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Combined mild chemical pretreatments for complete cadmium release and cellulosic ethanol co-production distinctive in wheat mutant straw†

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Wheat is a major food crop providing substantial biomass straw applicable for heavy metal phytoremediation and cellulosic ethanol production. In this study, we observed that wheat mutant straw could accumulate a maximum cadmium (Cd) concentration of 8.29 $\mu\text{g g}^{-1}$ dry straw, whereas wheat wild type (WT) contained Cd at a concentration of 6.43 $\mu\text{g g}^{-1}$, while grown in soil pots supplied with 75 mg CdCl₂ per kg soil. Due to the Cd accumulation, the wheat mutant and WT straws distinctively showed a reduced cellulose level and degree of polymerization (DP) with increased soluble sugar stock compared to the control (without Cd), which led to much improved cellulose surface accessibility for relatively higher biomass enzymatic saccharification and bioethanol production under various chemical pretreatments. Notably, compared to the one-step pretreatment of a strong alkali or acid, the two-step pretreatment of a mild alkali and acid could cause an effective wall polymer extraction for a complete Cd release and collection from the mature straw typical in the wheat mutant. Therefore, this study has demonstrated a green-like technology for Cd collection and cellulosic ethanol co-production by employing a mild chemical pretreatment. It has also suggested a potential biotechnology for Cd phytoremediation by selecting cell wall-altered mutants and transgenic crops.

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1. Introduction

Cadmium (Cd) is one of the major toxic heavy metals polluting agricultural land from the mining industry, phosphate fertilizers and other sources. However, its uptake by crops has been considered as a green-like phytoremediation for environmental protection.^{1,2} In particular, the crop straw could accumulate a large proportion of Cd due to its enormous lignocellulose residues. It thus becomes important to find an effective technology for Cd collection with additional benefits.

Crop straw represents a sustainable lignocellulose resource convertible for biofuels and other bio-chemicals. In principle, biochemical conversion of lignocellulose involves three major steps: initial physical and chemical pretreatments, sequential

enzymatic hydrolysis and final yeast fermentation for bioethanol production.^{3,4} Over the past few years, various chemical pretreatments have been performed in different biomass residues for enhancing lignocellulose enzymatic saccharification and bioethanol production.^{5–7} For instance, acid and alkali pretreatments such as H₂SO₄ and NaOH are applied for respectively splitting strong chemical bonds of wall polymers at high temperature and disassociating hydrogen and other bonds with cellulose microfibrils. In terms of crop straw that has accumulated heavy metals, however, much remains unknown about the optimal pretreatment technology for both high bioethanol production and complete Cd collection with relatively less secondary pollution to the environment.

Wheat is a major food crop world-wide providing enormous lignocellulose residues.^{8,9} In this study, we collected mature wheat mutant and wild type (WT, a native cultivar) straw that were grown in soil pots co-supplied with Cd, and examined the maximum Cd accumulated in the lignocellulose residue of wheat mutant straw. Then, this study compared various chemical pretreatments in order to find a green-like technology for high cellulosic ethanol production and efficient Cd phytoremediation in wheat by elucidating how Cd could be released during the lignocellulose process.

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2. Materials and methods

2.1. Wheat straw collection

A general experimental procedure is described in Fig. S1.† Wheat cultivars ZM9023 and the mutant ZT5 (from EMS chemical mutagenesis) were grown in soil pots co-supplied with CdCl₂ at different concentrations (0, 25, 50, 75, 100 mg Cd per kg dry soil). Each pot was added with 25 kg soil and plants were managed under routine conditions. After the plants matured, the straw was collected and ground through a 40 mesh screen, and the well-mixed powders were stored in a sealed dry container until use.

2.2. Cd assay

The dry biomass sample (0.1000 g) loaded into a crucible was gradually heated in a muffle furnace up to 200 °C for 1 h, and set at 600 °C for 6–8 h. The ash was dissolved with 1% HNO₃ (v/v) and washed with 1% HNO₃ 3 times, and all solutions were collected in a 25 mL volumetric flask. An atomic absorption spectrometer (Agilent 240Z GFAA) was applied for the measurement of Cd content, and all samples were measured in triplicate.

2.3. Wall polymer assay

A plant cell wall fractionation procedure was performed to extract soluble sugars, pectin, hemicellulose and cellulose fractions as previously described by Peng *et al.*¹⁰ and Jin *et al.*¹¹ A UV-VIS spectrometer (V-1100D, Shanghai MAPADA Instruments Co.) was used for hexose, pentose and uronic acid assays as previously described by Huang *et al.*¹² For cellulose assay, the sample was dissolved in 67% H₂SO₄ and hexoses were calculated by the anthrone/H₂SO₄ method. Hemicelluloses were calculated by determining total hexoses and pentoses of the hemicellulose fraction, and pectin was measured by calculating total hexoses, pentoses and uronic acids of the fraction. In addition, a two-step acid hydrolysis method was applied for total lignin assay according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory. All experimental analyses were performed in triplicate.

2.4. Monosaccharide determination

GC-MS (SHIMADZU GCMS-QP2010 Plus) was used for detection of the monosaccharide composition of the soluble sugar fraction as previously described by Fan *et al.*¹³ Trifluoroacetic acid was applied for oligosaccharide digestion of the soluble sugar fraction and *myo*-inositol was added as the internal standard.

2.5. Detection of cellulose features and accessibility

The degree of polymerization (DP) of the crude cellulose sample was measured as recently described by Li *et al.*¹⁴ All experiments were conducted in biological triplicate. The cellulose crystallinity index (CrI) was determined using the X-ray diffraction (XRD) method (Rigaku-D/MAX, Ultima III; Japan) as previously described by Xu *et al.*¹⁵ Congo Red (CR) stain was

applied to estimate the cellulose surface area as previously described by Wiman *et al.*¹⁶ The biomass samples (100 mg) were added to dye solution at a series of concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg mL⁻¹) in 0.3 M phosphate buffer (pH 6) with 1.4 mM NaCl, and incubated at 60 °C for 24 h. After centrifugation at 8000g for 5 min, the absorbance of the supernatant was measured at 498 nm and the maximum amount of adsorbed dye was calculated by subtraction of the free dye in the supernatant from the initial added dye according to:

$$A_e = (C_i - C_e) \times V / (M \times 1000) \quad (1)$$

and

$$[C]/[A] = 1/K_{\text{ads}}[A]_{\text{max}} + [C]/[A]_{\text{max}} \quad (2)$$

All experimental analyses were performed in triplicate.

2.6. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed to observe the chemical linkages in the raw and pretreated wheat samples using a PerkinElmer spectrophotometer (NEXUS 470, Thermo Fisher Scientific, Waltham, MA, USA). The well-dried biomasses were finely powdered to reduce scattering losses and deformations in the absorption band. The samples (2–4 mg) were dispersed in KBr at a weight ratio of 1 : 100 and subsequently pressed to produce a transparent pelletized disc by applying 1 MPa pressure for at least 2 min. The pelletized disc samples were positioned in the path of IR light and the spectra were recorded in absorption mode over 32 scans at a resolution of 4 cm⁻¹ in the range of 4000 to 400 cm⁻¹.

2.7. Chemical pretreatment and enzymatic hydrolysis

The general biomass process chart is described in Fig. S1.† One-step chemical (H₂SO₄, NaOH) pretreatments were respectively performed as previously described by Jin *et al.*¹¹ and Li *et al.*¹⁴ The acid pretreatment was conducted at 121 °C (15 psi) for 20 min, whereas the alkali pretreatment was undertaken at 50 °C and 150 rpm for 2 h. During the two-step chemical pretreatments, the first-step alkali pretreatment was carried out at 50 °C and 150 rpm for 2 h. After the pH of the pretreated residues was adjusted to neutral, the second-step acid pretreatment was conducted under the conditions described above.¹⁷ For enzymatic hydrolysis, the pretreated biomass residues were incubated with the final concentration of 1.6 g L⁻¹ mixed-cellulases (10.6 FPU g⁻¹ biomass) and xylanase (6.72 U g⁻¹ biomass) purchased from Imperial Jade Bio-Technology Co., Ltd co-supplied with 1% Tween-80 as recently described by Li *et al.*¹⁴ All experiments were conducted in triplicate.

2.8. Yeast fermentation and ethanol measurement

Yeast fermentation was conducted using the *Saccharomyces cerevisiae* strain (Angel Yeast Co., Ltd, Yichang, China) and ethanol was measured as previously described by Jin *et al.*¹¹ and Zahoor *et al.*¹⁸ The yeast fermentation used total hexoses released by enzymatic hydrolysis of pretreated biomass resi-

dues or total soluble sugars obtained from the plant cell wall fractionation procedure. All experimental assays were performed in triplicate.

2.9. Statistical analysis

Statistical Package for the Social Sciences (SPSS version 16.0, Inc., Chicago, IL) were applied for any types of calculations. Means were separated by a least significant difference (LSD) test at $P = 0.05$. Pair-wise comparisons were conducted between two measurements by Student's t -test. The line graphs were generated using Origin 8.5 software (Microcal Software, Northampton, MA). The average values were calculated from the original triplicate measurements for these analyses.

3. Results and discussion

3.1. Increased Cd accumulation in wheat mutant straw

As illustrated in the general experimental procedure (Fig. S1[†]), this study collected mature straw samples of wheat mutant ZT5 and wild type WT/ZM9023, a native cultivar grown in soil pots co-supplied with five concentrations of CdCl₂ (0, 25, 50, 75, 100 mg kg⁻¹ dry soil). Based on the chemical analysis, the wheat mutant and WT straw showed the highest Cd contents (8.29/6.43 μg g⁻¹ dry matter) from the pots co-supplied with 75 mg CdCl₂ per kg dry soil, but had reduced Cd contents (6.59/2.32 μg g⁻¹ dry matter) from the co-supplement with 100 mg CdCl₂ per kg dry soil (Fig. 1A; Table S1[†]), indicating that the wheat mutant straw could accumulate much more Cd by 29% and 184% than those of the WT from the co-supplements of two CdCl₂ concentrations. However, both wheat mutant and WT contained similar Cd contents in their straws harvested from soil pots co-supplied with other concentrations of CdCl₂. Meanwhile, this study examined that seeds of wheat

mutant and WT contained similar Cd contents harvested from soil pots co-supplied with different concentrations of CdCl₂, suggesting that the wheat mutant could distinctively accumulate more Cd in the straw tissue. In addition, regardless of how much Cd was accumulated in the wheat mutant, both wheat mutant and WT had similar straw yields from all CdCl₂ co-supplements (Fig. S2[†]). Therefore, the selection of the wheat mutant straw should be promising for Cd phytoremediation.

3.2. Enhanced biomass enzymatic saccharification and bioethanol production

Because wheat straw could accumulate the most Cd from the 75 mg CdCl₂ kg⁻¹ co-supplement, this study focused on collecting the related biomass samples from all the following bioethanol process experiments. Using the well-established chemical pretreatment approaches,^{17,19,20} we performed acid and alkali pretreatments in both wheat mutant and WT straw using H₂SO₄ and NaOH (Fig. 2A and B, Fig. S3[†]). After pretreatment with a series of concentrations of H₂SO₄, the wheat mutant ZT5 showed much higher hexose yields (% cellulose) than those of WT released from enzymatic hydrolysis, but had the highest hexose yield of less than 85% under 4% H₂SO₄ pretreatment (Fig. 2A). By comparison, the wheat mutant exhibited a complete enzymatic hydrolysis with a hexose yield of 100% under 2% NaOH pretreatments, whereas the WT had a 100% hexose yield from 4% NaOH pretreatment (Fig. 2B). Meanwhile, after being co-supplied with 75 mg CdCl₂ kg⁻¹, the wheat mutant maintained higher hexose yield than that of the WT from 2% H₂SO₄ pretreatment, but both mutant and WT had a complete enzymatic saccharification from 4% NaOH pretreatment (Fig. 2C; Table S2[†]), consistent with the previous reports where the alkali pretreatment leads to much higher biomass enzymatic digestibility than that of the acid pretreatment.^{21–23}

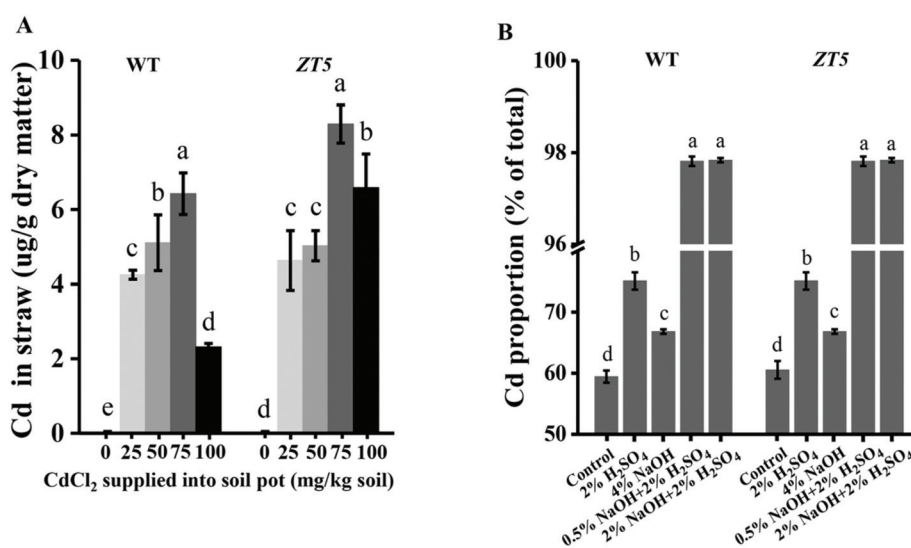


Fig. 1 Cd content of mature straw in wheat mutant ZT5 and wild type WT/ZM9023 grown in soil pots supplied with different concentrations of CdCl₂ (A). (B) Cd proportion (% of total) released from various chemical pretreatments. Data as means \pm SD ($N = 3$), and letters (a, b, c, d, e) as mean values for significant difference between each other by the LSD-test, respectively.

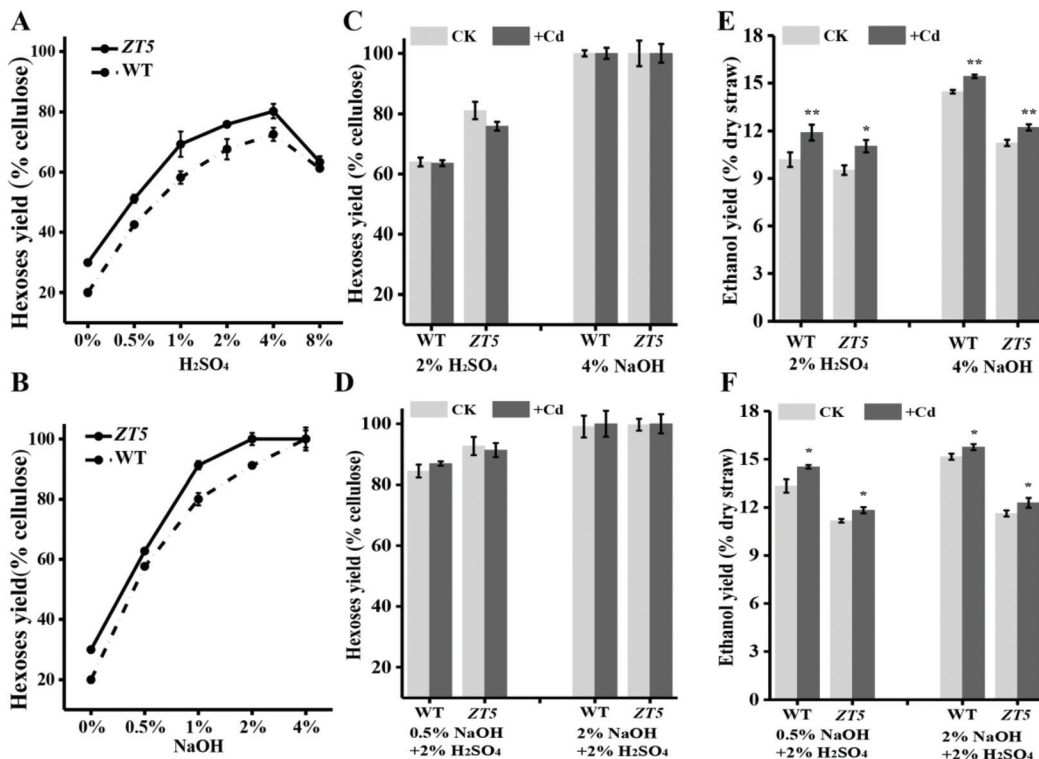


Fig. 2 Hexose and bioethanol yields in wheat mutant (*ZT5*) and WT. (A, B) Hexose yields (% cellulose) released from enzymatic hydrolysis after H₂SO₄ and NaOH at a series of concentrations. (C, D) Yields of hexoses released from enzymatic hydrolysis after one-step (H₂SO₄, NaOH) and two-step pretreatment (NaOH + H₂SO₄) with wheat straws supplied with 75 mg kg⁻¹ CdCl₂ or without CdCl₂ (CK). (E, F) Bioethanol yields (% dry straw) from yeast fermentation using total hexoses released from enzymatic hydrolyses and soluble sugars. Data as means ± SD (*N* = 3). * & ** as significant difference between two samples by the *t*-test at *P* < 0.05 and 0.01 (*N* = 3).

As the alkali pretreatment mainly disassociates lignin and the acid pretreatment typically digests hemicelluloses from plant cell walls, the two-step alkali and acid pretreatments have been examined for effectively extracting non-cellulosic polymers from biomass residues,^{17,24,25} which may also be powerful for heavy metal release. Hence, this study reported a two-step pretreatment using low concentrations of NaOH and H₂SO₄ (Fig. 2D). Based on our previous comparisons between one-step and two-step (alkali, acid) pretreatments,^{17,18} this study found that 2% H₂SO₄ pretreatment (v/v) was optional for relatively high hexose yield as compared to other acid concentrations. The two-step pretreatments were thus conducted including 0.5% NaOH + 2% H₂SO₄ and 2% NaOH + 2% H₂SO₄, which led to an almost complete biomass enzymatic saccharification in the wheat mutant (Fig. S3†). In detail, the two-step (0.5% NaOH + 2% H₂SO₄) pretreatments could cause much higher hexose yields than those of the one-step acid (2% H₂SO₄) pretreatment in all straw samples, but the wheat mutant maintained significantly higher biomass saccharification than the WT. In particular, the two-step (2% NaOH + 2% H₂SO₄) pretreatments led to a complete biomass enzymatic saccharification in all wheat straw samples, similar to the one-step alkali (4% NaOH) pretreatment (Table S3†).

Using total hexoses from the soluble sugar fraction and enzymatic hydrolyses of pretreated lignocellulose, we per-

formed yeast fermentation for bioethanol production in all wheat straw samples. In comparison, the two-step (0.5% NaOH + 2% H₂SO₄) pretreatments led to significantly increased bioethanol yields (% dry straw) compared to those of the one-step acid (2% H₂SO₄) pretreatment at *P* < 0.05 and 0.01 levels (Fig. 2E and F; Table S4†). Despite the varied hexose yields, the wheat mutant and WT co-supplied with CdCl₂ all showed significantly higher bioethanol yields than those of the controls (CK), which should be due to the higher soluble sugar accumulation from the CdCl₂ supplement as described below. In addition, the wheat mutant (*ZT5*) had relatively lower bioethanol yields against dry matter (% dry straw) than those of the WT under different pretreatments, mainly due to the relatively lower cellulose level against dry matter (% dry straw) in the mutant.

3.3. Reduced cellulose level and increased soluble sugars from CdCl₂ supplements

With respect to the Cd accumulation and bioethanol production in wheat, this study determined the cell wall composition and total soluble sugar contents in the mature wheat mutant and WT straw (Table 1). With four concentrations of CdCl₂, the wheat mutant and WT exhibited drastically increased amounts of soluble sugars up to 1.7–2.4 fold, but had significantly reduced cellulose and pectin levels in the dry

Table 1 Cell wall composition and soluble sugar content of mature wheat straw

Sample	CdCl ₂ supply (mg kg ⁻¹ soil)	Cellulose	Hemicellulose	Lignin	Pectin	Soluble sugars
WT	0	32.50 ± 0.87 ^a	31.57 ± 0.33	19.61 ± 0.29	3.67 ± 0.04 ^a	4.81 ± 0.10 ^c
	25	31.21 ± 1.36 ^a	31.94 ± 0.90	20.05 ± 0.24	3.63 ± 0.12 ^a	5.92 ± 0.19 ^d
	50	31.32 ± 0.14 ^a	31.92 ± 0.32	19.71 ± 0.26	3.43 ± 0.10 ^b	9.80 ± 0.34 ^c
	75	29.64 ± 0.19 ^b	31.34 ± 0.42	20.06 ± 0.28	3.40 ± 0.06 ^b	10.34 ± 0.21 ^b
	100	29.79 ± 0.32 ^b	31.74 ± 0.30	19.53 ± 1.12	3.33 ± 0.06 ^b	11.66 ± 0.26 ^a
ZT5	0	23.21 ± 0.71 ^a	35.74 ± 0.78	20.59 ± 0.62	3.97 ± 0.06 ^a	8.49 ± 0.26 ^d
	25	22.59 ± 0.64 ^{ab}	34.44 ± 0.49	21.25 ± 1.13	3.91 ± 0.11 ^a	14.03 ± 0.25 ^a
	50	21.38 ± 0.36 ^c	35.50 ± 0.96	21.99 ± 0.71	3.85 ± 0.07 ^a	13.70 ± 0.24 ^b
	75	22.02 ± 0.22 ^{bc}	34.98 ± 1.23	22.00 ± 0.21	3.83 ± 0.12 ^a	12.24 ± 0.29 ^{bc}
	100	21.73 ± 0.40 ^c	35.87 ± 0.95	21.37 ± 0.06	3.46 ± 0.16 ^b	11.99 ± 0.16 ^c

Data as means ± SD (*N* = 3) with the LSD-test for significant difference among all values marked as a, b, c, d, and e respectively.

straw. However, the lignin and hemicellulose contents were hardly affected by the CdCl₂ supplements, which was in contrast to the *Miscanthus* accessions showing significantly increased hemicellulose and pectin levels from Cd accumulation.²⁶ Meanwhile, monosaccharide composition analysis indicated that glucose proportions covered 82% and 85% of total soluble sugars in the mature straw of the wheat mutant and WT co-supplied with 75 mg CdCl₂ kg⁻¹, but had 57% and 50% in the controls (Table S5[†]). Regardless of the reduced cellulose rates of 5% and 10% from the 75 mg CdCl₂ kg⁻¹ co-supplement, the wheat mutant and WT respectively showed the increased glucose rates of 42% and 71% in the dry straw, which could partially explain why the wheat straw had significantly increased bioethanol yields (% dry straw) from the CdCl₂ co-supplements (Fig. 2E and F). On the other hand, compared with the WT, the wheat mutant had an increased glucose content by 14%, but contained a much lower cellulose content by 26% from the CdCl₂ co-supplements, which should mainly account for relatively lower bioethanol yields against dry matter (%) in the wheat mutant. Hence, although it has been reported that Cd accumulation could mainly inhibit cellulose production in plants,^{26,27} this study also examined high extractable glucose accumulation in wheat straw from the CdCl₂ co-supplement, suggesting that the reduced cellulose

and pectin production may contribute to the increased soluble sugars in mature wheat straw.

3.4. Complete Cd release and collection from wheat straw

In terms of the high Cd accumulation in wheat straw, this study examined the Cd release from biomass pretreatment and sequential enzymatic hydrolysis (Fig. 1B; Table 2). In general, the wheat mutant and WT straw respectively contained 61% and 59% of total soluble Cd (Table 2), which should not be associated with plant cell walls. In comparison, the one-step pretreatment with 2% H₂SO₄ or 4% NaOH could lead to 67–87% (% of total) Cd release from wheat mutant and WT straw, whereas the two-step pretreatments (0.5% NaOH + 2% H₂SO₄ or 2% NaOH + 2% H₂SO₄) released about 98% of total Cd (Fig. 1B; Table 2), indicating that the two-step pretreatments should be more effective for Cd release from wheat straw. Furthermore, the acid pretreatment could extract much more Cd than the alkali pretreatment, which explained why the 0.5% NaOH + 2% H₂SO₄ pretreatment showed a similar Cd extraction to the 2% NaOH + 2% H₂SO₄ pretreatment. As the one-step alkali and acid pretreatments could respectively extract lignin and hemicellulose or pectin,^{3,17,22} the two-step (alkali + acid) pretreatments could effectively extract those wall polymers. Furthermore, despite Cd being associated with wall

Table 2 Released Cd proportion and remaining residue weight after chemical pretreatment and sequential enzymatic hydrolysis in wheat straw

Total Cd level (μg g ⁻¹ dry matter)	Pretreatment	Residue weight (g g ⁻¹ dry matter) after pretreatment	Residue weight (g g ⁻¹ dry matter) after enzymatic hydrolysis	Cd proportion (% total) released from pretreatment and enzymatic hydrolysis
		WT	Control	0.80 ± 0.57
6.43 ± 0.56 ^b (3.82 ± 0.02) ^a	2% H ₂ SO ₄	0.69 ± 0.16	0.33 ± 0.19	99
	4% NaOH	0.81 ± 0.40	0.12 ± 0.22	99
	0.5% NaOH + 2% H ₂ SO ₄	0.64 ± 0.75	0.18 ± 0.16	100
	2% NaOH + 2% H ₂ SO ₄	0.61 ± 0.46	0.11 ± 0.31	100
	ZT5	Control	0.78 ± 0.35	0.54 ± 0.55
8.29 ± 0.51 ^b (5.02 ± 0.09) ^a	2% H ₂ SO ₄	0.62 ± 0.44	0.26 ± 0.26	99
	4% NaOH	0.76 ± 0.42	0.09 ± 0.34	98
	0.5% NaOH + 2% H ₂ SO ₄	0.62 ± 0.84	0.17 ± 0.47	100
	2% NaOH + 2% H ₂ SO ₄	0.54 ± 0.67	0.09 ± 0.31	100

Data as means ± SD (*N* = 3). ^aAs soluble Cd without accumulation into cell walls. ^b75 mg CdCl₂ kg⁻¹ co-supplement.

polymers,^{28–30} it could be efficiently released under acid conditions,^{31,32} which explained why two-step pretreatments led to 98% Cd release from the mature wheat straw in both WT and mutant. In addition, the sequential enzymatic hydrolysis led to a complete Cd release from the two-step pretreated wheat straws, but 1–2% Cd remained from the one-step pretreated ones. Therefore, the two-step pretreatment should be optimal for a complete biomass enzymatic saccharification and Cd release in wheat straw, due to its effective extraction of major wall polymers.

3.5. Distinct altered cellulose features and lignocellulose accessibility in wheat mutant straw

As the Cd accumulation led to a reduced cellulose level with little impact on other major wall polymers (hemicellulose and lignin), this study further detected two major cellulose features: cellulose crystalline index (CrI) and degree of polymerization (DP), which have been characterized as negative factors in biomass enzymatic saccharification under various pretreatments.^{33,34} Since the 0.5% NaOH + 2% H₂SO₄ pretreatment caused a sufficient Cd extraction similar to the 2% NaOH + 2% H₂SO₄ pretreatment as described above, the next experiments focused on comparing the raw material and the 0.5% NaOH + 2% H₂SO₄ pretreated residues (Fig. 3).

Compared with the raw materials, the two-step pretreated residues showed much reduced cellulose DP values by 22–36% at $P < 0.01$ ($N = 3$) in all wheat straw samples, but had relatively higher cellulose CrI values by 16–33% (Fig. 3A, Table S6†). The increased cellulose CrI values should be due to the effective extraction of wall polymers from two-step pretreatment, consistent with the previous reports.^{35–37} Notably, the wheat mutant showed much lower cellulose DP and CrI values than those of the WT in both raw materials and pretreated biomass residues (Table S6†), explaining why the wheat mutant had higher biomass enzymatic saccharification.^{3,34}

Meanwhile, the CdCl₂ co-supplement led to relatively reduced cellulose DP values in raw materials of wheat mutant and WT, probably due to the inhibition of cellulose biosynthesis by Cd.^{27,34} As the cellulose DP negatively affects biomass porosity,^{14,38} this study also measured cellulose surface accessibility using Congo red stain, and the two-step pretreated residues showed drastically increased Congo red areas by 1–1.4 fold compared to those of the raw materials in wheat mutant and WT (Fig. 3B), suggesting that the enhanced biomass saccharification should be mainly due to the increased cellulose accessibility for cellulase enzymes attack in the two-step pretreated wheat straw.³⁹ Furthermore, the increased cellulose accessibility should be partially due to much wall polymer

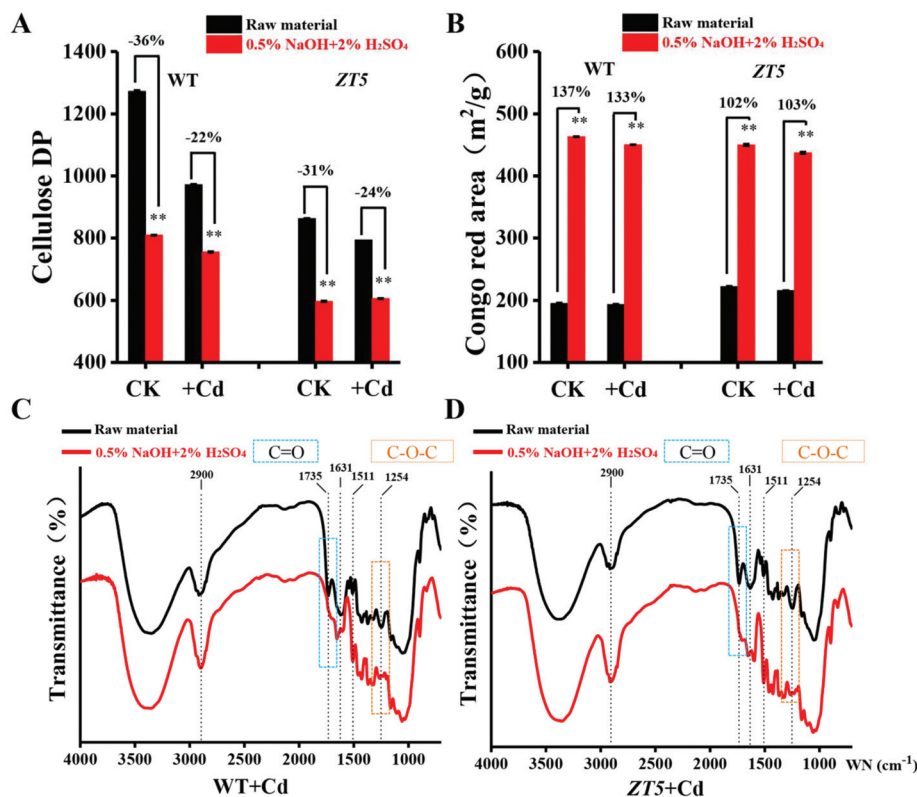


Fig. 3 Cellulose degree of polymerization (DP) in the raw material (A) and two-step pretreated residues of mature straw in wheat mutant and WT supplied with 75 mg kg⁻¹ CdCl₂ and without CdCl₂ (CK). (B) Cellulose surface accessibility by measuring the Congo red (CR) dye area (1 gram CR adsorbed corresponds to the area of 1055 m²). (A, B) Data as means ± SD ($N = 3$); **as significant difference between raw materials and two-step pretreated residues (0.5% NaOH + 2% H₂SO₄) by the t -test at $P < 0.01$. (C, D) Fourier transform infrared spectroscopic profiling among the raw materials (black) and two-step pretreated residues (red).

extraction from the two-step chemical pretreatment. In addition, this study observed the wall polymer linkage styles using Fourier transform infrared (FTIR) spectroscopy. Compared to the raw materials with or without CdCl₂ supplements, the two-step pretreated residues showed apparent variations of at least five peaks characteristic of three major wall polymer inter-linkage styles (C–O–C, C=C, C=O, C–H) in wheat mutant and WT (Fig. 3C and D; Fig. S4; Table S7[†]). For instance, the absorption bands located at 1735 cm⁻¹ were not seen in the pretreated residues of wheat mutant and WT, which were considered as either ester-linked acetyl and uronic groups of hemicelluloses or the carboxylic acid groups of ferulic and *p*-coumaric acids of lignin and hemicelluloses.⁴⁰ Hence, FTIR profiling confirmed the findings of effective wall polymer extraction and Cd release from the two-step pretreated wheat straw.

3.6. Mechanism of enhanced Cd phytoremediation and cellulosic ethanol production in wheat straw

Based on the major findings of this study and the previously-published articles,^{14,26,41} we thus proposed a mechanistic model elucidating how the Cd accumulation could distinctively reduce the cellulose content and features (DP, CrI) in wheat straw, leading to much increased cellulose accessibility particularly under the two-step pretreatments either for enhanced lignocellulose enzymatic saccharification or for complete Cd release (Fig. 4). Meanwhile, this model attempted to explain why the reduced cellulose level could lead to much soluble sugar accumulation directly fermentable for additional bioethanol production. The major reasons included: (1) despite the cellulose level being much reduced in wheat mutant straw in particular under Cd supplements, the non-cellulosic polysaccharides (hemicelluloses, pectin) were not relatively altered (Table 1), suggesting that the excess glucose not used for cellulose biosynthesis should be mainly stored as soluble sugars in straw under dynamic regulation of carbon partitioning *via* sucrose synthases or other enzymes.^{42–44} (2)

Due to its inhibited cellulose biosynthesis, the wheat mutant showed much reduced cellulose features (DP, CrI), consistent with the previous reports on rice mutant and transgenic plants.^{14,34,41} (3) As the alkali and acid pretreatments could respectively extract lignin and hemicelluloses,^{3,17,22} the two-step alkali and acid pretreatments of wheat straw could take advantage for complete Cd release into the supernatant for collection (Fig. 1B). (4) It was also understandable why the two-step pretreatment could lead to much increased cellulose accessibility for lignocellulose enzymatic saccharification. (5) Recent reports have indicated that the reduced cellulose DP could largely enhance biomass enzymatic saccharification, due to the increased ends of β-1,4-glucan chains of cellulose microfibrils enabled for cellulase enzyme attack,^{14,41} which should be an additional cause of why the wheat mutant had relatively higher enzymatic saccharification than that of the WT with/without Cd supplements (Fig. 2C and D). Therefore, this model highlights a potential strategy for effective Cd phytoremediation and high cellulosic ethanol co-production in wheat straw. In addition, once the wheat mutant (*ZT5*) gene is identified in the future, it should be applicable for transgenic crop selection for further enhancing the Cd accumulation capacity and cellulosic ethanol co-production in green-like biomass processing in wheat and other crops.

4. Conclusions

Using soil pots supplied with CdCl₂, this study examined a maximum Cd accumulation in wheat mutant straw. The Cd accumulation led to a reduction of cellulose level and DP with higher soluble sugar accumulation distinctive in wheat mutant and wild type straw. This fundamentally increased cellulose surface accessibility for enhanced biomass enzymatic saccharification and bioethanol production under chemical pretreatments. In particular, the two-step mild alkali and acid pretreatments could cause an effective wall polymer extraction and a complete Cd release from the mature straw. Hence, this study provided a green-like technology for Cd collection and cellulosic ethanol co-production in wheat straw and beyond.

Conflicts of interest

The authors have no conflicts of interest to declare.

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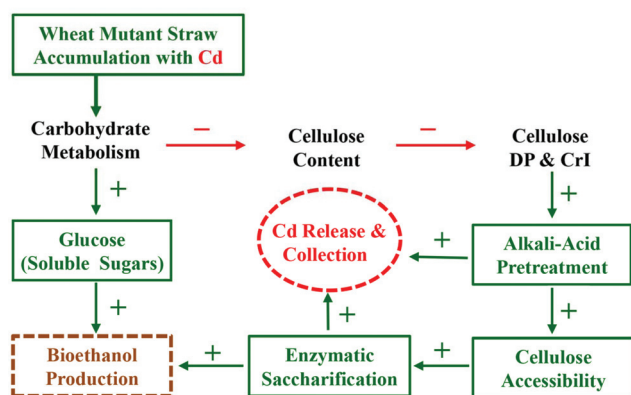


Fig. 4 A mechanism model links Cd phytoremediation and cellulosic ethanol co-production under two-step mild chemical biomass processing distinctive in wheat mutant (*ZT5*) straw. The “+”/“–” marks were respectively indicated as increased/decreased factors or impacts in this study.

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