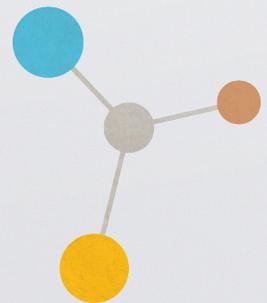


Between sugars and genes



Applied scientific knowledge of sugarcane
may prove useful in developing new
methods for ethanol production

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PUBLISHED IN OCTOBER 2012



Following a large number of genetic, physiological and agronomic studies of sugarcane conducted in recent years, our older colleagues might say that the plant is being turned on its head. Sugarcane is a member of the grass family and was brought to Brazil by the Portuguese in the 16th century. Today, scientists hope to gain a deeper understanding of sugarcane and its peculiarities with a view towards increasing its productivity. The ultimate goal is to produce more ethanol per hectare of land.

Efforts to achieve this increase have included research aimed at making sugarcane better adapted to the so-called second generation of alcohol production. In this technology, enzymes use the sugars recovered from crushed sugarcane, or bagasse, to form a type of broth, which is then used to produce more biofuel. For this reason, researchers from several Brazilian institutions are keeping one eye on basic research and the other eye on the future of industrial ethanol production processes. The first scientific advance came in 1999 with the launch of the Sugarcane Genome Project, financed by FAPESP. The most recent findings from that project confirm that sugarcane stalks and leaves have more sugars (the basic substances for creating ethanol) in the hemicellulose fraction than in the cellulose fraction. These findings could change the course of second-generation ethanol production in the future.

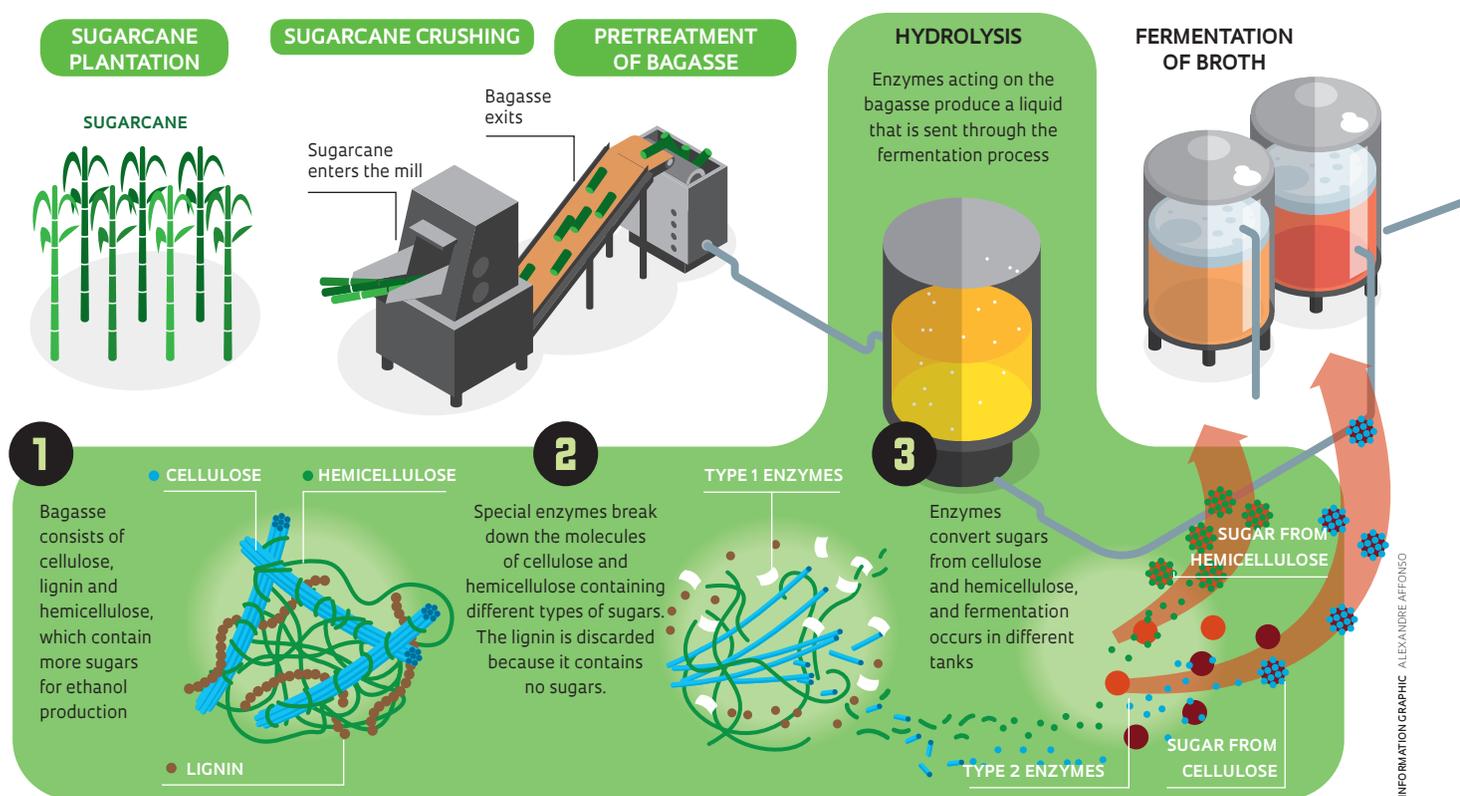
“Our studies of the cell walls of both the stalk and the leaves of sugarcane plants showed that about 30% of the sugars are present in the cellulose, 50% are in the hemicellulose, and 10% reside in the pectins. The technology now being designed for future second-generation ethanol was based only on cellulose, while the sugar polymers of hemicelluloses – which contain complex sugars such as arabinoxylans, beta-glucans and xyloglucans – are being ignored, as are the pectins, and together these represent 70% of the sugars in sugarcane cell walls,” says Marcos Buckenridge, a professor at the Biosciences Institute at the University of São Paulo and coordina-



Transgenic sugarcane seedlings at the Chemistry Institute of USP

The future of second-generation ethanol processes

After the sugarcane broth is used in the first-generation process, the bagasse and leaves are utilized in the hydrolysis process. The final stage involves traditional fermentation by yeasts that convert the sugars into ethanol



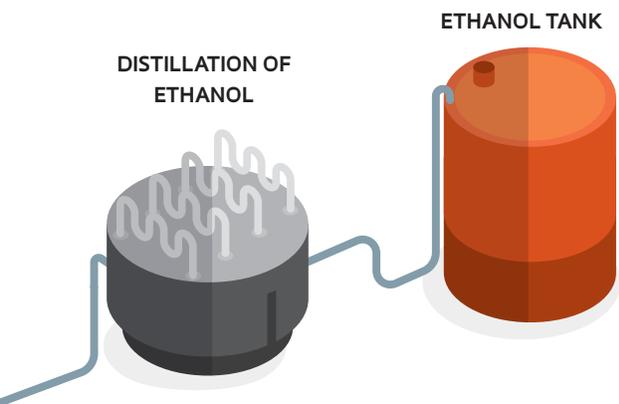
tor of Brazil's National Institute of Science and Technology (INCT) of Bioethanol, which encompasses 31 laboratories in five Brazilian states. In sugarcane cell walls, the hemicelluloses and pectins are located between the microfibrils, which are agglomerates of cellulose molecules. These microfibrils have many five-carbon sugars and are therefore not digestible by the yeasts (*Saccharomyces cerevisiae*) that are used to ferment sugarcane broth. These yeasts are accustomed either to sucrose (which is composed of glucose and fructose and found in sugarcane juice) or to glucose from cellulose or certain hemicelluloses that have six carbon atoms.

The future utilization, via hydrolysis, of pentoses (five-carbon sugars) from bagasse could drive up Brazilian ethanol production by an estimated minimum of five billion liters (see *Pesquisa FAPESP* No. 192) over the current output of 25 billion liters. Pentoses could also be utilized in biotechnology applications, thereby increasing the commercial value of bagasse. In second-generation processes, the enzymes form a liquid that also serves as food for the yeasts.

An estimated 5 billion more liters of ethanol can be produced using sugars from hemicellulose

“There have been attempts to produce *Saccharomyces* lineages capable of utilizing five-carbon sugars, including at the Brazilian Bioethanol Science and Technology Laboratory (CTBE) and at other institutions and companies in Brazil and abroad. In England and Sweden, scientists have already successfully shown that this is possible, although the work was done entirely in a sterile laboratory environment. For Brazilian production plants, however, that is not yet sufficient. The yeasts must be robust enough to survive in the presence of other microorganisms, such as bacteria, that exist in an unsterilized environment,” says Buckeridge, who is also the scientific director of the CTBE in Campinas, São Paulo.

Experiments on the most advanced stage, cellulose hydrolysis, show that many uncertainties still linger. “We now have a good understanding of the pretreatment process, but we still need to investigate the various options for performing hydrolysis in a way that the industry can quickly absorb, both economically and sustainably,” says Professor Rubens Maciel Filho of the University of Campinas (Unicamp), one of the coordinators of FAPESP's Program for Research on Bioenergy



(BIOEN), in which INCT Bioethanol also participates. “Techno-economic and sustainability assessments are needed – in this case, in analyses of water consumption and in the use of chemicals in the hydrolysis process,” Maciel Filho notes.

“In the current second-generation experiments, after the bagasse is discarded after the first generation when the sugarcane broth is extracted to make ethanol, it goes through a process of rupturing the cell walls to obtain the cellulose surrounded by hemicellulose and lignin, a polymer that has no sugar,” says Buckeridge. This rupturing is currently performed using a high-pressure steam process. During this process, the cell walls of the bagasse are relaxed, and solvents, acids and enzymes are used to separate the components. “It’s the use of force, in an effort to get rid of everything you have around the cellulose,” he adds. “Our idea is to start the hydrolysis process in the field. To produce sugarcane that is more second generation-ready, that makes hydrolysis easier and eliminates the need to wash the bagasse, which removes many sugars from the material.”

In an article to be published in the journal *BioEnergy Research*, Buckeridge and two other researchers from his group at USP, along with two researchers from the University of Georgia Complex Carbohydrate Research Center in the U.S., present their work in identifying the fractions of each sugar polymer in sugarcane. They also make observations about the complexity of the cell walls and the difficulty of finding chemical keys or codes that could better utilize the network of polysaccharides. These researchers also believe that new knowledge of the sugar composition of sugarcane could lead to modifications in the second-generation process. Buck-

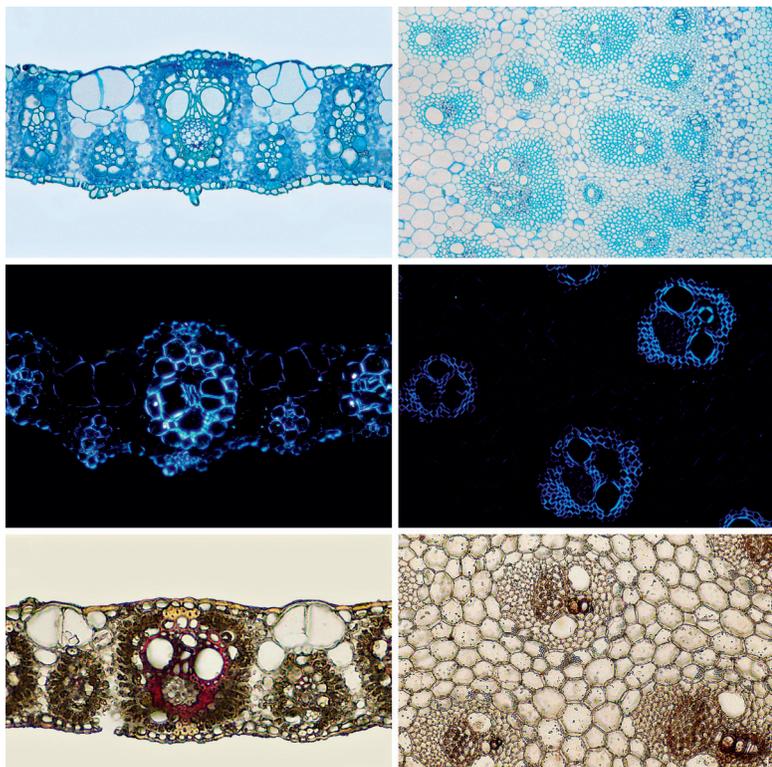
The sugarcane of the future would have some characteristics of the papaya, which is sweeter and softer after ripening and harvesting

eridge envisions a best-case scenario in which the sugarcane would be brought in whole for the hydrolysis process after the broth is extracted for the first generation process.

“The biology of the cell walls is the essence of these advances, and it is essential for technological progress to be made in the area of sustainable biofuels and biomaterials,” says Professor Leonardo Gomez of the York University Department of Biology in England. Gomez, who is Argentinian, spent time in Brazil in 2010 working with the CTBE. “In the opinion of many specialists, the development of second-generation biofuels has been made easier by the presence of a well-established first-generation industry. As a result, Brazil has the best environment for this to occur. But we are only talking about potential. Someone has to assume the risk and invest in biofuels on an industrial scale,” Gomez notes.

In the interest of advancing the process of obtaining second-generation ethanol, Buckeridge draws attention to a physiological pretreatment that leaves the plant more malleable and better-suited for processing by hydrolysis. “When this substance is applied in plantations while the plant is still very small, it inhibits an enzyme in the sugarcane that makes phenylpropanoids, which are the precursors of lignin, the substance that binds the sugars to the cell wall and gives the plant mechanical strength. We do not yet know for sure what happens, but with this compound, we were able to process 30% more xylans, which make up 50% of hemicelluloses,” Buckeridge says. The use of this substance, comprised of piperolynic acid in sugarcane, is the subject of a patent filed with the Brazilian Industrial Property Institute (INPI) by Buckeridge and his former post-doc Wanderley dos Santos. Santos is now a professor at the Federal University of Paraná (UFPR), and he is testing the product in the field. “We still have to improve it and try to reduce the cost,” Buckeridge notes.

Another solution for the second generation is being developed under BIOEN, a collaboration of 13 research groups. Their goal is to develop one or more agronomic and genetic varieties of sugarcane with high-quality characteristics for the first and second generations. One such characteristic is a greater capacity for photosynthesis. The researchers have already identified at least four genes that are responsible for capturing sunlight. These genes may be related to higher cellular growth and a consequent increase in sucrose production. The development of transgenic plants is one of the biotechnological tools being used to produce this supercane. In this case, transgenic methods would include not only introducing foreign genes into the plant but



Microscopic images for the analysis of sugarcane leaves (left column) and stalks (right). Fluorescence applied in cells (middle row) and the presence of lignin (in red, bottom row), where several intact circular-shaped cells full of juice can be identified

also activating or reversing the silencing of native sugarcane genes. “We could also develop plants with cell walls that are better accommodated to the second generation,” says Buckeridge. “These concepts might seem futuristic, but BIOEN has genes related to the modified cell wall of which we are thinking of making ‘sugarcane papaya,’ for example.” This proposed transgenic sugarcane would have some characteristics similar to papaya, which is sweeter and softer after ripening and harvesting.

“We now have 380 genes linked to sucrose and over a thousand related to drought resistance,” says Professor Glaucia Mendes Souza of the Chemistry Institute of USP (IQUSP), who will jointly head the sugarcane genome research at BIOEN with Professor Marie Anne van Sluys of the Biosciences Institute of USP. Also participating in the research will be Professor Marcelo Menossi of the Biology Institute of Unicamp. Of those 380 genes, 250 are now being tested in sugarcane seedlings placed in test tubes, tubs and pots at IQUSP or in greenhouses at the Luiz de Queiroz School of Agriculture at USP in Piracicaba. These experiments are being coordinated by Professor Helaine Carrer, who is studying gene expression. Also being tested is the expression of sugarcane genes in tobacco, a plant that is easy to manipulate in the laboratory and that serves as a model for this type of experiment. Two sugarcane genes linked to drought resistance have already been expressed in tobacco, and a patent for their use has been filed with the INPI.

Modifying a plant with genes of interest requires promoters, which are biotechnological tools in the form of the DNA sequences in which the gene will be expressed. It is in these molecules that researchers will modulate the superexpression or silencing of genes. “We filed a patent this year for 10 sugarcane promoters that will enable the genes to be expressed differently,” says Souza. In regard to sugarcane cell walls, she notes that she has already developed plants in which the genes for lignin production have been silenced. “Lignin upsets second-generation execution because it makes it difficult to extract polysaccharides, but switching off its production caused the plant to fall over in some experiments. We need to come up with varieties in which we can try for a midway point, reducing the presence of lignin but still keeping the plant upright,” explains Souza.

Academic research is also trying to improve hydrolysis through the use of enzymes that are more effective in breaking down sugarcane cell walls, extracting the sugars and preparing the material for ethanol production. However, which enzymes should be used to process the different polysaccharides that are present in the plants’ cell walls? Some enzymes used by the food industry, for example, are being tested with sugarcane, but in themselves they do not present a full solution. “These industrial enzymes are produced mainly by fungi,” says Professor Richard Ward of the Chemistry Department in the Ribeirão Preto School of Philosophy, Science and Letters at USP, and of the CTBE, who has successfully designed two multifunctional enzymes that act on hemicelluloses. Known as chimeric enzymes, these enzymes are produced by bacteria.

“We know that cellulose is more hidden than the other polysaccharides present in sugarcane cell walls, and our challenge is to create enzymes that are programmed to destroy and degrade the other components that are also important sources of sugar, until we get to the cellulose,” Ward explains. “It is important to develop the enzymes most appropriate for each polysaccharide. But it is still difficult to find good enzymes at a low cost. They are currently being marketed for tens of dollars per kilo. That may seem inexpensive, but we have to think about in-plant processing of hundreds or even thousands of tons of lignocellulosic material per day.” Ward says that the goal is to build chimeric enzymes such that each enzyme attacks more than one polymer of sugarcane bagasse. “That is especially important for hemicelluloses, which have a heterogeneous set of polysaccharides.”

While some of the enzyme research may seem unconventional, it is based on simple natural phenomena. In the search for enzymes that digest

The digestive tracts of cockroaches are being studied to find enzymes for second-generation ethanol processes

cellulose and lignocellulosic material, Professor Ednildo Machado of the Biophysics Institute of the Federal University of Rio de Janeiro (UFRJ) is studying the enzymatic composition of the digestive tract of two cockroaches. He has focused on two species: *Periplaneta americana*, which is common in large cities, and *Nauphoeta cinerea*, which was created to feed reptiles raised in captivity. “In lab experiments, I was only able to provide sugarcane bagasse to the cockroaches, and they fed on it. In other words, they were able to digest the cell walls of this material to survive very favorably,” Machado says. Consequently, he began to think about which enzymes in the digestive tracts of these insects could be useful in second-generation ethanol production.

Machado was introduced to Buckeridge during the Brazilian Conference on Biochemistry in 2010, and they formed a close collaboration. Buckeridge was at the CTBE, where a number of experiments were conducted. “We were able to identify a few enzymes that are produced by bacteria inside the digestive tract of cockroaches. We don’t yet know if these bacteria were already there, or if the insect acquired them from the material, in the case of bagasse.” The cockroach can also produce these enzymes through fungi and protozoa, can very easily feed on a wide variety of waste matter and can adapt easily to such diversity. “That characteristic enabled us to identify a number of enzymes in the insects that are excellent for various technological processes,” Machado says. The next step is to confirm the identity of the microorganisms that produce the enzymes. To do this, it will be necessary to sequence all of the DNA present in the cockroach intestines using a process called metagenomics. Metagenomics will allow researchers to identify the species and genes involved in the production of enzymes that specialize in breaking down cellulose and hemicellulose from sugarcane bagasse. Identifying these genes makes it possible to clone them into bacteria such as *Escherichia coli* and even facilitate the production of these enzymes on an industrial scale. Professor Ward is starting to use the same process in a laboratory to produce the enzymes that attack sugarcane cell walls.



Transgenic seedlings: biotechnological tools for testing strategies to silence or activate sugarcane genes

Such efforts are increasing the number of tools that could aid in producing more ethanol from sugarcane within a few years. “In the past 10 years, there has been an exponential increase in the amount of research and technological investment aimed at utilizing biomass as a renewable, sustainable substitute for oil,” says Professor Gomez of York University. “The current research in the field of biomass composition offers new potential for biorenewable energy.” With that goal in mind, the production of ethanol and high-performance chemicals from biomass is possible only with a detailed, multidisciplinary understanding of the biology and biochemistry of biomass. ■

Projects

1. National Institute of Science and Technology (INCT) of Bioethanol (nº 2008/57908-6); Thematic project of the FAPESP Program for Research on Bioenergy (BIOEN); **Coord.** Marcos Silveira Buckeridge/USP; **Investment** R\$2,896,588.59 and US\$303,342.92 (FAPESP);
2. Sugarcane signaling and regulatory networks (nº 2008/52146-0); Thematic project of the FAPESP Program for Research on Bioenergy (BIOEN); **Coord.** Gláucia Mendes Souza/USP; **Investment** R\$3,390,743.73 and US\$1,174,768.67 (FAPESP)
3. Identification, characterization and engineering of enzymes that degrade plant cell walls (nº 2010/18850); Thematic project; **Coord.** Richard John Ward/USP; **Investment** R\$491,952.05 and US\$313,495.03 (FAPESP).

Scientific articles

- DE SOUZA, A.P. *et al.* Composition and structure of sugarcane cell walls: implications for cell wall hydrolysis and second generation bioethanol. **BioEnergy Research**. In press. Sept. 2012.
- BEGCY, K. *et al.* A novel stress-induced sugarcane gene confers tolerance to drought, salt and oxidative stress in transgenic tobacco plants. **Plos One**. Vol. 7, No. 9, e44697. Sept. 2012.
- FURTADO, G.P. *et al.* A designed bifunctional laccase /b-1,31,4-glucanase enzyme shows synergistic sugar release from milled sugarcane bagasse. **Protein Engineering, Design & Selection**. In press. Sept. 2012.