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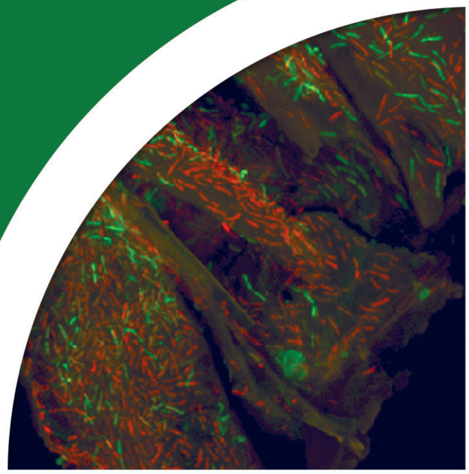
U.S. Department of Energy

BIOENERGY RESEARCH CENTERS



10-Year
Retrospective

Breakthroughs and Impacts 2007–2017

U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Biological and Environmental Research

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Websites for DOE Bioenergy Research Centers

BioEnergy Science Center (BESC)
bioenergycenter.org

Great Lakes Bioenergy Research Center (GLBRC)
glbrc.org

Joint BioEnergy Institute (JBEI)
jbei.org

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Cover images: Switchgrass courtesy Great Lakes Bioenergy Research Center. Microbe *Clostridium thermocellum* growing on poplar biomass courtesy Jennifer Morrell-Falvey, BioEnergy Science Center. Poplar courtesy Joint BioEnergy Institute.

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Bioenergy Research Centers 10-Year Retrospective Breakthroughs and Impacts 2007–2017

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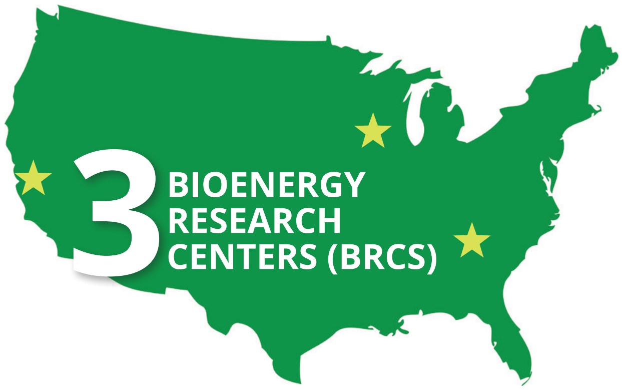


5 RESEARCH FOCUS AREAS

- Biomass Supply and Sustainability
- Biomass Development
- Biomass Deconstruction and Separation
- Conversion of Biomass to Biofuels
- Enabling Technologies

SCIENTIFIC STAFF*

GLBRC	BESC	JBEI
~325	~250	~150

CITATIONS AS OF DECEMBER 2017

>113,000

2,696 Publications

EDUCATION, OUTREACH

EDUCATORS	STUDENTS
>1,000	>650,000 and increasing

BRC RESEARCH SPANNED

35 Academic, Nonprofit, and Industrial Institutions

7 DOE National Laboratories

25 States

TECHNOLOGICAL OUTPUT

Invention Disclosures	619
Patent Applications	397
Patents	101
Licenses/Options	199
Company Startups	15

IMPACT ON FUTURE SCIENCE

MULTIDISCIPLINARY TRAINING PROVIDED FOR MORE THAN 970 ALUMNI

From 2007 to 2017, more than **970** graduate students and postdoctoral researchers participated in the BRCs before moving on to other positions in academia, national laboratories, industry, and nonprofit research organizations.

GLBRC	BESC	JBEI
60% Academia	45% Industry	40% Academia
25% Industry	40% Academia	40% Industry
15% Nonprofit Research Organizations	15% National Laboratories	10% National Laboratories
		10% Other

* Total number of scientists and research laboratory technicians (excluding graduate students and postdoctoral researchers) supported over the 10-year period at the Great Lakes Bioenergy Research Center (GLBRC), BioEnergy Science Center (BESC), and Joint BioEnergy Institute (JBEI).

U.S. Department of Energy Bioenergy Research Centers

Ten-Year Retrospective

The Genomic Science program of the Office of Biological and Environmental Research (BER) within the U.S. Department of Energy (DOE) Office of Science focuses on understanding microbes, microbial communities, and plants as integrated systems of relevance to DOE's energy and environmental missions. One aspect of this program seeks to develop the fundamental science, research technologies, and knowledgebase necessary to enable the cost-effective, sustainable production of advanced biofuels and bioproducts from plant biomass.

Lignocellulosic materials from plants provide the largest reservoir of raw materials for biofuels and biobased products, yet cost-effective use of these materials is hampered by their complexity and resistance to breakdown. Cellulose is contained within plant cell walls in the form of long, tightly bound chains of sugars (polysaccharides) that can be converted into biofuels and bioproducts by microbes and enzymes. Physically accessing these sugars, however, is difficult because the cellulose is embedded within a matrix of other polymers including hemicellulose and lignin, making it resistant to degradation (see Fig. 1. Three-Dimensional Illustration of Lignocellulose Meshwork, this page). This resistance, called recalcitrance, and a lack of efficient methods to convert lignocellulose to useful products are major impediments to the cost-effective production of biofuels and bioproducts from plant biomass.

To understand the challenges associated with lignocellulosic usage for biofuels, BER supported the initial three Bioenergy Research Centers (BRCs) from 2007 to 2017. The purpose of forming centers was to promote large, multidisciplinary, integrative efforts capable of addressing biofuel research from multiple angles and to accelerate transformational breakthroughs in the basic sciences needed to develop cost-effective and sustainable

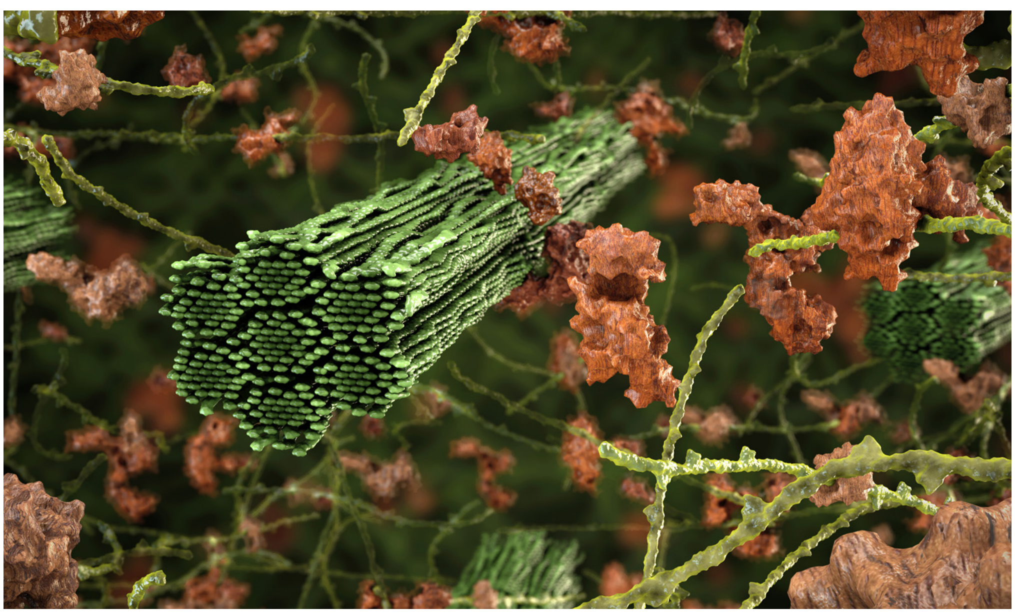


Fig. 1. Three-Dimensional Illustration of Lignocellulose Meshwork. Researchers are using computational modeling to gain a molecular-level understanding of the plant cell wall and its major components, including cellulose fibers (green), lignin molecules (brown wooden texture), and hemicellulose (light green). [Image courtesy Thomas Splettstoesser, www.scistyle.com, for Oak Ridge National Laboratory]

commercial production of cellulosic biofuels on a national scale (U.S. DOE 2006).

The three BRCs were based in the geographically diverse Southeast, Midwest, and West Coast regions and included the BioEnergy Science Center (BESC), led by Oak Ridge National Laboratory; the Great Lakes Bioenergy Research Center (GLBRC), led by the University of Wisconsin–Madison in close partnership with Michigan State University; and the Joint BioEnergy Institute (JBEI), led by Lawrence Berkeley National Laboratory. Center partners included experts from many areas of science and engineering, as well as economics, government, and industry.

Complementary and Collaborative Approach

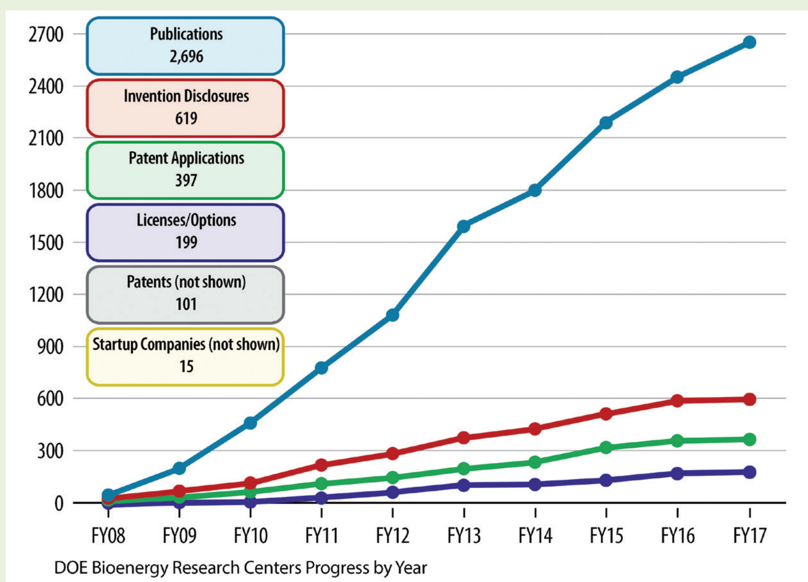
The mission of these BRCs, broadly stated, was to advance science, engineering, and technology to support conversion of lignocellulosic biomass into renewable liquid transportation biofuels.

Working toward this mission, the BRCs developed complementary and collaborative research portfolios to address the key technical and economic challenges in large-scale biofuel production from lignocellulosic biomass.

In this endeavor, the BRCs coordinated sustainable biofuels research along the entire pathway, from creating new energy crops and new methods for deconstructing lignocellulosic material into chemical building blocks to creating new metabolic pathways inserted into microbial hosts to produce ethanol or even hydrocarbon fuels. This center-scale approach allowed technology development specialists to design automated pipelines that streamlined workflows and increased research efficiencies, enabled the testing of research ideas from proof of concept to field trials, and allowed research breakthroughs in one area to immediately inform research direction in other areas (see sidebars, Accelerating Deployment of Biofuels Science and Commercialization, this page; and Startup Companies Stemming from the BRCs, p. 3).

Accelerating Deployment of Biofuels Science and Commercialization

Scientific advances made by the Department of Energy's Bioenergy Research Centers (BRCs) have provided crucial knowledge needed to develop new biobased fuels and products, methods, and tools that the emerging biofuel industry can use. Through intellectual property licensing agreements, partnerships, and targeted collaborative affiliations, the BRCs helped to speed the translation of basic research results to industry, contributing to clean energy (see figure at right).



Summary of Bioenergy Research Center (BRC) Scientific and Technological Output Through Fiscal Year 2017. The initial three BRCs collectively produced a portfolio of diverse and complementary scientific strategies that address the challenges of biomass conversion to biofuels on a scale far greater than any effort to date. The resulting knowledgebase is providing new insights to help industry meet the broad challenges of reducing the cost of and meeting the demand for advanced biofuels.

Startup Companies Stemming from the BRCs

Some examples of the transfer of Bioenergy Research Center (BRC) research to industry are included in the following list of startup companies. Each of these companies brings new technologies and products to the marketplace, and they all have roots in research initially conducted at the BRCs.

BioEnergy Science Center

- **Gate Fuels Inc.** is developing a low-cost platform for producing methyl ethyl ketone directly from pretreated cellulosic biomass.
- **Enchi Corporation** has licensed a custom-engineered microbe, *Clostridium thermocellum*, to digest and convert biomass into ethanol.
- **Vertimass LLC** is commercializing a "green" catalyst technology that converts ethanol into gasoline, diesel, and jet fuel–blend stocks that are compatible with the current transportation fuel infrastructure.

Great Lakes Bioenergy Research Center

- **Glucan Biorenewables LLC** is using gamma-valerolactone, a plant-based solvent, to help convert biomass into three streams of high-value industrial chemicals.
- **Hyrax Energy Inc.**, which existed from 2011 to 2018, used an improved method for extracting fermentable sugars from biomass to improve the economics of the cellulosic biofuel pipeline.
- **Kopess Biomass Solutions** is working to unlock the genetic regulators of cellulose biosynthesis in plants.
- **Lactic Solutions LLC** (purchased by Lallemand Inc.) genetically engineers lactic acid bacteria, the organisms that contaminate ethanol fermentations, to produce ethanol rather than lactic acid.
- **Xylome** uses newly developed yeast strains to transform hard-to-digest sugars into commercially viable, second-generation ethanol and biodiesel precursors and to synthesize a host of advanced biorenewable products.

Joint BioEnergy Institute

- **Afingen** develops and commercializes emerging synthetic biology technologies expected to have enormous impact on economics and environmental sustainability through new biological products and improved biological capabilities.
- **Cascade Fluidics Inc.** builds laboratory automation systems for high-throughput biological sample preparation.
- **Constructive Biology** develops engineered microbes to convert inexpensive, renewable sugars into high-value specialty chemicals.
- **Demetrix** develops engineered microbes to convert inexpensive, renewable sugars into high-value specialty chemicals.
- **Illium Technologies** produces ionic liquid solvents from lignin. The ionic liquids can be used for biomass pretreatment to produce cellulose and glucose for biomanufacturing.
- **Lygos** produces "bio-advantaged" chemicals, where petrochemistry cannot compete with biochemistry.
- **TeselaGen Biotechnology, Inc.** is developing a fully integrated solution for synthetic biology. Its DNA design and assembly platform makes genetic engineering cheaper and faster.

Companies that Have Licensed BRC Technologies

BioEnergy Science Center

- Forage Genetics International
- GreenWood Resources, Inc.
- Mascoma Corporation
- Enchi Corporation
- Aspen Machining and Molding, Inc.
- DuPont
- Dow AgroSciences
- ArborGen, Inc.
- Ceres, Inc.
- Kerafast, Inc.
- Glydia Biotech LLC
- Monsanto Company

Great Lakes Bioenergy Research Center

- Glucan Biorenewables LLC
- Hyrax Energy Inc.
- Xylome
- Lactic Solutions LLC
- Amphora
- FuturaGene

Joint BioEnergy Institute

- DuPont
- Novozymes
- POET

Fig. 2. Marginal Lands. Flower-dotted marginal land in the foreground contrasts sharply with more uniform traditional croplands of corn and alfalfa in the distance. Great Lakes Bioenergy Research Center (GLBRC) scientists have shown that perennial crops grown on marginal lands not currently in use for food production could provide large quantities of biomass and major ecological and environmental benefits. [Courtesy GLBRC]

The BRCs also trained scores of multidisciplinary scientists in the bioenergy field, and BRC outreach efforts have communicated the value of sustainable bioenergy to the public.

BRC Breakthroughs for Lignocellulosic Biofuels Production

From 2007 to 2017, the original three DOE BRCs made a number of important breakthroughs toward overcoming critical challenges to cost-effective production of biofuels from biomass (see sidebar, At a Glance: BRC Accomplishments from 2007 to 2017, p. 5). These challenges, identified by the BRCs in their first years, included biomass supply and sustainability, biomass development, biomass deconstruction and separation, conversion of biomass to biofuels, and enabling technologies.

The BRCs built their research portfolios around these challenges, and select advances from their collective efforts are highlighted in the following sections.

Top Accomplishments in Biomass Supply and Sustainability

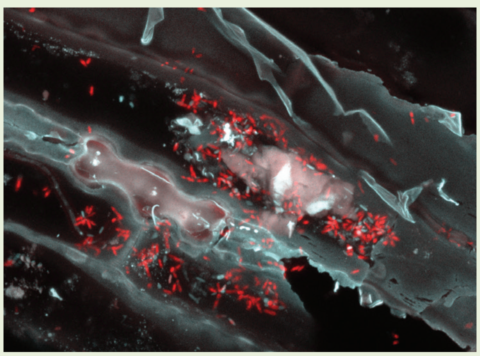
A key challenge when the BRCs began their research efforts was that the environmental, social, and economic consequences of growing biomass for biofuels needed to be thoroughly evaluated to ensure minimal, or even positive, impacts on food production and the environment (see Fig. 2. Marginal Lands, this page). For example, how far could biomass be transported from a field to a biorefinery before the fuel used in its transport exceeded the amount of fuel that could be obtained in the conversion to biofuels? The BRCs determined an analysis of the entire supply chain was necessary to understand the economic feasibility and logistics for collecting, transporting, and delivering this biomass to biorefineries. The BRCs also developed techno-economic modeling approaches to study how

At a Glance: BRC Accomplishments from 2007 to 2017

Great Lakes Bioenergy Research Center

The Great Lakes Bioenergy Research Center (GLBRC) developed a fundamental understanding of nitrogen and carbon cycling, which is essential for creating sustainable biofuel landscapes. GLBRC also pursued economic sustainability via biological and chemical routes to low-cost sugars, developing unique pretreatment methods that release lignin for potential conversion to fuel precursors and value-added coproducts (see figure at right). Producing biofuels and chemicals from both the sugar and lignin components of plant biomass has the potential to provide added value and increase the profitability of cellulosic biofuels.

Biomass Pretreatment for Cutting Enzyme Use, Boosting Biofuel Production. A liquid ammonia–based pretreatment, called extractive ammonia (EA), processes biomass such as switchgrass in a substance that helps unlock the plant's sugars. Simultaneously, EA separates out up to 45% of the lignin, while largely keeping its carbohydrates intact. [Courtesy Matthew Wisniewski, Great Lakes Bioenergy Research Center]



Clostridium thermocellum. A model of *C. thermocellum* engineered for greater ethanol tolerance during bioprocessing and licensed by the BioEnergy Science Center to Enchi Corporation. [Courtesy Bryon Donohoe, National Renewable Energy Laboratory]

BioEnergy Science Center

The BioEnergy Science Center (BESC) made crucial progress toward understanding, manipulating, and managing plant cell wall recalcitrance and conversion. Notably, the BESC team proved that multiple genes control plant cell wall recalcitrance and that manipulation of these genes could yield perennial bioenergy feedstocks with enhanced deconstruction properties. Furthermore, BESC successfully demonstrated the ability to combine the processes of cellulose digestion and fermentation of released sugars into biofuel in a single microbial organism (see figure at left). These discoveries not only represent significant progress toward the goal of developing improved feedstocks and microbial deconstruction technologies for advanced biofuel production, but they also have redefined the scientific basis of the phenomenon of biomass recalcitrance.

Joint BioEnergy Institute

The Joint BioEnergy Institute (JBEI) used the latest tools in synthetic biology and chemical engineering, including computational and automated high-throughput technologies, to transform biomass sugars into energy-rich fuels. JBEI successfully altered biomass composition in model plants and bioenergy crops, reducing inhibitors that impact downstream processing and making lignin more readily depolymerized. JBEI research has shown that new solvents, known as ionic liquids, permit near-complete dissolution and fractionation of plant biomass, thereby facilitating its enzymatic conversion to sugars at near-theoretical yields. JBEI's pioneering work in synthetic biology has enabled the realization of microbes that produce a variety of molecules from these sugars that can serve as renewable jet, diesel, and gasoline "drop-in" blendstocks (see figure at right).

Synthetic Biology. Joint BioEnergy Institute researchers are using the tools of synthetic biology to engineer new microbes as alternatives to yeast that can quickly and efficiently ferment complex sugars into advanced biofuels. [Courtesy Lawrence Berkeley National Laboratory] their fundamental, early-stage research findings might operate as mature technologies at commercial scale. Additionally, this modeling helped to pinpoint the areas of highest cost and thus where new advances would have the greatest impact.

Demonstrated Potential of Marginal Lands. Biomass production on marginal or nonagricultural lands can avoid the negative impacts of diverting farmland or crops for biofuels production, namely the food versus fuel conflict and indirect greenhouse gas emissions. Field experiments and modeling demonstrated that sufficient marginal land exists in the U.S. Midwest to grow a significant fraction of biomass for expected biofuel needs. These analyses were extended to a wider geographic region, incorporating techno-economic modeling to show reasonable potential configurations for future feedstock supplies (Gelfand et al. 2013).

Increased Understanding of Potential Economic Impacts of BRC Discoveries. Researchers modeled the economic impact of newly developed ionic liquid deconstruction processes in the context of a virtual, commercial-scale biorefinery to understand the capital and operating costs, net energy needs, and material balance. Subsequent modeling considered the entire supply chain to determine cradle-to-grave greenhouse gas emissions and water use. One of these techno-economic simulations compared the performance of biorefineries using newly developed ionic liquid–based pretreatment technologies, including a one-pot process (see research accomplishment, Developed and Demonstrated a One-Pot Ionic Liquid Deconstruction and Conversion Technology, p. 12), which dramatically reduces the need for water washing and energy-intensive separations. Calculations indicated that combining this process with the use of protic ionic liquids clears the way to producing cellulosic biofuels at a lower cost and cuts greenhouse gas emissions to less than one-tenth that of the previous state of technology for ionic liquids based on a water-wash process. Compared with gasoline, the one-pot process combined with a high-performing ionic liquid can reduce greenhouse gas emissions by 85%. These results highlight the dramatic impact that increasing biomass loading and minimizing water use in the biorefinery can have on the cost and environmental footprint of advanced biorefineries (Klein-Marcuschamer et al. 2011; Konda et al. 2014; Sun et al. 2017; Neupane et al. 2017).

Examined Feedstock Variability and Effects on Deconstruction and Microbial Conversion. Variation in environmental conditions

Fig. 3. "Super Yeast": Potential Improvement for Biofuel Economics. Great Lakes Bioenergy Research Center (GLBRC) scientists have found a way to nearly double the efficiency with which *Saccharomyces cerevisiae*, a commonly used industrial yeast strain, converts plant sugars to biofuel. The engineered super yeast (being examined by a GLBRC researcher) could boost the economics of producing ethanol and specialty biofuels and bioproducts. [Courtesy James Runde, GLBRC]

where bioenergy crops are grown can have significant detrimental effects on microbes' ability to ferment sugars to biofuels. In a field-to-fuel pipeline analysis, researchers studied the effects of variable precipitation on two bioenergy feedstocks—corn stover and switchgrass—with sampling from a major drought year and 2 years with average precipitation. The study examined effects on hydrolysate chemical composition and the ability of yeast (*Saccharomyces cerevisiae*) or bacteria (*Zymomonas mobilis*) to ferment the hydrolysates to ethanol (see Fig. 3. Super Yeast: Potential Improvement for Biofuel Economics, p. 6). Most were readily fermented to biofuel, but the growth of yeast was completely inhibited in hydrolysate generated from drought-stressed switchgrass. Biomass and compositional analysis revealed that drought-year switchgrass accumulated greater concentrations of soluble sugars, likely a stress response to drought, that were degraded to inhibitory compounds during pretreatment. These findings indicate that environmental variation can have significant effects on biomass hydrolysate properties and subsequent fermentation to biofuels. Integrating the effects of environmental conditions and microbial molecular signatures into a predictive model can guide pretreatment and biocatalyst choices to mitigate inhibitory effects (Ong et al. 2016).

Top Accomplishments in Biomass Development

In contrast to crops used for food and fiber, no crops had been developed specifically for use as cellulosic biofuel feedstocks when the BRCs began in 2007. Consequently, a key challenge was to identify optimal agronomic properties and develop fundamental knowledge of plant cell wall synthesis and structure to enable production of high-yield, sustainable feedstocks such as switchgrass and poplar that are easier to deconstruct and convert into biofuels. Part of this challenge involves lignin, which is one of the most recalcitrant biopolymers present in lignocellulose.

Demonstrated Potential for Doubling Biofuel Production with Transgenic Switchgrass. Researchers demonstrated the potential of developing dedicated bioenergy crops with specific engineered traits capable of thriving in the environment. Switchgrass engineered to express an altered lignin structure thrived in field trials over a 3-year period relative to controls (see Fig. 4. Real-World Performance of Low-Lignin Switchgrass, this page). The crops retained the reduced recalcitrance phenotype and yielded 25% to 35% more sugar release upon deconstruction, demonstrating the viability of a specifically designed bioenergy crop (Dumitrache et al. 2017).

Engineered and Field-Tested Plants with Modified Lignin. Using the bacterial enzyme 3-dehydroshikimate dehydratase (QsuB) in crops such as sorghum, tobacco, poplar, and switchgrass, researchers found that QsuB can reduce the recalcitrance of lignin and improve the energy yield of biomass in two ways: (1) The expression of QsuB reduces the amount of lignin in plant cell walls, enhancing breakdown into soluble sugars. (2) QsuB catalyzes the synthesis of protocatechuic acid, which can be converted into the platform chemical muconic acid, a precursor for several valuable bioplastics. These results highlight the potential

Fig. 4. Real-World Performance of Low-Lignin Switchgrass. Lignin in the cell walls of switchgrass (pictured) and other bioenergy feedstocks severely limits the accessibility of cell wall carbohydrates to enzymatic breakdown into sugars and their subsequent fermentation into biofuels. This first reported study of its kind evaluated the biofuel potential of transgenic switchgrass with reduced lignin content over several growing seasons. [Courtesy BioEnergy Science Center]

Fig. 5. Assay Tool for Characterizing Plant Sugar Transporters. A family of six nucleotide sugar transporters never before described were characterized in *Arabidopsis* (pictured), a model plant for research in advanced biofuels. [Courtesy Lawrence Berkeley National Laboratory]

impact of tailored bioenergy crops to decrease costs associated with biofuel production (Eudes et al. 2015, 2017; Wu et al. 2017).

Elucidated Genes Controlling Plant Cell Wall Recalcitrance. Significant advances were made in understanding, manipulating, and managing plant cell wall recalcitrance and conversion in studies of *Populus* and switchgrass (*Panicum virgatum*). Both are high-yield perennials and recognized as potential domestic biofeedstocks. Researchers showed that multiple plant genes control cell wall recalcitrance and that manipulation of these genes can yield lower recalcitrance in perennial biofeedstocks. These feedstocks were analyzed by a series of chemical, biochemical, molecular, and systems biology approaches. Findings included increased understanding of cell wall structure and biosynthetic pathways for four components—lignin, xylan, cellulose, and pectin—and that each could be manipulated to reduce recalcitrance. For lignin biosynthesis, researchers manipulated every gene in the pathway, demonstrating that it is a key influencer of biomass recalcitrance. The most surprising effect was from pectin, which, though small in quantity, also proved to be an important contributor to recalcitrance. The results provide mechanistic understanding of the molecular bases of recalcitrance, enabling a path for improving feedstocks by cisgenic manipulations, selection of the best natural variants, or genetically assisted breeding (Shen et al. 2013; Zhao et al. 2013; Barros et al. 2016; Peña et al. 2016; Urbanowicz et al. 2012, 2014; Mazumder et al. 2012; Mazumder and York 2010; Pattathil et al. 2010; Bar-Peled et al. 2012; Bali et al. 2016; Vandavasi et al. 2015; Payyavula et al. 2014; Tan et al. 2013; Atmodjo et al. 2013; Xie et al. 2018; Biswal et al. 2018a, 2018b; Kalluri et al. 2014).

Identified Nucleotide Sugar Transporters. Nucleotide sugars are the substrates for biosynthesis of cell wall polysaccharides. Most of the nucleotide sugars are produced in the cytoplasm and need to be transported in the Golgi lumen to be used for glycan biosynthesis. A novel method was developed to determine the transport activity of nucleotide sugars, and researchers identified the substrates for 22 of 44 transporters in *Arabidopsis* (see Fig. 5. Assay Tool for Characterizing Plant Sugar Transporters, this page). By comparison, only eight of the 32 human-mammalian transporters have been characterized at this level of detail. Identification of nucleotide sugar transporters is important for basic understanding of cell wall biology and provides powerful tools for engineering plants with modified biomass composition optimized for production of biofuels and bioproducts. Using this knowledge, researchers engineered expression of both uridine

Fig. 6. Largest-Ever Dataset of Genetic Variations in Poplar Trees. The BioEnergy Science Center (BESC) genome-wide association study dataset comprises more than 28 million single-nucleotide polymorphisms, or SNPs, derived from approximately 900 resequenced poplar genotypes. The information is proving useful to researchers in the fields of biofuels, materials science, and secondary plant metabolism. [Courtesy BESC]

diphosphate (UDP)-galactose and UDP-xylose transporters and increased the glucose-xylose ratio in the engineered *Arabidopsis* plants. The sugar stream produced from these plants is preferable for most of the microbes currently used in biorefineries that prefer glucose over xylose (Rautengarten et al. 2014, 2017; Ebert et al. 2015).

Identified Biomass Recalcitrance Regulators with Genome-Wide Association Studies (GWAS). To quickly identify the genes that control cell wall recalcitrance (resistance to sugar release), researchers conducted large-scale studies to understand natural variation in both switchgrass and *Populus*. These studies included high-throughput recalcitrance phenotyping and sequencing, along with other omics studies, for these natural variants as well as generated transgenics. Sequencing identified millions of unique variations called single-nucleotide polymorphisms (SNPs) in specific genes and in noncoding regions of the genome. Using a GWAS approach, many measurements (including growth, composition, and biomass convertibility) of the broad variation across the switchgrass and *Populus* populations were correlated with specific SNPs (see Fig. 6. Largest-Ever Dataset of Genetic Variations in Poplar Trees, this page). The GWAS mapping enabled researchers to discover a genetic regulator that modulates carbon flow among four major metabolic pathways in plants. Manipulation of the regulator leads to reduced lignin content, increased biofuels production, and increased digestibility. These results laid the foundation for using breeding and selection to identify better plant feedstocks (Serba et al. 2015; Muchero et al. 2015; Evans et al. 2014).

Greenhouse and field trials were conducted for a limited number of natural variant *Populus* and switchgrass lines with reduced recalcitrance and those arising from directed transgenic engineering. Researchers used multiple phenotypic characterization assays including sugar release, sugar and lignin composition, ethanol production, and crystallinity. A key discovery was the ability to achieve both lower recalcitrance and higher biomass simultaneously in certain lines (Biswal et al. 2015; Baxter et al. 2014, 2015; Dumitrache et al. 2017).

Used Genomic Approaches to Engineer Lignin. Redesigning lignin, the aromatic polymer fortifying plant cell walls, to be more amenable to chemical depolymerization can lower the energy required for industrial processing and increase sugar yields. Poplar trees were engineered to introduce ester linkages ("zips") in place of ether linkages into the lignin backbone by augmenting the monomer pool with monolignol ferulate conjugates (see Fig. 7. Zip-Lignin™, this page). This approach incorporates weak bonds that are easier to break apart, improving digestibility, but retains lignin's structural value to the plant. The plants also appear to grow normally compared with nontransgenic controls. Tailoring plants to use such conjugates during cell wall biosynthesis holds promise for producing plants designed for more efficient deconstruction (Wilkerson et al. 2014).

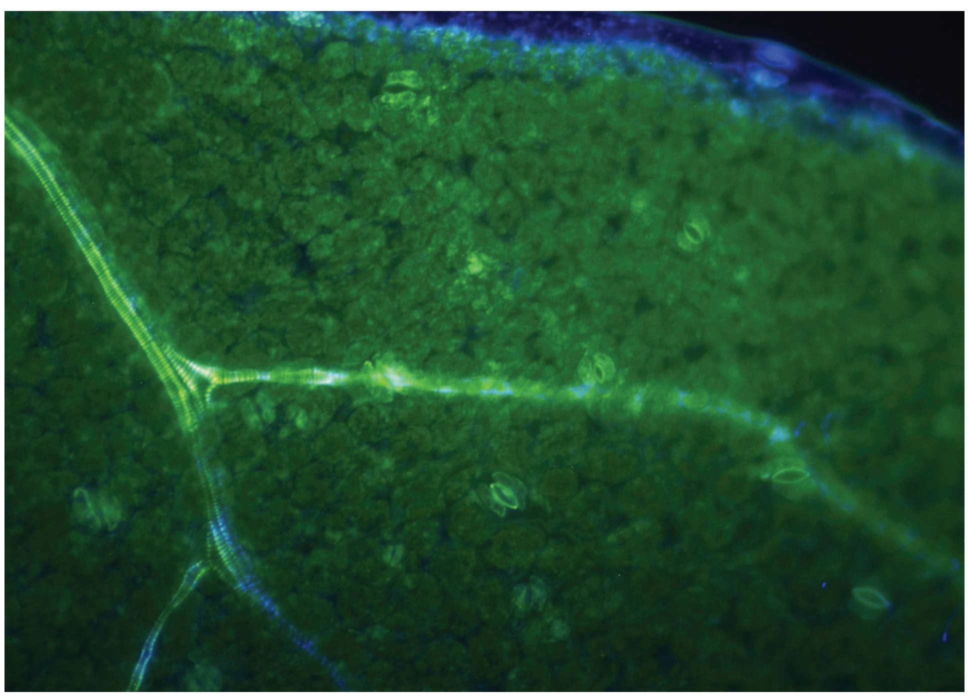
Additional genetic engineering approaches were identified for improved plant processing. For example, augmentation of available pools of soluble sucrose in poplar yields reduced lignin and altered cell wall structure, while mutation of a gene encoding a lignin biosynthetic enzyme increased zip-lignin levels in maize (Unda et al. 2017; Smith et al. 2017).

Top Accomplishments in Biomass Deconstruction and Separation

The BRCs identified deconstruction of lignocellulosic feedstocks into their constituent sugars, while minimizing the formation of inhibitors and using as few chemicals as possible, as the primary technical barrier to economical liquid biofuels. Scalable, sustainable, and affordable processes needed to be developed to handle diverse feedstocks at low cost.

Developed a Next-Generation Deconstruction Strategy. An innovative, next-generation biomass deconstruction method was developed that works with a wide variety of energy crops and avoids the use of expensive chemicals. The method is based on gamma-valerolactone, an organic solvent produced from plants, that can break down biomass into cellulose, hemicellulose, and lignin. These components can be

Fig. 7. Zip-Lignin™. The Great Lakes Bioenergy Research Center's Zip-Lignin™ technology allows for easier degradation of lignin, the most difficult part of the plant to break down. Here, the feruloyl-coenzyme A monolignol transferase is expressed following introduction of weak bonds into the lignin of poplar tissue. [Courtesy Shawn Mansfield, University of British Columbia]



further converted to useful biofuels, specialty chemicals, and lignin-based carbon foam, all commercially valuable products, as a basis for an integrated biorefinery process producing biofuels and chemicals from renewable biomass (Luterbacher et al. 2014; Alonso et al. 2017).

Discovered New Enzymes, Microbes for Breaking Down Lignocellulose. Microbes such as fungi and bacteria produce enzymes, called glycoside hydrolases, to acquire nutrients through the degradation of cellulose. Some of these enzymes can break down the polysaccharides and lignin that constitute plant cell walls and, therefore, could be especially effective at generating the intermediates needed for microbial production of biofuels and bioproducts. Researchers studied two distinct ecosystems known to be very efficient in breaking down cellulose—composts in California (see Fig. 8. Cellulose-Degrading Enzymes, this page) and rainforest soils in Puerto Rico. From these studies, they discovered (1) a glycoside hydrolase family 12 enzyme, produced by the bacterium *Thermobispora bispora*, that plays a previously underappreciated role in efficiently breaking down crystalline cellulose into glucose; (2) a complex of cellulose-degrading enzymes that performs well at higher temperatures than current commercial enzymes can tolerate; and (3) a previously unknown organism, *Enterobacter lignolyticus*, that can efficiently break down and grow on lignin. Researchers isolated a single gene from this organism that confers tolerance to the presence of ionic liquids to biofuel-producing hosts, such as *Escherichia coli*, thus paving the way to new consolidated approaches to biomass conversion (Khudyakov et al. 2012; Ruegg et al. 2014; Hiras et al. 2016).

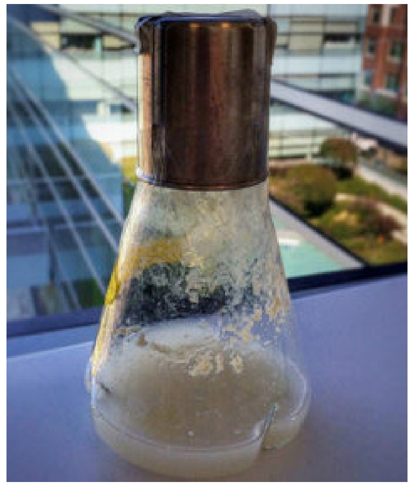


Fig. 8. Cellulose-Degrading Enzymes. This 50-ml flask contains a symbiotic mix of bacteria derived from compost that was maintained for 3 years. [Courtesy Joint BioEnergy Institute]

Developed an Innovative Pretreatment Method that Reduces Costly Enzyme Loadings and Increases Biofuel Yields. The recalcitrance of lignocellulosic biomass requires energy-intensive pretreatment and large amounts of expensive enzymes to release sugars for viable biochemical conversion to biofuels. Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) employs renewable tetrahydrofuran as a co-solvent to greatly enhance traditional dilute acid pretreatment. Testing showed that CELF dissolved a large portion of plant lignin, removed and recovered most of the hemicellulose sugars, and increased enzymatic digestibility of biomass. Overall, CELF pretreatment achieved about a 10-fold reduction in required enzyme loadings compared with traditional dilute acid pretreatment. It also removed a majority of biomass lignin (over 90% in wood) that could be precipitated as a high-purity product suitable for conversion to high-value chemicals and materials (Nguyen et al. 2015).

Developed a Feedstock-Agnostic Biomass Pretreatment Method. Lignocellulosic biomass is a natural resource distributed across the United States with significant variations as a function of genotype and geographical region. Most of the pretreatment technologies that have been studied and developed to date are highly effective in handling a specific range of feedstocks, but there are very few conversion technologies with a demonstrated ability to handle a wide range of feedstocks with minimal negative impact on efficiency and sugar yields. The ionic liquid pretreatment process is unique in that it efficiently handles softwoods, hardwoods, herbaceous materials, and agricultural residues as single and mixed feedstocks and generates high yields of sugars and biofuels (Shi et al. 2013; Torr et al. 2012; Li et al. 2010; Singh et al. 2009; Dibble et al. 2011).

Top Accomplishments in Conversion of Biomass to Biofuels

Using lignocellulosic biomass as a sustainable feedstock for producing biofuels and bioproducts requires a cost-effective, efficient process for transforming the polysaccharides in lignocellulose into sugars for bioconversion. These sugars are then fed to microorganisms that convert them into biofuels and bioproducts. A key challenge for the BRCs was developing more energy-efficient and economical conversion processes, along with synthesis processes for new fuels in addition to ethanol.

The BRCs also targeted lignin, which constitutes up to a third of typical biomass, as a key focus area. By concentrating on the structure of lignin, its modification, and potential use, the BRCs studied ways to mitigate lignin's effect on recalcitrance while also identifying opportunities to effectively use lignin for chemicals or fuels.

Developed and Demonstrated a One-Pot Ionic Liquid Deconstruction and Conversion Technology. Similar to the advantages of "one-stop" shopping in the retail and services industry, a "one-pot" processing system in which sugars could be extracted from biomass and turned into biofuels in a single reactor vessel could produce significant advantages for the biofuels industry. Researchers made a major step toward this goal with the development and demonstration of a one-pot, wash-free process for ionic liquid pretreatment and saccharification of switchgrass (see Fig. 9. Jet Fuel Brewed in One-Pot Recipe, this page). Ionic liquids are a unique and powerful class of solvents that are superior to conventional pretreatment approaches (e.g., dilute acid, ammonia, organic solvent, and hydrothermal). Researchers also discovered enzymes, microbes, and microbial communities that can tolerate ionic liquids used for pretreatment, with the goal of consolidating

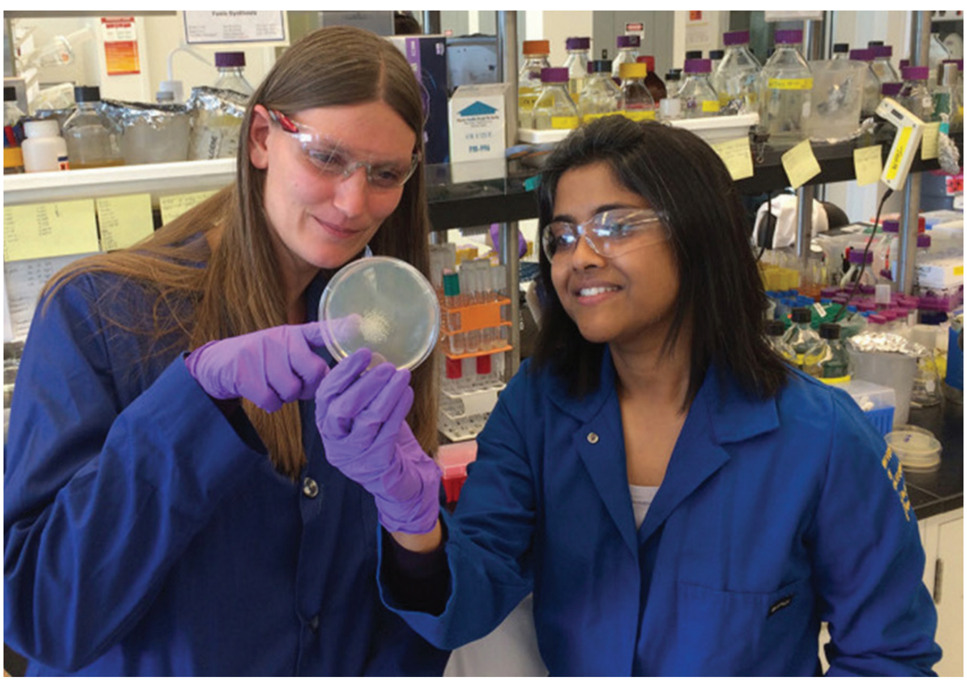


Fig. 9. Jet Fuel Brewed in One-Pot Recipe. Joint BioEnergy Institute scientists have engineered a strain of bacteria that enables a one-pot method for producing advanced biofuels from a slurry of pretreated plant material. [Courtesy Lawrence Berkeley National Laboratory]

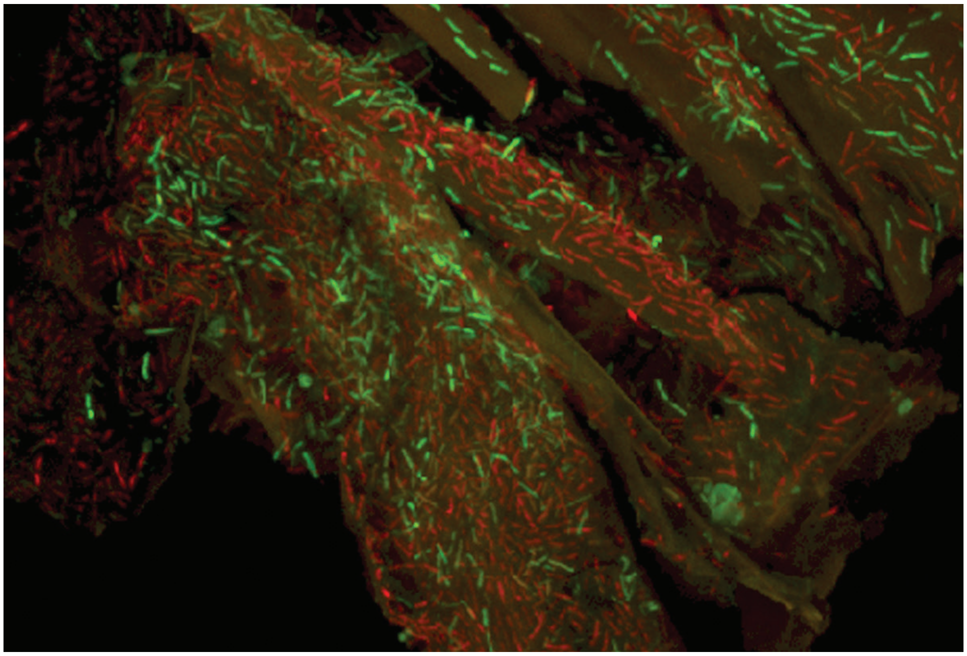


Fig. 10. Study of Nature's Best Biocatalysts for Biofuels Production. The microbe *Clostridium thermocellum* (stained green), seen growing on a piece of poplar biomass, was among several microorganisms evaluated in a comparative study analyzing their ability to solubilize potential bioenergy feedstocks. [Courtesy Jennifer Morrell-Falvey, BioEnergy Science Center]

the multiple reactors currently required into a single reactor, known as a one-pot configuration. A demonstration of this process at high solid loading levels resulted in unprecedented biofuel yields (~90%), while minimizing water use and waste disposal. This new one-pot process uses a combination of ionic liquid pretreatment, enzymatic saccharification, and yeast fermentation; and it eliminates the need for separations, recoveries, and other operational steps thereby generating significant capital and operating cost savings (Xu, F., et al. 2016; Sun, J., et al. 2016, 2017).

Identified and Validated Key Microbial Gene and Enzyme Targets for Consolidated Bioprocessing (CBP). CBP is a one-step approach by which microbes simultaneously digest biomass and convert it to biofuels and bioproducts without the need to add enzymes. This approach has the potential to greatly reduce capital costs by eliminating separate enzyme production and decreasing pretreatment severity (Lynd et al. 2017; Olson et al. 2012). A key focus was on improving product formation in thermophilic cellulolytic bacteria (e.g., *Clostridium thermocellum* and *Caldicellulosiruptor bescii*). Major advances include:

- In comprehensive comparative evaluations of biomass deconstruction, those microbes achieving the highest carbohydrate solubilization yields (several-fold higher than the industry standard fungal cellulose) were thermophilic anaerobes and *C. thermocellum* in particular (see Fig. 10. Study of Nature's Best Biocatalysts for Biofuels Production, this page; Paye et al. 2016; Lynd et al. 2016; Chung et al. 2014).
- Genetic tools for thermophiles were developed and used to initiate metabolic engineering of *C. thermocellum* and *C. bescii* (Cha et al. 2013; Guss et al. 2012; Olson and Lynd 2012; Lipscomb et al. 2016).
- Substantial advances were made in understanding and manipulating the metabolism of target CBP microbes. (1) Zhou et al. (2013) described nonstandard glycolysis

in *C. thermocellum*. (2) *Thermoanaerobacter saccharolyticum* was improved to produce economically recoverable ethanol concentrations at near-theoretical yield in hemicellulose fermenting (Herring et al. 2016). (3) Isobutanol was produced by adding key pathway enzymes in modified *C. thermocellum* at unprecedented yields and titers (Lin et al. 2015). (4) Ethanol titer and yield were increased in *C. thermocellum* by eliminating side products (Papanek et al. 2015; Tian et al. 2016; Biswas et al. 2016).

- Deconstruction enzymes targeting the major biopolymers of lignocellulosic biomass were identified. Fundamental enzymology found multifunctional cellulases in *Caldicellulosiruptor* species and *C. thermocellum* (Xu, Q., et al. 2016). Additionally, CelA, a multifunctional glycosyl hydrolase from *C. bescii*, was shown to be a particularly powerful hydrolytic enzyme (Brunecky et al. 2013; Kim et al. 2016).

- With co-treatment, *C. thermocellum* was able to achieve greater than 85% carbohydrate solubilization for *Populus* and switchgrass in the absence of added enzymes and thermochemical pretreatment, implying that *C. thermocellum* can attack all the major chemical linkages in representative woody and herbaceous lignocellulose crops when given sufficient physical access (Paye et al. 2016; Lynd et al. 2017; Holwerda et al. 2017).

Developed a Genome-Enabled Platform to Identify Valuable Coproducts from Biomass Hydrolysates. Processing plant biomass releases materials that have a negative effect on the ability of biofuel microbes to produce products. Consequently, researchers developed a genome-enabled platform to identify toxic compounds within biomass hydrolysates, determine their mode of action, and develop ways to bypass their negative impact on biofuel microbes. An unexpected result was the discovery of a compound (poacic acid) within biomass hydrolysates that is a potent antifungal agent (see sidebar, Non–Bioenergy Benefits of BRC Research, p. 15). Kilogram quantities of this compound have been prepared for field trials as a natural fungicide against agricultural pests. Poacic acid is a prime example of how genome- and systems biology–enabled advances can provide fuels and valuable products that can be produced by lignocellulosic biorefineries (Piotrowski et al. 2015).

Discovered and Optimized Metabolic Pathways and Hosts to Produce Advanced Biofuels. Researchers built on previous metabolic engineering advances to produce advanced jet-fuel, diesel, and gasoline-blending agents from cellulosic sugars. A fatty acid biosynthetic pathway was harnessed to produce diesel substitutes, fatty acid ethyl esters, and methyl ketones. Isoprenoid pathways were engineered in *E. coli* and *S. cerevisiae* to produce biodiesel (bisabolene), jet fuels (limonene, eucalyptol, and pinenes), and gasoline (isopentenol). Researchers used systems biology and host engineering to discover and develop mechanisms that improve host tolerance to final products and inhibitors from pretreated biomass. The research is a culmination of a series of metabolic engineering advances, laying a foundation for a broader biotechnology industry that produces biofuels and chemicals from renewable biomass (Steen et al. 2010; George et al. 2015; Ghosh et al. 2016; Yuzawa et al. 2016).

Developed Techniques to Valorize Lignin. Researchers found that lignin plays a key role in biomass recalcitrance (Ziebell et al. 2010, 2016; Li et al. 2016). Lignin removal would allow its valorization (Ragauskas et al. 2014), whether into fiber (Sun, Q., et al. 2016) or into value-added intermediates (Beckham et al. 2016).

Converting lignin into aromatics and other valuable products has the potential to improve the sustainability and economic viability of lignocellulosic biorefineries. Researchers took several approaches to enable lignin valorization, including (1) structural and biochemical analyses of bacterial enzymes that cleave lignin bonds (Helmich et al. 2016; Pereira et al. 2016);

Non–Bioenergy Benefits of BRC Research

Some of the discoveries made by the Bioenergy Research Centers also have important potential applications in other sectors, including sustainable agriculture, biomanufacturing, biodefense, and human health. Examples follow.

Plant-Derived Antifungal Agent

Great Lakes Bioenergy Research Center scientists discovered poacic acid through research focused on the small-molecule inhibitors present when cellulosic biomass is broken down for biofuel production. These inhibitors curb the growth of biofuel-producing microbes and are considered obstacles in the process of converting biomass to biofuels. Screening them for bioactivity against yeast revealed that one of the compounds, poacic acid, caused considerable cell death. Further testing against some common fungal pathogens found that a single application substantially reduced pathogen growth compared with control applications. If field tests are successful, the chemical compound eventually could be used as a natural fungicide in both sustainable and conventional agriculture.

Higher-Value Forage Plants

Working with a population of poplar trees, researchers at the BioEnergy Science Center identified a gene that regulates the production of lignin, the material that imparts plant rigidity. By altering lignin synthesis, the gene can decrease lignin content, making the plant easier to break down and convert into biofuels. The researchers also found that the reduced lignin content increases desirable flavonoids, improving the digestibility and nutritional value of animal feedstocks such as alfalfa, corn, and sorghum. Forage Genetics International, a major supplier of alfalfa seed, has licensed this plant gene and is evaluating it for commercial use in animal feed.

High-Throughput Microfluidics

Scientists and engineers working at the Joint BioEnergy Institute developed microfluidic devices for rapid, precise, and high-throughput characterization of the polysaccharides and lignin present in lignocellulosic biomass. This research included assay development at the interface of droplet microfluidics and mass spectrometry to streamline the discovery and optimization of biofuel metabolic pathways. These devices and assays have vastly superior performance compared to conventional approaches such as standard chromatography and colorimetric assays. They also have applications beyond bioenergy, such as biomanufacturing, agriculture, biodefense, and human health.

(2) chemical analysis of dimeric degradation products released by lignin depolymerization to verify and quantify components used in lignification (Yue et al. 2017); (3) utilization of formaldehyde to facilitate lignin monomer release during biomass depolymerization (Shuai et al. 2016); (4) identification of valuable extractable “clip-off” bioproducts like the flavonoid tricin, shown to be involved in lignification in monocots (Lan et al. 2015); and (5) identification of new and complex lignin compositions in palm species, including polyphenolic compounds and hydroxystilbenes, incorporated into lignin, and an unprecedented range of monolignol conjugates not previously known to be involved in lignification (del Rio et al. 2017). All these approaches help to inform understanding of lignification and enable lignin valorization, both through extraction of naturally occurring components and of compounds that have been engineered into bioenergy crops.

Top Accomplishments in Enabling Technologies

New technologies were needed to facilitate and accelerate BRC research, including, but not limited to, high-throughput laboratory technologies and computational and information systems, several of which have applications to biological research as a whole.

Developed Software Tools for Synthetic Biology and Metabolic Engineering. A powerful suite of synthetic biology approaches and computational tools was developed to facilitate the engineering of microbes to produce biofuels from sugars generated from lignocellulose deconstruction. This suite includes the establishment of a parts-based approach to DNA construction and quantitative tools to facilitate and improve the design of engineered pathways, such as the identification of metabolic flux–diverting side reactions as targets for metabolic engineering. Other newly developed resources include an open-source, web-based digital repository platform for microbial strains, sequences, and parts and plant seeds, as well as a web-based canvas for biological computer-aided design that enables users to visually design combinatorial DNA constructs (Chen et al. 2012; Hillson et al. 2012; Ham et al. 2012; Birkel et al. 2017).

Developed High-Throughput Methods for Rapid Analysis. The development of high-throughput methods for rapid analysis of pretreatment and enzymatic hydrolysis allowed for rapid identification of low-recalcitrance plant lines from thousands of natural and transgenic variants. These low-recalcitrance plant lines then could be characterized using multiple analytical and omics approaches, quickly advancing deeper understanding of the recalcitrance phenotype (Studer et al. 2011; Decker et al. 2015; Selig et al. 2011). Increased understanding of recalcitrance mechanisms also was supported by developing techniques such as glycome profiling (Pattathil et al. 2012) and improving the use of nuclear magnetic resonance spectroscopy for biomass (Foston and Ragauskas 2012; Peña et al. 2016; Pu et al. 2013, 2016; Kataeva et al. 2013).

Integrated Nanostructure-Initiator Mass Spectrometry (NIMS) with Microfluidics. Glycoside hydrolases are natively used by microbes to degrade cellulosic biomass, and they also are used in industrial production of biofuels from cellulosic biomass. These proteins are incredibly diverse, and relatively few examples from their more than 150,000 unique domain arrangements have been functionally characterized. The integration of *in vitro* expression and acoustic printing with NIMS technologies has proven to be a powerful combination for screening enzyme and microbial activities on lignocellulosic substrates. This analysis platform was used to characterize native plant biomass under a range of conditions. Product detection is achieved by coupling a unique chemical probe to the oligosaccharides using a stable oxime linkage; this process was used to characterize the functional diversity of glycoside hydrolases secreted by *C. thermocellum*, a model cellulolytic organism. New enzyme specificities were identified, and differences in individual enzyme activities were demonstrated in reactions with biomass. Analysis of the data suggested that use of multifunctional glycoside hydrolases can decrease the complexity of enzyme cocktails needed for liberating sugars from plant biomass during biofuel production. The NIMS method has been integrated with microfluidics technology to reduce sample volumes and greatly increase the number of assays that can be performed for enzyme cocktail optimization and strain optimization experiments (Reindl et al. 2011; Heinemann et al. 2017).

Developed New Cell Wall Chemical Imaging Approaches. New methods were developed to image the chemical components composing the cell wall. (1) Raman spectroscopy was used to image hemicellulose for the first time (Zeng et al. 2016). (2) Modified atomic force microscopy (AFM) techniques were used to chemically image the cell wall at the submicron level (Tetard et al. 2011). (3) Quantitative fluorescence confocal laser scanning microscopy (CLSM) and surface spectroscopy by time-of-flight secondary ion mass spectroscopy (TOF-SIMS) showed that, following microbial digestion, surface cellulose decreased while surface lignin increased, indicating that biomass recalcitrance may be controlled by surface characteristics (Dumitrache et al. 2017).

Integrated Omics and Software Tools. Key to advancing metabolic engineering beyond trial-and-error research are genetic parts with well-defined performance metrics that can be readily applied in a predictable way. Optimization of engineered microbes depends greatly on analytical tools that screen for target biofuel production across many thousands of samples because the number of design possibilities is so large. To help find the optimal microbe rapidly, researchers developed technologies to measure proteins and metabolites across different microbes and biofuels pathways. This analysis provides a systems-level view of the cell factory to yield quantitative information of dynamic processes between parts and the microbe that drive the next engineering steps to reach the highest biofuel production level. These methods have significantly reduced the time needed for optimizing microbes to produce large amounts of biofuel molecules by identifying bottlenecks resulting from low protein levels or metabolites that are toxic to the cell. Complementing the omics technologies is a suite of software tools developed to standardize procedures and data-type reporting for metabolic engineering research as well as to encourage sharing with the greater biofuels community. With this integrated toolbox, the large datasets, statistical analyses, and rule-based approaches help increase success rates for achieving high biofuel production (Morrell et al. 2017).

Integrated Omics Data for Key Processes. Integrated omics of microbial growth on complex lignocellulosic biomass over time provided a detailed view of the molecular machinery (metabolites and enzymes) and revealed temporal adaptation to a complex, lignocellulose substrate (Poudel et al. 2017). For *Populus*, profiling genotype-specific proteomes derived from ribonucleic acid sequencing data better defined the link between genotypes and phenotypes (Abraham et al. 2015).

DOE Bioenergy Research Centers: Next Iteration

Collectively, the original BRCs provided multiple proofs of concept for further capability improvements with the next generation of lignocellulosic biofuels and bioproducts, but remaining basic science challenges continue to limit the cost-effective conversion of plant biomass to advanced biofuels and bioproducts (U.S. DOE 2015).

Significant advances in plant breeding, molecular genetics, and genomic technologies provide unique opportunities to build on existing knowledge of plant biology and more confidently predict and manipulate functional properties of biomass feedstock crops. Similarly, continuing advances in omics-enabled technologies and synthetic biology approaches for microorganisms provide opportunities to further develop nonmodel microorganisms for applications in industrial biotechnology and for conversion of biomass into biofuels and bioproducts. Most importantly, integrating plant and microbial systems biology with cutting-edge research in chemical engineering, synthetic biology, and computational biology facilitates the scientific breakthroughs needed to foster the development of a sustainable bioeconomy.

Leveraging these opportunities in the next phase of DOE bioenergy research are four BRCs: (1) Center for Advanced Bioenergy and Bioproducts Innovation, led by the University of Illinois at Urbana-Champaign; (2) Center for Bioenergy Innovation, led by Oak Ridge National Laboratory in Oak Ridge, Tennessee; (3) Great Lakes Bioenergy Research Center, led by the University of Wisconsin–Madison in close partnership with Michigan State University; and (4) Joint BioEnergy Institute, led by Lawrence Berkeley National Laboratory.

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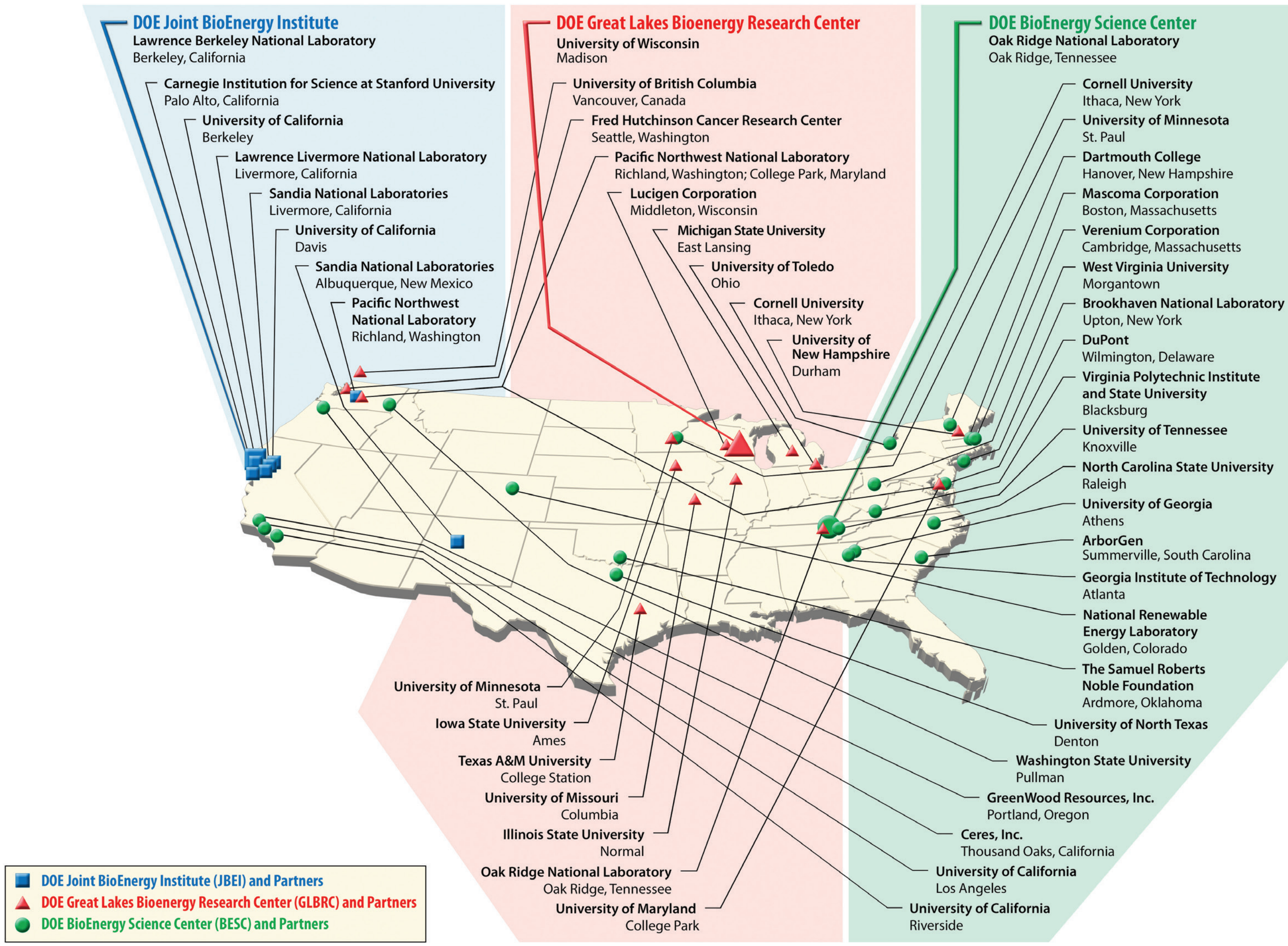

* See also numbers under "BRC Research Spanned," p. iv.