but remain unchanged after 24 h. This result indicated that D-lactic acid was predominantly degraded during the initial phase of fermentation and the oxidation might be inhibited by high concentrations of L-lactic acid after 24 h. This inhibition was consistent with the previous results (Li et al., 2017). However, *P. acidilactici* LJ2071 with D-lactic acid oxidation pathway converted more D-lactic acid than that by the control strain *P. acidilactici* ZY271 without D-lactic acid oxidation pathway (Fig. 4c). The residual D-lactic acid content in the final fermentation broth by *P. acidilactici* LJ2071 were always lower than the control strain, resulting in the increased L-lactic acid chirality (98.72 % vs. 97.28 % by *P. acidilactici* LJ2071 and *P. acidilactici* ZY271, respectively, with 100 % corn steep liquor usage). When the corn steep liquor addition was 60 % of the total nitrogen additives, the chirality of L-lactic acid increased to 99.12 % by *P. acidilactici* LJ2071. When the corn steep liquor addition was 40 % of the total nitrogen additives, the L-lactic acid chirality reached to 99.63 % by *P. acidilactici* LJ2071. The results suggested that when corn steep liquor replacement to the expensive yeast extract was 40 % in the cellulosic L-lactic acid fermentation using lignocellulose as feedstock, the L-lactic acid chirality still exceeded 99.5 %, which met the standard requirement for L-lactide synthesis.

There have been few reports of the application of D-lactate-degrading enzymes (D-lactate oxidase and D-lactate dehydrogenase) in the production of chiral L-lactic acid. Shapira et al., (2022) directly added crude enzyme of GOX2071 to the L-lactic acid fermentation broth using mixed food organic waste as substrate by *Bacillus coagulan*, achieving over 99.5 % chirality of L-lactic acid. However, the production of crude enzyme of GOX2071 would account for an extra cost. Chauliac et al., (2015) also reported over 99.99 % chirality of L-lactic acid using the prolonged adapted *E. coli* to complete degrade 8.7 g/L of D-lactic acid in totally 135 g/L lactic acid syrup obtained after thermochemical hydrolysis of PLA beads. In this study, we first reported a D-lactic acid degrading and L-lactic acid producing engineered strain, enabling the (partial) use of cheap nitrogen additive and lignocellulose feedstock containing high D-lactic acid derived from storage contaminations (Guo et al., 2024) or other inevitable pathways. However, a complete conversion of D-lactic acid using corn steep liquor as nitrogen additive was not achieved by overexpressing the available D-lactate oxidase gene in *P. acidilactici*. The future investigations are on the way to mine or engineer the D-lactate oxidases with significantly improved catalytic activity by artificial intelligence (AI) assisted methodology.

4. Conclusion

This study engineered a D-lactate oxidase expressing *P. acidilactici* LJ 2071 strain with 99.63 % chirality of L-lactic acid using wheat straw feedstock and 40 % of cheap corn steep liquor (CSL) to replace yeast extract as nitrogen additive. The degradation of trace D-lactic acid derived from fermentation source by engineered L-lactic acid producing strain would be a new strategy to improve the chirality of cellulosic L-lactic acid.

CRediT authorship contribution statement

Jiao Liu: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Chaolong Qu:** Writing – review & editing, Methodology, Investigation. **Bin Zhang:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Jie Bao:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Data Availability

Data will be made available on request.

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