Mechanism of lignocellulose modification and enzyme disadsorption for complete biomass saccharification to maximize bioethanol yield in rapeseed stalks†

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Rapeseed stalk provides an enormous biomass resource for bioethanol production, but its characteristic recalcitrance towards catalysis results in inefficient cellulose hydrolysis, with lower bioethanol yield compared with other major crop straws. Based on our previous analyses of large populations of rapeseed samples, in this study we selected three rapeseed stalks that showed distinct cell wall composition, and then performed steam explosion followed by mild chemical pretreatment to reduce the recalcitrance to lignocellulose degradation. As a result, three typical pretreatments were established to extract cell wall polymers (hemicelluloses, lignin, pectin) and also to specifically reduce the degree of polymerization of the β-1,4-glucans, leading to a remarkable increase in biomass porosity and cellulose accessibility in rapeseed stalks. Notably, steam explosion with mild CaO pretreatment (50 °C) generated an optimal lignocellulose substrate that was effective for almost complete enzymatic saccharification, while 1% Tween-80 was supplied to block lignin adsorption with cellulase enzymes. As a consequence, bioethanol yields from 18.8% to 20.5% (% dry biomass) were achieved due to relatively high sugar–ethanol conversion rates of 90–93%, which were much higher than those previously reported in rapeseed stalks. Furthermore, this study proposes a model mechanism to highlight why optimal lignocellulose modification could cause complete biomass saccharification leading to the maximum bioethanol yield achieved in rapeseed stalks, and how the surfactant plays a role in the enhancement of enzymatic hydrolysis of diverse lignocellulose substrates. Hence, this study demonstrates an effective strategy to potentially maximize bioethanol production with a low-cost and green-like biomass process in rapeseed, and other crop residues.

1. Introduction

Lignocellulose represents an enormous source of biomass for biofuels and chemical production, and, in particular, cellulose ethanol is an excellent additive to gasoline for partial replacement of petrol fuels and less net carbon release.1–5 In principle, cellulose ethanol production involves three essential biochemical conversion steps: biomass pretreatment, enzymatic hydrolysis and yeast fermentation.6,7 Lignocellulose recalcitrance results in a costly bioethanol process, with potential secondary waste release into the environment,1–3 and so it is important to explore optional technologies that reduce lignocellulose recalcitrance in order to achieve low-cost and high-efficiency bioethanol conversion.

As an initial step in the biomass process, various physical and chemical pretreatments have been performed to reduce lignocellulose recalcitrance by extracting cell wall polymers and destroying cell wall networks.8 For instance, acid (H2SO4) and
alkali (NaOH) have been applied as classic agents for chemical pretreatments, but they normally require strong conditions (high concentration, high temperature) for effective polymer extraction, leading to difficulties in avoiding secondary waste release.\[^{9,10}\] In contrast, lime (CaO) has been defined as a green-like and low-cost chemical for biomass pretreatment, but is of relatively low efficiency for polymer extraction due to its saturated nature.\[^{11,12}\] Steam explosion, as an environmentally friendly pretreatment, can effectively extract hemicelluloses and largely reduces the degree of polymerization of the glucans.\[^{13,14}\] Nevertheless, an integrated technology that can diminish lignocellulose recalcitrance for complete enzymatic saccharification leading to maximum bioethanol production remains to be found.

Efficient enzymatic saccharification has been evaluated as a crucial factor in bioethanol production.\[^{5,7}\] Over the past few years, surfactants such as Tween and PEG have been applied to enhance enzymatic hydrolysis in agricultural crop residues.\[^{7,11,14,16}\] However, despite the assumption that surfactants can lessen cellulase enzyme absorption with wall polymers or disassociate wall polymers,\[^{16-18}\] much is still unknown about the mechanism of surfactant enhancement for diverse lignocellulose substrates under various physical and chemical pretreatments.

Rapeseed (Brassica napus) is a major oil crop around the world and could produce large quantities of lignocellulose-rich stalks.\[^{19}\] Although various technologies have been attempted for bioethanol production in rapeseed stalks, the bioethanol yield remains relatively low compared with that achieved in other major agricultural crop straws.\[^{20-25}\] Hence, based on our previous analyses of large populations of rapeseed cultivars,\[^{19}\] in this study selected three rapeseed stalks that showed distinctive cell wall composition, and then performed steam explosion and chemical pretreatments using dilute H\(_2\)SO\(_4\) and CaO. In addition, Tween-80 was introduced into the enzymatic hydrolysis, resulting in complete biomass saccharification, maximizing bioethanol yield compared with previous studies. Notably, this work attempts to determine how maximum bioethanol yield can be achieved in rapeseed stalks by proposing a model mechanism that highlights the distinctively improved lignocellulose substrate from combined steam explosion and chemical pretreatment for efficient blocking of cellulase adsorption by surfactant.

2. Materials and methods

2.1. Biomass sample collection

Three representative Brassica napus plants were grown in a Wuhan experimental field, and mature stalks were collected and dried at 50 °C. The dried samples were powdered by passing through a 40-mesh screen and were stored in a dry container until use.

2.2. Wall polymer extraction and assay

The major wall polymers were extracted as previously described by Peng et al.\[^{26}\] and Alam et al.\[^{9}\] The soluble sugars, lipids, starch and pectin in the biomass samples were extracted sequentially using potassium phosphate buffer (pH 7.0), chloroform–methanol (1 : 1, v/v), DMSO–water (9 : 1, v/v) and ammonium oxalate 0.5% (w/v). The remaining residues were incubated with 4 M KOH (containing 1.0 mg mL\(^{-1}\) sodium borohydride) at 25 °C for 1 h, and, after centrifugation, the supernatants were collected as the KOH-extractable hemicelluloses. The remaining non-KOH-extractable residues were sequentially extracted with H\(_2\)SO\(_4\) (67%, v/v) at 25 °C for 1 h, and the hexoses and pentoses of the supernatants were detected as total cellulose and the non-KOH-extractable hemicelluloses, respectively. A UV-vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) was used to detect pentoses, hexoses and uronic acids, as previously described by Cheng et al.\[^{27}\]

A two-step acid hydrolysis method was applied for detection of lignin content according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory, as previously described by Fan et al.\[^{28}\] All experiments were performed independently in triplicate.

2.3. Assay of hemicellulose monosaccharides and lignin monomers

Hemicellulose monosaccharides and lignin monomers were determined by gas chromatography-mass spectroscopy (GC-MS) (Shimadzu GCMS-QP2010 Plus) and high-pressure liquid chromatography methods (1525, Waters Corp., MA, USA), respectively, as previously described by Xu et al.\[^{29}\]

2.4. Detection of cellulose crystalline index and degree of polymerization

Cellulose crystalline index (CrI) was measured using a Rigaku-D/MAX instrument (Ultima III, Japan), as previously described by Li et al.\[^{7}\] The CrI was estimated using the equation: CrI = 100 × \(I_{200} - I_{105}/I_{200}\). \(I_{200}\) is the intensity of the 200 peak (\(I_{200}, \theta = 22.5^\circ\)), which represents crystalline cellulose. \(I_{105}\) (\(I_{105}, \theta = 18.5^\circ\)) is the intensity at the minimum between the 200 and 110 peaks, which corresponds to amorphous cellulose. The degree of polymerization (DP) of cellulose samples was determined using the viscosity method, subject to the equation: DP\[^{90m}\] = 0.75[\(\eta\)], where [\(\eta\)] is the intrinsic viscosity, as described previously by Alam et al.\[^{6}\] All experiments were performed in triplicate at 25 ± 0.5 °C using an Ubbelohde viscosity meter.

2.5. Fourier-transform infrared (FTIR) spectroscopy scanning

A PerkinElmer spectrophotometer (Nexus 470, ThermoFisher Scientific, Waltham, MA, USA) was used to qualitatively monitor the biomass samples, and the FTIR spectra were recorded in absorption mode over 32 scans at a resolution of 4 cm\(^{-1}\) in the range 4000 to 400 cm\(^{-1}\), as previously described by Wu et al.\[^{11}\]

2.6. Measurement of biomass porosity and cellulose accessibility

Simons' stain was used to determine the lignocellulose porosity, as previously described by Alam et al.,\[^{6}\] with minor
2.7. Biomass pretreatment and enzymatic hydrolysis

Steam explosion. The well-dried rapeseed straw samples were treated in the steam explosion reactor (QBS-200, Hebi Zhengdao Machine Factory, Hebi, China), as previously described by Wiman et al. and Cheng et al. The biomass samples (100 mg) were incubated with dye solution at a series of concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 g L⁻¹) in 0.3 M phosphate buffer at 60 °C for 24 h. After centrifugation at 8000g, the absorbance of the supernatant was measured at 498 nm. The maximum dye adsorption capacity of the biomass was calculated using the monolayer Langmuir adsorption model. All measurements were conducted independently in triplicate.

$\begin{align*}
\text{Absorbance} &= \frac{\text{Dye concentration}}{\text{Maximum dye adsorption capacity}} \\
\text{Maximum dye adsorption capacity} &= \frac{\text{Dye concentration} \times \text{Volume of dye solution}}{\text{Mass of biomass}}
\end{align*}$

2.8. Yeast fermentation and bioethanol determination

Yeast fermentation was performed with the Saccharomyces cerevisiae (purchased from Angel Yeast Co., Ltd., Yichang, China) strain using total hexoses obtained from pretreatments and saccharification as the carbon source at 3% solid loading. The fermentation liquid was distilled at 100 °C to collect ethanol liquor. Ethanol was measured using the K$_2$Cr$_2$O$_7$ method, as described by Li et al. The sugar–ethanol conversion rate was calculated using the following formula: conversion rate (%) = $E/ \left( H \times 0.511 \right) \times 100$, where $E$ is the ethanol yield (% dry biomass) detected, $H$ is the total hexose yield (% dry biomass) used for yeast fermentation and 0.511 is the theoretical conversion rate of hexoses to ethanol (0.511 g ethanol per g hexoses). Absolute ethanol was used for standard curve plotting. All experiments were carried out independently in triplicate.

2.9. Soluble enzyme detection and SDS–PAGE

Total soluble protein was obtained by collecting the supernatant from the biomass enzymatic hydrolysis and determined using the Coomassie Brilliant Blue G250 assay, as described by Alam et al. The SDS–PAGE analysis of soluble cellulase enzymes was described by Li et al.

2.10. Statistical analysis

Correlation analysis was performed by the Spearman method for all measured traits across biomass samples from different treatments. Superior Performance Software Systems (SPSS version 16.0, Inc., Chicago, IL) was used for all types of calculations. Pairwise comparisons were conducted between two measurements using Student’s $t$-test. Means were separated by a least significant difference (LSD) test at $p < 0.05$. Graphs were generated using Origin 8.5 software (Microcal Software, Northampton, MA). Average values were calculated from the original triplicate measurements.

3. Results and discussion

3.1. Altered cell wall compositions of rapeseed stalks from steam explosion

In our previous study, a total of 19 rapeseed cultivars were determined, with large variations in biomass enzymatic saccharification in the mature stalks. In this work, we selected three rapeseed cultivars that showed distinct cell wall composition, including cellulose, hemicelluloses, lignin and pectin (Table 1). For instance, three rapeseed cultivars contained cellulose levels ranging from 28% to 33% (% dry matter), but they had lignin contents varying from 15% to 17%. Furthermore, two rapeseed cultivars (Bn13, Bn08) had similar hemicellulose content (at 15%), but the Bn15 cultivar contained 20% hemicelluloses. Notably, all three rapeseed cultivars contained much higher pectin contents (8–11%) compared with other major agricultural crop stalks.

Using our previously established conditions, we performed steam explosion with mature stalks of these three rapeseed cultivars (Table 1). The three steam-explored residues showed significantly lower levels of hemicelluloses than the raw
Table 1  Cell wall composition (% dry matter) of raw material and SE residues in three rapeseed stalks

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pretreatment</th>
<th>Cellulose</th>
<th>Hemicelluloses</th>
<th>Lignin</th>
<th>Pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bn13</td>
<td>Raw material</td>
<td>28.20 ± 1.28</td>
<td>15.35 ± 0.07</td>
<td>16.24 ± 0.64</td>
<td>9.43 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Steam-exploded</td>
<td>36.73 ± 1.30**</td>
<td>9.77 ± 0.12**</td>
<td>22.64 ± 0.26**</td>
<td>0.87 ± 0.12**</td>
</tr>
<tr>
<td></td>
<td>30%^C</td>
<td>-36%</td>
<td>-39%</td>
<td>8.34 ± 0.30</td>
<td>-91%</td>
</tr>
<tr>
<td>Bn08</td>
<td>Raw material</td>
<td>30.78 ± 0.84</td>
<td>15.58 ± 0.26</td>
<td>14.87 ± 0.35</td>
<td>8.34 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Steam-exploded</td>
<td>36.86 ± 0.60**</td>
<td>10.25 ± 0.21**</td>
<td>20.91 ± 0.75**</td>
<td>0.58 ± 0.11**</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>-34%</td>
<td>41%</td>
<td>9.77 ± 0.20</td>
<td>-93%</td>
</tr>
<tr>
<td>Bn15</td>
<td>Raw material</td>
<td>33.10 ± 0.69</td>
<td>20.43 ± 0.26</td>
<td>17.27 ± 1.11</td>
<td>10.91 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Steam-exploded</td>
<td>38.31 ± 0.50**</td>
<td>12.46 ± 0.23**</td>
<td>20.39 ± 0.32*</td>
<td>1.2 ± 0.17**</td>
</tr>
<tr>
<td></td>
<td>16%</td>
<td>-39%</td>
<td>18%</td>
<td>8.34 ± 0.31</td>
<td>-89%</td>
</tr>
</tbody>
</table>

* and ** indicate significant differences between raw material and SE residues by t-test at p < 0.05 and p < 0.01 (n = 3). † Percentage of increased or decreased rates between the raw material and SE residues by subtraction of two values divided by the raw material. Data indicated are mean ± SD (n = 3).
almost complete enzymatic saccharification, with hexose yields of 99.6–100%, with the raw materials having hexose yields of 71–83% (Fig. 2B). In addition, we determined the monosaccharide composition of the total sugars released from enzymatic hydrolysis using GC-MS (Table S2†). By comparison, the glucose proportion was 97–98% of total hexoses, with the other two hexoses (mannose and galactose) comprising less than 3% of the total. This indicates that the hexose yields released by the enzymatic hydrolysis were almost entirely accounted for by the cellulose digestibility examined in this study. In particular, the xylose content comprises more than 96% of the total pentoses released from enzymatic hydrolysis of the biomass residues. Furthermore, when pretreated with 10% or 15% CaO, the SE residues showed relatively lower hexose yields, probably due to partial sugar oxidation by the high concentration of alkali.6,36 Therefore, optimal, strong H2SO4 pretreatment could lead to almost complete enzymatic saccharification of SE residues in the preferred rapeseed cultivar, but optimal mild CaO pretreatment was even more effective for complete enzymatic hydrolysis in all rapeseed cultivars examined in this study.

3.4. Maximum bioethanol production under combined pretreatment

Using total hexoses released from enzymatic hydrolysis and pretreatment (Fig. S1†), we conducted a classic yeast fermentation to obtain bioethanol product in three rapeseed stalks (Fig. 3). The three rapeseed cultivars showed the highest bioethanol yields of 18.8–20.5% (% dry biomass) in the SE + 5% CaO residue samples obtained from combined steam explosion and CaO pretreatment, compared with all other optimal, pretreated samples (Fig. 3A–D and S2†). Notably, the SE + 5% CaO residue samples also showed relatively higher sugar–ethanol conversion rates (90–93%) than those of other optimal pretreated samples (69–87%, Fig. 3E–H), suggesting that, following CaO pretreatment, SE residues may release less toxic compounds with low inhibition of yeast fermentation. In contrast, relatively low sugar–ethanol conversion rates in the SE + 6% H2SO4 residue samples may be due to more toxic compounds generated under H2SO4 pretreatment at high concentration and high temperature, as previously reported.37,38 In addition, we assumed that the CaO may be able to adsorb some toxic compounds for a relatively high sugar–ethanol conversion rate,39 confirming that CaO pretreatment should be a green-like biomass process.

Furthermore, this study characterized the mass balance of the lignocellulose process for bioethanol production in the three rapeseed cultivars (Fig. 4, S3 and S4†). As a comparison, the Bn13 cultivar showed relatively higher bioethanol yields and sugar–ethanol conversion rates compared with the other two cultivars, in raw materials and in the five optimal pretreated residues. Further, in comparison to the previously reported ethanol yields in rapeseeds,20–25 the Bn13 cultivar showed the maximum bioethanol yield (20.5%) and a higher hexose–ethanol conversion rate (93%), for the SE + 5% CaO residue sample in particular (Table 2). Although high bioethanol yield (19.8%) has also been obtained in a previous study using both pentoses and hexoses as the carbon source for yeast co-

Fig. 2 Hexose yields released from enzymatic hydrolysis supplied with 1% Tween-80 after H2SO4 and CaO pretreatments. (A) H2SO4 pretreatment. (B) CaO pretreatment. Data indicated are mean ± SD (n = 3).
Fig. 3  Bioethanol yields released from yeast fermentation using total hexoses obtained from pretreatment and enzymatic hydrolysis. (A and E) Raw material. (B and F) SE residues. (C and G) SE residues followed by 6% H$_2$SO$_4$ pretreatment. (D and H) SE residues with 5% CaO. Letters (a, b and c) indicate multiple significant differences by LSD test at $p < 0.05$. Data indicated are mean ± SD ($n = 3$).

Fig. 4  Mass balance analysis for bioethanol production in the desirable rapeseed stalk (Bn13).
fermentation, here we achieved much higher bioethanol yields (26.9%) from xylose and hexose co-fermentation, based on in-theory estimation of the average pentose–ethanol conversion rate, as previously described.\textsuperscript{40,41} Because both steam explosion and CaO pretreatments have been accepted as green-like technologies, these results indicate that rapeseed stalks could be considered as a desirable bioenergy crop to produce maximum cellulosic bioethanol under a low-cost and green-like biomass process technology.

### 3.5. Distinct wall polymer extraction from three optimal pretreatments

To understand why complete enzymatic saccharification could be achieved for maximum bioethanol yield, this study examined cell wall polymer extraction from three optimal pretreatments using two rapeseed cultivars (Bn13, Bn08) that showed relatively higher bioethanol yields, as described above (Fig. 5A and S5A\textsuperscript{†}). Compared with the raw materials, the SE residues of the two rapeseed cultivars showed 62% hemicellulose extraction, with related loss of 22–28% cellulose and 15–16% lignin. However, the second-step H\textsubscript{2}SO\textsubscript{4} pretreatment with SE residues led to extraction of 83–84% hemicellulose and 50–54% cellulose, together with 32–40% lignin removal in the SE + 6% H\textsubscript{2}SO\textsubscript{4} residue samples. In terms of cellulose extraction, we assumed that 6% H\textsubscript{2}SO\textsubscript{4} pretreatment at high temperature mainly destroys non-crystalline cellulose.\textsuperscript{42} Importantly, the second-step, mild CaO pretreatment only extracted 21–24% lignin, along with small amounts of cellulose and hemicellulose removal in the SE + 5% CaO residue samples, compared with the SE samples.

With respect to cell wall polymer extraction, we detected polymer interlinkages in raw materials and three SE residues using FTIR spectroscopy (Fig. 5B and S5B\textsuperscript{†}). Compared with the raw materials, three optimal pretreated SE residue samples showed relatively smaller peaks (1241, 1600, 1735 cm\textsuperscript{-1}) characteristic of the functional groups C–O–C, C=O, and C=C associated with cell wall polymer (hemicelluloses, pectin and lignin) interlinkage (Table S3\textsuperscript{†}). Hence, the data from FTIR profiling confirmed distinct wall polymer extraction from three optimal pretreatments performed in this study.

![Fig. 5](image-url) Characterization of cell wall polymer extraction under three optimal pretreatments in the desirable rapeseed sample (Bn13). (A) Cell wall composition (based on 100 g raw material of rapeseed stalk). (B) FTIR spectroscopic profiling of raw material and three pretreated biomass residues of Bn13. Letters (a, b, c and d) indicate multiple significant differences by LSD test at $p < 0.05$. \textsuperscript{a} Indicates percentage of decreased rates between the raw material and pretreated residues by subtraction of two values divided by the raw material. Data indicated are mean ± SD ($n = 3$).
3.6. Improved wall polymer features from three optimal pretreatments

Characterization of the improvement of lignocellulose recalcitrance by wall polymer extraction has been performed. In this study, we examined major wall polymer features tightly associated with lignocellulose recalcitrance (Fig. 6 and S6†). In terms of cellulose features, we detected the cellulose crystalline index (CrI) using an X-ray diffraction method, and all three optimal pretreated SE residues showed relatively higher CrI values than the raw materials (Fig. 6A and S6A†), which should be accounted for by wall polymer extraction that disassociates hydrogen bonds. However, when CrI values were compared with absolute cellulose contents for each sample, both SE + 6% H₂SO₄ and SE + 5% CaO residue samples showed significantly lower CrI/cellulose values (Fig. 6B and S6B†). Meanwhile, in this study we found significantly reduced cellulose DP in all three optimal pretreated SE residue samples, at p < 0.01 levels (Fig. 6C and S6C†). In particular, the SE + 6% H₂SO₄ residue samples showed almost five-fold lower cellulose DP values than those of the raw materials, consistent with the assumption that 6% H₂SO₄ pretreatment at high temperature may largely extract non-crystalline cellulose, as discussed above. Because cellulose CrI and DP have been well examined as key negative factors for biomass enzymatic hydrolysis, such a significant reduction in CrI/cellulose and DP values should contribute to biomass saccharification in the three optimal pretreated residues of the rapeseed cultivars.

With regard to hemicellulose features, we determined the monosaccharide composition of the hemicelluloses (Table S4†). Due to the hemicellulose extraction, three optimal SE residue samples showed much reduced xylose (Xyl) levels, particularly in the SE + 6% H₂SO₄ samples (Fig. 6D and S6D†). Xyl and arabinose (Ara) are two major components of hemicelluloses, and this study detected a much higher Xyl/Ara ratio in the three optimal SE residue samples (Fig. 6E and S6E†) due to extraction of almost all the Ara by combined SE and chemical pretreatment. This suggested that the Xyl/Ara ratio should not be the crucial factor in biomass enzymatic saccharification in pretreated biomass residues. In addition, we determined a large variation in lignin compositions (S, G, H) in three optimal SE residue samples, probably due to their complicated wall structure and lignin–hemicellulose complexes (Table S5†). In particular, the SE + 6% H₂SO₄ and SE + 5% CaO residue samples showed relatively lower S/G ratios than those of the raw materials (Fig. 6F and S6F†). As the lignin S/G ratio has been examined with dual impacts on enzymatic hydrolysis, the much reduced S/G ratio in the optimal pretreated residues may contribute to enzymatic saccharification in this study.

3.7. Increased biomass porosity and cellulose accessibility

Wall polymer extraction has been characterized as increasing biomass porosity for enzyme loading and accessibility to cellulose surfaces. Using a recently improved Simons’ staining, a classic assay for biomass porosity, in this study we detected remarkably increased blue and yellow dyes in three

![Fig. 6 Detection of cell wall polymer features in three pretreated residues of the desirable sample (BnL3). (A) Cellulose crystallinity index (%). (B) Ratio of CrI value against cellulose level. (C) Cellulose DP. (D) Xylose proportion of hemicelluloses. (E) Xyl/Ara (xylose/arabinose) ratio of hemicelluloses. (F) S/G ratio of lignin. Letters (a, b, c, and d) indicate multiple significant differences by LSD test at p < 0.05. # Indicates percentage of increased or decreased rates between the raw material and pretreated residues by subtraction of two values divided by the raw material. Data indicated are mean ± SD (n = 3).](image-url)
optimal SE residue samples relative to the raw materials (Fig. 7A and S7A†). As yellow and blue dyes account for large and small pore sizes, respectively, of biomass residues,6,15 these data confirmed that the wall polymer extraction could effectively enlarge both small and large pores in three optimal SE residues. Furthermore, using Congo Red stain, a direct assay for the specific surface area of cellulose, we measured almost two-fold larger cellulose surface areas (m² g⁻¹) in both SE and SE + 5% CaO residue samples, compared with the raw materials (Fig. 7B and S7B†). This indicates remarkably enlarged cellulose accessibility upon one-step SE pretreatment or two-step SE and CaO pretreatment. Although the SE + 6% H₂SO₄ residue samples were of significantly increased cellulose accessibility, they showed much less surface area than the SE and SE + 5% CaO samples, suggesting that the subsequent 6% H₂SO₄ pretreatment at high temperature may lead to extraction of accessible non-crystalline regions of cellulose microfibrils.42

3.8. Effective blocking of cellulase enzyme adsorption from Tween-80 supply

To sort out why supply of Tween-80 mainly enhanced enzymatic saccharification, we measured total soluble cellulase enzymes remaining in the supernatants of the biomass enzymatic hydrolysis reaction at 50°C for 48 h that was performed in this study (Fig. 8 and S8†). Without Tween-80 supply, we measured a low dosage of soluble enzymes in the three optimal SE residue samples and raw materials (Fig. 8A and S8A†). However, when 1% Tween-80 was co-supplied into the enzymatic hydrolysis reaction, an extremely high abundance of soluble enzymes was produced.
determined in the three optimal SE residue samples and raw materials, and their soluble enzyme contents were even 2–7-fold higher than those of the controls (without Tween-80). Furthermore, the SE + 5% CaO samples showed almost 1.5-fold more soluble enzymes than the SE and SE + 6% H₂SO₄ samples. To confirm this finding, we carried out separation profiling of total soluble enzymes using SDS–PAGE (Fig. 8B and S8B†). With 1% Tween-80 supply, the three optimal SE residue samples exhibited two major bands corresponding to the standard mixed cellulases added into the enzymatic hydrolysis reaction, in particular on the SE + 5% CaO samples. By comparison, the controls (without Tween-80) of SE did not show clear bands, probably due to the much lower dosage of soluble enzymes applied in the SDS–PAGE. Meanwhile, the raw material samples with Tween-80 also showed relatively strong bands compared with their controls. Hence, this study provided direct evidence that Tween-80 supply could largely block cellulase enzyme adsorption with lignocellulose substrates.

To determine whether there is a connection between soluble cellulase enzyme levels and hexose yields, we performed a correlation analysis using all the data obtained. Significantly, the soluble cellulase levels showed a positive correlation with hexose yields, at $p < 0.01$ level ($n = 18$) with a high $r$ value of 0.87 (Fig. 8C), indicating that Tween-80 enhancement of the enzymatic saccharification should be mainly due to its blocking role to reduce cellulase adsorption with non-cellulosic polymers. Furthermore, we performed a correlation analysis between three major cell wall polymer contents and soluble enzyme levels in the diverse lignocellulose substrates examined (Fig. 8D). Without Tween-80 supply, three major wall polymers did not show any significant correlation with the soluble enzymes, consistent with the finding that small amounts of soluble enzymes were determined in all lignocellulose substrates. However, when supplied with 1% Tween-80, lignin levels in a total of 12 biomass samples were negatively correlated with their soluble enzyme content, at $p < 0.01$ level ($n = 12$), indicating that Tween-80 should mainly interact with lignin to block its adsorption with cellulase enzymes. Meanwhile, we determined a significant positive correlation between hemicellulose levels and soluble enzymes at $p < 0.05$ ($n = 12$), mainly due to complete hemicellulose digestion for all xylanase enzymes released into the supernatants. Taken together, these results demonstrate that Tween-80 surfactant could effectively block lignin adsorption with cellulase enzymes.

**Fig. 9** A hypothetical model for complete biomass saccharification and maximum bioethanol production in rapeseed stalks. The model elucidates distinct lignocellulose modification from combined steam explosion and chemical (acid, alkali) pretreatments for enhanced enzymatic saccharification by 1% Tween-80 supplementation, to block lignin adsorption with cellulases, leading to the maximum bioethanol yield achieved in the optimal lignocellulose substrate in rapeseed stalks. 1. The “−” means extracted cell wall polymers (hemicelluloses, lignin) or reduced cellulose DP level compared with raw materials. And different number of “−” indicates different levels of cell wall polymer extraction or cellulose DP reduction. The more “−” means more cell wall polymers are extracted or more cellulose DP is reduced. 2. The “+” indicated that compared with raw materials the biomass porosity and cellulose accessibility are increased under three different pretretments. More “+” means a greater increase.
in diverse lignocellulose substrates, resulting in efficient biomass enzymatic saccharification.

3.9. A model mechanism for distinct lignocellulose enzymatic saccharification and bioethanol production

Based on the data obtained in this study, a model mechanism is proposed to summarize the major findings about lignocellulose modification and enzyme desorption for enhanced enzymatic saccharification and bioethanol production under steam explosion, followed by chemical pretreatment in rapeseed stalks (Fig. 9). Under steam explosion, about 62% of hemicelluloses and almost all pectin could be extracted in three rapeseed stalks, and the cellulose DP was reduced by 20–29%, largely raising biomass porosity and cellulose accessibility, which should enhance soluble cellulase enzyme loading and accession into cellulose microfibril surfaces for efficient enzymatic hydrolysis. Tween-80 co-supply effectively blocked lignin adsorption with cellulase enzymes, leading to a hexose yield of 85% (% cellulose) from the enzymatic hydrolysis and a bioethanol yield of 13.4% (% dry biomass) from the final yeast fermentation.

Subsequent 6% H2SO4 pretreatment with the SE residue further extracted the remaining hemicellulose–lignin complexes, but may also diminish non-crystalline cellulose, resulting in the lowest cellulose DP and relatively lower cellulose accessibility compared with the other pretreatments examined. The remarkable reduction of cellulose DP should account for more reducing ends of cellulose for efficient enzymatic hydrolysis, with hexose yields of 96.5% in one desirable rapeseed. However, the bioethanol yield only reached 18.2% with a relatively low hexose–ethanol conversion rate (84%), probably due to formation of more toxic compounds from acid pretreatment under strong conditions.

Mild CaO pretreatment with the SE residue mainly extracted lignin, with small amounts of hemicellulose removal, but only slightly reduced cellulose DP and cellulose accessibility, which caused effective Tween-80 blocking with the remaining lignin residues to maintain large amounts of soluble cellulose applicable for complete enzymatic saccharification, with hexose yields of 99.6–100% in three rapeseed stalks. The final bioethanol yields achieved were therefore 18.8% to 20.5%, mainly due to relatively high sugar–ethanol conversion rates from 90%–93%, which were higher than the previously reported ones in rapeseed stalks. Hence, this integrated technology should be conventionally applicable for enhancing enzymatic saccharification of diverse lignocellulose substrates towards high sugar–ethanol conversion rates.

4. Conclusions

Using three distinct rapeseed stalks, we performed an optimal lignocellulose modification using steam explosion followed by mild CaO pretreatment, leading to almost complete enzymatic saccharification, with hexose yields of 99.6–100% (% cellulose) in three rapeseed samples when supplied with 1% Tween-80. As a consequence, bioethanol yields from 18.8% to 20.5% (% dry biomass) could be achieved with relatively high sugar–ethanol conversion rates of 90–93%, which is the highest yield obtained compared with those previously reported in rapeseed stalks. Furthermore, this study has proposed a hypothetical model to interpret how the optimal lignocellulose substrates were generated for complete biomass enzymatic hydrolysis. It also provides an effective strategy for low-cost and green-like bioethanol production in rapeseed stalks and other crop residues.

Authors’ contributions

JD and XZ did the major experiments and wrote the draft manuscript. PC and WZ detected wall polymer features. ST and XL participated in yeast fermentation and bioethanol assay. RZ, BH and ZL collected rapeseed (Brassica napus) samples and analyzed genetic characters. HK and LY analyzed the data and co-supervised the experiments. LP designed the project, supervised experiments and finalized the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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