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## **Approaches for More Efficient Biological Conversion of Lignocellulosic Feedstocks to Biofuels and Bioproducts**

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## **Abstract**

The future bioeconomy promises drop-in or performance-advantaged biofuels and bioproducts derived from lignocellulosic biomass, substantial greenhouse gas (GHG) emissions reductions in sectors with few or no alternatives, and increased domestic energy production in countries with sufficient biomass resources. Despite the slower than anticipated pace of commercializing next-generation biofuels, the research community continues to make dramatic improvements at every stage of production, from feedstock cultivation through conversion to final products. However, the interdisciplinary nature of bioenergy research, and the need for cross-coordination among biologists, chemists, agronomists, and engineers, make coordinating and optimizing these strategies challenging. This Perspective surveys recent advancements in bioenergy crop engineering, lignocellulosic biomass deconstruction and fractionation, catabolism of biomass-derived sugars and aromatics, and biological conversion to fuels and products. We organize major research approaches into broad categories and comment on which strategies offer synergies or trade-offs in the context of four approaches to improving the economics and carbon-efficiency of advanced biofuels and bioproducts: 1) maximize sugar conversion to a single product, 2) utilize diverse carbon sources for producing a single product, 3) convert lignin to high-value products, and 4) fractionate the hydrolysate to derive maximum value from each component.

## **Keywords**

Bioenergy, bioproducts, biomass engineering, plant cell walls, sustainability, technoeconomic analysis

## **Introduction**

Advanced biofuels and bioproducts derived from lignocellulosic biomass offer the promise of substantial reductions in greenhouse gas (GHG) emissions and increased domestic energy production in countries with sufficient biomass resources and/or land suitable for cultivating energy crops. Bio-derived liquid fuels may also be the most viable option for decarbonizing sectors that are challenging to electrify, such as heavy-duty freight and aviation.<sup>1,2</sup> However, the scale-up of second-generation biofuel production has fallen far short of expectations.<sup>3</sup> In the United States, the Energy Independence and Security Act (EISA) of 2007 set an annual blending target of 16 billion gallons of cellulosic biofuels by 2022, but 2017 production was less than 300 million gallons.<sup>4,5</sup> The scientific and technological progress made in the last two decades has addressed many of the challenges initially facing cellulosic fuels and bioproducts. Furthermore, the confluence of high-throughput and data-driven advanced technologies with disruptive tools in genome editing and genomics has set the research community on a path toward rapid discovery and improvement.<sup>3,6,7</sup> The goal of this Perspective is

to identify some of the most promising efforts and understand how they can fit together in integrated feedstock-to-fuel (and products) systems.

The interdisciplinary nature of bioenergy research, and the need for cross-coordination among biologists, chemists, agronomists, and engineers, makes optimizing any specific production system challenging. For example, tailoring feedstocks for ease of deconstruction could have unintended effects on their responses to biotic or abiotic stresses, or on biomass stability during long-term storage;<sup>8,9</sup> many of the most effective solvents for biomass pretreatment are incompatible with enzymes and microbial hosts required for downstream saccharification and bioconversion;<sup>10</sup> and microbes capable of producing advanced fuels or products at high yields often do not natively utilize pentose sugars present in hydrolysates.<sup>11,12</sup> Conversely, consolidated approaches aimed at decreasing costs, energy demand, and material inputs for biomass deconstruction also lend themselves to engineered hosts capable of utilizing a wide range of sugars and aromatics downstream.<sup>13,14</sup> Biological conversion has the inherent advantage of manufacturing compounds that are unobtainable using solely chemical methods - leading to new categories of fuels and bioproducts.<sup>15</sup> In short, some advances at the unit process or sub-system level may be more compatible than others, while some combinations may offer unexpected synergies. This Perspective encompasses published research on bioenergy crop engineering, lignocellulosic biomass deconstruction and fractionation, catabolism of

biomass-derived sugars and aromatics, and biological conversion to fuels and products. We comment on which strategies may offer synergies or trade-offs based on four general strategies for improving the economics and carbon-efficiency of advanced biofuels and bioproducts: 1) maximize sugar conversion to a single product, 2) utilize diverse carbon sources for producing a single product, 3) convert lignin to high-value products, and 4) fractionate the hydrolysate to derive maximum value from each component.

### **Plant engineering for cell wall optimization and *in-planta* production**

Delivered feedstock cost and the composition of biomass are some of the driving factors in the economics of the bioenergy and bioproducts. Cost is primarily driven by crop yield, a simple metric that is derived from many interlinking and complex underlying agronomic traits. The most desirable composition varies depending on how the biomass is handled and ultimately converted. For example, a process that only utilizes glucose will be best suited to feedstocks with minimal lignin and hemicellulose. In addition to natural variation in lignin, cellulose, and hemicellulose content, the presence of inhibitory compounds in hydrolysates and the resulting impacts on microbial conversion will vary. For example, Du et al.<sup>16</sup> explored different combinations of feedstocks and pretreatment and identified 40 potentially inhibitory compounds present in the resulting hydrolysates. Some engineering efforts have focused on reducing the presence of these otherwise naturally occurring inhibitors, such as cell wall-bound ferulic acid.<sup>17</sup>

Feedstock engineering can deliver biomass tailored to the end use, which can in turn have a dramatic impact on product yields, costs, and net environmental impacts. This requires a detailed knowledge of plant cell wall biosynthesis and carbon metabolism. This paper focuses on dedicated biomass crops, although similar strategies could be used to alter crop residue biomass if they do not negatively impact the yield and quality of valuable primary products such as corn grain.

Biomass is primarily composed of the secondary cell wall, which is laid down on the inside of the primary cell wall at the cessation of cell growth. It contains polysaccharides, which are a mixture of six-carbon sugars (hexoses) and five-carbon sugars (pentoses), and lignin, a complex cross-linked polymer constructed from aromatic monomers (Figure 1).<sup>18</sup> The exact composition of sugars, linkages, and phenolics is dependent on plant species, tissue type, as well as environmental factors. Many different strategies for biomass improvement have been proposed. Some, such as increasing the quantity of hexoses, would have broad applicability, given the far wider range of host microbes that natively utilize glucose relative to pentoses. Others, such as producing soluble co-products, may require highly tailored downstream processing and separations.

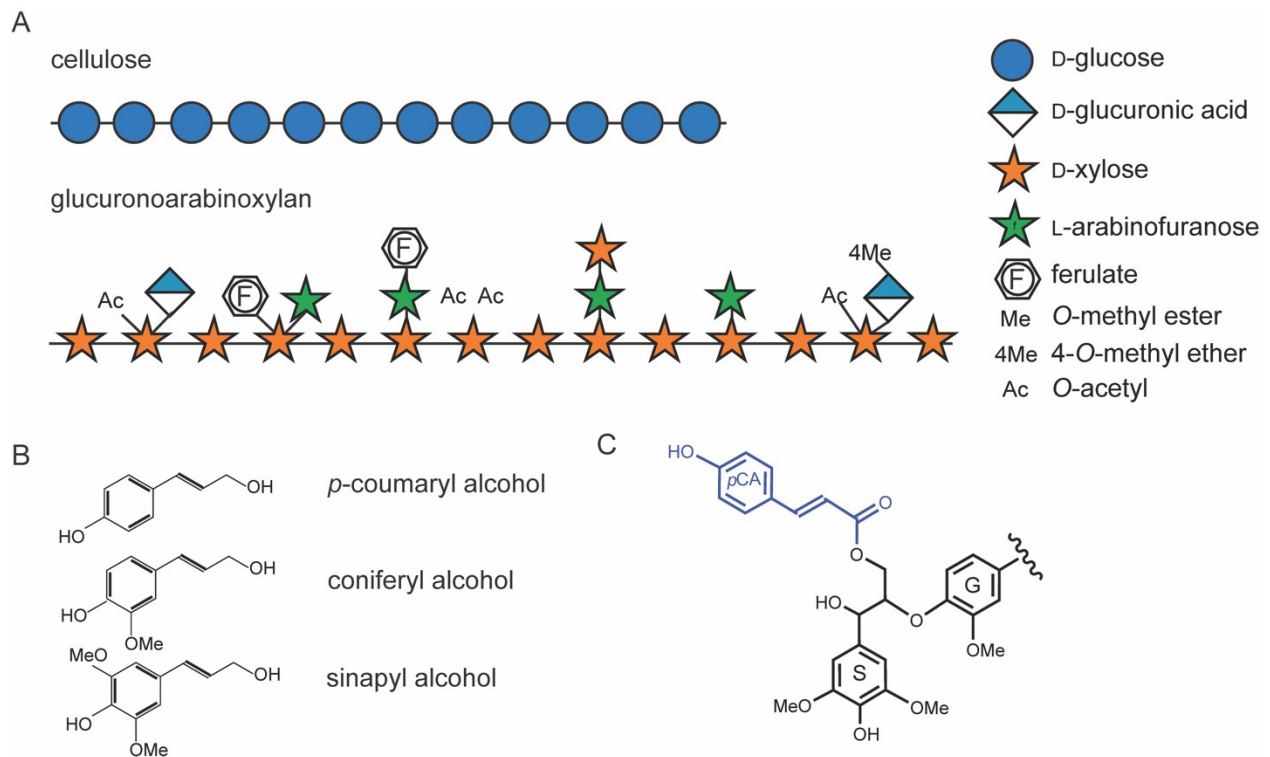


Figure 1. Major components of the grass secondary cell wall. (A) Example polysaccharide structures (B) and lignin monomers. (C) An example lignin fragment with *p*-coumarate acylation. Glucose is an example of six-carbon sugar or hexose while xylose is an example of a five-carbon sugar or pentose.

### **Redesigning Polysaccharides**

Most industrially used microbes preferentially or exclusively use hexose over pentose sugars.<sup>19</sup> Increasing the proportion of hexose sugars in the non-starch plant biomass fraction (i.e. the cell wall) is therefore a popular strategy, and can be achieved by many routes. The majority of hexose in the plant cell wall comes from the glucose in cellulose, which primarily forms crystalline microfibrils that are recalcitrant to enzymatic degradation. The



remaining cellulose is present in an amorphous form that is more readily depolymerized by enzymes. Efforts to increase the cellulose content in the commonly-studied woody feedstock poplar by overexpressing cellulose synthase genes have yet to be successful, as it resulted in silencing of the endogenous cellulose synthase genes and a massive decrease in cellulose content.<sup>20</sup> However, introduction of a mutated form of one of the cellulose synthase genes into another model plant, tobacco, increased the quantity of amorphous cellulose, which translated to a 40-60% increase in saccharification yields.<sup>21</sup>

A more promising approach has been to increase the synthesis of non-cellulosic hexose-containing polysaccharides: pectins and hemicelluloses.<sup>22</sup> The enzyme that synthesizes mixed linkage glucan, a soluble polymer of glucose found in grass cell walls, has been introduced in Arabidopsis (another model plant), producing a fourfold increase in non-cellulosic glucose, and a 42% increase in saccharification yield. Importantly, this increase was only achieved by expressing the enzyme under a senescence-related promoter, which avoided developmental defects observed when introducing mixed linkage glucan production in developing cells. Of course, these temporal engineering strategies will have implications for agronomic practices. This approach would require pre-harvest senescence, which is not always practical for all species across all possible locations.

A second strategy to enhance the hexose content was to increase the quantity of pectic galactan, which is made from the hexose galactose, and is usually a component of just the primary cell wall. Initial attempts to increase the quantity of galactan by overexpressing the galactan synthase had limited success.<sup>23</sup> However, by stacking the galactan synthase gene with other genes in the biosynthetic pathway, the galactose content was increased from ~25 ug/mg biomass to more than 100 ug/mg stem biomass.<sup>24,25</sup>

Strategies to increase the quantity of hexoses combined with a reduction in pentoses quantity can be particularly attractive. Although pentose sugars can be easier to liberate at near-theoretical yields in some pretreatment configurations and more difficult in others,<sup>13,26</sup> they are still a fairly universal challenge for microbial conversion, given that most promising hosts either do not natively utilize pentoses or preferentially utilize glucose.<sup>19</sup> Additionally, a non-negligible fraction of xylose is converted to furfural during common pretreatments such as with dilute-acid, which inhibit downstream microbial conversion.<sup>26,27</sup> The primary approach for decreasing pentoses is to decrease the quantity of the hemicellulose xylan, the dominant polysaccharide after cellulose.<sup>27</sup> Constitutive reduction in xylan has a severe effect on plant growth and development, primarily due to weakening and then collapse of the plant vasculature.<sup>28,29</sup> However, this penalty can be avoided by the use of cell-specific promoters, and targeting the fiber cells.<sup>25,30-32</sup> In Arabidopsis, this

resulted in a 20% reduction in xylan content, while maintaining normal growth.

### ***Modifying lignin monomeric composition and structure***

Lignin typically makes up 10-25%, and up to as much as 40%, of plant-based biomass.<sup>33,34</sup> This portion of the plant inhibits cell wall deconstruction and the lignin itself remains underutilized in subsequent biological conversion due to the heterogeneous nature of its aromatic/monomeric components. Most system-wide studies assume lignin is combusted to produce heat and electricity.<sup>27,35</sup> Valorizing lignin streams has remained a long-standing challenge for the pulp and paper industry, and thermochemical routes to high-value products have proven similarly challenging.<sup>34</sup> Given this context, plant engineering strategies can focus on reducing lignin content, achieving homogeneous lignin, or some combination of the two.

Homogenizing lignin monomeric composition is an appealing approach to achieve higher yields of aromatic monomers and simpler product mixtures after lignin depolymerization. Unusual catechyl lignin (C-lignin) present in the seed coats of vanilla<sup>36,37</sup> and various members of the Cactaceae<sup>36,37</sup> is an example of a lignin monomer that can be valorized. Such lignin, which features one single type of monomer and only  $\beta$ -O-4 interunit cleavable bonds, has been exploited to achieve high product selectivity using catalytic hydrogenolysis with specific catalyst and solvent combinations.<sup>38</sup> These

observations indicate that engineering lignin in bioenergy crops for the replacement of conventional G and S units with C units could represent a promising approach (Figure 1B). The engineering of lignin monomeric composition has been achieved in poplar, resulting in lignin containing up to 98% of S units and ~90% of  $\beta$ -O-4 linkages. Extraction of this S-rich lignin under acidic conditions, with concomitant stabilization with formaldehyde to prevent condensation reactions, allowed a high monomer yield (78%) under hydrogenolytic conditions.<sup>39</sup> Other engineering strategies in the model plant *Arabidopsis* led to important reductions in lignin content and enrichment of H units that resulted in improved biomass saccharification efficiencies,<sup>40,41</sup> and in some cases, allowed significant reductions of cellulase loadings (up 5 fold) to achieve sugar yields similar to those obtained with control non-engineered plants.<sup>42</sup> Nevertheless, these approaches have not yet been validated in bioenergy crops and implemented without compromising agronomic performance.

Alternatively, rather than modifying lignin content or its ratio of conventional G and S units, lignin structure has been successfully altered by enhancing the incorporation of exotic monomers that initiate lignin chain elongation (e.g. syringaldehyde, 4-hydroxybenzoic acid) or introduce labile linkages within the polymer (e.g., coniferyl ferulate, curcumin), resulting in increased biomass saccharification efficiencies. Such approaches have been demonstrated both in *Arabidopsis* and poplar.<sup>43-45</sup>

### ***Increasing lignin value via addition of valuable and readily-cleavable units***

*p*-Coumarate is a lignin monomer precursor that can decorate lignin through ester bonds (Figure 1C). For example, grass lignins can contain up to 10% to 15% by weight of *p*-coumarate esters.<sup>46</sup> *p*-Coumarate is particularly interesting because certain microbial hosts have the capacity to use it as a growth substrate. For highly reduced advanced biofuel targets, eliminating the need to use sugars as a carbon source can boost yields substantially. For example, using glucose alone to produce limonene (an attractive jet fuel precursor: C<sub>10</sub>H<sub>16</sub>) has a stoichiometric theoretical yield of 0.32 g limonene per g glucose.<sup>47</sup> If utilization of *p*-coumarate means that glucose is no longer needed as a source of reducing equivalents as well as carbon, the theoretical limonene yield increases to 0.45 g limonene/g glucose, and this increase in potential yield can dramatically reduce production costs.<sup>48</sup> A combination of complementary engineering approaches could be employed to enrich lignin with *p*-coumarate esters. Increasing carbon flux through the shikimate pathway by expressing a bacterial feedback-insensitive variant of the first enzyme in the pathway (3-deoxy-D-arabinoheptulosonate 7-phosphate synthase) was shown to increase 3-5 fold the amount of soluble *p*-coumarate-derived metabolites in *Arabidopsis*.<sup>49</sup> Moreover, expression of grass-specific coumaroyl-CoA:monolignol transferase - the enzyme that attaches *p*-coumarate moieties onto lignin monomers- resulted in an

increase of *p*-coumarate linked to lignin in Arabidopsis (~8% dry weight [DW]) and poplar (~2% DW).<sup>50,51</sup> Finally, the recent characterization of a plant-specific tyrosine ammonia-lyase, an enzyme that converts tyrosine into *p*-coumarate, represents another potential engineering target for increasing *p*-coumarate.<sup>52</sup> Simultaneously overexpressing these enzymes via a gene-stacking approach could enhance further increase the amount of *p*-coumarate ester-linked to lignin in bioenergy crops.

### ***Cell wall modification of pith tissue***

Minimizing the moisture content of biomass at harvest is crucial for reducing storage losses and transportation costs. This is true for bioenergy applications, and any other industry that requires long-term storage of biomass without an ensiling process (e.g. fiber production). For domestic use, industry experts note that a target of 20% moisture or lower is sufficient to ensure stability and further dry-down, while biomass meant for international export, biomass is compressed to reach closer to 15% moisture content.<sup>53</sup> Crops that senesce can reach these targets, although it is not always practical to wait for senescence before harvest and some feedstocks will not senesce, depending on the specific crop and location. Farmers will go to great lengths to ensure that biomass meant for long-term storage remains as low-moisture as possible, such as harvesting early to avoid the rainy season, even at the expense of yield. This challenge is particularly prevalent for sorghum, as it is well-known that sorghum stalks do not dry down well in

the field. Parenchyma cells in the pith are responsible for the storage of water in stems. Genetic engineering of these cells represents a promising approach to increase air porosity or hydrophobicity of pith tissues. For example, the *D* gene in sorghum, which was recently shown to encode for a transcription factor that induces programmed death of parenchyma cells, represent an interesting target.<sup>54</sup> A functional allele of the *D* gene results in dead, air-filled, pith parenchyma cells that reduce stem water content whereas non-functional alleles result in sorghum varieties that produce juicy stems. As another approach, identification and disruption (e.g., by gene-editing) of transcription factors that act as negative regulators of secondary cell wall deposition in the pith could be considered to increase lignification and thereby hydrophobicity of this tissue.<sup>55,56</sup>

### ***Improving agronomic traits***

In addition to the biomass modification approaches discussed above, including lignin engineering strategies that potentially benefit both forage and bioenergy sectors,<sup>1,2</sup> there is a wealth of agriculturally-relevant traits which, while not all specific to the bioenergy market, would be useful in ensuring consistently high-yielding, resilient crops. Since many proposed dedicated bioenergy crops are only partially domesticated, there is substantial room for improvement in many cases. These traits include drought tolerance, pathogen and pest resistance, and reduced lodging (collapse of the stem due to a plant's inability to support its own weight).

Recent research has also revealed a promising avenue for increasing photosynthetic efficiency in C3 crops: introduction of a synthetic pathway to metabolize the products of photorespiration increased yield in field-grown tobacco by as much as 40%.<sup>57</sup>

Tolerance to abiotic stresses, particularly drought, is likely to become increasingly important as rainfall becomes less predictable<sup>58,59</sup> and groundwater resources for agriculture become increasingly restricted.<sup>60</sup> Drought tolerance is a complex trait.<sup>61</sup> Much progress has been made on understanding the molecular/genetic components.<sup>62</sup> Probabilistic methods have been proposed for assessing the best combination of alleles depending on the drought scenarios,<sup>63</sup> as well as combinations of agronomic practices and genetics.<sup>64</sup> This is important, given that the ultimate impact of agronomically-relevant traits on yield and resilience cannot necessarily be evaluated while holding farming practices constant; farmers are likely to adapt their practices based on the needs of the crops in the specific environment where they are grown. Adoption of different agronomic practices may also require different physiologies to be developed in the elite cultivars, such as canopy shape and root architecture. It should also be noted that biomass engineering, at least in one case, has unexpectedly increased drought tolerance.<sup>30</sup> Drought can also alter biomass composition, for example decreasing lignin and structural glycans, while increasing ash



and extractives.<sup>59,65</sup> Biomass grown in drought conditions can also have unexpectedly negative effects on microbial growth and production.<sup>66</sup>

### ***Bioenergy crops as production platforms for chemicals***

*In-planta* production of valuable chemicals represents an appealing option to increase the value of biomass feedstocks and, in some cases, the overall titers of target compounds being produced biologically from other plant-derived components. Selecting targets that are simple to extract, are not metabolized by the microbial hosts (unless desired), and do not meaningfully increase the complexity of downstream product recovery is crucial. These chemicals produced *in-planta* can be metabolic intermediates from particular pathways implemented in microbial hosts or the target compound itself. The efficiency of *in-planta* production, assuming carbon is being diverted from polysaccharides or other biomass components, must also be weighed against the efficiency of producing the target microbially. This presents an interesting set of tradeoffs that have so far not been adequately addressed in the literature.

One promising example of a platform chemical is 4-hydroxybenzoic acid (4HBA), which is an important intermediate for several bioproducts with biotechnological applications.<sup>67</sup> Some engineering approaches have already proven to be successful for enhancing dramatically 4HBA content in plants,<sup>68</sup> and their translation to bioenergy crops could enable the manufacturing of 4HBA-based bioproducts. Protocatechuate (PCA) is another example of

value-added chemical that can be overproduced in plants for downstream microbial conversion into important compounds such as muconic acid.<sup>69</sup> A techno-economic analysis based on a sweet sorghum feedstock showed that *in-planta* production of PCA (i.e., ~10% of soluble sugars) for ultimate bioconversion into muconic acid, when combined with ethanol production from sugars, could provide a net reduction in the minimum selling price of ethanol.<sup>70</sup> Moreover, engineering strategies for the phytoproduction of muconic acid have been recently reported in the model plant *Arabidopsis*. Although achieved titers in plant biomass were low (~0.1% DW in stems), optimization of the proposed pathways in feedstock crops currently used for microbial production of muconic acid could enhance overall titers considering that muconic acid is readily extractable from lignocellulosic biomass via conventional pretreatment methods.<sup>71</sup> Lastly, a metabolic engineering strategy for overproduction of triglycerol, which can be used for biodiesel production, was recently achieved in vegetative tissues of sugarcane (4.7% DW in leaves).<sup>72</sup> It should be noted that production of bioproducts *in-planta* can have negative effects on plant growth, such as the polyhydroxyalkanoates (PHAs), a class of bioplastics.<sup>73,74</sup> Synthetic biology strategies, such as cell-, tissue- or life-stage specific expression are a promising approach to avoid these effects, as demonstrated for muconic acid production.<sup>71</sup>

## **Pretreatment and saccharification of lignocellulosic biomass**

Biomass pretreatment is a crucial step in the lignocellulosic conversion process. The overall function of pretreatment is to increase the accessibility of biomass-deconstructing enzymes to hemicellulose and cellulose to enable efficient depolymerization into fermentable sugars (Figure 2).<sup>75,76</sup> Historically, pretreatment has been one of the most expensive unit operations within the biomass conversion regime and, over the last two decades, many pretreatment techniques have been developed for biomass depolymerization and fractionation.<sup>77,78</sup> Despite technological advancements that have improved yield and overall economics, there is still much to be done to maximize process efficiency and reduce costs. There are several potential focus areas for improving these metrics, including maximizing sugar yields, valorizing lignin, and reducing the number of unit operations.

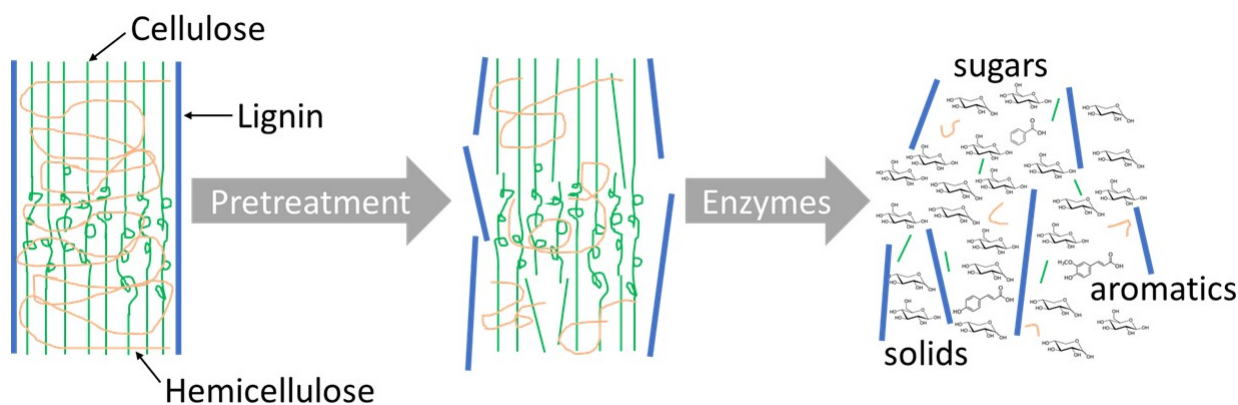


Figure 2. Schematic of pretreatment impact on lignocellulosic biomass

Pretreatment methods can be classified into three major categories: physical, chemical, and biological. In addition, two or more pretreatment

techniques from the same or different categories can be combined to generate a wide variety of different methodologies.<sup>79</sup> The real challenge is to determine which of these many strategies provides effective, efficient, and economical biomass depolymerization. An ideal pretreatment strategy can be characterized by several salient features: minimizing energy input, reducing biomass losses, having high biocompatibility with enzymes and microbes used downstream, and reducing biorefinery costs.<sup>80,81</sup> In addition, several other factors should be considered, like whether the process includes the possibility of lignin valorization, the ease of solvent recycling where required, ease of catalyst reuse, and subsequent downstream processing.<sup>34,82</sup> While developing a pretreatment technology, it is necessary to comprehensively consider all the aforementioned criteria.

Of the three main pretreatment categories, chemical pretreatment has been investigated the most.<sup>83-85</sup> Chemical pretreatments have been successfully implemented for many years in the pulp and paper industry for production of paper by delignification of cellulosic materials, and that work has laid the foundation for investigating this approach for developing lignocellulose conversion technologies.<sup>77,86</sup> The most common chemical pretreatment methods that have been investigated are based on exposure of biomass to alkali, acid, ammonia, organic solvents (organosolv),<sup>87-89</sup> and ionic liquids.<sup>89,90</sup> Historically, the primary goal of the majority of these methods has been improving cellulose depolymerization by removing lignin and/or

hemicellulose or reducing the degree of cellulose polymerization/crystallinity. However, given the biomass feedstocks currently available, any economically viable bioenergy and/or bioproduct route must make use of hemicellulose and lignin. Therefore, pretreatment research needs to focus on methods that enable complete hexose and pentose conversion, as well as provide a route to lignin valorization.

### ***Process integration of pretreatment with saccharification and fermentation***

Driving down the cost of biomass deconstruction requires a holistic approach that takes into account the intended downstream conversion. Many, if not most, chemical pretreatment methods involve chemicals or reaction conditions (temperature, pH) that are incompatible with enzymes and/or host microbes used downstream. Depending on the value or price of the pretreatment chemical, it may also be necessary to achieve high rates of recovery and recycling.<sup>10</sup> Separation and washing of pretreated biomass can reduce toxicity to downstream unit operations, and sequential pretreatment, saccharification, and bioconversion enables the use of highly optimized, commercially-available enzymes and strains, maximizing the efficiency of each unit operation. However, these benefits are counterbalanced by increases in process complexity, carbon lost to waste streams, and an overall reduction in process intensity.<sup>91</sup> Due to these inherent tradeoffs, continued development of efficient, resilient, and biocompatible deconstruction

technologies, paired with development and testing of more robust microbial hosts, is required to fully unlock the benefits of process integration.

Elimination of solid-liquid separation following both pretreatment and saccharification enables streamlined processing, with no intermediate losses and all separations consolidated downstream of fermentation. Successful implementation of separation-free bioprocessing requires a biocompatible pretreatment strategy, coupled with robust enzymes and host strains capable of withstanding toxicity introduced during biomass pretreatment. For ionic liquid-based pretreatment, elimination of these upstream separations creates substantial techno-economic benefits, potentially reducing total production cost by 40% due to reductions in water-washing of the pretreated biomass.<sup>92</sup> To fully leverage the benefits of an integrated process, significant improvements have been made in separation free “one-pot” processing using bio-derived ionic (“bionic”) liquids,<sup>10,92-95</sup> ionic liquid-tolerant enzyme cocktails,<sup>96</sup> and ionic liquid-tolerant strains.<sup>97,98</sup> A number of studies have now demonstrated successful bioconversion of whole slurry hydrolysate following saccharification, including conversion of ionic liquid-pretreated<sup>13</sup> and AFEX-pretreated<sup>99,100</sup> biomass.

Elimination of separations between saccharification and bioconversion creates opportunities to fully consolidate these two operations, through configurations such as simultaneous saccharification-fermentation (SSF) and

consolidated bioprocessing (CBP). These unit operations operate at ambient pressure over similar time spans (1-5 days), temperatures (30-50°C), and pH regimes (pH 4-5). Due to the long residence times required in each step, coupling these operations is a compelling way to both simplify and intensify the conversion process. As compared to sequential, separation-free processing, SSF and CBP both require full compatibility between saccharification and fermentation.

If commercial enzymes are used to saccharify lignocellulose, SSF can be implemented and the resulting lignocellulosic hydrolysate that then be converted to biofuels and bioproducts by a wide variety of organisms capable of consuming these carbon sources.<sup>93,101-104</sup> However, the use of commercial enzymes can add significantly to the overall cost within a biorefinery.<sup>105</sup> To avoid these costs, CBP can be performed by organisms like *Clostridium thermocellum* that can deconstruct and convert pretreated lignocellulose without the aid of exogenous enzymes.<sup>106-108</sup> Size reduction techniques have also been tested and yielded promising results in the context of CBP, such as in-situ ball milling.<sup>109</sup> While CBP promises significant reductions in enzyme cost, the technology is currently limited by reduced rates of saccharification, and by the relatively small number of well-characterized organisms with the demonstrated ability to directly deconstruct lignocellulose. This factor limits CBP to process conditions compatible with these organisms (e.g. anaerobic), and limits the range of

bioproducts that can be produced leveraging their metabolism.<sup>108,110</sup>

Therefore, the decision to implement SSF or CBP is highly dependent on the desired process and bioproduct.

### ***Pretreatment process intensification***

Primary methods for process intensification in the biomass to biofuels pathway include process consolidation (as described above) and improvements in the volumetric throughput of biomass. Increasing solids loading in biomass deconstruction enables increased sugar concentrations in the resulting hydrolysate, translating into higher volumetric productivity, higher product titers, and reduced separation and purification costs.<sup>10,13,111,112</sup>

Maximum biomass loading is dictated by rheological challenges and by the osmotic tolerance of the biocatalyst. Osmotic inhibition triggered by high sugar concentrations can be overcome through use of osmotically tolerant strains,<sup>113,114</sup> or through implementation of an SSF process configuration in which sugar consumption is matched to the rate of saccharification.

Rheological challenges during saccharification can be addressed via fed-batch configurations, in which pretreated biomass is introduced gradually to the saccharification vessel.<sup>10</sup> Moving forward, a significant opportunity exists for synergistic advances coupling feedstock engineering, enzyme engineering, and high-solids biomass deconstruction. In a fed-batch saccharification configuration, maximum hexose and pentose concentrations are limited by the accumulation of insoluble recalcitrant biomass composed of residual lignin, hemicellulose, and cellulose. Continued reductions in or



modifications of the lignin content of energy crops, coupled with improvements in saccharification efficiency, will serve to drive down the fraction of recalcitrant biomass, enabling a synergistic increase in biomass loading, sugar concentrations and ultimately in bioproduct titers.

### **Catabolism and conversion**

As the progress in renewable biomass deconstruction and processing produces cleaner streams of a range of sugars and lignin-derived intermediates, the downstream bioconversion must keep pace with this evolving source of carbon and energy. An economically-viable process requires that microbial systems be capable of utilizing all components of this feedstock as well as convert it efficiently to the desired final product. Uptake and metabolism of the carbon intermediates released by the deconstruction process can be approached using several strategies that result in different benefits and tradeoffs. Based on the microbial host selected or the metabolic engineering undertaken, a range of carbon intermediates can be catabolized, co-catabolized, and a large number of final compounds can be produced.

### ***Catabolism of plant-derived substrates***

Bioconversion of plant-derived substrates have focused on the conversion of glucose, a hexose sugar, through central metabolism. The classical host for glucose conversion is *Saccharomyces cerevisiae*, which can be cultivated in sugar solutions that are >100 g/L.<sup>115</sup> *Zymomonas mobilis*, a bacterium which has been isolated from alcoholic beverages, grows at comparable levels of

sugar to *S. cerevisiae*.<sup>116</sup> A key limitation of these hosts for bioconversion of plant-derived biomass is their inability to natively metabolize xylose, a pentose sugar, therefore substantial efforts have been undertaken to engineer *S. cerevisiae* and *Z. mobilis* to convert xylose through the oxidative pentose phosphate pathway.<sup>117,118</sup> *E. coli*, which is widely used for metabolic engineering, can grow on both glucose and xylose (though natively with a preference to glucose) but tolerates lower sugar concentrations than *S. cerevisiae* and *Z. mobilis*.<sup>119</sup> Because of the impact this lower tolerance has on the overall biorefinery economics, forward-looking techno-economic modeling efforts tend to favor other hosts; for example, the widely-cited report by the National Renewable Energy Laboratory (NREL) assumes a glucose and xylose-utilizing *Z. mobilis* strain.<sup>27</sup>

Recently, strategies have been developed to expand the range of substrates from plant biomass beyond sugars derived from plant polysaccharides. This is important, particularly given the recognition that lignin-derived compounds must be diverted to higher-value application than combustion for on-site heat and electricity generation. Some soil bacteria harbor pathways to convert lignin-related monoaromatics (*p*-coumarate, ferulate, vanillate, hydroxybenzoate) to central metabolic intermediates.<sup>120</sup> These monoaromatics are funneled through peripheral pathways to the beta-ketoadipate pathway, which converts the monoaromatics through

protocatechuate to acetyl-CoA and succinyl-CoA, where they enter the TCA cycle.

Strategies have been developed to depolymerize lignin and convert the resulting monoaromatics (such as *p*-coumarate) to polyhydroxyalkanoates in *Pseudomonas putida* and triacylglycerides in *Rhodococcus opacus*.<sup>14,121</sup> This catabolic process in *P. putida*, which proceeds through the beta-ketoadipate pathway, has also been engineered to convert monoaromatics to *cis,cis*-muconic acid, which is a precursor to adipic acid, a widely used polymer precursor.<sup>122</sup> An engineered *Novosphingobium aromaticivorans* DSM12444 converts plant-derived aromatic compounds into 2-pyrone-4,6-dicarboxylic acid, which is a potential polyester precursor.<sup>123</sup> Other microbial hosts with innate ability to catabolize broader ranges of plant biomass derived components, such as the fungal host *Rhodosporidium toruloides*<sup>104</sup> and *Corynebacterium glutamicum*<sup>124</sup> provide promising host for the production of biofuels. Further progress in the conversion of aromatics from biomass to biofuels and bioproducts will benefit from engineered lignin that can be depolymerized through either chemical or biological means to a defined set of aromatics that can be converted through bacterial pathway. Methods have also been developed to convert plant-derived proteins to products by engineering amino acid catabolic pathways in *E. coli*.<sup>125,126</sup>

Future metabolic engineering strategies for the catabolism of lignocellulose may be enhanced by engineering metabolic pathways to maximize the conversion of carbon in the plant to products. A commonly used strategy for improving the carbon conversion of glucose metabolism is to express bifidobacterial phosphoketolase, which shunts glucose metabolism by generating acetyl phosphate from pentose phosphate pathway intermediates (fructose-6-phosphate, xylulose-5-phosphate).<sup>127,128</sup> Acetyl phosphate is converted to acetyl-CoA by phosphoacetyltransferase. The bifidobacterial shunt bypasses central metabolism and lowers carbon loss as CO<sub>2</sub> from the conversion of pyruvate to acetyl-CoA during conventional glycolysis. Application of the bifidobacterial shunt in glycolysis could lead to 83% conservation of carbon from glucose, in comparison to a 66% conversion for intermediates that proceed through decarboxylation catalyzed by pyruvate dehydrogenase. An additional strategy segregates glucose metabolism from the metabolism of other plant derived products. In this method, nonphosphorylative metabolisms for pentose sugars (xylose, arabinose) and galacturonate, derived from pectin, were engineered into *E. coli*. These pathways enabled high titer and yield production of 1,4-butanediol,<sup>129</sup> which is derived from central TCA (tricarboxylic acid) cycle intermediates. The bioproduct 1,4-butanediol was produced from xylose (12.0 g/L, 43% yield), arabinose (15.6 g/L, 37% yield) and galacturonate (16.5 g/L, 70% yield). In this scenario, glucose is used for cell growth while the other plant components are used for bioproduct formation. Although the best approach

may vary depending on hydrolysate composition and target molecules, new conversion strategies that combine sugar and aromatic metabolism will improve the carbon efficiency of biomass conversion and lower the cost of biofuel and bioproduct formation.

### ***Conversion to bio-based fuels and products***

Due to several large projects aiming to generate bulk platform chemicals and fuels via bioconversion (e.g. the Bioenergy Research Centers in the United States, FAPESP Bioenergy Research Program in Brazil, and the European Technology and Innovation Platform Bioenergy), the last decade has seen significant improvements in the ability to engineer a range of microbial hosts to generate a broad set of compounds.<sup>127,130,131</sup> These compounds represent many different biosynthetic routes and underscore the potential of converting biomass-derived carbon sources into final products. However, while most of these proof-of-concept systems are reported with at least low grams per liter titers, they have not yet been examined for performance in terms of rate or yield, both of which are important for the economics and life-cycle greenhouse gas footprints. Determination of yields requires use of defined media where the mass balance can be calculated and determination of rate requires fed-batch or continuous cultivation that are also not the norm at the research laboratory development level. This leaves a considerable gap between results reported in the literature and any quantitative understanding of the performance, and long-term economic

viability, of these systems. A few representative/select examples of both fuels and platform chemicals that have been reported with some relevant performance data are listed in Table 1.

Even in cases where relevant performance data is reported, most pertinent examples of conversion to advanced biofuel and bioproduct pertain only to the hexose sugar, glucose. Many efforts have focused exclusively on optimising the use of cellulosic glucose for the production of ethanol<sup>106</sup> and other advanced biofuels.<sup>15</sup> However, as is evident from the discussions in previous sections, deconstructed plant biomass will contain a far more diverse range of compounds, including other hexose sugars (e.g galactose), pentose sugars (e.g xylose), and lignin-derived aromatics. The use of xylose and other intermediates have been demonstrated but are not as advanced as the processes using glucose. Xylose utilization is the most obvious target after glucose, as it is typically the second-most abundant single component in herbaceous biomass hydrolysates.

Recent examples of developing fungal systems for conversion of pentose sugars to biofuels include coupled use of cellulosic acetic acid and xylose in an engineered *S. cerevisiae* for bioconversion to ethanol.<sup>132</sup> Alternative approaches have used fungal hosts that are natively capable of utilizing mixed carbon sources, such as the use of *Rhodospiridium toruloides* to produce the jet-fuel precursor bisabolene at 2.2 g/ L from sorghum

hydrolysate in a 20-L one-pot process.<sup>13</sup> Equivalent approaches in bacterial systems involve engineering the ability to utilize xylose in *P.putida*, a host with other desirable phenotypes<sup>133</sup> or in *Clostridium acetobutylicum* for acetone/butanol/ethanol (ABE) production from xylose.<sup>134</sup> As discussed previously, some feedstock engineering strategies to increase the ratio of hexose to pentose sugars may increase galactose rather than glucose. Though some examples exist,<sup>135</sup> other hexose sugars such as galactose are not the preferred carbon source in laboratory-stage development. These examples provide the foundation for future conversions from mixed carbon sources, and highlight the need for further work.

Some of the most prominent progress in developing biological systems for bioconversion has been in the production of non-natural final products or natural products at levels that are not made naturally. Here, for biofuels and platforms chemicals, targets have ranged from aviation fuels<sup>48,136</sup> and gasoline targets<sup>137</sup> to polymer precursors<sup>138</sup> and other bulk chemicals.<sup>139</sup> Several recent reviews focus the range of compounds that can be made via microbial conversion.<sup>140</sup> In addition to the development of the biosynthetic pathways, optimization of the ancillary metabolism and the host are also critical and can be used to maximize the conversion yield. Interesting examples of pathway developments include the exploration of multiple pathways to make the same final product,<sup>106</sup> using metabolic bypasses to reduce cofactor using steps<sup>127</sup> or modifying steps that generate inhibitory

intermediates.<sup>141</sup> Improving the tolerance of a microbial system to precursor or final compounds is an important aspect,<sup>142</sup> as are demonstrations for titers at higher scales and in bioreactors.<sup>13,143,144</sup> Non-targeted methods involve the use of lab evolution<sup>145</sup> and high throughput methods to seek optimal pathway architectures.<sup>146</sup>

Industrial teams have achieved successful implementation of microbially produced bulk products such as ethanol (Braskem, Praj), 1,3-propanediol (Dupont), lactic acid (Cargill), trans- $\beta$ -Farnesene (Amyris), and 1,4 butanediol (Genomatica). In this review we model the potential of the next generation of commodity chemicals and fuels that show similar promise. It is also worth mentioning that the renewed vigor of the biotech sector brought about by data driven sciences, in turn facilitated by high throughput synthetic biology (Zymergen, Ginko Bioworks, Riffyn) - have important socio-economic impacts.

Table 1. Examples of promising fuels and commodity chemicals

<b>Compound</b>	<b>Uses</b>	<b>Microbes</b>	<b>Maximum titer</b>	<b>Scale</b>	<b>Carbon source</b>
Isopentenol <sup>147</sup>	Biogasoline Platform to isoprene	<i>E. coli</i>	2.2 g/L from 10g/L glucose		2% Glucose
Methyl Ketones <sup>148</sup>	Biodiesel, fragrances	Various bacteria	5.4 g/L	2L	Glucose and Hydrolysate
Fatty	Biodiesel	Fungal	6 g/L	2L	Glucose and



Alcohols <sup>103</sup>	Commodity chemical	systems			Hydrolysate
Isobutanol <sup>149</sup>	Fuel, platform chemical	<i>E. coli</i>	50 g/L in 72 h. From 55g/L glucose	1L	Glucose With product removal using gas stripping
Ethanol <sup>106</sup>		<i>Clostridium</i>	22.4 ± 1.4 g/L ethanol		from 60 g/L cellulose
Short chain Ketones <sup>137</sup>	Novel branching in gasoline target Ideal RON MON* numbers were shown. Platform chemicals	<i>S. albus</i>	> 1 g/L	2L	Pure sugars and Hydrolysate

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\*RON: Research Octane Number, MON: Motor Octane Number

### **Integrating Research Approaches for Cost and Emissions Reductions**

Each of the scientific research approaches described in previous sections offers advantages over the current state of technology and many represent fruitful avenues of research that are likely to yield basic scientific discoveries and practical process improvements as the work progresses. However, a constant challenge, even for highly-integrated interdisciplinary research programs, is the assembly of individual research efforts into broader, synergistic strategies that convert biomass to fuels and products. For example, approaches that work well in model plants or even greenhouse experiments with bioenergy crops may result in different results when tested in the field.<sup>150</sup> Within the biorefinery, key unit processes such as separations,

are often not explored until scale-up and commercialization efforts begin, and this makes estimating system-wide impacts more challenging.

Researchers must depend largely on simulations to estimate the downstream impacts of variations in feedstock composition, increased solids loading, differing hydrolysate fractionation requirements, or varying end product(s). For example, Humbird et al.<sup>151</sup> used selected experimental data to develop an empirical correlation between glucose yield and solids loading, enzyme loading, and temperature. Studies that make use of currently costly chemical inputs, such as ionic liquids and  $\gamma$ -valerolactone, have simulated the potential impacts of high rates of recovery and recycling.<sup>10,152</sup>

Separations can be even more challenging in the context of bioproducts, where product purity specifications upwards of 99.9% and multiple azeotropic mixtures add significant process complexity. Both Wu et al.<sup>153</sup> and Markham et al.<sup>154</sup> simulated ethylene production routes with potentially costly product recovery systems. Although simulations can offer valuable insights, the research community's reliance on them introduces a level of unavoidable uncertainty for early-stage research.

In the face of inherent uncertainty that accompanies the translation of bench-scale research to large-scale, fully-designed bioenergy and bioproduct systems, the research community continues to pursue a wide variety of strategies with an eye towards cost reduction and minimized environmental impacts. Some of the research approaches outlined in this review are likely

to offer net benefits regardless of the specific biorefinery configuration or targeted end products, whereas others may help to minimize costs and improve efficiency in some configurations but will be neutral or counterproductive in others. With some exceptions, most recent and ongoing work on biochemical routes to fuels and products can be categorized as falling into one or more of four broad strategies: 1) maximize sugar conversion to a single product, 2) utilize diverse carbon sources for producing a single product, and 3) convert lignin to high-value products, and 4) fractionate the hydrolysate to derive maximum value from each component (Figure 3). We attempt to evaluate the compatibility of research strategies spanning feedstock engineering, deconstruction, and catabolism and conversion, with dual objectives of reducing costs and net greenhouse gas emissions.

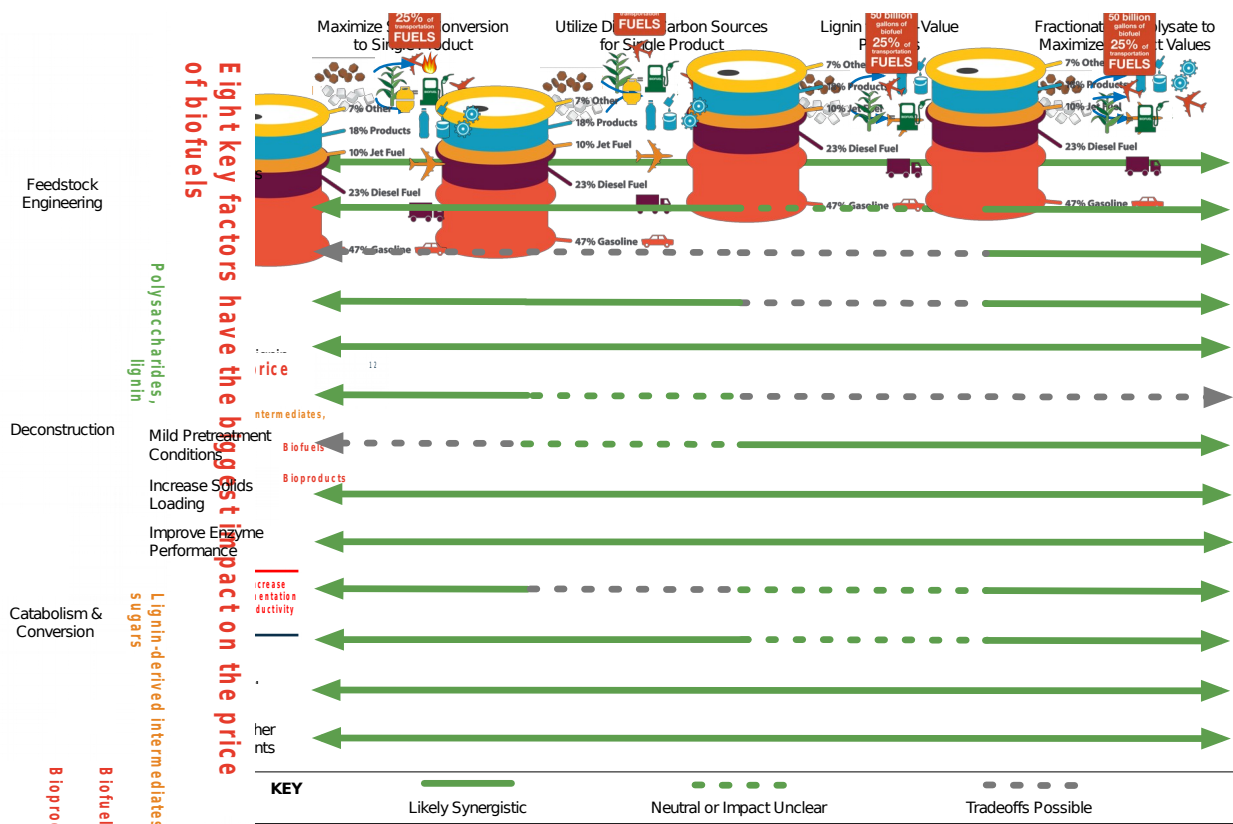


Figure 3. Compatibility of Selected Research Approaches with Biorefinery Process Strategies

### Engineering Biomass Feedstocks

Across the broad range of feedstock engineering research being undertaken, it is likely that any significant improvement in agronomic traits is likely to yield a net benefit, even if there is a minor negative impact on composition. Increased yield, reduced lodging, reduced moisture at harvest, and improved resilience to biotic and abiotic stressors will all impact the market price of biomass, which is the single largest operating cost for cellulosic biorefineries. For example, in a recently simulated biomass sorghum-to-jet fuel production facility, the biomass feedstock comprised 47% of the total materials cost and

23% of the overall minimum fuel selling price.<sup>48</sup> In the case of corn stover-to-cellulosic ethanol, the corn stover feedstock is responsible for 34% of the minimum selling price of ethanol.<sup>27</sup> Improvements in yield and/or reduction in crop losses will also result in GHG emissions improvements, particularly if nitrogenous fertilizer inputs can remain constant. Of course, this broad generalization will not apply to some agronomic traits. For example, if only cost and GHG emissions are of interest, improvements in water use efficiency are likely to have a minimal impact. The net economic impact of deficit irrigation regimes is uncertain and very crop, region, and water pricing-dependant.<sup>155</sup>

In terms of feedstock engineering approaches aimed at altering composition, the system-wide impacts depend on any likely agronomic tradeoffs (e.g. if decreasing lignin content results in increased lodging or reduced yield) and the compatibility with downstream conversion routes. Tailored, homogenized lignin appears to have little downside, given the potential improvements in deconstruction efficiency and increased ease with which a more homogeneous lignin stream can be used as a carbon source for host microbes. Increasing the ratio of hexose to pentose sugars is another nearly-universally advantageous strategy, as long as it does not negatively impact agronomic traits important for feedstock production costs and environmental impacts. However, to realize the intended yield improvements, any non-glucose hexoses (galactose or fructose) would ideally be easily utilized by

selected host microbe(s) or at least preferable to the pentoses being replaced. Given the limited research on galactose utilization to-date, this is an area requiring further study.

Another feedstock engineering strategy dependent on the effectiveness of research efforts downstream is lignin minimization. In the context of the current state of technology, reducing the lignin content in bioenergy feedstock crops is very likely to be advantageous for improving sugar and downstream product yields. Increasing the ratio of carbohydrates (hexoses and pentoses) to overall plant dry matter from 59 to 70% alone results in a 12% reduction in minimum fuel selling price for isopentanol and a 9% decrease in GHG emissions.<sup>156</sup> However, in scenarios where lignin-derived monoaromatics become either an energy source for host microbes or a precursor to valuable co-products, reducing lignin content may no longer be desirable. It may also be challenging to gauge the agronomic impacts of lignin minimization. Field studies of sorghum indicate that low-lignin mutants (e.g Brown Midrib) do suffer from increased lodging relative to other varieties.<sup>157</sup> Similarly, accumulation of *in-planta* intermediates may offer value in specific biorefinery configurations, although the outcome is dependent on where and how carbon is diverted to generate these products and the net impact on downstream separations. Limonene is an interesting example to explore, as it can be accumulated in some plant tissues at relatively high concentrations<sup>158</sup> and can also be microbially produced from

sugars.<sup>136</sup> The question of whether engineering a bioenergy crop to accumulate limonene *in-planta* can reduce costs will depend on the net impact on biomass yield, plant carbohydrate content, the stability of limonene during deconstruction, and the maximum achievable limonene yield from sugars.

### ***Deconstructing Biomass for Downstream Conversion***

Efficient biomass deconstruction to sugars, lignin intermediates, and other monomers is central to achieving cost-competitive and sustainable biofuel production. The deconstruction strategy is often the driving factor in the overall process configuration because the solvents used and reaction conditions impact all downstream unit operations. It is also inextricably linked to choice about downstream conversion, since individual components of the hydrolysate may be useful carbon sources or undesirable inhibitors depending on the host microbe(s) employed during bioconversion. Across all system-wide strategies outlined in Figure 3, increasing solids loading and improving enzyme performance during deconstruction result in lower costs and emissions. Moving from 20% solids to 30% can reduce the minimum fuel selling price for an ionic liquid-based isopentenol production process by 8% and cut GHG emissions by more than 30%.<sup>156</sup>

Establishing broad categories that appropriately capture the wide variety of pretreatment and saccharification strategies, from dilute-acid to steam

explosion, ionic liquid pretreatment, and CBP, is challenging and each has a unique set of tradeoffs. For the purposes of this review, we separate strategies on the basis of harsh vs. mild pretreatment conditions, where harsh pretreatment strategies are assumed to generate higher sugar yields but may degrade lignin and other plant-derived intermediates in unpredictable or undesirable ways. For example, steam explosion operates at temperatures from 180 to 240°C.<sup>159</sup> Acid pretreatment can span both categories, ranging from 60 to 200°C, and the same is true for AFEX, where high sugar yields were reported for 140°C at relatively low pressures.<sup>99,159</sup> These conditions are well-suited to biorefinery configurations that aim to maximize the conversion of sugars, and may also be appropriate for microbial hosts capable of utilizing a wide variety of carbon sources, such as *R. toruloides*, as long as the process generates minimal inhibitory compounds. Mild pretreatment conditions, conversely, may have lower sugar yields but leave other components intact. Ionic liquid pretreatment operates close to ambient temperatures, although the resulting xylose yields are generally lower than compared to dilute acid or ammonia pretreatment.<sup>99</sup> Ultimately, the ideal deconstruction strategy is highly dependent on the host and molecular targets to be produced downstream.

### ***Converting Intermediates to Bio-Fuels and Products***

Much progress has been made in the catabolism and conversion of plant-derived sugars, aromatics, and other components. In particular, the



emphasis on pathways and hosts that enable the use of lignin-derived monomers and other compounds present in hydrolysates are likely to improve costs and reduce emissions regardless of the specific biorefinery configuration. In a consolidated process with little or no fractionation upstream, the microbial host can be used to convert every carbon source available to a single product. This simplifies the process and reduces carbon losses, but can present a challenge if any high-value solvents are inadvertently metabolized before they can be recovered. For example, the ionic liquid [Ch][Lys], can be metabolized by the *Pseudomonas* species.<sup>160</sup> Utilizing lignin also allows biorefineries to avoid the need for on-site combustion or export for co-firing with other solid fuels. Eliminating the need for a solids boiler on-site and instead relying solely on biogas produced during on-site wastewater treatment (plus any supplemental natural gas required) can reduce capital costs for the combined heat and power section by more than 60%.<sup>35</sup>

The question of whether utilizing a diverse range of carbon sources is preferential to optimizing for the use of sugars alone depends on the feedstock composition as well as tradeoffs in titer, rate, and yield. For herbaceous feedstocks, pentose sugars are the second-most abundant substrate behind glucose. For example, xylan alone makes up 15-23% of corn stover's dry mass compared with glucan at 27-38% and lignin at 11-18%.<sup>27</sup> Crop residues are unlikely to be dramatically altered for bioenergy

purposes if those engineering strategies impact the primary product (e.g. corn grain). However, if next-generation herbaceous bioenergy feedstocks can be engineered to further reduce lignin content to < 10%, there may be at least some target molecules for which the most practical option is to optimize solely for maximal yield from sugars. In these cases, *S. cerevisiae* and *Z. mobilis* are likely to be attractive hosts because of their ability to tolerate higher sugar concentrations than *E. coli* and the efforts that have already been undertaken to engineer them for co-utilization of pentoses. Several common strategies exist for engineering or improving pentose utilization in microbes, and future efforts may expand the breadth of hosts that can convert hexoses and pentoses.<sup>161</sup> That said, while both native and engineered pentose utilizers can effectively consume xylose, improving the hexoses-to-pentoses ratio in herbaceous feedstocks still offers significant process benefits due to the difficulty of achieving simultaneous rather than sequential pentose and hexose consumption,<sup>119</sup> and due to the reduced yields for many bioproducts when utilizing pentose sugars.

In contrast with herbaceous material, lignin is generally more abundant than hemicellulose in woody feedstocks, with commonly studied feedstocks such as *Eucalyptus saligna*, Monterey Pine, and poplar varieties ranging from 21 to 29% lignin on a dry mass basis.<sup>162</sup> Poplar varieties, for example, have lower xylan, ranging from 13 to 19% of dry mass.<sup>162</sup> Minimizing lignin will remain an important goal for these feedstocks, barring any breakthroughs

that result in very high-value lignin-derived co-products, but the higher starting values for lignin suggest that any viable strategy for converting woody biomass must make use of lignin for either fuel or bioproduct production. In strategies with minimal or no fractionation of the hydrolysate, *R. toruloides*, which utilizes mixed carbon sources natively or *P. putida*, which has been engineered to utilize lignin-derived aromatic monomers, may be the most appropriate hosts to pursue. However, if some substrates are utilized more slowly than others, and the net impact of using mixed hydrolysates can be a significantly increased residence time, this will increase capital costs and GHG emissions. The power demand associated with bubbling air through bioconversion reactors during aerobic conversion is one of the primary contributors to the GHG footprint of advanced microbially-produced fuels.<sup>163</sup> For example, reducing bioconversion time from 63 to 36 hours has a minimal impact on costs but cuts the GHG footprint of IL-based isopentenol production by 40%.<sup>156</sup>

Regardless of feedstock type, another option is to use hosts optimized for the catabolism and conversion of specific hydrolysate components. Certainly there are far more hosts and metabolic pathways available for the conversion of glucose to novel fuels and products, whereas the options for utilization of lignin-derived monomers and pentoses are more limited. Perhaps a host such as *R. toruloides* should be used to convert lignin intermediates and pentoses, leaving glucose available for conversion using a

different host and pathway. Lignin is already recovered through solid-liquid separation in most process configurations documented in the literature,<sup>10,27,48,151</sup> although the question of where and how lignin will be deconstructed to monomers or other easily-catabolized intermediates is not as well-established. Separation of hexose from pentose sugar streams, while less common, is far from new given the interest in using xylose for xylitol production (a low-calorie sweetener).<sup>164,165</sup> In a dilute acid configuration, for example, liberated xylose could be separated after pretreatment in a solid-liquid separation, with remaining solids going to simultaneous saccharification and bioconversion before lignin is separated through a second solid-liquid separation step. The only difference in this process relative to what is described in Humbird et al.<sup>27</sup> is the pentose sugar recovery after pretreatment. A few studies have actually explored utilizing only hemicellulose from sugarcane bagasse due to the ease of deconstruction, sending cellulose for on-site combustion or other lower-value applications.<sup>166,167</sup> However, as Zhang et al.<sup>168</sup> point out, achieving high xylose yields may require not only solid-liquid separation, but also washing and post-hydrolysis to ensure breakdown of remaining xylooligomers. This kind of fractionation also means that biorefineries must either take in a very consistent feedstock or risk under- or over-sizing equipment for each conversion process if the proportion of lignin, cellulose, and hemicellulose vary seasonally and annually. These kinds of system-wide tradeoffs are

important to consider, but the optimal approach may vary on a case-by-case basis depending on the location and feedstock mix available.

## Conclusions

Over the last decade, impressive advancements have been made in fundamental research related to tailoring biomass feedstocks for bioenergy and bioproduct applications, efficiently deconstructing plants to useful intermediates, and converting those intermediates to a wide range of valuable products. Some advanced conversion strategies are already being implemented using first-generation starch and sugar feedstocks such as ethanol, 1,3-propanediol, lactic acid, trans- $\beta$ -Farnesene, and 1,4 butanediol. In the long term, low oil prices and competition from other renewable energy carriers mean that a successful bioeconomy must make use of every carbon source available and generate renewable fuels and products for markets in which few or no viable alternatives exist. Achieving this goal requires strategies that reach far beyond the traditionally-envisioned cellulosic ethanol facilities.

In response to this challenge, the research community continues to pursue a broad suite of approaches, beginning with feedstock traits that make cultivation, harvest, and long-term storage of high-yielding biomass as low-cost and low-input as possible. Tailoring feedstock composition to increase the relative proportion of carbohydrates and favor more easily-converted substrates, such as hexose sugars and homogenous lignin, offers significant reductions in cost and emissions across a wide range of biorefinery configurations. Reducing overall lignin content also facilitates the

intensification of otherwise costly deconstruction, allowing for high solids loading, lower solvent inputs, and milder conditions. Simpler hydrolysates with lower concentrations of inert materials that otherwise inhibit saccharification and bioconversion will benefit biorefinery configurations across the spectrum, from consolidated one-pot processes to extensively fractionated, targeted conversion of each plant-derived component. Finally, utilization of a more diverse range of carbon sources beyond glucose, and conversion of those substrates to fuels and products that offer performance advantages or meet the needs of a market without other alternatives, will enable biorefineries of the future to derive the greatest value from every tonne of biomass arriving at the facility gate.

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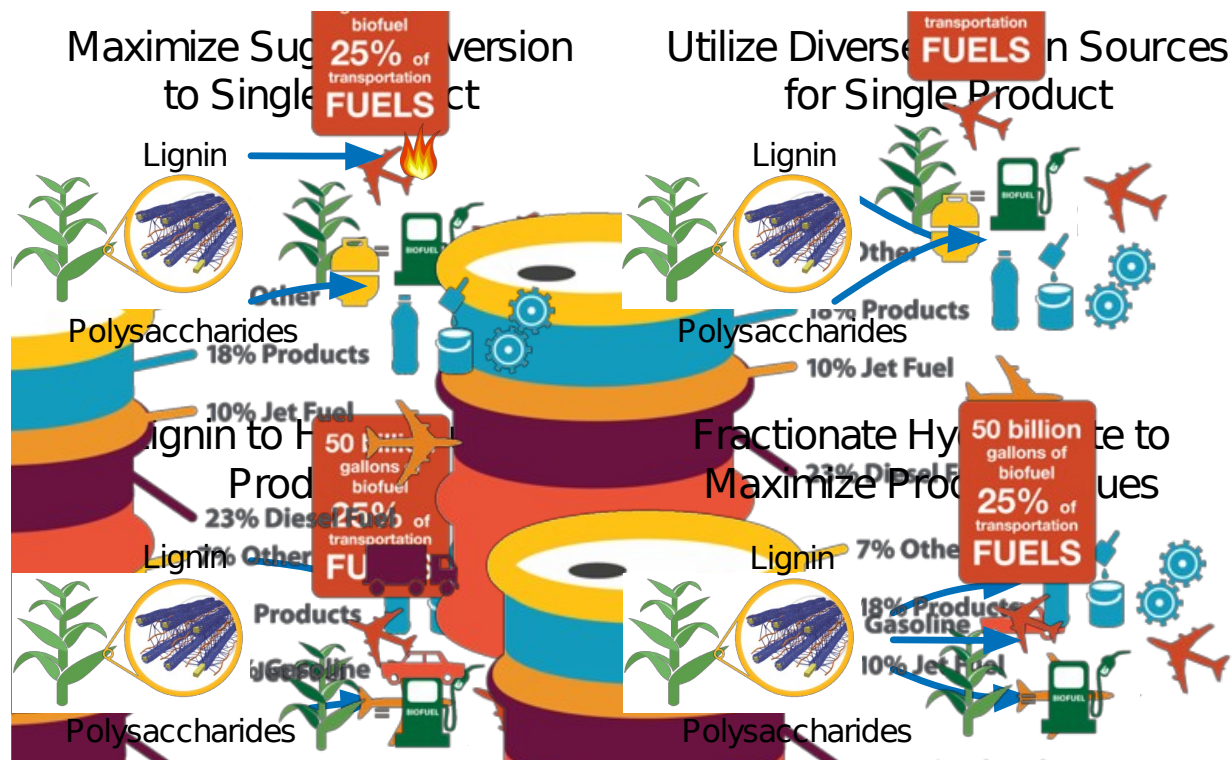
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**TOC/Abstract Graphic:**



**Synopsis**

A more competitive, efficient, and sustainable bioeconomy requires tailored feedstocks, intensified deconstruction, and robust omnivorous microbial hosts.

## **Author Photos and Biographies**

Nawa Raj Baral: Nawa Raj Baral is a postdoctoral fellow in Life-cycle, Economics, & Agronomy Division at the Joint BioEnergy Institute, Lawrence Berkeley National Laboratory. His primary research interests include techno-economic analysis and life-cycle assessment of biomass conservation into biofuels, biochemicals, and bioproducts.





Eric R. Sundstrom: Eric Sundstrom is a Research Scientist at Lawrence Berkeley National Laboratory, where he serves as a principal investigator at the Advanced Biofuels Process Development Unit (ABPDU). At ABPDU, his research focuses on biomanufacturing, with an emphasis on utilization of lignocellulosic and waste feedstocks for production of novel biofuels, biochemicals, biomaterials, and foods. Prior to joining ABPDU in 2015 he served as Principal Research Engineer at Algae Systems, where he developed new technologies coupling algal wastewater treatment with biofuel production. Eric received his Ph.D. in Environmental Engineering from Stanford University in 2013, and holds M.E. and B.S. degrees in Civil and Environmental Engineering from Rice University.



Lalitendu Das: Lalitendu Das received his PhD degree in 2015 from North Carolina State University, Raleigh (USA) for his research in catalytic valorization of lignin to value added chemicals. He then spent two years as a postdoctoral scholar at the University of Kentucky, Lexington (USA) where he worked on catalytic depolymerization of lignin in ionic liquids and deep eutectic solvents. Since April 2018, he is a postdoctoral fellow at Sandia National Laboratories and US Department of Energy's Joint BioEnergy Institute (JBEI). His current research focusses on the biomass fractionation and lignin valorization using deep eutectic solvents.



John Gladden: John Gladden received his PhD at UC Berkeley in 2005 in molecular and cell biology and has since worked at the Joint BioEnergy Institute (JBEI) where as a post-doctoral student he used advanced 'omic techniques to identify and characterize a variety of lignocellulose-degrading bacterial enzymes and then used them to develop enzyme mixtures for lignocellulose deconstruction. In 2011, John then moved to Sandia National Lab where he continues his work at JBEI and is currently Director of two teams in the Deconstruction Division. The first team is focused on developing ionic liquid-based technologies for efficient lignocellulose pretreatment and deconstruction. The second team is focused on developing fungal systems to either produce lignocellulose-degrading enzymes or convert lignocellulose into biofuels and bioproducts.



Aymerick Eudes: Aymerick Eudes received his Ph.D. in 2005 in Plant Cellular and Molecular Physiology at the University of Paris XI. His work, conducted under the supervision of Dr. Lise Jouanin at the The French National Institute for Agricultural Research (INRA), focused on the identification of genes involved in lignin biosynthesis in the model plant *Arabidopsis*. Dr. Eudes then spent three years as a Postdoctoral Fellow in Prof. Andrew Hanson's lab in the Department of Horticultural Sciences at the University of Florida to study B-vitamins metabolism in plants and bacteria. In 2009, he moved to the Lawrence Berkeley National Laboratory (LBNL) to join Dominique Loqué's group in the Feedstocks Division of the Joint BioEnergy Institute (JBEI) to develop novel genetic engineering approaches to reduce plant biomass recalcitrance. In 2016, Dr. Eudes became a Biological Engineer Research Scientist at LBNL in the Biosciences Area. At JBEI, Dr. Eudes is the Deputy Director of the Cell Wall Biology & Engineering group in the Feedstocks Division. His research interests focus on the design of new strategies to modify lignin in plants and the development of bioenergy crops for sustainable production of fuels and chemicals.



Jenny C. Mortimer: Dr. Jenny Mortimer is a Staff Scientist at LBNL. After completing graduate work at Cambridge University, UK, she began studying the plant cell wall as a postdoc with Prof. Paul Dupree. Research included re-engineering the cell wall to improve its bioenergy-related properties as part of the BBSRC Bioenergy Centre. This was followed by a fellowship at RIKEN Yokohama, Japan, hosted by Prof. Taku Demura and the Cellulose Production Research Team.

At LBNL, she leads the Plant Systems Biology Group and is the Feedstocks Division Deputy VP at JBEI. Her group seeks to understand how plants use the products of photosynthesis - simple sugars - to build complex glycans. We are applying this knowledge to produce renewable, sustainable, fuels and biochemicals from plant biomass.

Dr. Mortimer is also co-leading the LBNL EcoPOD team, developing highly-instrumented mesocosms to bridge the scale and complexity gap between laboratory and field plant-microbe-soil research. EcoPODs will help researchers to test and model sustainable agriculture, and promote healthy soils. She has co-authored 50+ peer reviewed articles. She was selected as a World Economic Forum Young Scientist in 2016 and 2017, where she contributed to the WEF Code of Ethics for Researchers. Find her on Twitter @Jenny\_Mortimer1.



Steven W. Singer: Steve Singer is a Senior Scientist at Lawrence Berkeley National Laboratory and Director of Microbial and Enzyme Discovery at the Joint BioEnergy Institute in Berkeley. His group studies ways to convert plants, methane and carbon dioxide to biofuels.



Aindrila Mukhopadhyay: Aindrila Mukhopadhyay received her Ph.D. from the University of Chicago (USA) in 2002 for her work in understanding two-component signal transduction in the industrially important microbe *Agrobacterium tumefaciens*, in the laboratory of Prof. David. G. Lynn. She continued her research as a postdoc at the Lawrence Berkeley National Laboratory (LBNL) in the laboratory of Prof. Jay D. Keasling, developing systems biology and functional genomics approaches to examine environmentally important microbes. She was appointed a Career Scientist at LBNL in 2004 and expanded her work to include engineered microbial systems. She is currently a Senior Scientist in the BioSciences Area of LBNL where her group uses systems and synthetic biology in natural and engineered microbial systems. Her studies span several microbial systems including fungi, bacteria and archaea. Her group has developed tools and engineered scalable microbial systems for clean energy solutions and to reduce the need for petrochemically derived products. These include strains for sustainable production of biogasoline, biodiesel, jet fuels and bioproducts; as well as for bioremediation. She also serves as the Vice President of the Biofuels and Products Division of the US Department of Energy Bioenergy research center, the Joint BioEnergy Institute, located in Emeryville, CA, USA.





Corinne D. Scown: Corinne Scown is the vice-president for life-cycle, economics and agronomy and director of life-cycle and technoeconomic analysis at the US Department of Energy's Joint BioEnergy Institute (JBEI). She received a PhD in civil engineering from UC Berkeley and completed an MS at UC Berkeley in May 2008 in civil engineering, and a BS in civil engineering with a double major in engineering and public policy (EPP) at Carnegie Mellon University in December 2006. Her current work focuses on the lifecycle assessment and technoeconomic analysis of advanced biofuel and bioproduct production, national-scale fine-resolution scenario modeling of biofuel/bioproduct scale-up strategies, and bioenergy crop production, and scenario development for waste-to-energy systems in California.

