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Dry biorefining maximizes the potentials of simultaneous saccharification and co-fermentation for cellulosic ethanol production

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Abstract

Despite the well-recognized merits of simultaneous saccharification and co-fermentation (SSCF) on relieving sugar product inhibition on cellulase activity, a practical concomitance difficulty of xylose with inhibitors in the pretreated lignocellulose feedstock prohibits the essential application of SSCF for cellulosic ethanol fermentation. To maximize the SSCF potentials for cellulosic ethanol production, a dry biorefining approach was proposed starting from dry acid pretreatment, disk milling, and biodetoxification of lignocellulose feedstock. The successful SSCF of the inhibitor free and xylose conserved lignocellulose feedstock after dry biorefining reached a record high ethanol titer at moderate cellulase usage and minimum wastewater generation. For wheat straw, 101.4 g/L of ethanol (equivalent to 12.8% in volumetric percentage) was produced with the overall yield of 74.8% from cellulose and xylose, in which the xylose conversion was 73.9%, at the moderate cellulase usage of 15 mg protein per gram cellulose. For corn stover, 85.1 g/L of ethanol (equivalent to 10.8% in volumetric percentage) is produced with the overall conversion of 84.7% from cellulose and xylose, in which the xylose conversion was 87.7%, at the minimum cellulase usage of 10 mg protein per gram cellulose. Most significantly, the SSCF operation achieved the high conversion efficiency by generating the minimum amount of wastewater. Both the fermentation efficiency and the wastewater generation in the current dry biorefining for cellulosic ethanol production are very close to that of corn ethanol production, indicating that the technical gap between cellulosic ethanol and corn ethanol has been gradually filled by the advancing biorefining technology.

KEYWORDS
biodetoxification, cellulosic ethanol, dry acid pretreatment, lignocellulose, simultaneous saccharification and co-fermentation (SSCF), wastewater generation

Gang Liu and Qiang Zhang contributed equally to this work.

1 | INTRODUCTION

Lignocellulose biorefining for ethanol production includes steps of prehandling, pretreatment, detoxification (conditioning), hydrolysis, fermentation, and recovery (Lynd, Laser, & Bransby, 2008). Pretreatment operation disrupts lignocellulose structure and partially hydrolyzes hemicellulose, but also generates various inhibitor compounds such as furan aldehydes, phenolic aldehydes, and weak organic acids (Balan, 2014; Jorgensen, Kristensen, & Felby, 2007). The fast and complete removal of the inhibitors (detoxification) from pretreated lignocellulose feedstock is crucially important for achieving the highly efficient enzymatic hydrolysis and ethanol fermentation (Jing, Zhang, & Bao, 2009; Klinke, Thomsen, & Ahring, 2004). However, the conventional water washing or overliming detoxification methods inevitably company with the heavy loss of xylose (Humbird et al., 2011; Liu & Chen, 2016). The existence of inhibitor compounds leads to the reduced cellulase activity and poor fermenting cell growth and/or metabolism, but the low xylose content after water washing or overliming makes the co-fermentation of xylose less meaningful on increasing ethanol yield (Koppram, Tomas-Pejo, Xiros, & Olsson, 2014). Therefore, despite the well-recognized advantage of simultaneous saccharification and co-fermentation (SSCF) on relieving product (glucose) inhibition on cellulase activity, as well as the fact that various xylose assimilation strains are available (Jin et al., 2017; Kawaguchi, Hasunuma, Ogino, & Kondo, 2016; Ohgren, Rudolf, Galbe, & Zacchi, 2006), the potentials of SSCF operation had not been fully demonstrated due to the concomitance difficulty of xylose with inhibitors in the pretreated lignocellulose feedstock.

To maximize the SSCF potentials for cellulosic ethanol production, a dry acid pretreatment and biodetoxification approach (DryPB) is proposed as illustrated in Figure 1. DryPB starts from the dry sulfuric acid pretreatment under extremely high lignocellulose solids ratio (70%, w/w), followed by a brief disk milling to remove the extra-long fibers to avoid the blockage in pipes and valves. The inhibitor compounds generated from the dry acid pretreatment are all accumulated onto the pretreated lignocellulose solids because of no generation of wastewater streams during the pretreatment. Then a "biodetoxification" operation is conducted on the pretreated lignocellulose solids by a unique fungus *Amorphotheca resinae* ZN1 with the priority of inhibitor utilization to xylose. The inhibitor free and xylose conserved lignocellulose feedstock is sent to the SSCF and a record high ethanol titer is achieved at the moderate cellulase usage and the minimum wastewater generation.

The current SSCF refined wheat straw into 101.4 g/L (equivalent to 12.8% in the volumetric percentage) of ethanol with the overall yield of 74.8% from cellulose and xylose in which the xylose conversion was 73.9% at the moderate cellulase usage of 15 mg protein per gram cellulose. The refining of corn stover produced 85.1 g/L(equivalent to 10.8% in the volumetric percentage) of ethanol with the overall yield of 84.7% from cellulose and xylose in which the xylose conversion was 87.7% at the minimum cellulase usage of 10 mg protein per gram of cellulase. Most significantly, the SSCF operation achieved the high conversion efficiency by generating the minimum amount of wastewater. Both the fermentation efficiency and the wastewater generation in the current dry biorefining for cellulosic ethanol production are very close to that of corn ethanol production, indicating that the technical gap between cellulosic ethanol and corn ethanol has been gradually filled by the advancing biorefining technology.

2 | MATERIALS AND METHODS

2.1 | Raw materials

Five lignocellulose feedstocks were collected from their dominant growing regions in China. Corn stover was harvested from Bayan Nur,

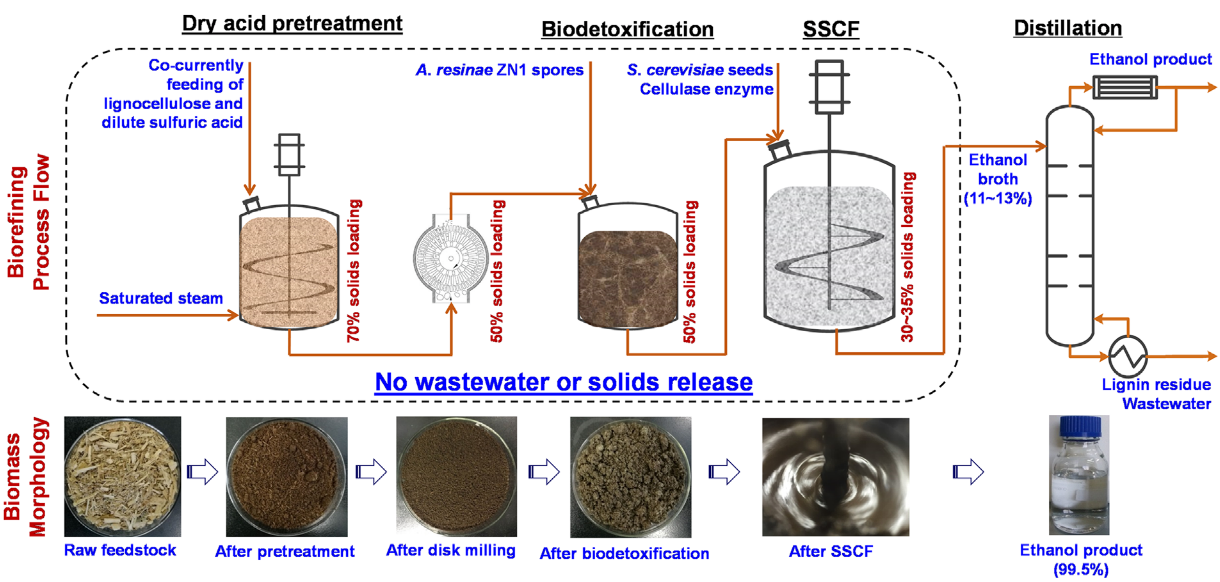

FIGURE 1 Diagram of DryPB biorefining process for cellulosic ethanol production

Inner Mongolia, China in fall 2015. Wheat straw was harvested from Dan Cheng, Henan, China in fall 2013. Rice straw was harvested from Chang Zhou, Jiangsu, China in fall 2014. Sugarcane bagasse was obtained from the sugar plant of Bei Hai, Guangxi, China in fall 2014. Italian poplar sawdust was obtained from the wood factory in Yan Cheng, Jiangsu, China in fall 2015. The field dirt, sands, metal pieces, and other impurities were carefully avoided during the collection and then screened during the prehandling. The collected corn stover, wheat straw, rice straw, and sugarcane bagasse were ground coarsely using a beater pulverizer and screened through a mesh with the circle diameter of 10 mm. Poplar sawdust was used directly without grinding.

The composition of the lignocellulose feedstocks was measured by the two-step acid hydrolysis method according to National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008, 2012). The cellulose, hemicellulose, lignin, and ash content of the five lignocellulose feedstocks on the dry weight base (w/w) are shown in Table 1.

2.2 | Enzyme and reagents

Commercial cellulase enzyme Cellic CTec 2.0 was kindly provided by Novozymes (China) Investment Co., Beijing, China. The filter paper activity was 203.2 FPU per milliliter of cellulase determined according to the NREL protocol LAP-006 (Adney & Baker, 1996). The cellobiase activity was 4,900 CBU per milliliter of cellulase determined using the method of Ghose (1987). The total protein concentration was 87.3 mg/ml of cellulase determined by Bradford (1976) method using bovine serum albumin (BSA) as protein standard. The cellulase enzyme was used based on the total protein weight per gram of cellulose substrate in the lignocellulose feedstock.

The reagents KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, H_2SO_4 were purchased from a local provider Lingfeng Chemical Reagent Co., Shanghai, China. Yeast Extract was from Angel Yeast Co., Yichang, China. Agar was from Aladdin BioChem Co., Shanghai, China.

2.3 | Strains and media

Biodetoxification fungus *A. resinae* ZN1 was isolated in our previous work and stored in China General Microorganism Collection Center (CGMCC, Beijing, China) with the registration number 7452 (Zhang, Zhu et al., 2010). *A. resinae* ZN1 was maintained on potato-dextrose-agar (PDA) slant prepared by boiling 200 g of peeled and sliced potatoes in one liter deionized water for 30 min with the addition of 15 g of agar.

Ethanol fermentation strain *Saccharomyces cerevisiae* XH7 was derived from the wild-type diploid *S. cerevisiae* strain BSIF through rationally designed genetic modifications combined with adaptive evolution in xylose including genomic integration of the novel gene Ru-*xylA* encoding xylose isomerase, overexpression of *XKS1* encoding endogenous xylulokinase and four genes of non-oxidative pentose phosphate pathway, and the inactivation of two genes *GRE3* and *PHO13* encoding aldose reductase and alkaline phosphatase, respectively (Li et al., 2015, 2016). The strain was cultured in YPD medium (20 g/L of glucose, 20 g/L of peptone, and 10 g/L of yeast extract) or corn stover slurry for activation and adaption procedure.

TABLE 1 Composition of the five lignocellulose feedstocks on the dry weight base (w/w)

Feedstocks	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Corn stover	35.4	24.6	16.1	3.5
Wheat straw	38.7	25.9	14.9	5.2
Rice straw	35.3	18.4	22.5	9.3
Sugarcane bagasse	38.8	23.9	26.4	1.3
Poplar sawdust	39.7	16.6	29.4	3.2

2.4 | Dry sulfuric acid pretreatment and biodetoxification

Corn stover, wheat straw, rice straw, sugarcane bagasse, or poplar sawdust were pretreated using the dry acid pretreatment method (He, Zhang, Zhang, & Bao, 2014; He, Zhang, & Bao, 2014; Zhang, Wang, Chu, He, & Bao, 2011). Briefly, 1,200 g of feedstock (dry base) and approximately 500–600 g of 5% (w/w) dilute sulfuric acid solution (depending on the moisture content of the feedstock) were co-currently fed into the pretreatment reactor for 3 min at the solids/liquid ratio of 2:1 (w/w). The reactor was 20 L in the inner volume and thermally insulated. A single helical ribbon impeller was installed under the mild agitation rate (50 rpm). The sulfuric acid concentration in the dilute acid solution was adjusted in a narrow range according to the measured moisture content of the feedstocks. The saturated water steam (1.6 MPa, 201°C) was produced from a steam generator machine (HX-36D, Huazheng Boiler Co., Shanghai, China). The pretreatment operation started when the hot steam was jetted onto the feedstock bulk in the reactor to 175 ± 1°C for 5 min under the mild helical agitation (50 rpm). Then the pretreated solid feedstocks were discharged gravitationally from the bottom outlet port. All the dilute acid solution and the condensed water were completely adsorbed into the solids to form approximately 50% (w/w) of the dry pretreated feedstock solids with the pH around 2.0, and no free wastewater stream was generated. The sulfuric acid in the pretreated biomass solids was neutralized to 5.5 by the addition of 20% (w/w) $Ca(OH)_2$ suspension slurry. The pretreated biomass solids were briefly milled by a disk milling machine (PSB-80JX, Fleck Co., Nantong, Jiangsu, China) to remove the extra-long fibers to avoid the blockage of pipelines and valves in the downstream flow of the hydrolysate slurry and broth.

The pretreated solids were aerobically biodetoxified in a 15 L bioreactor to remove the inhibitors generated during the dry acid pretreatment operation (He, Zhang, & Bao, 2016; Zhang, Zhu et al., 2010). Briefly, *A. resinae* ZN1 fungus was grown on potato-dextrose-agar (PDA) slant at 28°C for sporulation. The spores were collected and diluted to approximately $5–6 \times 10^6$ per milliliter of the spore suspension and inoculated onto the pretreated lignocellulose solids at the weight ratio of 10% (the ratio of the spore suspension weight to the pretreated feedstock weight) for 5 days as the biodetoxification seeds. Then the seed solids were inoculated onto the freshly pretreated solid feedstock at 10% (w/w) inoculation ratio. The biodetoxification was conducted at 28°C and the water saturated aeration of 0.8 vvm (the ratio of the air input rate in liter per minute to the pretreated biomass volume) for 36–48 hr. The major inhibitors including furfural, 5-hydroxymethylfurfural (HMF), acetic acid and phenolic aldehydes were completely assimilated and degraded. Xylose and glucose released during the pretreatment were preserved without observable loss because of the priority of inhibitors as substrates to the sugars by *A. resinae* ZN1. No cellulose degradation was observed by *A. resinae* ZN1 during the biodetoxification period.

2.5 | Simultaneous saccharification and co-fermentation (SSCF)

The pretreated and biodetoxified lignocellulose feedstock solids were pre-hydrolyzed into liquid hydrolysate slurry in a short period (12 hr) at 50°C, pH 4.8 in the specially designed 5 L bioreactors equipped with helical ribbon impeller (Zhang, Chu et al., 2010). Then glucose and xylose were co-fermented into ethanol simultaneously with the hydrolysis of cellulose and oligomer sugars at the high solids loading (25–35%, w/w) at 30°C for 96 hr by inoculating the shortly adapted yeast seed cells *S. cerevisiae* XH7 into the hydrolysate at 10% (v/v). pH was increased to at 5.5 by adding 5 M NaOH solution. Then the SSCF operation was scaled up by 10-folds to a 50 L bioreactor with the same mixing structure and helical ribbon impeller with the working volume of 30 L at the solids loading of 30% (w/w). Higher pH lessens the adsorption of cellulase on lignin (Lan, Lou, & Zhu, 2013; Leu and Zhu, 2013; Lou, Zhu, Lan, Lai, & Qiu, 2013; Wang, Lan, & Zhu, 2013). Therefore, the SSCF was conducted at pH value 5.5 instead of the optimal pH 4.8 for cellulase activity. However, the hydrolysis yield was not affected by the varying pH value in the short period of pre-hydrolysis (12 hr) (Figure S1; Zhao et al., 2013) and the pre-hydrolysis was still conducted at pH 4.8 then switched to 5.5 for the 96 hr SSCF.

The *S. cerevisiae* XH7 seed broth was prepared in a two-step adaption procedure using the pretreated and biodetoxified corn stover as the carbon source, instead of glucose for reducing the cost of seed culture (Qureshi, Zhang, & Bao, 2015). One vial of the frozen culture of *S. cerevisiae* XH7 was activated in 20 ml of YPD medium at 30°C for 12 hr as the adaption seed. In the first adaption step, the seed culture was inoculated into the corn stover slurry containing 5% (w/w) of the pretreated and biodetoxified corn stover and the cellulase dosage of 10 mg protein per gram of cellulose. In the second step adaption, the culture from the first adaptation was inoculated into the corn stover slurry containing 10% (w/w) of the same corn stover and cellulase dosage. The first and second step adaptions were lasted for 12 and 24 hr at 30°C, pH 5.5. 10% (v/v) of the adapted broth was inoculated into the SSCF fermentation system with the nutrient addition of 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, and 10 g/L of yeast extract.

Samples were periodically withdrawn and for analysis of glucose, xylose, ethanol, glycerol, acetic acid, furfural, and HMF.

2.6 | Calculation of ethanol yield

The ethanol yield (%) in SSCF was calculated based on the method by Zhang and Bao (2012):

$$\text{Ethanol yield}(\%) = \frac{[\text{Ethanol}] \times W}{976.9 - 0.804 \times [\text{Ethanol}]} \times \frac{1}{0.511 \times ([\text{Cellulose}] \times 1.111 + [\text{Xylose}]) \times [\text{Solids}] \times M} \times 100\%$$

where [Ethanol] is the ethanol concentration in the liquid portion of the fermentation broth at the end of SSCF (g/L); *W* is the total water input in both the hydrolysis and the SSCF periods (g); *M* is the total weight of SSCF system at the beginning of the operation (g); [Cellulose] is the cellulose content in the dry pretreated feedstock (g/g); [Xylose] is the xylose content in the dry pretreated feedstock (g/g); [Solids] is the pretreated solids content of the hydrolysis and SSCF system on the dry base (g/g); 976.9 is the ethanol correction factor (g/L) between the mass concentration (g/g) and the volumetric concentration (g/L); 0.804 is the dimensionless factor in calculating water loss in SSCF; 0.511 is the dimensionless conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast; 1.111 is the dimensionless conversion factor for cellulose to equivalent glucose.

Xylose conversion is calculated by measuring the percentage ratio of the decreased xylose concentration in the hydrolysate at the beginning and the end of the SSCF operation over the total xylose concentration.

2.7 | Inhibitors, sugars, and ethanol analysis

Glucose, xylose, ethanol, glycerol, furfural, HMF, acetic acid were analyzed on HPLC (LC-20AD, Shimadzu, Kyoto, Japan) equipped with Bio-rad Aminex HPX-87H column (Bio-rad, Hercules, CA) and RID-10A detector (Shimadzu, Kyoto, Japan). A total of 5 mM H_2SO_4 solution was used as flow phase at the flow rate of 0.6 ml/min. Furans were analyzed on HPLC (LC-20AT, Shimazu, Kyoto, Japan) equipped with YMC-Pack ODS-A column (YMC, Tokyo, Japan) and an SPD-20A UV detector (Shimadzu, Kyoto, Japan).

The yeast cell viability in the simultaneous saccharification and co-fermentation (SSCF) was assayed by counting the colony forming units (CFU) on the YPD petri dish when the 100 µl of the 10^{-5} or 10^{-6} diluted fermentation broth withdrawn at different time points were stretched and cultured for 48 hr at 30°C.

3 | RESULTS

3.1 | Minor tuning of pretreatment and detoxification parameters to changing feedstocks

Five lignocellulose biomass including corn stover, wheat straw, rice straw, sugarcane bagasse, and poplar sawdust were dry acid pretreated and biodetoxified before the simultaneous saccharification and co-fermentation (SSCF). The varied biorecalcitrance requires the adjustment of catalyst (sulfuric acid) usage in a small range to achieve an optimal hydrolysis yield and fermentability (Table 2). Intensive pretreatment led to the higher hydrolysis yield but suppressed ethanol fermentability, thus a compromise was made among the hydrolysis efficiency, inhibitor generation and ethanol fermentability by regulating the pretreatment severity, biodetoxification intensity, and fermentation parameters (Figure 2a). The adjusted sulfuric acid usage was 2.0–2.5 g/100 g of dry feedstock, in which 2.0 g for corn stover, sugarcane bagasse, and poplar sawdust, and 2.5 g/100 g for wheat straw and rice straw. Biodetoxification time was also varied from 36 hr (for wheat straw and rice straw) to 48 hr (for corn stover) or 60 hr (for sugarcane bagasse and poplar sawdust) according to the inhibitor generation levels. The ethanol titer was in the range of 71.9–88.0 g/L and the xylose conversion was of 77.2–90.6% for corn stover, wheat straw, rice straw, sugarcane bagasse, and poplar sawdust (Table 2). 10–15 g/L of glycerol was generated due to the relatively high initial glucose and xylose concentration after the prehydrolysis of the pretreated lignocellulose feedstocks (Figure S2).

3.2 | SSCF under varying feedstock solids loadings

Higher feedstock solids loading directly leads to the higher ethanol titer but also leads to the higher initial sugar inhibition on cellulase activity and fermenting strain. Three solids loadings of the pretreated and biodetoxified corn stover and wheat straw in the range of 25%, 30%, 35% (w/w) were tested (Figures 2b and 2c). For the maximum solids loading of 35% (w/w), minor adjustments were made including to divide 2/3 of the cellulase (10 mg/g cellulose) to the pre-hydrolysis stage and 1/3 (5 mg/g cellulose) to the SSCF stage to reduce the initial glucose concentration in the prehydrolysis, as well as to extend the biodetoxification time to 72 hr for the complete removal of the higher inhibitors with the observable xylose loss (approximately 7%). In the yeast seed culture step, the pretreated corn stover or wheat straw was used as the carbon source, instead of pure glucose, by adding cellulase in the seed culture flasks or bioreactors for minimizing the overall cost, and the yeast seed slurry containing residual cellulose, hemicellulose, as well as inert lignin residues were inoculated into the main fermentation bioreactors for SSCF.

At the solids loading of 25%, 30%, and 35% (w/w), corn stover produced 71.2, 78.7, 90.3 g/L of ethanol with the overall yield of 90.6%, 78.6%, 73.5%, and the xylose conversion yield of 89.2%, 80.7%, 59.5%, respectively (Figure 2b); wheat straw produced 79.9, 88.0, 101.1 g/L were produced with the overall yield of 87.1%, 79.9%, 74.8%, and xylose conversion yield of 95.6%, 89.9%, 73.9%, respectively (Figure 2c). The ethanol yield decreased with increasing solids loading because of the declining cell viability indicated by the colony forming units (CFU) and the increased formation of glycerol (12, 14, 20 g/L for corn stover, and 14, 16, 21 g/L for wheat straw at 25, 30, 35% solids loadings, respectively) due to the increased osmotic pressure of the initial glucose (Figures S2b and S2c). The

TABLE 2 Biorefining evaluation by minor tuning of pretreatment and biodetoxification parameters on different feedstocks

	Structural composition									SSCF	
Varying parameters	Cellulose (%, w/w)	Xylan	Glucose (mg/g DM)	Xylose	Acetate	Furfural	HMF	Hydrolysis yield(%)	Detoxification time (h)	Ethanol titer (g/L)	Xylose conversion (%)
(a) Corn stover at varying sulfuric acid dosage (H_2SO_4 mg/g DM) in the dry acid pretreatment											
25.0	36.5 ± 0.5	4.5 ± 0.4	29.7 ± 0.9	155.1 ± 2.1	21.7 ± 0.2	4.0 ± 0.0	3.6 ± 0.4	85.4 ± 0.7	96	78.9	82.0
22.5	37.0 ± 0.4	4.3 ± 0.3	20.1 ± 1.8	139.6 ± 1.8	15.1 ± 0.3	3.2 ± 0.1	3.2 ± 0.1	83.0 ± 0.9	48	78.0	71.4
20.0	37.6 ± 0.6	4.4 ± 0.0	22.1 ± 1.2	138.0 ± 1.7	15.3 ± 0.2	3.3 ± 0.0	2.8 ± 0.8	77.1 ± 0.6	36	77.1	84.2
17.5	38.6 ± 0.4	6.7 ± 0.0	13.3 ± 1.1	102.3 ± 0.9	14.2 ± 0.5	1.9 ± 0.1	1.7 ± 0.4	68.7 ± 1.1		Not conducted	
15.0	39.0 ± 0.7	8.0 ± 0.5	9.9 ± 0.6	73.3 ± 0.8	11.3 ± 0.3	1.2 ± 0.0	1.1 ± 0.0	53.8 ± 0.3		Not conducted	
(b) Operation at minor-tuned conditions of dry acid pretreatment and biodetoxification (sulfuric acid dosage, 20 mg/g DM for corn stover, bagasse, poplar sawdust; 25 mg/g DM for wheat straw, rice straw)											
Corn stover	37.6 ± 0.6	4.4 ± 0.0	22.1 ± 1.2	138.0 ± 1.7	15.3 ± 0.2	3.3 ± 0.0	2.8 ± 0.8	77.1 ± 0.6	48	78.7	77.2
Wheat straw	40.2 ± 0.1	1.6 ± 0.0	32.6 ± 1.6	165.2 ± 3.8	12.5 ± 0.0	4.9 ± 0.0	2.7 ± 0.1	98.7 ± 0.4	36	88.0	89.9
Rice straw	40.7 ± 0.2	4.8 ± 0.0	35.9 ± 0.8	115.7 ± 4.0	16.3 ± 0.0	4.7 ± 0.0	4.9 ± 0.3	98.1 ± 0.8	36	71.9	90.6
Bagasse	38.8 ± 0.5	2.7 ± 0.0	43.0 ± 0.4	187.9 ± 0.3	22.5 ± 0.0	4.5 ± 0.0	2.1 ± 0.3	78.5 ± 0.9	60	78.3	89.7
Poplar sawdust	43.7 ± 1.1	0.9 ± 0.0	43.0 ± 2.5	164.0 ± 3.3	21.6 ± 0.3	3.4 ± 0.0	3.0 ± 0.9	72.4 ± 1.4	60	79.1	80.8

Biorefining evaluation on corn stover, wheat straw, sugarcane bagasse, rice straw, Italian poplar sawdust feedstocks were conducted at the conditions: Pretreatment at 175°C for 5 min with the sulfuric acid dosage of 15–25 mg per gram of dry feedstock; Detoxification: 28°C for 36, 48, 60, or 96 hr with the aeration rate of 0.8 vvm. Prehydrolysis conditions: 50°C and pH 4.8 for 12 hr, 15 mg cellulase protein per gram cellulose, 30% (w/w) of the dry solids loading. Fermentation conditions: *S. cerevisiae* XH7, 10% (v/v) of inoculum ratio, 30°C and pH 5.5 for 96 hr.

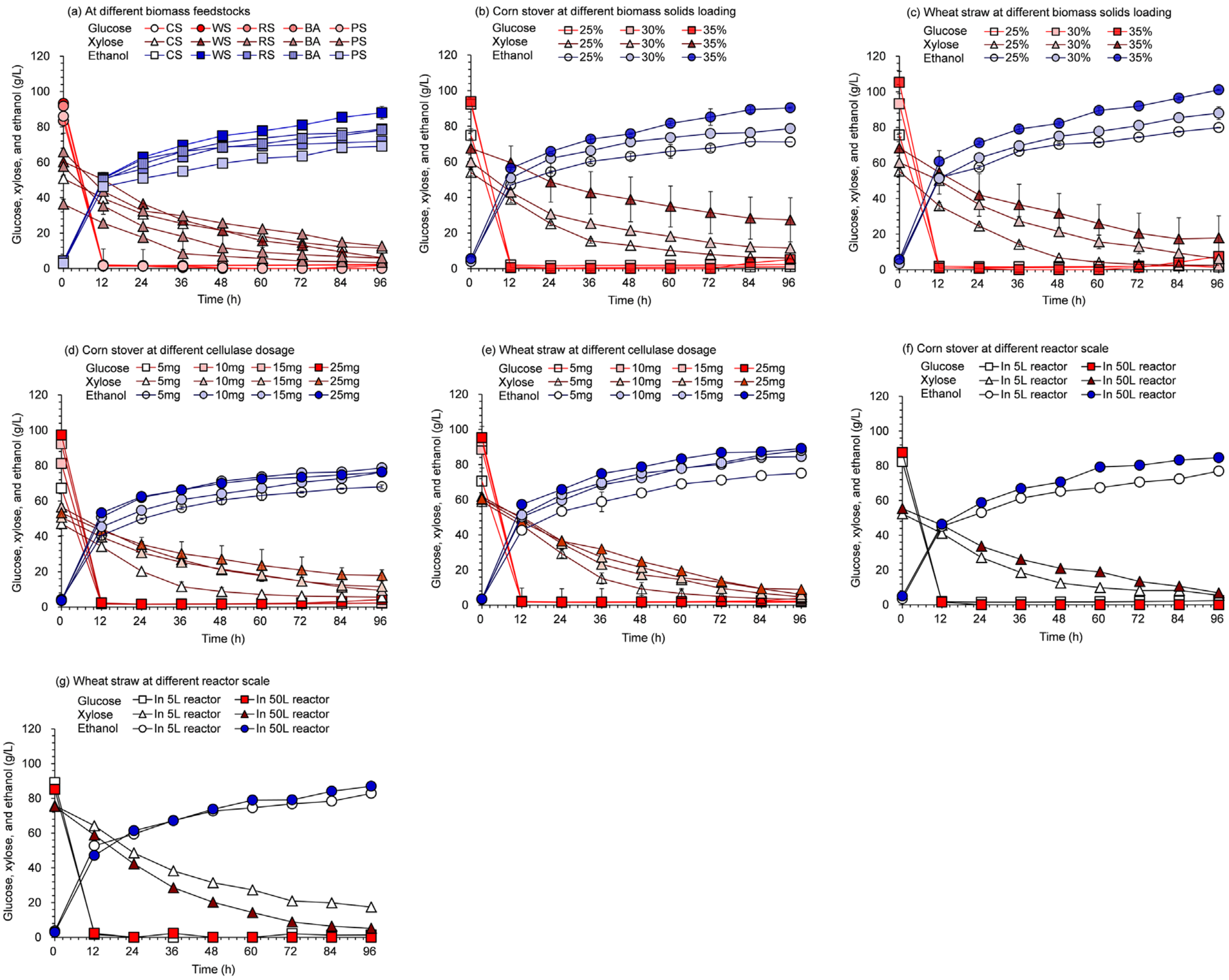

FIGURE 2 Simultaneous saccharification and ethanol co-fermentation (SSCF) of dry acid pretreated and biodetoxified lignocellulose feedstocks. (a) At different biomass feedstocks including corn stover (CS), wheat straw (WS), sugarcane bagasse (BA), rice straw (RS), Poplar sawdust (PS) at the cellulase dosage of 15 mg total proteins per gram of cellulose, 30% (w/w) of solids loading in 5 L bioreactor; (b) corn stover and (c) wheat straw at varying lignocellulose solids loading from 25%, 30%, and 35% (w/w) at the cellulase dosage of 15 mg total proteins per gram of cellulose in 5 L bioreactor; (d) corn stover and (e) wheat straw at varying cellulase enzyme dosage from 5, 10, 15, and 25 mg total protein per gram of cellulose, 30% (w/w) of solids loading in 5 L bioreactor; (f) corn stover and (g) wheat straw at varying bioreactor scales of 5 and 50 L, at the cellulase dosage of 10 mg total protein per gram of cellulose and 30% (w/w) of solids loading. Pretreatment was conducted at 175°C for 5 min with 20 or 25 mg of sulfuric acid per gram of dry feedstock matter (DM). Detoxification was conducted at 28°C for 36, 48, or 60 hr with the aeration rate of 0.8 vvm by *A. resinae* ZN1 unless mentioned elsewhere. Pre-hydrolysis was conducted at 50°C and pH 4.8 for 12 hr using cellulase enzyme Cellic CTec 2.0 on the basis of total protein weight per gram of cellulose in the pretreated and biodetoxified lignocellulose feedstock. SSCF was conducted using *S. cerevisiae* XH7 at 10% (v/v) of the inoculum ratio, 30°C and pH 5.5 for 96 hr

ethanol titer of 90.3 g/L (11.4%, v/v) for corn stover and of 101.1 g/L (12.8%, v/v) for wheat straw at the moderate cellulase dosage of 15 mg/g cellulose are already very close to corn ethanol titer (12–15%, v/v) (Koppram et al., 2014; Taylor, Kurantz, Goldberg, McAloon, & Craig, 2000), indicating that the present DryPB process for cellulosic ethanol production behaves the competing potential to corn ethanol on the conversion efficiency.

The differed ethanol generation between corn stover and wheat straw may be explained by the higher cellulose content (40.18% vs. 37.64% of cellulose in pretreated wheat straw and corn stover, respectively) and the lower inhibitor in SSCF (2.5 g/L vs. 4.5 g/L of acetic acid for wheat straw and corn stover, respectively). The cell viability was depressed and the glycerol formation was increased with increasing of cellulase dosage and the initial glucose concentration (Figure S2).

3.3 | SSCF under varying cellulase enzyme dosage

Cellulase enzyme takes 20–50% of the overall cost of cellulosic ethanol production depending on cellulase enzyme supply chain (Liu et al., 2016). Four cellulase dosages of Cellic CTec 2.0 enzyme from 5, 10, 15, to 25 mg total protein per gram of cellulose in corn stover and wheat straw were tested under the solids loading of 30% (w/w).

In the pre-hydrolysis stage, glucose increased with increasing cellulase dosage, but only slightly increased when the cellulase was above 15 mg/g due to the high sugar product (mono- or oligo-saccharides) inhibited on cellulase activity. Xylose was relatively constant with increasing cellulase dosage (approximately 60 g/L for wheat straw and 55 g/L for corn stover) because the majority of xylan was already converted to xylose and oligo-xylan in the pretreatment step.

In the consequent SSCF stage, the initial glucose was quickly converted into ethanol within 12 hr then started to utilize glucose from cellulose hydrolysis. At the cellulase dosage of 10 mg/g, wheat straw and corn stover produced 84.6 and 76.3 g/L of ethanol, respectively. When cellulase dosage increased to 15 mg/g, only slight increase of ethanol was observed for both corn stover and wheat straw. Xylose conversion decreased with the increasing of cellulase dosage due to the stronger inhibition by the higher initial glucose. Approximately, half ethanol came from the initial glucose released from the pre-hydrolysis, the other half was from xylose and the glucose released during the SSCF.

3.4 | SSCF scale up

The SSCF operation was scaled up by 10-folds from 5 L bioreactor to 50 L bioreactor with the working volume of 30 L at 10 mg/g of cellulase dosage and 30% (w/w) solids loading of the same dry acid pretreated and biodetoxified corn stover and wheat straw. In the 50 L bioreactor, corn stover produced 85.1 g/L (equivalent to 10.8%, v/v) of ethanol with the ethanol yield of 84.7% and the xylose conversion of 87.7% (Figure 2f); wheat straw produced 87.0 g/L (11.0%, v/v) of ethanol with the overall yield of 82.8% and the xylose conversion yield of 93.1% (Figure 2g). The cell viability, inhibitor conversion, and glycerol formation in the scale up operation were similar to that in the smaller reactors (Figure S2). Both the results were slightly better than that in the 5 L bioreactor, confirming that the present SSCF process from the DryPB refined lignocellulose feedstock is applicable to the process scale up.

4 | DISCUSSION

Several typical biorefining technologies for cellulosic ethanol fermentation were summarized in Table 3. These technologies are indicated by their unique pretreatment methods including ammonia fiber explosion (AFEX) (Kim & Dale, 2015; Uppugundla et al., 2014), dilute acid (DAP) (Humbird, Mohagheghi, Dowe, & Schell, 2010; Humbird et al., 2011), deacetylation, mechanical refining (DMR) (Chen et al., 2015, 2016), and the present dry acid pretreatment and biodetoxification (DryPB). DryPB produces the

TABLE 3 Biorefining performance of the typical biorefining processes for ethanol production from corn stover or wheat straw

Processing technology	DryPB (this study)				DAP[a]	AFEX[b]	DMR[c]
Pretreatment	Dry acid pretreatment (DryAP)				Dilute acid pretreatment (DAP)	Ammonia fiber explosion (AFEX)	Deacetylation, mechanical refining (DMR)
Detoxification	Biodetoxification				Ammonia treatment	No detoxification	Water washing
Hydrolysis and fermentation[d]	SSCF				SHCF	SHCF	SHCF
Hydrolysis	50°C for 12 hr				48°C for 84 hr	50°C for 72 hr	50°C for 120 hr
Fermentation	30°C for 96 hr				32°C for 36 hr	33°C for 120 hr	33°C for 22 hr
Strain	*S. cerevisiae* XH7				*Zymomonas mobilis* 8b	*S. cerevisiae* 424A	*Zymomonas mobilis* 13-H-9-2
Cellulase enzyme used	CTec2				Spezyme CP	CTec2 + HTec2	CTec3 + HTec3
Feedstock	Corn stover		Wheat straw		Corn stover	Corn stover	Corn stover
Initial solids loading (%, w/w)	30	35	30	35	20	18	28
Total cellulose content (%, w/w)[e]	11.0	12.9	12.1	14.5	6.6	6.0	12.2
Total xylan content (%, w/w)[f]	4.6	5.4	5.3	6.4	3.4	4.5	9.3
Cellulase dosage (mg/g cellulose)	10	15	10	15	20	30	20
Ethanol titer (g/L)	85.1	90.3	87.0	101.1	54	40	86
Ethanol titer (% v/v)	10.8	11.4	11.0	12.8	6.8	5.1	10.9
Ethanol yield (%)	84.7	73.5	82.8	74.8	85.5	63.9	81.0
Xylose conversion (%)	87.7	59.5	93.1	73.9	85.0	80.0	46.9

[a]Refer to Humbird et al. (2010, 2011).
[b]Refer to Uppugundla et al. (2014) and Kim and Dale (2015).
[c]Refer to Chen et al. (2015, 2016).
[d]SHCF, separate hydrolysis and co-fermentation; SSCF, simultaneous saccharification and co-fermentation.
[e]All the glucose, cellobiose, and cellulose in the pretreated biomass were converted to the cellulose content according to the stoichiometric equivalence.
[f]All the xylose, xylo-oligomers, xylan in the pretreated biomass were converted to the xylan content according to the stoichiometric equivalence.

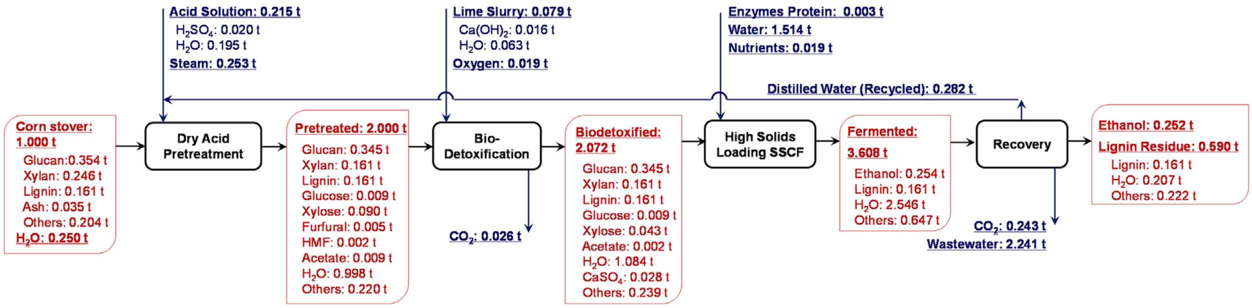

FIGURE 3 Mass balance of cellulosic ethanol production in DryPB process (based on one metric ton of dry corn stover with the humidity of 20%, w/w)

maximum ethanol (91.4 g/L for corn stover and 101.3 g/L for wheat straw) using the least cellulase dosage (10–15 mg/g cellulose), the highest solids (30–35%, w/w) at the similar cellulose and xylan content (12.9% + 5.4% for corn stover, 14.5% + 6.4% for wheat straw). As the comparison, AFEX obtains 40 g/L of ethanol from 6.0% of cellulose and 4.5% of xylan using 30 mg/g of cellulase; DAP obtains 54 g/L of ethanol from 6.6% of cellulose and 3.4% of xylan using 20 mg/g of cellulase; DMR obtains 86 g/L of ethanol from 12.2% of cellulose and 9.3% of xylan using 20 mg/g of cellulase. The hydrolysis and fermentation time of DryPB is shorter (108 hr) than that of AFEX (192 hr), DAP (120 hr), and DMR (142 hr). The current cellulosic ethanol titer of 10.8–12.8% (v/v) at the moderate cellulase dosage of 10–15 mg protein per gram of cellulose is very close to corn ethanol titer of 12–15% (v/v) (Kopppram et al., 2014; Taylor et al., 2000).

The key merit of this study comes from the successful implementation of simultaneous saccharification and co-fermentation (SSCF). DryPB solved the concomitance difficulty of xylose with inhibitors perfectly by applying biodetoxification to quickly and completely remove the inhibitors from the pretreated lignocellulose feedstock by *A. resinae* ZN1 with the priority of inhibitor utilization to xylose. Benefit from the use of the unique biodetoxification fungus *A. resinae* ZN1 and the xylose utilizing yeast *S. cerevisiae* XH7, the xylose conversion yield is around 90% and the overall ethanol yield approaches 85% in the high solids loading SSCF of the DryPB refined lignocellulose feedstock. The bioreactor with the single helical ribbon stirrer ensures a well mixing of the large bulk of solids (up to 30–35%) with the small cellulase amount and seed culture liquids (2–3%) in a short time (less than 1 min for liter scale reactor and 1–3 min for hundred cubic meter scale) and low power input (Hou, An, Zhang, & Bao, 2016). This vertical helical ribbon impeller design is superior to the horizontal bioreactor in the space utilization efficiency and more accepted for commercial scale operation: 250 kg of dry lignocellulose solids is filled in one cubic meter of the vertical helical ribbon reactor (25 kg of 50% solids feedstock in the 50 L reactor), while only 33 kg of dry lignocellulose solids is filled in one cubic meter of the horizontal reactor (1 kg of 30% solids in 9 L reactor) (Chen et al., 2015, 2016).

Generally, the wastewater generation is the price for high enzymatic hydrolysis and fermentation yields of cellulosic ethanol production. Efforts of this study were made on elevating conversion efficiency of SSCF while maintaining the wastewater generation close to the corn ethanol production. Figure 3 shows the detailed mass balance of one metric ton of dry corn stover in the DryPB refining chain based on the 50 L scale SSCF result. A total of 0.252 ton of ethanol is produced by per ton corn stover and no wastewater generate in the core steps of pretreatment, detoxification, hydrolysis, fermentation. Refining one metric ton of dry corn stover generates 2.241 tons of wastewater, equivalent to 8.89 tons of wastewater per one metric ton of fuel ethanol. As the comparison, the mature corn ethanol generates approximately 8–12 tons of wastewater per metric ton of ethanol (Wilkie, Riedesel, & Owens, 2000; Willington and Marten, 1982). Obviously, the wastewater generation of 8.89 tons per metric ton of ethanol is comparable to the level of corn ethanol process.

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CONFLICT OF INTEREST

All authors have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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