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COVER STORY

Seeking Better Biofuels

Researchers at the Joint BioEnergy Institute transform biomass into energy-rich fuel molecules

By ***Melody M. Bomgardner***

SUPERIOR SLUDGE
JBEI researcher Rajit Sapar examines cellulose that has been treated with a new microbe capable of making fuel from cellulose.

Credit: Lawrence Berkeley National Laboratory

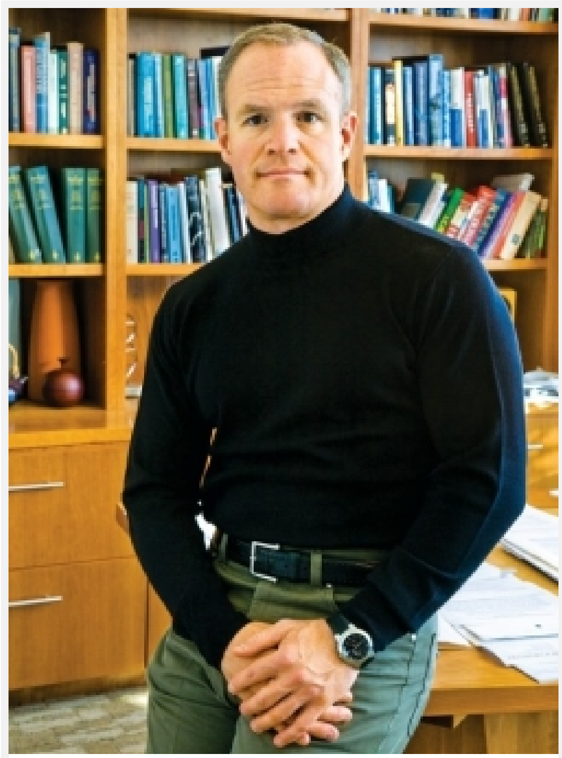
In this season of cheap oil, the promise of running our energy-intensive economy on plants rather than fossil fuels can seem further out than ever. Yes, there's a dollop of corn ethanol in our gasoline, but that is not exactly the energy transformation we were promised. Maybe the whole idea behind our bioenergy future needs to be completely rethought.

Jay Keasling, the principal investigator and chief executive officer of the Joint BioEnergy Institute at Lawrence Berkeley National Laboratory, has been doing that kind of rethinking for a decade now. **JBEI <http://www.jbei.org>** , pronounced "jay bay" by those in the know, is a bustling hub of 150 scientists and staff whose purview starts with the basics of plant biology and extends to making fuels with molecules that have never been fuels before.

"We are a basic science research institute but are focused on the particular problems of biomass-to-biofuels transformations," Keasling says, just after greeting a visitor to the lab. And he is very particular about which such problems he wants his team to tackle. "We work on risky future stuff—what no company in its right mind would take on. It's not corn, yeast, and ethanol—those are not interesting to me."

JBEI at a glance

- **Year founded:** 2007
- **Location:** Emeryville, Calif.
- **Budget:** $25 million per year from the Department of Energy
- **Founder:** Jay Keasling
- **Number of researchers:** 150
- **Patent applications:** 100
- **Patents issued:** 9
- **Technologies licensed:** 63
- **Spin-off companies:** 4, with 5 more pending



Keasling
Credit: Lawrence Berkeley National Laboratory

Instead, JBEI researchers are zeroing in on concepts that others have decided are too difficult or would take too long to prove. Can you engineer a plant's cell walls to release its sugar building blocks? Is there a new chemistry that can break cell walls down? Can you identify a new fuel candidate by its chemical structure and then engineer a microbe capable of producing it from sugar?

At almost every turn, synthetic biology promises answers to these questions. Keasling, who is also a professor at the University of California, Berkeley, is a native of that burgeoning field. He founded Amyris and LS9, two of the earliest biobased chemical firms that use engineered microbes. JBEI scientists say Keasling has given the institute an entrepreneurial vibe; several have started their own companies.

JBEI is one of three Department of Energy-funded Bioenergy research centers established in 2007 by the Office of Science to accelerate research breakthroughs related to advanced biofuels. The other two are at Oak Ridge National Laboratory and the University of Wisconsin, Madison.

The institute hosts postdocs and visiting scientists from around the globe. It has a research partnership with three other national labs—Lawrence Livermore, Pacific Northwest, and Sandia. And it has academic partnerships with UC Berkeley, UC Davis, and the Carnegie Institution for Science. But unlike in most joint research groups, all JBEI scientists work together on one floor of offices and labs in Emeryville, Calif.

Early this month, JBEI hit two milestones—it filed its 100th patent application and published its 500th paper. But its future is uncertain. At the end of 2017 it will reach the end of its second funding cycle. Keasling has plans to set up a new version of the institute that will apply the science it is developing to problems beyond fuels.

Peering Inside Plants

Combinations of heat, acids, bases, and enzymes can be used in many ways to extract sugars from biomass. Yet no technique is efficient, quick, and cheap. After many years of effort, the people who work with biomass still have the same complaint: Plant cell walls are recalcitrant and don’t want to release their valuable sugar molecules.

And that’s no accident. Plants have evolved clever ways to protect their valuable tissues from insect and microbial pests, and those methods work equally well against humans who wish to make renewable fuels and chemicals. Most of a plant’s sugar is bound up in cellulose and hemicellulose—complex polysaccharides that give cell walls their structure. The polysaccharides are cross-linked by lignin, a phenolic polymer that is resistant to water.

To make economical use of biomass, scientists need a better understanding of the underlying biology of the plant cell wall, according to Jennifer C. Mortimer, JBEI’s director of plant systems biology. “We have very, very limited knowledge to help answer questions such as, ‘Why are plant cell walls so hard to break down?’ ” she says.

Mortimer’s group studies the molecular architecture of cell walls, in particular the shapes of various forms of cellulose, hemicellulose, and lignin and how they are arranged relative to each other. These arrangements are responsible for the mechanical properties of the cell wall—and for their resistance to chemical deconstruction.

Recently, Mortimer and her team used solid-state nuclear magnetic resonance to decipher the shapes of cellulose, lignin, and the hemicellulose xylan in the stem cell walls of the model plant *Arabidopsis thaliana* (*Biochemistry* 2015, DOI: **10.1021/bi501552k <http://cgi.cen.acs.org/cgi-bin/cen/trustedproxy.cgi?redirect=http://pubs.acs.org/doi/abs/10.1021/bi501552k?source=cen>**). The NMR techniques resolved the locations of carbon sites of the three materials and their spatial relationships to each other.

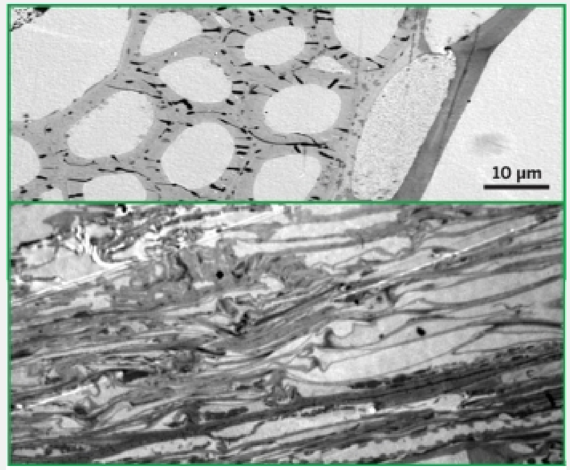
In the near term, understanding plant cell wall architecture means Mortimer can generate hypotheses "to tell the deconstruction guys what to go after." But she'd also like to know how plants make sugars, transport them to cells, and assemble them into cellulose and other macromolecules. "If we understand how plants are made, we can use synthetic biology to reroute certain pathways to make simpler versions of the polymers with fewer types of bonds," she suggests.

Indeed, scientists are beginning to understand there is more than one way to build a successful plant. For example, Mortimer's team is learning how to grow *Arabidopsis* with mutations that make it lignin-light. Early results have been promising—the scientists grew plants that could survive with 30% less of the cross-linking polymer, and after processing, generate higher yields of sugar.

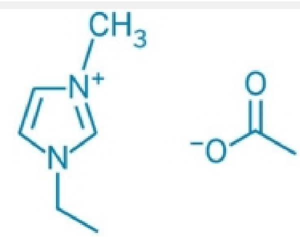
The team has graduated to testing versions of *Arabidopsis* with altered lignin content in just the leaves, rather than in the stems, where lignin provides structure for the system that moves water through the plant. Plants altered this way grow to roughly the same size as wild-type plants, Mortimer says.

Plants, Deconstructed

Low-lignin energy crops are only one approach to biobased fuels in JBEI's crosshairs. Researchers at the institute also have developed a process that uses ionic liquids to attack cell walls. Ionic liquids are molten salts that are liquid at room temperature. Unlike ionic solutions such as salt water, which contains neutral water molecules in addition to ions, ionic liquids have only cations and anions. They can harness their strong polarity to invade stubborn plant cell walls.



BREAKDOWN
Intact plant cell walls (top) are degraded after 40 minutes of treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate (bottom).
Credit: Lawrence Berkeley National Laboratory



1-Ethyl-3-methylimidazolium acetate

Normally, biomass must be treated by enzymes that have been customized for a particular plant material—a major expense that has hindered biofuels development, explains Nathan Hillson, JBEI's director of synthetic biology. "But treating biomass with ionic liquids softens up the cell walls, regardless of the type of feedstock," he says. And whereas standard processes that use heat, acids, and expensive enzymes primarily act on cellulose, ionic liquids dissolve hemicellulose and some portion of lignin. Studies suggest the ions form strong hydrogen bonds at the hydroxyl groups in cellulose. Less is known about how they dissolve lignin.

Once dissolved, the polysaccharide components of biomass can be precipitated out of the liquid in a simpler form. The partially unwound molecules can be quickly converted into simple sugars via treatment with enzymes. The result is "very clean sugar that even picky microbes love," Keasling says.

Keeping microbes happy is important because they serve as chemical factories that turn sugar into fuel and chemical molecules. Unfortunately, most microbes and enzymes do not perform well in the presence of ionic liquids. In addition, ionic liquids such as JBEI's lab standard, 1-ethyl-3-methylimidazolium acetate, are expensive and produced from nonrenewable resources.

Teams led by JBEI's Blake Simmons, vice president of deconstruction, are pursuing several avenues to make ionic liquid pretreatment commercially viable. They are looking for cheaper ionic liquids, designing recovery and recycling processes, and creating biologically friendly ionic liquids from dissolved lignin (*Proc. Natl. Acad. Sci. USA* 2014, DOI: **10.1073/pnas.1405685111 <http://dx.doi.org/10.1073/pnas.1405685111>**).

Another approach is to find ionic-liquid-tolerant microbes and enzymes—or to design them that way using synthetic biology. That's why biochemist Steven Singer is a connoisseur of compost, although in smaller quantities than consumed by the typical gardening enthusiast. “We can get free compost in Berkeley, but I was conspicuous because I came with a cup and not a truck,” he says.

Singer also gets samples of microbial communities from the San Francisco salt flats and from rain forests, where the organisms are heat tolerant. Such microbes produce interesting active compounds in response to environmental conditions such as heat, toxics, and competition with other organisms. For example, Singer has found enzymes that tolerate both high temperatures and ionic liquids. He’s also found microbes with cellular protein pumps that can expel ionic liquids.

But it’s tough to find the genes responsible for these abilities from a sample containing a variety of species and strains. “It’s a puzzle made of multiple puzzles with no image to guide you,” Singer laments. His team has to do a great deal of analysis and write their own genetic algorithms, part of a new field called metagenomics (*Microbiome* 2014, DOI: **10.1186/2049-2618-2-26 <http://dx.doi.org/10.1186/2049-2618-2-26>**).

The algorithms guide the selection of useful genetic sequences that can be adapted to function in a biofuel system. They can then be put into yeast to create strains that can convert sugar into fuels when ionic liquids are present or inserted into fungi to efficiently produce new commercial enzymes.

Pathways To Fuels And Chemicals

At JBEI, bacteria and yeast make some very interesting molecules. JBEI researchers have engineered *Escherichia coli* to produce methyl ketones, which are the main chemicals responsible for the flavor of blue cheese and butter. Certain aliphatic methyl ketones in the C_{11} to C_{15} range, they found, also make excellent diesel blendstocks.

Harry Beller, biofuels pathways director, led a team that used synthetic biology to modify *E. coli* to overproduce aliphatic methyl ketones, resulting in a 700-fold increase in yield (*Appl. Environ. Microbiol.* 2012, DOI: **10.1128/aem.06785-11 <http://dx.doi.org/10.1128/aem.06785-11>**). “With engineering, our metabolic diversity is incredible,” Beller says. “Whatever your wildest imaginings of transformations, you can find a bug that does it.”

Bisabolane (+)-α-Pinene Isoamyl alcohol

The universe of natural products that can be used as fuels is enormous, according to Beller and Taek Soon Lee, JBEI's director of metabolic engineering. In addition to methyl ketones, they have targeted the family of natural molecules that includes fatty acid alkyl esters, alkanes, and α-olefins.

Isoprenoids, a family of natural products normally associated with plants, are also targets for Beller and his collaborators. For example, they have produced isoamyl alcohol—a precursor to banana oil—and the terpenes α-pinene and bisabolane. "Whether an isoprenoid would make a good fuel depends on it having to fit narrow constraints in terms of energy density, chain length, combustion heat, efficiency, lubricity, and stability," Beller explains. He points out that fuels made from isoprenoids can have very low freezing temperatures.

Pinene, usually extracted from pinewood, has a ring structure that makes it a potential precursor to high-energy fuels for jets and rockets (*ACS Synth. Biol.* 2014, DOI: **10.1021/sb4001382 <http://cgi.cen.acs.org/cgi-bin/cen/trustedproxy.cgi?redirect=http://pubs.acs.org/doi/abs/10.1021/sb4001382?source=cen>**).

Bisabolane has a similarly handy carbon ring. Lee says it has promise as a biosynthetic alternative to diesel. His team has been manufacturing the precursor bisabolene by engineering the mevalonate pathway in microbes. The pathway is responsible for the C_5 starting materials for isoprenoids. It also has been engineered to produce the biobased chemical intermediate farnesene and the antimalarial drug artemisinin (*Nat. Commun.* 2011, DOI: **10.1038/ncomms1494 <http://dx.doi.org/10.1038/ncomms1494>**). Amyris, the start-up founded by Keasling, developed the artemisinin pathway and is commercializing farnesene.

Lee successfully inserted the genomic pathway for bisabolene into *Saccharomyces cerevisiae,* a hardy yeast used in industrial production of ethanol. He'd also like the yeast to host an enzymatic step to hydrogenate bisabolene to bisabolane.

Before a new molecule can enter the market as a component in fuel, it must be certified by a standards agency such as ASTM, and that generally requires industry partnerships. In 2014, for example, Total joined with Amyris to get approval for 10% farnesene to be used in jet fuel. Also last year, bisabolane was named one of nine advanced molecules that could revolutionize jet fuel by Jim Lane, editor of *Biofuels Digest*.

With all these elegant and useful pathways, it's no surprise that JBEI scientists are itching to go beyond the institute's core fuels mandate and make higher-value products.

"Fuels are chemicals. We go to all this trouble to make a beautiful molecule just to burn it up in your car," says Leonard Katz, a JBEI collaborator and director of research in synthetic biology at UC Berkeley. Katz's expertise is harnessing a family of multienzyme complexes called polyketide synthases, or PKSs, to make useful chemicals.

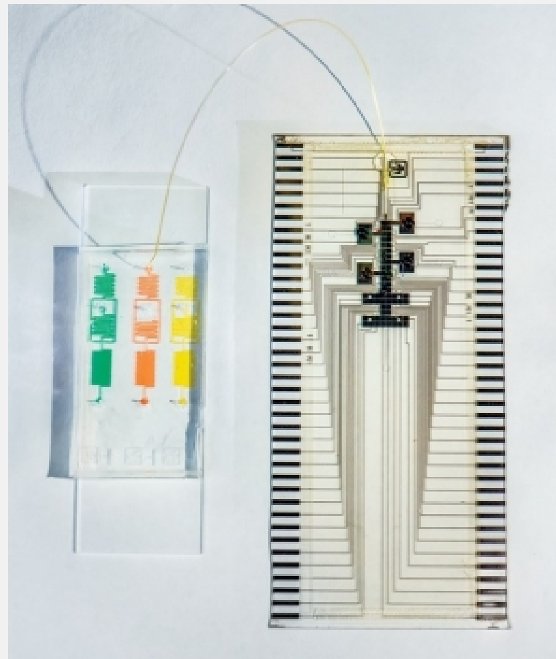
PKSs are responsible for making polyketide drugs including the antibiotic tetracycline. They work like modular assembly factories. "By repurposing PKS, we make fuels and chemicals where the intermediates don't flow freely but are tied to the enzymes," Katz says. By linking PKS modules so that each performs a transformation, it is possible to make chemicals such as adipic acid and customized polymers with advanced properties.

Yet to bring down the costs of these microbially produced fuel and chemical molecules, researchers need to increase yields from tens or hundreds of milligrams per liter to several grams or more. They also must be able to predict how well sequences will work in industrial organisms, particularly yeast. "We need to get an even better biological understanding so that the metabolic systems are not a black box," Hillson says.

Building A Knowledge Engine

Faster, higher, stronger. Like Olympic athletes, scientists hoping to see new biobased chemicals and fuels hit the market must optimize every discovery and quickly learn from their successes and failures. That can require doing tens of thousands of experiments a week, building knowledge along the way.

“We do a lot of proteomics and metabolomics because they are the bread and butter of pathway engineering,” says Paul Adams, JBEI’s vice president for technology. With the ability to detect 8,000 proteins made in *E. coli* per day, he’s able to help synthetic biologists rationally engineer higher-yielding metabolic pathways.



TINY ASSAYS
In this microfluidics device, a droplet generator (left) makes single cell-sized droplets, which can be combined with chemicals in various channels on the chip (right).

Credit: LBNL

Adams’s group assists JBEI scientists with high-throughput assays, high-resolution analytics, and other tools to process and analyze huge quantities of biomass, metabolic products, and engineered enzymes and microbes. The rest of the time the team invents new technologies.

For example, to analyze what happens to plants when they are engineered to make less lignin, Adams’s team developed a new instrument to measure the mechanical strength of plant stems. The team used scanning electron microscopy to validate that it works. Because the method doesn’t require sample preparation, it speeds up the analysis process.

Recently, the group created a new device that marries a droplet generator to a microfluidics chip to perform multistep assays. The generator creates droplets containing a single microbe cell, and the channels in the chip move the droplets and combine them to do parallel experiments.

In one set of experiments, researchers used the device to evaluate the effect of four different ionic liquids at four concentrations on the growth rates and ethanol production of yeast (*Lab Chip* 2015, DOI: **10.1039/c4lc00794h <http://dx.doi.org/10.1039/c4lc00794h>**).

Much of what Adams works on is methods to speed up experiments with thousands of samples and analyze them more quickly. That creates a huge amount of data—which JBEI posts on its website—that can be mined to design future experiments.

Reinventing JBEI

There's little doubt that many of the discoveries and technologies developed at JBEI have a bright future. Indeed, some already have been licensed to companies large and small. But the institute's current funding from the Department of Energy ends after 2017.

Keasling is anticipating a call for proposals from the same agency for research and development of biobased chemicals as well as fuels. Seeking to participate, he and his colleagues unveiled plans in July for the next iteration of JBEI, called the FutureBio Institute.

At the heart of the new institute is a set of capabilities called the FutureBio Foundry.

"The Foundry is an engine built to drive energy discoveries; it can also build other solutions," Keasling explains. "You want to make sure you get value from the chemicals made from sugar. And biology can make very interesting molecules that you can't make with chemistry." Those molecules could be part of the solution to problems such as climate change, sustainable food production, and environmental pollution, he adds.

Hillson, the architect of the FutureBio Foundry, says it will be an outward-facing biological engineering resource that will collaborate with industry partners in agriculture, materials, and even automotive manufacturing. "It represents our ability to design, build, test, and learn from experiments," he says. "The goal is to scale up technology that works and shorten the time to commercialization."

Escaping The Lab

Four start-ups build on discoveries that span the breadth of JBEI research.

Afingen: Getting more sugar from plants

Lignin helps plants take up water and gives wood its strength. But it also stands in the way of affordable chemicals made from biomass. Afingen, founded in 2012 by JBEI plant biology and genomics scientists Henrik Scheller, Dominique Loqué, and Miguel Vega-Sanchez, is designing crops that contain less lignin and more sugar.

Afingen is building on JBEI research on how master transcription factors regulate the expression of genes that turn on or off biosynthesis in plant tissues. Although that work showed that some low-lignin plants produce higher yields of sugar in the lab, it was unknown whether modified energy crops would grow well. Using tissue-targeted engineering, Afingen has developed low-lignin switchgrass, an energy crop.

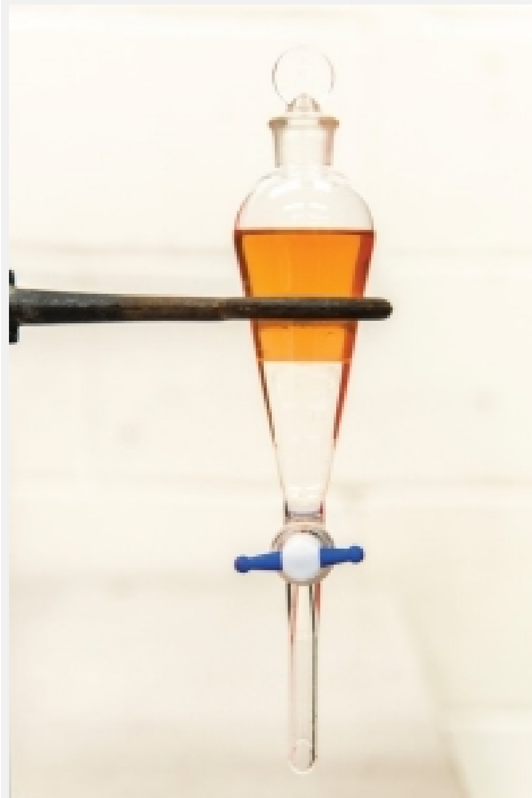
The plants contain lignin only where it is needed. “Our switchgrass is extremely healthy, very robust, and actually grows faster than controls,” explains Ai Oikawa, Afingen’s managing director.

Afingen is also engineering yeast strains to boost production of fine and specialty chemicals made from switchgrass sugars. The company has begun a pilot project at the Advanced Biofuels Process Demonstration Unit, a scale-up facility located downstairs from the JBEI labs.

Illium: Ionic liquids from lignin

Although most advanced biofuels researchers view lignin as a problem, Aaron M. Socha, CEO of Illium Technologies, takes a different view. Illium is working to deconstruct the phenolic polymer into monomers and then use them to design useful solvents called ionic liquids.

Ionic liquids, solvents that contain only cations and anions, are pricey and normally made from petroleum precursors. But Socha is taking the biobased route. “Some lignin monomers have aldehyde groups that can be functionalized—they can be easily converted to ammoniums to make the cation of your ionic liquid,” he says.



LIGNIN LAYER
Illium scientists made this brownish amine solution, an ionic liquid precursor, from lignin. The clear layer is water.
Credit: Luis Lopez/Bronx Community College

In turn, those ionic liquids can be used to degrade biomass, making it easier and faster to get the sugars out. Socha is also targeting other bulk markets, such as heat transfer fluids, that call for large quantities of ionic liquids but do not require high purity.

Lygos: Microbes that make chemicals

By engineering yeast, it’s possible to convert sugar into a huge variety of chemicals. But only a handful of these chemicals can be produced more cheaply by microbes than via conventional means. One of those, according to Lygos founder Eric Steen, is malonic acid, a dicarboxylic acid precursor to specialty polyesters.

Formed in 2010, Lygos was JBEI's first spin-off. Steen is a former postdoctoral researcher at the institute. It was the skills he learned there that formed the basis of the company. "The underlying technology is physically how you manipulate DNA to predictively program the cell's blueprint," Steen says.

But there is a lot of work to make a business from such abilities. "Getting from just a sniff of a chemical to a commercially viable scale requires a lot of optimization and data analytics," Steen explains. He hopes to find partner companies to get biobased malonic acid into products such as low-energy-curing coatings, resins, and adhesives.

TeselaGen Biotechnology: Building better DNA sequences

Consumer product designers use computer-assisted design and rapid-prototyping tools to quickly turn ideas into physical items. Michael Fero, founder of TeselaGen, wants to bring similar tools to the folks who work in synthetic biology.

"It's about speed. The companies that are most interested in us are interested in accelerating their research," Fero says. "Cloning is brutally slow and fraught with errors, complications, and dependencies."

Fero and cofounder Nathan Hillson, JBEI's director of synthetic biology, started the company in 2011 to build on a Hillson invention that puts DNA pieces together without overlapping sequences. TeselaGen's computational tools are built on new, commonly used protocols for composing new DNA sequences. "Otherwise people are piecing it together with spreadsheets," Fero says.

TeselaGen already has customers for its products, including the pharmaceutical maker Amgen and the biobased chemical company Genomatica. And a yet-to-be-named chemical giant has hired it to build an entire synthetic library, Fero says.

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Comments

S. Vatcha **(October 26, 2015 5:56 PM)**
JBEI's successor FutureBio Institute should consider merging with the Energy Biosciences Institute (EBI) (http://www.energybiosciencesinstitute.org/, http://vcresearch.berkeley.edu/research-unit/energy-biosciences-institute), which is seeking new external sources of funding (http://alumni.berkeley.edu/california-magazine/just-in/2015-02-20/not-so-fast-uc-berkeley-biofuel-research-takes-hit-bp-oil).
» **Reply**

Bruce Walck **(October 27, 2015 3:07 AM)**
Take a look at the Intrexon /Dominion energy conversion tech. The duo is skipping plant based biomass in lieu of natural gas as a far cheaper feedstock. Isobutanol is the first target and likely aimed at the ethanol market since output is less corossive and can be shipped via existing plumbing. Tech will likely be expanded into lubricants and exotics (jet fuel) if successful in its first iteration.
» **Reply**

R. S. Berry **(October 30, 2015 10:21 PM)**
We are making this article available to the students in our class on Energy and Energy Policy. They are upper-class undergraduates and graduate students in a wide range of fields, who work in interdisciplinary teams.
» **Reply**