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Transformation of lignocellulose to starch-like carbohydrates by organic acid-catalyzed pretreatment and biological detoxification

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Abstract

Corn dry milling provides a mature model for lignocellulose biorefinery process. To copy this technical success, a crucial step is to transform lignocellulose into starch-like carbohydrates (SLC), similar to milled corn grain and in a similar fashion to corn dry milling. The transformation process should be zero wastewater generation and sufficient fermentable sugar conservation; the product should be in solid particle form, free of toxic residues, and high enzymatic hydrolysis yield and fermentability. Here we designed and verified a SLC transformation process by (i) biodegradable oxalic acid-catalyzed pretreatment, and (ii) simultaneous biodegradation of inhibitors and oxalic acid catalyst. The oxalic acid catalyst was effective on disrupting the lignocellulose structure and also biodegradable at low pH value. The biodetoxification fungus *Paecilomyces variotii* FN89 was capable of degrading the furan/phenolic aldehydes and oxalic acid simultaneously and ultimately, while the fermentable sugars were well preserved. The obtained SLC from wheat straw and corn stover were similar to dry milled corn meal in terms of morphological properties, fermentable sugar contents, enzymatic hydrolysis yield, elemental contents, and free of inhibitors and acid catalyst. The bioconversion of starch-like wheat straw and corn stover produced 78.5 and 75.3 g/L of ethanol (9.9% and 9.5%, v/v) with the yield of 0.47 and 0.45 g ethanol/g cellulose/xylose, respectively, compared with 78.7 g/L (10.0%, v/v) from corn meal and the yield of 0.48 g ethanol/g starch. Mass balances suggest that the ethanol yield, wastewater generation, and elemental recycling of the SLC from lignocellulose were essentially the same as those of corn meal.

KEYWORDS
biodegradable catalyst, biodetoxification, biofuels, dry milling, lignocellulose, starch-like carbohydrates

1 | INTRODUCTION

The commercialization of cellulosic ethanol production has experienced a significant stagnation in recent years owing to the technical barriers including large-scale wastewater discharge, substantial sugar loss, and low conversion efficiency, and with that economics hurdels (Almuth & Rachel, 2018; Irshad, 2016; Liu & Bao, 2017b; Sarantis, 2018). Overcoming these technical barriers is a prerequisite for the future revival of this highly promising technology into commercial-scale practice. As a technical model for lignocellulose biorefining process, corn dry milling provides a mature process for biofuels production. To copy the success of corn ethanol production

by corn dry milling technology, the crucial step is to transform lignocellulose feedstock into starch-like carbohydrates (SLCs), similar to dry milled corn grain and in a similar fashion to corn dry milling process.

The core step of SLC transformation of lignocellulose is pretreatment. Overcoming biorecalcitrance of lignocellulose biomass makes the most significant difference between lignocellulose and corn refining (Mosier, 2015; Yang & Wyman, 2008). Various pretreatment methods have been extensively investigated (Hassan et al., 2018; Mosier et al., 2005; Rabemanolontsoa & Saka, 2016), among which, dilute acid pretreatment has been practically applied in large-scale demonstration operations (Aden et al., 2002; Humbird et al., 2011; Vasconcelos et al., 2020; Wooley et al., 1999). However, the conventional dilute acid pretreatment is not appropriate for performing SLC transformation owing to its high wastewater generation, high inhibitor generation, and significant reactor corrosion. A modified acid pretreatment method, dry acid pretreatment, was used in this study (He, Zhang, Zhang, et al., 2014; Liu & Bao, 2017b; Liu et al., 2018; Shao et al., 2017; Zhang et al., 2010). In this dry acid pretreatment operation, lignocellulose feedstock is fed into the pretreatment reactor in dry particles form under high solids content (~70% w/w) and left the reactor in dry particles form containing 40%–60% (w/w) solids with the co-current feeding of minimum acid catalyst solution. For this reason, this "dry" to "dry" pretreatment method is designated as "the dry acid pretreatment." All inhibitory compounds are accumulated into the solid pretreated feedstocks to very high levels and a solids-surface biodetoxification culture is followed to completely and ultimately degrade all the inhibitory compounds into CO_2 and water (Yi et al., 2019). This dry acid pretreatment operation has been effectively applied to various lignocellulose feedstocks, such as corn stover, wheat straw, rice straw, and cotton stalk for the production of cellulosic ethanol (Liu et al., 2018). But the mineral acid catalyst used such as sulfuric acid is not biodegradable and the neutralization generates either highly concentrated sulfate wastewater (ammonia or sodium sulfates), or solid calcium precipitates (calcium sulfate) as the potential sulfur oxide source in downstream lignin combustion (Kumar & Murthy, 2011). To solve this problem, the acid catalyst should be effective in disrupting the lignocellulose structure and biodegradable at an extremely low pH value before neutralization, while the fermentable sugars should be well preserved.

This study aimed to construct an SLC transformation to obtain clean, easily hydrolyzable, and fermentable lignocellulose carbohydrates under the criteria of (i) highly efficient pretreatment operation with zero wastewater generation, (ii) complete removal of both inhibitors and acid catalyst, and (iii) minimum loss of fermentable sugars. The results show that lignocellulose feedstock was successfully transformed into SLC, similar to dry milled corn meal in morphology, fermentable sugar content, enzymatic hydrolysis yield, element contents, and ethanol fermentability. Mass balance calculations further suggest that the ethanol conversion yield, wastewater generation, elemental recycling during the starch-like transformation and ethanol conversion were essentially the same as those of corn feedstock in the dry milling process.

2 | MATERIALS AND METHODS

2.1 | Raw materials

Wheat straw and corn stover were harvested from Nanyang City, Henan Province, China in fall 2018. The raw materials were coarsely chopped, washed to remove field dirt and stones then air-dried, and milled to pass through the mesh with 10 mm in diameter. In industrial-scale biorefining, a de-dusting step is conducted in the pre-handling step to remove part of dust by mechanical approaches as in the National Renewable Energy Laboratory (NREL) report (Humbird et al., 2011). In lab bench scale, this mechanical step is not practical thus we used water washing instead. The composition was measured according to NREL protocols (Sluiter et al., 2008, 2012). The wheat straw contained 38.1% of cellulose, 30.4% of xylan, 17.2% of lignin, and 7.7% of ash on dry weight base (w/w). The corn stover contained 34.4% of cellulose, 27.6% of xylan, 18.2% of lignin, and 7.4% of ash on dry weight base (w/w).

Corn grains were harvested from Changchun City, Jilin Province, China in spring 2019. Corn grains were dry milled into fine corn meal by milling pass through the 60 mesh screen with the bore diameter of 0.3 mm. The starch content was determined by Ewers polarimetric method (International Standard: ISO 10520, 1997). The xylan content was determined using the NREL protocols after the starch was removed by a two-step enzymatic hydrolysis. The protein content was determined by Bradford method (Bradford, 1976). Corn meal contained 78.2% of starch, 14.6% of protein, and 1.8% of xylan on dry weight base (w/w). The corn meal also contained a small amount of lignin in its corn fiber fraction.

2.2 | Enzymes and reagents

The commercial cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes. The filter paper activity, cellobiase activity, and protein content were measured to be 203 FPU/ml, 4900 CBU/ml, and 87.3 mg/ml, respectively, according to the previously reported methods (Adney & Baker, 1996; Bradford, 1976; Ghose, 1987).

The α-amylase HTAA and glucoamylase GA-L NEW were purchased from Genencor with 21,000 and 103,900 U/ml, respectively. Yeast extract was purchased from Oxoid. Oxalic acid dihydrate was purchased from Tianchem. Glucose and other chemical reagents were of analytical reagent grade and purchased from Sinopharm Chemical Reagent.

2.3 | Microorganisms and culture procedures

Biodetoxification fungus *Paecilomyces variotii* FN89 was isolated from the contaminated microbial colony growing on the sulfuric acid

pretreated corn stover. *P. variotii* FN89 was stored in the Chinese General Microorganism Collection Centre (CGMCC, www.cgmcc.net) by the registration number of 17665. *P. variotii* FN89 was cultured on the synthetic medium (SM) agar slant compose of 20 g/L glucose, 1 g/L yeast extract, 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, and 1 g/L $MgSO_4{\cdot}7H_2O$. The control biodetoxification strain *Amorphotheca resinae* ZN1 (CGMCC 7452C) used followed the same culture protocols as in our previous publications (He et al., 2016; Yi et al., 2019; Zhang et al., 2010). The morphology of the fungi was photographed by digital camera E330 (Olympus, Japan).

The ethanol fermentation strain *Saccharomyces cerevisiae* XH7 used followed the same culture protocols as in our previous publications (Li et al., 2016; Liu et al., 2018).

2.4 | Pretreatment and biodetoxification

The acid pretreatment was according to the previously published protocols (He, Zhang, Zhang, et al., 2014; He, Zhang, & Bao, 2014; Liu et al., 2018; Zhang et al., 2011). The pretreatment was conducted in a 20-L helical ribbon impeller-driven reactor (He, Zhang, Zhang, et al., 2014). Briefly, 1200 g of wheat straw or corn stover (dry base) and 500–600 g of acid solution (adjusted based on the moisture content of the feedstock, and finally equivalent to the dry solid weight to the acid liquid weight of 2:1) were co-currently fed into the reactor without impregnation (He, Zhang, & Bao, 2014). After stirring for 3 min at 50 rpm, the hot steam was then jetted into the reactor and maintained at 175°C for 5 min. The pretreated wheat straw or corn stover solids (pH ~ 2.3) were discharged from the bottom outlet port of the reactor without free wastewater generation, then briefly milled to move the extra-long fibers (Liu et al., 2018; Zhang et al., 2011). This pretreatment can effectively pretreat various kinds of agriculture residual lignocellulose feedstocks, such as wheat straw, corn stover, rice straw, and cotton stalk (Liu et al., 2018).

The acid catalyst usage for pretreatment was adjusted according to the method previously described (Han & Bao, 2018). A suspension slurry was prepared by mixing 2 g of raw feedstock (dry base) with 100 ml deionized water in a 250 ml flask, and then shaken vigorously at 30°C for 30 min, and the pH value of the slurry was measured by pH sensor. The suspension slurry of wheat straw or corn stover containing different ash was titrated by 4% (w/w) sulfuric acid or oxalic acid to the base pH value (~2.3). This adjusted acid usage (mg acid/g dry matter) was applied to the practical acid pretreatment. In this study, the acid dosage for pretreatment was adjusted in the range of 24–27 mg/g DM (Table 2).

The solid-state biodetoxification was conducted in a 15-L bioreactor to remove the inhibitory compounds and acid catalyst. The spore suspension of *P. variotii* FN89 was firstly collected by washing the SM agar plate using 0.05% (w/v) Tween 80 solution. The biodetoxification seed was prepared by inoculating *P. variotii* FN89 spores to the freshly pretreated wheat straw or corn stover solids with the concentration of 10^6 spores/g feedstock, and cultured at 37°C for 48 h. Then the seed was inoculated into pretreated wheat straw or corn stover solids at 10% (w/w) mass ratio, and incubated at 37°C for 36-48 h with the aeration rate of 1 vvm (air volume per culture volume per min). The mixture of inoculum and pretreated feedstocks occupied 2/3 volume of the bioreactor. The stirring was conducted at 50 rpm every 12 h for 5 min. The difference between the present biodetoxification and the previous one was that no alkaline neutralizer reagent and nutrients were added during the biodetoxification (He et al., 2016; Yi et al., 2019).

2.5 | Enzymatic hydrolysis

One gram of corn meal (dry base) was added into deionized water to prepare 20 ml of 5% (w/w) solids slurry in 100 ml of flask. The enzymatic hydrolysis of corn meal was assayed by a two-step of liquefaction (α-amylase HTAA with the enzyme loading of 44 FPU/ml at 90°C and 200 rpm for 12 h) and saccharification (GA-L NEW with the enzyme loading of 200 FPU/ml at 60°C and 200 rpm for 24 h).

The pretreated and biodetoxified wheat straw and corn stover were assayed by enzymatic hydrolysis following the NREL LAP-009 (Brown & Torget, 1996). One gram of pretreated feedstock (dry base) was added into 10 ml of deionized water in 100 ml flask and then adjusted to pH 4.8 by adding 5 M NaOH solution. 0.1 M citrate buffer (pH 4.8) was then added to prepare a 5% (w/w) solids slurry. The cellulase dosage was 20 FPU/g DM (dry matter) and the hydrolysis lasted for 72 h at 50°C and 150 rpm of shaking.

2.6 | Ethanol fermentation

The separate hydrolysis and fermentation was used for corn ethanol production. The corn meal hydrolysate was firstly prepared by a two-step of liquefaction (α-amylase HTAA with the enzyme loading of 44 FPU/ml at 90°C and 200 rpm for 12 h) and saccharification (GA-L NEW with the enzyme loading of 200 FPU/ml 60°C and 200 rpm for 24 h) at ~20% solids loading. The ethanol-producing strain *S. cerevisiae* XH7 was activated in 20 ml of YPD medium at 30°C and 180 rpm for 12 h. The primary seed of *S. cerevisiae* XH7 was prepared by inoculating the activated seed into 5% (w/v) solids loading corn meal hydrolysate. The secondary seed was prepared by inoculating the primary seed into 10% (w/v) solids loading corn meal hydrolysate. The primary and secondary seeds were cultured at 30°C and 180 rpm for 12 and 24 h. The nutrients were added into hydrolysate at the beginning of fermentation.

The simultaneous saccharification and co-fermentation was conducted out for cellulosic ethanol production (Liu et al., 2018). The primary seed of *S. cerevisiae* XH7 for cellulosic ethanol fermentation was obtained by inoculating the activated seed into the slurry containing 5% (w/v) of the biodetoxified lignocellulose biomass and the cellulase at the dosage of 10 mg protein per gram of dry matter. Then the secondary seed was prepared by inoculating the primary seed into the slurry containing 10% (w/v) of

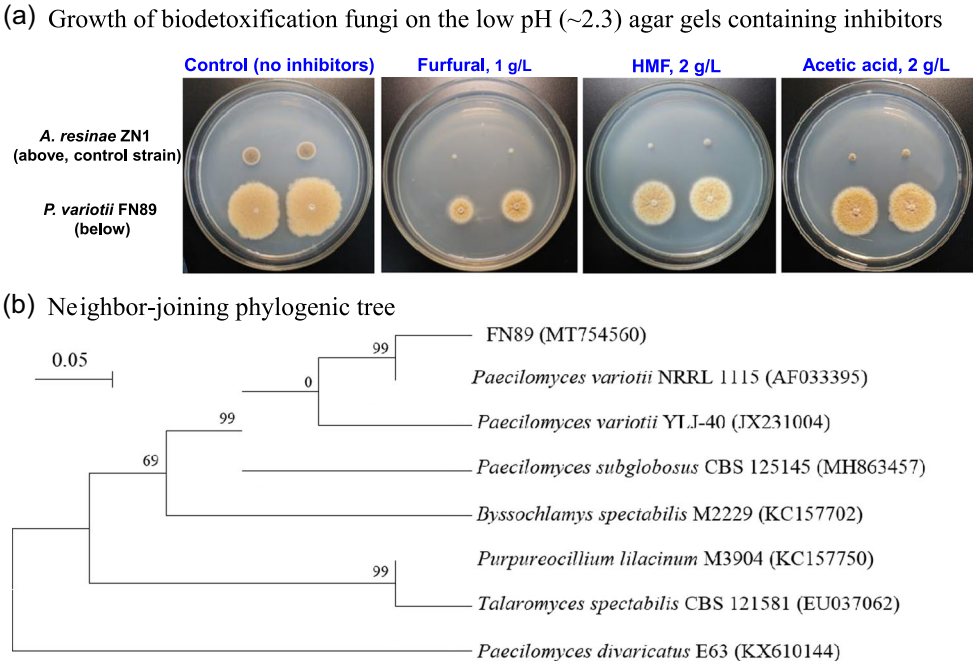

FIGURE 1 *Paecilomyces variotii* FN89 growth at pH 2.3 and its internal transcribed spacer (ITS) identification. (a) *P. variotii* FN89 growth (below) with the control strain *A. resinae* ZN1 (above). Each colony was initially cultivated from ~10^4 spores and cultured for 96 h. The pH was adjusted by sulfuric acid. (b) Phylogenetic tree of *P. variotii* FN89 by MEGA 7 software according to the neighbor-joining method with 1000 bootstrap replicates. The scale bar represents 0.05 nucleotide substitutions per position. The sequence of ITS fragment with 624 nucleotides was submitted to Genbank (accession number of MT754560). The identity of ITS sequence to *Paecilomyces variotii* NRRL 1115AF was 100% based on the NCBI blast

the same biomass and cellulase. The primary and secondary seeds were also cultured at 30°C and 180 rpm for 12 and 24 h. The pretreated and biodetoxified feedstocks were pre-hydrolyzed into slurry in the specially designed 5-L bioreactor equipped with helical ribbon impeller at 50°C by fed-batch within 12 h (Zhang et al., 2009). The cellulase dosage for SSCF was 4 mg protein/g dry matter. The fermentable sugars were co-fermented into ethanol simultaneously with the hydrolysis of cellulose and oligomer sugars at high solid loading (~25%. w/w), 30°C for 96 h by inoculating the secondary seed. The broth pH was controlled at 5.5 by adding 5 M NaOH. The practical ethanol yield in SSCF at high solids loading is according to the method described by Zhang and Bao (2012).

2.7 | Analysis methods

Glucose, xylose, ethanol, furfural, 5-hydroxymethylfurfural (HMF), and acetic acid were analyzed by Shimadzu HPLC system equipped with a Bio-rad Aminex HPX-87H column and RID-10A detector. Twenty microliters of the sample was subjected and analyzed at 60°C using 5 mM H_2SO_4 as eluent with flow rate of 0.6 ml/min (Liu et al., 2018).

Vanillin, syringaldehyde, and their derivatives (vanillic acid, vanillyl alcohol, syringic acid, and syringyl alcohol) were measured by Shimadzu HPLC system equipped with a YMC-Pack ODS-A column and UV/Vis detector SPD-20A. The UV detection wavelength was set at 270 nm. The mobile phase was 100% acetonitrile (pump A): 0.1% (w/v) formic acid (pump B) (9:1, v/v) with flow rate of 1.0 ml/min at 35°C. Furoic acid, furfuryl alcohol, HMF acid, and HMF alcohol were analyzed using 50% (w/v) acetonitrile as the mobile phase at 35°C and 1.0 v/min and the detection wavelength of 220 nm (Yi et al., 2019).

Oxalic acid was analyzed by Shimadzu HPLC system equipped with a Bio-rad Aminex HPX-87H column and UV/Vis detector SPD-20A. The UV detection wavelength was set at 210 nm. Twenty microliters of the sample was subjected and analyzed at 55°C using 5 mM H_2SO_4 as eluent with the flow rate of 0.4 ml/min.

The element contents were measured by inductively coupled plasma atomic emission spectrometry (725 ICP-OES, Agilent) using the simultaneous CCD detector at 1.2 kW, 15 L/min of plasma gas flow, 1.5 L/min of auxiliary gas flow, 0.75 L/min of nebulizer flow, 15 rpm of pump speed, 35 s of sample delay time, and 10 s of stabilization time (Han & Bao, 2018). The samples were prepared by mixing 0.1 g of the ions containing solids or hydrolysate with 3 ml of nitric acid and 1 ml of perchloric acid, then boiled on an electric furnace for 4 h. All the liquid was then transferred into a flask and diluted with deionized water to 25 ml before used for detection.

3 | RESULTS

3.1 | Isolation of biodetoxification microorganism with extreme low-pH tolerance

The first effort of this study focused on isolating microorganisms for biodegradation of both acid catalyst and inhibitory compounds generated from pretreatment at extremely low pH value, while the fermentable sugars (mainly xylose) were well preserved.

Fortuitously, we observed a rarely occurring microbial community growing on the sulfuric acid pretreated corn stover (pH ~2.3) containing free sulfuric acid and high inhibitor contents. The microbial community was then transferred and cultured on the synthetic medium agar gel at pH 2.3 (adjusted by sulfuric acid). A specific fungus was isolated owing to its relatively stronger growth performance than other microbial counterparts. The tolerance of the new isolate under the low pH and high inhibitor content (furfural, 5-hydroxymethylfurfural (HMF), and acetic acid) is shown in Figure 1a, with the control strain *A. resinae* ZN1. The low-pH biodetoxification performance of the new isolate was clearly superior to that of *A. resinae* ZN1 according to the colony size observed.

The isolate was identified as *Peacilomyces variotii* species and assigned as *P. variotii* FN89 by phylogenetic affiliation analysis with deoxyribonucleic acid internal transcribed spacer (ITS) sequencing (Figure 1b) and deposited as CGMCC 17665.

Promising strong organic acids used as pretreatment catalysts were selected and assayed with biodegradable potential by *P. variotii* FN89 (Table 1), including maleic acid, malonic acid, oxalic acid, fumaric acid, citric acid, and succinic acid. *P. variotii* FN89 showed good growth behaviors with 2 g/L of oxalic acid, fumaric acid, citric acid, or succinic acid as the sole carbon source, and the pH of the medium increased from 2.3 to 3.5 to 5.0–6.0, indicating *P. variotii* FN89 was capable of degrading oxalic acid, fumaric acid, citric acid, and succinic acid. No fungus growth and pH change were observed when maleic acid or malonic acid was used as the sole carbon source. Balancing the acidity and biodegradability of the acids used, oxalic acid was selected as the pretreatment catalyst.

The simultaneous inhibitor detoxification, oxalic acid degradation, and fermentable sugar preservation of *P. variotii* FN89 were further investigated in the presence of glucose or xylose (Figure 2). The results show that the oxalic acid and all the inhibitors in the medium were completely degraded by *P. variotii* FN89 within 48 h (with vanillin the only exception, taking 60 h), while over 90% of glucose or xylose was preserved when half of the inhibitor was degraded. The furan and phenolic aldehyde inhibitors (furfural, HMF, vanillin, and syringaldehyde) were ultimately degraded into the corresponding intermediate metabolite alcohols and acids, then into the central metabolic pathways without alcohols or acids residues (Yi et al., 2019). The biodegradable oxalic acid catalyst and low-pH tolerant *P. variotii* FN89 strain provided the essential tool for the SLC transformation of lignocellulose.

3.2 | Oxalic acid-catalyzed pretreatment and low-pH biodetoxification

The SLC transformation of two typical lignocellulose feedstocks, wheat straw and corn stover, were performed by oxalic acid-catalyzed pretreatment, followed by solid-state biodetoxification driven by *P. variotii* FN89 at low pH value. The sulfuric acid pretreatment was taken as the control. The pretreatment was conducted at high solid content (~70%). No aqueous water was released during the pretreatment, because the pretreated feedstocks absorbed all the free water (both the acid catalyst solution and the condensed water from vapor steam). The pretreated feedstocks were in solid particles form, instead of slurry form.

Table 2 shows the compositions of oxalic acid and sulfuric acid pretreated wheat straw and corn stover. The tendency of the compositional changes by the two acid catalysts was similar, in which most of the xylan was hydrolyzed and the cellulose remained unchanged. The inhibitors (furfural, HMF, and acetic acid) content by oxalic acid pretreatment was much lower than that by sulfuric acid pretreatment. This pretreatment operation was clean without any free wastewater generation. However, all the inhibitory compounds were accumulated into the pretreated solid feedstocks in this scenario. When the feedstock loading is high (till 30% of the total fermentation solids weight) to yield the practically reasonable ethanol titer (70–80 g/L, approximately equivalent to 10% by volume percentage), the inhibitor contents are considerably high. Although yeast strains are tolerant to one or several inhibitors at low concentrations (Palmqvist et al., 1999), the combinational inhibitors derived from the real lignocellulose hydrolysate after intensive pretreatment under

TABLE 1 Growth behavior of *P. variotii* FN89 with free organic acids as sole carbon source

	Maleic acid	Malonic acid	Oxalic acid	Fumaric acid	Citric acid	Succinic acid
Microbial growth	–	–	+	++	+++	+++
Initial pH	2.4	2.7	2.3	2.8	3.1	3.4
Ending pH	2.4	2.7	5.6	5.6	5.2	5.6

The growth of *P. variotii* FN89 in SM medium with free organic acid as sole carbon source. "+++" indicates the excellent cell growth; "++" indicates the well growth; "+" indicates the normal growth; "–" indicates no cell growth. Conditions: 10% (v/v) inoculation, 37°C, 200 rpm, 48 h.

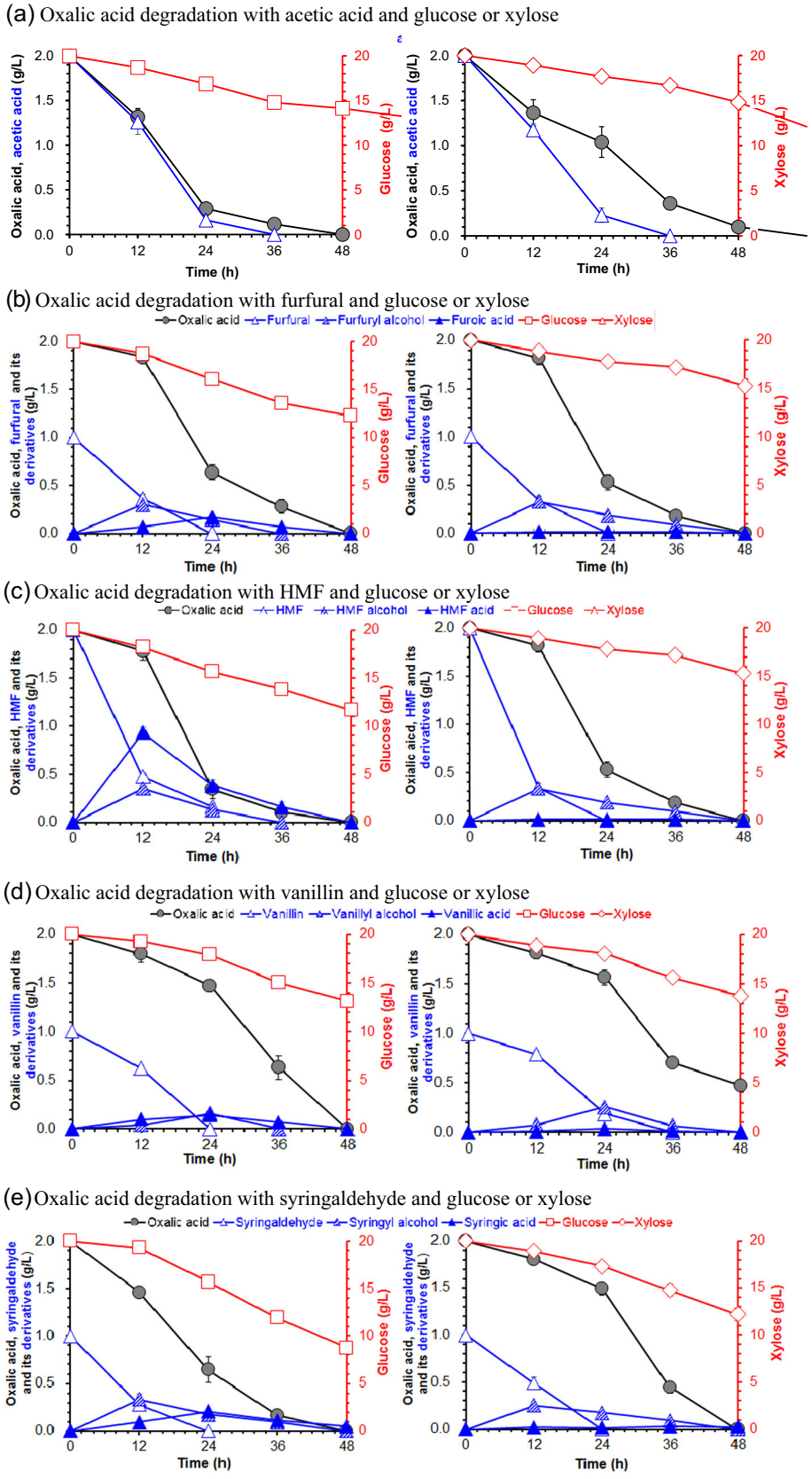

FIGURE 2 (See caption on next page)

TABLE 2 Compositions of the pretreated wheat straw and corn stover

Feedstock	Catalyst dosage (mg/g DM)[a]	Compositions (mg/g DM)[b]								
		Polysaccharides		Oligosaccharides		Monosaccharides		Inhibitors		
		Cellulose	Xylan	Glu-oligo	Xylo-oligo	Glucose	Xylose	Furfural	HMF	Acetate
Wheat straw	H_2SO_4 26.8	326.5 ± 11.2	2.6 ± 1.1	32.1 ± 0.2	96.6 ± 3.3	9.9 ± 2.7	195.3 ± 7.5	3.3 ± 0.4	1.4 ± 0.1	15.2 ± 2.8
	Oxalic acid 24.8	321.6 ± 13.1	3.5 ± 1.6	38.4 ± 2.1	155.9 ± 8.6	10.3 ± 1.2	132.4 ± 1.0	1.6 ± 0.1	0.6 ± 0.1	15.2 ± 0.4
Corn stover	H_2SO_4 26.7	294.2 ± 3.5	1.7 ± 1.3	7.5 ± 0.1	112.5 ± 2.0	30.1 ± 0.9	95.6 ± 2.6	5.2 ± 0.2	7.1 ± 0.4	22.1 ± 0.1
	Oxalic acid 24.6	312.3 ± 8.4	4.4 ± 1.8	8.0 ± 1.8	144.6 ± 17.6	11.2 ± 5.0	82.1 ± 8.6	1.9 ± 0.3	1.6 ± 0.4	18.6 ± 0.5

[a]Acid catalyst dosage was determined by the method in Han and Bao (2018).

[b]The lignin content in pretreated feedstocks is similar to raw feedstocks, which is ~170 mg/g DM for wheat straw, and ~180 mg/g DM for corn stover.

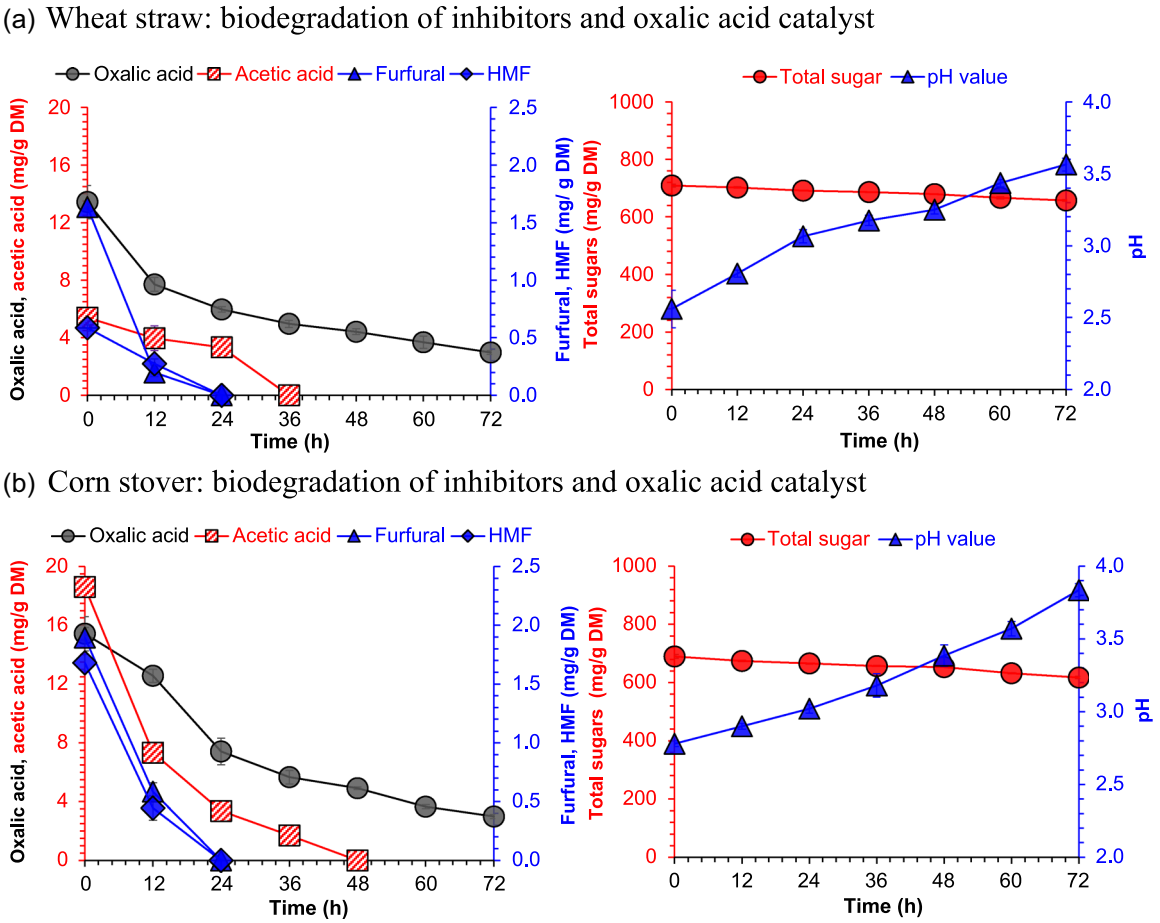

FIGURE 3 Biodetoxification of oxalic acid pretreated wheat straw and corn stover by *Paecilomyces variotii* FN89. (a) Total sugar consumption, pH value change, inhibitors biodetoxification and oxalic acid degradation of pretreated wheat straw. (b) Total sugar consumption, pH value change, inhibitors biodetoxification and oxalic acid degradation of pretreated corn stover. No pH adjustment and nutrients addition. Total sugars included cellulose, xylan, glu-oligosaccharide, xylo-oligosaccharide, glucose and xylose

FIGURE 2 Simultaneous biodegradation of inhibitors and oxalic acid catalyst by *Paecilomyces variotii* FN89 at low pH 2.3. The synthetic medium contained 2 g/L oxalic acid, 20 g/L glucose or 20 g/L xylose, and (a) 2 g/L acetic acid, (b) 1 g/L furfural, (c) 2 g/L HMF (d) 1 g/L vanillin, or (e) 1 g/L syringaldehyde as carbon source. The furan and phenolic aldehyde inhibitors' derivatives are their corresponding intermediate metabolite alcohols and acids. The initial pH was ~2.3. Fifty milliliters of the medium was added to 250 ml flask and incubated at 37°C, 100 rpm for 60 h. Each experiment was performed in triplicate. The error bar represents the standard deviation

high solids loading harshly inhibit their cell growth and metabolisms. Therefore, the complete removal of inhibitors is a prerequisite and extremely important step for well growth and high fermentation performance (Palmqvist & Hahn-Hägerdal, 2000a; 2000b).

In the consequent biodetoxification step by *P. variotii* FN89, the simultaneous biodegradation of the inhibitors and the oxalic acid catalyst in the pretreated wheat straw and corn stover was conducted without the addition of alkaline neutralizer reagent and nutrients (Figure 3). The pretreated feedstocks were directly biodetoxified in the way of solid surface culture by inoculating *P. variotii* FN89 fungus. Furfural, HMF, and acetic acid were completely degraded (after 36 h for wheat straw and 48 h for corn stover). Oxalic acid was biodegraded by 63.0% and 68.0% (36 h for wheat straw and 48 h for corn stover) simultaneously with the biodegradation of inhibitors. The residual oxalic acid (~5.0 mg/g DM) was neutralized by calcium hydroxide into insoluble oxalates. Only 3.2% and 5.3% of the total fermentable sugars were consumed during the biodegradation of oxalic acid and inhibitors of wheat straw and corn stover, respectively. The removal of oxalic acid and acetic acid resulted in the increase of pH value from 2.3 to 3.2–3.5. After a slight adjustment of pH value to above 4.0, the biodetoxified feedstocks can be used for high solids loading enzymatic saccharification and fermentation. The insoluble oxalates could be mixed with lignin residues. The solid residues containing insoluble oxalates and lignin residues were burnt for electricity generation (Liu & Bao, 2017a). In such a combustion scenario, even slight decomposition of calcium oxalate to $CaCO_3$ and CO_2 generates no sulfate oxides toxins as in the case of sulfuric acid as pretreatment catalyst (Sawada & Murakami, 2005).

3.3 | Biofuel production evaluation of SLCs from lignocellulose feedstock

The properties of morphology, compositions, enzymatic hydrolysis yield, and ethanol fermentability of the oxalic acid pretreated and biodetoxified wheat straw and corn stover were compared with that of dry milled corn meal. Then the mass and elemental balances of the SLC transformation of lignocellulose were calculated and compared with that of corn dry milling process.

The final morphology of wheat straw and corn stover was in the dry granular solid particle form with light or dark yellow color, similar to dry milled corn meal (Figure 4a). Although all the free water (both the acid catalyst solution and the condensed water from vapor steam) and lignocellulose-derived inhibitors from pretreatment were accumulated into the pretreated lignocellulose feedstocks leading to the higher water content (~50%, w/w) of pretreated feedstocks than that of corn meal (13.2 ± 3.5%), the biodetoxified feedstocks were still in granular form with no inhibitors detected (Figure 4a), owing to its favorable hygroscopicity and efficient biodetoxification operation.

The total fermentable carbohydrates content of C6-sugars (cellulose, glu-oligosaccharides, glucose) and C5-sugars (xylan, xylo-oligosaccharides, xylose) in wheat straw and corn stover were 65.2 ± 4.2% and 56.5 ± 6.0% (w/w, dry base), respectively, approximately 20% lower than the C6-sugars (starch) content of 78.2 ± 1.8% (w/w, dry base) in corn meal, but still comparable (Figure 4b). The enzymatic hydrolysis capacity test was further implemented to evaluate the sensitivity of polysaccharides in feedstocks to hydrolase. Almost 90% of polysaccharides (cellulose and xylan) in wheat straw and corn stover were enzymatically hydrolyzed into the fermentable sugars under regular conditions, similar to that of corn meal (Figure 4c).

The contents of the major elements Na, K, Ca, Mg, Al, Fe, S, and P in wheat straw and corn stover were measured and compared with those in corn meal (Figure 4d). The detection of major elements in the biorefinery chain can help control pollution emissions (e.g., sulfide) as well as recycle to farmland (e.g. phosphorus and potassium) (Liu & Bao, 2019). The sodium (Na) content in wheat straw and corn stover was approximately the same as that in corn meal, while the Mg, Ca, Al, and Fe contents were greater due to the greater ash content. The high potassium (K) and phosphorus (P) in corn meal perhaps was due to the favorable accumulation in grain section than in straw section (Cassidy, 1970; Trevizam et al., 2013). Corn meal contained more sulfur (S) owing to its higher protein content.

The mass balances were calculated with the corn dry mill process as control based on the above experimental results (Figure 4e). The mass balance starts with 100 kg (dry mass) of corn grain, wheat straw, or corn stover. The relative errors between the actual measured values and the theoretical values were shown in red to indicate the weight loss in pretreatment and biodetoxification steps (1.4%–7.5%). Corn grain is dry milled without fermentable sugar (starch) loss and no water stream generation. One hundred kilograms (dry mass) of wheat straw or corn stover contains 68.5 or 59.0 kg of the total fermentable sugars (cellulose and xylan), approximately equivalent to 75%–85% of corn grain. After oxalic acid pretreatment and low-pH biodetoxification, 209.0 kg of pretreated wheat straw and 201.5 kg of biodetoxified wheat straw were produced, respectively. Pretreatment generated 0.2 kg of furfural, 0.1 kg of HMF, and 1.5 kg of acetic acid, and then completely removed in the biodetoxification operation. A similar mass balance occurs for corn stover transformation, 220.0 kg of pretreated corn stover and 210.5 kg of biodetoxified corn stover were produced. The inhibitors generated in pretreatment were completely removed. The oxalic acid catalyst used is decomposed by 44% in pretreatment, biodegraded by 36%–39% in biodetoxification, and the residual oxalic acid is neutralized into insoluble oxalates by calcium hydroxide. The fermentable sugars were highly conserved in the SLC transformation with a gross loss of less than 8%. Overall, the SLC transformation generates the clean (acid catalyst and inhibitors free), granular solid particles, high fermentable sugar content (~65%), and favorable hydrolysis yield (~90%) under the processing constraints of no wastewater and solids stream generation, which is highly similar to corn meal feedstock from the mature dry milling process.

The ethanol production performance of the starch-like transformed wheat straw and corn stover was evaluated by simultaneous saccharification and co-fermentation (SSCF) with the dry milled corn

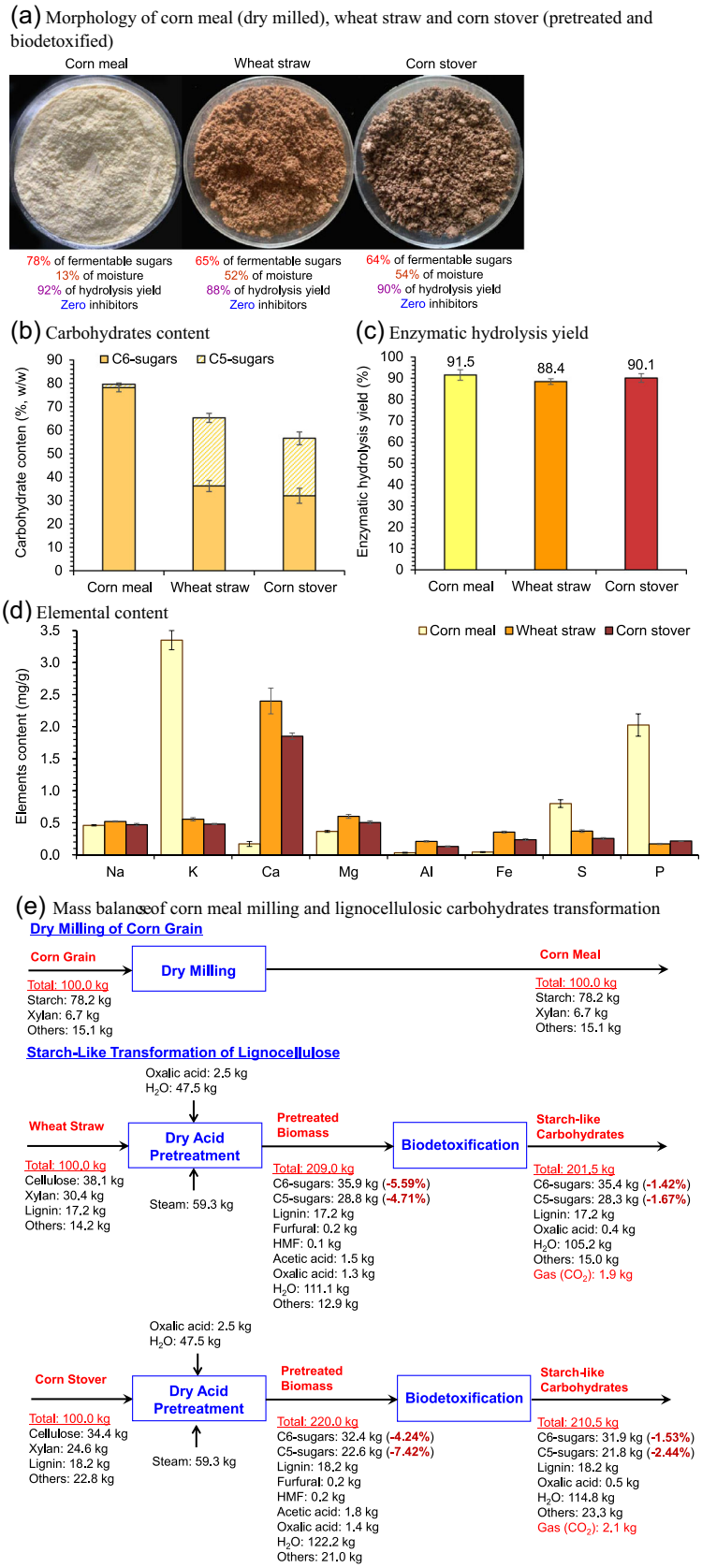

FIGURE 4 Starch-like carbohydrates transformation of lignocellulose with the comparison of corn dry milling. (a) Morphology. (b) Carbohydrates content. C6-sugars and C5-sugars in corn meal includes glucan (starch) and xylan (corn fiber components), respectively. While in corn stover and wheat straw, C6-sugars include glucan (cellulose), gluco-oligosaccharide, and glucose; C5-sugars include xylan (hemicellulose), xylo-oligosaccharide and xylose. (c) Enzymatic hydrolysis yield. Corn meal was by two-step liquefaction and saccharification as in Section 2. Wheat straw and corn stover were according to the protocol of National Renewable Energy Laboratory (NREL) LAP-009 (Brown & Torget, 1996). (d) Elemental contents. (e) Mass balances of corn dry milling and starch-like carbohydrates transformation of wheat straw or corn stover. The other components in raw wheat straw and corn stover included ash (~8%), hemicellulose factions (arabinan, mannn, galactan, acetyl groups, etc., ~7%), and protein (~3%) of the dry mass by weight percentage according to NERL report (Aden et al., 2002). The others components in corn meal include protein and lipid in corn germs and a small amount of lignin in corn fiber. The relative errors between the actual measured values and the theoretical values were shown in red to indicate the weight loss in the pretreatment and biodetoxification steps

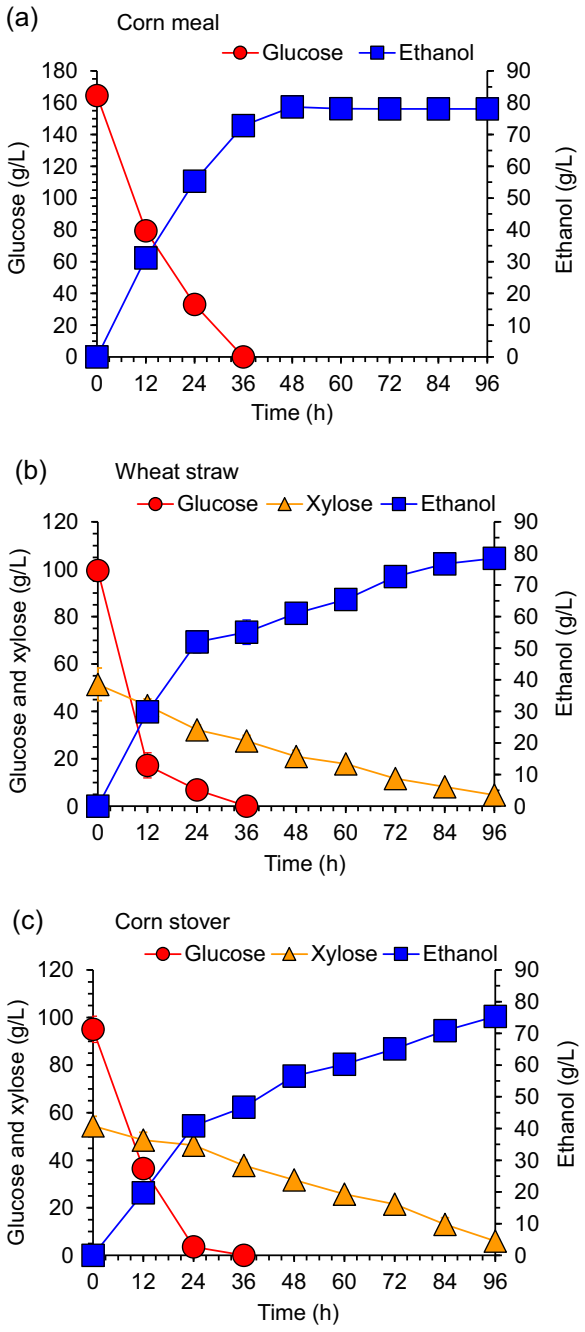

FIGURE 5 Simultaneous saccharification and co-fermentation (SSCF) of oxalic acid pretreated and low-pH biodetoxified wheat straw (b) and corn stover (c) for ethanol production with the comparison of corn meal control (a). SSCF conditions: 30°C, pH 5.5. Biodetoxification: pH was slightly adjusted to about 4.0 using lime before SSCF

meal feedstock as control (Figure 5). The solids loading of wheat straw and corn stover (~25%, w/w, dry base) was adjusted to the same fermentable sugar content with that of corn meal feedstock (21.1%, w/w, dry base). For corn ethanol production (control case), the initial glucose concentration of corn meal hydrolysate was 164.5 ± 1.8 g/L after enzymatic hydrolysis of corn meal and the final ethanol titer was 78.7 ± 1.7 g/L (10.0%, v/v) with the ethanol yield of 0.48 g/g starch (Figure 5a). For cellulosic ethanol production, the pre-saccharification produced 99.5 ± 3.7 g/L of glucose and 51.4 ± 6.9 g/L of xylose for wheat straw (totally 150.9 ± 10.6 g/L) (Figure 5b), and 95.0 ± 5.5 g/L of glucose and 54.5 ± 4.0 g/L of xylose for corn stover (total of 149.5 ± 9.5 g/L) (Figure 5c). The consequent simultaneous saccharification and co-fermentation (SSCF) produced 78.5 ± 1.3 g/L of ethanol (9.9%, v/v) from wheat straw and 75.3 ± 1.1 g/L (9.5%, v/v) from corn stover, with the ethanol yield of 0.47 and 0.45 g/g cellulose/xylose, respectively. These results indicated that both the ethanol quantity and yield of the biodetoxified wheat straw and corn stover were similar to dry milled corn meal, although the productivity of cellulosic ethanol was smaller than that of corn ethanol due to the slower xylose utilization rate of the fermenting strain *S. cerevisiae* XH7 and the weaker glucose release from cellulose than that of starch.

The overall mass balances of ethanol production start from 100 kg of dry corn grain, wheat straw, or corn stover are shown in Figure 6a. As for corn ethanol, 37.37 kg of corn ethanol is produced. The overall ethanol yield from dry corn meal was 84.5% of the theoretical value, or 0.48 g ethanol/g starch. 91.5 kg of distillers dried grains with solubles and 389.2 kg of wastewater, equivalent to 2.45 kg of solid residue and 10.36 kg of wastewater per kg of ethanol produced. As for cellulosic ethanol, 27.1 or 24.4 kg of cellulosic ethanol is produced from 100 kg of dry wheat straw or corn stover, with an overall yield of 69.3% and 72.5% of the theoretical value, or 0.40 and 0.41 g/g cellulose/xylan (approximately 83% of corn ethanol). 128.9 or 147.5 kg of lignin residue and 250.7 or 218.5 kg of wastewater are also generated respectively, equivalent to 4.76 kg or 6.04 of solid residue and 9.27 or 8.95 kg of wastewater per kg of ethanol produced. Here we only consider the generation of wastewater and the wastewater recycling network is not taken into account as in the practical ethanol plant.

Take corn meal and wheat straw as examples, the overall elemental mass balances from 100 kg of dry corn meal or wheat straw were also calculated as shown in Figure 6b. The major elements of K, Na, Ca, Mg, S, and P in soluble and insoluble forms are taken into account based on the measured element contents. Potassium (122.9 g) and phosphorus (42.5 g) in 100 kg of wheat straw are only 1/3 and 1/6 of those in 100 kg of corn grain (potassium, 382.9 g; phosphorus, 231.4 g), respectively, while the sulfur content in wheat straw (80.5 g) and corn grain (91.4 g) approximately are the same. Wheat straw contains significantly more calcium (469.3 g vs. 19.4 g) and magnesium (111.7 g vs. 41.7 g) than corn grain due to the greater insoluble ash content in agricultural residues. Based on ethanol production (per kg of ethanol), the sodium and sulfur release from cellulosic ethanol (9.07 and 3.52 g) are similar to those from corn ethanol (9.58 and 4.19 g). Only half potassium (6.68 g) and 1/3 of phosphorus (2.33 g) are released in cellulosic ethanol production comparing with those in corn ethanol production (K, 12.72 g; P, 7.43 g). More calcium (22.19 vs. 0.90 g) and magnesium (7.21 vs. 3.56 g) are accumulated into the solid residues in cellulosic ethanol production than in corn ethanol production.

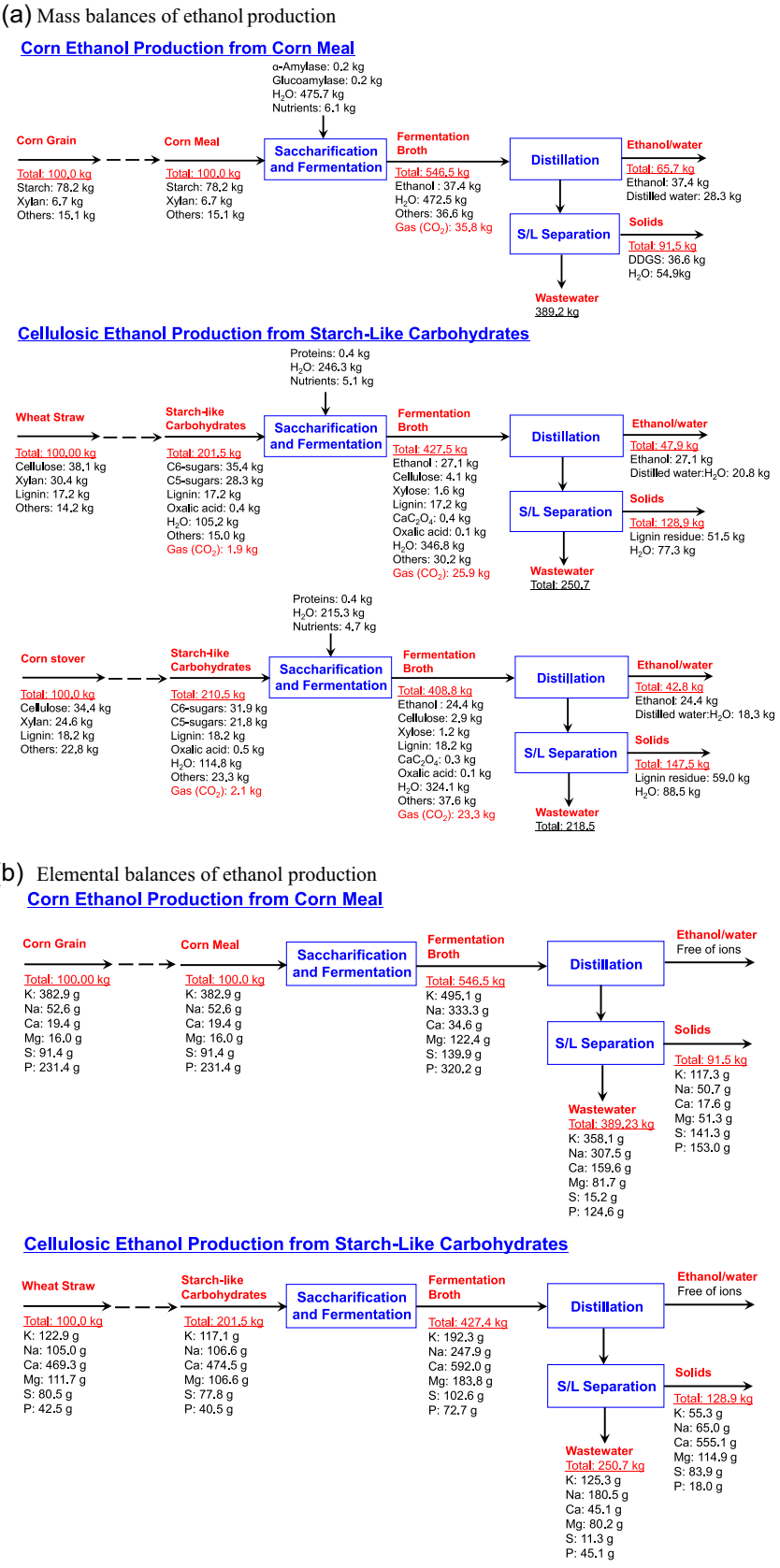

FIGURE 6 Mass and elemental balances of corn ethanol and cellulosic ethanol production. (a) Mass balances of ethanol production from corn meal or starch-like carbohydrates. The biodetoxification seed was cultured in the pretreated feedstock without adding water and nutrients, therefore, the inoculation in the biodetoxification step was not shown. For ethanol fermentation, the quality of inoculation was converted to the final fermentation broth. Ethanol distillation is a mature and conventional industrial operation composed of beer column, rectification column, and molecular sieve adsorption column to obtain the fuel ethanol product (99.5% purity). This study conducted the first distillation operation in bench scale with a glass column of 25 mm in diameter. A distillation system equipped with a heat exchanger and spiral condenser was used. The volumetric ethanol concentration in the distillate of the glass column was ~72%. The mass balance was the simplified calculation based on the conventional distillation operation. (b) Elemental balances of ethanol production from corn meal or wheat straw

In summary, though the biodetoxified feedstocks contain much higher content of lignin (170–190 mg/g DM) and higher moisture (over 50% DM) than corn meal, the essential features of the oxalic acid pretreated and low-pH biodetoxified lignocellulose feedstocks are similar or same to the dry milled corn meal in terms of solid particles morphology, fermentable sugar contents, elemental contents, enzymatic hydrolysis yield, and ethanol fermentability. The mass balance results suggest that the transformation leads to a similar and comparable conversion yield and wastewater generation for fuel ethanol production from lignocellulose feedstock to corn feedstock. The elemental mass balance results further indicate that the transformation and cellulosic ethanol production was acceptable in terms of pollution release and controlling potassium and phosphorus recycling. The purpose to copy the technical success of corn dry milling in lignocellulose biorefinery has been successfully achieved by the transformation as described in this study. In other words, the lignocellulose feedstocks are transformed into "starch-like carbohydrates (SLC)."

4 | DISCUSSIONS

Biorefinery processing technology developed in the past two decades has encountered serious technical barriers, failures, economic loss when it is applied to commercial-scale practice (Almuth & Rachel, 2018; Irshad, 2016; Sarantis, 2018). These barriers include large-scale wastewater and toxin discharge, high sugar loss, and low conversion efficiency among other difficulties. Overcoming these technical barriers is the prerequisite for the future revival of this highly promising technology into commercial practice. This study demonstrated a practical solution for cellulosic biofuel production by transforming lignocellulose biomass into SLC, similar to dry milled corn meal and in a similar fashion with corn dry milling process in almost all measurable performances.

We started with the isolation of a unique biodetoxification fungus, *P. variotii* FN89, for biodegradation of inhibitors and organic acid catalysts at extremely low pH value. A specific organic acid with strong acidity and available biodegradability, oxalic acid, was selected for dry acid pretreatment, then followed by *P. variotii* FN89-driven biodetoxification to generate clean, hydrolysis-favorable carbohydrates from wheat straw and corn stover. The SLC transformation perfectly met the requirements of (i) highly efficient pretreatment efficiency with high hydrolysis capacity and free of wastewater generation; (ii) complete removal of both inhibitory compounds and the oxalic acid catalyst to produce the clean and solids form carbohydrates; and (iii) minimum fermentable sugars loss (less than 8%) during the transformation and no generation of highly concentrated soluble salts or insoluble solids from neutralization. The obtained biodetoxified wheat straw and corn stover feedstocks were similar to dry milled corn meal in terms of morphological observation, fermentable sugar content, enzymatic hydrolysis yield, elemental contents, ethanol fermentability, and wastes generation, indicating a successful transformation of lignocellulose feedstock into SLC.

Previous studies showed that organic acids catalyzed pretreatment such as maleic acid, fumaric acid, and oxalic acid resulted in the similar hydrolysis efficiency of lignocellulose with sulfuric acid with lower inhibitors generation (Kootstra et al., 2009a, 2009b; Qing et al., 2015). Not only oxalic acid is biodegradable after the catalyst use, but also is sustainable in its production by glucose oxidation (Betiku et al., 2016; Sawada & Murakami, 2005). The glucose could be derived from lignocellulose as the feedstock for oxalic acid production, enabling a fully converged oxalic acid production and consumption. Meanwhile, the biological production of oxalic acid from fermentable sugars can further reduce the cost in the future (Betiku et al., 2016). In the practical demonstration installation, a unit of oxalic acid in-situ production can be built to reduce the cost of acid catalyst. Furthermore, according to the NREL report (Humbird et al., 2011), the cost of sulfuric acid (~85 $/ton, website: www.1688.com) catalyst for cellulosic ethanol production is 2.4 cents/gal ethanol. The cost of oxalic acid (~500 $/ton, industrial grade) can be calculated accordingly as 14.1 cents/gal ethanol. However, the total ethanol cost only increased by ~5.4% (from 2.15 to 2.26 $/gal) without considering the benefits of using oxalic acid as catalyst. On balance, therefore, the increased cost of replacing sulfuric acid with oxalic acid catalyst is acceptable.

The simultaneous biodegradation of oxalic acid catalyst and inhibitors at low pH (without neutralization) by *P. variotii* FN89 shows a favorable priority of inhibitors and oxalic acid to fermentable sugars (Figures 2 and 4), ensuring well preservation of most fermentable sugars for biofuel production in the consequent hydrolysis and fermentation steps. This unique tolerance of *P. variotii* FN89 toward low-pH biodegradation of inhibitors and organic acids remains under investigation to elucidate the mechanism.

5 | CONCLUSION

We designed and verified a biodegradable oxalic acid catalyzed pretreatment with zero wastewater stream generation and high enzymatic hydrolysis yield. A unique biodetoxification fungus *P. variotii* FN89 was isolated and applied to biodegrade oxalic acid and inhibitors simultaneously in the pretreated feedstocks at low pH (without neutralization). Two typical lignocellulose feedstocks, wheat straw and corn stover, were successfully transformed into a SLC feedstock, similar to dry milled corn meal in morphological observation, fermentable sugar content, enzymatic hydrolysis yield, elemental contents, free of inhibitors and acid catalyst, and ethanol fermentability. The ethanol production from wheat straw and corn stover after the starch-like transformation was 78.5 and 75.3 g/L with the yield of 0.47 and 0.45 g ethanol/g cellulose/xylose, respectively, comparing with 78.7 g/L and 0.48 g ethanol/g starch yield from dry milled corn meal. The mass balance calculations suggest that the ethanol conversion yield, wastewater generation, and elemental recycling in the starch-like

transformation were highly comparable with these of corn feedstock in dry milling process. The cellulosic ethanol production using SLC maximizes the potentials to compete with corn ethanol on the aspects of conversion rate, emissions, especially in the control of potassium and phosphorus recycling.

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AUTHOR CONTRIBUTIONS

Jie Bao conceived and directed the study. Faryal A. Khushik and Jie Bao isolated *P. variotii* FN89; Bin Zhang and Faryal A. Khushik performed the biodetoxification; Bin Zhang and Baorui Zhan performed the pretreatment; Bin Zhang and Jie Bao designed research and drafted the manuscript; Bin Zhang analyzed the data; all the authors edited and approved the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All study data are included in the article.

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