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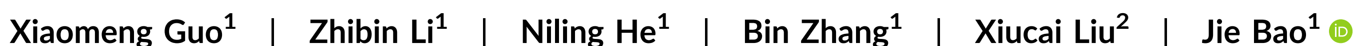
Received: 1 June 2023 | Revised: 5 September 2023 | Accepted: 1 October 2023
DOI: 10.1002/bit.28583

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING WILEY

Detection and elimination of trace D-lactic acid in lignocellulose biorefining chain: Generation, flow, and impact on chiral lactide synthesis



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Funding information
National Natural Science Foundation of China, Grant/Award Number: 21978083; Yangfan Project of Science and Technology Committee of Shanghai Municipality, Grant/Award Number: 23YF1409900

Abstract

High chiral purity of lactic acid is a crucial indicator for the synthesis of chiral lactide as the primary intermediate chemical for ring-open polymerization of high molecular weight polylactic acid (PLA). Lignocellulose biomass is the most promising carbohydrate feedstock for commercial production of PLA, but the presence of trace D-lactic acid in the biorefinery chain adversely affects the synthesis and quality of chiral lactide. This study analyzed the fingerprint of trace D-lactic acid in the biorefinery chain and found that the major source of D-lactic acid comes from lignocellulose feedstock. The naturally occurring lactic acid bacteria and water-soluble carbohydrates in lignocellulose feedstock provide the necessary conditions for D-lactic acid generation. Three strategies were proposed to eliminate the generation pathway of D-lactic acid, including reduction of moisture content, conversion of water-soluble carbohydrates to furan aldehydes in pretreatment, and conversion to L-lactic acid by inoculating engineered L-lactic acid bacteria. The natural reduction of lactic acid content in lignocellulose feedstock during storage was observed due to the lactate oxidase-catalyzed oxidation of L- and D-lactic acids. This study provided an important support for the production of cellulosic L-lactic acid with high chiral purity.

KEYWORDS
biorefinery chain, chiral lactic acid, D-lactic acid, elimination, fingerprint, lignocellulose

1 | INTRODUCTION

Production of biodegradable polylactic acid (PLA) is composed of multiple steps including fermentative production of chiral lactic acid (L- or D-lactic acids), polycondensation/depolymerization of chiral lactic acid to cyclic lactide, and ring-opening polymerization of lactide to PLA (Balla et al., 2021; Lasprilla et al., 2012; Van Wouwe et al., 2016). Currently, chiral lactic acid is predominantly produced by fermentation using glucose derived from corn or other food crops as carbohydrate feedstock. However, this dependence significantly limits the potential for replacing petroleum-derived polymer materials with PLA due to the limited quantity of food crops and policy restrictions on food crop use (Abdel-Banat et al., 2010). Lignocellulose biomass is a highly abundant and cost-effective alternative to food crop carbohydrates for commercial production of chiral lactic acid. Previous studies have demonstrated that dry biorefining technology converts lignocellulose into L- or D-lactic acids with similar technical key performance indicators and enables the synthesis of cyclic L- and D-lactides with high chiral and chemical purities from chiral lactic acids (He et al., 2022, 2023; Liu et al., 2018; Qiu et al., 2018; Yi et al., 2016; Zhang et al., 2021).

Xiaomeng Guo, Zhibin Li, and Niling He contributed equally to this study.

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High chiral purity (higher than 99.5%) of lactic acid is a crucial indicator for the synthesis of chiral lactide as the primary monomer for ring-open polymerization of high molecular weight PLA (Chen et al., 2001). When L-lactic acid is used for cyclic L-lactide synthesis, the presence of D-lactic acid inside L-lactic acid will result in the generation of meso-lactide and consequently affect the physical and chemical properties of PLA (Van Wouwe et al., 2016). Our earlier studies reveal that trace amounts of D-lactic acid (typically ranging from 0.2% to 0.6%) were frequently discovered in L-lactic acid fermentation broth when lignocellulose feedstock was used (He et al., 2022; Zhang et al., 2022).

Completely removing trace amounts of D-lactic acid from L-lactic acid broth in downstream processes is not a feasible option. The apparent viscosity of cellulosic lactic acid fermentation broth is more than an order of magnitude greater than that of corn lactic acid broth, due to the presence of lignin solid particles (Hou et al., 2019). Separating L- and D-lactic acids by chiral chromatography is not only expensive but also practically not possible for solid particles containing lactic acid broth. The elimination of trace D-lactic acid by L-lactic acid fermentation strains is also a great challenge for metabolic engineering, and to date, no successful results have been reported. Therefore, the most practical method to eliminate the D-lactic acid in L-lactic acid broth is to identify and completely cut off its generation pathways in the biorefinery processing chain.

Three potential pathways may allow the generation of D-lactic acid in the biorefinery chain: (i) synthesis by L-lactic acid fermentation strains; (ii) introduction from external sources; and (iii) synthesis within lignocellulose feedstock during collection and storage. Regarding the first pathway, most L-lactic acid fermentations currently use engineered strains with D-lactate dehydrogenase (LDH) gene(s) knocked out (Kong et al., 2019; Qiu et al., 2018; Tian et al., 2021). This modification allowed for an over 99.6% chirality of L-lactic acid as in our earlier cases (Qiu et al., 2018); thus, this pathway should be safely eliminated. The second pathway for D-lactic acid generation primarily involves the supplementations of various nitrogen and nutrient substances during fermentations, such as corn steep liquor (CSL) containing mixed L- and D-lactic acids up to 20% of dry weight (Zhang et al., 2022). However, this risk has been well acknowledged and nutrient materials containing lactic acid have been banned completely, such as the use of CSL. Thus, the possibility from the second pathway should be insignificant. The risk of D-lactic acid generation by the third pathway has been underestimated. Lignocellulose feedstocks such as corn stover or wheat straw usually contain 4%–12% of water-soluble carbohydrates (glucose, fructose, and sucrose) (Chen et al., 2007; Niu et al., 2016), as well as diverse wild lactic acid bacteria (LABs) naturally occurring on lignocellulose biomass (Fang et al., 2022). Most LABs are racemic microbes, generating both L- and D-lactic acids with varying ratios of L- and D-lactic acids. The presence of water-soluble carbohydrates and wild-type LABs in lignocellulose could result in the generation of both L- and D-lactic acids. As a result, the chiral purity of L-lactic acid in the fermentation broth could be reduced by 5%–10% (He et al., 2022; Qiu et al., 2018).

In this study, trace D-lactic acid fingerprint in the biorefinery processing chain was identified from the collection of lignocellulose feedstocks to the transportation and storage of raw material, pretreatment, biodetoxification, saccharification, and lactic acid fermentation. Lignocellulose feedstock was found to be the primary source of D-lactic acid generation due to the existence of naturally occurring LABs and water-soluble carbohydrates. Three strategies were proposed and tested to eliminate the D-lactic acid generation in the biorefinery chain. This study provides strong technical support for the production of high chiral purity L-lactic acid from lignocellulose feedstocks.

2 | MATERIALS AND METHODS

2.1 | Raw materials, enzymes, and reagents

Wheat straw and corn stover were harvested from Nanyang, Henan, China, in autumn 2021 and 2020, respectively, and rice straw was harvested from Yichun, Jiangxi, China, in summer 2019. The feedstocks were coarsely chopped and milled to pass through a mesh 10 mm in diameter and then sealed into a plastic bag and stored at ambient temperature.

Corn fiber was obtained from Juneng Golden Corn Co. in 2020 as the by-product from wet milling of corn. Bamboo powder was collected from a bamboo processing factory in Yixing in 2020.

Commercial cellulase Cellic CTec2.0 was purchased from Novozymes. The filter paper activity, cellobiase activity, and protein content were 203.2 filter paper unit/mL (Adney & Baker, 1996), 4900 cellobiase unit/mL (Ghose, 1987), and 87.3 mg/mL (Bradford, 1976), respectively. Glucoamylase GA-L NEW was purchased from Genencor and the enzymatic activity was 103,900 WU/mL according to the manufacturer's instructions.

Peptone and yeast extract were purchased from Oxford. Soybean meal was purchased from China Oil and Foodstuffs Co. Cottonseed meal was purchased from Jiahui Feed Co. The other chemical reagents were purchased from Sinopharm Reagent Co.

2.2 | Microorganisms and culture media

Pediococcus acidilactici ZY271 (CGMCC 13611) (Qiu et al., 2018) was used for converting water-soluble monosaccharides into L-lactic acid during feedstock storage and producing cellulosic L-lactic acid.

P. acidilactici DQ2 (CGMCC 7471) (Zhao et al., 2013) was used for converting water-soluble monosaccharides into mixed L-lactic acid and D-lactic acid during feedstock storage.

P. acidilactici strains were cultured at 42°C with shaking at 150 rpm in a simplified Man–Rogosa–Sharp (MRS) medium containing 20.0 g/L glucose, 10.0 g/L peptone, 10.0 g/L yeast extract, 5.0 g/L sodium acetate, 2.0 g/L ammonium citrate dibasic, 2.0 g/L dipotassium phosphate, 0.58 g/L magnesium sulfate heptahydrate, and 0.25 g/L manganese sulfate monohydrate (He et al., 2022).

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Glucoamylase was also added at 1% (v/v) during the seed culture to avoid cell flocculation (Liu et al., 2015).

Paecilomyces variotii FN89 (CGMCC 17665) (Zhang et al., 2021) was used as a biodetoxification strain to remove the inhibitory compounds. A vial of the fungus was cultured on potato dextrose agar medium at 37°C for 2–3 days. Then, the spores (~3×10^8) were transferred into a synthetic medium (20 g/L glucose, 1.0 g/L yeast extract, 2.0 g/L potassium dihydrogen phosphate, 1.0 g/L ammonium sulfate, and 1.0 g/L magnesium sulfate heptahydrate) and cultured at 37°C with shaking at 300 rpm for 16 h as the biodetoxification seed.

2.3 | Isolation and identification of LABs

To isolate lactic acid-producing microbials from lignocellulose feedstocks, 10 g of wheat straw, corn stover, rice straw, bamboo powder, or corn fiber was mixed with 140 mL of sterile water in 250 mL shake flask. The mixtures were incubated at 37°C with shaking at 150 rpm for 1 h. A volume of 10 mL of the extracted supernatant liquid was collected and added to 50 mL of MRS medium and then cultured at 42°C with shaking at 150 rpm for 12 h. The MRS medium contained 22.0 g/L glucose, 10.0 g/L peptone, 4.0 g/L yeast extract, 8.0 g/L beef extract, 3.0 g/L sodium acetate, 2.0 g/L ammonium citrate dibasic, 2.0 g/L dipotassium phosphate, 0.2 g/L magnesium sulfate heptahydrate, 0.05 g/L manganese sulfate monohydrate, and 1 mL/L Tween-80.

The enriched culture broth was diluted ranging from 10^{-1}- to 10^{-9}-fold and then spread on the MRS agar gel containing 1% (w/v) $CaCO_3$ and 20 g/L agar. Later, the MRS agar gel plates were incubated for 2–3 days at 42°C for the growth of microbial colonies. Colonies with lactic acid generation were initially identified and observed to form transparent circles by dissolving the fine particles of $CaCO_3$ within the gel. Eight single colonies with transparent circles were picked from the dispersed gels of five types of lignocellulose feedstocks (wheat straw, corn stover, rice straw, bamboo powder, or corn fiber).

Each isolated colony was inoculated into a test tube containing 5 mL MRS medium and incubated for 12 h at 42°C. Samples were collected to measure the generation of lactic acid. Then, the culture broth was restreaked onto the agar plates and incubated at 42°C for 2–3 days to complete the first round of purification culture. This process was repeated two more times, resulting in three rounds of purification culture to obtain the purified single colony.

These single colonies were cultured in the MRS medium and then the cells were collected to extract the genomic DNA by TIANamp Bacteria DNA Kit (Tiangen Biotech). The 16S ribosomal DNA (rDNA) genes were amplified by PCR using universal primers 27F (5′-AGAG TTTGATCCTGGCTCAG-3′) and 149R (5′-GGTTACCTTGTTACGAC TT-3′) and then were sequenced and blasted in the NCBI GenBank (Kang et al., 2020; Kanklai et al., 2020; Meidong et al., 2021). The evolutionary distance was calculated using the Kimura 2-parameter model in MEGA 11 software, and the phylogenetic tree was constructed based on the neighbor-joining method.

2.4 | Storage and lactic acid removal operation

The freshly harvested wheat straw contained 22.75 ± 1.77% of water. Based on the dry weight of wheat straw, the glucose content was 14.65 ± 0.32 mg/g dry wheat straw matter (DM) and the fructose was 24.22 ± 3.06 mg/g DM. The L-lactic acid and D-lactic acid contents were 1.18 ± 0.06 and 0.54 ± 0.01 mg/g DM, respectively. According to NREL LAP-002 and LAP-005, the contents of cellulose, xylan, lignin, and ash in wheat straw were measured as 34.31 ± 0.14%, 21.30 ± 1.78%, 22.12 ± 0.07%, 10.63 ± 0.28%, respectively (Sluiter et al., 2008, 2012).

Two lactic acid removal treatments were conducted on the feedstocks: (i) inoculating 10% (v/w) of *P. acidilactici* ZY271 seed culture onto the freshly harvested wheat straw; (ii) completely washing the wheat straw in a washing machine (packing wheat straw into laundry bags) till no water-soluble carbohydrates or lactic acids were detected and then air-drying the wheat straw. Glucose, fructose, lactic acids, or lactic acid bacterium seed were added onto the clean wheat straw and well-mixed according to the experimental design. Then, the wheat straw was sealed into 80 × 60 cm airtight bags with the air pumped out. Samples were collected regularly from the stored wheat straw and then water-extracted by shaking at 150 rpm for 1 h at 30°C. The extracted supernatant was obtained after centrifuging at 11,167*g* for 5 min and the contents of lactic acid and residual sugar were determined.

For the seed solution, 3.82×10^{-3} mg/g DM D-lactic acid and 9.07×10^{-3} mg/g DM L-lactic acid were added on the raw material with a moisture content of 8%; 0.953 mg/g of DM glucose, 0.725 mg/g DM of total lactic acid, 0.181 mg/g DM of D-lactic acid, and 0.499 mg/g DM of L-lactic acid in the solution was added on the raw material with a moisture content of 15% and 25%. The experiments were conducted in parallel and the final data was calculated by taking the average.

2.5 | Dry biorefinery processing and L-lactic acid fermentation

Wheat straw was dry acid pretreated in a 20 L reactor with a single helical stirrer and steam jetting. The wheat straw was mixed with the dilute sulfuric acid solution at a weight ratio of 2:1 (w/w) and the sulfuric acid usage was 3.8% (w/w) of the dry wheat straw weight. The temperature was maintained at 175°C for 5 min with moderate mixing.

The pretreated wheat straw was adjusted to pH 5.5 by adding 20% (w/w) calcium hydroxide slurry and then enzymatically hydrolyzed in a 5 L bioreactor for 12 h at 50°C. The cellulase dosage was 5 mg protein/g DM and the wheat straw solid loadings was 30% (w/v). Then, the slurry was transferred into a 3 L bioreactor and inoculated with 10% (v/v) of the *P. variotii* FN89 seed. The biodetoxification was conducted at 37°C with an aeration rate of 1.0 vvm and then vigorously mixed by a Rushton stirrer till acetic acid,

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5-hydroxymethylfurfural (HMF), and furfural were not detected on high-performance liquid chromatography (HPLC).

L-Lactic acid fermentation was conducted by simultaneous saccharification and co-fermentation (SSCF) by *P. acidilactici* ZY271. Specifically, 10% (v/v) of the seed culture was inoculated and the nutrients were added with 10.0 g/L peptone, 15.0 g/L yeast extract, 2.0 g/L ammonium citrate dibasic, and 0.25 g/L manganese sulfate monohydrate. The fermentation was carried out for 72 h and samples were collected every 24 h. The pH was controlled at 5.5 by adding 25% (w/w) calcium hydroxide slurry.

2.6 | L-Lactide synthesis

The synthesis of L-lactide was conducted according to the method in our previous study (He et al., 2022). Briefly, a mixture of L-lactic acid and D-lactic acid in varying proportions was added to a 250 mL round bottom flask and heated at 80°C, 0.01 MPa for 30 min. After returning to normal pressure, 1.0% (v/w) stannous octoate was added. The temperature was slowly increased to 140°C and held for 1.5 h to remove the residual water. The temperature was then rapidly raised to 230–240°C under vacuum conditions (0.098 MPa) until no liquid remained in the flask and the resulting pale-yellow distillate crystallized into crude lactide. The crude lactide was dissolved in absolute ethanol at 50°C, recrystallized twice at 4°C, and then dried using a vacuum freeze dryer.

2.7 | Analysis methods

The contents of soluble components such as glucose, fructose, xylose, lactic acid, acetic acid, HMF, and furfural were assayed by HPLC with refractive index detector RID10A (Shimadzu) and Aminex HPX-87H (BioRad) at 65°C. The mobile phase was 5 mM sulfuric acid solution at a flow rate of 0.6 mL/min.

The optical purity of lactic acid was assayed using the D/L-Lactic Acid Kit (Megazyme International).

The melting point of lactide was assayed using Synchronous Thermal Analyzer (STA 449 F5 Jupiter) at a heating rate of 5°C/min under nitrogen protection.

3 | RESULTS AND DISCUSSION

3.1 | Fingerprinting the trace D-lactic acid in the biorefinery process chain

A complete biorefinery chain for chiral lactic acid production from lignocellulose biomass includes feedstock collection, storage, pretreatment, saccharification, detoxification, and fermentation. D-Lactic acid generation may occur at each step and will directly affect the chiral purity of the final L-lactic acid product. Here, we fingerprinted the trace D-lactic acid in the biorefinery chain from the initial feedstock collection to L-lactic acid fermentation for identification of the origins of D-lactic acid.

Table 1 shows that all five selected lignocellulose feedstocks (wheat straw, corn stover, rice straw, corn fiber, and bamboo powder) contained different levels of racemic lactic acid (both L- and D-lactic acids). The newly harvested wheat straw, corn straw, and rice straw contained 0.54, 0.18, and 0.38 mg of D-lactic acid/g DM, respectively. These D-lactic acid contents were equivalent to 243.0, 81.0, and 169.1 mg/L of D-lactic acid in the final fermentation broth or 0.188%, 0.0628%, and 0.131% of the L-lactic acid product in the typical dry biorefinery process cases (He et al., 2022). Corn fiber and bamboo powder contained less D-lactic acid (0.013 and 0.027 mg/g DM), which were equivalent to 5.84 and 12.0 mg/L in the fermentation broth or 0.045% and 0.093% of the L-lactic acid product. A surprising

TABLE 1 L- and D-Lactic acid contents in lignocellulose feedstocks, fermentation additives, and enzymes in the biorefinery chain.

	L-Lactic acid (mg/g DM)	D-Lactic acid (mg/g DM)	Equivalent D-lactic acid in L-lactic acid broth [a] (mg/L)	Equivalent D-lactic acid in L-lactic acid product (mg/g)
Raw feedstocks				
Wheat straw	1.18	0.54	243.0	1.88
Corn stover	0.31	0.18	81.0	0.628
Rice straw	0.53	0.38	169.1	1.31
Corn fiber	2.88	0.027	12.0	0.093
Bamboo powder	0.018	0.013	5.84	0.045
Feedstock after long-term storage[b]				
Wheat straw	6.14	5.16	2322.0	17.98
Nitrogen additives[c]				
Yeast extract	1.84	1.50	16.50	0.128
Peptone	1.23	0.92	10.12	0.0784
Cottonseed meal	ND	ND	ND	ND
Soybean meal	ND	ND	ND	ND
Enzymes[d]				
Cellulase	ND	ND	ND	ND
Glucoamylase	2.860	3.160	3.16	0.0245

Abbreviations: DM, dry matter of wheat straw; HPLC, high-performance liquid chromatography; ND, not detected by D/L-Lactic Acid Kit and HPLC; SSCF, simultaneous saccharification and co-fermentation.

[a]L-Lactic acid fermentation by SSCF using wheat straw at 30% (w/w) solid loadings and 10% (v/v) inoculation of *P. acidilactici* ZY271 seed.

[b]Wheat straw at 25% (w/w) moisture content was sealed in plastic bags and stored for 2 months.

[c]Dosage of yeast extract, peptone, cottonseed meal, and soybean meal were 11, 11, 22, and 22 g/L in wheat straw hydrolysate, respectively.

[d]One percent (v/v) of glucoamylase in the seed culture and 4 mg cellulase protein/g dry feedstock were added in the fermentation, respectively.

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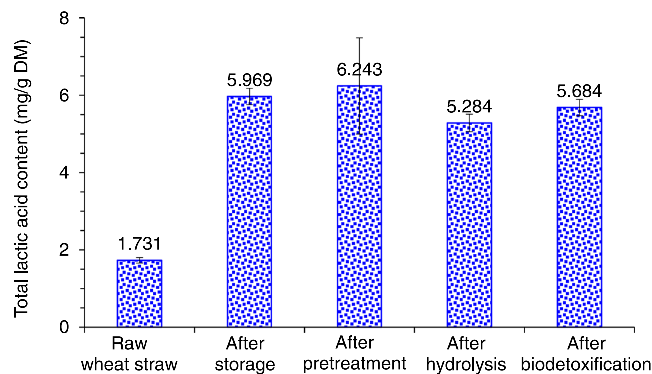

FIGURE 1 Total lactic acid contents in the biorefinery chain based on dry weight of wheat straw. The raw and pretreated wheat straw samples were withdrawn in each step of the biorefinery chain and soaked with deionized water at 30°C at 150 rpm for 1 h and then the supernatant was collected by centrifuging at 11,167*g* for 5 min. The hydrolysate after saccharification and detoxification was sampled separately and centrifuged at 11,167*g* for 5 min. The supernatant obtained from each step was collected to determine the lactic acid content.

result in Table 1 is that the high moisture content (25%) in wheat straw led to a sharp increase of D-lactic acid by an order of magnitude (from 0.54 to 5.16 mg/g DM) during the 2-month storage period, which was equivalent to 2.32 g/L of D-lactic acid in the fermentation broth or ~1.8% of the L-lactic acid product. This D-lactic acid level would negatively affect the chiral purity of L-lactic acid. Considering that humid storage conditions in real storage scenarios are commonly encountered, the result might indicate that the presence of mixed lactic acids (both L- and D-lactic acids) in lignocellulose feedstocks is inevitable.

The lactic acid contents in the enzymes and nitrogen nutrient additives were also detected (Table 1). No D-lactic acid was detected in the cellulase enzyme (CTec2.0). A relatively high D-lactic acid content (3.16 g/L) was found in the glucoamylase (GA-L NEW) used in the seed culture of *P. acidilactici* to avoid flocculation. However, due to the low usage (~1% v/v) of glucoamylase in the seed culture step, the equivalent D-lactic acid titer in the fermentation broth was only 3.16 mg/L and the content in L-lactic acid product was only 0.00245%. Nitrogen nutrients pose a high risk of introducing D-lactic acid due to the complex composition and exposure to the environment. Thus, CSL is not suitable for L-lactic acid fermentation because of its high D-lactic acid content (Zhang et al., 2022). Yeast extract and peptone contained 1.50 and 0.92 mg/g DM of D-lactic acid, equivalent to 16.50 and 10.12 mg/L of D-lactic acid in the fermentation broth, or 0.0128% and 0.00784% of L-lactic acid, respectively. No lactic acid was detected in the cottonseed meal and soybean meal used. The results suggest that the addition of enzymes and nitrogen nutrients did add some risks for introducing D-lactic acid into the biorefinery chain. However, the impact was minimal mainly because the D-lactic acid in the additives was present at low content. The primary pathway for D-lactic acid generation continues to be the one in lignocellulose feedstocks.

The total lactic acid content in each step of the biorefinery chain was calculated based on the typical case of L-lactic acid production from wheat straw by dry biorefinery processing (He et al., 2022) (Figure 1). The most significant change occurred during the storage period, where the lactic acid content in the freshly harvested wheat straw (1.73 ± 0.07 mg/g DM) was increased by 3.4-folds (up to 5.97 ± 0.21 mg/g DM). In the subsequent biorefinery steps of pretreatment, hydrolysis, and biodetoxification, the lactic acid content remained approximately constant: after subsequent pretreatment, 6.24 ± 1.25 mg/g DM; after enzymatic hydrolysis, 5.28 ± 0.23 mg/g DM; after biodetoxification, 5.68 ± 0.21 mg/g DM. These results suggest that neither the generation nor the degradation of lactic acid occurred during the conversion steps of the biorefinery chain.

Among the three potential pathways of D-lactic acid generation (fermentation strains, external sources, and lignocellulose feedstocks), the D-lactic acid generation in lignocellulose feedstocks should be the major cause of the reduced chiral purity of L-lactic acid. The D-lactic acid generated in the raw feedstock and during the storage will eventually enter the cellulosic L-lactic acid fermentation broth to ~18 mg/g L-lactic of D-lactic acid (Table 1).

3.2 | Mechanism of D-lactic acid generation in lignocellulose feedstocks

The formation of lactic acid in lignocellulose feedstocks requires at least two preconditions: the presence of lactic acid-producing microbes and water-soluble carbohydrates. Previous studies have shown that both conditions are available in lignocellulose feedstocks under appropriate storage conditions, along with trace amounts of vitamins, proteins, and lipids essential for the synthesis of lactic acid (Cui et al., 2020; Han et al., 2018, 2021; Tian et al., 2022; Zhao et al., 2019). An example is the L-lactic acid fermentation strain used in this study, *P. acidilactici* ZY271, which has its wild-type origin from corn stover hydrolysate (Zhao et al., 2013).

The five typical lignocellulose feedstocks (wheat straw, corn strove, rice straw, bamboo powder, and corn fiber) were used to isolate lactic acid-producing microbes according to the methods described in Section 2 (Figure 2). Figure 2a shows the transparent circles on the MRS agar gel with 1% (w/v) $CaCO_3$ nearby the microbial colonies. Eight single colonies with transparent circles were picked from the agar gels inoculated with the feedstock extracts. Then, the colony isolates were purified by three consecutive cultures. The isolates WS1–WS8 were from wheat straw extract, CS1–CS8 from corn stover, RS1–RS8 from rice straw, BS1–BS8 from bamboo powder and CF1–CF8 from corn fiber. The 16S rDNA fragments of these isolates were amplified, sequenced, and blasted on NCBI to construct a phylogenetic tree (Figure 2b). The results show that the 16S rDNA sequences of the isolates WS1 and CS1–CS8 were similar to those of *Lactobacillus murinus* and *Enterococcus faecalis*; WS2–WS5, RS1–RS8, and CF1–CF8 were similar to *Enterococcus faecium*; BP2–BP4 were similar to *Enterococcus gallinarum* with the

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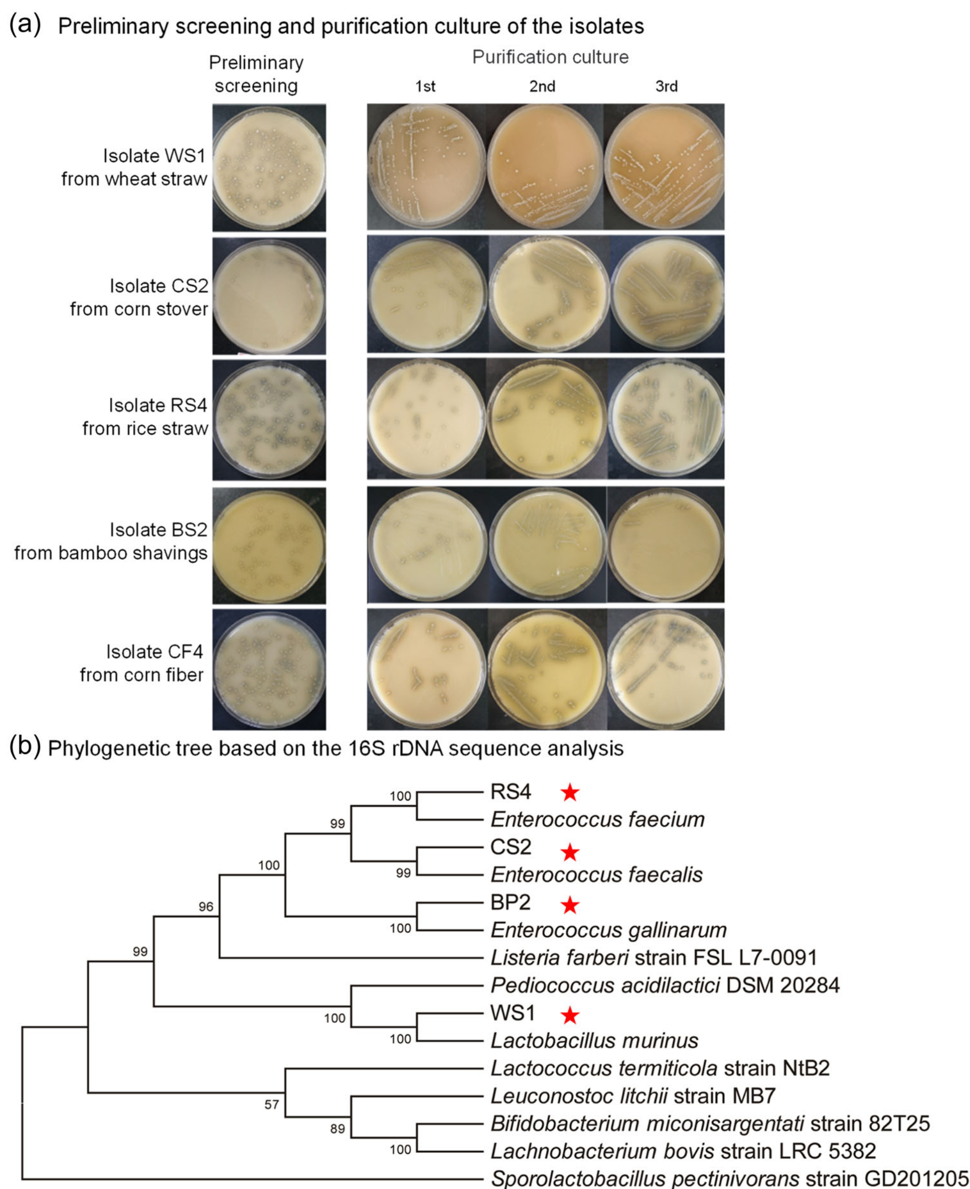

FIGURE 2 Isolation and identification of wild-type lactic acid bacteria in lignocellulose feedstocks. (a) Isolation and purification as described in Section 2. (b) Phylogenetic tree of the isolates by 16S ribosomal DNA on NCBI blast. MEGA 11.0 by neighbor-joining. The length of branches indicates the evolutionary distance. The data at branches (e.g., 93, 92) represent the confidence coefficient. BP, bamboo powder; CF, corn fiber; CS, corn stover; RS, rice straw; WS, wheat straw.

similarity of 16S rDNA sequences greater than 99.7%. The four types of isolates were named *L. murinus* WS1, *E. faecium* RS4, *E. faecalis* CS2, and *E. gallinarum* BP2, respectively. These wild LABs are all heterogeneous lactic acid producers, generating racemic lactic acids (both L-lactic acid and D-lactic acid) in varying ratios.

Plants generally contain varying amounts of water-soluble carbohydrates such as glucose, fructose, and sucrose inside their stems and leaves (Niu et al., 2016; Tian et al., 2022). The contents of water-soluble carbohydrates in wheat straw, corn strove, rice straw, bamboo powder, and corn fiber were detected by water extraction as described in Section 2 (Table 2). The newly harvested corn stover and wheat straw contained 4%–5% glucose and fructose to their dry biomass weight. Rice straw, bamboo powder, and corn fiber contained about 1% of water-soluble carbohydrates, among which the rice straw had been stored for 2 years and certain amounts of sugars may have been consumed by microbes.

These water-soluble carbohydrates in wheat straw were estimated to generate 40 mg of D-lactic acid/g of L-lactic acid and lead to a 5% decrease in chiral purity of L-lactic acid in the typical dry biorefinery process cases (He et al., 2022). Obviously, the presence of wild LABs and water-soluble carbohydrates enables the conversion of soluble monosaccharides into both L- and D-lactic acids during feedstock storage, raising a high risk of chiral purity reduction to chiral lactic acid products.

Racemic lactic acid content in lignocellulose feedstock increased sharply during the storage period in a humid environment (Table 1).

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TABLE 2 Analysis of soluble sugars in typical biomass feedstocks.

Feedstocks	Glucose (mg/g DM)	Fructose (mg/g DM)	Total sugars (mg/g DM)
Wheat straw	14.65 ± 0.32	24.22 ± 3.06	38.87 ± 3.39
Corn stover	23.35 ± 2.05	31.40 ± 0.85	54.75 ± 2.90
Rice straw	3.17 ± 0.31	6.55 ± 069	9.72 ± 0.37
Bamboo powder	0.87 ± 0.08	3.38 ± 0.87	4.25 ± 0.94
Corn fiber	3.16 ± 0.69	8.50 ± 1.75	11.66 ± 2.43

Note: Lignocellulose feedstocks were extracted using water at 30°C at 150 rpm for 1 h. The supernatant was collected by centrifugation at 11,167*g* for 5 min to determine the concentration of soluble sugars.

Therefore, the racemic lactic acid generation from water-soluble carbohydrates (glucose and fructose) in wheat straw was investigated under different moisture content (Figure 3). The moisture content in freshly harvested straw is about 10%–20% (Humbird et al., 2011) and may increase to 20%–30% after long-term storage in humid conditions (Karunanithy et al., 2013), or even higher if exposed to rainfall or snowfall, or less than 10% in dry seasons or after drying treatment. The moisture contents of 25%, 15%, and 8% (w/w) were selected to represent the cases of wheat straw at long-term storage in humid conditions, freshly harvested state, and dry conditions, respectively. The wheat straw used was cleaned and inoculated with 10% (v/w) of the wild-type *P. acidilactici* DQ2, which was isolated from corn stover hydrolysate slurry (Zhao et al., 2013). Varying water, glucose, and fructose were added and then stored in sealed bags for 5 months. Figure 3a,b shows that at 8% moisture content, the least sugars were consumed in the first month of storage and no further sugar consumption occurred in the consequent storage period, while almost no lactic acid was generated; at 15% moisture content, the sugar consumption rate was accelerated significantly, but the total lactic acid generation only increased slightly from 0.68 to 0.72 mg/g DM; at 25% moisture content, both the sugar consumption rate and the total lactic acid generation sharply increased for approximately 20-folds. On the other hand, the D-lactic acid ratio in the total lactic acid generated was around 40% without significant change, in which the dry condition case (8%) had a large relative error because of the very low total lactic acid content (Figure 3c). The maximum D-lactic acid content in the wheat straw after 5-month storage at 25% moisture content reached 5.16 mg/g DM, which was approximately equivalent to 2% reduction of the chiral purity of L-lactic acid in the typical dry biorefinery process case (He et al., 2022).

The results suggest that the moisture content of lignocellulose feedstock was highly correlated with the D-lactic acid generation. To eliminate this effect, drying treatment immediately after harvest is an ideal procedure but not a practical option. In most cases, a long storage period up to a complete harvest season (1 year) is inevitable. The closed or semiclosed storage measures only avoid extreme conditions such as being directly rinsed by rains, snows, and fogs. The possibility of moisture content increasing to ~25% moisture content occurs most likely in practical scenarios.

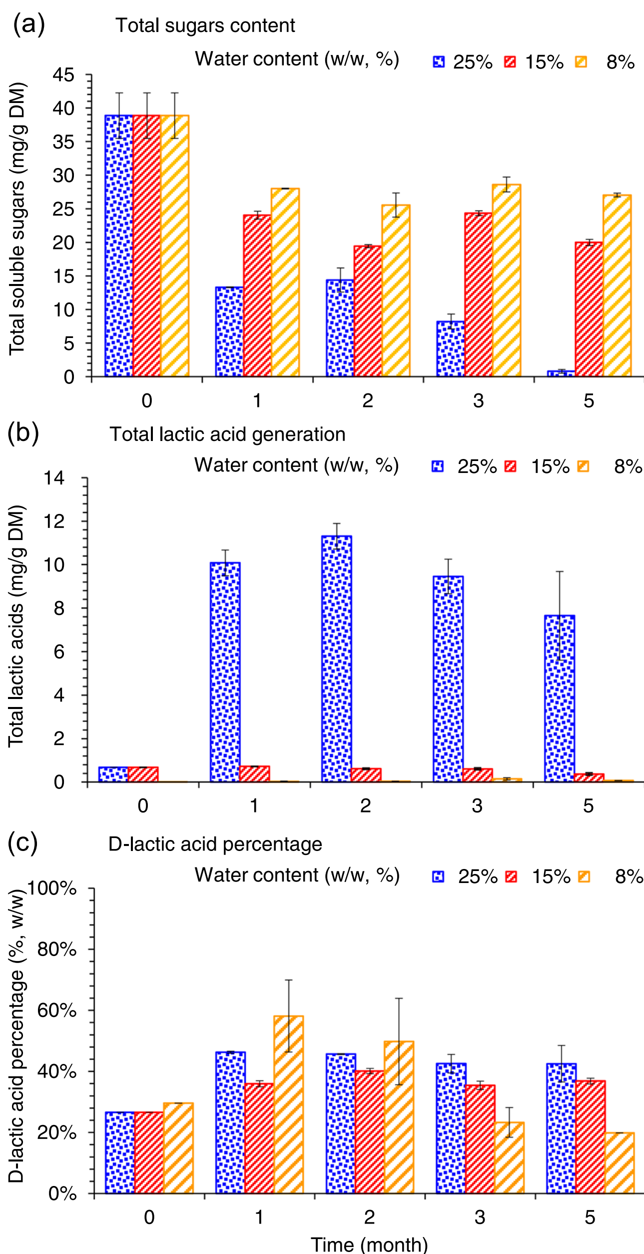

FIGURE 3 Water-soluble carbohydrates consumption and lactic acid generation in wheat straw during 5-month storage under varying moisture conditions. (a) Total sugars content. (b) Total lactic acid generation. (c) D-Lactic acid ratio in total lactic acid generated. *Pediococcus acidilactici* DQ2 was inoculated at 10% (v/w). Glucose and fructose were added corresponding to the contents in the newly harvested wheat straw.

3.3 | Effect of trace D-lactic acid presence on L-lactide synthesis

The presence of D-lactic acid in L-lactic acid on the subsequent chiral lactide was tested (Table 3). Trace amounts of D-lactic acid were added into L-lactic acid and the mixed D/L-lactic acids were used for the synthesis of L-lactide. Three D-lactic acid levels were set to 1.7, 15, and 40 mg D-lactic acid/g of L-lactic acid for polycondensation

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and depolymerization to cyclic L-lactide, corresponding to the D-lactic acid content in wheat straw at 8%, 15%, and 25% (w/w) moisture contents stored for 2 months, respectively. The melting point of the synthesized L-lactide was measured to evaluate the impact of trace D-lactic acid in L-lactic acid on the properties of L-lactide.

The melting point of pure L-lactide or D-lactide ranged from 95°C to 98°C, while the meso-lactide ranged from 53°C to 54°C and the racemic lactide (mixture of D-lactide and L-lactide) from 122°C to 126°C (Masutani & Kimura, 2014). Table 3 shows that the melting point of L-lactide synthesized from pure L-lactic acid was 99.2°C, which agreed with the reported 95–98°C. D-Lactic acid content in L-lactic acid at 1.7 mg/g resulted in an increase in the melting point of L-lactide (113.9°C), and a further increase in D-lactic acid content led to a sharp decrease in the melting point of L-lactide. When the D-lactic acid content in L-lactic acid reached 40 mg/g, the melting point of L-lactide decreased to 85.9°C, it is highly possible that meso-lactide was synthesized. The results indicate that the existence of trace D-lactic acid in the regularly collected and stored lignocellulose significantly affected the properties of the synthesized L-lactide. Effective and feasible strategies need to be taken to cut off the generation of D-lactic acid inside lignocellulose feedstock.

TABLE 3 Melting point analysis of L-lactide samples synthesized from L-lactic acid containing trace amounts of D-lactic acid.

D-lactic acid in L-lactic acid monomer (mg/g)	Melting point of synthesized L-lactide (°C)
Control	95–98 (Masutani & Kimura, 2014)
0.0	99.2
1.7	113.9
15.0	86.6
40.0	85.9

Note: Control: The commercial L-lactide using starch-derived glucose as feedstock.

3.4 | Elimination of the conversion of water-soluble carbohydrates to D-lactic acid

D-lactic acid in lignocellulose feedstocks is synthesized from water-soluble carbohydrates on the biomass by wild-type LABs growing on the biomass. Three methods were proposed to cut off this conversion: (i) reduction of moisture content of feedstocks; (ii) conversion of water-soluble carbohydrates into furan aldehydes in pretreatment; and (iii) conversion of water-soluble carbohydrates into L-lactic acid by inoculating engineered L-lactic acid bacterium strain during storage.

3.4.1 | Strategy 1: Reduction of moisture content of feedstocks

The first measure to avoid D-lactic acid generation and prevent water-soluble carbohydrate degradation is to store lignocellulose feedstock in a dry environment. Figure 3 suggests that both sugar consumption rate and D-lactic acid generation were significantly reduced at low moisture content. When the moisture was below 15%, the D-lactic acid generation was reduced by 98.7% compared with that at 25% of moisture content. When the moisture was reduced to 8%, almost no visible sugar consumption and D-lactic acid generation. A shielded warehouse could avoid direct rinse by rains and snows and maintain the moisture content around 15%–20%

TABLE 4 D-Lactic acid generation in wheat straw at varying stages of storage and the effect on L-lactic acid chirality.

Wheat straw at different stages	Wheat straw composition (mg/g DM)					D-Lactic acid in L-lactic acid broth[b] (g/L)	L-Lactic acid chirality (%, w/w)
	Water-soluble sugars	Lactic acids	HMF	Furfural	Acetic acid		
Newly harvested (no sugar conversions)	Glucose, 15.0 Fructose, 24.0	L-, 0.0 D-, 0.0	5.46 ± 0.26	7.24 ± 0.97	17.75 ± 0.44	0.53 ± 0.068	99.61
Initial stage of storage (half sugars converted to lactic acids)	Glucose, 7.5 Fructose, 12.0	L-, 13.0 D-, 6.5	4.05 ± 0.16	9.09 ± 0.77	18.16 ± 0.59	4.76 ± 0.36	96.18
Late stage of storage (all sugars converted to lactic acids)	Glucose, 0.0 Fructose, 0.0	L-, 26.0 D-, 13.0	3.08 ± 0.41	8.46 ± 0.012	18.32 ± 0.98	7.90 ± 0.17	94.03
Control (completely washed) (no sugars or lactic acids contained)	Glucose, 0.0 Fructose, 0.0	L-, 0.0 D-, 0.0	1.23 ± 0.091	3.26 ± 0.092	16.34 ± 0.24	0.62 ± 0.091	99.53

Abbreviations: DM, dry matter of wheat straw; SSCF, simultaneous saccharification and co-fermentation.

[a]Wheat straw was pretreated using 3.8% (g/g DM) sulfuric acid at 175°C for 5 min.

[b]L-Lactic acid broth was obtained by SSCF carried out in a 3 L bioreactor at 42°C, 300 rpm, pH 5.5, and 10% (v/v) inoculation size with 30% (w/w) solid content.

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(Humbird et al., 2011), but a further reduction of moisture content to 8%–10% needs additional drying operations. This further drying operations might be a tough job in practical scenarios since the collection, transportation, and storage of biomass are time-consuming and labor-intensive. A 15% moisture content is expected to be the most optimistic scenario within a harvest cycle (1 year).

3.4.2 | Strategy 2: Conversion of water-soluble carbohydrates into furan aldehydes in pretreatment

Pretreatment is the key step to overcome the biorecalcitrance of lignocellulose for the next conversion to fermentable sugars. Water-soluble carbohydrates (glucose and fructose) in lignocellulose feedstock will be converted into HMF and partial furfural during pretreatment, and then these furan aldehydes will be degraded into CO_2 and water in the subsequent biodetoxification step (Yi et al., 2019). In this way, the D-lactic acid generation from water-soluble carbohydrates could be effectively blocked.

Wheat straw with different sugars and lactic acid addition was selected for dry acid pretreatment and the D-lactic acid generation outcome was examined (Table 4). The freshly harvested wheat straw was represented by containing 15.0 mg/g DM of glucose, 24.0 mg/g DM of fructose, and no lactic acid. The wheat straw at the initial stage of storage was represented by containing half of the original sugars (7.5 and 12.0 mg/g DM glucose and fructose), as well as 13.0 and 6.5 mg/g DM of L- and D-lactic acids (generated from half of the original sugars at the L/D ratio of 2:1), respectively. The wheat straw at the late stage of storage was represented by containing 26.0 and 13.0 mg/g DM of L- and D-lactic acid (all water-soluble carbohydrates were converted into lactic acid) with no glucose or fructose left. The thoroughly washed wheat straw was used as a control.

Table 4 indicates that the freshly harvested wheat straw after pretreatment generated the highest amounts of HMF, but the least D-lactic acid (0.53 ± 0.068 g/L) after biodetoxification for inhibitor removal and SSCF for L-lactic acid production, similar to that of the control (0.62 ± 0.091 g/L). The wheat straw at the initial stage of storage generated less HMF but more furfural due to the assisted enhancement of pretreatment severity by lactic acid, but D-lactic acid concentration increased by approximately one order of magnitude compared with that of the freshly harvested wheat straw, resulting in a considerable decrease of L-lactic acid chirality from 99.61% to 96.18%. The wheat straw at the late stage of storage generated a further reduced HMF and similar furfural, resulting in higher D-lactic acid generation and further reduction of L-lactic acid chirality (94.03%).

This strategy completely blocks the conversion of water-soluble carbohydrates to D-lactic acid with a slight yield loss (water-soluble carbohydrates were not converted to lactic acid) due to the uniqueness of the biodetoxification step in dry biorefinery technology (He et al., 2022; Zhang et al., 2021). Though effective and practically feasible, however, this strategy requires the pretreatment to be conducted immediately after harvest of lignocellulose feedstock, to ensure that a minimal D-lactic acid is generated before pretreatment. In a practical scenario, lignocellulose feedstock takes a long journey of harvest, collection, transportation, and storage to a central biorefinery plant before pretreatment. The results also suggest that an early biorefinery process of lignocellulose feedstock is the best way to assure the high chirality of L-lactic acid product.

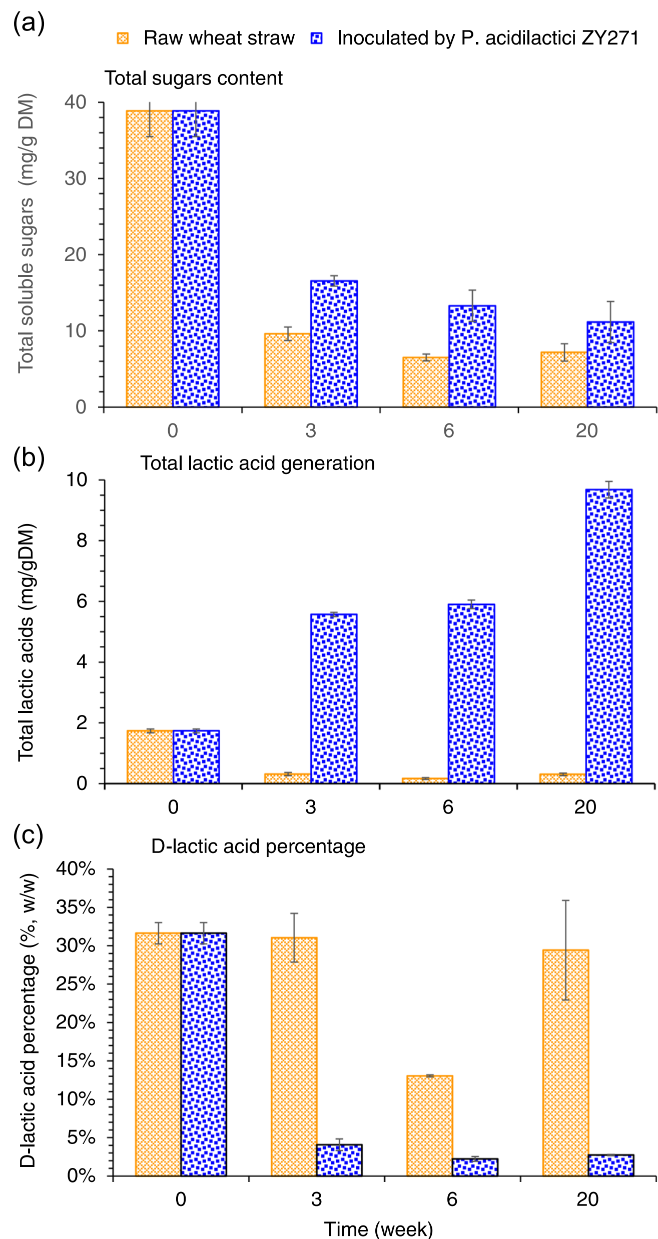

FIGURE 4 Sugar and lactic acid content changes in wheat straw with inoculation of *Pediococcus acidilactici* ZY271 during anaerobic storage. (a) Total sugars; (b) total lactic acid generation; (c) D-lactic acid percentage. The wheat straw was inoculated by *P. acidilactici* ZY271 at 10% (v/w) inoculation size and then evenly mixed and anaerobically stored. The added bacterial solution had 1.10 mg/g DM of L-lactic acid and 1.14 mg/g DM of glucose, and no fructose. The untreated wheat straw was used as the control group.

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3.4.3 | Strategy 3: Conversion of water-soluble carbohydrates into L-lactic acid by inoculating engineered strain during storage

Wild-type LABs in lignocellulose feedstocks generally are racemic LABs producing both L- and D-lactic acid. The third strategy is to convert the naturally occurring water-soluble carbohydrates into L-lactic acid only by inoculating engineered L-lactic acid bacterium cells onto the feedstocks during storage.

Figure 4 shows the changes of sugars and lactic acid contents in the freshly harvested wheat straw (without any treatment) during 20-week storage after uniformly spraying the seed broth of L-lactic acid fermenting strain *P. acidilactici* ZY271 at an inoculum of 10% (v/w). The sugar consumption was effectively curbed (Figure 4a) and the L-lactic acid generation was significantly increased (Figure 4b,c) compared with the control without inoculation of *P. acidilactici* ZY271. The ratio of D-lactic acid in the total lactic acids generated during the storage was also reduced by one order of magnitude (from 29.4% to 2.7%) due to the increasing generation of L-lactic acid (Figure 4c). The generation of L-lactic acid resulted in a reduced pH of the feedstocks, which also benefited to the inhibition of bacteria growth and D-lactic acid generation.

This strategy converts the water-soluble carbohydrates to L-lactic acid by inoculating a new engineered strain; thus, the generation of D-lactic acid by wild-type LAB is effectively inhibited. Not only the L-lactic acid chirality was improved but also more L-lactic acid was produced (although slightly). The major barrier of this method for practical application is the cost, including seed cell culture, solids mixing, and storage under relatively anaerobic conditions.

3.5 | Preliminary pathway analysis of lactic acid metabolism during feedstock storage

A phenomenon was frequently observed during the storage of lignocellulose: the lactic acid (both L-and D-lactic acid) converted from water-soluble carbohydrates gradually decreased after glucose and fructose were depleted (Figures 3b and 4b). An experiment was designed to elucidate the phenomenon by culturing the isolated wild-type LABs in the MRS medium containing both glucose and L-lactic acid. Similar to the lactic acid decrease in solid feedstock, the four LABs, including *L. murinus* WS1, *E. gallinarum* BP2, *E. faecium* RS4, and *E. faecalis* CS2, consumed glucose as a carbon source first and then lactic acid was consumed gradually after glucose was depleted with the generation of acetic acid (Supporting Information S1: Figure 1a). The result suggests that the wild-type LABs were capable of lactic acid assimilation into acetic acid for maintaining the bacteria growth when glucose was depleted (Duwat et al., 2001).

Three enzymes may be involved in the lactic acid metabolism of wild-type LABs: lactate racemase, LDH, or lactate oxidase (LOX). Lactate racemase is an α/β-sheet nickel-dependent enzyme responsible for interconversion between L- and D-lactic acid (Desguin et al., 2017). Since both L- and D-lactic acid contents were decreased during lignocellulose

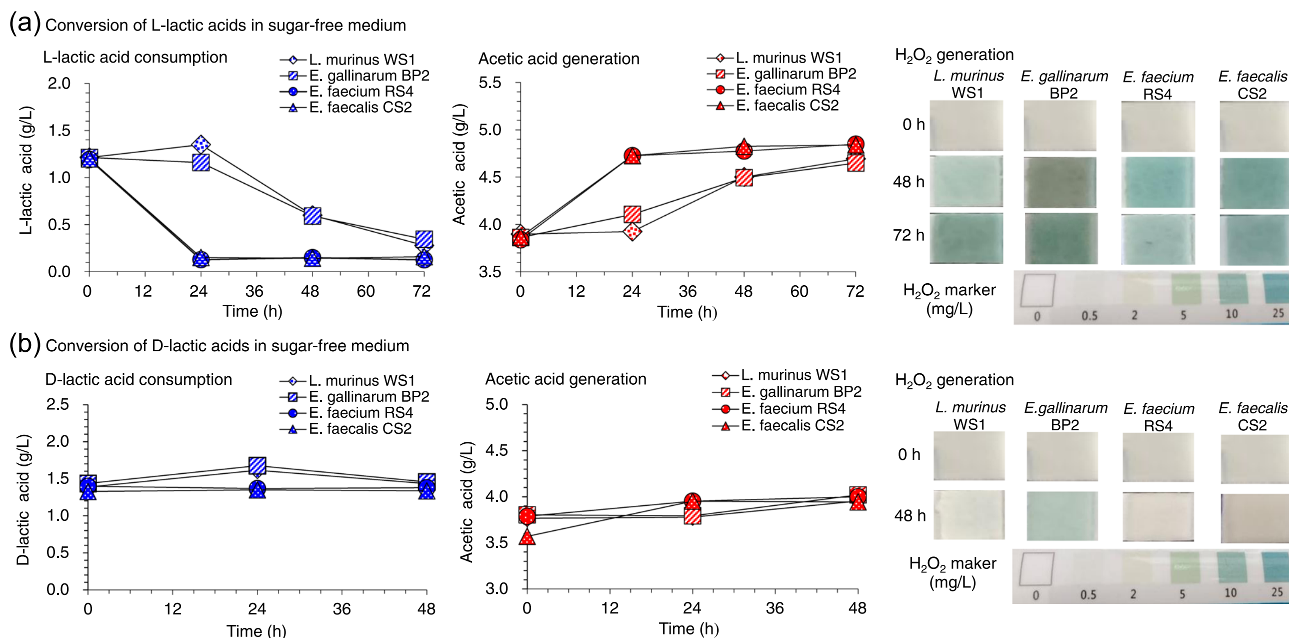

FIGURE 5 Conversion of L/D-lactic acid in sugar-free MRS medium. (a) Medium containing 1.0 g/L L-lactic acid. (b) Medium containing 1.0 g/L D-lactic acid. Bacterial cells were collected by centrifuging and washing and then transferred into a sugar-free MRS culture medium containing 1 g/L of L- or D-lactic acid. Fermentation: 42°C, 150 rpm for 48 or 72 h. Hydrogen peroxide detection: Immersing the hydrogen peroxide test sheets into a 10-mL broth sample for 10 s, drying the liquid and standing for 60 s, and then comparing the sheet with the standard color card of hydrogen peroxide concentrations.

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storage and liquid medium culture by the LABs, instead of a L-/D-lactic acid interconversion, the lactate racemase pathway can be eliminated (Figures 3 and 4b). LDH is the major enzyme to catalyzes the conversion of pyruvate to lactic acid or the vice versa reaction (Goffin et al., 2006; Guo et al., 2017). LDH pathway is also less likely to play a major role because (i) sugar metabolism is decreased and the LDH activity is inhibited when glucose is depleted (Garrigues et al., 1997; Ma et al., 2007; Thomas et al., 1979; Yamada & Carlsson, 1975); (ii) the conversion from lactate to pyruvate works only at high concentration of lactic acid (Ma et al., 2007).

The most possible pathway of lactic acid metabolism is by the LOX-catalyzed oxidation. LOX catalyzes the oxidation of lactate to pyruvate and reduces molecular oxygen to hydrogen peroxide (Sheng et al., 2015, 2016). A preliminary verification experiment was designed by culturing the four isolated wild-type LABs in the sugar-free MRS medium with the addition of 1 g/L L-lactic acid or D-lactic acid (Figure 5). Figure 5a shows that the four LABs consumed L-lactic acid with the generation of acetic acid and hydrogen peroxide. When glucose was used as the sole carbon source, no hydrogen peroxide generation was observed (Supporting Information S1: Figure 1b); only when lactic acid was the sole carbon source was the hydrogen peroxide generated. The results suggest that L-lactic acid may assimilate by LOX-catalyzed oxidation to pyruvate with the generation of hydrogen peroxide and pyruvate is further converted to acetic acid (Eckhardt et al., 2013; Kaneda, 2001; Nordkvist et al., 2003) (Supporting Information S1: Figure 1c).

The slow D-lactic acid decrease after the depletion of glucose may also be assimilated by D-LOX (Figure 3b). A low hydrogen peroxide titer was detected when *E. gallinarum* BP2 was cultured in the MRS medium (Figure 5b), indicating that D-LOX may take a role in D-lactic acid oxidation with a relatively low rate compared with L-LOX.

In summary, LOX is speculated to play a primary role in the lactic acid metabolism of wild-type LAB based on the experimental observation. When sugar is depleted, lactic acid becomes the new carbon source and is converted to pyruvate by the catalysis of LOX and then further converted to acetic acid or other products. A further mechanism is still needed for verification.

AUTHOR CONTRIBUTIONS

Xiaomeng Guo, Zhibin Li, and Niling He conducted the experiments. Jie Bao, Xiaomeng Guo, Bin Zhang, and Niling He designed the study. Jie Bao and Xiucai Liu conceived the study. Xiaomeng Guo, Zhibin Li, and Jie Bao wrote the manuscript. All authors contributed to the revisions of the manuscript.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (21978083) and the Yangfan Project of Science and Technology Committee of Shanghai Municipality (23YF1409900).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Guo, X., Li, Z., He, N., Zhang, B., Liu, X., & Bao, J. (2024). Detection and elimination of trace D-lactic acid in lignocellulose biorefining chain: Generation, flow, and impact on chiral lactide synthesis. *Biotechnology and Bioengineering*, *121*, 670–682. https://doi.org/10.1002/bit.28583

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