



# The Race for New Biofuels

by Frances H. Arnold

New technology, developed just over the last few years, has now allowed us to design microorganisms—almost from the ground up—that will provide new sources of fuel. Transportation fuel counts for about 28 percent of the U.S. total energy use, 22 percent of world's energy use, and 27 percent of global carbon emissions. Most of that fuel comes from petroleum. We get about 40 quads of petroleum (enough energy to burn a 100-watt lightbulb for more than 13 million years) from domestic and imported sources, of which 60 percent goes to transportation.

Instead of chewing up fossil fuels for transportation, we need a more sustainable strategy for two main reasons, the first of which is the security of supply. About 60 percent of our oil is imported, much of it from countries that don't like us very much and lie in unstable parts of the world. The total economic and societal costs of these imports—including, for example, the costs

of defending interests in the Middle East—are not reflected in the already very high cost of oil today, which has reached more than \$130 a barrel. Clearly, the less we import, the better.

The other reason for developing biofuels is climate change. We're interested in fuels derived from plant materials because overall, they're carbon neutral. Green plants take solar energy, water, and carbon dioxide, and convert them into biomass, which is what we call the plant material we can use for fuel. Then, some magnificent microorganisms turn the biomass into liquid fuel. When you burn the fuel, growing plants eventually reabsorb the emitted carbon dioxide, giving you a carbon-neutral cycle. Growing plants is a simple system for capturing carbon, and as long as you don't use up a lot of fossil fuels during any part of the process—for example, by using gasoline to truck biomass to the ethanol facility or using a great deal of fertilizer—you come out even. The key to assessing

this technology is to look at whether you get more energy out than you're putting in with fossil fuels.

That's the essence of the debate over biofuels—most notably corn ethanol—that you might be reading about. I won't go into that debate, other than to tell you that corn ethanol probably creates as many problems as it solves, and is likely to be only a short-term solution to jump-start a biofuels industry. To turn corn into fuel, you take the 72 percent of the corn kernel that's starch and break it down into a simple sugar, called glucose, with the aid of an enzyme. Then, using a process that we've all been fond of for thousands of years—that is, brewing beer with the help of microorganisms—we

Ethanol may be good in certain drinks, but it isn't necessarily the best fuel we can conceive of. We make ethanol simply because we know how.

ferment the glucose. However, growing and processing the corn requires substantial amounts of resources like energy, water, and fertilizer. The yield is low, because you only get a small amount of biomass that you can convert to ethanol—about five tons per acre of corn. And, because you're turning food crops into fuel, corn ethanol production helps to drive up prices of food, like the cost of tortillas in Mexico. That trade-off is unacceptable. Ethanol may be good in certain drinks, but it isn't necessarily the best fuel we can conceive of. We make ethanol simply because we know how.

The alternative to using corn or other food-based crops for fuel is to use cellulosic feedstock. In the short term, we're talking about things like rice straw; corn stover, which consists of the leaves, stalks, and other waste matter from corn; bagasse, which is what's left over after you extract the juice from sugar cane; and corn fiber, the byproduct of milling corn into syrup. In the long term, we need to move toward dedicated energy crops, such as switchgrass or miscanthus, which not only produce as much as 30 tons of biomass per acre, but also need minimal water and nutrients and grow

very rapidly. A study by the Department of Energy and the Department of Agriculture estimates that the United States could produce 1.3 billion tons of cellulosic biomass per year, without having a grossly negative impact on food supply. A back-of-the-envelope calculation shows that 1.3 billion tons is the energy equivalent of some three billion barrels of oil—a considerable fraction of the total U.S. annual consumption of about seven billion barrels. Biofuels aren't going to solve the energy problem alone, but their contribution can be significant.

So the good news is that there's a lot of energy stored in biomass and the United States is in a very good position to be making renewable fuels. The bad news is that photosynthesis isn't very efficient. In the midlatitudes, where we live, only 1.2 percent of the sun's energy, averaged over the course of a year to allow for daily and seasonal fluctuations and weather patterns, is converted to chemical energy in the form of biomass. Additionally, growing biofuels requires a lot of land—we'd have to use much of the currently marginal and unused farmland to grow energy crops. There are also major engineering problems, the biggest being that biomass in its natural form is not something you can put into your automobile. You have to convert biomass to liquid fuel.

So why is this so darn hard? Why isn't there a biomass-to-fuel factory on every block? The reason is that plants have evolved to defend their structural integrity. They eventually give up and degrade, but they're pretty robust. Some 25 percent of the plant is this stuff called lignin, which is chemically similar to asphalt, and very few things can break down asphalt. They say you can make anything from lignin except money. It's basically trash—you can't break it down, and you can't convert it into anything useful, at least not in an economical fashion. So it just gets burned to provide energy for the rest of the process.



Miscanthus, a tropical grass, grows up to 11 feet tall.

Fortunately for us, about 70 percent of a plant is cellulose and hemicellulose, polymers that can be broken down into sugars microbes can use. (Wood and plant fibers like cotton are mostly cellulose.) The cellulose's sugars, however, are physically inaccessible. The glucose units in the polymer chains form tightly packed layers in a crystalline structure. The cellulose chains, in turn, form tight bundles called microfibrils in which regions of glucose units in this crystalline array alternate with regions of glucose chains in an amorphous configuration. Any large enzyme that could break down the cellulose into its glucose units if the cellulose were in solution would have trouble getting in there in the first place, unless some substantial and expensive pretreatment were done to make the chains physically accessible. There's no easy chemical solution to this problem. Right now people are using grinding and treatment with acids to break up the microfibrils, but you'd need an awful lot of acid to make an impact on our oil consumption. Also, too much acid can ruin the sugars. We need to use more biology and less chemistry to develop environmentally friendly methods for making this cellulosic glucose accessible to the microbes that make the fuel.

Brazil, a major ethanol producer, ferments glucose straight from sugar cane—a process that's both easy and, especially with current high oil prices, profitable. Brazil basically runs its auto fleet on domestically produced ethanol, and has become a supplier of ethanol to the rest of the world. In the United States, ethanol is primarily made from corn. Corn kernels are mostly starch, so processing them requires an enzyme to break the starch down into sugars that can be fermented. This extra step makes it more expensive. When we begin producing dedicated biofuel crops such as switchgrass, which don't even have a high starch content, we'll have to go through even more mechanical and chemical steps to break down the cellulose, and

this will drive up the capital cost of the biofuel facility. The good news is that the feedstock, which is a major factor in fuel costs, will be less expensive than sugar or corn.

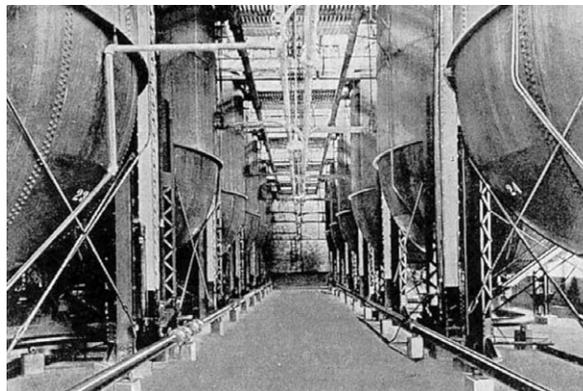
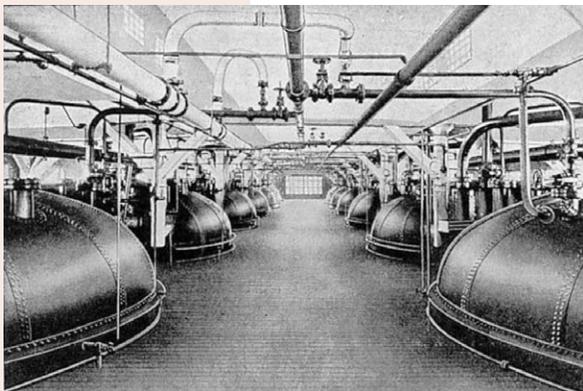
If you take a step back, you see that ethanol isn't even the most attractive of fuels. The energy content of ethanol is a lot lower than gasoline, delivering only .7 times the mileage. Moreover, the existing fuel distribution infrastructure cannot be used to store and deliver ethanol. Ethanol has a high vapor pressure, and because of its high affinity for water, it readily takes up water and corrodes the tanks and pipelines that carry oil. Also, ethanol can only be blended with gasoline up to about 10 percent before car engines need to be modified.

As a result of these problems, and the fact that ethanol can't be used to fuel trucks or jets, many people are interested in alternatives to ethanol. In fact, alternatives are becoming possible because of the genetic-engineering revolution. Within the last 30 years or so, biologists, chemical engineers, and just about everybody else have become able to tinker with DNA. Even high-school students do molecular-biology experiments with kits that can be ordered from any chemical supply house. We can engineer bacteria to produce all kinds of molecules, so we can sit back and ask ourselves, if we don't want ethanol, what do we want? What could be supplied in a biologically friendly and environmentally friendly fashion? Hydrocarbons that look like petroleum, of course, would be very nice, but nobody has demonstrated such a technology that's close to being practical yet. One good possibility, however, is ethanol's bigger cousin, butanol.

**Corn, switchgrass, and miscanthus grow side by side in experimental plots in Urbana, Illinois. These fields are two years old.**



Andrew Lenkey



Founded at the end of World War I, the Commercial Solvents Corporation was a leader in making butanol with Weizmann's fermentation methods. The left photo shows the tops of the 50,000-gallon tanks at a butanol plant. The tank bottoms are shown on the right.

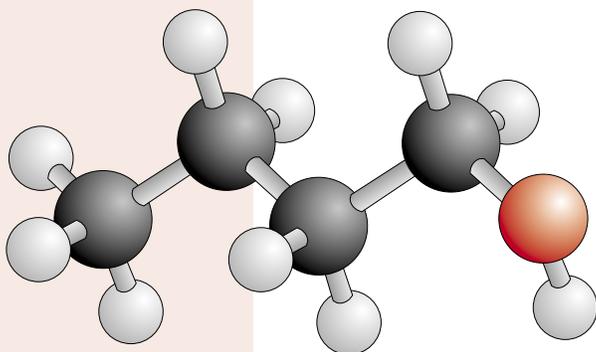
### BETTER BIOFUELS WITH BUTANOL?

Ethanol has only two carbons while butanol has four, which makes it more energy-rich. Butanol's low water content (it has less affinity for water than ethanol) and high energy content are right up there with gasoline. Butanol can be distributed and stored in existing pipelines and tanks. It burns cleanly, without any kind of modification to gasoline engines, and it can be blended with gasoline at any ratio. Furthermore, you can make other fuels—gasoline, diesel, or jet fuel—from butanol using well-known chemical processes. Butanol was, in fact, one of the most important commercial fermentation processes in the mid-20th century. Because the last butanol plants were closed down just before the genetic-engineering revolution, the organisms that were used to make butanol commercially have not been genetically modified to improve their productivity.

Like everything else, making butanol started off as a defense application. Chaim Weizmann, a chemist who later became the first president of Israel, was awarded a patent in 1916 for his method of producing acetone from the bacterium *Clostridium acetobutylicum*. The acetone was used to make an artillery-shell propellant called cordite,

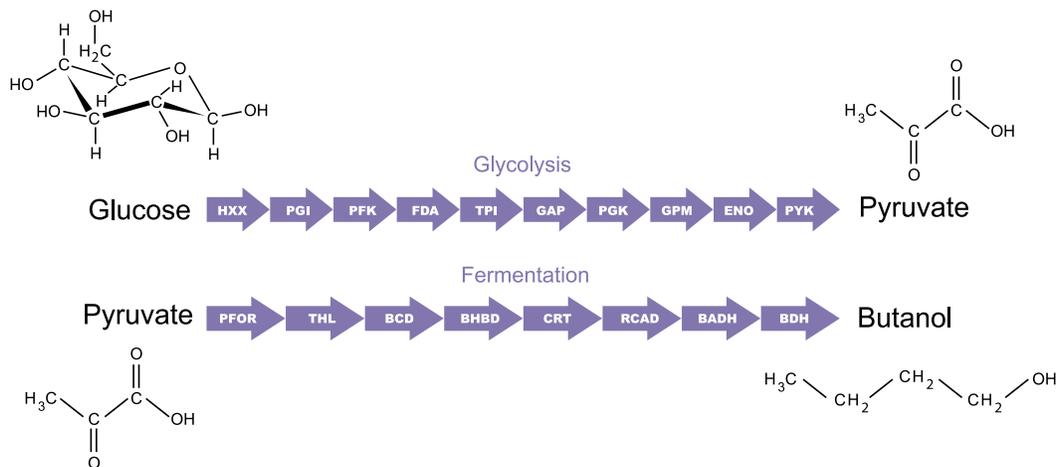
also known as smokeless powder, for World War I. The bacteria also produced butanol and ethanol as by-products. By 1927, people had recognized that butanol was a good motor fuel and solvent, and had started breeding strains of microbes to increase the butanol content of the product mix. By the next decade, there were large plants containing big wooden vats of fermenting clostridia that produced butanol from molasses, which was a waste product of sugar processing, or from potatoes. But in the 1950s, petroleum became a cheaper source of fuel and of chemical feedstocks, which was the other main use of butanol. Butanol plants in the West closed in the 1960s, and the last remaining ones in the Soviet Union and South Africa closed in the 1980s. However, the organisms that were used to make butanol are now sitting in the freezer of a South African researcher who moved to New Zealand more than 20 years ago.

We could just pull those microbes out of the fridge, put them back in those huge 50,000-gallon tanks, and start making butanol again on an industrial scale. The problem is that these microbes put too many of their resources into survival and reproduction, which is what evolution has bred them to do. The old manufacturing method was very good at converting your investment into a lot of organisms and just a little bit of money. The butanol is produced as a metabolic by-product, in relatively low yield, as part of a mixture with acetone and ethanol. Butanol is also toxic to the organisms that make it, so making it in bulk is not at all attractive to them. The organisms that make ethanol, on the other hand, can produce a broth that's over 10 percent ethanol; sake is about 12 to 18 percent ethanol. We've been breeding those guys for thousands of years, and they can tolerate a lot of ethanol. We haven't been working as hard on butanol, because you can't drink the stuff. We could try to improve on these organisms, but the process would be slow and difficult.



A ball-and-stick rendering of butanol. The black spheres are carbon atoms; white, hydrogen; and red, oxygen.

Inside the bacterium that ferments butanol, a series of enzymes (arrows) converts glucose into pyruvate; another chain of steps then turns pyruvate into butanol.

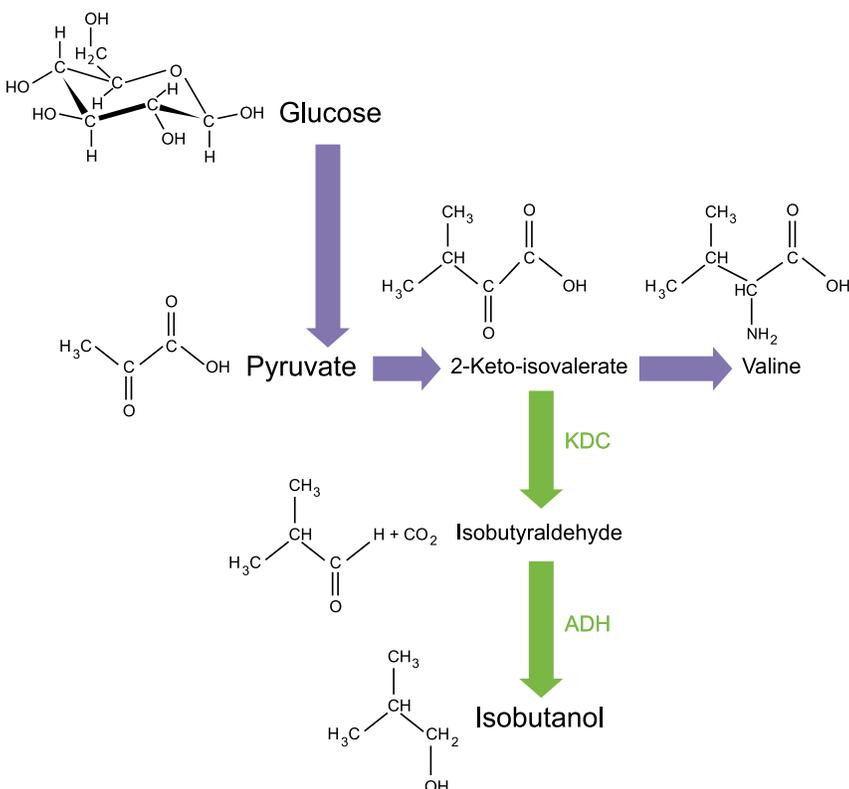


*E. coli* has a series of catalytic steps that turns pyruvate into an amino acid called valine. Jim Liao at UCLA hijacked that pathway, and by introducing two new enzymes, keto-acid decarboxylase (KDC) and alcohol dehydrogenase (ADH), he engineered *E. coli* that produced isobutanol instead of valine.

A butanol-producing clostridium, to a first approximation, is a little bag of catalysts that takes up glucose and converts it into a molecule called pyruvate, which is the cell's major source of energy. Pyruvate is an intermediate in a biochemical pathway inside the organism that's common to both ethanol and butanol production. Producing ethanol from pyruvate just takes a couple of steps, but making butanol is a long, tortured process involving intermediates that are quite costly to the cell in terms of the energy and catalysts required. Some of these intermediates also lead to ethanol, further lowering the yield.

People like me—we call ourselves synthetic biol-

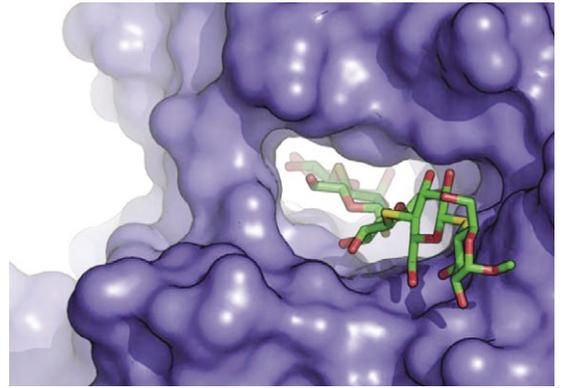
ogists now, that's our new marketing term—view microbes as the chemical factories of the future. Our plan is to start more from scratch and create an organism that is specifically designed to make butanol. My colleague, Jim Liao at UCLA, had a great idea. He pointed out that the cells already have a pathway—a series of catalytic steps—that turns pyruvate into an amino acid called valine. He envisioned a branch off of that pathway that makes a form of butanol called isobutanol in just two additional steps. Not only is this pathway not very toxic to the cell, it's also one that the cell is predisposed to use, because cells can make valine at high levels.



And with the revolution in genetic engineering, we can actually build this organism. Jim chose first to modify the *E. coli* bacterium because we already know a lot about it, and have identified all its genes and the vast majority of its pathways. It's a ready-made chassis, if you will. But that doesn't necessarily mean *E. coli* is going to be a useful butanol maker. However, it's a great proof of concept.

Remember that the catalysts for each of these steps, the enzymes, are all encoded in the bacterium's DNA. When I talk about reprogramming these cells, I'm talking about modifying their DNA so that they make the enzymes that I want them to make. To do that, we have to figure out the DNA code for those enzymes (and any associated controllers) and then synthesize that DNA. Not too many years ago, I had to actually synthesize little pieces of DNA by hand. It would take me hours, and I could only make short pieces, and those pieces were full of errors. Today I can just punch in a sequence and e-mail it to my favorite supplier. For less than a dollar a base pair, they will synthesize the gene and send it back to me in a few days or a few weeks. Basically, we can make any DNA we want and insert it into the bacteria.

Jim Liao's group did exactly that. He hijacked the valine pathway at an intermediate molecule called 2-keto-isovalerate. Remember, I said it takes



**An enzyme called cellobiohydrolase II (blue) pulls a cellulose polymer chain (green) through a hole in the enzyme. The enzyme is one of many that breaks down cellulose.**

only two steps from there to make isobutanol. The first step is turning 2-keto-isovalerate into isobutyraldehyde, which the 2-keto-acid decarboxylase, or KDC, enzyme does. Then you convert isobutyraldehyde to isobutanol with an enzyme called alcohol dehydrogenase, or ADH. So Jim took a KDC gene from a bacterium called *Lactococcus lactis* and the ADH gene from *Saccharomyces cerevisiae*, better known as baker's yeast or brewer's yeast, and put them in *E. coli*. And lo and behold, the bacteria started making isobutanol. But they could also make many other things from pyruvate, so he optimized the yield by getting rid of those competing pathways by knocking out the DNA that encodes them, which diverted more of the flow to isobutanol. (He had to do that very carefully, because some of that DNA encodes things that are important for the growth of the organism.) He was able to make up to 20 grams of isobutanol per liter of the microbial broth—86 percent of the theoretical yield, which was better than the best reported butanol production from any natural organism, even at the height of industrial butanol production.

#### CUTTING THROUGH THE CELLULOSE

So far, everything I've told you is about turning glucose into butanol. But could we get (iso)butanol from cellulose? To have the simplest and cheapest possible process, we'd like to consolidate all the steps into a single organism that degrades the biomass into glucose, and then converts the glucose into butanol, all in a single pot.

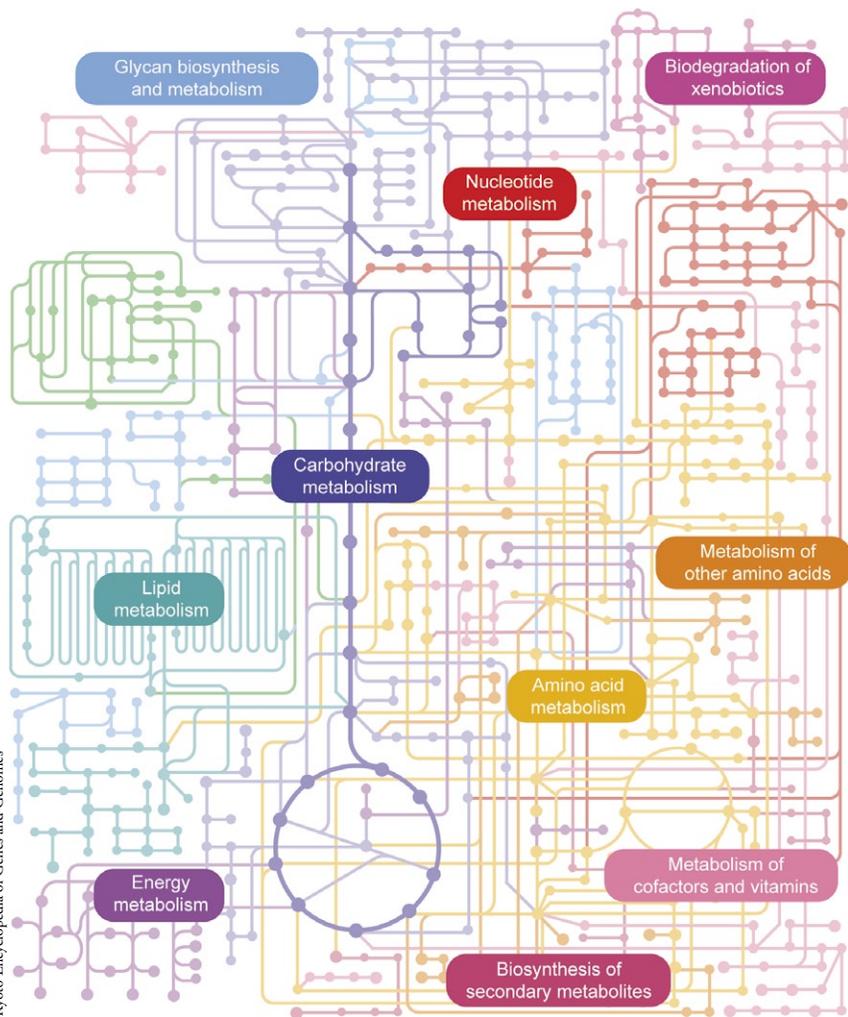
Cellulose degradation, however, is the most complicated and difficult step of producing butanol—or any other biofuel. If cellulose broke down easily, plants would turn into sugary ooze when licked, and they don't do that. The problem is that cellulose-degrading enzymes have a hard time attacking those bundled cellulose polymers. So after the cellulose is pretreated to make it more amorphous

and accessible, there's a concerted attack involving a whole bunch of enzymes doing different things simultaneously to break the cellulose down into glucose. These enzymes are collectively called cellulases.

A subset of cellulases called endoglucanases cuts the middle of the cellulose polymer chains in the amorphous regions. This frees up the ends of the crystalline areas for another group of enzymes, called cellobiohydrolases, to attack. A cellobiohydrolase is a fascinating molecular machine that pulls a chain through a hole in the middle of the enzyme to bite off two sugar units at once, making a chemical unit called cellobiose. The two ends of the crystalline chain are different, and each is degraded by a different cellobiohydrolase. You're now left with a bunch of cellobioses, and an enzyme called beta-glucosidase comes and cuts them into individual sugar molecules, which the microbes can then convert into biofuel.

Breaking down cellulose takes a lot of enzymes and time, and is expensive, so researchers across the world are trying to discover novel cellulases—or engineer new ones—that will do a better job. Right now, industry uses a cellulase system that was discovered during World War II when someone's tent got chewed up in the jungles of Borneo by a fungus called *Trichoderma reesei*. We haven't moved to a substantially better system since then. The engineering and production have been improved, but the enzymes themselves have hardly been modified.

Some bacteria package all their cellulase enzymes into little molecular factories called cellulosomes on their cell surfaces. One could envision constructing such cellulose-chewing factories on the surface of your butanol-producing organism—which would be a lot of fun to do. The cellulases would break down cellulose and deliver it straight to the organism for fuel production. So Caltech and UCLA have started a synthetic-biology challenge to create such a microbe. We are working



**This map of a cell's metabolic pathways could be taken for a circuit diagram or a particularly elaborate subway map. Boosting the cell's isobutanol yield required shutting off some pathways and rerouting others.**

together to combine powerful, new cellulases from Caltech with the isobutanol pathway discovered at UCLA.

None of this is as easy as I've made it seem, however. Even a supposedly simple organism like *E. coli* is a complex beast. When I said we understand how *E. coli* is programmed, I lied. All those simple one-way pathways through which we convert glucose to isobutanol with this series of catalysts are very nice, but all these catalysts are sitting inside a cell that's got a whole bunch of other molecules working at the same time as it reproduces, grows, and responds to its environment. All of the pathways involved in breaking down cellulose or producing isobutanol regulate and interact with each other and with other pathways the cell needs to survive. It's an incredibly complex system that we really don't understand well, and if we're able to make isobutanol from cellulose, we're darn lucky. Furthermore, Jim made 20 grams of isobutanol per liter of the microbial broth, but we're going to need to make even more than that if this system is going to be practical.

## WHO NEEDS MELVILLE?

Some people think we can turn biology into an engineering discipline, making analogies between engineering living systems and engineering other forms of matter, from buildings to circuit boards. In this view, bacteria are little robots, programmed by their DNA to respond to their environment. The set of interactions among the genes and proteins in a regulatory network is like an integrated circuit in a silicon chip that embodies a function in its hardware. We should be able to have a little parts book where we can pull devices—a little piece of a circuit or a catalyst, or a little controller—from a parts list and construct the butanol-production pathway. We would write the DNA program by finding the desired code—the parts from our parts list—on a website, assembling them into one long sequence, and then sending it to a DNA-synthesis facility. We'd get our custom-made DNA in the mail the next day, and we'd put it into the bacteria, and they'd start doing what we've programmed them to do. This is the dream of the synthetic-biology community.

On the other hand, that dream belies the complexity of biology, and the fact that these systems are highly dynamic and interacting. There's a lot of redundancy in how DNA codes for a particular function, and there are many ways to tweak it and subtly alter its function. Unfortunately, we don't know what DNA sequence will encode a particular outcome. We don't know how to write down the DNA sequence that would make a "super" cellulase do exactly what we want. If we can't even make a single cellulase, how are we going to make synthetic biology so predictable that we can build a new organism that will efficiently make large quantities of new biofuels?

Every year, a set of tongue-in-cheek awards called the Ig Nobel Prizes is given for dubious scientific achievements. Real Nobel laureates and other scientific luminaries attend the award ceremonies, which are held at Harvard and feature a science opera and "nano-lectures," in which people are challenged to describe a technical topic in 24 seconds and to write a corresponding abstract that anybody can understand in seven words or less. For the human genome project, in which the complete DNA sequence that goes into making a human being was determined, MIT's Eric Lander wrote, "Genome. Bought the book. Hard to read." In other words, even if you know the sequence, you don't know what it does—there are too many possible outcomes, and the details matter.

It's the same in synthetic biology—if you can't even read a genome, how are you going to write it? If I'm ever asked to give a nano-abstract about engineering these organisms, I'm ready: "Genome. Great story! Hard to write." So what do you do when you've got writer's block? You get a good editor, of course. Creating the genome for an artificial

