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Altered carbon assimilation and cellulose accessibility to maximize bioethanol yield under low-cost biomass processing in corn brittle stalk

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Abstract:

Although cellulosic ethanol has been regarded as a perfect additive for transportation fuels, lignocellulose recalcitrance fundamentally determines an expensive bioethanol process unacceptable for commercial production. Here, this study collected the brittle stalk of corn mutant bk1 that showed similar biomass and seed yields to its wild type (elite cultivar). We then detected significantly reduced cellulose content and degree of polymerization of β-1,4-glucans, leading to a 74% increase in directly-fermentable hexoses accumulation in the brittle stalk by reducing sucrose production and altering carbon assimilation. Notably, under two green-like pretreatments (20 min liquid hot water, 15% CaO at 50 °C), the brittle stalk exhibited remarkably improved cellulose accessibility for almost complete biomass saccharification, resulting in achieving the highest bioethanol yield of 19.3% (% dry matter), compared to the historical records under strong corn stalk pretreatments. Furthermore, even though without any pretreatment, we evaluated that the brittle stalk could
obtain the bioethanol yield of 20.3%, if total xylose and hexoses from the brittle stalk were combined for yeast co-fermentation. This study has thus provided a perspective model of the green-like bioethanol industry that integrates engineered bioenergy crops and yeast strains with a cost-effective biomass processing.

1. Introduction

Cellulosic ethanol has been considered a promising solution for the partial replacement of fossil fuels and reduced net carbon release.¹⁻³ Although lignocellulose represents the most renewable biomass resource, there are three major factors that decide an unacceptable costly bioethanol production: intrinsic lignocellulose recalcitrance, extrinsic bioenergy crop planting and collection, and potential secondary pollution of wastes and byproducts.⁴ Hence, it becomes vital to explore an integrated strategy for a cost-effective and green-like biomass process with dedicated bioenergy crops.⁵

Lignocellulose recalcitrance is basically determined by diverse cell wall compositions, specialized wall polymer features and complicated wall-network configurations.⁶⁻⁹ To reduce recalcitrance, genetic modification of plant cell walls has been implemented in bioenergy crops. In particular, cellulose is directly targeted to increase its accessibility by reducing the degree of polymerization (DP) of β-1,4-glucans and the crystalline index (CrI) of lignocellulose.¹⁰⁻¹² Importantly, although cellulose is the major wall polymer of all biomass resources, its mild modification has shown a slight impact on plant growth and biomass or grain yield, indicating a feasible approach for cell wall modification in bioenergy crops.

Furthermore, various chemical pretreatments have been employed to breakdown lignocellulose recalcitrance for sequentially enhancing enzymatic saccharification.¹³⁻¹⁴ However, most pretreatments lead to a costly biomass process with consistent waste and byproduct formation.¹⁵ Comparatively, CaO
is a low-cost alkali chemical, but it requires recycling in another industry.\textsuperscript{65} Given that liquid hot water (LHW) is a green-like pretreatment, it could cause
much enhanced enzymatic saccharification in most biomass residues examined.\textsuperscript{67} Therefore, combined one-time genetic lignocellulose modification
with green-like pretreatment may lead to a cost-effective biomass process for
bioethanol production.

Plant photosynthesis is the characteristic process that enables the capture
of carbon for the storage of solar energy in the form of lignocellulose, starch,
and soluble sugars in plants. In most plants, sucrose metabolism plays a key
role in carbon assimilation from sources (soluble sugars) to sinks (starch,
cellulose, others).\textsuperscript{18,19} Unlike lignocellulose-based bioenergy crops, sugar- and
starch-derived bioenergy crops have been commercially employed for
bioethanol production, but their large-scale consumption faces a conflict with
food and feed security.\textsuperscript{4,20}

Among the widely cultivated food crops worldwide, corn is a highly
photosynthetic-efficient C4 plant that annually produces billions of tons of grain
and lignocellulose residue.\textsuperscript{21} However, except for corn grain, its lignocellulose
residue is rarely employed for commercial bioethanol production. In this study,
we selected a brittle-stalk mutant (\textit{bk1}) of corn containing much higher levels
of soluble sugars and reduced cellulose. We then determined the highest
bioethanol yield by yeast co-fermentation with the soluble sugars and hexoses
released from enzymatic hydrolysis under two green-like pretreatments (CaO,
LHW) of the brittle stalk, compared to the previous records of cellulosic ethanol
under strong pretreatment conditions. Notably, this study also obtained high
bioethanol yield even though without any pretreatment in the brittle stalk.
Based on carbohydrate metabolism analysis, this study presents a
hypothetical model that highlights potential cost-effective and green-like
biomass processing to maximize bioethanol yield in the brittle stalk of corn that
alters carbon flux.
2. Materials and methods

2.1. Collection of brittle stalk in bk1 mutant

The brittle stalk mutant (single recessive gene, termed bk1) was selected from the F_2 seeds of Zong31 (wild type/WT, a Chinese maize inbred) crossing with MuDR transposon W22::Mu. The homozygous seeds of WT and bk1 were obtained by self-fertilization and their stalk samples were consistently collected in the experimental fields of Huazhong Agricultural University, Wuhan from years of 2013-2017. The 11th-leaf stalk was under manually bending for brittle phenotype observation and the mature stalk (without leave, seeds and roots) were dried, grounded through a 40 mesh screen and stored in a dry container for cell wall composition analysis and biomass enzymatic hydrolysis.

2.2. Scanning electron microscopic observation

The top internodes of corn stalk at the 11th-leaf stage were collected and preserved in the formalin-acetic acid-alcohol solution. The plant cell walls were observed using scanning electron microscopy (SEM JSM-5610/LV, Hitachi, Tokyo, Japan). The pretreated biomass residues were dried at 50 °C, and imaged using SEM. Each sample was observed for 8-10 times in this study.

2.3. Wall polymers extraction and assay

The cell wall fractionation was conducted as previously described by Peng et al., Jia et al. and Li et al. The well-mixed biomass powders were initially ground with potassium phosphate buffer (pH 7.0), followed with chloroform–methanol (1:1, v/v), DMSO–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate to remove lipid, starch and pectin. The residual was extracted with 4 M KOH with 1.0 mg/mL sodium borohydride as KOH-extractable hemicelluloses, followed by H_2SO_4 (67%, v/v) to completely dissolve cellulose and non-KOH-extractable hemicelluloses. Cellulose content was calculated by
determining hexoses of the cellulose fraction using the anthrone/H$_2$SO$_4$ method.$^{23}$ Total hemicelluloses were calculated by determining hexoses and pentoses using orcinol/HCl method.$^{24}$ Lignin content was measured by two-step acid hydrolysis method according to NREL analytical LAP protocol.$^{25}$ All experimental analyses were performed in biological triplicates.

2.4. Soluble sugars analysis

The biomass sample (0.3 g) was added with 6 mL distilled water and shaken at 150 rpm for 2 h at 50 °C. After centrifugation at 3,000 g for 5 min, the supernatant was collected for soluble sugar analysis. The pentoses and hexoses of soluble sugars were respectively detected as described above, whereas the fructose, glucose and sucrose were measured using High Performance Liquid Chromatography (HPLC, SHIMADZU, LC-20A with RID-10A detector).

2.5. Fourier transform infrared spectroscopy scanning

A Perkin-Elmer spectro-photometer (NEXUS 470, Thermo Fisher Scientific, Waltham, MA, USA) was used to qualitatively monitor the biomass samples 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$^{-1}$ region.

2.6. Cellulose DP and CrI detection

The cellulose DP and CrI were respectively detected as previously described by Li et al.$^{11}$ and Hu et al.$^{17}$. The cellulose DP detection was performed using the crude cellulose samples extracted with 4 M KOH (containing sodium borohydride at 1.0 mg/mL) and 8% (w/v) NaClO$_2$. All experiments were conducted in triplicate. The X-ray diffraction method was performed to detect cellulose crystalline index (CrI) using the RigakuD/MAX instrument (Ultima III, Japan). Technical standard errors of the CrI method
were measured at ± 0.05 ~ 0.15 using five representative samples in triplicate.

2.7. Lignocellulose accessibility detection

The cellulose accessibility was estimated by performing Congo red (CR) staining as previously described by Wiman et al.\textsuperscript{26} with minor modification. The biomass samples (100 mg) were treated with dye solution at a series of concentrations (0.25, 0.50, 1.0, 2.0, 3.0, 4.0 mg/mL) in 0.3 M phosphate buffer (pH 6) with 1.4 mM NaCl, and incubated at 60 °C for 24 h. After centrifugation at 8000 \( g \), the absorbance of the supernatant was measured at 498 nm and the maximum amount of adsorbed dye was calculated by subtraction of free dye in the supernatant from the initial added dye. All measurements were conducted in biological triplicate.

2.8. Hemicelluloses monosaccharide and lignin monomer determination

Monosaccharide composition of hemicellulose was determined by GC-MS (SHIMADZU GCMSQP2010 Plus) according as previously described by Xu et al.\textsuperscript{6} and Li et al.\textsuperscript{10}. The lignin monomers were examined by HPLC (LC-20A, SHIMADZU) as previously described by Li et al.\textsuperscript{11} and Jia et al.\textsuperscript{8}. Standard chemicals: p-Hydroxybenzaldehyde (H), vanillin (G) and syringaldehyde (S) were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.9. Transcriptome sequencing and metabolism pathways analyses

The transcriptome sequencing and metabolism pathway analyses were conducted as previously described by Wang et al.\textsuperscript{27}. The 3rd leaf vein of corn plant at the eleven-leaf stage was flash frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent. The sequencing library for each sample was constructed using the NEBNext mRNA Library Prep Master Mix Set for Illumina (E6110; New England Biolabs) and the NEBNext Multiplex Oligos for Illumina (E7500; New England Biolabs). Paired-end sequencing was
performed on an Illumina HiSeq 2500 (Illumina). Genes with fold change $\geq 2.0$
and significance level $p \leq 0.05$ were considered to be differentially expressed.
Differentially expressed genes (DEGs) were annotated against GO and KEGG
database (www.genome.jp/kegg/) for function annotation; category enrichment
was performed using Blast2GO, with FDR value 0.05 by Fisher’s Exact Test,
or $p$ value $\leq 0.05$ by student’s $t$-test. Each sample (mutant, wild type) was
conducted with biological triplicate.

2.10. Biomass pretreatment and enzymatic hydrolysis

Three biomass pretreatments and sequential enzymatic hydrolysis were
performed as previously described by Jia et al.$^8$, Hu et al.$^{17}$ and Li et al.$^{28}$ with
modification of chemical concentration and incubation time. Acid pretreatment:
the well-mixed biomass powder (0.300 g) was added with 6 mL H$_2$SO$_4$ at
different concentrations (0.5%, 1%, 4%, 6%, v/v) at 5% solid loading, and
heated at 121 °C for 20 min in autoclave (0.15 Mpa). Liquid hot water (LHW)
pretreatment: The well-mixed biomass powder (0.300 g) was added into
well-sealed stainless steel bombs (1:8, W/V) at 12.5% solid loading, and
heated at 200 °C under 15 rpm shaking for 5, 10, 20, 25 min, respectively.
Alkali pretreatment: the biomass power (0.300 g) was added with 6 mL CaO at
different concentrations (5%, 10%, 15%, 20%, w/w) with 5% solid loading, and
incubated at 50°C under shaken at 150 rpm for 48 h. Control (without
pretreatment): the well-mixed powder was added with 6 mL distilled water at
5% solid loading and shaken at 150 rpm for 2 h at 50 °C.

The pretreated biomass residues were washed once with 10 mL of
mixed-cellulase reaction buffer. The washed residues were incubated with 6
mL (2 g/L) of mixed-cellulases (containing cellulases at 10.60 FPU g$^{-1}$
biomass and xylanase at 6.72 U g$^{-1}$ biomass from Imperial Jade
Bio-technology Co., Ltd) containing with 1% Tween-80 at 5% solid loading,
and shaken under 150 rpm for 48 h at 50 °C. The samples were centrifuged at
3,000 g for 5 min, and the supernatants were collected for hexoses and pentose assay. All experiments were carried out in biological triplicate.

2.11. Yeast fermentation and ethanol measurement

Yeast fermentation and ethanol measurement were conducted as previously described by Li et al.\textsuperscript{11} and Hu et al.\textsuperscript{17}. Yeast \textit{Saccharomyces cerevisiae} strain (purchased from Angel Yeast Co., Ltd., Yichang, China) was suspended with 0.2 M phosphate buffer (pH 4.8) for 30 min for activation prior to use. The yeast powder was then added with the phosphate buffer to achieve in final concentration of 0.5 g/L in all fermentation tubes, and the fermentation was conducted at 37 °C for 48 h in tubes. Ethanol was measured using K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} method. The experiments were performed with biological triplicate.

3. Results and discussion

3.1. Reduced cellulose and increased soluble sugars in the \textit{bk1} mutant

Using mutator-inserted technology, this study selected a genetically stable brittle-stalk mutant (\textit{bk1}) of corn by crossing elite corn inbred Z31 (wild type/WT, an elite Chinese cultivar) with \textit{MuDR} transposon (\textit{W22::Mu}). Compared with the WT, the homozygous \textit{bk1} mutant consistently exhibited normal growth with slightly affected biomass and grain yields in three-season field experiments (Fig. 1A; Fig. S1\textsuperscript{†}), which was similar to our previously identified rice brittle-culm mutant showing normal growth and high biomass and grain yields\textsuperscript{10}. However, as the \textit{bk1} mutant was of a brittle-stalk phenotype under manual bending, we observed thinner sclerenchyma cell walls under scanning electron microscopy (Fig. 1B-C). With respect to the thinner cell walls of brittle stalk, the \textit{bk1} mutant contained significantly lower cellulose levels than that of WT at $p < 0.01$ (n =3), with a relatively reduced rate of 29%, but both mutant and WT did not show significantly different hemicelluloses and lignin levels at $p > 0.05$ (Fig. 1D). In addition, this study found that the \textit{bk1}
mutant and WT had a similarity with either the monosaccharide composition of hemicellulose or the monomer constituent of lignin (Tables S1-S2†), suggesting that the thinner cell walls of the brittle stalk could be mainly due to its reduced cellulose level, rather than the non-cellulosic polymers in the \textit{bk1} mutant. Notably, unlike the rice brittle-culm mutant, the \textit{bk1} mutant contained much higher levels of soluble sugars than those of the WT, with a 65% increase in the brittle stalk (Fig. 1D). Among the total soluble sugars increased in the \textit{bk1} mutant, we further examined that the hexose and pentose levels were increased by 74% and 25% (Fig. 2A), respectively, indicating that significantly altered carbon flux may occur in the \textit{bk1} mutant. Because sucrose is the end product of plant photosynthesis,\textsuperscript{29} the brittle stalk of the \textit{bk1} mutant contained a reduced sucrose level by 15% with increased fructose and glucose by 6% and 20% (Fig. 2B), respectively, suggesting that altered sucrose metabolism may dynamically regulate carbon partitioning and allocation in the brittle stalk of the \textit{bk1} mutant.

3.2. Enhanced hexoses and ethanol yields in brittle stalk under three pretreatments

Using our previously established approach,\textsuperscript{8,11,12,17} this study examined biomass enzymatic saccharification of the brittle stalk by measuring hexose yields (% cellulose) released from enzymatic hydrolyses under three distinct biomass pretreatments (Fig. 3A-C). In comparison, the \textit{bk1} mutant constantly exhibited much higher hexose yields than those of the WT either from the acid pretreatment with various H\textsubscript{2}SO\textsubscript{4} concentrations (0.5%, 1%, 4%, 6%) or from the LHW pretreatment over a time course (5, 10, 20, 25 min). In particular, the 20 min LHW could lead to an almost complete biomass enzymatic saccharification with a hexose yield of 96% in the brittle stalk. In addition, under 5% and 10% CaO pretreatments, the \textit{bk1} mutant showed significantly higher hexose yields than those of the WT, but pretreatment with saturated
CaO (15%) achieved a hexose yield of 100% in both mutant and WT. Consequently, this study performed a classic yeast fermentation for bioethanol production using total soluble sugars and hexoses released from enzymatic hydrolysis under three optimal pretreatments (Fig. 3D-G). Compared to the WT and the control (without pretreatment), the bk1 mutant exhibited significantly higher ethanol yields with increased rates from 10% to 37% at the \( p < 0.05 \) or 0.01 level (n=3) under three optimal pretreatments, consistent with its higher levels of soluble sugars and hexose yields as described above. In particular, the bk1 mutant had the highest ethanol yield of 19.3% (% dry matter) under two optimal green-like pretreatments (20 min LHW,15% CaO), compared to the previously reported ethanol yields (16.2%, 13.0%) obtained from the corn stalk process even though under strong pretreatment conditions (Table 1).\(^{30-32}\) Despite ammonia fiber expansion pretreatment of corn stover has led to the ethanol yield of 19.1%, it is due to a yeast co-fermentation of hexoses and xylose.\(^{33}\) Provided all xyloses obtained from soluble sugars and hemicellulose hydrolysis were co-fermented by an engineered yeast strain in the brittle stalk, this study roughly estimated that the bk1 mutant could respectively achieve the ethanol yields of 26.6% and 28.5% under two optimal green-like pretreatments, based on the average xylose-ethanol conversion rate as previously reported (Table 1). Hence, the data revealed that combining the green-like LHW pretreatment with the brittle stalk could achieve much higher bioethanol production without any secondary chemical waste release to the environment. Notably, without any pretreatment, the brittle stalk remained to achieve a high bioethanol yield of 15.2%, but it could even have the ethanol yield of 20.3% if adding all xyloses into the yeast co-fermentation (Table 1), providing a cost-effective bioethanol production technology without any chemical process. This was mainly due to increased soluble sugars (hexoses, pentoses) accumulation and higher hexoses yield released from a direct enzymatic hydrolysis in the brittle stalk, but it may also
be subjected to less inhibitor formation from the direct enzymatic hydrolysis of
the brittle stalk towards an efficient sugar-ethanol conversion during yeast
fermentation.\textsuperscript{15,34} In addition, this study only performed low solid loading
experiments, and thus it requires to find out the optimal green-like technology
for bioethanol production at high concentration (g/L) by increasing solid
loading of corn brittle stalk, along with high-dosage cellulases enzyme
supplement and engineered yeast strain applied for xylose and hexoses
co-fermentation in the future.

3.3. Decreased cellulose DP and improved biomass accessibility in the
brittle stalk

To understand why the bk1 mutant was of much enhanced biomass
enzymatic saccharification for bioethanol production, this study detected the
cellulose DP values of stalks in both mutant and WT (Fig. 4). In general, the
bk1 mutant was detected with significantly reduced cellulose DP values than
those of WT at the $p < 0.01$ level (n=3) in all raw stalk materials (without
pretreatment) and three optimal pretreated biomass residues (Fig. 4A). In
particular, all pretreated biomass residues also had much lower cellulose DP
values than those of the raw stalk materials in both mutant and WT. Because it
has been well characterized that cellulose DP negatively affects lignocellulose
enzymatic hydrolysis in various biomass residues examined,\textsuperscript{11,12} the reduction
of cellulose DP values in the bk1 mutant should be a major factor accounting
for its increased biomass enzymatic saccharification. Similarly, the bk1 mutant
was also detected with consistently reduced lignocellulose CrI values in all
samples, but the pretreated residues had a relatively higher lignocellulose CrI
values than those of the raw stalk materials (Fig. S2†), which should be due to
partial wall polymer extractions from the pretreatments.\textsuperscript{35} Hence, although
lignocellulose CrI is a negative factor on biomass enzymatic hydrolysis,\textsuperscript{6,7,10} it
should be limited to account for the increased enzymatic saccharification of the
pretreated biomass residues in both bk1 mutant and WT relative to their raw stalk materials.

As cellulose accessibility is a direct parameter accounting for biomass enzymatic hydrolysis, this study further performed Congo red staining with all biomass samples, which has been well used to measure the specific surface area of cellulose.\textsuperscript{26,35} Significantly, the bk1 mutant exhibited much larger Congo red staining areas than those of the WT in all samples examined, in particular on three pretreated biomass residues with increased rates from 22\% to 34\% (Fig. 4B), indicating a remarkably improved cellulose accessibility for high biomass enzymatic saccharification in the brittle stalk of the bk1 mutant. Furthermore, since correlation analysis has been widely applied to account for the wall polymer feature impacts on biomass enzymatic saccharification in various lignocellulose samples examined,\textsuperscript{36-38} this study characterized that cellulose DP values were negatively correlated with hexoses yields at the $p < 0.01$ level ($n = 24$), whereas cellulose accessibility had a significantly positive correlation, with a high $r$ value at 0.887 (Fig. 4 C-D), which confirmed that largely enhanced biomass saccharification of the brittle stalk should be mainly due to its significantly reduced cellulose DP and increased accessibility under three optimal pretreatments. In addition, this study examined that cellulose DP values were negatively correlated with cellulose accessibility (Fig. S3 †), consistent with the previously proposed assumption that the reduced cellulose DP should reflect increased amorphous regions of cellulose microfibrils enabled more cellulase enzyme access and loading during lignocellulose hydrolysis.\textsuperscript{11,12}

### 3.4. Unaltered wall polymer extraction in brittle stalk from pretreatments

With regard to the bk1 mutant being of slightly altered hemicellulose and lignin levels in brittle stalk, this study also examined wall polymer extraction upon optimal three pretreatments and estimated the overall mass balances
among three optimal pretreatments, subsequent enzymatic hydrolysis, and final yeast fermentation (Fig. 5; Fig. S6-S8†). Similarly, both bk1 mutant and WT showed a major hemicellulose extraction from 4% H2SO4 pretreatment or 20 min LHW pretreatment with a predominant removal of lignin from 15% CaO pretreatment with little cellulose extraction (Table S3†). Using Fourier transform infrared spectroscopy, we observed that the pretreated biomass residues exhibited characteristic alteration of peaks in both bk1 mutant and WT compared to their controls/without pretreatment (Fig. 6).39,40 For example, the peaks at 1727 cm\(^{-1}\) were almost absent in all three optimal pretreated residues of the mutant and WT referred to as (C=O) either ester-linked acetyl and uronic groups of the hemicellulosc or carboxylic acid groups of ferulic and \(p\)-coumaric acids of lignin and hemicellulosc (Table S4†), suggesting an almost complete removal of hemicellulosc-lignin linkages from three optimal pretreatments.41 The absorption peaks at 1247 cm\(^{-1}\) showed much reduced intensities, particularly in the LHW pretreated residues, which is referred to as the (C-O-C) aryl–alkyl ether bond in lignin.42 However, we did not observe much differences in the characteristic peaks between the bk1 mutant and WT in the pretreated biomass residues relative to their raw stalks. In addition, the bk1 mutant and WT did not display much different topology of the pretreated biomass residues under scanning electron microscopy (Fig. S4†). Taken together, these data indicate that the bk1 mutant predominately altered cellulose features (DP, accessibility) in its raw stalk material and pretreated biomass residues with little alteration of non-cellulosic polymers (hemicellulose, lignin) compared to the WT.

3.5. Altered sucrose metabolism pathway and carbon flux

To understand why the bk1 mutant was characteristic for cellulose reduction and soluble sugar increase in its brittle stalk, we performed a transcriptomic analysis to observe differentially expressed genes (DEGs) between the bk1
mutant and WT (Fig. S5A†). As a result, approximately 387 down-regulated
and 312 up-regulated genes were sorted out among a total of 46430 genes
identified in the bk1 mutant (Fig. S5B†). Based on the enrichment annotation
of DEGs, most DEGs were found to be involved in sucrose metabolism,
carbohydrate transport, cellulose biosynthesis, cell wall formation and other
related biological processes (Fig. S5C†). By profiling sucrose synthesis and
the related carbohydrate metabolism (Fig. 7; Table S5†), several
representative genes, including the sucrose synthase (SUS) gene, were
identified to regulate the sucrose synthetic pathway enabled for reducing
sucrose and UDP-glucose products, leading to increasing soluble sugar
(hexoses) accumulation in the brittle stalk of the bk1 mutant (Fig. 7A-B). Since
UDP-glucose is the unique substrate for cellulose biosynthesis,43-45 the
reduced substrate should directly affect cellulose production in the brittle stalk
(Fig. 7D). Furthermore, because it has been recently characterized that
overproduction of β-glucosidases could lead to reduced cellulose DP and CrI
in transgenic rice,11,12 the up-regulated β-glucosidase may play a similar role in
cellulose features (DP, CrI) reduction, which may lead to soluble glucose
accumulation in the brittle stalk (Fig. 7C). Therefore, the transcriptomic
analysis briefly interpreted why the brittle stalk of the bk1 mutant contained
significantly reduced cellulose levels and DP with increased soluble sugar
allocation.

Despite genetic modification of plant cell walls has been implemented by
targeting non-cellulosic polymers (hemicellulose, lignin, pectin) for reducing
lignocellulose recalcitrance in various bioenergy crops,5,48-52 it has more
recently been reported that slight cellulose alteration could lead to a direct
improvement of cellulose accessibility in genetic mutant and transgenic plants
with little impact on plant growth and biomass/grain yield.10-12 Although our
preliminary results indicated that the bk1 mutant is the candidate gene with a
characteristic defect of sucrose synthesis, this study briefly sorted out other
several important genes involved in carbon flux regulation and cellulose feature improvement as described above. Therefore, those genes could be combined with the mutant candidate gene for comprehensively genetic engineering of bioenergy corn and other crops using advanced synthetic biological approaches in the near future, which may be more precise for integrative enhancements of both soluble sugar allocation and lignocellulose enzymatic digestibility in bioenergy crops.

3.6. A hypothesis model for low-cost and green-like bioethanol production

Taken all data together, this study proposed a hypothesis model that highlights the cost-effective and green-like biomass process enabled to obtain maximum ethanol production in the brittle stalk of corn mutant, based on the following evidences (Fig. 8): (1) With respect to the starch-rich grain/seeds of corn potent for food/feed or bioethanol production, its lignocellulose utilization could save planting costs compared to the dedicated bioenergy crops (switchgrass, Miscanthus, etc.); (2) Due to the reduced cellulose level and improved accessibility of brittle stalk, the green-like LHW pretreatment was sufficient for complete lignocellulose enzymatic hydrolysis, which suggested the possibility of directly using solar energy for more-green LHW pretreatment; (3) Using soluble sugars and hexoses released from direct enzymatic hydrolysis of brittle stalk without any pretreatment, this study also achieved high bioethanol yield, and the remaining pure cellulose residue could be applied as value-added material for other industries, indicating a potential technology for much low-cost and benefit-added biomass process without any chemical waste release. However, it remains to sort out the key factors/parameters accounting for green-like bioethanol production in the corn brittle stalk and other bioenergy crops in the future. Therefore, this study provided a powerful strategy for the green-like bioethanol industry by
combining engineered bioenergy crops and yeast strains with low-cost (LHW or no pretreatment) biomass process.

4. Conclusions

Using the brittle stalk of corn \( bk1 \) mutant showing significantly reduced cellulose content and DP with remarkably increased soluble sugars, this study determined an almost complete biomass enzymatic saccharification for the highest bioethanol yield of 19.3% (% dry matter) achieved under two green-like pretreatments enabled for distinctively enhancing cellulose accessibility. Furthermore, even though without any chemical pretreatment, the brittle stalk could obtain the ethanol yield of 20.3% if total xylose and hexoses from soluble sugars and direct lignocellulose enzymatic hydrolysis were joined for yeast co-fermentation. Therefore, this study has revealed a hypothesis model of the green-like bioethanol production that combines desirable bioenergy crops and engineered yeast strains with low-cost biomass processing by dynamically altering carbon assimilation for high fermentable sugar accumulation in crop stalk.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Table 1. Bioethanol yields (% dry matter) obtained in the \( bk1 \) mutant and other corn
### Samples under different pretreatments

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Hexoses fermentation only</th>
<th>Hexoses &amp; pentoses co-fermentation</th>
<th>Solid loading for enzymatic hydrolysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without pretreatment</td>
<td>15.24%</td>
<td>20.28%(^c)</td>
<td>5%</td>
<td>This study</td>
</tr>
<tr>
<td>4% H(_2)SO(_4)</td>
<td>18.71%</td>
<td>23.75%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>20 min LHW</td>
<td>19.29%</td>
<td>24.33%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>15% CaO</td>
<td>19.35%</td>
<td>24.39%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>AFEX(^a)</td>
<td></td>
<td>19.15%</td>
<td>17.6%</td>
<td>[33]</td>
</tr>
<tr>
<td>MBSP(^b)</td>
<td>16.15%</td>
<td></td>
<td>10%</td>
<td>[30]</td>
</tr>
<tr>
<td>1% H(_2)SO(_4) + steam explosion</td>
<td>12.99%</td>
<td></td>
<td>10%</td>
<td>[31]</td>
</tr>
<tr>
<td>Ionic liquid</td>
<td>7.22%</td>
<td></td>
<td>6%</td>
<td>[32]</td>
</tr>
</tbody>
</table>

\(^a\) Ammonia fiber expansion.
\(^b\) Magnesium bisulfite pretreatment.
\(^c\) Based on the average xylose-ethanol conversion rate of 35% as previously reported by Rodrussamee et al. (2018)\(^{33}\) and Valinhas et al. (2018)\(^{34}\).
**Figures:**

(A) The *bk1* mutant and its wild type (WT) showing a normal plant growth. (B) A brittle-stalk phenotype in the *bk1* mutant under manual bending. (C) Relative thinner cell walls observed in the brittle stalk of *bk1* mutant under SEM. (D) Increased soluble sugars and reduced cellulose contents (% dry matter) in the *bk1* mutant. **As significant differences between the *bk1* and WT by Student’s *t*-test at *p* < 0.01 (n=3) with the increased/decreased (-) percentage of the *bk1* mutant relative to the WT, and data as means ± SD (n=3).
Fig. 2. Soluble sugar composition of the brittle stalk in the bk1 mutant.

(A) The bk1 mutant containing significantly increased hexoses and pentoses relative to the WT. (B) The bk1 mutant containing relatively high fructose and glucose and low sucrose. **As significant differences between the bk1 and WT by Student's t-test at $p < 0.01$ (n=3) with the increased percentage of the bk1 mutant relative to WT, and data as means ± SD (n=3).
Fig. 3. Enhanced biomass saccharification and bioethanol production of the brittle stalk in the *bk1* mutant.

(A), (B), (C) Hexoses yields (% cellulose) released from enzymatic hydrolysis under acid (H$_2$SO$_4$, A), liquid hot water (LHW, B) and alkali (CaO, C) pretreatments. (D), (E), (F), (G) Bioethanol yields (% dry matter) obtained from yeast fermentation using soluble sugars and hexoses released from enzymatic hydrolysis under three optimal pretreatments (D, control/without pretreatment; E, 4% H$_2$SO$_4$; F, 20 min LHW; G, 15% CaO). *and** As significant differences between the *bk1* and WT by Student's *t*-test at *p* < 0.05 and 0.01 (n=3) with the increased percentage of the *bk1* mutant relative to the WT, and data as means ± SD (n=3).
Fig. 4. Cellulose DP and accessibility impacts on biomass enzymatic saccharification.

(A),(B) Significantly reduced cellulose DP (A) and raised cellulose accessibility (B) in the raw stalk material and three optimal pretreated biomass residues of bk1 mutant. Cellulose accessibility (B) defined by Congo red stain area (m²/g). *and** As significant differences between the bk1 and WT by Student’s t-test at p < 0.05 and 0.01 (n=3) with the increased/decreased (-) percentage of the bk1 mutant relative to the WT, and data as means ± SD (n=3). (C),(D) Correlation analysis between the cellulose DP (n=22) (C) or accessibility (n=16) (D) and hexoses yields released from enzymatic hydrolysis under three optimal pretreatments and control (without pretreatment). ** As significant correlation at p < 0.01.
Fig. 5. Mass balance for lignocellulose conversion to ethanol between Z31 (left) and bk1 (right) under 20 min LHW pretreatment.
Fig. 6. Fourier transform infrared spectroscopic profiling of raw stalk materials (without pretreatment) and pretreated biomass residues. (A),(B),(C) WT samples. (D),(E),(F) bk1 samples. The characteristic perks referred in Supplementary Table 4.
Fig. 7. Sucrose and carbohydrate metabolism pathways alteration in the bk1 mutant.

(A) Down-regulated sucrose synthesis. (B),(C) Increased glucose allocation and accumulation. (D) Down-regulated cellulose synthesis. Red box highlighted as up-regulated gene/enzyme; green box as down-regulated gene; yellow box as dual up/down-regulated gene, grey and white boxes as insignificantly altered genes. The representative genes/enzymes referred in Supplementary Table 5.
Fig. 8. A hypothetical model to highlight a green-like strategy for the bioethanol industry by integrating engineered bioenergy crop and yeast strain with low-cost biomass process. The engineered bioenergy crop should be of significantly improved cellulose accessibility for an efficient enzymatic saccharification with much high soluble sugars accumulation for a direct ethanol fermentation. The engineered yeast strain should be able to use both hexoses and pentose (xylose) as carbon source for co-fermentation. (-) and (+) respectively as reducing and enhancing strategy for biomass process and bioethanol production in the model; “SUS” as sucrose synthase.
References


Graphical Abstract

Altered carbon assimilation and cellulose accessibility to maximize bioethanol yield under low-cost biomass processing in corn brittle stalk