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Cascading of engineered bioenergy plants and fungi sustainable for low-cost bioethanol and high-value biomaterials under green-like biomass processing

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ABSTRACT

Plant cell walls contain the most abundant sustainable biomass resource for biofuels and biomaterials on the earth. However, lignocellulose recalcitrance generally requires a costly biomass process unacceptable for large-scale bioethanol production with the potential formation of secondary wastes. To address this bottleneck-like biomass recalcitrance issue, this review attempts to connect recent innovation progress regrading up-stream lignocellulose modification, middle-stream cellulases production and down-stream biomass processing. Particularly, the site-specific gene editing is demonstrated for precise and mild modification of plant cell walls to generate recalcitrance-much-reduced cellulose nanofibers, which not only leads to little impact on plant strength and biomass yield, but also causes remarkably enhanced enzymatic saccharification in major bioenergy crops. By selecting the size-reduced cellulose nanofibers of engineered bioenergy crops as enzyme-inducing substrate, fungal strains are then engineered to secret the optimal cellulases enzymes cocktails enabled for complete enzymatic saccharification of diverse lignocellulose residues from cost-effective biomass pretreatments. Consequently, engineered yeast strains could use both hexoses and xylose released from complete saccharification as carbon sources for maximum bioethanol production by an efficient co-fermentation. Finally, the green-like processing technology is introduced to generate biomaterials and biochemicals by using the remaining lignin-rich residues. Therefore, this work has originally proposed a novel strategy that dynamically cascades the engineered bioenergy crops and fungal strains with the advanced biomass process technology, which should be considered as next generation of integrated biotechnology for both cost-effective biofuels production and value-added bioproducts with minimum waste releases into the environment.

1. Introduction

Energy security and environmental change have drawn attention to exploring renewable and sustainable biomass resources for biofuels and biochemicals. In general, lignocellulose-based biomasses are derived from forestry, agricultural straw and daily life wastes without compromising food security. Cellulosic ethanol has been recently evaluated as an excellent supplement for petrol [1]. However, because lignocellulose exhibits recalcitrance against enzyme degradation, the current technology requires strong biomass pretreatments and costly enzymatic saccharification for bioethanol production [2,3]. Hence, various genetic engineering approaches have been implemented to reduce lignocellulose recalcitrance in bioenergy crops, increase enzymes production and catalytic activity in fungi, and enhance co-fermentation of hexoses and xylose in *S. cerevisiae* for effective enzymatic saccharification and bioethanol production [4–7].

The selection of bioenergy crops has been considered to be a powerful approach for reducing lignocellulose recalcitrance by genetic

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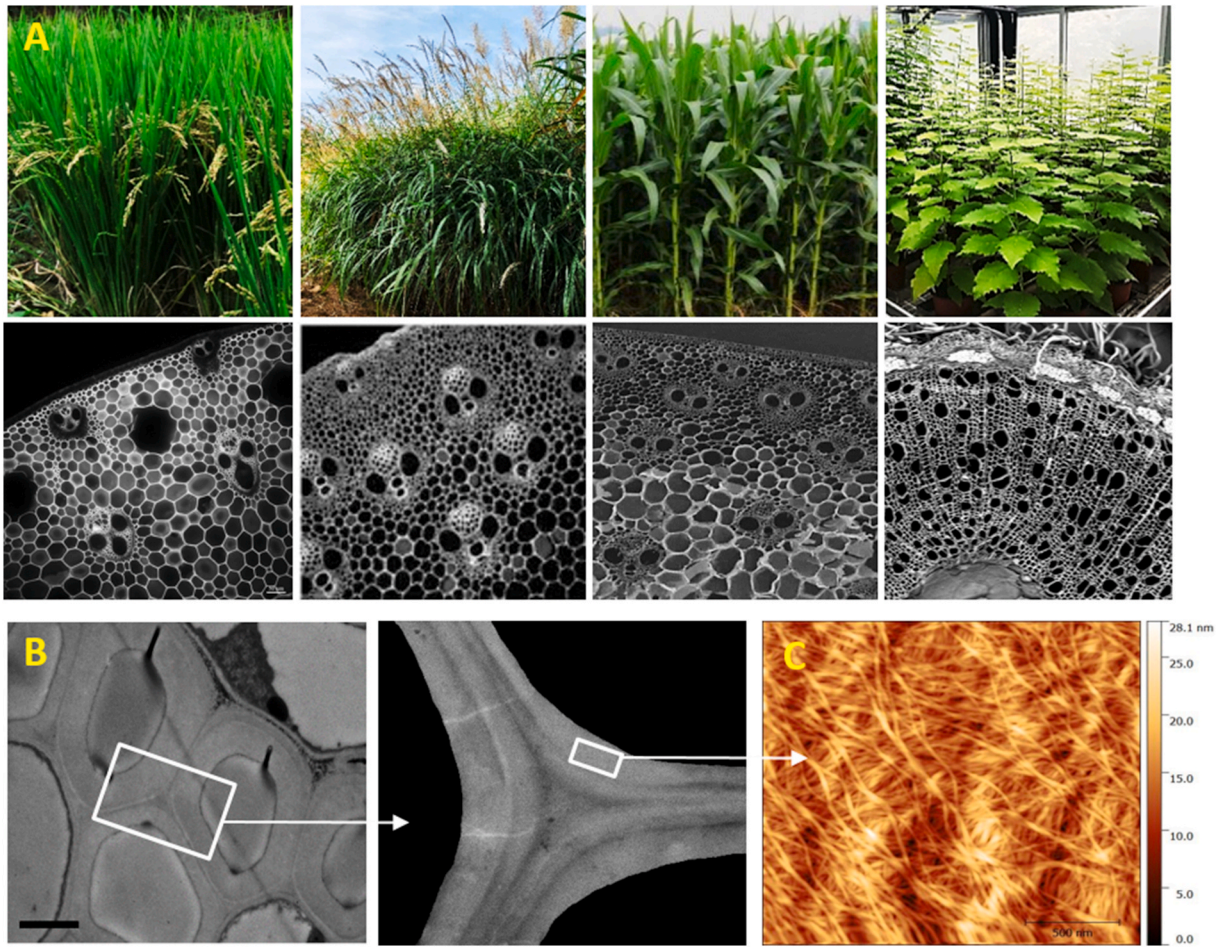

Fig. 1. Distinct bioenergy crops for dedicated lignocellulose. (A) Four types of bioenergy crops (rice, *Miscanthus* [205], corn [145], popular) and their cell wall structures; (B) Primary and secondary cell walls; (C) Cellulose microfibrils and nanofibers.

manipulation of one or more genes involved in cell wall biosynthesis and assembly [8]. As plant cell walls are composed of numerous complex polymer networks with essential functions in the life cycle of plant, genetic modifications of plant cell walls can simply cause defects in plant growth, mechanical strength and biomass yield [9]. Because thousands of genes are required for plant cell wall biosynthesis and wall network construction, it is important to determine an optimal biotechnology that can address three crucial issues associated with the selection of bioenergy crops: (1) genetic manipulation is simple and practicable, (2) improvement of lignocellulose recalcitrance is sufficient for complete biomass enzymatic hydrolysis under cost-effective and green-like processing, and (3) lignocellulose modification has minor impacts on plant growth and mechanical strength, as well as biomass yield.

Enzymatic hydrolysis of lignocellulose has emerged as the most promising technology for converting biomass into fermentable sugars by using fungal-secreted cellulases. To increase cellulase productivity and enzymatic activity, advanced technology has been applied to engineer fungal strains, in particular *Trichoderma reesei* [10]. In addition, as xylose is the second largest quantity of monosaccharide released from the hydrolysates of lignocellulosic feedstocks, converting xylose to ethanol can be a major addition to final bioethanol production. Therefore, the metabolic engineering of *Saccharomyces cerevisiae* to ferment xylose along with improved robustness for salt and sugar tolerance is also important for high bioethanol production [11,12].

Lignin is the major component of residues remaining after yeast co-fermentation of both hexoses and xylose released from cellulose and hemicellulose hydrolyses; the enhancement of lignin value is achieved by performing a series of chemical processing steps to produce various by-products such as glues, adsorbents, bioplastics and carbon materials [13]. Meanwhile, the remaining neutral sugars of hemicellulose have been used as foods and medicine additives [14]. In addition to cellulose ethanol production, it has thus become noteworthy to obtain the high-value bioproducts derived from lignin and hemicellulose without any wastes released into the environment.

In this review, we initially described the innovative biotechnology such as recently-invented gene editing approach for selecting the desirable bioenergy crops with significantly reduced recalcitrance and slightly affected plant growth and biomass yield after being subjected to a precise modification of plant cell walls. This work then updated the progress regarding genetic engineering of fungal and yeast strains for consistently enhanced cellulases productivity and yeast fermentation capacity by using modified lignocelluloses such as size-reduced nanofibers as optimal inducing substrates. Furthermore, we presented potential advanced technologies to generate diverse bioproducts using all lignin-rich residues remained from the biomass process without any wastes release. Therefore, this study proposed a novel strategy that dynamically combines engineered bioenergy crops and yeast strains for green-like lignocellulose processes towards low-cost bioethanol production and high-value bioproducts.

2. Precise engineering of bioenergy crops

2.1. Bioenergy crops for diverse dedicated lignocellulose resource

Bioenergy crops can provide diverse lignocellulose resources for biofuels, biochemicals and biomaterials and include herbaceous grasses,

Table 1
Three major wall polymer composition and estimated bioethanol production of bioenergy plants.

Plant species	Cell wall composition [a]			Biomass yield (t/ha)	Bioethanol yield (% w/w)	Theoretical bioethanol yield (kg/ha) [b]	Ref
	Cellulose	Hemicelluloses	Lignin				
C3 plant (straw)							
Rice	25	19	15	5–9	15–18	1620	[20–22]
Wheat	31	31	22	5–9	9–10	900	[23,24]
Rapeseed	29	17	22	12–14	8–10	1400	[25–27]
C4 plant (straw)							
Maize	29	27	18	13–30	11–17	5100	[28–30]
Sweet sorghum	32	30	19	15–35	10–16	5600	[31,32]
Miscanthus	36	29	20	20–30	14–19	5700	[18,33,34]
Woody plant							
Poplar	42	22	18	7–11	8–12	1320	[35–37]
Pine	40	28	36	3–13	6–10	2300	[38,39]
Eucalyptus	42	20	29	10–20	7–11	2200	[40,41]

[a] Indicates average wall polymer level (% of dry weight, w/w) which was calculated using the data obtained from cited references.
[b] Theoretical bioethanol yield was calculated based on the biomass yield and bioethanol yield obtained from references. t as initial of ton; ha as hectare.

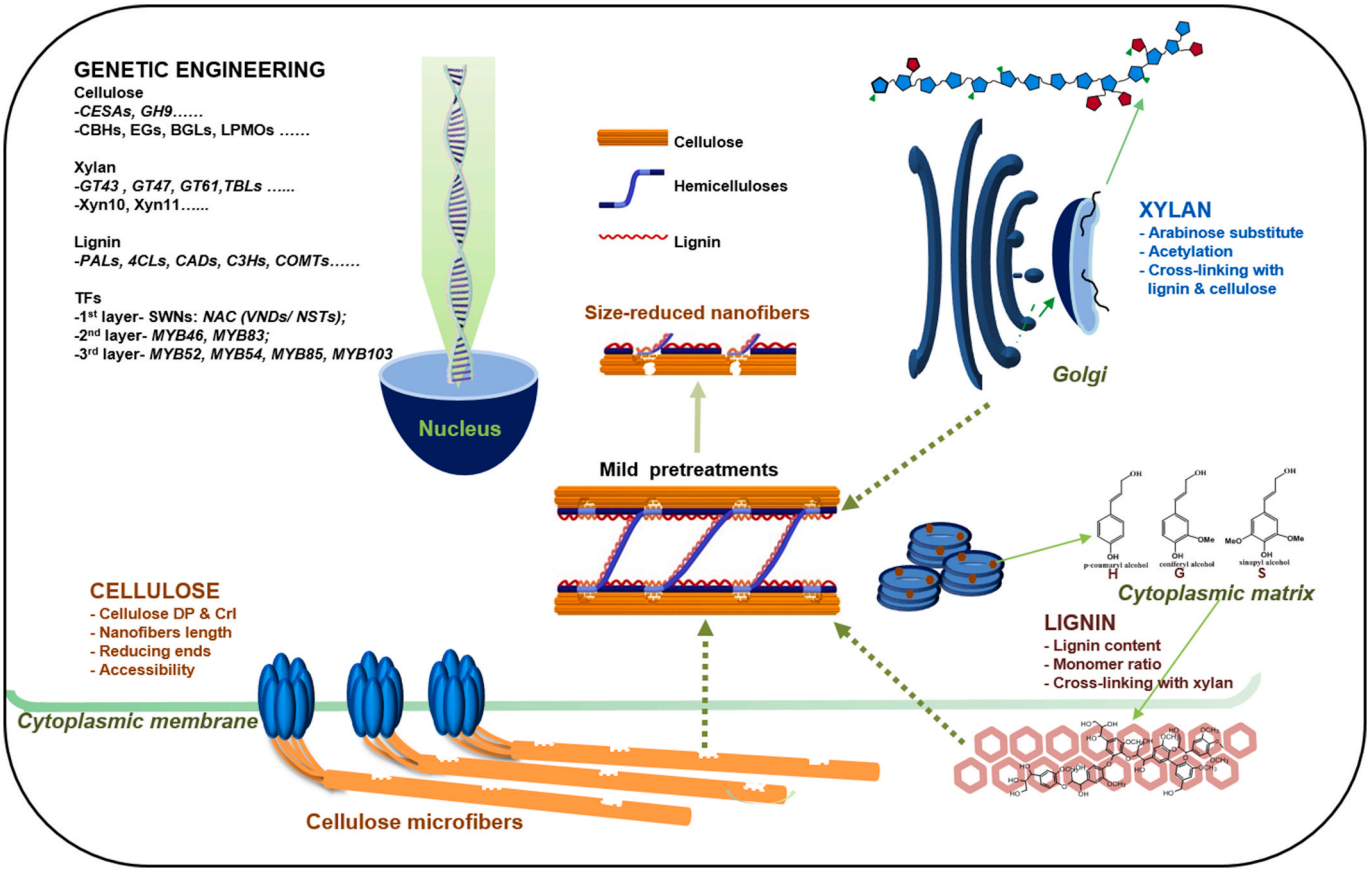

Fig. 2. Genetic modification of plant cell walls.

food crop straws and woody plants. Lignocellulose properties are largely determined by the architecture of vascular tissue systems (e.g., xylem and phloem), which provide mechanical support and lignocellulosic biomass. For instance, C4 grasses, such as maize, sweet sorghum and *Miscanthus,* typically have vascular bundles rich at lignocellulose, compared to C3 grasses (Fig. 1). As the major components of biomass, plant cell walls contain cellulose, hemicellulose, lignin and minor pectin (Table 1). Lignocellulose recalcitrance has been defined by determining major wall component levels, wall-polymer features and wall-network styles [15]. In most grass plants examined, the crystalline index (CrI) and degree of polymerization (DP) of cellulose are the key negative factors for lignocellulose recalcitrance [4], whereas the arabinose (Ara) substitution degree of hemicellulose is another factor positively affecting biomass saccharification [16]. As woody plants are rich in lignin, total lignin levels and three monolignol proportions mainly account for lignocellulose recalcitrance in woody plants [17]. More recently, biomass porosity and lignocellulose accessibility have been demonstrated as the determinants of lignocellulose saccharification in diverse biomass resources [18,19].

2.2. Precise modification of cellulose microfibrils for size-reduced nanofibers

Cellulose is composed of the β-1,4-glucans that assemble as microfibrils via hydrogen bonds and others [42]. Because cellulose microfibrils exhibit crystalline and insoluble properties to provide plant

Table 2
Genetic approaches for improving lignocellulolytic enzymes production in *T. reesei*.

Engineering strategies	Genes	Approaches	Effect on production	Ref
Artificial transcriptional activators	*ACE3*	Replacing the native *ACE3* with truncated *ACE3*	20–30% increase in FPase	[85]
	XYR1	Replacing the activation domain of Gal4 by Xyr1	50.5% increase in endoglucanases	[86]
	XYR1/ACE2/ ACE1	Constructed *XYR1VP, ACE2VP* and *ACE1VP* by linking C terminus with VP16	Improved cellulase and/or xylanase production	[87]
	CTF1	Deleting the repressor gene *CTF1*	36.9% increase in cellulase production	[88]
	XYR1/YPR1/ YPR2	Fusion Xyr1 and transactivation domain of either Ypr1 or Ypr2	Enhanced the cellulase and xylanase	[89]
	TRVIB-1	Overexpressed	200% and 219% increase in cellulase production and protein secretion	[90]
	ACE2/CRE1	Expressed DBD_{ace2}-*VP16*/DBD_{cre1}-*VP16*	Improved cellulase production	[91]
	CEL3A/SUC1	Heterologous expressed *CEL3A/UC1* in *ace1* mutants	Increased cellulase by 71-fold and hemicellulose by 42-fold	[92]
	ACE2/XYR1	Designing new *cbh1* promoters by changing the binding sites of *ACE1* to those of *ACE2* and *Xyr1*	Improved the promoter transcription efficiency	[93]
Gene manipulations	*VP1*	Heterologous expressed	Significant improvement in the yield of total reducing sugar and delignification	[94]
	CRE1	Knock out the homologous genes in hyper-cellulolytic mutant	Significantly increased in cellulase activity	[95]
	EGL1	Overexpressed *egl1* at *ace1* locus	90% and 132.7% increase in the activities of total cellulases and endoglucanases	[96]
	BGL3I	Deletion of the *bgl3i*	Significantly increased cellulase activities	[97]
	BGL1	Heterologous expressed *bgl1* under *xyn3* promoter	Increased cellobiase activity	[98]
	CBHI	Heterologous expressed *cbh1*	Increased the cellobiohydrolase activities by 4.1-fold	[99]
	AABG1/CBHI	Heterologous expressed *aabg1* under *cbh1*or *egl1* promotors	20–30% increase in saccharification	[100]
	BG/CBHII	Co-expressed *bg* and *cbhII*	Improved filter paper activities	[101]
	Cel7A	Heterologous expressed *Te-Cel7A*	28.8% enhancement in cellobiohydrolase activity, 65.2% increase in filter paper activity	[102]
	TRE	Deletion of target proteases by one-step genetic transformation	78% decrease in protease activity, 6-fold increase in cellulase activity	[103]
Coenzymes/cofactors	*LPMO*	Expressed *Trcel61A (LPMO)* in *T.reesei*	75% increase in saccharification	[104]
	LPMO	Pretreated samples with LPMO	Released of C1-oxidized oligosaccharides	[105]
	LPMO	Heterologous expressed *AA9 LPMO* from *Thermoascus aurantiacus* in *T.reesei*	More efficient in the degradation of Avicel and delignified corncob residue	[106]
	SWO1	Overexpressed *Swo1* in *T.reesei*	Increased endoglucanases and cellobiohydrolases activities	[107]
	SWO1	Overexpressed *Swo1* in *xyr1* mutant	Boosted slightly the reducing sugar release from a native grass substrate	[108]
	SWO1	Heterologous expression *AfSwo1* in *T.reesei*	Increased facilitation saccharification of Avicel	[109]

mechanical strength, genetic engineering of cellulose biosynthesis could simply affect plant growth and biomass yield [43]. However, site-specific mutations of cellulose synthase (CESAs) genes have generated significantly enhanced biomass yields by 25%–41% and have improved the plant lodging resistance at significant level in a natural mutant, *Osfc16*, which is an integrated parameter accounting for plant growth and strength [20], thus indicating an applicable and powerful approach for cellulose modifications (Fig. 2). Notably, the *Osfc16* mutant exhibits significantly reduced cellulose features (e.g., CrI and DP) and increased cellulose accessibility for considerably size-lessened cellulose nanofibers under mild chemical pretreatments, thus leading to remarkably enhanced biomass enzymatic saccharification by up to 2.3-fold and ethanol productivity by 34%–42%. Notably, our preliminary results have shown that CRISPR/cas9-mutated plants (e.g., *OsCesA4, 7, 9)* are of normal plant growth phenotypes, shorter cellulose nanofibers and higher biomass enzymatic saccharification, which are similar to the *Osfc16* mutant (data not shown). Those findings demonstrate that the recently-established gene editing technology should be practicable for precise modification of cellulose microfibrils in bioenergy crops.

Meanwhile, overproduction of the hydrolytic enzymes derived from fungi and rice has caused significantly-decreased cellulose DP and CrI values, as well as size-lessened nanofibers for remarkably raising biomass saccharification of engineered rice straws and it has also enhanced biomass yields [21,22], which confirm that specific size-reduction of cellulose nanofibers should be a novel strategy that is optimal for genetic engineering of bioenergy crops. In addition, because the lytic polysaccharide monooxygenases (LPMOs) are specific to crystalline cellulose digestion [44], overproduction of LPMOs could be another option or a plus to generate optimal cellulose nanofibers in bioenergy crops.

2.3. Specific increase of hemicellulosic Ara and pectin's uronic acids

As the major non-cellulosic polysaccharides with diverse monosaccharides composition, hemicelluloses exhibit remarkable variability in different types of plant cell walls. Recent progress suggests that genetic modification of hemicellulose can be targeted to improve biomass processing (Fig. 2) [45]. In general, xylans are the major hemicelluloses in the lignified secondary cell walls of woody plants or in both primary and secondary cell walls of grass plants including *O*-acetylglucuronoxylan, arabinoglucuronoxylan, *O*-acetylglucuronoarabinoxylan, and O-acetylarabinoxylan [46]. In particular, the total Ara level and its substitution degree of xylans could positively affect lignocellulose hydrolysis upon physical and chemical pretreatments in diverse biomass resources examined [16]. The interpretation of these results is that the branched Ara may have interactions with the non-crystalline cellulose microfibrils and cause significantly decreased nanofibers lengths/sizes, which are accountable by relatively reduced cellulose CrI and DP values [4,16]. Hence, overexpression of *XATs (GT61)* genes may largely enhance Ara substitution degrees for high biomass enzymatic saccharification in transgenic bioenergy crops. Meanwhile, acetylation modification of xylans plays an important role in wall polymer interactions, but transgenic plants are defective in plant growth and biomass yields [47]. In addition, although pectin occurs at small amount in plant cell walls of mature crop straws, the uronic acids (mainly from pectin) positively affect lignocellulose enzymatic digestibility by decreasing cellulose CrI [48]. Therefore, potential increases of pectin levels should be another engineering approach to simply reduce lignocellulose recalcitrance in bioenergy crops.

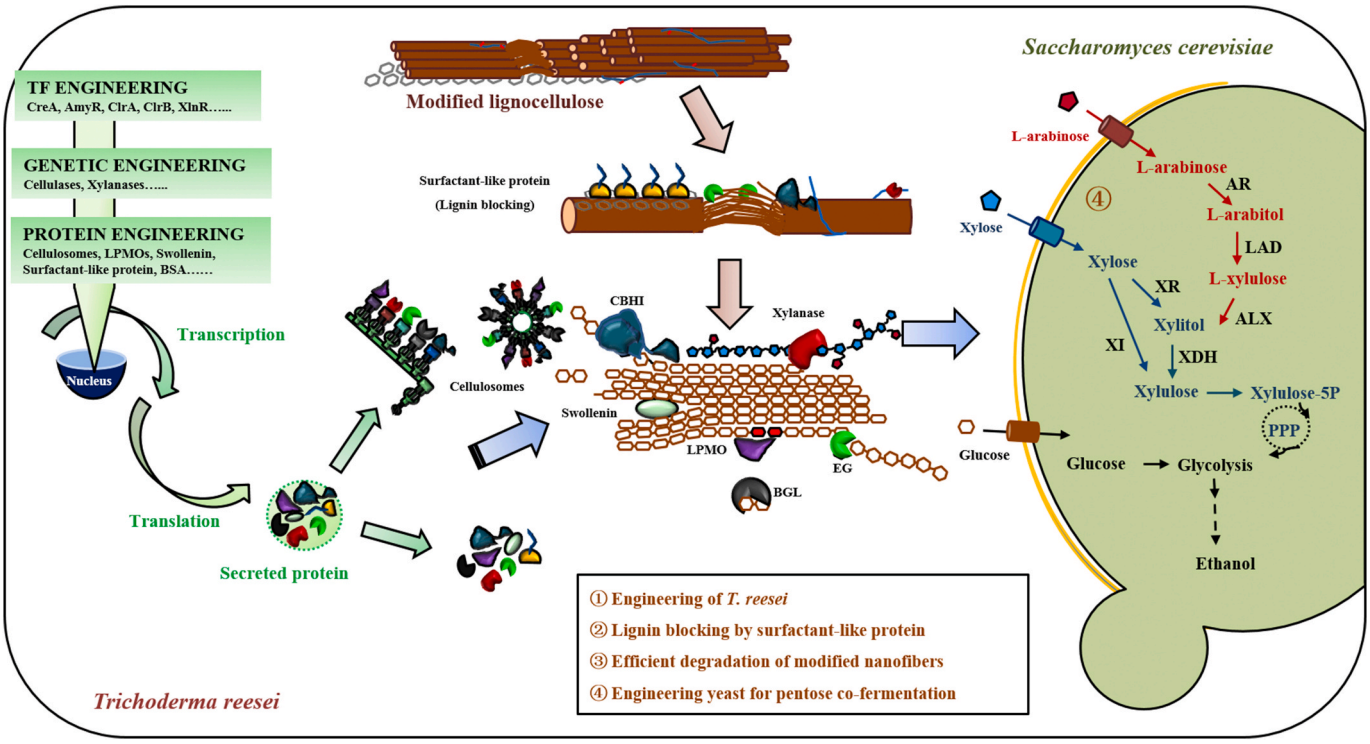

Fig. 3. Engineering of *T. reesei* for high cellulases and xylanase production and of *S. cerevisiae* for pentoses and hexoses co-fermentation.

2.4. Mild disruption of lignin-polysaccharide interlinkages

Lignin is the most complicated heteropolymer containing *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers and is mainly deposited in the secondary cell walls of vascular plants. As lignin is strongly associated with hemicelluloses to form a solid barrier against cellulase enzyme accession, its deposition significantly contributes to lignocellulose recalcitrance in bioenergy crops [49–51]. On the other hand, as lignin deposition is essential to maintain plant cell integrity and mechanical strength, genetic engineering of lignin biosynthesis could simply affect plant growth and biomass yield [52]. However, based on the gene editing technology as described above, site-mutations of lignin biosynthesis genes (such as *PALs,* 4CLs*, CADs, C3Hs,* and *COMTs*) may slightly reduce lignin levels but could effectively disrupt wall polymer networks to expose accessible space for enzyme loading after mild biomass pretreatments [53,54]. This network disruption may help to expose cellulose nanofibers, which should be one of the major causes for efficient lignocellulose enzymatic saccharification (Fig. 2).

2.5. Multiple regulation of plant cell wall biosynthesis

The regulation networks of transcription factors (TFs) have been characterized for secondary cell wall biosynthesis in plants [55,56]. For instance, SWNs as a subgroup of NAC TFs, serve as master switches and directly regulate downstream TFs. SWNs are composed of VNDs and NSTs in the first regulatory layer and are specifically expressed in vessels and fibers. Particularly, SWNs are functionally conserved across nearly all bioenergy crop species including maize, rice, *Miscanthus*, switchgrass, cotton, sorghum and poplar [57–63]. Recently, genetic manipulations of SWNs have been implemented for the specific regulation of secondary cell wall biosynthesis by using heterologous expressions of the rice *OsSWN1* gene in *Populus* or by generating synthetic novel transcription factors and promoter panels in transgenic plants [64].

Furthermore, *MYB46* and *MYB83* TFs work as master switches in the second layer and they are directly regulated by *VNDs* (*VND6/VND7*) and *NSTs* (*NST1/NST3*). The *MYBs* are specifically expressed in fibres and vessels and their major targets are the secondary cell wall-related genes and other downstream genes or TFs for secondary cell wall thickening [65]. As downstream TFs of *MYB46/MYB83*, the TFs *MYB52, MYB54, MYB85 and MYB103* function as the third layer involved in secondary cell wall biosynthesis and assembly [66]. Hence, slightly engineering downstream TFs has been examined to significantly reduce lignocellulose recalcitrance in several bioenergy crops [67–69]. In addition, genetic manipulation of the genes associated with primary cell wall synthesis and UDP-Glc bioconversion has enhanced secondary cell wall deposition for high biomass yields in transgenic crops [70–74]. However, this study suggests that the gene editing of TFs should be more precise for regulating plant cell wall biosynthesis and wall network construction, thus providing multiple options to select the desirable bioenergy crops that are of specifically modified lignocellulose and size-reduced cellulose nanofibers (Fig. 2).

3. Subsequent engineering of fungal strains for optimal mixed-cellulases production and bioethanol fermentation

After initial physical and chemical pretreatments, the sequential enzymatic hydrolysis of biomass residues is an essential step to release soluble sugars for ethanol fermentation and other biofuel production. Genetic engineering of fungal strains has been implemented for the highly-efficient and cost-effective lignocellulolytic enzymes systems (Table 2), which is required for the effective utilization of biomass materials [75]. The high-performance lignocellulolytic enzymes systems typically consist of active cellulases, xylanases, coenzymes and lignin-modifying enzymes that act synergistically (Fig. 3). Three major types of lignocellulolytic enzymes include exo-1, 4-β-D-glucanases (CBHs), endo-1, 4-β-D-glucanases (EGs) and 1, 4-β-D-glucosidases (BGLs), which are required to hydrolyse cellulose into glucose monomers, as well as endo-1, 4-β-D-xylanases and 1, 4-β-D-xylosidases are the core enzymes specific for xylan degradation [76]. Moreover, LPMOs, which cleavage the glycosidic bonds in cellulose and other polysaccharides using an oxidative mechanism [77], and swollenins which are capable of breakdown of the hydrogen bonds among the wall

Table 3
Genetic manipulations for improving xylose and hexoses co-fermenting in *S. cerevisiae*.

Engineering Strategies	Genes	Approaches	Effect on production	Ref
Construction of xylose-assimilating pathways	*XR/XDH/XK*	Overexpressed	Consumed D-xylose, produced glutathione	[113]
	XYL1	Heterologous expressed	Revealed significant higher ethanol yield	[114]
	XR	Overexpressed	Fast xylose consumption	[115]
	XR	Overexpressed	Increased in xylose consumption	[116]
	TAL1/TKL1	Overexpressed PPP genes in *xi* mutants	Increased the xylose consumption rate	[117]
	XI	Heterologous Co-expressed *XI*	Result in 0.44 g ethanol/g xylose	[118]
	XI/XR-XDH	Introduced *XI/XR-XDH* into L-arabinose cofermenting strain	Increased ethanol yield by 30%	[119]
	XI/XR-XDH	Introduced *XI/XR-XDH* into robust industrial strains	Improved xylose consumption	[120]
	XI/ARA	Assembly of *XIs* and *araA/araB/araD*	Resulted in 1.38 g/g DW/h ethanol production	[121]
	LpXI	Expressed randomly mutagenized *LpXI* in *IR-2* strain	Produce 53.3 g/L ethanol by nearly 85 g/L D-glucose and 35 g/L D-xylose	[122]
	RPE1/ASC1	Expressed pentose phosphate pathway	Simultaneous co-fermentation	[123]
	SZ2/Gal2	Constructed artificial complex between endogenous sugar transporter and heterologous xylose isomerase	Increased in xylose conversion and ethanol production	[124]
	Hxk2p	Expressed nuclear-localized hexokinase2	Increased the xylose consumption rate and ethanol yield	[125]
	CiGXS1	Mutagenesis and selection based on *FIM* mutant strain	Substantially improved xylose transport rates	[126]
	BsAraE	Heterologous expressed *BsAraE* with full *hxt*	Increased in xylose consumption and xylitol production	[127]
Enhancing microbial tolerance to inhibitors	*RCK1*	Overexpressed	Improved resistance to acetic acid stress	[128]
	YCR102C	Overexpressed	Improved resistance to 40 °C and 3.6 g/L acetic acid stress	[129]
	QDR3	Adaptive laboratory evolution	Improved resistance to dicarboxylic acids, muconic acid and glutaconic acid	[130]
	TPS1/ARI1	Co-overexpressed *tps1* and *ari1* in *nth1* deletion mutant	Improved ethanol tolerance up to 14% of ethanol	[131]
	YlILT1	Overexpressed	Improved growth rate and ethanol production	[132]
	YHB1/SET5	Overexpressed	Improved resistance to osmotic and heat stress	[133]
	SNF1	Overexpressed	Improved resistance to high glucose, ethanol, and heat stresses	[134]
	CDS1/CHO1	Overexpressed	Improved resistance to salt stress	[135]
	GRE2	Expressed *Gre2p* with native promotor	Improved resistance to the toxic stress	[136]

polysaccharides without hydrolysis, could act synergistically with lignocellulolytic enzymes for complete lignocellulose degradation [78]. In addition, lignin-blocking proteins such as soybean proteins, bull serum albumin (BSA) and surfactant-like proteins have been applied to block the non-productive binding of cellulase enzymes with lignin [79]. Importantly, for individual biomass-degrading enzymes, cellulosomes are extracellular supramolecular multi-enzyme complexes that are produced by anaerobic cellulolytic bacteria [80]. Cellulosomes can efficiently degrade cellulose and hemicelluloses by improving substrate uptake, tightening specific interactions with certain substrates and enhancing the synergistic activity and processivity of cellulases [81]. To date, several enzymes and proteins have been utilised for plant biomass degradation.

Recent advances have allowed exploitation of various synthetic biology approaches for constructing metabolic pathways and have produced a series of value-added bioproducts from lignocellulose residues [82–84]. To date, various genetic manipulations have been improved for fungal strains (Table 2) such as the construction of artificial transcription factors, specific enhancement of the catalytic activity of cellulase enzymes and enzyme production, expression of coenzymes/cofactors (e.g., LPMO, lignin-blocking protein, and swollenins), and reconstruction of cellulosome complexes (Fig. 3). However, in terms of the modified lignocellulose substrates with size-lessened nanofibers after mild chemical pretreatments, the optimal proportion of cellulases and xylanase production for complete biomass enzymatic saccharification by using engineered fungal strains to directly incubate with the optimal nanofibers substrates remains to be determined, which may be the direction to select next generation of the optimal cellulases cocktails enabled for complete enzymatic degradation of diverse lignocellulose substrates/residues at low cost and high efficiency.

Provided that a breakthrough efficient sugar release could be achieved by integrating the modified lignocellulose for size-reduced nanofibers substrates and the engineered fungal strains for optimal lignocellulolytic enzyme systems, the final conversion of all released sugars into bioethanol is limited by the common yeast strains that could not use xylose as a carbon source for ethanol fermentation [110]. In principle, xylose assimilation requires the isomerization of xylose into xylulose by using two distinct pathways, namely the oxidoreductase pathway and the isomerase pathway. Xylulose is then channelled into the glycolytic pathway through innate xylulokinase and the pentose phosphate pathway [110]. A variety of phosphorylated sugars such as GA-3-p, ribulose-5-p, and fructose-6-p, which are derived from the pentose phosphate pathway, serve as intermediates of glycolysis, which is the main route of ethanol fermentation [111]. Recently, recombinant *S. cerevisiae* strains engineered with the heterologous pentose assimilation pathway have shown efficient ethanol fermentation abilities from pure pentose or lignocellulosic hydrolysates and the metabolic engineering for efficient xylose utilization has been achieved [112]. Meanwhile, other biotechnologies have been attempted to enhance xylose utilization by introducing high-affinity xylose transporters or by abolishing metabolic restrictions to boost xylose utilization and enhance the

Table 4
Green-like biomass process.

Pretreatments	Species	Reaction conditions	Effect on biomass	Glucose yield (% cellulose)	Ref
Liquid hot water	Sweet sorghum	190 °C, 20 min	Remove lignin and hemicellulose	86	[142]
	Poplar	180 °C, 18–70 min		39–70	[37]
	Beech wood	190–220 °C, 15 min.		67	[143]
	Hazelnut tree	190–210 °C, 45 min		55–60	[144]
	Corn straw	200 °C, 20min		95	[145]
Ionic liquid	Ashless filter paper	[AMIM][Cl]; [EMIM][Cl]	Decrease cellulosic crystallinity	~100	[146]
	Rice straw	Microwave-[BMIM]Cl		61	[147]
	Rice straw	$[C_2MIM]Cl–K_2CO_3$		86	[148]
	Corn cob	[EMIM][OAc]/ethanol (water)		80	[149]
	Mustard stalk	$[C_2MIM][OAc]$		98	[150]
Steam explosion	Hybrid pennisetum	275 °C, 2.5 MPa, 10 min	Reduce cellulosic crystallinity and extract hemicelluloses	68	[151]
	Hippophae rhamnoides	162–240 °C, 12–30 min		27–30	[152]
	Rice straw	1.2 MPa, 3 min		63	[153]
	Corn straw	1.0 MPa, 30 min		100	[154]
	Miscanthus	2.5 MPa, 180 s		17–40	[33]
	Corn stover	1.5 MPa, 9 min		86	[155]
	Wheat straw	225 °C,2.5 MPa, 3 min		44–52	[24]
Mild chemical incubation	Rice straw	1% NaOH at 50 °C for 2 h	Remove lignin and hemicellulose	70	[22]
	Rice straw	0.5% NaOH at 50 °C for 2 h	Remove lignin and hemicellulose	50	[21]
	Corn Straw	15% CaO, 2 h	Remove lignin and hemicellulose	100	[145]
	Wheat straw	PHP ($H_3PO_4 + H_2O_2$)	Remove lignin	100	[156]
	Wheat straw	10% H_2O_2	Remove lignin	99	[157]
	Sweet sorghum straw	10% H_2O_2	Remove lignin	66	[158]
	Tobacco stalk	Ball-milling and dilute alkali cooking	Remove lignin and hemicellulose	94	[159]
	Corncob	Magnetic carbon-based solid acid catalyst	Remove hemicellulose	90	[160]
Ammonia-based	Sugarcane bagasse	25% NH_4OH, 180 °C for 1.78 h	Remove 71% lignin	45	[161]
	Miscanthus	0.1 ammonia, 120 °C for 12 h	Remove 84% xylan	95	[162]
White rot fungi	Corn stover	75–85% H_2O, several white rot fungi	Degrade lignin	67	[163]
	Corn cob	Mixed with food waste	Decrease cellulose crystallinity	83	[164]

co-fermentation capability of glucose and xylose (Table 3). In addition, recent advancement of either salt/sugar-tolerance-capacity engineering or evolutionary engineering with yeast strains should provide another platform to obtain the desirable host strains for efficient bioethanol fermentation (Table 3). Hence, this study has proposed a novel strategy that dynamically connects the modified lignocellulose for efficient soluble sugars releasing and the engineered fungal strains for optimal cellulases cocktails and effective co-fermentation of all released hexoses and xylose, along with a cost-effective and green-like biomass processing (Fig. 3).

4. Green-like biomass process technology

For the initial biomass process, various physical and chemical pretreatments have been conducted with diverse lignocellulose residues by effectively extracting the major wall polymers and by specifically improving lignocellulose features, which lead to significantly reduced lignocellulose recalcitrance and increased cellulose accessibility for the pretreated biomass residues [137–140]. Due to lignocellulose recalcitrance, however, nearly all pretreatments require extreme conditions such as high temperatures, high pressures and long incubation times and in particular, chemical pretreatments are conducted at high chemical concentrations, which can cause either formation of toxic compounds that inhibit yeast fermentation or the release of secondary wastes. Alternatively, environment-friendly pretreatments have been explored by using liquid hot water, ionic liquids, steam explosion, CaO, and fungi (Table 4), but most pretreatments have limitations to some degree in reducing lignocellulose recalcitrance or enhancing enzymatic

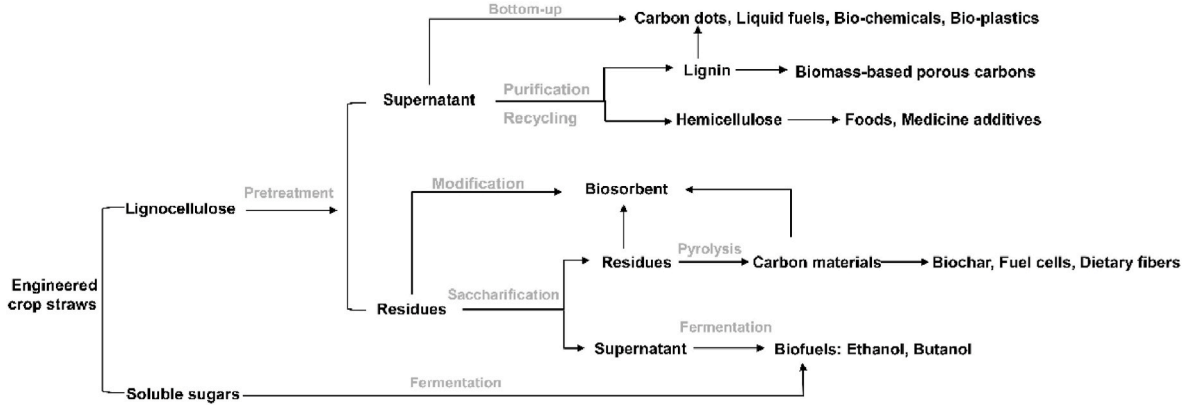

Fig. 4. Flowchart of byproducts generated using the lignin-rich residues from biomass pretreatment, sequential enzymatic hydrolysis and final yeast fermentation in engineered bioenergy crops.

Table 5
Lignin-based bioproducts following the biorefinery process.

Bioproducts	Carbon sources	Modifications	Characterization/Application	Ref
Porous carbon	Fermentation residues	Hydrothermal carbonization, KOH, 800 °C	High specific capacitance of 312 F g^{-1}, 81% retention at 80 A g^{-1} in the aqueous electrolyte.	[176]
Porous carbon nanoflakes	Enzymatic hydrolysis residues	$ZnCl_2$, $FeCl_2$, 900 °C	Surface area 2026 m^2 g^{-1}, mesopore ratio 86%, electric conductivity 5.4 S cm^{-1}	[177]
Porous carbon	Fermentation residues	N_2, KOH, microwave heating	Mesopore ratio: 65.8%, surface oxygen content: 16.5%, energy density 23.0 kW kg^{-1} at 10 A g^{-1}, 71.1% retention at 10 A g^{-1}	[178]
Porous carbon	Rice husk	PVA, KOH, 750 °C	263.4 mg g^{-1} of sorption capacity	[179]
Porous carbon	Fermentation residues	N_2, KOH, microwave heating,	Highest specific capacitance of 254.6 F g^{-1} at 0.5 A g^{-1}, 75.6% retention at 10 A g^{-1}.	[180]
Hydrogel	Alkaline lignin	Mixed with chitosan solution	Biocompatible	[181]
Hydrogel	Enzymatic hydrolysis residues	PVA, 75 °C	Adsorption: rhodamine 6G 196 mg g^{-1}, crystal violet169 mg g^{-1}, methylene blue dyes 179 mg g^{-1}	[182]
Hydrogel	Sulphuric acid soluble lignin	Mixed with NaOH/NH_4OH and PMVE/MA	High methylene blue removal efficiency, which ranged from 12% to 96%	[183]
Nanoparticles	Enzymatic hydrolysis residues	Dissolved in tetrahydrofuran	Contributed to the cellular uptake and the accumulation of the drug within HeLa cells	[184]
Nanofiber membrane	Alkaline lignin	PVA, pyrolysis at 250 °C, carbonized at 800 °C	Improved spinning stability and fiber quality, High-performance absorbent for water purification	[185]
3D prints	Organosolv isolated lignin	Acylation with methacrylic anhydride	Increased ductility and high-resolution prints	[186]
3D prints	Softwood kraft lignin	Dispersed into the methacrylate resin	Increased the tensile strength by 46–64% and Young's modulus by 13–37%	[187]
Nanocomposites	Lignin from pulp	TiO_2, Sol gel	Thermally stable up to 300 °C, well-defined edges	[188]
N-L-CQDs	Alkali lignin	Mixed with betaine and lactic acid, carbonization at 300 °C	Quantum yield of 7.95%, exhibited excellent optoelectronic properties, excitation-dependent and pH stability	[189]
L-CQDs	Alkali lignin	Mixed with different molar ratios of citric acid and ethylenediamine	Showed low cytotoxicity and good cellular biocompatibility	[190]
L-CDs	Fermentation residues	Ethanol solution, dialysis	Showed good cellular biocompatibility	[191]
Carbon fibers	Alkali lignin	PVA, KOH, 200 °C	Adsorption: toluene 169.41 mg g^{-1}, methanol 133.06 mg $g^{-1}$and acetone105.48 mg g^{-1}	[192]
Carbon fibers	Alkali lignin	PVA, KOH, 200 °C	Higher specific surface area (1147.16 m^2 g^{-1}) and greater toluene adsorption (463 mg g^{-1})	[193]

PVA, Polymer polyvinyl alcohol. PMVE/MA, Methyvinyl ether co-maleic acid. L-CQD, Lignin carbon quantum dots. L-CD, Lignin carbon dots. N-L-CQD, Nitrogen-doped lignin/des carbon quantum dots.

saccharification [141]. However, progress has been made by performing green-like or mild chemical pretreatments with the modified lignocellulose residues in the genetic mutants and in the transgenic crops to produce the size-lessened cellulose nanofibers, thereby resulting in complete lignocellulose enzymatic hydrolysis for obtaining the highest bioethanol yields in the history [21,22,145,165]. It has also been interpreted that the green-like pretreatments performed are sufficient to further address lignocellulose recalcitrance by reducing cellulose features (e.g., DP or CrI) and by increasing lignocellulose accessibility [24,33,166,167]. Furthermore, the modified lignocellulose samples have shown significantly improved sugar-ethanol conversion rates due to the formation of less-toxic compounds from the green-like pretreatments performed [21]. In addition, chemical surfactants or non-chemical plant proteins at low dosages could be added into the enzymatic hydrolysis for largely enhanced biomass saccharification by their effective blocking with the lignin of biomass substrates, particularly for the modified lignocellulose residues [168–171]. Based on the modern systems biology and synthetic biology, therefore, combining engineered bioenergy crops with engineered fungal strains should be considered as the next generation of integrated biotechnology for complete biomass saccharification to achieve maximum bioethanol yield under cost-effective and green-like processing.

5. Complete biomass processing for diverse value-added bioproducts

Although lignocellulose residues from engineered bioenergy crops can be completely degraded for bioethanol production under green-like biomass process as described above, large amounts of lignin-rich residues could be used to generate value-added bioproducts (Fig. 4). Hence, the effective enhancement of lignin value is another crucial issue for pursuing more sustainable and competitive biorefineries [172,173]. Over the past years, various lignin-based bioproducts have been examined by following the biorefinery process for bioethanol production, which depends on lignocellulose fractionation, lignin depolymerisation and upgrading for desirable chemicals [13,174]. There are three lignin resources within a biorefinery: initial pretreatment hydrolysates, sequential enzymatic hydrolysates and final yeast fermentation residues [175]. The potential high-value products derived from lignin include carbon dots, biochar, biosorbents, biomass-based porous carbons, carbon nanofibers, bioplastics, thermoplastic elastomers, polymeric foams, and nanomaterials (Table 5). The variations of lignin structure, polydispersity and chemical reactivity can be further exploited to produce specialized biochemicals and biomaterials with extremely high value [194,195]. In addition, despite that the major monosaccharide (xylose) from hemicellulose can be converted for bioethanol using engineered yeast strains, the remaining Ara and other sugars can be used for food, medicine and the chemical industries [196–198]. This work has thus considered various advanced technologies for potential utilizations of all lignocellulose components to produce diverse high-value bioproducts without any wastes release.

6. Practical biotechnology implications and challenges

To find out the practical biotechnologies, this study presents the most approaches that have been recently applied in biomass process for biofuels and bioproducts (Table 6). Although various engineering approaches are implicated for genetic modifications of three major wall polymers in genetic-model plants and bioenergy crops [4,199], it remains to test if those modifications could consequently affect plant growth, mechanical strength and biomass yield in the most samples examined. However, this study has indicated that the gene editing for site-specific mutations of cellulose synthase genes could not only avoid plant growth defects in rice, but also enhance biomass production and

Table 6
Overview of biotechnological approaches in lignocellulosic-bioethanol conversion processes [4, 7, 8, 74, 83, 84, 110, 140, 203, 204].

Objective	Strategies	Classical methods	Advantages	Disadvantages
Reducing recalcitrance of biomass substrates	●Genetic modification of plant cell wall			
	Mutagenesis and bioenergy crops selection	T-DNA insertion EMS mutagenesis	Simple principle Low cost	Randomness Less chances of getting desired phenotype
	Precise engineering of plant cell wall	Regulation of transcription factors Knock out/down the synthesis genes Overexpression of degradation enzymes	Purposeful Efficiently reducing recalcitrance	In the laboratory stage High cost
	●Pretreatments			
	Physical	Ball milling Microwave	Easy handing Low fermentation inhibitors	High energy input Some require high cost
	Chemical	Acid Alkali Organosolv Ionic liquids	Low temperature and pressure Easier operation	Long reaction time Some cause secondary pollution
	Physical-Chemical	Steam-explosion Liquid hot water Wet oxidation	Improved enzymatic hydrolysis Little inhibitors generation	High energy input, Some cause secondary pollution
	Biological	White rot treatment Bacterial treatment Enzymatic treatment	Low temperature costs Less or no wastes generation No environment pollution	Long pretreatment time Poor stability of ligninolytic enzymes in industrial process
Enhancement of biomass digestibility	**●Genetic engineering of *T. reesei***			
	Increased in lignocellulolytic enzymes production	Transcription factor engineering, Secretory pathway modification Sit directed mutagenesis Construction of cellulosomes	Efficiently enhancing cellulase productions	Enzymes need to be evaluated in practical contexts
	Engineering the performance of enzyme mixtures	Supplementation of exogenous components De novo reconstitution of enzyme mixtures	Improved cellulase thermostability activity and other properties	
	Mutagenesis and evolution	UV Chemical mutagenesis	Generate mutant strains library No need structural information but bases on natural selection Have been used at industrial level	Reduced growth rate Sporulation defects or genomic instability Require multiple generation cycles
	●Co-enzymes/Additives	*LPMOs* Swollenins Surfactant: Tween 80, PEG Protein: BSA, Soybean protein	Increased of non-crystal cellulose Lignin-blocking Decreased non-specific adsorption of cellulases	Should work with cellulose mix High cost
Enhancement of lignocellulose-derived sugar conversion	**●Genetic engineering of *S. cerevisiae***			
	Construction of xylose-assimilating pathways	Channeling xylose source to cell metabolism Introducing high-affinity xylose transporters	Improved xylose-fermenting capabilities	Unstable genetic inheritance Need to be evaluated in practical contexts
	Enhancing microbial tolerance to inhibitors	Screening target genes for stress tolerance Genetic and metabolic engineering for improving tolerance	Beneficial to industrial scale	May bring extra burden or expense
	Evolutionary engineering	Classical mating Random mutagenesis UV-induced mutations Stress- induced mutation	Increased various industrial stress tolerances Enhanced leavening ability Most widely used and very cost-effective	Randomness Require multiple generation cycles Less chances of getting desired phenotype

enzymatic saccharification as described above. This may be due to the slight and precise modification of cellulose microfibrils in rice straw with a typical silicon accumulation, and increased silicon deposition should compensate its mechanical defect to some degree in the rice mutants [200,201]. Hence, it is important to examine whether the site-specific mutation is practicable for other cellulose-high C4 grassy crops and lignin-rich woody plants in the future. Even though it remains challenging to maintain plant strength and biomass yield in the site-mutated bioenergy crops, overproduction of Ara-rich hemicellulose or uronic acids-rich pectin or small molecular of lignin into the mutants may be a compensation solution.

Over the past years, engineered fungi strains have been selected to produce distinct cellulases cocktails (Table 6), but it remains difficult to obtain the mixed-cellulases that are of high enzymatic activities for consistently efficient hydrolyses of distinct lignocellulose substrates in all major bioenergy crops. This study has thus proposed to use the size-reduced cellulose nanofibers of engineered bioenergy crops as inducing substrate with fungi, which may secret optimal cellulases cocktails enabled for complete enzymatic hydrolysis of all distinct lignocellulose residues [202]. Once the optimal cellulases cocktails are achieved, it should provide insights into further engineering of fungi strains using synthetic biology approach.

For biofuel production at large scale, it has been defined to find out a cost-effective and green-like biomass process technology. Despite that physical and chemical pretreatments have been previously conducted with distinct lignocellulose residues of bioenergy crops, the most technologies require extreme incubation conditions for enhancing sequential enzymatic saccharification, leading to an unacceptable costly biomass

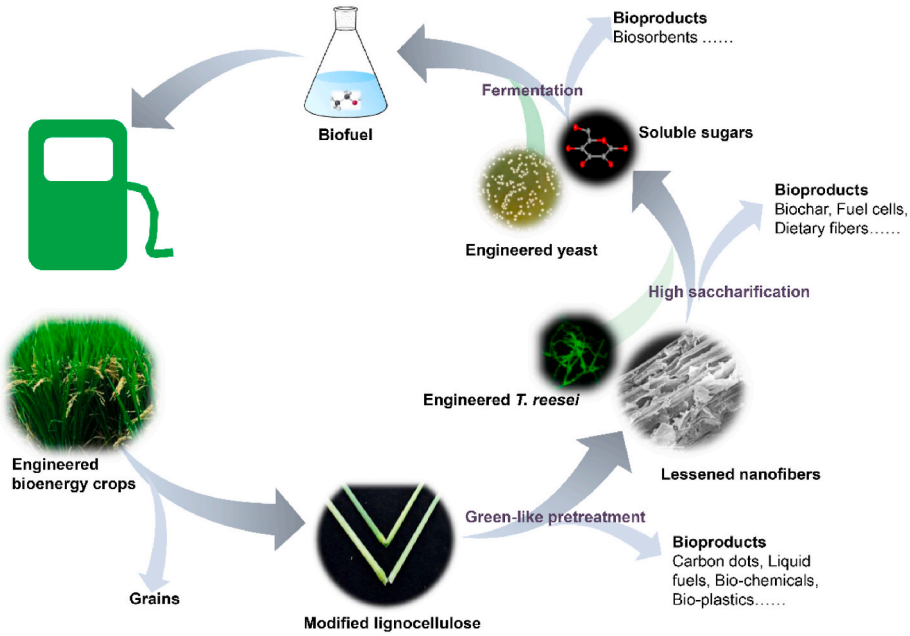

Fig. 5. A strategy integrating engineered bioenergy crops and fungi strains with the green-like biomass process for bioethanol production and diverse biochemicals and biomaterials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

processing with potential secondary wastes formation (Table 6). However, our recent studies have indicated that mild and green-like chemical pretreatments are sufficient for complete biomass saccharification to maximize bioethanol yields in the engineered bioenergy crops, even though only crude mixed-cellulases are added into the enzymatic hydrolyses [20–22,24,145,165]. Using recalcitrance-reduced cellulose nanofibers of the site-mutated bioenergy crops and their inducing optimal cellulases cocktail, this study predicts that much mild biomass processing should be practicable for cost-effective bioethanol production at large scale. In addition, provided that the remaining lignin-rich residues can be applied to generate value-added bioproducts for non-waste release, its total biomass quantity is limited for large scale productivity. Hence, this study also suggest that the optimal cellulose nanofibers of engineered bioenergy crops could be directly applied to generate the desirable carbon materials and graphene-like substances applicable for fuel cells and other extremely-high-value products, which may be a great plus of benefit for the biofuel production in the industry scale.

7. Conclusion

Based on the recently achieved progress described above, this study proposes a novel strategy that dynamically combines engineered bioenergy crops and engineered fungal strains with cost-effective and green-like processes for complete biomass enzymatic saccharification towards maximum bioethanol production along with diverse value-added bioproducts generated from remaining lignin-rich residues (Figure 5). (1) Precise lignocellulose modification not only maintains plant normal growth and biomass yields but also greatly enhances lignocellulose saccharification to maximize bioethanol yields in engineered bioenergy crops. (2) The size-decreased cellulose nanofibers of engineered bioenergy crops should be optimal for fungal inducing to secrete very large quantities of cellulases and xylanases at high enzyme activity. (3) Engineered yeast strains are a great plus for co-fermentation of xylose and hexoses to increase bioethanol yields. (4) Various bioproducts are generated using the lignin-rich residues remained from green-like pretreatments, sequential enzymatic hydrolysis and final yeast fermentation and in particular for high-value biochemicals and biomaterials, which should be considered as next generation of integrated biotechnology for low-cost biofuel production and high-value bioproducts under a green-like process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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