Combining Agriculture with Microbial Genomics To Make Fuels

Efficient use of biomass will depend on crops adapted to marginal land, novel enzymes, and recapture of carbon dioxide

Nadathur S. Govind and Arup Sen

Despite major challenges, we believe that cellulosic sugars in agricultural waste are the best sustainable source for biofuels to meet worldwide energy needs. One key challenge involves dealing with diverse cellulosic sugars, many with high levels of lignin. Other tough challenges include converting both 5- and 6-carbon sugars efficiently into ethanol and recycling carbon dioxide from fermentations back into useful biomass. We would like to develop "integrated biorefineries" that will use residual biomass from conventional crops and also harness microbial genomics to identify enzymes with which to process biomass produced in marginal lands with much greater efficiency than is now possible.

In particular, we hope to meet sustainable agriculture goals by focusing on a perennial crop belonging to the Malvaceae family. These plants grow in marginal lands, producing both starch (crown) and cellulosic (stem and leaves) biomass, while their blossoms contain high-value antioxidants. We also are evaluating novel varieties of sugarcane, called "energy cane," that produce both molasses for food and feed along with substantially higher levels of biomass (bagasse) than do traditional sugar canes.

More generally, we are exploring diverse ecosystems in Caribbean coastal areas for microbes that produce robust enzymes, with which to create cocktails for processing biomass and waste. We are also examining yeast isolates that are indigenous to certain crops for genetic pathways that ferment a broad variety of sugars. Cellulose and hemicellulose represent the primary forms of stored sugars in lignocellulosic biomass. Their heterogeneity is greater in mixtures of biomass from different sources. However, if biomass from different crops and from municipal solid wastes could be used to feed a single fermentation process for biofuel, the costs and risks of transporting that biomass might be substantially reduced. Further, although enzymes can degrade any known polysaccharide, effective processing will require enzymes that work under harsh conditions—a strategy that likely will depend on having large numbers of enzymes on hand with which to custom blend "cocktails" to meet processing needs. Microorganisms that thrive in harsh conditions are likely sources for such enzymes. This comprehensive approach of exploring and developing recombinant microbes should benefit the pursuit of renewable energy.

Summary

- Key challenges include dealing with diverse cellulosic sugars and finding ways to recycle carbon dioxide back into useful biomass.
- Sugar cane and Hibiscus varieties from the Malvaceae plant family are sources of cellulosic biomass that do not require highly productive agricultural lands.
- Microorganisms associated with termites and shipworms might harbor useful enzymes for processing biomass, while yeasts associated with Malvaceae plants are also worth investigating.
- Microalgae varieties from the Caribbean coast are being evaluated as potential producers of oils that could be used for fuel.

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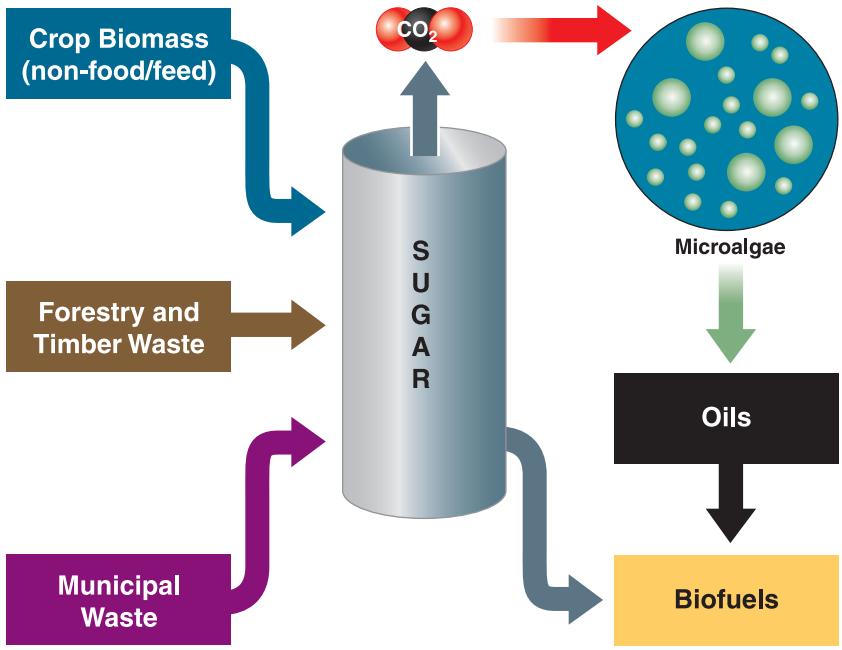
FIGURE 1



Stored carbon from plants (crops/forestry) and plant-derived products are degraded to sugars that are fermented to produce fuel alcohol and carbon dioxide; using sunlight as the source of energy, microalgae recycle the carbon dioxide, and produce oils that are converted to diesel fuels.

Integrated Biorefinery Derives Fuel from Lignocellulosic Biomass

Sugars in plants and waste materials that derive from plant materials make up a largely untapped resource for producing renewable energy. All plants contain stored sugar polymers, mainly starch and cellulose. Many common household wastes, including plant clippings, paper, and cardboard, as well as discarded industrial materials such as waste timber, contain large amounts of cellulosic sugars. Harvesting these sugar polymers and processing them into simple sugars to produce energy and chemical feedstocks that microbes ferment into ethanol or other useful intermediates would provide an opportunity to replace fossil fuels on which we now depend (Fig. 1). We would further benefit if there were ways to capture and recycle carbon dioxide emitted from coal and ethanol fermentation plants.

Like all other prospective sources of stored sugar that serve as alternatives to fossil fuel, use of lignocellulosic biomass presents several challenges and opportunities. The primary challenges include that it competes for land with other plants used to produce food and feed, its conversion to fuel depends on commercially unproven technologies, the difficulties in handling and converting diverse sources of biomass, and the unmet need to use different types of sugars and to capture carbon dioxide to achieve environmental benefits.

However, in the face of those challenges, there are also several opportunities to be realized in pursuing lignocellulosic biomass. They include the abundant availabiity of agricultural leftovers and solid wastes with significant cellulose content, the possibilities of producing multiple products by integrating technologies, recycling carbon dioxide to produce additional biomass for biofuels (and other products), advances in technologies, and growing legislative support for federal funds to establish sustainable businesses that supply alternative fuels.

Our program is focused on several goals, including propagating high-biomass crops, identifying robust carbohydrate-degrading enzymes on the basis of environmental genomics, developing proprietary microorganisms that convert 5- and 6-carbon sugars into ethanol, and identifying microalgae to produce oils from recycled by-products from ethanol fermentations.

High-Biomass Crops, Marginal Lands

Agriculture is the primary source of stored sugar that can be used for producing fuel and biomass. However, as a practical concern, we are experiencing the social and economic effects of our prior reliance on corn starch as a source for fermentable sugars. Thus, as more corn is diverted into producing fuel, less is available as a feed for livestock, raising the cost of food. To avoid this impact on food costs, it would be ideal to use for biomass either the leftover organic matter from crops after the feed and food components are harvested, or another crop that is not used for such purposes but can grow on poorer-quality land.

With those effects in mind, we are focusing on two crops, sugar cane and Malvaceae. The first

Govind: a Belief in Nature Entails a Search for Productive Microorganisms

Nadathur Govind believes in nature. "Whatever process we scientists want to develop, nature has already done it, and done it well," he says. "We can learn a lot from observing how nature does it." His attitude reflects his scientific focus, which entails looking for microbes that degrade biomass to produce useful chemicals and energy, with the added goal of recombining genes from diverse organisms to improve the overall efficiency of renewable energy processes.

To work on such challenges, Nadathur, a native of India, left California in 1993 for the University of Puerto Rico, where he is now a professor in the department of marine sciences. "My major reason for moving was the rich microbial diversity present in tropical and subtropical regions," he says. "When I first arrived here, we did an initial survey of whether one could go about isolating tropical microbes that could perform certain degradative activities, such as biomass, bioplastics, and hydrocarbons. We found that there were indeed microbes that could be isolated that were quite efficient with these kinds of reactions. Ever since then, my laboratory has been dedicated to looking for such organisms, or their genetic material to exploit, and to set up biomass degradation."

Nadathur, 53, also advises the university on strategic planning and has consulted with the Department of Commerce and Economic Development in Puerto Rico. Born in Chennai, formerly known as Madras, he grew up in Ahmedabad, in west central India, about 200 miles north of Mumbai. His late father was a senior official in India's largest nationalized bank, his late mother a housewife. His father began working in a bank when he could no longer afford to continue his education. Thus, he later paid for his two sons to continue their schooling, and insisted that Nadathur and his brother, now a mechanical engineer in Hong Kong, forsake jobs during their years of study. "After [my father] retired, he finished university and got his B.A. in English literature," Nadathur says.

When it came to a career, "the stress was always that we needed to study science," Nadathur says. "My dad wanted one son an engineer, and one in biology or medicine. I never got animated to study medicine, but biology has always intrigued me. When I started reading about the history of microbiology with the wonderful work of people such as Jonas Salk, Pasteur, Robert Koch, Salvador Luria, and Max Delbruck—the golden era of microbiology—I was hooked."

Nadathur received his B.S. in microbiology in 1975 and his M.S., also in microbiology, in 1977, both from Gujarat University, in Ahmedabad. In 1982 he earned a Ph.D. in microbiology from Maharajah Sayajirao University, in Baroda, India. Shortly thereafter he moved to the University of Seville, in Spain, as a postdoctoral associate in the department of genetics. In 1985, he left Spain for La Jolla, Calif., where he spent one year as a postdoctoral associate at the Scripps Clinic and Research Foundation. In 1987, he moved to the Marine Science Institute and department of biological sciences at the University of California, Santa Barbara, conducting research there until he departed for Puerto Rico.

Nadathur allows himself a little time for hobbies and travel. "In my spare time I read, fiction mostly, or watch basketball," he says. "We, as a family also like to travel to different parts of the world. We have been traveling with my daughter ever since she was 2 months old, and she absolutely loves it."

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of these two, sugar cane, is used widely to produce molassess and sucrose for food and feed, while leftover material, called bagasse, is a source of cellulosic biomass. Recently, new varieties of sugar cane have been developed that produce more than 100 tons biomass per acre without reducing the brix, or dissolved sugar, and thus can still meet molasses production standards set for food and feed. These varieties are especially suitable for Caribbean countries and some parts of the United States.

Meanwhile, among members of the Malvaceae plant family, several hibiscus varieties produce blossoms that are excellent sources of high-value nutrients, while the remainder of the plant yields desirable lignocellulosic biomass. Additionally, these plants can grow in marginal lands not customarily used for food or feed

FIGURE 2

Stored carbon from plants (crops/forestry) and plant-derived products are degraded to Pine grids placed in the magrove area in southwestern Puerto Rico after four months of exposure. On the right, shipworm isolated from the wood tentatively identified as *Teredo* sp.

crops. With this crop, growers can choose between harvesting only the above-ground portion of the plant, leaving the remainder underground to regrow without replanting, or also harvesting the entire plant as a source of starch for fermentation into bioethanol.

Of course, we need to continue using other sources of lignocellulosic biomass, including timberland wastes, leftover materials from other crops such as corn stover and rice husks, and municipal solid wastes containing cellulosic materials that otherwise becomes landfill. Economic pressures and the demand for environmental benefits require us to focus on crops that do not readily compete for use of highly productive agricultural lands and also to make better use of alternative sources of cellulosic biomass.

Termites and Shipworms, Potential Sources of Enzymes for Processing Biomass

If we are to recover stored sugars from lignocellulosic biomass to produce ethanol and to convert other material into fermentable feedstocks, we will need processing steps that are versatile and that enable us to use diverse lignocellulosic materials. We look forward to harnessing genetic capabilities in nature—specifically investigating termites and shipworms that degrade lignocellulosic materials under harsh conditions and then adapting their enzymes to process biomass in an environmentally safe manner.

Termites voraciously consume and degrade biomass, serving as a keystone species at the base of the food chain for tropical terrestrial ecosystems. To degrade such materials, termites establish nutritional symbioses with microorganisms, enabling them to consume plant cellusosic materials. Environmental genomic analyses that focus on the microbial symbionts in termite guts reveal many genes encoding enzymes that degrade complex carbohydrates into fermentable, simple sugars.

Recently, we began isolating genes from bacterial flora associated with the termite *Nasutitermes acajutlae* that is found in mangroves in southwestern Puerto Rico. We enriched the bacterial populations from termites before extracting, partially digesting, and cloning the bacterial DNA into a λzap vector. We then screened approximately 5×10^5 independent clones before isolating genes encoding four cellulases and four xylanases. The sequence data for one of the cellulases indicate that the enzyme shares only about 80% identity at the protein level to another cellulase gene that was isolated from the termite *Nasutitermes* sp. This structural disparity suggests either that different species of termite harbor different associated bacteria or that the same species of termite from different locations harbor different bacteria that could provide us with unique enzymes.

The marine wood-boring mollusks (bivalves) of the families Teredinidae and Xylophagainae, commonly called shipworms, are well-known for the damage that they cause to wharves and other wood that is exposed to the sea. By the 1920s, scientists realized that these worms digest both cellulose and hemicellulose. Such mollusks contain a specialized organ, called the Deshayes gland, in their gills with ducts leading to the digestive tract. In nitrogen-limiting environments, such mollusks survive with the help of *Teredinobacter turnerae* bacteria, which are cellulolytic. A 60-kDa endoglucanase from this bacterium can break interior linkages of cellodextrin chains that are larger than cellotriose. Its optimum temperature of 60°C and optimum pH of 5–7 suggest that this endoglucanase is rugged, and thus suitable for industrial uses.

The cellulases that have been isolated and studied derive mainly from organisms that have been cultivated. Yet, difficult-to-culture extremophiles tend to be good sources for commercially valuable enzymes, suggesting that cul-

ture-independent methods that utilize isolated DNA from different environments of interest could prove a fruitful approach for identifying unique carbohydrate-degrading enzymes. Although this metagenome-based approach would likely prove helpful, to our knowledge it has not been used to search for genes encoding carbohydrate-degrading enzymes from bacteria associated with shipworms.

One major challenge in studying wood-boring shipworms is in trying to collect large numbers to enrich for bacteria associated with them. We know areas in southwestern Puerto Rico where such mollusks are abundant, and we also developed a technique for placing wooden grids strategically in mangroves for monitoring on a monthly basis for enrichments (Fig. 2). This approach can be adapted in such a way that the grids expose the shipworms that they catch to a particular biomass blend, thus enriching for the best enzyme candidates to degrade a particular mixture of carbohydrates in that biomass sample.

Our observations suggest that a broad-based search for enzymes among tropical organisms, especially those that avidly degrade lignocellulosic biomass, will yield novel carbohydrate-degrading enzymes. Such enzymes should provide a more effective means to produce fermentable sugars from diverse sources of lignocellulosic biomass.

Converting Lignocellulosic Feedstocks into Ethanol, Recycling Carbon Dioxide

Humans began using microbes to make ethanol from grains and fruits long ago. Some microorganisms that grow on grapes are so highly efficient at producing alcohol that they are prized in the wine industry. With that example in mind, we began a comprehensive search for fermenting organisms that associate with blossoms of Malvaceae plants and with high-biomass sugar cane varieties, and we soon identified unusual fermenting microorganisms that we are continuing to characterize. We anticipate using some of these organisms directly or incorporating genes from some of them into other more fully characterized microbes used in industrial fermentation.

Carbon dioxide, the major by-product of ethanol fermentations, is not only reusable but also poses a heavy burden to the environment unless it is sequestered or recaptured. One approach that would meet this goal and also would greatly boost the efficiency of fermentations involves cultivating photosynthetic microalgae, which also are referred to as diatoms, green algae, blue-green algae, and golden algae. Their biomass contains three main components: proteins, carbohydrates, and natural oils.

By completing a closed loop for carbon recycling, microalgae could become another source of biomass and oil as part of an overall biomass-to-ethanol production process. Microalgae, among the most photosynthetically efficient plants because of their simple cell structure, accumulate high levels of natural oils, use carbon dioxide as their sole carbon source, and grow in both marine and freshwater environments. Some experts consider algal biodiesel fuel the most energy-positive and environmentally beneficial end-product possible from high-volume carbon dioxide reuse schemes.

When grown in aqueous suspension, algae can produce up to 30 times the amount of oil per unit land compared to terrestrial oilseed crops, according to the National Renewable Energy Laboratory. Algae grown on a mere 200,000 hectares, which is less than 0.1% of the arable land mass in the United States, could produce one quad of fuel, which is 10^{15} British thermal units (BTUs). An intensive program to produce algal biodiesel could easily yield several quads of fuel—substantially more than what existing oilseed crops provide. Harvesting methods would need optimizing if algae-based fuel production were to become commercially viable.

Microalgae systems also use far less water than do traditional oilseed crops. Some species of algae appear to be particularly well suited for producing biodiesel due to their high oil content, with some varieties containing more than 50% oil, exceeding terrestrial plants. Moreover, some algal varieties grow extremely fast. Typically classified according to pigmentation, life cycle, and cellular structure, the four most abundant groups of algae are *Bacillariophyceae*, *Chlorophyceae*, *Cyanophyceae*, and *Chrysophyceae*. Individual species vary considerably in terms of growth rate, nutrient requirements, and oils produced.

We are analyzing microalgae varieties from different ecosystems along the Caribbean coast and along the southeastern U.S. mainland, with

a special focus on microalgae that grow in harsh environments. We are characterizing isolates with respect to growth vigor, nutritional requirements, and oils they produce. We are also establishing indoor and outdoor large-scale growth facilities for evaluating various means of harvesting the algae via low-energy-requiring processes and with minimal loss of water. As part of this program, we characterized five species of microalgae from tropical habitats that produce oils with chain lengths that meet the requirements for making jet fuel. We are also evaluating algae for their capacity to produce hydrogen.

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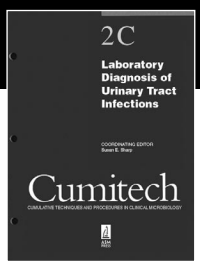

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