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Development of Lignocellulosic Biorefinery Technologies: Recent Advances and Current Challenges

Green Chemistry within the Biorefinery Concept

Antonella Amore,[A,C,D] *Peter Ciesielski*,[A,C] *Chien-Yuan Lin*,[A,C] *Davinia Salvachúa*,[B,C] *and Violeta Sànchez i Nogué*[B,C]

[A]Biosciences Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA.

[B]National Bioenergy Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA.

[C]All authors equally contributed to this work.

[D]Corresponding author. Email: antonella.amore@nrel.gov

Recent developments of the biorefinery concept are described within this review, which focuses on the efforts required to make the lignocellulosic biorefinery a sustainable and economically viable reality. Despite the major research and development endeavours directed towards this goal over the past several decades, the integrated production of biofuel and other bio-based products still needs to be optimized from both technical and economical perspectives. This review will highlight recent progress towards the optimization of the major biorefinery processes, including biomass pretreatment and fractionation, saccharification of sugars, and conversion of sugars and lignin into fuels and chemical precursors. In addition, advances in genetic modification of biomass structure and composition for the purpose of enhancing the efficacy of conversion processes, which is emerging as a powerful tool for tailoring biomass fated for the biorefinery, will be overviewed. The continual improvement of these processes and their integration in the format of a modern biorefinery is paving the way for a sustainable bio-economy which will displace large portions of petroleum-derived fuels and chemicals with renewable substitutes.

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Biomass deconstruction and conversion of sugar and lignin to high-value products will be essential steps in the implementation of next-generation integrated lignocellulosic biorefineries for manufacturing bio-based fuels and chemicals. For selective utilisation of all biomass components, understanding and exploiting the

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interplay between conversion processes and the structure of lignocellulosic feedstocks is a critical point for the development of the modern biorefinery, warranting the development of feedstock engineering techniques and advanced characterization methods, in tandem with the deployment of tailored deconstruction and upgrading processes.

Introduction – The Biorefinery Concept

The International Energy Agency (IEA) recently coined a formal definition for biorefinery, as 'the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, and/or materials) and bioenergy (biofuels, power, and/or heat)'.[1] Nearly all types of biomass can represent a viable feedstock to be used within a biorefinery as long as efficient methods of conversion and valorization are available: biomass from forestry, agriculture, aquaculture (algae and seaweeds), waste residues from industry and households, and other organic residues derived from plant and animals.[2] An ideal biorefinery should be a close-to-zero-waste process, which exploits sequential processes of fractionation and extraction followed by a combination of biochemical and thermal processing with continual recycling of energy and waste streams. As a consequence, a biorefinery requires the combination of interdisciplinary expertise including chemical engineering, chemistry, biology, biochemistry, biomolecular engineering, and materials science. While biomass has long been used as a source of heat and construction materials, modern products from a biorefinery include liquid transportation fuels and commodity chemicals. The delivery of these bio-based chemicals and products will offer economic and environmental benefits; however, technical challenges still impede the widespread industrial adoption of the biorefinery concept.[2–5] The ideal biorefinery concept is an inherent manifestation of the principles of 'green chemistry' since it is centred on sustainable, catalytic transformation of renewable feedstocks while striving for atom and energy efficiency at minimal detriment to the environment.

Considerable research and development efforts are still required in order to make the lignocellulosic biorefinery a sustainable and economically viable reality. Thus far, several generations of the biorefinery concept have been articulated in the literature as recently reviewed by Clark and Deswarte:[6]

• *Phase I biorefinery* (single feedstock, single process, and single major product);

• *Phase II biorefinery* (single feedstock, multiple processes, and multiple major products);

• *Phase III biorefinery* (multiple feedstocks, multiple processes, and multiple major products).

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Table 1 reports a list of some existing technologies, which differ based on the heterogeneity of the feedstocks, the feedstock structure and chemistry, and the streams of products.

Our current efforts at the National Renewable Energy Laboratory as 'Young Scientists Advancing Green Chemistry' are aimed at facilitating the deployment of advanced, modern biorefineries through: i) improving our understanding of the lignocellulosic structure at the molecular, macromolecular, and bulk scales; ii) engineering of the lignocellulosic structure and composition to facilitate lignin removal and enable simpler, more efficient biomass deconstruction processes; iii) developing advanced biocatalysts and gaining further insight into the function of current biocatalysts which are currently employed in biorefineries to improve sugars yield to be converted in fuels; and iv) developing methods for the utilisation of the entire biomass feedstock, including minor or typically underutilised streams such as lignin, for further upgrading to chemical precursors.

In this review, a survey of recent advances in modern biorefinery development is reported (Fig. 1), emphasising some recent achievements within several of the main biorefinery unit operations developed under the biochemical conversion platform at NREL. We describe the structure of lignocellulose, and discuss several sophisticated techniques currently used for characterization of biomass feedstocks. Attention is focussed on advances in feedstock improvement since it can favour lignocellulose breakdown and lead to more easily upgradable biomass streams. The remainder of the review describes progress achieved within some of the main unit operations of the biorefinery. In particular, advanced techniques used in pretreatment are described, highlighting the need to improve overall fractionation selectivity towards a more holistic biorefinery where sugars and lignin are both valorized. Recent findings regarding the structure–function relationship of the cellulase Cel7A from *Trichoderma reesei* are reported, as this is the workhorse enzyme in cellulose deconstruction. Also, an overview of the biological conversions of sugars into advanced biofuels is reported, along with the description of chemicals' production from sugars and lignin.

Biomass Structure and Properties

Hierarchical Structure of Biomass

Biomass is a composite of several energy-rich biopolymers that exhibit a hierarchical structural organisation that spans many orders of magnitude. This structure can vary considerably with the species of origin and even between genetic variants of the same species. At the bulk scale, biomass obviously exhibits the macrostructure of an organism (i.e. tree branches, grass stalks,

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etc.), although these are virtually always subjected to some form of mechanical comminution for particle size reduction before entering any conversion process. At the microscale, the porous structure of biomass is the dominant feature. This microporosity is highly species dependent, and is an artefact of the cellular tissue structure of the living organism that was used to transport water and nutrients. In the context of biomass conversion, this microscale porosity aids in the transport of conversion catalysts and chemical products throughout biomass particles.[7] The mesostructure of biomass is considered the higher-order assembly of nanoscale constituents, which make up the cell walls. Cell walls can generally comprise three ultrastructural domains consisting of the middle lamella, primary cell wall (PCW), and secondary cell wall (SCW). The SCW can further contain multiple distinct layers which can be seen by transmission electron microscopy (TEM) of cell walls from the vascular tissue of *Arabidopsis* as shown in the left panel of Fig. 2. The middle lamella, which acts as an adhesion layer between adjacent cells, often may not be clearly delineated from the PCW and thus the collection of these two regions is termed the 'compound middle lamella'. In woody biomass, all mature tissue contains SCWs, while in herbaceous plants pithy tissue contains only PCWs, which are not lignified, but the vascular tissue is reinforced with lignified SCWs. While non-lignified PCWs are relatively easy to deconstruct via treatment with saccharification enzyme cocktails, the majority of the mass, and thus energy content of the biomass, is found in SCWs. This material, often termed lignocellulose, consists of cellulose nanofibrils, crosslinking hemicellulose, and lignin which are arranged into a highly evolved structure that imparts strength to the material as well as protection to its valuable carbohydrate constituents from other organisms like fungi and bacteria. A schematic of the meso-/nano-structure of lignocellulose is presented in the right panel of Fig. 2. The resistance of this material to various conversion strategies is often termed 'biomass recalcitrance' and poses one of the major barriers to economically feasible biofuel production.[8]

While the composition of plant cell walls may be probed relatively easily by bulk analytical methods, much work has been devoted to determining the precise configuration of various biopolymers within plant cell walls. The PCW has been the focus of the plant biology due to its relevance to biosynthesis and has resulted in generally accepted models for PCW structure and morphology.[9,10] However, SCWs have been the major concern of the forest products industry, and more recently the biofuels community, due to their importance to engineering applications of wood[11] and high energy content relative to the PCW.[8] Specialised imaging techniques have proven exceptional tools for understanding the structure of SCWs. Transmission electron tomography has emerged as a powerful technique that has been used to investigate the configuration of cellulose and lignin in woody[12] and herbaceous biomass.[13] An excellent

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complement to this technique is offered by X-ray scattering methods which can provide ensemble measurements of the arrangement of cellulose fibrils within a specimen.[14,15] Atomic force microscopy (AFM) is another useful technique due to its ability to image soft materials with minima sample prep.[16] These multimodal methods, coupled to quantitative image analysis and computational investigations, are beginning to provide a more holistic view of the structure and properties of plant cell walls.

Biopolymer Components of Biomass: Cellulose, Hemicellulose, and Lignin

As mentioned above, the mature cell walls of biomass are principally composed of SCWs, which is made of three major components: cellulose, hemicellulose, and lignin. The variation of biopolymers among different plant species have been characterized in detail [17–31] and are listed in Table 2. In this section, the structure and the biosynthesis process of the three biomass components are briefly introduced.

Cellulose, as the most abundant biopolymer in plants, is the fundamental building block of microfibrils of the plant cell wall. It is composed of linear, unbranched, and parallel chains of several hundred to over ten thousand β-(1,4)-linked D-glucose units. In its natural form, cellulose is highly crystalline due to strong hydrogen bond and van der Waals interactions between the cellulose chains.[32] In 1980, Brown proposed that cellulose biosynthesis occurs on a plasma membrane, through a rosette form of a cellulose synthase (CesA) complex by a freeze–fracture technique.[33] Later on, these CesA genes have been identified and their functions have also been discovered (Table 3).[34–48]

Hemicelluloses are traditionally classified as non-cellulosic polysaccharides.[49] Hemicelluloses are typically easily hydrolyzed by alkali and acid treatments.[50–53] Hemicellulose principally consists of β-(1,4)-linked glycans, showing a complex combination of glycosyl substituents, such as D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-*O*-methyl-D-glucuronic acid, D-galacturonic acid, and D-glucuronic acid or, rarely, L-rhamnose and L-fucose.[54] Based on the types of substituents, it is possible to classify hemicelluloses in xyloglucans (XGs), xylans, mannans, and glucomannans.[49] Xylan is the major hemicellulose in dicots with 20–30 % glucuronoxylan (GX), while galactoglucomannan (GGM) mainly exists in gymnosperms.[55,56] For example, *O*-acetylgalactoglucomannan and arabino-4-*O*-methylglucuronoxylan are the two major hemicelluloses in softwood, and there are *O*-acetyl-4-*O*-methylglucuronoxylan (85 %) and glucomannan (15 %) in hardwood.[42,57] Hemicellulose synthesis is proposed to occur in the golgi via cellulose synthase-like (Csl) enzymes which belong to the glycosyltransferase family 2 (GT2).[34,49,58] According to their homology, Csl enzymes in plants are divided into nine sub-

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families, namely Csl A–H and J.[59,60] The distribution and function of each Csl are listed in Table 4.[42,49,60–65] Besides Csl, the enzymes involved in the biosynthesis of xylan include members from other GT families, such as IRX9 and IRX14 from the GT43 family, IRX10, FRA/IRX7, and F8H from GT47, and IRX8/GAUT12 and PARVUS from GT8.[55]

In contrast to cellulose and hemicellulose, lignin is a heterogeneous phenolic polymer that interweaves with cellulose microfibrils and hemicelluloses, providing mechanical support to the cell wall and facilitating water transport through xylem due to its hydrophobic characteristics.[66] Lignin is composed of three principal phenylpropanoid subunits, the 4-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits. The H, G, and S subunits are polymerized from 4-coumaryl, coniferyl, and sinapyl alcohols, respectively.[57] Lignin is inter-linked by ether bonds, with ferulic acid and *p*-coumaric acid attached to G and S subunits, respectively,[67,68] where *p*-coumaric acid and ferulic acid are the most common hydroxycinnamates in the wall of grass species besides H, G, and S subunits.[23]

The composition of lignin varies among plant species: in angiosperms, such as *Populus trichocarpa*, the ratio between S and G subunits (S/G) is ~2, with traces of H subunits (up to 0.5 %),[69] whereas in most gymnosperms, such as pine, the G unit is the main subunit.[66] In grasses, higher amounts of H subunits (<~5 %) can be found, with similar amounts of G and S subunits,[70,71] such as the case of mature maize stalks with 4 % H.[23] Moreover, in the same species, the content and composition of lignin can vary among different cell types including xylem vessels, tracheary elements, and fibres in the xylem tissue of woody plants.[72,73] Other phenolic compounds can be incorporated into lignin, such as hydroxycinnamaldehydes which are found in the lignin of cinnamyl alcohol dehydrogenase (CAD) deficient mutants of pine and transgenic tobacco,[74,75] and the 5-hydroxylated guaiacyl (5-OH-G) subunit which is identified in the lignin of 5-hydroxyconifer-aldehyde-*O*-methyltransferase (AldOMT) transgenic plants and deficient maize mutants.[76–82]

Lignin is often considered as the hindrance of biomass for economic processing.[83,84] Both lignin content and composition influence plant recalcitrance during delignification for paper and pulp or biofuel production, thus the development of a biorefinery. Lowering the lignin content or increasing the S/G ratio in lignin composition have shown positive correlations with the yield of fermentable sugars and the economic value of plant biomass for industrial needs.[28,85–88]

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Biorefinery Process

Pretreatment/Fractionation

In the context of biochemical conversion processes, pretreatment of biomass generally refers to the application of a thermochemical and/or mechanical process to the biomass feedstock before its subjection to enzymatic saccharification. Effective pretreatments facilitate penetration of enzymes into the cell wall interior by loosening the cell wall matrix as well as relocating and/or removing lignin. Common pretreatments include dilute sulfuric acid, liquid hot water, steam explosion, and various alkaline processes. Dilute sulfuric acid presents an attractive technology because of the low cost of the acid and its propensity to hydrolyze the majority of the hemicellulose for subsequent recovery in an aqueous stream before saccharification.[89] Processes that incorporate explosive decompression with dilute acid[90] or ammonia chemical treatments (i.e. ammonia fibre explosion, AFEX)[91] introduce additional mechanical disruption of the biomass, which further enhances the digestibility of the resultant material by causing delamination of nanofibrillation at the cell wall scale.[15] TEM images showing the effects of dilute acid/steam explosion pretreatment on corn stover cell walls are presented in Fig. 3. More recent economic evaluations of various biorefinery concepts have identified the importance of lignin valorization, and therefore the need of processes that effectively fractionate biomass into streams that predominantly contain one biopolymer before subsequent conversion steps.[92] Chemical pretreatments with Organosolv[93] and sodium hydroxide[94] have been shown to effectively fractionate lignin derivatives into a separate, easily recoverable stream, while the remaining carbohydrate components are particularly well suited for subsequent enzymatic or chemical conversion processes which will be discussed in detail later in this review.

Enzymatic Saccharification

Enzymatic saccharification, along with any chemical hydrolysis that occurs during the pretreatment step, leads to the production of fermentable sugars from the major biomass polysaccharides such as cellulose and hemicellulose. These monomeric sugars are the key molecules for the production of bio-based fuels and chemicals. Enzymatic saccharification requires a range of hydrolytic and oxidative enzymes that act in a synergistic manner to obtain high concentrations of both pentose and hexose sugars. Cellulases are the primary enzymes involved in cellulose hydrolysis and their properties and mode of action will be discussed here. In contrast, due to its high heterogeneity, hemicellulose hydrolysis requires a more complex enzymatic cocktail comprising xylanases, arabinofuranosidases, mannanases, and xylosidases. Researchers have also recently focussed on a different type of lytic enzymes dubbed as

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polysaccharide monooxygenases (LPMOs), oxidative enzymes which have been demonstrated to be involved in cellulose deconstruction through oxidative chain breakage.[95]

Basic Cellulase Paradigms

Cellulases are key enzymes involved in lignocellulose conversion into high-value products within the saccharification step of the lignocellulosic biorefinery. Three types of cellulases take part in the hydrolysis of cellulose, namely i) the endo-β-(1,4)-glucanases (EGs) or β-(1,4)-D-glucan-4-glucanohydrolases (EC 3.2.1.4), which randomly hydrolyze soluble and insoluble β-(1,4)-glucan substrates; ii) the exo-β-(1,4)-D-glucanases, including both the β-(1,4)-D-glucan glucohydrolases (EC 3.2.1.74) and β-(1,4)-D-glucan cellobiohydrolases (CBHs) (EC 3.2.1.91), which liberate D-glucose from β-(1,4)- -glucans and D-cellobiose from β-(1,4)-glucans, respectively. The β-D-glucosidases or β-D-glucoside glucohydrolases (EC 3.2.1.21) release D-glucose units from cellobiose.[96]

Cellulases can exist as a ‘free enzyme system’ or as a ‘multienzyme cellulosomic system’. These represent the two basic cellulase paradigms on which researchers have focussed so far.[97] Free cellulases are generally composed of a catalytic domain (CD) and a carbohydrate-binding module (CBM), which are connected by a flexible linker. They act as independent catalytic units, with the EGs acting on the cellulose amorphous region, the CBHs converting the cellulose crystalline region into cellobiose molecules, and the BGs finally converting the cellobiose into glucose monomers. This cellulose paradigm is generally exemplified by the cellulase system of the fungus *Trichoderma reesei*. In contrast, cellulosomes are composed of a non-catalytic scaffolding-based module and a variable large number of active enzymes, which are assembled by multiple cohesin modules. The first discovered and most representative cellulosome is that from the anaerobic rumen bacterium *Clostridium thermocellum.*

Importance and Role of CBHs

Fungal CBHs are recognized as the principal cellulolytic enzymes since they are the most prevalent cellulases in fungal secretomes. Fungal cellulases are now being utilised industrially to depolymerize cellulose in ethanol biorefineries. In particular, CBHs from Glycoside Hydrolase Family 7 (GH7) are the primary enzymes in the industrial enzymatic cocktails. The most representative fungal CBHs are those from *T. reesei* which produces both CBH I (Cel7A), acting on the reducing ends of crystalline cellulose, and CBH II (Cel6A), acting on the non-reducing ends of crystalline cellulose. Particular attention is generally focussed on the highly processive *T. reesei* Cel7A (TrCel7A), which acts through a highly complex series of events where all the

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catalytic and non-catalytic domains take part: i) adsorption to the crystalline cellulose surface; ii) cellulose chain decrystallization; ii) chain threading through the binding tunnel or direct binding; iv) hydrolysis; and v) product expulsion and desorption. This review is focussed on the most recent advances on understanding TrCel7A, the industrial workhorse enzyme involved in the production of glucose, the building block molecule used for ethanol and other high-added value products obtainment. NREL research has been focussing on the study of this enzyme for years, leading worldwide the understanding of the structure–function relationship featuring the enzyme.

Recent Advances in TrCel7A Research

As previously described, TrCel7A is composed of a CBM and CD connected by a flexible, glycosylated linker. These linkers have long been thought to simply serve as a chain between structured domains or to act in an inchworm-like fashion during catalytic action. To examine linker function, Payne et al.[98] performed molecular dynamics (MD) simulations of TrCel7A bound to cellulose. This showed that the glycosylated linkers bind directly to cellulose, suggesting a previously unknown role in enzyme action. In particular, the highly disordered glycosylated linkers in carbohydrate-active enzymes aid the dynamic binding during the enzymatic deconstruction of plant cell walls.

Native glycosylation of family 1 CBMs has been shown to substantially impact multiple physical properties, including thermal and proteolytic stability and cellulose binding affinity. Chen et al.[99] explored the specificity of *O*-glycosylation in enhancing the stability and cellulose binding affinity of family 1 CBMs. In particular, a library of 20 CBMs was synthesized with mono-, di-, or tri-saccharides at each site for comparison of binding affinity, proteolytic stability, and thermostability. This study showed that glycosylation positively affects the CBM, favouring an overall protection to thermolysin proteolysis, increasing thermostability and enhancing the binding affinity to cellulose.

To gain molecular insights into the changes in CBM properties upon glycosylation, Happs et al.[100] studied solution structures of two glycoforms of a *T. reesei* family 1 CBM by liquid-state 2D homonuclear NMR spectroscopy, showing that family 1 CBM structures are strongly affected by covalent attachment of monosaccharides, in agreement with computational investigation results previously reported.[101,102] Despite the well recognised importance of CBM in determining the cellulase binding to the substrate, Várnai et al.[103] proposed that the water content in the hydrolysis reaction moiety could actually drive the binding of cellulases to the substrate. In detail, they found that the decrease of water content in the hydrolytic system reduces the positive effect

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of CBMs, increasing the affinity of cellulases to substrates, as a consequence of the higher probability of enzyme–substrate interaction.

In order to further understand the TrCel7A processivity, Payne et al.[104] developed a mathematical relationship, which quantitatively relates the binding free energy to the kinetic parameters of the enzyme. This way, it was possible to demonstrate that the ligand binding free energy is a key parameter in comparing the activity and function of GHs. In fact, the degree of processivity is directly correlated with the binding free energy of cello-oligosaccharide ligands to GH7s. This study offers a molecular-level theory of carbohydrate processivity in TrCel7A, which can be transfer to other GH7s.

Besides studying the effects of glycosylation, as the main post-translational modification affecting both the stability and the binding of TrCel7A, researchers are interested in investigating and overcoming the inhibition effects arising from cellobiose, the main end-reaction product. In fact, experiments and computational simulations have demonstrated that family 7 CBHs are the most sensitive cellulases to cellobiose inhibition.[105–109] Impairing interactions between cellobiose and CBHs by site-directed mutagenesis constitutes the most straightforward strategy to overcome these issues. For instance, in their recent paper, Silveira and Skaf[110] used MD simulations to investigate the effects of eight key mutations in the product-binding site, computationally demonstrating how these mutations can affect the cellobiose–enzyme interactions before hydrolysis. In particular, they showed that mutation of charged residues in the TrCel7A product-binding site causes perturbations that affect the structure of the loops forming the substrate-binding tunnel of the enzyme and, hence, may affect TrCel7A function in other steps of the hydrolysis mechanism.

Along with the end product's inhibition (i.e. cellobiose inhibition), inhibition effects are also related to the lignin that, at different concentrations and with different structures properties depending on the kind of biomass pretreatment, contaminates the substrates. Hydrophobic interactions have been identified as a major driving force for enzyme adsorption on lignin,[111] in addition to electrostatic interactions.[112] Nakagame et al.[113] suggested that native differences in lignin structures might cause differences in the inhibitory properties. In particular, they demonstrated that lignin isolated from woody species gives rise to stronger inhibition effects in Avicel hydrolysis, than lignin isolated from corn stover.

The aim of the recent studies by Rahikainen et al.[114] was to further elucidate the effect of lignin chemistry on cellulase adsorption. By means of a quartz crystal microbalance, they demonstrated that the positive role of steam explosion pretreatment in opening the cell wall

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matrix can be easily compromised by the structural changes of lignin that increase non-productive enzyme adsorption. Nordwald et al.[115] reported a novel approach that reduces lignin inhibition along with improving tolerance to ionic liquids, which are leading candidates as alternative solvents to improve the rate of hydrolysis of untreated cellulose at high concentrations (i.e. 20–30 % (w/v)). Engineering TrCel7A charge by succinylation led to a nearly two-fold enhancement in cellulose conversion in 15 % (v/v) 1-butyl-3-methylimidazolium chloride, and to a greater than two-fold increase in Avicel conversion after 170 h in buffer with 1 % lignin. These data show that TrCel7A, as well as other GH7s, with highly negative surface charge densities may be characterized by an improved activity, as a consequence of lignin repulsion.

Site-directed mutagenesis has been shown a powerful tool to study the process of crystalline cellulose degradation. For example, Nakamra et al.[116] demonstrated that Trp-40 is involved in recruiting individual substrate chains into the active site tunnel to initiate processive hydrolysis. These data are in accordance with previous high-speed atomic force microscopy studies, which showed that mutation of Trp-40 at the entrance of the catalytic tunnel drastically decreases the ability to degrade crystalline cellulose.[117]

High speed atomic force microscopy has been exploited by different groups to retrieve more details about the complex heterogeneous hydrolysis of TrCel7A. For instance, Igarashi et al.[118] had previously revealed that TrCel7A works as a ‘linear molecular motor’, and that the productive bindings to the reducing ends of the crystalline cellulose results in translational movement to the non-reducing end. By means of the same technique, Jung et al.[119] were able to visualise single TrCel7A molecules on cellulose Iα and III_I. This way, it was possible to show that the susceptibility of cellulose Iα and III_I to TrCel7A hydrolysis is highly dependent on enzyme concentration.

O’Dell et al.[120] also studied the processivity of TrCel7A towards cellulose fibrils by means of atomic force microscopy, demonstrating the contrasting digestibility of cellulose Iα fibrils from *Gluconacetobacter xylinus* and *Cladophora aegagropila* by TrCel7A. The bacterial cellulose (BC) fibrils from *G. xylinus* were rapidly totally hydrolyzed in 120 h, while TrCel7A hydrolysis of the algal cellulose (AC) fibrils from *C. aegagropila* was slow and limited (around 30 %). This was explained by the different structure of the fibrils, which can hinder or not the enzyme access to the available reducing ends. In particular, the AC microfibrils have compact cross-sections which give rise to the formation of tightly associated fibrils hampering enzyme access; on the other hand, the BC microfibrils have a flat, ribbon cross-section which results in more loosely associated fibrils which favour the accessibility of the enzyme and the overall digestion.

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To better understand and improve the catalytic performances of the workhorse enzyme involved in the saccharification step of the modern lignocellulosic biorefinery, new advanced tools are needed. Very recently, Brady et al.[121] have developed an optical tweezers-based single-molecule (SM) motility assay for precision tracking of TrCel7A, this paving the way to more insights of the motors mechanistic action of TrCel7A. Interestingly, through the SM of the single domains composing TrCel7A, it was shown that the catalytic domain alone is sufficient for processive motion, with the CBM sometimes even hindering the translocation.

The studies so far reviewed enlarge the general understanding of TrCel7A and pave the way to improve its hydrolytic performances. Many efforts have also been focussed on the improvement of TrCel7A production in a recombinant manner. Several expression systems can be used to produce high titres of TrCel7A, even if production of Cel7A with native-like properties has proven difficult. It is worth reporting within this review that at NREL, Linger et al.[122] have recently developed a constitutive protein expression system in *T. reesei*, useful for production and secretion of heterologous CBHs from GH family 7. This system is based on the use of the *eno*-promoter which allows the growth of *T. reesei* transformants in the presence of glucose. This results in an overall repression of the native cellulase system, and as a consequence, in a clean background, which makes easier and less expensive the downstream process.

Sugars Conversion for Fuels Production

Once sugars have been liberated from biomass feedstocks via pretreatment and saccharification they become available for subsequent conversion into desirable fuels and chemicals. Among biofuels, ethanol is the major contributor to the global demand for transportation fuel. Today, the majority of bioethanol is produced using corn or sugarcane as a feedstock. The production of this so-called ‘first generation bioethanol’ is a commercially well established process where considerable amounts of sugars are easily obtained from the raw material.[123] However, an important criticism directed towards first generation bioethanol is that crops and lands could be used for food and animal feed instead. The use of lignocellulosic feedstocks to produce biofuels – known as ‘second generation biofuels’ – represents an alternative to the use of sucrose- and starch-based biomass. The ‘third generation biofuels’ refers to biofuel derived from algae. Among these, the biogas obtained through anaerobic degradation of biomass, and the biodiesel produced from lipid accumulation in the algal cells are receiving the main attention. The current situation in the production of third generation biofuels has recently been reviewed by Alaswad and co-workers[124] and will not be reviewed in detail here. The following will focus on second

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generation biofuels including cellulosic ethanol plants and some other potential molecules that could be upgraded to fuels.

Cellulosic Ethanol

Over the last decades, extensive research has been conducted in the development of microorganisms for the production of ethanol. Regardless of the organism of choice, the following challenges need to be tackled in the fermentation step: i) the slower consumption rate of sugars other than glucose present in the lignocellulosic hydrolysate, ii) the generation of degradation compounds highly inhibitory for the fermenting microorganism, and iii) the lack of sterility in the production facility. Glucose is the main abundant sugar in lignocellulosic biomass and the preferred sugar for most microorganisms. But its consumption reduces the rate of synthesis of enzymes encoding for the utilisation of other sugars, leading to a fermentation of all other sugars much slower than that of glucose. Some sugars are consumed sequentially due to metabolic regulation and competition for transporters.[125,126] Therefore, there is a need to improve co-fermentation, particularly to reduce the fermentation times. In the case of *Saccharomyces cerevisiae*, metabolic engineering for pentose fermentation is required as Baker's yeast is not able to naturally consume these sugars. Several xylose pathways have been successfully expressed, including strategies that involved redox cofactor engineering and protein engineering to reduce cofactor imbalance in xylose-engineered *S. cerevisiae* strains.[127] Additional genetic manipulations, such as over-expression of the genes of the non-oxidative branch of the pentose phosphate pathway, deletion of native aldoreductases, or expression of heterologous transporters/facilitators have been shown to improve the abilities to ferment xylose. Recent advances of yeast strain development for improved xylose metabolism have been reviewed by Kim and co-workers.[128] As a complementary strategy to rational engineering approaches, the xylose metabolism of several yeast strains has been further developed through evolutionary engineering strategies.[129,130]

The need to increase the efficiency of the fermentation step under industrial conditions implies, in addition to the pathway engineering, the development of strains with improved robustness to the inhibitors present in lignocellulosic hydrolysates. The effects of several inhibitors, such as sugar degradation compounds (furans), weak acids (namely acetic, levulinic, and formic acid) and lignin-derived compounds, have been extensively studied over the last decades.[131,132] Recent advances for improved tolerance through metabolic engineering involve gene modifications related to stress responses[133,134] as well as cofactor availability.[135–137] In addition to rational

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engineering strategies, evolutionary engineering approaches have also been conducted to obtain strains with improved fermentation performances.[138–140]

Although *S. cerevisiae* is generally the organism of choice for ethanolic fermentation, other microorganisms, both yeasts and bacteria, have been extensively studied. For instance, NREL has pioneered the use of the yeast *Zymomonas mobilis* by developing acid tolerant strains able to ferment pentose sugars by means of genetic engineering.[141] The latest improvements on pentose fermentations have also been achieved via continuous adaptation. In the case of xylose, the resulting strain obtained through adaptation on dilute acid pretreated corn stover hydrolysate, displayed a higher xylose utilisation rate and produced more ethanol than the parent strain.[142] In the case of arabinose, the combination of genetic strategies with transposon based mutagenesis and selection was used to generate a strain with improved arabinose metabolism not only in defined media but also in corn stover hydrolysate.[143]

The lack of sterility in large-scale fermentation systems can represent another important issue, as the fermentation microorganisms must compete with other microorganisms for sugar utilisation and possible growth factors. Therefore, the development of highly efficient and robust production strains represents a competitive advantage against the microbial contaminants.

Other Advanced Biofuels

There are many different molecules that can be upgraded to fuels[144] for instance:

i) Alkanes or alkenes: these hydrocarbons are of particular interest due to their similarity to the petro-based fuels currently used (i.e. diesel substitutes); moreover they are superior to other biofuels, having a 30 % higher energy content than ethanol. Choi and Lee[145] reported the development of engineered *Escherichia coli* capable to produce short chain alkanes suitable for petrol, by engineering fatty acid biosynthesis and degradation pathways..

ii) Alcohols: alcohols other than ethanol can also be used as gasoline alternatives, such as butanol. Butanol is not miscible with water and presents more energy content than ethanol. Green et al.[146] recently reviewed the current industrial perspectives for the production of butan-1-ol where efforts are being made to re-commercialize the acetone–butanol–ethanol fermentation process with *Clostridium* species used as production organisms.

iii) Carboxylic acids: production of carboxylic acids such as propionic,[147] butyric,[148] or hexanoic acid[149] using biological routes represents an excellent platform for the generation of renewable fuel precursors, as all of them can be further catalytically upgraded to fuels. On the other hand, fatty acid produced using oleaginous yeasts represents a direct diesel

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alternative. Qiao and co-workers[150] recently reported significant improvements in terms of lipid titre and productivity using an engineered lipid overproducer *Yarrowia lipolytica* strain. These results could represent an important step towards the development of an efficient and cost-effective process.

Production of Chemicals from Different Biorefinery Streams

As mentioned above, there are multiple types of biomass deconstruction processes that enable either partial or complete fractionation of biomass into hemicellulose, cellulose, and lignin-enriched streams. This ability to fractionate biomass into separate constituents underpins the concept of the integrated biorefinery to manufacture multiple product streams, including both fuels and chemicals simultaneously in a single facility. Overall, this concept mirrors that of a petroleum refinery wherein fuels and chemicals are produced alongside one another, with chemical production enabling the economic viability of fuel production.[144,151,152]

Some examples of biomass deconstruction processes and the streams generated from these pretreatments are detailed here (Fig. 4). Specifically, hydrothermal or dilute-acid pretreatments enable the fractionation of hemicellulose-enriched hydrolysates, which are typically high in xylose concentration as well as other minor sugar components such as glucose, arabinose, and galactose. This hydrolysate can be fractionated from the cellulose-enriched solids, thus enabling upgrading of both streams separately (Fig. 4a). Conversely, these processes also facilitate keeping the resulting liquor together with the solids during enzymatic hydrolysis, resulting in a cellulose and hemicellulose-enriched stream that can be removed from the residual, lignin-enriched solids. Regardless, as mentioned before, acid or hot water-based pretreatments produce highly toxic hydrolysates, which can be overcome by robust downstream (bio)catalyst selection or by hydrolysate clean-up methods,[153,154] even if the latter is typically costly, adds capital costs, and results in lower sugar yields due to losses. Although it will be detailed later, it is worth mentioning that succinic acid has already been generated from these C5-enriched streams using organisms that are able to detoxify some of the inhibitors in the hydrolysate, such as furfural and hydroxymethylfurfural (HMF) in batch fermentation[155] and continuous fermentation on a bench scale (Fig. 4a). However, as a further demonstration of the integrated biorefinery concept, one could envision using the C6-enriched stream to produce fuels.

Alkaline-based approaches, such as dilute-NaOH or $Ca(OH)_2$ pretreatments can result in highly delignified biomass and a lignin- and acetate-enriched liquor stream, alongside solids enriched in hemicellulose and cellulose[94,156,157] (Fig. 4b); however, significant losses of hemicellulose in alkaline-based pretreatments are a major concern, which has driven down the severity of alkaline

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pretreatments, leading to the need for further types of pretreatment. However, for lignin valorization, these are relatively straightforward streams to work with because they are in the liquid phase compared with the solid residual lignin coming from the dilute acid pretreatment or that based in deacetylation and mechanical refining explained below. This type of lignin-enriched alkaline liquors have already been used to generate chemical precursors such as polyhydroxyalkanoates (PHA) and muconic acid[158–160] (Fig. 4b) as described in the section below about lignin upgrading, but significant challenges remain in their utilisation.

Recently, the concept of low-severity alkaline pretreatment, originally motivated by the need to remove the ethanologen inhibitor acetate, combined with mechanical refining has received significant attention.[160] This pretreatment generates lignin-enriched streams that can be isolated both upstream during pretreatment as a liquor, but also as enriched solids after polysaccharide deconstruction (Fig. 4c).[92,161] Regarding lignin from this pretreatment, it can be highlighted that its structure is native-like, which enhances the possibility of it being utilised by organisms able to uptake lignin monomeric compounds in nature. In conclusion, despite the many existing and emerging variations of biomass deconstruction and fractionation processes, these need to be closely co-designed and tailored to the downstream upgrading processes in order to produce both fuels and chemicals.

Chemicals from Sugars

The previous section described various pathways for the production of fuel precursors and chemicals from sugars and lignin; here several biological and catalytic upgrading pathways pursued at NREL are described. For the biological conversion of biomass sugar streams, one can envision manufacturing products that are near to central carbon metabolism, which are typically high in carbon efficiency (as a means of maximum carbon conversion from sugars to the targeted product), such as TCA cycle intermediates (e.g. succinic acid, malic acid, fumaric acid, citric acid, or itaconic acid), or those near the TCA cycle (e.g. lactic acid, butanediols, volatile fatty acids such as acetic, propionic, and butyric acids, and acetone–butanol–ethanol mixtures). Many of these products can be used as polymer precursors or industrial solvents, or can serve as building block chemicals, such that they can be chemically upgraded to many other species of interest. Moreover, these types of molecules can often be produced in anaerobic fermentation conditions, which offer substantial capital and operating cost savings in comparison to aerobic cultivation, with natural microbes, or via the addition of exogenous, engineered enzymes. With the exception of ethanol, fewer reports describe the production of these chemicals from biomass-derived sugars in a rigorous integrated fashion. As a more detailed example, it has been recently

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demonstrated that succinic acid can be produced from a fractionated, xylose-rich stream in both batch and continuous fermentation using a native succinic acid producing microbe, *Actinobacillus succinogenes*,[155,162] at near industrially relevant titres, rates, and yields – commonly the three most important variables in a bioprocess to manufacture a chemical of interest.

Beyond products near central carbon metabolism, products derived from isoprenoid or fatty acid pathways can also be manufactured, either in microbes that are already effective at producing these compounds (e.g. fatty acids in oleaginous microbes, as mentioned above, or polyhydroxyacids) or in engineered microbes.[163] Many types of bioplastics, which may find use as biodegradable plastic materials, can be obtained from polyhydroxyacids as recently reviewed by Agnew et al.[164] Oleochemicals produced via fatty acid biosynthesis will mainly find use as personal care products, surfactants, lubricants, and potentially (as discussed above) as fuel precursors. Both polyhydroxyacids and most of the oleochemicals are primarily intracellular carbon storage products, thus often requiring cell lysis to isolate them as intermediates or products. Cell lysis requires special handling procedures and adds significant cost, such that finding new solutions (e.g. enhanced cell lysis or product secretion) are significant areas of research and development.

Chemicals from Lignin

Lignin represents another, as-of-yet underexploited resource in the integrated biorefinery relative to biomass sugars. Currently lignin is mainly burnt to generate heat and power but it is a rich energy dense aromatic polymer with lots of possibilities.[165] As mentioned above, lignin can be isolated upstream in fractionation processes and isolated downstream in biochemical conversion processes as a solid residue (Fig. 4).[92,161] Strategies for lignin valorization have been pursued for many decades[166] and can be categorized as strategies that i) maintain the polymeric structure of lignin for use as a material or resin (e.g. carbon fibres[167]), ii) employ reductive catalytic routes towards benzene, toluene, and xylenes (BTX) or gasoline-range aromatic fuels,[166] iii) employ oxidative routes towards aldehydes (e.g. vanillin[168]) or acids,[169] or iv) use thermal and acid- or base-catalyzed processes towards mixtures of aromatic compounds that can then be further biologically upgraded. The production of materials and fuels from polymeric lignin could offer a more near-term valorization strategy, as these approaches do not require pure, chemically homogeneous substrates, which is inherently an issue with most lignin substrates. To produce chemicals from lignin, where purity is a key driver, will require approaches to either separate single components from highly heterogeneous mixtures or enhance the ability to convert lignin into a few compounds. Recent catalytic strategies on hardwoods point to the ability to do

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this.[170–173] The production of a fairly homogeneous distribution of upgradable alkyl-phenolics from for example birch and aspen lignin, demonstrates a potential route for a solely catalytic strategy to valorize some lignin feedstocks, especially those that exhibit a high syringyl/guaiacyl ratio (and thus have a significant number of labile C–O linkages).

Recently at NREL, attention has focussed on the development of a biological upgrading route for lignin valorization wherein depolymerized lignin-rich streams are fed to native aromatic-catabolic microbes such as *Pseudomonas putida* KT2440, which can 'biologically funnel' heterogeneous slates of aromatic molecules into single central intermediates through known 'upper pathways'.[158,174] These approaches have led to the ability to produce polyhydroxyacids,[158] fatty acids,[175] muconic acid,[159] and lactic acid[176] from lignin, but are still quite nascent in their development. Muconic acid in particular represents a highly atom efficient product from the intra-diol oxidative ring cleavage of catechol, and a particularly attractive chemical target. Muconic acid can be readily separated from residual aromatic substrates via activated carbon, acidification, crystallization, and resolubilization[159] and hydrogenated to adipic acid, which is the primary precursor to nylon-6,6. It can also be converted into terephthalic acid, another ubiquitous plastic used today on a large scale.[177] Muconic acid will likely find other uses as a functional replacement molecule given its inherent reactivity.

Biological solutions for lignin valorization will rely heavily on the ability to effectively depolymerize lignin to species that can be catabolized biologically. For this purpose, the use of biological agents such as ligninolytic enzymes and bacteria[175] is a key research area. At NREL, it has been demonstrated that ligninolytic enzymes secreted from bacteria are able to simultaneously depolymerize and catabolize aromatics to produce target molecules. This has been demonstrated to date in some bacteria such as *P. putida*, *Rhodococcus jostii*, and *Amycolatopsis* sp. Understanding the interplay between ligninolytic enzymes, specificity of aromatic catabolic microbes, and lignin substrate will be key to designing optimal biological systems for lignin valorization.

Overall, the amount of lignin to be generated in next-generation lignocellulosic biorefineries will be vast. Given its impending large-scale production, the valorization of lignin will become a more acute problem. Solutions ranging across a broad range of materials, chemicals, and fuels will likely be required.

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Genetic Improvement of Crops for Production of Biofuels and Biochemicals

While bioconversion technology has advanced substantially in recent years, many researchers have recognized the opportunity to improve the yields of these processes by engineering plants before they enter the biorefinery.[178] Indeed, plant engineering is a historical result from the development of agriculture and human civilization. The main purpose was to meet the increasing human population food demand, which had been estimated to increase up to 70 % by 2050.[179] Renewable bioenergy can also benefit from plant engineering efforts in order to meet our increasing energy demands and facilitate a transition away from fossil fuels. Instead of competing with food resources, scientists are now focussing on genetic engineering to make non-edible biomass, especially lignocellulosic material, more susceptible to thermochemical and/or biochemical conversion and enhance yields of desired conversion products.[159,180,181] In contrast to conventional breeding, scientists are improving biomass through genetic engineering using RNA interference (RNAi) or ectopic expression.[181–184] In this section, recent progress on genetic improvement of crops for biofuel production are reviewed.

Cellulose and Hemicellulose Modification

As the two major components of the plant cell wall, cellulose and hemicellulose content and composition in the cell wall have been engineered for greater yield of fermentable sugars. The effort to modify the composition of wall carbohydrates is because hexoses (glucoses) derived from cellulose are preferable monosaccharides for fermentation microbes than pentoses (mainly xylose and arabinose) derived from hemicellulose, which is due to the suppression of glucose catabolism by microbes during fermentation.[180,185] As far as increasing cellulose content is concerned, several studies had shown that ectopic expression of CesA results in silencing both transgenes and endogenous CesA *in planta*, which indicates the cellulose biosynthesis is tightly regulated and hard to fine-tune. Over-expression of barley cellulose synthase (HvCesA) resulted in silencing the transgenes and endogenous SCW HvCesA genes, and sometimes it has aberrant phenotypes including brittle nodes and dwarfing phenotype.[186] A similar result was previously reported in transgenic aspen.[187] Over-expression of cellulose synthase (PuCesA6) from Ussuri Poplar (*Populus ussuriensis*) in tobacco also exhibited a dwarf phenotype.[188] Instead of manipulating the expression level of CesA directly, successful attempts were made by indirect approaches. Over-expression of the *Gossypium hirsutum* sucrose synthase (SuSy) gene in hybrid poplar (*Populus alba* × *P. grandidentata*) significantly increased cellulose content (2 to 6 %) without influencing plant growth.[189] Moreover, the over-expression of UDPglucose pyrophosphorylase (UGPase) that produces UDPglucose for cellulose synthesis – like CcUGPase

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from Jute (*Corchorus capsularis*)[190,191] or LgUGPase from larch (*Larix gmelinii*)[192] – can increase the content of soluble sugars and cellulose. Interestingly, manipulation of cellulose biosynthesis by introducing a missense mutation of AtCesA3 under the heterologous M24 promoter in tobacco can increase the enzymatic saccharification yield by 54–66 and 40–51 % in leaf and stem, respectively.[193]

Additional efforts have focussed on modifying the composition of cell wall carbohydrates. Recently, it has been demonstrated that over-expressing arabinofuranosidase (ARAF) in rice decreased the arabinose content and increased the amount of glucose.[194] Over-expression of rice CslF6 under senescence-associated promoters in *Arabidopsis* increased the amount of MLG in the wall, with four times more glucose in the matrix cell wall fraction and an increase of saccharification yield up to 42 %.[195] Down-regulation of IRX8 in *Populus deltoids* by RNAi showed an increase of saccharification yield up to 4–8 %, with 25–47 % reduction in galacturonic acid and 17–30 % reduction in xylose.[196]

Lignin Modification

Genetic modulation of the overall lignin content[197] and monomeric composition[198] of plant cell walls has been shown to alter degradability. Unfortunately, genetic variants with reduced lignin content typically suffer from lower overall biomass production.[199,200] Subsequent approaches have developed altered lignin polymers that do not result in dwarfism,[201,202] which are more feasible to produce large quantities of tailored biomass for industrial biorefineries. Genetic modification of lignin biosynthesis has been shown to improve the conversion of *Arabidopsis*,[203] alfalfa,[86] switchgrass,[31] and poplar.[204] A recent study has elucidated the structural impacts of modifying the monomeric composition of lignin, wherein it was demonstrated that *Arabidopsis* genetic variants with an increased percentage of S-type subunits were more susceptible to acidic thermochemical deconstruction than the wild-type.[205] Fig. 5 presents TEM images of cell walls from *Arabidopsis* genetic variants with differing monomeric compositions following exposure to mild thermochemical treatment by maleic acid. These images illustrate how cell walls with a high content of S-type subunits experience enhanced deconstruction in the form of delamination and disjoining at the middle lamellae, while the wild-type and high-G cell walls display relatively little disruption in response to this pretreatment. Additional advances in modification of the lignin biosynthetic pathway have produced an exciting result involving plants that make predominantly H-type lignin. While previous attempts to produce plants with this type of lignin resulted in severe dwarfism, this recent study demonstrated that stacking an additional mutation, specifically disruption of the Mediator complex, rescued the

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dwarf phenotype and produced biomass that was highly digestible without any pretreatment.[206] Another example of stacking an additional mutation to engineer SCW deposition resulted in the effective rewiring of the secondary cell network in *Arabidopsis*. An artificial positive feedback loop (APFL) for enhancement secondary cell biosynthesis has been created in a *c4h* mutant using a promoter of VND6 (a vessel-specific transcription factor) to over-express cinnamate 4-hydroxylase (C4H), and a promoter of IRX8 to over-express NST1 (a fiber transcription factor). The results showed 20 % lignin reduction without growth defects and enhanced cell wall deposition that improves sugar release from enzymatic hydrolysis compared with wild-type.[207]

Moreover, recent studies have shown that monolignol biosynthesis in *P. trichocarpa* is regulated by complex mechanisms involving both metabolic and post-transcriptional controls. For instance, the two intermediate metabolites, 4-coumaroyl and caffeoyl shikimic acids, are identified as inhibitors of 4-coumaric acid:coenzyme A ligase 3 and 5 (4CL3 and 4CL5) in *P. trichocarpa* and may play important regulatory roles when these inhibitors accumulate.[208] This inhibitory effect may also exist in other plant species.[209] Protein phosphorylation was identified at the two serine sites (Ser^{123} or Ser^{125}) of AldOMT2. The phosphorylation at these sites is important for *O*-methyltransferase activity and this study reveals a rapid and energetically efficient regulation for S subunit monolignol biosynthesis.[210] Therefore, genetic engineering that targets the network in SCW, metabolic controls, or post-transcriptional modifications in the monolignol biosynthesis may provide additional opportunities for lignin reduction and structure alteration for future biomass improvement.

In Planta *Addition of Catalysts for Crop Improvement*

During the thermochemical pretreatment of biomass, the reduction of biomass and the enhancement of sugar yield can be achieved by incorporating iron ions as co-catalysts in the dilute acid pretreatment or cell wall-degrading (CWD) enzymes in the high temperature process.[211] In order to overcome the time-consuming procedure and the limitation of ion diffusion, an alternative approach that over-expresses soybean ferritin intracellularly in *Arabidopsis* under 35S promoter showed iron ion accumulation in the plant under both normal and iron-augmented watering conditions.[212] The resulting plant showed enhanced pre-treatability and a glucose and xylose release 13–19 % higher than the control.[212] An alternative approach to facilitate biomass pretreatment consists in the incorporation of thermostable xylanase, as in the case of intein-modified XynB (iXynB) expression in maize.[213] The result, obtained by temperature-regulated xylanase activation and hydrolysis in a cocktail of commercial CWD enzymes, showed >90 % theoretical glucose and >63 % theoretical xylose yields.[213]

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Targeted Genome Engineering

Besides gene knockdown (down-regulation) by RNAi in plants, modern approaches using targeted genome editing for gene knockout have been emerging *in planta*. Targeted genome engineering such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, more recently, clustered regularly interspaced short palindromic repeat (CRISPR)/Cas are promising technologies for future crop improvement. Among these approaches, the one based on the CRISPR/Cas cluster identified in *Streptococcus pyogenes* SF370, is easier to construct and has fewer target site limitations, requiring only the Cas9 protein and a target site with the protospacer-adjacent motif (PAM), 5′-NGG, preceding a 20–24 bp specific sequences for sgRNA targeting.[214–216] The CRISPR/Cas knockout system has been successfully used for genome editing in plants, such as rice,[217,218] wheat, *Arabidopsis*, tobacco,[219,220] sorghum,[221] sweet orange,[222] maize,[223] soybean, and *Medicago truncatula*.[224] The future practice of CRISPR/Cas for crop improvement will likely be a fruitful and promising strategy for increasing production of biofuels and biochemicals that will complement improvements to biorefinery unit operations.

Conclusions

The recent advances highlighted in this review constitute considerable progress towards enhanced economic sustainability and profitability of the modern biorefinery. However, despite significant research and development, considerable challenges remain to be addressed in biorefinery development from both the economical and the environmental points of view. Specifically, optimizations of biomass pretreatment, fractionation, saccharification, and conversion of the resultant sugar and lignin streams conversion into desired fuel and chemical products could greatly accelerate the widespread deployment of modern biorefineries.

Advancements in pretreatment technologies have shown that strategies that incorporate mechanical maceration with chemical deconstruction greatly enhance accessibility of cellulose fibrils to hydrolytic enzymes, and future efforts should seek to optimize the synergy of these physiochemical phenomena, in order to enhance the efficacy of hydrolytic enzymes and produce more homogenous sugar and lignin streams for subsequent conversion processes.

Improvements in enzyme functionality and unit operations can be met my modifications to the feedstock before it enters the biorefinery. While genetic modifications to lignin content and composition have already been shown to have a significant impact on the outcome of the biorefinery process, obtaining better genetic control of carbohydrate biosynthesis is expected to further enhance the utility of next-generation energy crops.

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Improving the hydrolytic performances of cellulases and hemicellulases included in the saccharification cocktails can increase sugar yields and accelerate hydrolysis kinetics. The workhorse enzyme Cel7A from Trichoderma reesei, which has been recognised as the main cellulase involved in cellulose conversion into glucose, could possibly be further improved through a better understanding of structure–function relationships, paving the way for the development of biocatalysts with improved processivity and performance. In particular, a lot of attention has been focussed on the role of glycosylation, a post-translational modification which potentially affects the enzyme properties of binding and processivity. Improving the knowledge of TrCel7a is a key step to make the biomass conversion an even more sustainable process from an economical point of view. While cellulosic ethanol has been recognised as the main biofuel to date, the modern biorefinery will likely include new alternative biofuels in its product suite.

Alkanes or alkenes are hydrocarbons of particular interest due to their similarity to the petro-based fuels currently used (i.e. diesel substitutes); they are superior to other biofuels, having a 30 % higher energy content than ethanol. Alcohols other than ethanol can also be considered as gasoline alternatives. For instance, butanol has significant industrial perspectives and many efforts are being made to re-commercialize the acetone–butanol–ethanol fermentation process with Clostridium species used as production organisms. Carboxylic acids such as propionic, butyric, or hexanoic acid may also represent a platform for the generation of renewable fuel precursors. Last but not least, fatty acids produced using oleaginous yeasts represent a direct diesel alternative.

Lastly, the valorization of lignin represents a major technical hurdle in the biorefinery. Despite significant work in lignin conversion over the last century, innovative chemical and biological processes are being developed, facilitated by advances in modern materials science for catalysis applications and synthetic biology for the design of sophisticated microbial hosts.

Continued efforts and progress in the areas described within this review will help the economic and environmental prospects of the biorefinery to become a reality.

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Fig. 1. Simplified scheme of a lignocellulosic biorefinery.

Fig. 2. (Left) TEM image of vascular cell walls from *Arabidopsis*. CL, cell lumen; SCW, secondary cell wall; PCW, primary cell wall; CML, compound middle lamella. (Right) Schematic depiction of the structure of lignocellulose. Image and schematic courtesy of P. Ciesielski, NREL Biomass Surface/Structure Characterisation Laboratory.

Fig. 3. TEM images showing the effects of corn stover cell walls following pretreatment by dilute acid with steam explosion. (a) Native (untreated) corn stover cell walls. (b) Pretreatment causes disjoining of cells at the middle lamella and introduces intra-cell wall void regions which facilitate the penetration of enzymes and other conversion catalysts. (c) High magnification of pretreated cell walls showing delamination and nanofibrillation of the cellulose. TEM images and schematic courtesy of P. Ciesielski, NREL Biomass Surface/Structure Characterisation Laboratory.

Fig. 4. Example of different biorefinery organizations considering diverse pretreatments developed at NREL: (a) dilute-acid, (b) alkaline, and (c) deacetylation and mechanical refining pretreatment. For the

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NREL mission, an ideal biorefinery will produce fuels alongside chemicals, the latter obtained from both sugars and lignin.

Fig. 5. Cell walls of several *Arabidopsis* genetic variants following exposure to mild maleic acid treatment. (a) Wild type cell walls, composed of both G and S-type monomeric units, show mild deconstruction in response to the pretreatment. (b) Cell walls with a high content of G-type monomeric units display enhanced resilience to deconstruction relative to the other variants. (c) Cell walls with a high content of S-type are severely deconstructed relative to the other variants when subjected to the same pretreatment. This enhanced disruption of the cell wall interior facilitated enhanced conversion of the material in subsequent enzymatic saccharification. TEM images and schematic courtesy of P. Ciesielski, NREL Biomass Surface/Structure Characterisation Laboratory.

Table 1. Examples of companies currently developing the biorefinery concept

Company	Site	Feedstock	Product stream
'Les Sohettes' complex	Pomacle, France	Sugar beet Wheat	Ethanol, straw-based paper, succinic acid.
Cargill Inc.	Nebraska, USA	Corn crops	Corn oil, sugar, ethanol, lactic acid, polylactic acid
DuPont Tate & Lyle BioProducts	Tennessee, USA	Corn	Propan-1,3-diol
BALI biorefinery	Borregaard, Norway	Bagasse, spruce, eucalyptus and wheat straw	Cellulosic ethanol, vanillin, lignosulfonates
GranBio	Alagoas, Brazil	Sugarcane straw and bagasse	Ethanol, expected to produced other biobased products
Abengoa's biorefinery	Kansas, USA	Corn stover, wheat straw	Ethanol and power
Beta Renewables biorefinery	Crescentino, Italy	Mainly *Arundo donax* (giant cane)	Ethanol and power
CIMV built	Pomacle, France	Agricultural by products (cereal straw, bagasse from sugarcane and sweet sorghum); fibre crops (hemp, flax, Provence cane, Miscanthus); forestry residues.	Lignin, C5 sugar syrup, paper grade cellulose pulp, bioethanol
Finnish energy company St1	Kajaani, Finland	Biowaste and process residues from local bakeries	Ethanol
Lanzatech	1. Auckland, New Zealand; Shanghai, China 2. Soperton, Georgia 3. Aurangabad, India	1. Steel mill off gases 2. Biomass syngas 3. Municipal solid waste	Ethanol, 2,3-BDO/butadiene, acetic acid, isobutylene
Novamont	Novara, Italy	Corn starch	Several biopolymers, among which: biodegradable polyesters (Origi-Bi) and starch-derived thermoplastics (Mater-Bi)

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Roquette site	Lestrem, France	Cereal grains	Polyols, native and modified starches, proteins and derivatives, cyclodextrins, organic acids and resins, more than 600 carbohydrate derivatives
ZeaChem	Port of Morrow in Boardman, Oregon.	Woody biomass Agricultural residues	Expected to produce bio-based fuels, C2 chemicals (acetic acid, ethyl acetate, ethanol and ethylene) and C3 chemicals (propionic acid, propanol and propylene)
SP Processum	Sweden	Wood	Energy, chemicals and material
POET	Iowa	Corn grain	Ethanol, corn germ and a protein-rich dried distillers grains (used as animal feed)
West Fraser	Canada/North American	Forestry wood	Umber (spruce/pine/fir ('SPF') and southern yellow pine ('SYP')), panels (plywood, MDF and LVL), pulp (NBSK and BCTMP), energy, newsprint and wood chips.

* Some information was previously collected by de Jong and Jungmeier.[2].

Table 2. Variation in biopolymer components of biomass: cellulose, hemicellulose and lignin

Plant	Cellulose [%]	Hemicellulose [%]	Lignin [%][A]	Protein [%]	Others (%)	Ref.
Arabidopsis thaliana	38.9	35.6	14.5 (0.41)	N.A.[B]	N.A.	[17]
Rice straw (*Oryza sativa*)	41.7	26.4	15.3 (0.29)	N.A.	11.0	[18,19]
Cotton stalk (*Gossypium hirsutum*)	39.9	17.2	25.6 (0.7)	N.A.	N.A.	[20,21]
Corn Stover (*Zea mays*)	39.2	33.4	14.8 (1.02–1.61 for stalk) (0.20–0.55 for leave)	3.7	8.9	[22,23]
Pinus radiata	39	23.3	28.8 [0.03]	N.A.	N.A.	[24]
Loblolly pine (*Pinus taeda*)	45.5	23.0	28.0 [0.01]	N.A.	2.8	[25]
Betula platyphylla	43.4	33.8	22.5 (1.2)	N.A.	N.A.	[26,27]
Aspen (*Populus tremuloides*)	50.6	5.3	21.5 (2.3)	N.A.	2.7.	[28,29]

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Populus trichocarpa	38.3	22.1	23.7 (2.1)	N.A.	10.9	[30]
Switchgrass (*Panicum virgatum*)	40.5	18.1	24.8 (0.71)	N.A	N.A	[31]

[A]Parenthesis in lignin column indicates the S/G ratio and brackets indicates the H/G ratio.

[B]N.A., not available.

Table 3. Cellulose (CesA) gene family *in planta*

Plant	Number of genes	Genes	Function	Ref.
Arabidopsis thaliana	10	*AtCesA1, AtCesA3,AtCesA6-like CesAs (AtCesA2, AtCesA5, AtCesA6, AtCesA9)*	Primary cell wall synthesis	[34–37]
		AtCesA4, AtCes7, AtCesA8	Secondary cell wall synthesis	
Oryza sativa	11	*OsCesA1, OsCesA3, OsCesA8*	Primary cell wall synthesis	[38]
		OsCesA4, OsCesA7, OsCesA9	Secondary cell wall synthesis	
Gossypium hirsutum	N.A.[A]	*GhCESA3, GhCESA5, GhCESA6, GhCESA9, GhCESA10*	Primary cell wall synthesis	[48]
		GhCESA1, GhCESA2, GhCESA7, GhCESA8	Secondary cell wall synthesis	
Zea mays	12	*ZmCesA10, ZmCesA11, ZmCesA12*	Secondary cell wall synthesis	[39]
Pinus radiata	8	*PrCesA2, PrCesA10*	Primary cell wall synthesis	[45,46]
		PrCesA1, PrCesA3, PrCesA7, PrCesA11,	Secondary cell wall synthesis	
Pinus taeda	10	*PtaCesA1, PtaCesA2, PtaCesA3*	Secondary cell wall synthesis	[40,47]
Betula platyphylla	N.A.	*BplCesA3*	Primary cell wall synthesis	[44]
		BplCesA4, BplCesA7, BplCesA8.	Secondary cell wall synthesis	
Populus tremuloides	N.A.	*PtdCesA4, PtdCesA5, PtdCesA6, PtdCesA7*	Primary cell wall synthesis	[43]
		PtdCesA1, PtdCesA2, PtdCesA3	Secondary cell wall synthesis	
Populus trichocarpa	17	*PtrCesA4, PtrCesA7-A, PtrCesA7-B,PtrCesA8-A, -PtrCesA8-B*	Secondary cell wall synthesis	[41,42]

[A]N.A., not available.

Table 4. Hemicellulose (Csl) gene family and their function *in planta*

Csl family	Distribution	Function	Ref.
CslA	All land plants	(Gluco)mannan synthesis	[61,42]
CslC	All land plants	Xyloglucan (XyG) synthesis	[62]
CslD	All land plants	Arabinoxylan (AX) structure	[63]
CslB and CslG	Eudicotidae	unknown	[63]

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CslE	Angiosperms	unknown	
CslF and CslH	Poaceae and closely related grasses	Mixed-linkage glucan synthesis	[64,65]
CslJ	Cereals (not in rice or Brachypodium)	Mixed-linkage glucan synthesis	[60]